Coordination Chemistry of Antimicrobial and Anticancer Agents

by

Katja Dralle Mjos

M.Sc. (Dipl.-Chem.), Carl von Ossietzky University, Oldenburg, Germany, 2007

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Abstract

The World Health Organization has named the resistance of microbes to known antimicrobial drugs as an increasingly serious threat to global public health. Isolates of the ESKAPE pathogens (*E. faecium*, *S. aureus*, *K. pneumonia*, *A. baumanii*, *P. aeruginosa*, and *Enterobacter* species) are responsible for many nosocomial infections each year that require complicated, and therefore expensive, medical treatment, often leading to death in immune-compromised patients. Over the past 50 years, (fluoro-)quinolone antimicrobial agents have been widely used in the clinic as broad-spectrum antibiotics, but lately growing resistance against this drug class has been reported.

Combining metal ions with known organic small-molecule drugs is one strategy to overcome such developed resistances. Previously, the antimicrobial properties of copper(II) and gallium(III) had been investigated, leading to Greek mythology comparisons for their mechanism of action: Cu^{2+} as the "Achilles Heel", Ga^{3+} as the "Trojan Horse" subterfuge for Fe³⁺. In this thesis, gallium(III) and copper(II) coordination complexes of (fluoro-)quinolone antimicrobial agents, and derivatives thereof, were synthesized in an attempt to combine the antimicrobial potency of Cu^{2+} and Ga^{3+} with that of the quinolone antimicrobial agents in one molecule. The antimicrobial susceptibility of these coordination complexes was evaluated against five isolates of the ESKAPE pathogens; combinational effects between the metals and the quinolone ligands were not observed. While the combination of metal ions with small, organic drug molecules may lead to novel potent metallodrugs, the interaction of metal ions with drugs *in vivo* is often associated with toxic side-effects of medical treatment, for which the iron(III)-mediated cumulative-dose cardiotoxicity of doxorubicin is one example. Vosaroxin is a first-in-class anticancer quinolone derivative in clinical trials. Unlike the anthracycline anticancer drug doxorubicin, vosaroxin is minimally metabolized *in vivo*. Spectrophotometric titrations and further studies of the isolated tris(vosaroxino)iron(III) and -gallium(III) complexes supported a strong coordination of the metal ion suggesting that vosaroxin treatment may not result in cardiotoxicity.

Preface

The research work for this thesis has been conducted by Katja D. Mjos (KDM) under the immediate supervision of Dr. Chris Orvig and co-supervision of Dr. Michael J. Abrams from September 2009 until August 2014 at The University of British Columbia (UBC). KDM designed the projects and performed all syntheses, analytical and biological experiments, if not stated otherwise below. Characterization data of all compounds were obtained by KDM, besides high-resolution mass spectrometry and elemental analyses, which were conducted at UBC's Mass Spectrometry Centre, as well as 600 MHz nuclear magnetic resonance (NMR) spectra, which were recorded by Dr. Maria Ezhova at UBC's NMR Facilities.

<u>Chapters 1 and 6</u> are an adaptation of published work, reproduced in part, with permission from Mjos, K.D. and Orvig, C.: Metallodrugs in Medicinal Inorganic Chemistry; *Chem. Rev.* **2014**, *114*, 4540-4563, Copyright 2014 The American Chemical Society. The manuscript benefited from discussions between KDM and Dr. Michael J. Abrams; it was written by KDM with editing from Dr. Chris Orvig.

<u>Chapters 2 and 3</u> are an adaptation of the manuscript in preparation for publication, Mjos, K.D.; Cawthray, J.F.; Polishchuk, E.; Abrams, M.J.; Orvig, C.: Testing the "Trojan Horse" Theory: Gallium(III) and Iron(III) Complexes of Quinolone Antimicrobials. The first idea for this project, to synthesize a tris(ciprofloxacino)gallium(III) complex and test its antimicrobial properties, came from Dr. Michael J. Abrams. KDM expanded the project from there, planned and performed all experiments. Dr. Jacqueline F. Cawthray modelled one possible stereoisomer of the tris(ciprofloxacino)gallium(III) complex using density functional theory (DFT) calculations. The manuscript was written by KDM with editing from Dr. Chris Orvig.

<u>Chapter 4</u> is an adaptation of the manuscript in preparation for publication, Mjos, K.D.; Polishchuk, E.; Abrams, M.J.; Orvig, C.: Syntheses, Characterization, and Evaluation of the Antimicrobial Potential of Copper(II) Coordination Complexes with Quinolone and Xylenyl-Linked Quinolone Ligands. The manuscript was written by KDM with editing from Dr. Chris Orvig.

<u>Chapter 5</u> is an adaptation of published work, and is reproduced in part, with permission from Mjos, K.D.; Cawthray, J.F.; Jamieson, G.; Fox, J.A. and Orvig, C. Iron(III)-Binding of the Anticancer Agents Doxorubicin and Vosaroxin; *Dalton Trans.*, **2015**, *44*, 2348-2358, Copyright 2015 The Royal Society of Chemistry. KDM and Dr. Jacqueline F. Cawthray designed the spectrophotometric titration experiment and titrated the iron(III)vosaroxin system, KDM recorded all data for the iron(III)-doxorubicin system, accordingly. Data fitting and DFT calculations were performed by Dr. Jacqueline F. Cawthray, who prepared the respective figures. Dr. Maria Ezhova carried out the temperature dependent NMR-study at 400 MHz. Together with Dr. Zhicheng (Paul) Xia, Dr. Maria Ezhova supported KDM with the design and execution of the NMR titration experiment. Caterina Ramogida and Jeff Therien assisted KDM with the cyclic voltammetry (CV) measurements. The results of this project were disseminated to *Sunesis Pharmaceuticals, Inc.* in form of a technical report in March 2014, which was written by KDM with input from Dr. Jacqueline F. Cawthray. This technical report laid the foundation for the manuscript which was written by KDM with input from Dr. Gene Jamieson and Dr. Judith A. Fox, Sunesis Pharmaceuticals, Inc.. Dr. Jacqueline F. Cawthray edited earlier versions of the manuscript; Dr. Chris Orvig edited the final version.

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List of Abbreviations

A adenine.

- **AAS** atomic absorption standard.
- **AD** Alzheimer disease.
- ADME concept concept of absorption, distribution, metabolism and excretion.
- **ADP** adenosine triphosphate.
- **AIDS** acquired immunodeficiency syndrome acquired through infection with human immunodeficiency virus (HIV).
- **APL** acute promyelocytic leukaemia.
- **AQD** anticancer quinolone derivative.
- ar aromatic ring system.
- ATO arsenic trioxide.
- **ATP** adenosine triphosphate.
- ATRA all-trans retinoid acid.
- **BAL** 2,3-dimerceptopropanol, H_2 DMPA.

BBB blood brain barrier.

BEOV bis(ethylmaltolato)oxovanadium(IV).

BMOV bis(maltolato)oxovanadium(IV).

BP bipolar disorder.

 ${\bf br}\,$ broad.

BSAC British Society for Antimicrobial Chemotherapy.

BSS bismuth subsalicylate.

 ${\bf Caco-2\ cells\ human\ color\ colorectal\ adenocarcinoma\ cells.}$

CBS colloidal bismuth sub-citrate.

CDC U.S. Centers for Disease Control and Prevention.

CKD-MBD chronic kidney disease-mineral and bone disorder.

CLSI U.S. Clinical and Laboratory Standards Institute.

CNS central nervous system.

COST European Cooperation in Science and Technology.

COSY correlated spectroscopy.

CTCL cutaneous T-cell lymphoma.

 ${\bf CV}\,$ cyclic voltammetry.

 \mathbf{d} doublet.

- $\mathbf{d}\mathbf{d}$ doublet of doublets.
- **DFO** desferrioxamine B.
- DFP deferiprone, 1,2-dimethyl-3-hydroxypyridin-4-one.
- **DFT** density functional theory.
- **DM** diabetes mellitus.
- **DMARD** disease-modifying anti-rheumatic drugs.
- **DMSO** dimethyl sulfoxide.
- **DNA** deoxyribonucleic acid.
- **DSB** double/strand breaks.
- **DTA** differential thermal analysis.
- E. coli Escherichia coli.
- E. faecalis Enterococcus faecalis.
- **EA** elemental analysis.
- ECDC European Centre for Disease Prevention and Control.
- **EMA** European Medicines Agency.
- **ESCI** electrospray and chemical ionization.
- EU European Union.
- EUCAST European Committee for Antimicrobial Susceptibility Testing.

FDA U.S. Food and Drug Administration.

 ${\bf FT}\,$ Fourier transformation.

G guanine.

GI gastrointestinal.

H. pylori Helicobacter pylori.

H₃DMPS D,L-2,3,-dimercaptopropane-1-sulfonic acid.

 H_4DMSA meso-2,3,-dimercaptosuccinic acid.

 $\mathbf{H_4DOTA}$ 1,4,7,10-tetra
aza-cyclododecane-1,4,7,10-tetracetic acid.

 $\mathbf{H_4EDTA}$ ethylenediaminetetra
acetic acid.

 $\mathbf{H_5DTPA}$ diethylenetriaminepenta acetic acid.

Hcipro ciprofloxacin.

HD Huntington disease.

Hdox doxorubicin.

Henox enoxacin.

 ${\bf Hflex}\,$ fleroxacin.

HFR High Flux Reactor.

HIV human immunodeficiency virus.

Hlevox levofloxacin.

Hlomx lomefloxacin.

 $\mathbf{Hma}\ \mathrm{maltol}.$

HMBC heteronuclear multiple bond correlation.

Hnofx norfloxacin.

Hnxa nalidixic acid.

Hoxa oxolinic acid.

Hpia pipemidic acid.

HPLC high performance liquid chromatography.

HR-ESI high-resolution electrospray ionization.

HSA human serum albumin.

HSQC heteronuclear single quantum coherence spectroscopy.

HSV-1 herpes simplex virus type 1.

 $\mathbf{Hvox}\xspace$ vosaroxin.

 ${\bf ICL670}$ deferasirox.

IDSA Infectious Disease Society of America.

IL-2 interleukin-2.

 ${\bf IR}\,$ infrared.

IRTK insulin receptor tyrosine kinase.

K. pneumonia Klebsiella pneumonia.

KDM Katja D. Mjos.

m multiplet.

m/z mass-to-charge ratio.

MD Menkes disease.

 $\mathbf{md}\xspace$ moderate.

MDR multi-drug resistant.

MIC minimum inhibitory concentration.

 \mathbf{mp} melting point.

 $\mathbf{MPAC}\ \mathrm{metal-protein}\ \mathrm{attenuating}\ \mathrm{compound}.$

MRI magnetic resonance imaging.

MS mass spectrometry.

MTD maximum tolerated dose.

NCCLS U.S. National Committee for Clinical Laboratory Standards.

NCI U.S. National Cancer Institute.

NIH U.S. National Institutes of Health.

NMR nuclear magnetic resonance.

NRU National Research Universal.

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P. aeruginosa Pseudomona aeruginosa.

PD Parkinson disease.

PDT photodynamic therapy.

PET positron emission tomography.

pip 1,4-piperazinyl ring in C_7 position on aromatic ring system.

POM polyoxometalates.

prop propyl ring in N_1 position on aromatic ring system.

PTH parathyroid hormone.

 ${\bf q}\,$ quartet.

 ${\bf RA}\,$ rheumatoid arthritis.

RBC ranitidine bismuth citrate.

RNA ribonucleic acid.

ROS reactive oxygen species.

 ${\bf s}$ singulet.

S. aureus Staphylococcus aureus.

SALEN 2,2'-ethylenebis(nitrilomethylidene)diphenol.

 ${\bf sh}$ should er.

 ${\bf siRNA}\,$ small interfering ribonucleic acid.

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 ${\bf SNP}\,$ sodium nitroprusside, ${\rm Na}_2[{\rm Fe}({\rm CN})_5{\rm NO}]\cdot 2\,{\rm H}_2{\rm O}.$

SOD superoxide dismutase.

SPECT single photon emission computed tomography.

 ${\bf st}$ strong.

TETA 2,2,2-trientine.

 ${\bf Tf}$ transferrin.

 ${\bf TGA}\,$ thermogravimetric analysis.

 ${\bf TSS}\,$ toxic shock syndrome.

tt triplet of triplets.

UBC The University of British Columbia.

 ${\bf USA}\,$ United States of America.

 ${\bf UV-Vis}$ ultraviolet-visible.

 ${\bf w}\,$ weak.

WD Wilson disease.

WHO World Health Organization.

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Motivated through my great experience in South Africa, I started my personal endeavour of travelling and working as a chemist with stops along the way at EMD Merck (Germany), in the research group of Professor Dr. Rüdiger Beckhaus at the Carl von Ossietzky University in Oldenburg (Germany), and at DNV Energy (Norway) gaining experience in a variety of areas, thanks to many skilled colleagues who shared their knowledge with me. The idea of doing my PhD in Canada, however, had manifested itself in my mind. Thanks to Professor Dr. Chris Orvig, my dream came true in 2009, when he accepted me into his group. Boss, thank you very much for letting me run freely in all directions. Thanks to your support of my career aspirations, I was able to not only deepen my knowledge of chemistry but to learn more about intellectual property law and technology transfer in my PhD years, too. I am as well grateful that Dr. Michael J. Abrams came on board officially as my co-supervisor in 2012. Mike, whenever we met, I left our discussion motivated, inspired and ready to return to the academic lab with a clearly defined goal in mind. Both of you, thank you very much for being my *Doktorväter*.

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Dedication

Für vier starke Frauen — Four Women — Edith, Elfriede, Else, Gerda

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Chapter 1

Introduction

This thesis describes studies of the coordination chemistry of antimicrobial and anticancer agents. The aim of this research is to find novel metallodrugs to fight the growing resistance of bacteria to common antibiotics, as well as to understand the interaction of small-organic molecule drugs with metals present in the human body, which can reduce the potency of an administered drug or even lead to toxic side-effects. In this chapter, an impression of the current field of metallodrugs in the discipline of medicinal inorganic chemistry is given. The focus lies on therapeutic metallodrugs which are currently approved in the United States of America (USA) and/or countries of the European Union (EU), but some of the most widely used diagnostic metallodrugs are briefly introduced as well. In addition, promising novel metallodrugs which are currently in clinical trials are discussed, next to general strategies and challenges of metallodrug research and development. Numerous review articles and books have been published on medicinal inorganic chemistry,¹² the field of metallodrugs,³⁴⁵⁶⁷⁸⁹ and especially on anti-cancer treatments;¹⁰¹¹¹²¹³, therefore, the objective of this chapter is to be neither repetitive nor comprehensive, it is merely setting the stage for the following chapters of research on this topic.

1.1 Medicinal Inorganic Chemistry

Metal ions play important roles in biological processes,¹⁴ and the field of knowledge concerned with the application of inorganic chemistry to therapy or diagnosis of disease is medicinal inorganic chemistry.¹⁵ Among the natural sciences, medicinal inorganic chemistry is still considered a rather young discipline by many, but this is contrary to the historically proven use of metals in pharmaceutical potions, which traces back to the ancient civilizations of Mesopotamia, Egypt, India and China.¹⁶¹⁷¹⁸

The introduction of metal ions or metal ion binding components into a biological system for the treatment of diseases is one of the main sub-divisions in the field of bioinorganic chemistry.¹⁹ Such an intentional introduction of metal ions into the human biological system has proven to be useful for both diagnostic and therapeutic purposes. Figure 1.1 presents selected examples of some successful therapeutic and diagnostic metallodrugs. The latter have led to an increased understanding and early detection of diseases through the imaging of the living body. Nowadays, contrast agents containing radioactive metal isotopes are produced and administered daily in many medium sized hospitals around the world in single photon emission computed tomography (SPECT) scans of the human body. Magnetic resonance imaging (MRI) contrast also uses metal ions (Gd^{3+}). In Canada 1.7 million MRI scans, 63,000 positron emission tomography (PET) scans and over a million SPECT scans were performed in 2011-2012, and the numbers are growing internationally.²⁰ Thanks to these diagnostic methods malignant growth, cardiologic diseases and atherosclerosis in patients can be detected early; furthermore, such imaging agents enhance research as they, for example, enable researchers to visualize the activity of the brain in vivo.

One of the first therapeutic metallodrugs was salvarsan, an arsenic-based antimicrobial agent developed by Paul Ehrlich under the working name 606, a mixture of 3-amino4-hydroxyphenyl-arsenic(III) compounds. In 1912, Paul Ehrlich published his results of salvarsan as an effective treatment against syphillis.²¹ Salvarsan provided an effective demonstration for Ehrlich's belief that it is possible to fight infectious diseases through a systematic search for drugs that kill invading microorganisms without damaging the host, his idea of "Magic Bullets". Although model structures for salvarsan have been elucidated recently,²² the exact composition of salvarsan is still unknown; despite that fact, it has been used widely in humans. With the addition of mercury and bismuth, salvarsan remained the standard remedy for syphilis until it was replaced by penicillin after World War II.²³

Although Ehrlich's Salvarsan is widely regarded as the birth of modern chemotherapy and often cited as the beginning of modern research and development of metallodrugs, the star drug of the field until today is the anticancer agent cisplatin (Platinol), which was discovered serendipitously in 1965 while Barnett Rosenberg and Loretta Van Camp at Michigan State University were studying the effect of an electric current on *Escherichia coli*.²⁴ It was found that cell division was inhibited by the production of *cis*diamminedichloroplatinum(II) from the platinum electrodes.²⁴²⁵ Further studies on this platinum-agent indicated that it possessed antitumor activity, and this finding led to ongoing research and development of anticancer metallodrugs.²⁶

Despite the immense success of cisplatin and the fact that some inorganic formulated drugs such as dietary supplements and antacids have been readily available over the counter for centuries, the majority of all drugs on the market today is of organic or biological origin. It seems that from historical experience the know-how and expertise of the pharmaceutical industry rest almost entirely in these areas. Even today metal containing medicinal agents are often discovered in an academic research setting, before risk friendly start-up companies develop the actual metallodrug candidate further, moving it into first clinical trials.²⁷



Diagnostic



Figure 1.1: Selected examples of successful therapeutic and diagnostic metallodrugs.

1.2 Diagnostic Metallodrugs

Radiopharmaceuticals play an important role in medical diagnostics and therapy (see as well Section 1.3.1.2).²⁸ Diagnostic radiopharmaceuticals are a powerful tool in the diagnosis of cancer, cardiological disorders, infections, kidney or liver abnormalities, and neurological disorders.^{29 30} For imaging specific biological targets at low concentrations, they have unprecedented advantages over other less sensitive diagnostic methods. Over the past fifty years the imaging quality of medical scans has improved tremendously through novel di-
agnostic metallodrugs entering the market as well as through the development of imaging devices with higher sensitivity and enhanced resolution. Abnormal growth can now be easily detected, and in the diagnosis of cancer, for example, it is often possible to differentiate between carcinogenic tissue and healthy tissue based on the visual imaging impression before an actual tissue sample is taken.

To image a variety of medical conditions, a diversity of different imaging agents can be employed that specifically target a certain organ or body fluid. Table 1.1 presents an overview of the diagnostic radiopharmaceuticals currently approved by the U.S. Food and Drug Administration (FDA). The dominant isotope in diagnostic imaging is technetium-99m, which has been called the "Workhorse of Diagnostic Nuclear Medicine".³¹ A total of sixty-seven ^{99m}Tc imaging agents have been approved over the years by the FDA alone; currently, a total of twenty-eight ^{99m}Tc imaging agents are FDA-approved.³² Its dominance of the imaging market is reflected in the annual sales of the two leading ^{99m}Tc diagnostic imaging agents Cardiolite, and Myoview, both heart imaging agents, which amounted to 675 million USD in 2007.³³ In general, worldwide sales for a diagnostic drug vary between 100-400 million USD per year,³⁴ which makes this area one of the financially most rewarding in the field of metallodrugs.

Since the first technetium-99m radiotracers were developed at the University of Chicago in 1964, ^{99m}Tc has revealed itself to be the optimal metal isotope for imaging with commercial γ -cameras, because it conveniently emits a 140 keV γ -ray with 89% abundance and activities of > 1.11 GBq, and it can be injected with a low radiation exposure to the patient.³⁵ The nine different oxidation states of technetium, from -I (d⁸) to +VII (d⁰), together with its diversified stereochemistry spanning from coordination number 4 to 9, open up a variety of target-specific tuneable platforms for the development of radiopharmaceuticals.^{36 37} A key challenge of the technetium-99m isotope, however, is the ongoing shortage in its production. Long blackout periods in the two obsolete nuclear reactors which have been generating more than 70% of the global market of molybdenum-99, the parent nuclide of technetium-99m, have contributed to a medical isotope crisis. The National Research Universal (NRU) reactor, built in 1957 in Chalk River, Canada, has been providing 45% of the world's supply of ⁹⁹Mo; the High Flux Reactor (HFR), built in 1961 in Petten, The Netherlands, has been supplying 30%. The reduced availability of ^{99m}Tc has sparked the search for possible future alternatives in radiochemistry.

One alternative to technetium-99m are isotopes gallium-67 for SPECT and gallium-68 for PET imaging. The many advantages of ⁶⁸Ga radiopharmaceuticals³⁵ and especially the easy generation of ⁶⁸Ga through mobile ⁶⁸Ge/⁶⁸Ga-generator systems have been discussed³⁸ and questioned for many years.³⁹ Although no such generator is currently approved by the FDA or the European Medicines Agency (EMA), the *préparation magistrale* of imaging agents is possible in many European countries. German authorities have granted manufacturing authorization for pharmacological ⁶⁸Ge/⁶⁸Ga generators for use in clinical studies in 2012.⁴⁰ Already in 2011 the EMA had given orphan drug designation to gallium-68 pasireotide tetraxetan (SOMscan), which is developed as a PET imaging agent for gastro-entero-pancreatic neuroendrocrine tumors.⁴¹

Metal chelating agents such as diethylenetriaminepentaacetic acid (H_5DTPA) and 1,4,7,10-tetraaza-cyclododecane-1,4,7,10-tetracetic acid (H_4DOTA) complexed to the highly paramagnetic $4f^7$ Gd³⁺ ion are used as injectable macrocyclic contrast agents for MRI scans (Figure 1.1);⁴² the imaging agent [Gd(DTPA)]²⁻ (Magnevist, Magnegita) obtained FDA approval in 1988, while [Gd(DOTA)]⁻ (Dotarem, Gadovist) was approved in March 2013. The use of these macrocyclic chelators in SPECT or PET radiopharmaceuticals opens the gateway to theranostic agents. Theranostics implies the quantitative molecular imaging diagnosis of a disease with a diagnostic pharmaceutical followed by a personalized treatment with a therapeutic radiopharmaceutical analog.⁴³ Preliminary clinical results for an example of such a theranostic approach based on the ⁶⁸Ga radionuclide for diagnostic imaging followed by therapeutic treatment with ⁹⁰Y have been successful, and European wide trials will start soon.⁴¹

radioisotope	radiation	active ingredient	trade name	diagnostic imaging
67 Ga	γ	Ga-67 citrate		Hodgkin's disease, lymphoma, bronchogenic car-
				cinoma
82 Rb	β^+	Rb-82 chloride	Cardiogen-82	myocardium
99m Tc	γ	Tc-99m bicisate	Neurolite	stroke
99m Tc	γ	Tc-99m disofenin	Hepatolite	cholecystitis
99m Tc	γ	Tc-99m exametazime Ceretec		stroke, abdominal infection
99m Tc	γ	Tc-99m macroaggregated albumin		pulmonary perfusion, shunt patency
99m Tc	γ	Tc-99m mebrofenin	Choletec	hepatobiliary system
99m Tc	γ	Tc-99m medronate	MDP-Bracco	bone
99m Tc	γ	Tc-99m mertiatide	Technescan MAG3	kidney
99m Tc	γ	Tc-99m oxidronate	Technescan HDP	bone
99m Tc	γ	Tc-99m pentetate		brain, kidney
99m Tc	γ	Tc-99m pyrophosphate	Technescan PYP	bone, myocardium, blood pool
99m Tc	γ	Tc-99m red blood cells	UltraTag	blood pool
99m Tc	γ	Tc-99m sestamibi	Cardiolite	myocardium, breast
99m Tc	γ	Tc-99m sodium pertechnetate	Technelite	brain, thyroid, blood pool, urinary tract, naso- lacrimal drainage system
99m Tc	γ	Tc-99m succimer		kidney
$^{99m}\mathrm{Tc}$	γ	Tc-99m sulfur colloid		lymphatic system, liver
$^{99m}\mathrm{Tc}$	γ	Tc-99m tetrofosmin	Myoview	myocardium
$^{99m}\mathrm{Tc}$	γ	Tc-99m tilmanocept	Lymphoseek	lymphatic system
111 In	γ	In-111 capromab pendetide	ProstaScint	prostate cancer
111 In	γ	In-111 chloride	Indiclor	radiolabeling of ProstaScint
111 In	γ	In-111 oxyquinoline		leukocytes, inflammation
111 In	γ	In-111 pentetate		brain, spinal canal
111 In	γ	In-111 pentetreotide	Octreoscan	neuroendocrine tumors
201 Tl	γ	Tl-201 chloride		myocardium, thyroid

Table 1.1:	FDA	approved	diagnostic	metalloradio	pharmaceuticals ³²
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1.3 Therapeutic Metallodrugs

This section provides an overview of metallodrugs which have been approved for the medical treatment of human diseases or are currently in clinical trials.

1.3.1 Anticancer Metallodrugs

1.3.1.1 Anticancer Therapeutics

The World Health Organization (WHO) names cancer as a leading cause of death worldwide, accounting for 7.6 million deaths (around 13 % all deaths) in 2008 and projected to rise above 13.1 million deaths in 2030.⁴⁴

One of the oldest and best-known metallodrugs is the anticancer drug cisplatin, *cis*diammine-dichloroplatinum(II) (Platinol), a square planar Pt^{2+} complex (Figures 1.1 and 1.2).⁴⁵ First synthesized by Peyrone in 1844,⁴⁶ its anticancer properties were discovered by Rosenberg and co-workers in the 1960s,^{24 47} further explored,^{25 48 49} patented,⁵⁰ and approved by the FDA in December 1978; cisplatin was the first metal-based medicinal agent to enter into worldwide clinical use for the treatment of cancer. Used alone or in combination against different types of cancers, cisplatin is a blockbuster drug and one of the most successful therapeutic metallodrugs even today;²⁶ it was amongst the top revenue-generating licensed products,⁵¹ which provided Michigan State University with a large gross revenue from licensing royalties⁵² until its second patent⁵³ was invalidated in litigation on the ground of obviousness-type double patenting.⁵⁴

These days, cisplatin therapy can be considered part of a standard treatment against many forms of cancer. After the initial surgical removal of malignant tissue, the patient undergoes cycles of intravenous injections of cisplatin. During treatment the patient experiences major unpleasant side effects of the drug because cisplatin is highly cytotoxic. The Pt^{2+} of the $\{Pt(NH_3)_2\}^{2+}$ unit binds covalently to deoxyribonucleic acid (DNA), more specifically, to the N-7 of either guanine (G) or adenine (A) in the dinucleotide sequences GG and AG to form interstrand crosslinks and 1,2- or 1,3-intrastrand crosslinks.⁵⁵ Such cisplatin-DNA adducts, together with cellular pathways activated in response to cisplatin, lead to replication arrest, transcription inhibition, cell-cycle arrests, DNA repair and apoptosis.⁵⁶ For many years, cisplatin's mechanism of action has been described to involve activation by aquation inside cells due to varying Cl⁻ concentration.⁵⁷⁵⁸ Research on platinum drugs for anticancer therapy embraced this concept and tried to overcome its drawbacks, mainly lowering the level of cytoxicity, with second generation platinum drugs as oxaliplatin and carboplatin. All anticancer platinum-metallodrugs that have been approved by the FDA or are currently in clinical trials in the USA are presented in Figure 1.2. Carboplatin, cis-diammine-dicyclobutane-1,1-dicarboxylato-platinum(II) (Paraplatin), was reported by Cleare and Hoeschele in 1973, ⁵⁹⁶⁰ patented in 1979⁶¹ and approved by the FDA in 1989. The chelate effect of the six-membered ring reduces its chemical reactivity and possible side effects as well as damage to the ear (otoxicity) and the kidneys (nephrotoxicity). Oxaliplatin, (1R, 2R)-(N, N'-1, 2-diamminocyclohexane)-(O-O')-ethanedioato)platinum(II) (Eloxatin), ⁶²⁶³ received European approval in 1999 and approval by the FDA in 2002. Drugs of a similar design are nedaplatin, lobaplatin and heptaplatin, which are currently in clinical trials in the USA but are already in clinical use in Japan, China and South Korea, respectively. In addition, novel liposome nanoparticle formulations of cisplatin (Lipoplatin) and oxaliplatin (Lipoxal), which appear to reduce serious adverse reactions allowing a better exploitation of the anticancer activity of the platinum agent,⁶⁴⁶⁵ are currently undergoing clinical trials.⁶⁶⁶⁷

The attractive advantage of satraplatin, bis(acetato)amminedichloro(cyclohexylamine)platinum(IV) (JM216, Orplatna), is its oral availability; it can be administered in pill form which is convenient for the patient and reduces health care costs. JM216 contains the mononuclear platinum(IV) core, which in the blood stream is reduced by metal-containing redox proteins⁶⁸ to the active Pt(II) complex (JM118).⁶⁹ Presently, satraplatin is still in clinical trials against various common cancers.

BBR3464, triplatin tetra nitrate, is an unusual trinuclear platinum complex with an overall charge of +4.⁷⁰ In phase II clinical trials, lung cancer patients did not show a significant response to BBR3464 while experiencing toxicity associated side-effects such as neutropenia and diarrhea, therefore, further clinical development was stopped.⁷¹

Nevertheless, satraplatin as well as BBR3464 have proven that breaking with the limiting conditions initially set for platinum drugs for cancer therapy (platinum(II) and *cis*conformation) can open up ways to novel lead compounds. Developing novel nonclassical structures among current platinum complexes⁷² and fully understanding their mechanism of action might be the solution to the problem of acquired or intrinsic resistance facing all platinum formulations currently on the market.⁷³ Recent advances in cancer research have shown that even the most successful targeted therapies lose potency with time. Even if an initial response occurs, acquired resistance due to mutations and epigenetic events limits efficacy.⁷⁴ Combination therapy or "Cocktail Therapy", the co-administration of two or more drugs simultaneously, is another approach to promising results; in the majority of cases an additive therapeutic effect is achieved, because each agent acts via a different mechanism of action or targets different pathways.⁷⁵

In addition to the vast amount of research that is still undertaken on platinum-based anticancer drugs, coordination complexes of gallium and organometallic complexes of ruthenium have moved into the focus for anticancer therapy since the 1990s. KP46, tris(8hydroxyquinolinato)gallium(III),⁷⁶ contains the metal chelating agent 8-hydroxyquinoline, which itself has anticancer properties.⁷⁷ An oral formulation of KP46 (NKP2235) is scheduled to start phase I clinical trials in the USA soon.⁷⁸ An advantage of ruthenium-based anticancer agents is their effectiveness against metastasis and their potency against a wide range of tumors, which might be due to their two core properties: ruthenium agents are activated by reduction of the ruthenium(III) core and selectively transported via the transferrin pathway,⁷⁹ their exact mechanism of action, however, remains elusive despite numerous mechanistic hypotheses.^{80,81}

Already in the 1950s Dwyer started working on bacteriostatic and anticancer ruthenium coordination complexes.⁸² The anticancer agent NAMI-A, imidazolium *trans*-tetrachloro-(dimethylsulfoxide)imidazole-ruthenate(III), developed by Alessio, Mestroni, Sava and co-workers was the first ruthenium compound to enter into clinical trials followed by the coordination compound of the Keppler group, KP1019, *trans*-tetrachlorobis(1H-indazole)ruthenate(III) or its 35-fold better soluble sodium salt (N)KP1339 which is used in clinical trials for the preparation of KP1019.⁸³⁸⁴ Figure 1.3 shows the anticancer metallodrugs which are currently in clinical trials.

With ruthenium and gallium compounds still in clinical trials, arsenic is the only other non-radioactive metal ion approved for the treatment of cancer. In traditional Chinese medicine, solutions containing crude arsenic oxide have been administered for thousands of years to treat different illnesses. Since the 20th century, injectable solutions of arsenic trioxide (ATO), commercially sold as Trisenox, are used in the treatment of acute promyelocytic leukaemia (APL).⁸⁵ Until now ATO is the treatment of choice for APL patients who relapse after the first line treatment of all-*trans* retinoid acid (ATRA) combined with chemotherapy, but recent clinical studies have shown that the novel, chemotherapy-free combination therapy of ATRA and ATO is not inferior to the standard ATRA-chemotherapy treatment in non-high-risk APL patients.⁸⁶ Darinaparsin, S-dimethylarsino-glutathione (DAR, ZIO101), is a novel arsenic-based anticancer agent currently in clinical trials.⁸⁷



Figure 1.2: Anticancer platinum metallodrugs approved and in clinical trials in the USA.



Figure 1.3: Anticancer metallodrugs in clinical trials.

radioisotope	radiation	active ingredient	trade name	indications
89 Sr	β	Sr-89 chloride	Metastron	skeletal metastases
90 Y	eta	Y-90 ibritumomab tiux- etan	Zevalin	non-Hodgkin's lymphoma
$^{153}\mathrm{Sm}$	β	Sm-153 lexidronam pen- tasodium	Quadramet	osteoblastic skeletal metastases
²²³ Ra	lpha	Ra-223 dichloride	Xofigo	castration-resistant prostate can- cer, symptomatic bone metas- tases

Table 1.2: Approved therapeutic metalloradiopharmaceuticals

1.3.1.2 Therapeutic Radiopharmaceuticals

Approved metal-based therapeutic radiopharmaceuticals are often employed as a measure of last resort in advanced stages of prostate cancer, breast cancer, lung cancer, bladder cancer and thyroid cancer where the cancer has spread to the bone tissue, as they are able to deliver cytotoxic doses of ionizing radiation directly to the local targeted tissue.⁸⁸ Metastatic bone cancer is extremely painful and restricts the mobility of patients. Table 1.2 provides an overview of injectable salt solutions of radium-223 dichloride, pentasodium samarium-153 N.N.N'.N'-tetrakis(phosphonato-methyl)ethane-1,2-diamine and strontium-89 chloride approved for the palliative pain treatment of metastatic bone cancer; yttrium-90 is a conjugated antibody used in the treatment of non-Hodgkin's lymphoma. Furthermore, several formulations of holmium-166, rhenium-186, rhenium-188, bismuth-213, actinium-225 and lutetium-288 are currently in clinical trials against a variety of cancers. β -emitting radionuclides ¹⁵³Sm, ⁸⁹Sr, ⁹⁰Y, ^{186/188}Re and ²¹³Bi have traditionally been used in clinical radionuclear therapy, because β -particles range far in biological tissue (50–1000 cell diameters) which makes them suitable for treating larger or poorly vascularized tumors.⁸⁹ In contrast, ²²³Ra and ²²⁵Ac are α -emitting radionuclides with a much shorter effective range $(<10 \text{ cell diameters}).^{90}$

1.3.1.3 Photochemotherapeutic Metallodrugs

Photochemotherapy is as well referred to as photoradiation therapy, phototherapy, or photodynamic therapy (PDT). Currently, PDT is used clinically for the treatment of obstructing esophageal cancer, and obstructing or microinvasive endobronchial non-small-cell lung cancer; additionally, several indications such as prostate cancer and non-resectable or inoperable cholangiocarcinoma are under investigation. As compared to traditional invasive cancer treatments, such as surgery and radiotherapy, PDT is not associated with radical side-effects, such as surgical removal of parts of the lung or complete excision of the bladder, and can be seen as a quite effective treatment option for localized cancers.⁹¹⁹²

PDT is a two-step treatment. First, a photosenitizer agent is administered either topically or intravenously. Secondly, after a couple of hours or days, depending on the drug-to-light interval of the drug, light of a specific wavelength is shone on the area to be treated. Because the light has to reach the deeper tissue layers, red light is usually chosen over short wavelength light in PDT.⁹¹ The light photoactivates the photosensitizing agent, and via its excited triplet state the agent generates highly reactive singlet oxygen $({}^{1}O_{2})$ from ground state oxygen $({}^{3}O_{2})$ within the tumor blood vessels. ${}^{1}O_{2}$ reacts further and a variety of reactive oxygen species (ROS) are produced which subsequently react with cellular components, leading to vasoconstriction, platelet aggregation, clotting and, ultimately, tumor vascular occlusion.⁹³

Porfimer sodium (Photofrin) has been approved as a photosensitizing agent by the FDA in 1995, and the palladium-based padeliporfin (WST11, Tookad Soluble) is currently in phase III clinical trials (Figure 1.4).⁹⁴



Figure 1.4: Metallodrugs for photodynamic therapy (PDT).

1.3.2 Antimicrobial and Antiparasitic Metallodrugs

Some of the first metallodrugs used in therapy were antimicrobial and antiparasitic agents based on arsenic.^{95 96} In 1907 Breinl and Thomas studied the use of atoxyl, arsanilic acid, for the treatment of trypanosomiasis (sleeping sickness).⁹⁷ Inspired by their findings, Ehrlich and co-workers began their work on arsenic antimicrobials,⁹⁸ which led to the discovery of salvarsan and marks the beginning of chemotherapy as outlined in 1.1. Although arsenicals, arsenic-based pharmaceuticals, were widely used in medicine in the beginning of the 20th century, most of them have been superseded by less toxic drugs. One arsenic drug that is still used against trypanosomiasis today, despite its severe side effect of encephalopathy, is melarsoprol, 2-(4-amino)-(4,6-diamino-1,3,5-triazin-2-yl)-phenyl-1,2,3dithiarsolan-4-methanol (Mel B, Arsobal), discovered in 1949. The WHO lists melarsoprol as a second stage treatment for both forms of human African sleeping sickness.⁹⁹

The other two heavier pnictogens, antimony and bismuth, have been in medical use against microbes and parasites as well. Antimony-based drugs have been prescribed against cutaneous and mucocutaneous leishmaniasis since the parasitic transmission of the tropical disease was understood in the beginning of the 20th century. The Brazilian physician Gaspar Vianna was the first to treat mucocutaneous leishmaniasis with antimony(III) tartar metic, potassium antimony tartrate.¹⁰⁰ Shortly afterwards, the activity of arsenic against visceral leishmanisis was confirmed in Italy and India, which led to the synthesis of an array of arsenic containing parasitic agents, among them the less toxic pentavalent antimonials: Stibosan, Neostibosan and Ureastibamine.¹⁰⁰ Other antimony(IV) drugs followed: sodium stibogluconate (Pentostam) and melglumine antimoniate (Glucantim, or Glucantime); both continue to be in use today despite their toxic side effects and increasing loss in potency due to the growing resistance of the parasite against antimony.^{101 102}

While one has to weigh the toxicity against the therapeutic benefit for arsenic and antimony, bismuth is nontoxic and well tolerated at high doses.¹⁰³ Since the 18th century bismuth has been used internally as its subnitrate or subcitrate. The history of bismuth drugs is closely connected to gastrointestinal disorders (Section 1.3.8), but bismuth is also co-administered in the fight against the bacterium Helicobacter pulori (H. pulori). A H. *pylori* infection can lead to gastritis (type-B, bacterial), ulcers in the gastrointestinal tract, and gastric cancer. Bismuth preparations as colloidal bismuth sub-citrate (CBS), sold as De-Nol, or ranitidine bismuth citrate (RBC), sold as Pylorid or Tritec, are used to treat peptic ulcers that are often associated with *H. pylori*. Clarithromycin has been the antibiotic of choice to kill the bacterium, but its strength is diminishing with an increasing resistance of the bacterium. This acquired resistance can be partly overcome through the co-administration of clarithromycin together with CBS or RBC alone, or in combination with a second antibiotic (amoxicillin) and a proton pump inhibitor (omeprazole). In the so-called bismuth-based triple therapy bismuth subcitrate potassium (Pylera) or bismuth subsalicylate (Helidac) are included in the cocktail together with metronidazole and tetracycline hydrochloride.¹⁰⁴ In cases where the two described first-line treatments have failed, the quadruple therapy can be highly effective against H. pylori: bismuth subcitrate potassium is administered together with metronidazole, tetracycline and omeprazole in one single capsule.^{105 106}

The external use of tribromophenatebismuth(III), xeroform, because of its antimicrobial properties was first described at the end of the 19th century. In the past, xeroform was often used as a substitute for iodoform in the treatment of wounds. Nowadays, occlusive petrolatum gauze readily impregnated with 3% bismuth tribromophenate is sold under the name Xeroform. Bismuth-thiol compounds have been widely studied for their antimicrobial properties and are currently marketed as a treatment of chronic wounds, such as diabetic foot ulcers; Microbion, is supposed to prevent the formation of biofilm growth in wounds.¹⁰⁷

Another metal that has been widely used in the treatment of wounds and their infection managements is silver.¹⁰⁸ Topical sulphonamide ointments such as silver sulphadiazine (Silvadene, Silverex, Silvazine, SSD, Thermazene) are applied as a cream formulation or aqueous solution (1% silver salt) to prevent and treat infections of second or third degree burns, although it appears that the use of silver preparations in burn treatment is traditionally rooted, and its effectiveness has been questioned¹⁰⁹ and criticized¹¹⁰ lately. Since 1976, cerium nitrate-silver sulphadiazine (Flammacerium) has been employed as a topical treatment for most cutaneous burns not undergoing immediate excision;¹¹¹ it is believed to reduce the inflammatory response to burn injury, decrease bacterial colonization and provide a firm eschar for easier wound management.¹¹² Figure 1.5 presents approved antimicrobial and antiparasitic drugs as well as drug candidates currently in clinical trials.

Many different applications of silver drugs are currently in clinical trials. Tested treatments range from the use of silver fluoride to treat hypersensitivity in teeth to the use of silver nitrate in the healing of cysts and abcesses. Silver ions are incorporated into surgical



Figure 1.5: Antimicrobial and antiparasitic drugs approved and in clinical trials.

wound dressing cloths (e.g., Acticoat) and catheters (e.g., SilverSoaker) for infection prevention or into textiles for the treatment of acute neurodermitis. Silver alginate (Algidex) is even studied for the prevention of central line infections in very low birth weight infants, while at the same time the toxicity of such silver biomaterials for clinical applications is still under evaluation.¹¹³

Two other promising metallodrug candidates that are currently undergoing phase II

clinical trials are the antimalaria agent ferrochloroquine (ferroquine, SSR97193, Figure 1.5) and the antifungal agent VT1161. Through the combination of ferrocene with the known antimalaria drug chloroquine, the resistance against chloroquine, which the malaria pathogen *Plasmodium falciparum* has developed, can be overcome.¹¹⁴¹¹⁵ VT1161 is currently in phase II clinical trials for the oral treatment of onychomycosis and candidiasis, this chelating agent of unspecified structure selectively inhibits the microbial metalloenzyme lanosterol demethylase (CYP51) involved in the synthesis of fungal cell wall sterols.¹¹⁶

1.3.3 Antiarthritic Metallodrugs

Up to 2% of the global population is affected by the chronic, systemic, inflammatory autoimmune disorder rheumatoid arthritis (RA). Although the aetiology of arthritis is not completely elucidated, it is a complex interplay of environmental and genetic factors that eventually leads to the inflammation of joints, which marks the beginning of the disease. Over time, the inflammatory condition leads to the progressive destruction of the joints, which restricts the movement of patients and leaves them in pain.¹¹⁷

In the 1930s, Forestier realized the potential of gold compounds in the treatment of RA.¹¹⁸ This is another example of a lucky drug discovery: during the years of 1925–1935, which have been described as the "Gold Decade",¹¹⁹ gold compounds, mainly gold(I) cyanide and thiosulfates, were used for the treatment of pulmonary tuberculosis, a medical approach that was more based on hope than on evidence.¹²⁰ Back then, arthritis was also believed to be a bacterial infection. Many of the gold thiosulfates still in clinical use today were introduced into therapy during the early 20th century: sodium aurothiomalate (Myochrysine, Myocrisin, Tauredon), aurothioglucose (Aureotan, Solganal, Solganol, Auromyose), sodium aurothiopropanol sulfonate (Allochrysine) and sodium aurothiosulfate (Sanochrysin). All of the named gold(I) compounds are charged, polymeric, and administered as water-soluble injectables directly into muscle tissue, while auranofin, tetraacetyl-

beta-D-thioglucose-gold(I)-thioethylphosphine (Crisinor, Crisofin, Ridaura), a much newer gold(I) compound that received FDA approval in 1985, is a monomeric, neutral coordination compound that is lipophilic and administered orally in capsule form. These gold drugs, classified as disease-modifying anti-rheumatic drugs (DMARD), slow the progression of RA and act by inhibiting several cathepsins implicated in RA, depending on the ligand system.¹²¹ Approved gold(I) antiarthritis metallodrugs are shown in Figure 1.6.

Despite the good therapeutic response gold drugs have shown in the clinic, chrysotherapy, the use of gold compounds in medical therapy, has been controversial over the many years gold drugs have been in use. In 1960, the Empire Rheumatism Council conducted a study on the efficacy of chrysotherapy and came to the conclusion that gold drugs do have a medicative effect,¹²² but the toxicity of gold(I), its slow clearance from the body, the not clearly defined structure of the intramuscular gold solutions, and the still not fully elucidated mode of action of gold(I) compounds against arthritis are often cited as counterarguments.¹²³ The market of DMARD has seen many new additions during the past ten years, these are mainly drugs based on active small organic molecules, or biologicals such as monoclonal antibodies or proteins. This development has seen traditional gold drugs being pushed down in priority and being prescribed for patients when other drugs have failed to provide sufficient relief. In these cases, practising physicians prefer intramuscular gold preparations over the orally available auranofin for the treatment of RA,¹²⁴ solely or in combination with other DMARD,¹²⁵ because gold is readily absorbed intramuscularly.¹²⁶

It remains to be seen if nanotechnology can revive the area of gold-pharmacology,¹²⁷ and if gold beads in knee osteoarthritis¹²⁸ or chemo-photothermal treatments¹²⁹ will rejuvenate gold treatment of arthritis.

During the 1950s, the intra-articular injection of aqueous solutions of osmium tetroxide, osmic acid, as a chemical synovectomy procedure for the treatment of RA in the knees,



Figure 1.6: Approved gold(I) antiarthritis metallodrugs.

moved into focus in Scandinavian countries.^{130 131} This beneficial procedure has been in clinical practice ever since,^{132 133} and lately it was shown that osmium tetroxide, as a fast mimic of superoxide dismutase, very efficiently catalyzes the dismutation of superoxide anion radical, one of the primary inflammatory species.¹³⁴

1.3.4 Antidiabetes Metallodrugs

An estimated 347 million people worldwide have diabetes mellitus (DM), and the numbers are increasing globally with more than 80% of diabetes deaths occurring in low- and middle-income countries.¹³⁵ Vanadium salts and coordination compounds have demonstrated various insulin-enhancing and antidiabetic effects; although they are not able to fundamentally substitute for the lack of insulin necessary in type I diabetes, they have shown to manage blood sugar levels in type II diabetes patients in a convenient oral formulation.¹³⁶

In 1899, Lyonnet recorded that the administration of sodium vanadate to his

diabetes patients had a positive effect on their health.¹³⁷ In 1977, Josephson realized that vanadate has an inhibitory effect towards phosphatases.¹³⁸ et al. In 1985, McNeill and co-workers reported that adding sodium orthovanadate to drinking water of experimentally diabetic rats could reverse most of the diabetic symptoms.¹³⁹ These findings triggered extensive research on the biological functions of vanadium itself,¹⁴⁰ as well as on vanadium(IV, V) coordination complexes with a variety of organic ligands such as naglivan,¹⁴¹ maltol, kojic acid, picolinic acid, acetylacetonate, dicarboxylate esters or 2,2'-ethylenebis(nitrilomethylidene)diphenol (SALEN).¹⁴² Bis(maltolato)oxovanadium(IV) (BMOV)¹⁴³ and its ethylmaltol analog bis(ethylmaltolato)oxovanadium(IV) (BEOV)¹⁴⁴, depicted in Figure 1.7, arose as lead compounds, showing an increased bioavailability over vanadyl sulfate in vivo. Both were carefully studied in animals and BEOV (AKP020) completed clinical trials phases I and II.^{145 146} Their insulin-enhancing effect is thought to originate from the activation of the insulin receptor through the inhibition of insulin receptor tyrosine kinase (IRTK) associated phosphatases. Unfortunately, although nowadays many vanadium preparations are available over the counter, for example, vanadyl sulfate is advertised as sports supplement (Vana Trace) and even available on Amazon.com, the story of an BMOV antidiabetes vanadium drug ends here due to patent expiry and side effects affecting the kidney of the patients:¹⁴⁷ however, the story of BMOV continues. Under the management of CFM Pharma, BMOV (compound CFM10, Vanadis) is currently being developed into a therapeutic for the prevention, stoppage and reparation of secondary tissue injury caused by fire, accidents (road traffic, brain trauma) or a heart attack.¹⁴⁸

A challenge with the first generation vanadium complexes BMOV and BEOV has been the high dose necessary to achieve a therapeutic effect. Further generation ligand systems such as that in bis((5-hydroxy-4-oxo-4H-pyran-2-yl)-methyl benzoatato) oxovanadium(IV)



Figure 1.7: Promising metallodrug candidates for the treatment of diabetes.

(BBOV) show half the acute oral toxicity compared to BMOV,¹⁴⁹ and through novel formulations the dose can be apparently lowered by a factor of 1000.¹⁵⁰ Vanadium formulated with Aonys, for the treatment of metabolic discorders has successfully completed phase I clinical trials in the European Union. The reverse-micelle emulsion containing vanadium is applied to the mucous membranes lining the inside of the mouth (buccal mucosa) with a spray pump, which reduces active doses from the mg/kg to the μ g/kg level and avoids side effects associated with high doses of vanadium in the earlier oral formulations.¹⁵⁰ In animal models, sodium tungstate (Na₂WO₄) reduced glycemia¹⁵¹ and adiposity¹⁵² without any significant side effects associated with long-term applications;¹⁵³ however, sodium tungstate did not show any efficacy as a pharmacological agent in the treatment of human obesity.¹⁵⁴

1.3.5 Antiviral Metallodrugs

There are currently no metallodrugs approved for the treatment of virus diseases, although two compounds have successfully proven to be effective against viri in the clinic. Bis(2-methylimidazole)-[(bis(acetylacetone)(ethylenediimine)]cobalt(III), CTC-96 (Doxovir) shown in Figure 1.8, has successfully completed phase II clinical trials for the treatment of *Herpes simplex labialis* and phase I clinical trials for the treatment of two viral eye infections (ophthalmic herpetic keratitis, adenoviral conjunctivitis).¹⁵⁵ In *in vitro* studies CTC-96 has shown to be active against herpes simplex virus type 1 (HSV-1) by preventing the entry of virus into cells through inhibition of membrane fusion events;¹⁵⁶ this resonates with findings that (acacen)cobalt(II) complexes bind covalently to histidine residues of zinc finger domains, and therewith prevent binding of the protein to its recognition oligonucleotide.¹⁵⁷

In 1985, Rozenbaum *et al.* were the first to administer polynuclear, transition-metal oxyanions, so-called polyoxometalates (POM), to patients with an acquired immunodeficiency syndrome acquired through infection with HIV (AIDS), and their study showed that the therapy with compound HPA-23 (ammonium-21-tungsto-9-antimonate) decreased levels of HIV in the patients.¹⁵⁸ POM are globular or spherical polyanionic structures containing bridging oxygen atoms, where the individual anionic charge is carried by the oxygen atoms on the periphery. A variety of POM structures exists (Lindquist, Keggin, Dawson, Anderson, Waugh and Silverton) incorporating a variety of transition-metals (vanadium, tungsten, molybdenum, niobium), all of which inhibit different families of enzymes.¹⁵⁹ This has shown to decrease activities of HIV, severe acute respiratory syndrome coronavirus, influenza virus, herpes simplex virus and hepatitis B virus *in vivo*.^{160 161} Despite their activity against ribonucleic acid viri and their favourable selectivity profile *in vitro*, so far no POM have been advantageous enough to surpass small organic molecule drugs currently in clinical use (such as aztreonam or ribavirin)¹⁶², and toxicity, especially deposition in the liver during long-term treatments, has been a concern.¹⁶³

It should also be noted that aluminium and mercury have been used as adjuvants in vaccines since the beginning of the 20th century. Aluminium hydroxide, aluminium phosphate and potassium alum (KAl(SO_4)₂ · 12 H₂O) help to stimulate the immune response via poorly understood mechanisms while displaying an excellent safety profile. ¹⁶⁴ Sodium-2-ethylmercurithio-benzoate (Thiomersal, Thimerosal) is mainly added as a preservative (see Figure 1.8). The ethylmercurithio cation of thiomersal, binds readily to thiol-groups



Figure 1.8: A promising metallodrug candidate (CTC-96) and a long-time vaccine adjuvant (sodium thiomersal) for antiviral therapy.

in protein structures blocking their enzymatic activity. The many applications of mercury and its high neurotoxicity have been controversial for years, culminating in January 2013, when governments participating in the WHO Intergovernmental Negotiating Committee agreed to the text of the "Minamata Convention on Mercury", a global legally binding instrument on mercury use, opened for signature October 2013.¹⁶⁵ Yet, like "large measuring devices where currently there are no mercury-free alternatives," ¹⁶⁵ vaccines and dental fillings will be excluded from the treaty, and the debate on the use of mercury in medical applications continues.

1.3.6 Metallodrugs Addressing Deficiencies

Insufficient concentrations of essential metals lead to deficiency syndromes. Mild forms of nutrient deficiency caused mostly by micronutrient malnutrition can be treated temporarily or over longer periods of time with dietary supplements comprising one single metal ion or a mix of several essential metal ions, until levels considered as normal by the medical community are reached. Worldwide, iron deficiency is the most prevalent nutritional deficiency affecting more than 2 billion people and is a priority area within the global micronutritient initiative program.¹⁶⁶ It should also be noted that in industrialized countries, many dietary supplements are taken as self-medication and not under medical surveillance. A large collection of dietary supplements is available in a variety of convenient oral preparations (capsule, drink powder, chewy tablet) with some appearing almost too convenient, as especially children can be in danger of acute metal intoxication from such preparations.¹⁶⁷ The demand for dietary supplements for medical and increasingly personal reasons is high and the market lucrative: the vitamin and supplement manufacturing industry is expected to grow its revenue with a rate of 2.4% annually to a total of 15.8 billion U.S. dollars in 2018.¹⁶⁸

Certain metal deficiencies result from genetic metabolic disorders (acrodermatitis enteropathica, Menkes disease (MD)) or arise as complications in cases of gastric atrophy or chronic kidney disease. Acrodermatitis enteropathica is an autosomal-recessive metabolic disorder affecting the uptake of zinc; there is no cure, and patients depend lifelong on zinc supplements to survive.

MD is caused by a mutation on the gene encoding Cu^{2+} -transporting ATPase that leads to a dysfunction of several copper-dependent enzymes and overall copper deficiency. Treatment must start in the first 2–3 months of life to avoid brain damage. Copper histidine is currently in phase II clinical trials for therapy in Menkes Disease; the copper replacement is injected directly into the body to bypass the normal route of absorption through the gastrointestinal tract, though severe cases of MD do not gain a therapeutic effect from copper-replacement therapy.¹⁶⁹

Severe iron-deficiency (anemia) or vitamin B_{12} -deficiency (Biermer-Addison's anemia, older name: pernicious anemia) can arise from chronic kidney disease or gastric bypass surgery, respectively. Treatment options for both diseases are based on replacing the missing metal ion (Fe²⁺) and coordination complex (vitamin B_{12}) through intravenous injections. Iron dextran (Proferdex, Dexferrum, InFed) or iron sucrose (Venofer) are administered intravenously to treat severe iron-deficiency, while the cobalt(III)-containing cyanocobalamin (CN-Cbl) and hydroxycobalamin (OH-Cbl) are available in form of a nasal spray (Nascobal) or parenteral injection (Vibisone) for the therapy of vitamin B_{12} -deficiency.

A common problem in hospitalized cancer patients is hypercalcemia, the imbalance between the net resorption of bone and urinary excretion of calcium. Through infusions of gallium(III) nitrate (Ganite) the calcium resorption from bone is reduced, as gallium(III) exerts a hypocalcemic effect.¹⁷⁰

Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to enhanced bone fragility and consequently a higher risk in bone fractures.¹⁷¹ In the majority of cases, such osteoporotic fractures affect the hips and knees of postmenopausal women, but men and children can as well be struck by osteoporosis. In the treatment of this chronic disease, a variety of nutrients are monitored and adjusted as necessary: calcium, magnesium, phosphorous, fluorine, vitamin D, and proteins. Besides the common hormone therapy, calcium supplements (e.g., Calcitrate) and strontium ranelate (Osseor, Protelos) are metal-based drugs employed in the management of osteoporosis. Strontium ranelate is approved in some European countries and Australia for the treatment and prevention of osteoporosis in postmenopausal women, but its use is becoming increasingly restricted after complications in patients with acute venous thromboemboli,¹⁷² hypertonus or other cardiovascular diseases.^{173 174} Because calcium preparations can cause hypercalcemia in patients (possible complications resulting from hypercalcemia are discussed in the context of hyperphosphatemia in kidney disease in Section 1.3.10.1), new treatment options are needed.

1.3.7 Metallodrugs for the Treatment of Cardiovascular Disorders

Metallodrugs for the treatment of cardiovascular diseases focus on the regulation of nitric oxide (NO) and dioxygen (O₂) in the blood vessels. Vasolidation, the widening of blood vessels, increases the blood flow in the body. Nitric oxide can be used therapeutically to adjust vasolidation. Sodium nitroprusside, Na₂[Fe(CN)₅NO] \cdot 2 H₂O (SNP), sold as Nitropress, rapidly decreases arterial pressure and total peripheral resistance.¹⁷⁵ One downside of SNP is the fact that, in parallel with NO, toxic cyanide (CN⁻) is released into the blood system as well. New NO coordination complexes of ruthenium¹⁷⁶ and photoactive iron complexes have also been explored for the treatment of parasitic diseases.¹⁷⁸ In some medical conditions, such as toxic shock syndrome (TSS), the blood pressure is extremely low and needs to be raised quickly to stabilize the patient. Here, metal complexes that absorb excess NO in a swift manner might be useful.¹⁷⁹

Dioxygen is essential for our survival, but failures in processing of O_2 can lead to the formation of superoxide anion $(O_2^{-\cdot})$ or hyperoxyl (HO₂) in acidic regions. Both $O_2^{-\cdot}$ and HO₂, are highly damaging ROS that not only protect the cells from invading organisms but also initiate auto-oxidation reactions *in vivo* that damage membrane lipids, tissue and DNA. To avoid any of the detrimental chain reactions, the superoxide dismutase (SOD) carefully control and limit $O_2^{-\cdot}$ levels in the cells by catalytically disproportionating it into molecular oxygen and hydrogen peroxide, the latter being further disproportionated to water and molecular oxygen by glutathione peroxidase or catalase.¹⁸⁰ Three types of these first-line-of-defense-metalloenzymes have been characterized: two isoforms of CuSOD/Zn-SOD are located either intracellularly in cytoplasm and nucleus (SOD1) or extracellularly (ECSOD, SOD3), while MnSOD (SOD2) acts in the mitochondria and appears to be the SOD most critical for mammals.¹⁸¹ However, in cases of disease or trauma, the production of harmful superoxide species might increase above the capacity of allocatable SODs to enforce dismutation.

In such cases of extreme oxidative stress, SOD-mimicking metallodrugs may assist the autoimmune defense of the body in disarming superoxide species. Macrocycles of porphyrins, phthalocyanines, porphyryzines as well as cyclic polyamines and SALEN coordinated to iron(II), copper(II), and manganese(II) have been widely studied as SODmimics.¹⁸² Compared to copper(II) and iron(II), manganese(II) macrocycles seem less fragile because toxic side effects, such as radical formation or Fenton chemistry starting from "free" iron or copper ions, have not been observed for manganese, and the overall toxicity of manganese(II) macrocycles is lower as compared to free aquatic forms of manganese.¹⁸² Compound M40403, a SOD mimicking manganese(II) (pentaaza)macrocycle shown in Figure 1.9, possesses advanced selectivity, as it can quench superoxide anions while not impacting NO, H_2O_2 or hypochlorite;¹⁸³ furthermore, it displayed the highest SOD-activity in a comparison study with other manganese(II) macrocycles.¹⁸⁴ Phase I/II clinical trials for the prevention or reduction of hypotension in patients receiving interleukin-2 (IL-2) therapy with M40403 have been suspended for now, but the possible application of manganese(II) macrocycles in pain management *in vivo* has gained some attention already.¹⁸⁵

1.3.8 Metallodrugs for the Treatment of Gastrointestinal Disorders

Minor stomach pain and digestion problems have been treated with metallodrugs for centuries. Oral antacid preparations of sodium(I), magnesium(II), calcium(II), and aluminum(III) as their basic carbonate, hydrogen carbonate or hydroxide salts increase the pH in the stomach and reduce the secretion of acid by gastric cells leading to a neutralization of excessive acidity in the stomach and a relief from heartburn symptoms. Brand products, for example, Alka-Seltzer (chew tablet containing NaHCO₃ and KHCO₃), Maalox (solid or liquid formulation of Al(OH)₃ and Mg(OH)₂), or Rennie (chew tablet containing



Figure 1.9: SOD-mimicking macrocycle (M40403), a promising metallodrug candidate for the treatment of cardiovascular disorders (left); bismuth sub-salicylate (BSS), a widely used metallodrug for the treatment of gastrointestinal disorders (right).

 $CaCO_3$ and $MgCO_3$), as well as a variety of generic antacid products are available over the counter worldwide and are safe to use even for pregnant women. Magnesium hydroxide, in vernacular language known as "Milk of Magnesia", is both an antacid as well as a laxative; epsom salt (Mg_2SO_4) helps in cases of constipation, too. Known in many countries around the world as the "Pink Stuff", bismuth subsalicylate (BSS) or Pepto-Bismol, shown in Figure 1.9, was developed in 1901 and is still used to self-medicate an upset stomach and symptoms of diarrhea, heartburn indigestion and nausea. Despite the fact that BSS is sold across the globe and has been used safely by many people for over 100 years, its chemical structure and mechanism of action are still not fully understood.

1.3.9 Metallodrugs as Psychotropics

Bipolar disorder (BP) is a psychiatric disorder that demonstrates itself as times of mania alternating with episodes of depression. Patients showing such strong mood disorders have been treated and maintained with lithium carbonate since lithium became recognized as a modern psychopharmacological agent in the 1950s.¹⁸⁶ While Eskalith, Lithane are listed by the FDA as discontinued, Lithobid, an oral lithium carbonate formulation first approved in 1979 is still on the market in the USA. Lithium cations have proven to reduce suicide risk and mood swings in bipolar disorder patients; however, the high dose causes a variety of unpleasant side effects which leave lithium drugs with a narrow therapeutic window between beneficial therapeutic and detrimental toxic effects.¹⁸⁷ Moreover, treatment responses to lithium drugs vary, and a genetic component to this has been discussed.¹⁸⁸ Offspring of bipolar parents often inherit their manifested classical mood disorders,¹⁸⁹ and response to lithium appears as well to be a family trait.¹⁹⁰ Although the mechanism of action of lithium ions has not been completely illuminated, it is understood that they act on multiple levels regulating neurotransmission and actively modulating cellular and intracellular changes in the second messenger systems.¹⁹¹ Furthermore, lithium ions exert a neuroprotective effect on amygdala, hippocampus and prefrontal cortical regions.¹⁹² This neuroprotective role of lithium bears a tremendous benefit for the treatment of neurodegenerative diseases; such a treatment however would require the life-long intake of sufficient amounts of lithium, and this could not be reached with the current lithium carbonate preparations without serious toxic interferences. A novel Aonys, formulation of lithium citrate tetrahydrate claims to achieve similar therapeutic effects as traditional oral lithium carbonate preparations containing a 150–400 times lower dose of lithium cation and is currently undergoing clinical trials for the treatment of Huntington disease (HD).¹⁵⁰ HD is an inherited neurodegenerative disorder affecting muscle coordination and cognitive abilities which leads to long-time physical deterioration accompanied by emotional turmoil and eventually death. Today's approved treatment options for HD can only relieve the symptoms of the disease such as involuntary movements, anxiety or depression, but NP03, an Aonys, water-in-oil micro emulsion drug delivery vector in which a low-dose of lithium has been incorporated, proved to be successful in a HD mouse model,¹⁹³ and phase I clinical trials have been completed.¹⁵⁰

1.3.10 Chelating Proligand Drugs

1.3.10.1 In the Treatment of Overload Disorders

In all living organisms, metal ion homeostasis consists of a variety of highly complex transactions, and some metals are essential for surviving.¹⁹⁴ In cases of acute intoxication or chronic disease, the concentration of foreign or essential metal ions increases above normal values recommended by the medical community. Such unwanted metal ions can be redistributed or removed through chelation therapy, which refers to the administration of chelating agents as drugs. Different from all other metallodrug examples presented in this chapter, these chelating agents are in principle proligand drugs. To effectively treat a metal sequestering disorder, the chelating ligand prodrug finds a free metal ion in the body, complexes it strongly (sequestering it), and promotes its excretion from the body. Because chelating agents do not selectively complex unwanted metal ions, problems during the treatment may arise, because biologically essential metal ions are excreted from the body as well. In metal intoxication therapy, one differentiates between two medical conditions: while the proligand drug should not compete with any natural metal binding sites in case of chronic intoxication diseases, the drug must excel in its metal-binding properties above any of the natural metal-binding sites in cases of acute metal intoxications to avoid any further toxic uptake of unwanted metal.

Acute intoxication is often caused through the adventitious exposure to metals and metalloids such as aluminum, antimony, arsenic, bismuth, cadmium, cobalt, copper, gold, iron, lead, mercury, nickel, organic tin compounds, thallium or zinc; the acute overload usually occurs by overnutrition, exposure to pesticides, or environmental or occupational exposure.^{195 167} Research on possible treatments for metal intoxication was in the beginning fuelled by the need to mitigate the toxicity of lead and of arsenic compounds, which were the standard prescription against syphillis in the first half of the 20th century. First, intravenous infusions of calcium or zinc polyamine carboxylic acids such as ethylenediaminetetraacetic acid (H_4EDTA), and $Ca_2(EDTA)$ respectively, shown in Figure 1.10, or H_5 DTPA, and ZnNa₃(DTPA) respectively, were developed, but their metal-complexes were poorly absorbed in the gastrointestinal tract. During World War II, British Anti-Lewisite, 2,3-dimerceptopropanol, H₂DMPA (BAL), was used as an antidote to the chemical weapon Lewisite, dichlorovinylarsine, the so-called "Dew of Death". Meso-2,3,-dimercaptosuccinic acid (H_4 DMSA) and D,L-2,3,-dimercaptopropane-1-sulfonic acid (H_2DMPS) from the 1950s are known for their high biological stability. With the growing understanding of chronic intoxication diseases, sequestering agents for iron and copper ions moved into focus. Desferrioxamine B (DFO), sold as Desferal, a siderophore isolated from *Streptomycin pilosus* by the Ciba-Geigy AG in 1960, ¹⁹⁶ and the orally active deferiprone, 1,2-dimethyl-3-hydroxypyridin-4-one (DFP), sold as Ferriprox, penicillamine $(H_2DPA, Cuprimine, Depen)$ and 2,2,2-trientine (TETA), sold as Syprine, chelate excess copper and iron well; moreover, they have been used successfully to treat aluminium¹⁹⁷ and arsenic intoxications.¹⁹⁸ Other chelating agents are more specific; in severe cases of cyanide poisoning the patient is given hydroxycobalamin, a precursor to cyanocobalamin which binds cyanide ion and forms cyanocobalamin which is then excreted by the kidneys (Cyanokit, FDA-approved since 2006). The newest iron-chelator on the market approved by the FDA (2005) and by the EU (2006) for use in children is defension (ICL670), sold as Exjade.¹⁹⁹

Chronic metal intoxications are genetically conditioned (Thalassemia, Wilson Disease), have been connected to neurodegenerative diseases (Alzheimer Disease, Parkinson Disease), and often eventuate as a side effect of organ failure (e.g., chronic kidney disease-mineral bone disorder).

Thalassemia is known as an autosomal-recessive bequeathed disorder manifesting it-



Figure 1.10: Approved metal chelating prodrugs.

self in the insufficient production of hemoglobin. Depending on the levels of hemoglobin one distinguishes between a mild disorder or a major defect causing severe anemia; both lead to iron overload either from iron-rich foods or from complications of frequent blood transfusions during treatment. Iron-chelating therapies with multidentate ligands are the treatment of choice for β -thalassemia, which is also known as transfusion-dependent thalassemia.²⁰⁰ Parenteral administered desferrioxamine and oral doses of deferiprone, alone or combined, are the first line treatment.²⁰¹

Wilson disease (WD) is caused by homozygous or compound heterozygous mutations in the ATP7B gene (OMIM-606882) on chromosome 13q14 (OMIM-277900). It is an autosomal recessive disorder characterized by a disfunction of several copper-dependent enzymes that leads to a toxic accumulation of copper primarily in the liver and the brain, resulting in growth defects, neurological defects and psychiatric symptoms.²⁰² Currently, three treatment options for WD are available: two are chelating agents (D-penicillamine or TETA) assisting with the excretion of copper, while oral preparations of zinc acetate (Galzin, Wilzin) work as transmetallation agents and successfully block the absorption of copper ions in the intestinal tract.²⁰³ Promising metallodrug agents in development, which inhibit copper trafficking proteins through metal cluster formation, are based on the active copper-depleting agent tetrathiomolybdate (TM, MoS_4^{2-}). Ammonium tetrathiomolybdate [(NH_4)₂(MoS_4)]²⁰⁴ and bis(choline)-tetrathiomolybdate (ATN-224, Decuprate) have been tested in clinical trials against WD and cancer, and the latter has received orphan drug designation from the EU in 2013.

Many questions and uncertainties are still surrounding neurodegenerative diseases such as Alzheimer disease (AD) and Parkinson disease (PD). Worldwide nearly 36 million people live with dementia, and this number is expected to grow rapidly over the next forty years;²⁰⁵ 1% of the world population suffer from motor impairment and dementia caused by PD. Although AD and PD are connected to the longevity of the population and aging processes in the brain and have been assumed to occur sporadically, a monogenic form of PD exists which occurs in about 5–10% of PD patients and their families as a genetic disorder.²⁰⁶ Nowadays, it is widely accepted that dyshomeostasis and overall miscompartmentalization of metals such as copper, zinc, and iron lead to disfunctions in the AD and the PD brain, with accumulation of copper and zinc in amyloid- β deposits and accumulation of iron in plaque-associated neurons, while the influence of aluminum in AD is still a controversial subject of ongoing debate.²⁰⁷ There is no cure for these neurodegenerative diseases; current therapies merely aim at symptomatic relief (e.g., reduction of tremor), and in good cases the cognitive decline is decelerated. Potential medicinal inorganic treatment options focus on the chelation and removal of copper, zinc and iron from the brain.²⁰⁸ The fact that the



Figure 1.11: Two metal chelators in clinical trials for the treatment of neurodegenerative diseases.

proligand drugs have to pass the blood brain barrier (BBB) to be able to reach the brain is a major challenge. Otherwise successful classic iron-chelators based on the desferrioxamine moiety fail to stand up to this challenge, and novel ideas²⁰⁹ such as Feralex, DP-109, JKL-169, or ligands designed the basis of natural products²¹⁰ have been investigated. Clioquinol, 5-chloro-7-iodo-quinolin-8-ol, a known oral antifungal and antiprotozoal drug, crosses the BBB and inhibits zinc and copper ions from binding to amyloid- β (Figure 1.11).²¹¹ It has completed a pilot phase II clinic trial for the treatment of AD through chelation therapy, in which patients reported improved cognition and showed lower plasma levels of amyloid- β_{42} .²¹² In addition, its metal-sequestering action is useful in the managing of PD, because chelating free metals in the brain prevents metal-mediated production of hydrogen peroxide and other radical oxygen species. A second generation of such a metal-protein attenuating compound (MPAC) with improved metal-peptide attenuating effects is the orally available 8-hydroxyquinoline (PBT2, Figure 1.11) which has completed phase II clinical trials 213 without showing a significant reduction in the levels of β -amyloid plaques in the brains of AD patients, but will proceed into phase IIb trials for the treatment of HD in the USA, while clinical trails against PD are in preparation.²¹⁴ PBT2 however is not acting as a metal chelator but rather as an ionophore; it increases the permeability of membranes leading to a more normal neuronal function.²¹⁵

Patients with a chronic kidney disease-mineral and bone disorder (CKD-MBD) show abnormalities in their calcium and phosphorus metabolism as well as in their parathyroid hormone (PTH) and vitamin D levels; in addition, they show abnormalities in bone turnover, mineralization, strength and growth (e.g., calcifications of adjacent tissue).²¹⁶ The progressive loss of kidney function leads to increased serum phosphate levels, and hyperphosphatemia is one of the clinical consequences that accompany end stage renal disease. A variety of treatment options for CKD-MBD are available targeting the down regulation of phosphate levels without disturbing levels of calcium ion; many of these pharmacological treatments are metal-based.²¹⁷ Aluminium hydroxide (Alu-Cap) is a potent and cheap phosphate binder but highly insoluble, often leading to constipation and an overall increased risk of aluminium toxicity; therefore, calcium salts such as calcium carbonate (Calcichew, Titralac) and calcium acetate alone (Phosex, PhosLo) or in combination with magnesium carbonate (Renepho, OsvaRen) have almost superseded aluminium hydroxide in the treatment of hyperphosphatemia, but such associated risks as hypercalcemia and calcification are observed in the clinic.²¹⁸ Lanthanum carbonate (Fosrenol) avoids any problems of calcium overloading or aluminium toxicity and does not cause digestive issues; in addition, it conveniently requires the intake of fewer pills per day than the leading small organic molecule drug sevelamer (Renagel, Renvela).²¹⁹ It should be noted that all these metallodrug therapies focus on binding any excess phosphate, while several biological therapeutics are available on the market that selectively target the vitamin D receptor and the parathyroid gland (e.g., Zemplar).

1.3.10.2 In the Treatment of Cancer, Microbial and Parasitic Infections

According to the nutritional immunity theory, parasites or bacteria in a host can be killed by reducing nutrients and therewith depriving the invading organism, which under such limiting conditions cannot proliferate, and eventually dies. The use of iron-deficiency, especially in the context of malaria prevention, has been the controversial for more than forty years.²²⁰²²¹ Although such iron chelating prodrugs as desferrioxamine and deferiprone have been used against malaria in clinical studies, the data has been evaluated as insufficient for supporting the use of iron-chelating agents as adjuncts in the treatment of malaria.²²²

In addition, macrocyclic chelating agents such as nonactin and valinomycin take this line of defence by complexing potassium ion, while crown ethers such as 15-crown-5, dibenzo-18-crown-6, and 24-crown-8 provide a good fit for the smaller sodium ion. After the macrocycle has wrapped up the metal ion, the coordination complex is transported through the cell membrane. *In vitro* studies have shown that in this way these agents change the permeability of the membrane to potassium ions, disrupting oxidative phosphorylation and inhibiting the processing of some proteins resulting in an overall antimicrobial effect.²²³

A spin-off from the POM research presented in Section 1.3.5 led via the detected anti-HIV activity of bicyclams to another serendipitous drug discovery; the metal-chelating agent AMD3100, [1,1'-[1,2-phenylene-bis(methylene)]-bis(1,4,8,11-tetraazacyclotetradecane)octahydrochloride dihydrate] (JM3100, Plerixafor, Mozobil) depicted in Figure 1.12, is an EMA and FDA approved selective CXCR4 chemokine receptor antagonist used to mobilize hematopoietic stem cells to the peripheral blood for collection and autologous transplantation in patients with non-Hodgekin's lymphoma or multiple myeloma.²²⁴ Vorinostat, N-hydroxy-N'-phenyloctanediamide (Zolinza), is a histone deacetylase inhibitor approved for the treatment of cutaneous T-cell lymphoma (CTCL).²²⁵ Other Zn-chelating agents currently in clinical trials against cancer are PXD101, (2E)-3-[3-(anilinosulfonyl)phenyl]-N-hydroxyacrylamide (Belinostat), and givinostat, 6-[(diethylamino)methyl]-naphthalen-2-yl-[methyl(4-hydroxycarbamoyl)phenyl]-carbamate.



Figure 1.12: Metal-chelators for cancer therapy.

1.4 Strategies for the Design of Metallodrugs

Many of the metallodrugs currently on the market have been discovered by chance, as the discovery stories for some of the prominent metallodrugs in previous sections reflect. On the basis of such first generation serendipious hits (e.g., cisplatin), further generations of drugs have been developed by carefully studying, analyzing, and partly guessing the mechanism of action as well as reasons for unwanted side effects of the first generation drug to be able to iron out these flaws in the second and often third generation of drug molecules. Staying with the example of platinum drugs for cancer therapy, these would be carboplatin (second generation), satraplatin (third generation) and subsequent agents such as nedaplatin, lobaplatin and heptaplatin. During the past years, medicinal bioinorganic chemists have focused strongly on moving the drug development process from the initial serendipity discoveries, which undoubtedly laid the foundation for this field, to a more rational drug design process. This section describes the drug design and development
process in general including advantages and challenges that arise from bringing a metal ion into the game.

1.4.1 Finding a Druggable Target

A rational approach of designing a metallodrug is in its principles not very different from designing a drug based on a small organic molecule or a biological molecule. The first step is the overall identification of a disease target and the specific elucidation of a molecular target associated with this disease's etiology and pathology.

To define putative targets, traditional medicinal chemists employ the disciplines of genomics and proteomics. Complementary to genomics and proteomics, bioinorganic medicinal chemists call on the growing field of metallomics to support their target validation. Metallomics refers to the characterization of the entirety of metal and metalloid species present in a cell or tissue type, as well as their interactions with the genome, transcriptome, proteome, and metabolome.²²⁶ The ultimate goal of this novel field is to understand comprehensively metal uptake, trafficking, function, and excretion in biological systems.²²⁷ Species of interest for metallomics are complexes of trace elements and their compounds with endogenous or bioinduced biomolecules such as organic acids, proteins, sugars, or DNA fragments.²²⁸

Seven of the twenty-one amino acids, the building blocks of peptides and proteins, possess appropriate donor atoms such as nitrogen, oxygen, or sulfur in their side-chains, giving them the opportunity to interact with a metallodrug. These seven amino acids are aspartic acid, cysteine, glutamic acid, histidine, lysine, methionine, and tyrosine. Moreover, specific metal-binding sites are located in the N-terminus of many naturally occurring proteins; one of these is the amino terminal copper(II)- and nickel(II)-binding ATCUNmotif, which is formed in proteins from a histidine in the third position, its proceeding residue, and the free N-terminus, providing a total of three N-atoms for interaction with a metal.^{229 230} If a metal ion is purposefully administered to the human body in form of a metallodrug, the metal ion can bind to a protein, possibly resulting in an altered protein structure and therewith loss or alteration of its function. On the other hand, the metal ion at the core of metalloenzymes is essential for their catalytic activity, for example, Zn^{2+} in zinc-enzymes or Cu^{2+} ;²³¹ if a proligand drug is administered, the chelating agent can bind strongly to the metal ion at the center of the metalloenzyme and remove it, which renders the metalloenzyme inactive. Emerging protein targets for metallodrugs have been recently reviewed.²³²

Another target for metallodrugs is DNA itself. All four bases contain nitrogen and oxygen as donor atoms to which a metal ion can bind, the N-7 of adenine and guanine in the major groove of double-helical DNA being among the most important binding sites. In a coordinative, covalent binding interaction with DNA, a metal ion can connect both strands to form an intrastrand crosslink, bind solely to bases on the same strand in an interstrand crosslink fashion or build a link with amino acid side-chains of a neighbouring protein, a so-called protein-DNA crosslink. Moreover, small, planar and mostly hydrophobic drug molecules can slide into the inside of the helix where they can intercalate between the base pairs in a non-covalent fashion. So-called dual mode DNA binding metallodrugs not only bind covalently to the DNA but additionally intercalate as well, while other metallodrugs selectively target a specific sequence.

Biological targets of metallodrugs have been comprehensively reviewed and critically evaluated quite recently.^{233 234} Nucleic acids, proteins, and DNA are commonly expressed by all kind of cells and are, therefore, rather unselective targets. In the current postgenomic era, in which the life sciences are being transformed by gene sequencing knowledge and advanced techniques, metallodrug research is as well progressing towards selective targeting. A specific tumor type and its unique chemical pathways²³⁵ or one molecular target in parasite biology 236 can be clearly identified and selectively attacked with a specifically designed metal-based molecule.

Besides macromolecular structures such as proteins and DNA, metal ions can react with various other small molecules contained in the body's fluids.²³⁷ In human blood the concentration of chloride amounts to 104 mM. In addition to chloride, human body fluids contain phosphate and carbonate in high concentrations, two other anions which could potentially bind the metal ion delivered with the metallodrug molecule. Because many metallodrugs are administered intravenously, it is important to understand what happens to the metallodrug molecule once it is surrounded by a variety of small anions such as chlorides, phosphates or carbonates.

Research in the area of biophysical chemistry increasingly focuses on the improved understanding of metal ion metabolism in the human body, often coupled with a variety of pharmacological methods. This has led to diverse novel bioanalytical methodologies for studying the mode of action of metallodrugs and therewith identifying their specific targets.²³⁸ For example, such approaches that comprise a variety of biophysical and pharmacological techniques span probing the interaction of metal ions with proteins²³⁹ to the application of different analytical techniques such as mass spectrometry,²⁴⁰ other hyphenated techniques,²⁴¹ and capillary electrophoresis.²⁴²

In classic drug development, it is critical to gain as much detailed information about one specific target as possible, because all this information can flow into the design of a drug molecule, which has a perfect fit and therefore preference for binding to a single target instead of interacting with various other molecules and their competing binding sites in the body, resulting in less unwanted side effects. On the other hand, such a one molecule-one target approach not only limits the number of possible side effects, but as well limits the ability to combat complex neurodegenerative diseases such as Alzheimer or Parkinson disease, for which more radical approaches of multifunctional metal chelators aiming at multiple neurological targets are needed.²⁴³ Another recent example from AD research has shown the danger that lies in developing a drug candidate on a diffusely defined target: the drug candidate tramiprosate (Alzhemed), which had been designed to selectively block the aggregation of amyloid- β plagues, was stopped in phase III clinical trial stage, when the statistical model for evaluating the drug based on cognitive efficiency data and brain volume data showed large variations and was therefore unable to support clinical efficacy.²⁴⁴

1.4.2 The Advantage of Variety: Designing Metal Complexes for the Perfect Fit

Compared to the structural features that can be built around a metal ion, the possibilities of small organic molecules and biological molecules seem almost drab. While such drug molecules rely purely on carbon, their binding geometry in space is dictated by the principles of hybridization -sp (linear), sp^2 (trigonal-planar) and sp^3 (tetrahedral) compared to the diverse geometry in 3D space open to metal ion-containing drugs. Besides linear, square-planar, and tetrahedral geometries, pyramidal, trigonal bipyramidal, and octahedral shapes can be created, the latter being of tremendous importance for biological processes. With the growing number of substituents around the metal center, the variety in stereoisomers and stereochemical flexibility in general increases to open up a diversity in 3D structures.²⁴⁵ Modification of these substituents or ligands tailors them to manifold functions and specific targets.²⁴⁶ Although ligand exchange reactions are often calculated mechanisms of action in medicinal inorganic chemistry, the metal ion itself is at the heart of action. The metal ion orchestrates the ligand coordination according to precise 3D configurations. With its fine-tuned redox-chemistry, the metal ion can participate in biological redox actions, and transition metals such as ruthenium or iron, which have multiple stable



Figure 1.13: Overview of various design possibilities for metallodrugs.

oxidation states, offer catalytic potential. Moreover, the metal ion introduces a distinct spectroscopic handle that can be exploited in a variety of techniques, some of which are not accessible for purely organic molecules, for example, Mössbauer spectroscopy. In addition, a metal ion can add magnetic properties to the metal-ligand complex and, if needed, radioactivity for utilizing elements with appropriate isotopes. Despite their described structural complexity, metal-ligand complexes are still quite small and lightweight as compared to some macrocyclic biological organic molecules. All of these tuneable design components (see Figure 1.13) create indefinite possibilities for metal-ligand complexes with novel and unprecedented properties.²⁴⁷

An extensive study by the U.S. National Cancer Institute (NCI) from 2005 mirrors the

design diversity for metallodrugs for the treatment of cancer. About one-thousand metal or metalloid containing compounds with potential anticancer activity were included. The aim of this study was to establish correlations between specific cytotoxic responses and differential gene expression profiles to expand the knowledge base for evaluating, designing, and developing new target-specific metallo-anticancer drugs. Although the study confirmed a large variety of possible mechanisms of action for metal-based compounds, four fundamental response classes were identified on the basis of preference of (1) binding to biological sulfhydryl groups, (2) chelation, (3) generation of reactive oxygen species and (4) production of lipophilic ions.²⁴⁸ These four categories are extremely broad, but only demonstrate once more the variety of targets affected by metallodrugs. Similarly, one metallodrug might be active against a variety of diseases. Gold(I) compounds (sodium aurothiomalate, auranofin) that have been traditionally employed in the therapy of rheumatoid arthritis (Section 1.3.3) are becoming more and more known for their anticancer properties, which are currently being tested in clinical trials.

Instead of such drug repositioning, one can take inspiration from naturally occurring molecules and carefully study the binding pocket of proteins to which they bind, perhaps with the assistance of computational methods,²⁴⁹ to discover a specific synthetic structure that nicely docks onto the protein. For example, PIM kinases are enzymes located on the proviral insertion site of the moloney murine leukaemia virus that can be selectively inhibited by inert half-sandwich ruthenium-indolocarbazole complexes.²⁵⁰ These organoruthenium complexes have demonstrated an extremely good fit in the adenosine triphosphate (ATP) binding pockets of PIM1 and PIM2 inactivating these PIM kinases, which in return leads to restored apoptosis in drug-resistant cancer cells.²⁵¹ The high potential of neutral or cationic arena ruthenium complexes for the development of anticancer metallodrugs has been widely discussed;²⁵² organometallic arene-ruthenium(II) complexes such as RM175,



Figure 1.14: Organometallic ruthenium(II) complexes with promising anticanceractivity.

 $[(\eta^{6}\text{-biphenyl})\text{-}(\text{ethylene-diamine})\text{ruthenium(II})\text{chloride}],^{253\,254}$ RAPTA-C, $[(\eta^{6}\text{-paracymene-}(1,3,5\text{-triaza-7-phosphaada-mantane})\text{-ruthenium(II})\text{dichloride}],^{255\,256}$ and NP309, $[(\eta^{6}\text{-cyclopentadiene})\text{-}[\text{N,N-}(9\text{-hydroxy-pyrido})\text{-}(2,3\text{-a-pyrrolo})\text{-}(3,4\text{-c-carbazole})\text{-}(5,7\text{-}dione)]\text{-ruthenium(II})\text{carbonyl}],^{250}$, depicted in Figure 1.14, have shown promising results in various *in vitro* and *in vivo* studies and continue to fuel research into organometallic Os(II) and Ir(III) complexes.^{257}

Certainly, the perfect fit for a defined target in the body is important, but first the drug molecule has to reach its target. Many metallodrugs are injected into the bloodstream or the muscle tissue, and during their passage through the blood and eventually into the cells, the drug molecule comes in contact with biological substances that can modify its composition through ligand exchange reactions; often serum proteins are their first binding partners.²⁴² For many metallodrugs, human serum albumin (HSA), 0.65 mM, acts as a reservoir, which can be exploited for delivery purposes. HSA-conjugates have been shown to accumulate in tumor tissue due to their enhanced permeability and clearance retention effect.²⁵⁸ Another protein with a strong affinity for metal ions is *apo*-transferrin (Tf), 0.037 mM, which can not only bind two equivalents of iron(III) but interacts with a variety of main group,²⁵⁹ transition group²⁶⁰ and lanthanide²⁶¹ metal ions. When the

concentration of a drug in the plasma does not correlate with its expected therapeutic effect, it must be assumed that the supposed drug molecule was only a prodrug and the active drug metabolite was only generated in a biological interaction *in vivo*.²⁶² This is not necessarily negative, as prodrugs can be an efficient way to deliver an active compound across barriers,²⁶³ for example, the successful *cis*-platinum drugs used in cancer therapy are prodrugs.

Because of their diverse structures, metallodrugs can act through different mechanisms of action compared to small organic molecule or biological drugs, such as targeted ligandexchange with biological molecules *in vivo*, giving a variety of novel drug targets and transport pathways. This notion gives hope that for therapeutic areas in which drug resistance is growing, metallodrugs can overcome developed resistance; 264 examples of overcome drug resistance from malaria research 265 and cancer research 266 suggest this hope may not be in vain. In Chapters 3 and 4 of this thesis, the possibility of overcoming the growing resistance of microbes to known antibiotic drugs with coordination complexes of Ga³⁺ and Cu²⁺ will be explored.

1.4.3 Exploring the Druggability of the Target

Once a target is found, its druggability needs to be explored. Therefore, screening systems are established that test the novel potential therapeutic agent against the desired target. In drug research, such testing of larger compound libraries is often performed in the form of high-throughput screening or high content cell-based assays. There are two complementary common approaches to validate a target. In the chemical approach, small molecule inhibitors can be used to modulate the functional activity of a target, providing insights into chemical evidence for druggability of the target or favourable selective toxicity against the pathogen versus the host (cell, tissue or whole animal). The genetic approach can be classified into target gene knockout (often mouse models) and target ribonucleic acid (RNA) knockdown methodologies, often using small interfering ribonucleic acid (siRNA).²⁶⁷

Because drug discovery is in general an expensive process, it is important to recognize early problematic drug candidates and undruggable molecular targets that will most probably fail (many genes are for example not druggable) to save costs and in return allow these resources to be used for the drug candidates that will most probably succeed. The most critical point in this regard is improving the suitability and robustness of the agents that enter the clinic.²⁶⁸ This relates directly to the thermodynamic and kinetic stability *in vivo*. It is essential to understand how the drug molecule affects the body, as well as how in return the body effects the drug molecule. Developing such an understanding is even more challenging in metallodrug-research, as metallodrugs can interact with a variety of biological molecules inside the body as was illustrated in Section 1.4.2. As an example from the clinic, the *in vivo* interaction of Fe³⁺ with the FDA approved anticancer drug doxorubicin and the newly developed anticancer agent vosaroxin, which is currently in phase III clinical trials, will be discussed in detail in chapter 5.5

1.4.4 Pharmacokinetics: Thermodynamic Stability and Kinetic Lability

A drug that is unable to reach its molecular target in the body possesses poor pharmacokinetics. The pharmacokinetic characteristics are defined by the concept of absorption, distribution, metabolism and excretion (ADME concept) properties of the potential drug molecule. Knowledge of ADME concept properties of the drug and its metabolites in humans (as well as in animals used for the toxicology assessments) is crucial to understand differences in effect among species and to optimize drug dosing in general.

It appears that the pharmacokinetic characteristics of a drug are strongly related to its physicochemical properties such as solubility, lipophilicity and stability, which can be determined by measuring the octanol-water partition coefficient (log P) and p K_a s. These measurements are useful in predicting protein binding, tissue distribution, and absorption in the gastrointestinal tract.²⁶⁹

Lipinski defined²⁷⁰ five rules for the lipophilicity, and therewith a measurable value for how easily a molecule can pass through the blood brain barrier, from empirical experience. According to "Lipinski's Rule of 5",²⁷⁰ poor absorption and permeation are more likely when the molecule has (I) more than 5 hydrogen bond donors, expressed as the sum of OHs and NHs, (II) more than 10 hydrogen bond acceptors, expressed as the sum of nitrogen and oxygen atoms in the molecule, (III) a molecular weight of over 500, and (IV) a partition coefficient of log P > 5.²⁷⁰ Although Lipinski's rules are helpful to evaluate and identify orally bioavailable drugs, bioinorganic medicinal chemists should bear in mind that these rules have been empirically found in approved organic small-molecule drugs and may not necessarily apply to metallodrugs in the same way.²⁷¹

The possibility of interactions of a metallodrug molecule with other biomolecules which are available at high concentrations in the human body has been discussed in Section 1.4.2. How likely a metallodrug is to undergo a structure-altering process such as ligand exchanges or transmetallations is determined by the strength of the metal-ligand bond(s) under physiological conditions.²⁷² Stability constants (log β_n), as defined in equation 1.1, are a measure of metal chelation, in which M represents the metal ion and L symbolizes the free ligand.

$$\log \beta_n = \log \left(\frac{[ML_n]}{[M][L]^n}\right) \tag{1.1}$$

This principle relationship can as well be expressed as protonation constants (K_a, pK_a) , dissociation constants (K_d, pK_d) , effective binding constants (K_{eff}) or free metal ion concentration pM.²⁷³

During the drug development process the stability of the metallodrug candidate against the two dominant proteins, *apo*-Tf and HSA, under biological conditions in 0.15-0.16 M

aqueous sodium chloride solution at 37 o C can be measured and compared with evaluations from potentiometry or spectrophotometric studies.

For orally administered drugs, adequate absorption and bioavailability must be achieved,²⁷⁴ which seems to be a challenge for metallodrugs. Many metallodrugs are given intravenously due to their limited solubility in oral formulation, the need to administer only small amounts of metal ion to avoid toxic side effects, and the lack of stability of metal-ligand complexes on their way through the various pH levels in the stomach and intestines. Novel approaches for the delivery of metallodrugs are required and have recently been reviewed;²⁷⁵ among them nanoparticles open up new vistas of improved delivery, cell uptake, and targeting.^{234,276} Micelle emulsions¹⁵⁰ and liposomal formulations also appear promising.^{277,278}

1.4.5 Pre-Clinical Studies

Besides target validation and pharmacological assessment, a first set of studies on the *in vitro* metabolism of the drug candidate and some initial toxicity studies are often included in the preclinical assessment of whether a drug candidate is suitable for the clinic or not. Drug metabolism can be studied on liver cells (heptatocytes) and cytochrome P450 enzymes, while cell permeability is often tested on MDCK and/or human colon colorectal adenocarcinoma cells (Caco-2 cells). The Caco-2 cells permeability assay has been widely adopted for understanding the gastrointestinal drug absorption process. At this stage, toxicity is evaluated in the *in vitro* cytotoxicity studies and eventually single acute dose studies in animals (mouse, rat, dog) to establish the maximum tolerated dose (MTD).²⁷⁴ Drug development candidates that satisfy these initial tests and any further extensive toxicological studies are deemed safe enough to proceed into clinical trials.

1.4.6 Clinical Studies

Testing of the drug candidate in the clinic starts with phase 0. This exploratory investigational new drug study of a few healthy individuals, in which these volunteers receive less than 1% of the therapeutic dose of the investigational drug over the course of maximum seven days, is followed by phase I during which the preliminary pharmacokinetics and toxicology are evaluated in healthy individuals in a primarily safety screening.

The drug candidate is tested for the first time in patients suffering from the targeted disease in phase II clinical trials. At this stage, the efficacy of the investigational drug is established against a placebo. The decisive challenge in phase II clinical trials lies in the design of the study itself. How can the desired outcome of the study be clearly described? What is the definite endpoint of success? Which patients can be recruited for the study? Often these questions are heatedly discussed until the respective proof-of-concept criteria for a clinical study finally can be clearly defined. Especially in oncology and in diseases of the central nervous system (CNS), it has proven to be difficult to establish clear efficacy signals. For example, in the early times of anticancer drug research, the efficacy goal was to shrink the tumor tissue, and for metallodrugs such as cisplatin this was an acceptable (and facile) way to evaluate the drug's performance. In contrast, new drug developments such as the ruthenium-based compounds, NAMI-A and KP1019, do not aim exclusively at reducing the malignant tissue but, moreover, are targeting angiogenesis to avoid metastasis. In addition, financial factors must be considered, because investors may fear that narrowly defined indications translate into a narrow market for the drug, which, coupled with safety concerns, was the reason the clinical development of MRI contrast agent ferumoxide, based on iron oxide nanoparticles (Combidex, Sinerem), was halted.³⁴

These practical examples illustrate that the design of a clinical trial to prove the principle action of the new drug is of vital importance and must be addressed already in the early stages of the drug development process. Should it prove to be impossible to demonstrate the desired action of the drug in the clinic through a carefully defined screening procedure, the best idea for a drug is worthless, because government agencies such as the FDA or EMA expect clear and complete data to grant approval. Attrition rates in 2011-2012 show that efficacy was stated as the cause of failure in 59% of all drug development projects killed in phase II clinical trials and 52% in phase III clinical trials, while the overall failure rates were highest in the therapeutic areas of oncology (29.5%) and CNS (14%),²⁷⁹ which once more illustrates the difficulty to establish clear efficacy signals in these therapeutic areas.

The major costs of clinical trials occur in phase III studies that are performed to confirm the safety, established in phase I, and the efficacy, established in phase II. This is usually the final step before the application for approval of the drug candidate can be filed with the respective government agencies.

1.5 Conclusion & Thesis Outlook

With a good overview of the diagnostic and therapeutic metallodrugs currently approved by the FDA and EMA, an impression of the biological challenges of metallodrug research and development as well as potential strategies to overcome these, we now set out to study the coordination chemistry of antimicrobial and anticancer agents. In Chapter 2, the drug class of the (fluoro-)quinolones is introduced, and the properties of nine members of this class are discussed in detail. In addition, the question of their stability in Iso-Sensitest broth, a growth medium for bacteria, is addressed, and their antimicrobial susceptibility against the five most commonly reported causative pathogens in nosocomial diseases is tested *in vitro*. Results once more show the growing resistance of microbes against commonly prescribed antibiotic drugs, and we, therefore, take these alarming results as motivation to develop novel antimicrobial agents based on a coordination chemistry approach in the following two chapters.

In an attempt to combine the anticipated antimicrobial properties of gallium(III) ions with the antimicrobial potential of the nine quinolones presented in Chapter 2, novel tris(quinolono)gallium(III) complexes and their respective iron(III) analogs are synthesized, chemically characterized, and their antimicrobial properties tested against the five selected bacteria, in Chapter 3. The following chapter, Chapter 4, expands on this strategy by acting on novel designs in quinolone antimicrobial research and complexing these ligands to copper(II), another metal ion that has been explored for its antimicrobial potential.

The agent vosaroxin is currently under development by *Sunesis Pharmaceuticals, Inc.* for the treatment of cancer; it is as well a quinolone. The leading anticancer drug on the market nowadays is doxorubicin, which is not only known for its anticancer potency but as well for its cardiotoxicity, a common side-effect of treatment with this drug that is believed to be partially caused through the interaction with the essential metal iron *in vivo*. Chapter 5 discusses the behaviour of vosaroxin and doxorubicin towards iron(III) based on results of a comparative spectrophotometric stability study under *in vivo* conditions. Moreover, the coordination chemistry of vosaroxin is explored further through the novel tris(vosaroxacino)iron(III) and its respective gallium(III) complex.

Chapter 6, summarizes the research results of this thesis and modestly addresses the future of the field of metallodrugs in medicinal inorganic chemistry.

Chapter 2

Introduction to Quinolone Antimicrobial Agents

In this chapter, the drug class of quinoline antimicrobial agents will be introduced. Nine selected quinolones will be characterized, their stability in Iso-Sensitest medium will be discussed, and their antimicrobial susceptibility will be evaluated.

2.1 Quinolone Antimicrobial Agents

With his discovery of the bactericidal properties of the naphthyridine agent nalidixic acid in 1962, George Y. Lesher laid the foundation for a highly successful class of antimicrobial agents.²⁸⁰ Quinolones have become a major group of popular synthetic antibacterial agents with activity against a diversity of Gram-positive and Gram-negative bacteria.²⁸¹ Due to their excellent penetration of most bodily tissue fluids, their clinical use dominates in bacterial infections of the genitourinary, respiratory, and gastrointestinal tracts,²⁸² while ciprofloxacin hydrochloride is also approved by the FDA for treatment of the inhaled form of anthrax.²⁸³

Quinolones have either a quinoline or a 1,8-naphthyridine aromatic ring at their core

Core structures





quinoline

OH

1st generation

nalidixic acid (Hnxa)



oxolinic acid (Hoxa)



pipemidic acid (Hpia)



2nd generation



enoxacin (Henox)



ciprofloxacin (Hcipro)



lomefloxacin (Hlomx)

Figure 2.1: Aromatic core structures of quinolone antimicrobial drugs and molecular structures of the nine quinolone agents selected for this study.



fleroxacin (Hflex)

F



levofloxacin (Hlevox)

(Figure 2.1). The carboxylic acid group on C_{ar3} , together with the carbonyl group on C_{ar4} are key in their antimicrobial mode of action.²⁸⁴ Although their exact mechanisms of action still remain elusive, the quinolones most probably interact through hydrogen binding via the 3-carboxyl-4-oxo modality with their microbial enzymatic targets, DNA gyrase (topoisomerase II) or/and topoisomerase IV, depending on the bacterium.^{285 i}

These enzymes are essential for orchestrating the supercoiling of cellular DNA.^{286 287 288} Quinolones bind to the formed enzyme-DNA-complex rendering the respective enzyme inactive, ²⁸⁹ and this disruption of the supercoiling process by the quinolone is bactericidal.²⁹⁰ The influence of the bivalent metal cations Mg^{2+} and Ca^{2+} in the process of establishing contact between the quinolone molecule and the enzyme-DNA-complex has been discussed for many years;^{291 292 293} recent studies state a water-metal bridge between the quinolone molecule and the topoisomerase IV to be crucial for this interaction.²⁹⁴

Over the past fifty years, a large array of activity related quinolone-core based structures has been developed with fine-tuned differences in their spectrum of activity and potency as well as adverse side-effects.^{295 296 297 298} These are commonly sorted into different drug generations. In the late 1970s, quinolones of the second generation, such as norfloxacin and enoxacin, bearing a fluorine in the 6-position, showed improved enzyme inhibition and a broadened spectrum of activity, leading to the dogma that the 6-fluorine was an essential feature and stamping another name for this drug class, fluoroquinolones.ⁱⁱ Even nowadays, quinolone antibacterial agents still gain attention,²⁹⁹ and medicinal chemists continue to tweak their chemical structure, and therewith pharmacological properties, to develop novel

ⁱFor quinolone targets, bacteria can be sorted into three categories (I) Gyrase only: human pathogens that lack a close homologue to topoisomerase IV, e.g., *Mycobacterium tuberculosis, Helicobacter pylori*; (II) Gyrase more sensitive a target than topoisomerase IV: Gram-negative bacteria, e.g., *Escherichia coli*; (III) Equal sensitivity for Gyrase and toposimerase IV: Gram-positive bacteria, e.g., *Staphylococcus aureus*.²⁸⁵

ⁱⁱBecause this study comprises (fluoro-)quinolones from different generations, including partly fluorinated and non-fluorinated derivatives, compounds based on the core structures shown will be simply referred to as quinolones, including mono-, bi- and tri-fluorinated species (Figure 2.1).

antibiotic drugs against the looming threat of growing antimicrobial resistance.³⁰⁰

Although common clinical quinolone drugs have been widely reviewed, and their syntheses, determination, and pharmacological data, as well as clinical data have been discussed for the past fifty years, we were surprised to find no complete set of chemical characterization data in the literature. Searching the published data, it proved difficult to find reliable chemical characterizations, as reported data came from many different sources and had been obtained in different laboratories, on a variety of instruments, under various conditions, and in different decades according to the best analytical standards of that time. A combination of all these factors stands presumably behind the fact that conflicting information on quinolone compounds has been published, particularly for ultraviolet-visible (UV-Vis) and NMR measurements. For these reasons, we saw the need for a single source collection of chemical characterization. In this chapter, the obtained spectroscopic and spectrometric data, including results of elemental analysis for sake of completion, of nine selected quinolones from three drug generations is reported (Figure 2.1): ciprofloxacin (Hcipro), enoxacin (Henox), fleroxacin (Hflex), levofloxacin (Hlevox), lomefloxacin (Hlomx), nalidixic acid (Hnxa), norfloxacin (Hnofx), oxolinic acid (Hoxa), and pipemidic acid (Hpia).

Another issue that has been raised in regard to quinolone antibiotic agents is their stability in standardized susceptibility testing settings, especially their behaviour towards metal ions in the test medium, and how such metal ions might affect their bactericidal performance. In their comparison of cation-adjusted Mueller-Hinton broth with Iso-Sensitest broth following the broth micro dilution method of the U.S. Clinical and Laboratory Standards Institute (CLSI), formerly known as U.S. National Committee for Clinical Laboratory Standards (NCCLS), Koeth *et al.* found that the four quinolone antimicrobial agents that they tested, amongst others ciprofloxacin and levofloxacin, showed slightly higher minimum inhibitory concentration (MIC) values in Iso-Sensitest than in Mueller-Hinton medium.³⁰¹ It has been known for over forty years that the Ca^{2+} and Mg^{2+} content of biological media can have a major effect on the bactericidal activity of antimicrobial agents, e.g., against *Pseudomonas aeruginosa*,³⁰² and can therefore significantly influence the results of susceptibility tests.³⁰³ When Turel *et al.* directly compared the antimicrobial activity (MIC) of their synthesized bis(ciprofloxacino)magnesium(II) complex with that of ciprofloxacin, they had to conclude that the magnesium(II)-quinolone complex possessed a two fold lower activity than the reference compounds ciprofloxacin and ciprofloxacin hydrochloride.³⁰⁴ Before the CLSI defined a specific concentration range for cations and other possible inhibitors in Mueller-Hinton media, batch variance, and therewith variance of metal ion concentrations, had been one of the core issues with Mueller-Hinton media. Although batch variance and too high metal ion concentrations are an issue in *in vitro* laboratory practice, current research results report as well that Mg^{2+} and Ca^{2+} play an important role in the mechanism of action of quinolone antimicrobial agents, ²⁹¹292294 as was mentioned earlier.

Compared to Mueller-Hinton, Iso-Sensitest is a synthetic and therewith chemically welldefined medium containing only minimal amounts of variable nutrients (for media formulae see Table A.1 and A.2). Traditionally, Iso-Sensitest has been used in Europe, where it has proven itself as a reliable medium with lesser reported problems than Mueller-Hinton.³⁰⁵ Several European National Committees have been advising the use of Iso-Sensitest, such as the British Society for Antimicrobial Chemotherapy (BSAC), but lately the European Committee for Antimicrobial Susceptibility Testing (EUCAST) has been recommending Mueller-Hinton in an attempt to unify susceptibility testing procedures to reach comparable test results across Europe and the world.³⁰⁶ Most probably, this decision will only feed the fire of the ongoing controversial discussion of Iso-Sensitest vs. Mueller-Hinton media.^{307 308}

In our lab, we prefer the use of Iso-Sensitest over Mueller-Hinton, because of its better

defined chemical content. To ensure the stability of the nine selected quinolones (Figure 2.1) throughout the antibacterial susceptibility single-disk test procedure, we have studied their behaviour in Iso-Sensitest broth via UV-Vis spectroscopy over time. Moreover, the results of antimicrobial susceptibility single-disk tests of these nine quinolones in Iso-Sensitest medium against five pathogens are reported, including Gram-positive and Gram-negative microorganisms, which are a common cause of nosocomial infections.

2.2 Materials & Methods

2.2.1 Chemicals

All chemicals were purchased from commercial sources: ciprofloxacin, enoxacin, levofloxacin, norfloxacin, oxolinic acid, and pipemidic acid were from Sigma-Aldrich, while fleroxacin, nalidixic acid, and lomefloxacin hydrochloride were from TCI America. Aqueous solutions were prepared from deionized water, purified through a ELGA PURELAB ultrapure water system with a resistivity of 18 M Ω ·cm (25°C).

2.2.2 Instrumentation

All melting point (mp) measurements were conducted in triplicate on a DigiMelt SRS melting point apparatus by Stanford Research Systems and are uncorrected. Ultaviolet-Visible spectra were obtained on a Hewlett Packard 8453 instrument run by UV-Vis ChemStation Software (version B.04.01[61], Agilent Technologies, 2001–2010) in methanol with up to 2% dimethyl sulfoxide (DMSO), in water, in Iso-Sensitest broth, and in aqueous sodium chloride solution (0.16 M). All maximum absorption bands and extinction coefficients (ϵ) are listed. Infrared (IR) spectra were recorded neat in the solid state on a Thermo Scientific Nicolet 6700 Fourier transformation (FT) IR spectrometer in the range of 4000–450 cm⁻¹ and analyzed with OMNIC software (version 7.4.127, Thermo Scientific). Bands were interpreted using the following abbreviations: strong (st), moderate (md), weak (w), broad (br), and shoulder (sh). Nuclear magnetic resonance spectroscopy was conducted on Bruker Avance 300 and 600 spectrometers running Topspin 2.1 software on Redhat Linux. The Bruker Avance 600 spectrometer contained a Bruker TCI-Z-5mm cryoprobe for detection of the ${}^{13}C$ and the ${}^{1}H$ nuclei with high sensitivity at signal-to-noise ratios of 6000/1 (${}^{1}H$) and 600/1 (¹³C). ¹H NMR, ¹³C NMR spectra as well as correlated spectroscopy (COSY), heteronuclear single quantum coherence spectroscopy (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra, and ¹⁹F spectra where applicable, were recorded at room temperature with the residual solvent signal of the deuterated solvent (d_6 -DMSO or D_2O) as internal standard, ³⁰⁹ referencing all chemical shifts (δ) in ppm against tetramethylsilane $(\delta = 0)$ and against trichlorofluoromethane $(\delta = 0 \text{ ppm})$ as applicable. The software iNMR (version 5.1.2, Mestrelab Research) was employed for spectral analysis, and the following abbreviations were used for description: aromatic ring system (ar), doublet (d), doublet of doublets (dd), multiplet (m), 1,4-piperazinyl ring in C_7 position on aromatic ring system (pip), propyl ring in N_1 position on aromatic ring system (prop), quartet (q), singulet (s), triplet of triplets (tt). Low-resolution mass spectrometry (MS) was performed on a Waters ZQ spectrometer equipped with an electrospray and chemical ionization (ESCI) source and MassLynx Mass Spectrometry software (version 4.00.00, Waters). Characteristic signals are listed as dimensionless numbers of their mass-to-charge ratio (m/z), with their intensity related to the base signal. Microanalyses for C, H, and N (elemental analysis (EA)) were recorded at the UBC Mass Spectrometry Centre on a Carlo Erba Elemental Analyzer EA 1108.

2.2.3 Biological Studies

All biological experiments were performed in UBC's Biological Services Laboratory. Iso-Sensitest agar and broth were manufactured by Oxoid. All one-time-use articles were bought from Fisher-Scientific, only the filter disks ($^{1}/_{4}$ inch diameter, approx. 0.6 mm) were obtained from Schleicher & Schüll, Germany. Antimicrobial susceptibility single-disk tests were performed against *Enterococcus faecalis* (ATCC-51575), methicillinsusceptible *Staphylococcus aureus* (MSSA-476, ATTC-BAA-1721), both Gram-positive; *Escherichia coli* (ATCC-25922), *Klebsiella pneumonia* (ATCC-13883), *Pseudomona aeruginosa* (ATCC-27853), all Gram-negative. Methanol and DMSO were purchased from commercial sources, the DMSO was only used after filtration through Millex-FG (0.20 μ m). Please see Appendix A for a detailed test procedure.

2.2.4 Chemical Characterization

2.2.4.1 Ciprofloxacin, Hcipro

Appearance: off-white solid. **Mp**: 253–255°C (brown). **UV-Vis** (CH₃OH with 1.5% DMSO): λ [nm] (ε) [M⁻¹cm⁻¹] = 289 (19900), 317 (12500), 331 (11800). **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3044 (m, br), 2844 (w, br) 1614 (st), 1587 (st), 1540 (md), 1498 (st), 1472 (md), 1448 (sh), 1372 (st, br), 1329 (md), 1310 (w), 1284 (st), 1260 (sh), 1172 (md), 1146 (st), 1130 (sh), 1102 (w), 1076 (w), 1035 (st), 1022 (st), 978 (w), 934 (st), 891 (md), 868 (st), 833 (st), 822 (md), 784 (st), 721 (st), 707 (md), 652 (md), 622 (st), 565 (st), 553 (sh), 543 (st), 494 (st), 479 (sh), 443 (md). **NMR**: **δ**_H (600 MHz, 298 K, d₆-DMSO) [ppm] = 14.68 (br s, 1H, COOH); 8.67 (s, 1 H, C_{ar2}H); 7.93 (d, J³_{H,F} = 13.0 Hz, 1 H, C_{ar5}H); 7.60 (d, J⁴_{H,F} = 7.4 Hz, 1 H, C_{ar8}H); 3.86 (tt, J³_{H,H} = 5.0 Hz, 4 H, C_{pip2,6}H₂); 3.27 (t, J³_{H,H} = 5.0 Hz, 4 H, C_{pip3,5}H₂); 1.34–1.31 (m, 2 H, C_{prop}H_{b,b'}); ³¹⁰ 1.20–1.17 (m, 2 H, C_{prop}H_{a,a'}). **δ**_C (125 MHz, 298 K, d₆-DMSO) [ppm] = 176.4 (s, C_{ar4}); 165.9 (s, COOH); 152.9 (d, J¹_{C,F} = 207.9 Hz, C_{ar6}); 148.2 (s, C_{ar2}); 144.3 (d, J²_{C,F} = 7.9 Hz, C_{ar7}); 139.1 (s, C_{ar8'}); 119.2 (d, J³_{C,F} = 5.9 Hz, C_{ar4'}); 111.2 (d, J²_{C,F} = 19.1 Hz, C_{ar5}); 108 (from HMBC, C_{ar3}); 106.8 (s, C_{ar8}); 46.8 (s, C_{pip2,6}); 42.8 (s, C_{pip3,5});

36.0 (s, C_{prop} H); 7.6 (s, C_{prop} H₂). δ_F (282 MHz, 298 K, d_6 -DMSO) [ppm] = -121.8 (s, 1 F, $C_{ar6}F$). **MS** (ES+, CH₃OH): m/z (%) = 332 (100) [HL + H⁺]. m/z (%)= 685 (100) [(HL)₂ + Na⁺]. **EA**: Anal. Calcd. (found) [%] for C_{17} H₁₈FN₃O₃: C, 61.62 (61.76); H, 5.48 (5.52); N, 12.68 (12.46).

2.2.4.2 Enoxacin, Henox

Appearance: off-white, fine crystalline solid. Mp: 226–228°C (yellow). UV-Vis (CH₃OH with 1.1% DMSO): λ [nm] (ϵ) [M⁻¹cm⁻¹] = 287 (14900), 345 (17800). **IR** (neat): $\tilde{\nu} \, [\mathrm{cm}^{-1}] = 3390 \, (\mathrm{md, \, br}), \, 2835 \, (\mathrm{st, \, br}), \, 2773 \, (\mathrm{md, \, br}), \, 2556 \, (\mathrm{md, \, br}), \, 1625 \, (\mathrm{st}), \, 1577$ (st), 1468 (sh), 1440 (st), triple crown motiv [1403 (md), 1365 (st), 1340 (st)], 1271 (st, br), 1172 (md), 1144 (md), 1107 (md), 1037 (md, br), 942 (st, br), 918 (sh), 826 (st), 790 (md), 729 (md, br), 681 (md), 639 (w), 622 (st), 546 (w), 561 (w), 524 (w), 474 (md, br). **NMR**: δ_H (300 MHz, 298 K, d₆-DMSO) [ppm] = 8.93 (s, 1 H, C_{ar2}H); 7.98 (d, $J_{H,F}^3 =$ 13.8 Hz, 1 H, $C_{ar5}H$); 4.45 (q, $J_{H,H}^3 =$ 7.1 Hz, 2 H, CH_2CH_3); 3.73 (dd, $J_{H,H}^3 =$ 5.9, 4.1 Hz, 4 H, $C_{pip2,6}H_2$); 2.84 (dd, $J_{H,H}^3 = 5.9$, 4.1 Hz, 4 H, $C_{pip3,5}H_2$); 1.37 (t, $J_{H,H}^3 = 7.1$ Hz, 3 H, CH₂CH₃). δ_C (75 MHz, 298 K, d₆-DMSO) [ppm] = 176.2 (d, $J_{C,F}^4$ = 2.3 Hz, C_{ar4}); 168.0 (s, $C_{ar8'}$); 165.9 (s, COOH); 149.9 (d, $J_{C,F}^2$ = 9.0 Hz, C_{ar7}); 147.5 (s, C_{ar2}); 146.2 $(\mathbf{d},\,J_{C,F}^1=\,204.2~\mathrm{Hz},\,C_{ar6});\,119.2~(\mathbf{d},\,J_{C,F}^2=\,22.0~\mathrm{Hz},\,C_{ar5});\,112.2~(\mathbf{d},\,J_{C,F}^3=\,3.6~\mathrm{Hz},\,C_{ar4'});\,112.2~\mathrm{Hz},\,C_{ar4'});\,112.2~\mathrm{Hz},\,C_{ar4'},\,C_{ar4'});\,112.2~\mathrm{Hz},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'});\,112.2~\mathrm{Hz},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'});\,112.2~\mathrm{Hz},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'});\,112.2~\mathrm{Hz},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{ar4'},\,C_{a$ 108.0 (s, C_{ar3}); 48.2 (d, $J_{C,F}^4$ = 7.9 Hz, $C_{pip2,6}$); 47.1 (s, CH_2CH_3); 45.6 (s, $C_{pip3,5}$); 14.6 (s, CH₂CH₃). δ_F (282 MHz, 298 K, d_6 -DMSO) [ppm] = -127.3 (s, 1 F, C_{ar6}F). MS (ES+): m/z (%) = 321 (100) [HL + H⁺]. m/z (%)= 664 (100) [(HL)₂ + Na⁺]. **EA**: Anal. Calcd. (found) [%] for $C_{15}H_{17}FN_4O_3 \cdot 1.5 H_2O$: C, 51.87 (51.60); H, 5.80 (5.67); N, 16.13 (15.86).

2.2.4.3 Fleroxacin, Hflex

Appearance: white solid. Mp: 260°C (decomposed, pale yellow). UV-Vis (CH₃OH with 1.5% DMSO): λ [nm] (ϵ) [M⁻¹cm⁻¹] = 294 (27500), 320 (12400), 330 (11500). IR

(neat): $\tilde{\nu}$ [cm⁻¹] = 3054 (md), 2941 (md), 2796 (md), 1716 (st), 1622 (st), 1556 (md), 1542 (sh), 1513 (md), 1474 (st, br), 1447 (st), 1408 (md), 1390 (w), 1375 (md), 1360 (md), 1327 (md), 1279 (st, br), 1244 (md), 1228 (md), 1214 (md), 1205 (md), 1142 (st), 1122 (sh), 1098 (md), 1061 (st), 1036 (st), 1019 (sh), 1010 (st), 970 (st), 941 (st), 925 (st), 869 (md), 852 (md), 816 (sh), 806 (st), 783 (md), 754 (sh), 741 (st), 672 (w), 656 (md, br), 573 (md), 550 (md), 532 (w), 504 (md), 450 (md, br). **NMR**: δ_H (600 MHz, 298 K, d₆-DMSO) [ppm] = 14.77 (br s, 1 H, COOH); 8.84 (s, 1 H, C_{ar2}H); 7.86 (d, $J_{H,F}^3 = 11.9$ Hz, 1 H, $C_{ar5}H$; 4.97–4.84 (m, 4 H, (CH₂)₂); 3.34 (br s, 4 H, $C_{pip2,6}H_2$, overlaid with water); 2.45 (br s, 4 H, C_{pip3,5}H_2); 2.23 (s, 3 H, CH_3). $\pmb{\delta_C}$ (125 MHz, 298 K, d_6-DMSO) [ppm] = 175.6 (s, C_{ar4}); 165.5 (s, COOH); 154.5 (d, $J_{C,F}^1 = 208.2$ Hz, C_{ar6}); 152.1 (s, C_{ar2}); 146.0 (d, $J_{C,F}^1 =$ 212.2 Hz, C_{ar8}); 133.7 (two overlapping d, $J_{C,F}^2 =$ 13.9, 11.7 Hz, C_{ar7}); 127.3 (d, $J_{C,F}^2 = 9.1 \text{ Hz}, C_{ar8'}); 120.1 \text{ (d}, J_{C,F}^3 = 35.1 \text{ Hz}, C_{ar4'}); 107.0 \text{ (d}, J_{C,F}^2 = 19.2 \text{ Hz}, C_{ar5}); 106.0 \text{ (d}, J_{C,F}^2 = 19.2 \text{ Hz}, C_{ar5});$ (from HMBC, C_{ar3}); 82.1 (d, $J_{C,F}^1 = 138.2$ Hz, CH_2CH_2F); 57.8 (two overlapping d, $J_{C,F} = 138.2$ Hz, $J_{C,F} = 138.2$ Hz, 15.8, 12.2 Hz, CH_2CH_2F); 55.1 (s, $C_{pip2,6}$); 50.3 (s, $C_{pip3,5}$); 46.0 (s, CH_3). δ_F (282 MHz, 298 K, $d_6\text{-}\mathrm{DMSO})\;[\mathrm{ppm}]=\text{-}119.2\;(\mathrm{d},\,J_{F,F}^4=11.9\;\mathrm{Hz},\,1\;\mathrm{F},\,\mathrm{C}_{ar6}F);\,\text{-}127.6\;(\mathrm{q},\,J_{F,F}=5.9\;\mathrm{Hz},\,1\,\mathrm{HZ},\,1\,\mathrm{HZ}$ 1 F, C_{ar8}F); -224.1 (d, $J_{F,F}^6$ = 5.9 Hz, 1 F, (CH₂)₂F). **MS** (ES+): m/z (%) = 370 (100) $[\text{HL} + \text{H}^+]$, 762 (80) $[(\text{HL})_2 + \text{Na}^+]$. **EA**: Anal. Calcd. (found) [%] for C₁₇H₁₈F₃N₃O₃: C, 55.28 (55.14); H, 4.91 (4.90); N, 11.38 (10.98).

2.2.4.4 Levofloxacin, Hlevox

Appearance: pale yellow solid. Mp: 224–226°C (dark brown). UV-Vis (CH₃OH with 1.5% DMSO): λ [nm] (ϵ) [M⁻¹cm⁻¹] = 299 (25100), 318 (10200). IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3247 (md, br), 2935 (md), 2884 (md), 2848 (md), 2802 (md), 1720 (st), 1619 (st), 1538 (md), 1518 (md), 1492 (md), 1468 (sh), 1439 (st, br), 1414 (sh), 1394 (st), 1359 (md), 1340 (st), 1315 (sh), 1289 (st), 1240 (st), 1207 (sh), 1195 (md), 1163 (md), 1136 (st), 1116 (md), 1086 (st, br), 1066 (sh), 1048 (md), 1004 (st), 963 (sh), 951 (md, br), 903 (sh), 873 (st),

839 (md), 800 (st), 778 (md), 755 (md), 741 (st), 727 (md), 695 (w), 666 (w), 650 (st), 578 (w), 559 (st), 490 (md), 459 (md). **NMR**: δ_{H} (600 MHz, 298 K, *d*₆-DMSO) [ppm] = 15.20 (br s, 1 H, COO*H*); 8.96 (s, 1 H, C_{*ar*2}*H*); 7.56 (d, $J_{H,F}^{3}$ = 12.4 Hz, 1 H, C_{*ar*5}*H*); 4.91 (d, $J_{H,H}$ = 6.8 Hz, 1 H, C*H*); 4.58 (dd, $J_{H,H}$ = 11.5, 1.7 Hz, 1 H) and 4.36 (dd, $J_{H,H}$ = 11.5, 2.3 Hz, 1 H) (OC*H*₂CH); 3.33-3.25 (m, 4 H, C_{*pip*2,6}*H*₂); 2.43 (br s, 4 H, C_{*pip*3,5}*H*₂); 2.23 (s, 3 H, NC*H*₃); 1.44 (d, $J_{H,H}^{3}$ = 3.3 Hz, 3 H, CHC*H*₃). δ_{C} (125 MHz, 298 K, *d*₆-DMSO) [ppm] = 176.4 (s, *C*_{*ar*4}); 166.1 (s, *C*OOH); 155.5 (d, $J_{C,F}^{1}$ = 206.3 Hz, *C*_{*ar*6}); 146.2 (s, *C*_{*ar*2}); 140.1 (d, $J_{C,F}^{3}$ = 5.7 Hz, *C*_{*ar*8}); 132.1 (d, $J_{C,F}^{2}$ = 11.9 Hz, *C*_{*ar*7}); 124.8 (s, *C*_{*ar*8'}); 119.6 (d, $J_{C,F}^{3}$ = 7.7 Hz, *C*_{*ar*4'}); 106.6 (s, *C*_{*ar*3}); 103.3 (d, $J_{C,F}^{2}$ = 20.4 Hz, *C*_{*ar*5}); 68.0 (s, OCH₂CH); 55.3 (s, *C*_{*pip*2,6}); 54.8 (s, CH); 50.1 (s, *C*_{*pip*3,5}); 46.1 (s, NCH₃); 17.9 (s, CH(*C*H₃)). δ_{F} (282 MHz, 298 K, *d*₆-DMSO) [ppm] = -120.2 (s, 1 F, C_{*ar*6}*F*). **MS** (ES+): *m/z* (%) = 362 (100) [HL + H⁺]. *m/z* (%) = 541 (100), 745 (60) [(HL)₂ + Na⁺], 1173 (30). **EA**: Anal. Calcd. (found) [%] for C₁₈H₂₀FN₃O₄: C, 59.83 (59.44); H, 5.58 (5.66); N, 11.63 (11.43).

2.2.4.5 Lomefloxacin, Hlomx

Appearance: white solid. Mp: >260°C. UV-Vis (CH₃OH with 1.1% DMSO): λ [nm] (ϵ) [M⁻¹cm⁻¹] = 291 (34500), 320 (15800), 332 (13100). IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3055 (w), 2936 (md), 2842 (w), 2756 (sh), 2698 (st, br), 2456 (md), 1721 (st), 1611 (st), 1543 (sh), 1524 (md), 1491 (st), 1471 (sh), 1448 (st, br), 1411 (w), 1392 (st), 1328 (st), 1299 (md), 1281 (md), 1253 (st), 1205 (st), 1182 (w), 1166 (w), 1141 (md), 1114 (md), 1093 (st), 1065 (w), 1041 (st), 1021 (md), 1006 (st), 979 (md), 928 (st), 892 (st), 844 (md), 821 (md), 807 (st), 791 (sh), 756 (sh), 738 (st), 653 (md), 578 (w), 555 (md), 545 (md), 534 (md), 513 (st), 488 (md), 476 (md), 452 (md). NMR: δ_H (600 MHz, 298 K, D₂O) [ppm] = 8.55 (s, 1 H, C_{ar2}H); 7.46 (d, $J_{H,F}^3$ = 11.4 Hz, 1 H, C_{ar5}H); 4.48 (d, $J_{H,H}^3$ = 6.0 Hz, 2H, CH₂CH₃); 3.70-3.53 (m, 5 H, C_{pip2,6}H₂ and C_{pip3}H); 3.42-3.38 (m, 2 H, C_{pip4}H₂); 1.49 (t, $J_{H,H}^3$ = 7.1 Hz, 3 H, CH₂CH₃); 1.40 (d, $J_{H,H}^3$ = 6.6 Hz, 3 H, CH₃). δ_C (125 MHz, 298 K, d_6 -DMSO) [ppm] = 175.3 (s, C_{ar4}); 168.3 (COOH); 154.9 (d, $J_{C,F}^1$ = 208.6 Hz, C_{ar6}); 150.6 (s, C_{ar2}); 146.1 (d, $J_{C,F}^1$ = 210.3 Hz, C_{ar8}); 133.0 (two overlapping d, $J_{C,F}^2$ = 11.6, 11.6 Hz, C_{ar7}); 127.0 (d, $J_{C,F}^2$ = 5.6 Hz, $C_{ar8'}$); 120.8 (d, $J_{C,F}^3$ = 7.3 Hz, $C_{ar4'}$); 106.8 (d, $J_{C,F}^2$ = 19.1 Hz, C_{ar5}); 106.1 (s, C_{ar3}); 55.0 (d, $J_{C,F}^4$ = 13.5 Hz, C_{pip2}); 53.4 (s, CH₂CH₃); 51.7 (s, C_{pip6}); 46.7 (s, C_{pip3}); 43.5 (s, C_{pip5}); 15.3 (s, CH₂CH₃); 14.9 (s, CH₃). δ_F (282 MHz, 298 K, d_6 -DMSO) [ppm] = -118.6 (d, $J_{F,F}^4$ = 10.7 Hz, 1 F, $C_{ar6}F$); -128.6 (d, $J_{F,F}^4$ = 11.3 Hz, 1 F, $C_{ar8}F$). **MS** (ES+): m/z (%) = 352 (100) [HL + H⁺]. m/z (%)= 769 (100), 725 (60) [(HL)₂ + Na⁺], 1143 (30). **EA**: Anal. Calcd. (found) [%] for $C_{17}H_{20}F_2N_3O_3 \cdot 1$ HCl: C, 52.65 (52.79); H, 5.20 (5.17); N, 10.84 (10.56).

2.2.4.6 Nalidixic acid, Hnxa

Appearance: white solid. **Mp**: 228–230°C (soft pink). **UV-Vis** (CH₃OH with 1.1% DMSO): λ [nm] (ε) [M⁻¹cm⁻¹] = 320 (13500), 328 (13700). **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3044 (md, br), 2987 (w, br), 2948 (w), 1707 (st, br), 1614 (st, br), 1562 (w), 1538 (w), 1518 (md), 1465 (sh), 1440 (st, br), 1384 (w), 1370 (md), 1353 (md), 1327 (w), 1294 (md), 1270 (sh), 1252 (st), 1227 (st), 1129 (st), 1102 (w), 1051 (w), 1034 (w), 971 (st, br), 875 (md), 803 (st), 777 (sh), 706 (md), 656 (md), 634 (md), 563 (w), 539 (md), 505 (w), 485 (st), 454 (md). **NMR**: **δ**_H (300 MHz, 298 K, d₆-DMSO) [ppm] = 9.18 (s, 1 H, C_{ar2}H); 8.60 (d, $J_{H,H}^3$ = 8.2 Hz, 1 H, C_{ar5}H); 7.59 (d, $J_{H,H}^3$ = 8.2 Hz, 1 H, C_{ar2}H); 4.64 (q, $J_{H,H}^3$ = 7.1 Hz, 2 H, CH₂CH₃); 2.71 (s, 3 H, CH₃); 1.42 (t, $J_{H,H}^3$ = 7.1 Hz, 3 H, CH₂CH₃). **δ**_C (75 MHz, 298 K, d₆-DMSO) [ppm] = 178.2 (s, C_{ar4}); 165.6 (s, C_{ar8'}); 164.7 (s, COOH); 149.7 (s, Car2); 148.3 (s, C_{ar7}); 135.6 (s, C_{ar4}); 122.6 (s, C_{ar6}); 118.3 (s, C_{ar4}); 108.6 (s, C_{ar3}); 46.8 (s, CH₂CH₃); 2.5.1 (s, CH₃); 1.5.0 (s, CH₂CH₃). **MS** (ES+): m/z (%) = 255 (100) [HL + Na⁺], 233 (60) [HL + H⁺]. m/z (%) = 786 (100) [(HL)₃ + Na⁺], 1040 (30) [(HL)₄ + Na⁺], 1294 (10) [(HL)₅ + Na⁺]. **EA**: Anal. Calcd. (found) [%] for C₁₂H₁₂N₂O₃: C, 62.06 (62.32); H, 5.21 (5.18); N, 12.06 (11.94).

2.2.4.7 Norfloxacin, Hnofx

Appearance: pale vellow solid. Mp: $221-223^{\circ}$ C (vellow). UV-Vis (CH₃OH with 1.5% DMSO): λ [nm] (ϵ) [M⁻¹cm⁻¹] = 290 (21900), 317 (9900), 330 (8700). **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3046 (w, br), 2944 (md), 2827 (md, br), 1722 (st), 1614 (st), 1519 (md), 1471 (st), 1439 (st), 1401 (w), 1373 (md), 1350 (md), 1323 (w), 1300 (md), 1272 (w), 1248 (st), 1210 (md), 1198 (st), 1148 (md), 1127 (md), 1102 (st), 1090 (sh), 1050 (w), 1025 (md), 978 (w), 945 (st, br), 885 (st), 838 (st), 826 (sh), 804 (st), 783 (md), 748 (st), 712 (w), 698 (st), 664 (md), 638 (md), 620 (md), 557 (md), 514 (md), 498 (md), 485 (w), 450 (md). NMR: δ_H (300 MHz, 298 K, d_6 -DMSO) [ppm] = 8.92 (s, 1 H, $C_{ar2}H$); 7.85 (d, $J_{H,F}^3$ = 13.5 Hz, 1 H, $C_{ar5}H$); 7.12 (d, $J_{H,F}^4$ = 7.3 Hz, 1 H, $C_{ar8}H$); 4.57 (q, $J_{H,H}^3$ = 7.1 Hz, 2 H, CH_2CH_3); $3.22 \text{ (dd, } J^3_{H,H} = 5.9, \, 3.8 \text{ Hz}, \, 4 \text{ H}, \, \mathbf{C}_{pip2,6}H_2); \, 2.89 \text{ (dd, } J^3_{H,H} = 5.9, \, 3.8 \text{ Hz}, \, 4 \text{ H}, \, \mathbf{C}_{pip3,5}H_2);$ 1.41 (t, $J_{H,H}^3$ = 7.1 Hz, 3 H, CH₂CH₃). δ_C (75 MHz, 298 K, d₆-DMSO) [ppm] = 176.1 (d, $J_{C,F}^4 = 2.7$ Hz, C_{ar4}); 166.1 (s, COOH); 152.5 (d, $J_{C,F}^1 = 207.5$ Hz, C_{ar6}); 148.3 (s, C_{ar2}); 145.9 (d, $J_{C,F}^2 = 9.7$ Hz, C_{ar7}); 137.2 (s, $C_{ar8'}$); 118.9 (d, $J_{C,F}^3 = 7.7$ Hz, $C_{ar4'}$); 111.0 (d, $J_{C,F}^2 = 23.1$ Hz, C_{ar5}); 107.0 (s, C_{ar3}); 105.4 (d, $J_{C,F}^3 = 3.7$ Hz, C_{ar8}); 50.8 (d, $J_{C,F}^4 = 4.8$ Hz, $C_{pip2,6}$); 49.0 (s, CH_2CH_3); 45.4 (s, $C_{pip3,5}$); 14.3 (s, CH_2CH_3). δ_F (282 MHz, 298 K, d_{6} -DMSO) [ppm] = -121.3 (s, 1 F, C_{ar6}F). **MS** (ES+): m/z (%) = 320 (100) [HL + H ⁺]. m/z (%)= 662 (70) [2 HL + Na⁺]. **EA**: Anal. Calcd. (found) [%] for C₁₆H₁₈FN₃O₃: C, 60.18 (60.02); H, 5.68 (5.75); N, 13.16 (12.92).

2.2.4.8 Oxolinic acid, Hoxa

Appearance: white solid. Mp: >260°C. UV-Vis (CH₃OH with 2.2% DMSO): λ [nm] (ϵ) [M⁻¹cm⁻¹] = 322 (7600), 336 (7700). IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3061 (md), 2984 (md), 2930 (md, br), 1698 (st), 1632 (st), 1573 (st), 1504 (md), 1440 (st, br), 1384 (md), 1350 (sh), 1301 (md) 1259 (st), 1222 (w), 1204 (w), 1186 (md), 1127 (md), 1094 (w), 1075 (md), 1036 (st), 936 (st, br), 876 (st), 856 (st), 807 (st), 773 (md), 754 (md), 690 (md), 645 (st), 605 (md), 556 (md), 498 (md), 447 (md). **NMR**: δ_{H} (300 MHz, 298 K, d_{6} -DMSO) [ppm] = 8.89 (s, 1 H, C_{ar2}H); 7.63 (s, 1 H, C_{ar8}H); 7.61 (s, 1 H, C_{ar5}H); 6.29 (s, 2 H, OCH₂O); 4.53 (q, $J_{H,H}^{3}$ = 7.1 Hz, 2 H, CH₂CH₃); 1.38 (t, $J_{H,H}^{3}$ = 7.1 Hz, 3 H, CH₂CH₃). δ_{C} (75 MHz, 298 K, d_{6} -DMSO) [ppm] = 176.0 (s, C_{ar4}); 166.3 (s, COOH); 153.7 (s, C_{ar7}); 147.10 (s, C_{ar6}); 147.0 (s, C_{ar2}); 136.9 (s, $C_{ar8'}$); 121.3 (s, $C_{ar4'}$); 107.3 (s, C_{ar3}); 103.3 (s, OCH₂O); 101.8 (s, C_{ar5}); 97.2 (s, C_{ar8}); 49.6 (s, CH₂CH₃); 14.6 (s, CH₂CH₃). **MS** (ES+): m/z (%) = 284 (100) [HL + Na⁺], 262 (10) [HL + H⁺]. m/z (%) = 589 (90) [(HL)₂ + Na⁺], 873 (100) [(HL)₃ + Na⁺], 1156 (30) [(HL)₄ + Na⁺]. **EA**: Anal. Calcd. (found) [%] for C₁₃H₁₁NO₅: C, 59.77 (59.83); H, 4.24 (4.24); N, 5.36 (5.37).

2.2.4.9 Pipemidic acid, Hpia

Appearance: white, fine powdered solid. Mp: 258–260°C (orange-brown). UV-Vis (CH₃OH with 2.2% DMSO): λ [nm] (ϵ) [M⁻¹cm⁻¹] = 288 (13500), 325 (7300), 342 (5400). IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3365 (md, br), 3028 (w), 2979 (w), 1615 (st), 1577 (st), 1532 (md), 1510 (md), 1471 (st), 1429 (st), 1378 (sh), 1357 (st, br), 1309 (md), 1279 (md), 1238 (st, br), 1259 (w), 1159 (w), 1147 (w), 1127 (st), 1092 (md), 1078 (md), 1044 (md) 1022 (st), 975 (md), 940 (md), 914 (st), 903 (md), 867 (md), 832 (st), 802 (md), 783 (md), 743 (st), 715 (md), 655 (w), 608 (w), 540 (st), 489 (md), 453 (md). NMR: δ_H (300 MHz, 298 K, d_6 -DMSO) [ppm] = 9.15 (s, 1 H, C_{ar5}H); 8.93 (s, 1 H, C_{ar2}H); 4.36 (q, $J_{H,H}^3$ = 7.1 Hz, 2 H, CH₂CH₃); 3.84 (d, $J_{H,H}^3$ = 17.1 Hz, 4 H, C_{pip2,6}H₂); 2.78 (br s, 4 H, C_{pip3,5}H₂); 1.35 (t, $J_{H,H}^3$ = 7.1 Hz, 3 H, CH₂CH₃). δ_C (75 MHz, 298 K, d_6 -DMSO) [ppm] = 177.1 (s, C_{ar4}); 165.3 (s, COOH); 160.5 (C_{ar7}); 160.1 (s, C_{ar5}); 155.1 (s, C_{ar8'}); 150.6 (s, C_{ar2}); 109.5 (s, C_{ar4'}); 108.3 (s, C_{ar3}); 45.8 (s, CH₂CH₃); 45.3 (br s, C_{pip2,3,5,6}); 14.4 (s, CH₂CH₃). MS (ES+): m/z (%) = 304 (50) [HL + H⁺], 326 (70) [HL + Na⁺], 348 (100) [HL + CO₂ + H⁺]. m/z (%) = 673 (40) [(NaL)₂ + Na⁺], 999 (95) [(NaL)₃ + Na⁺]. EA: Anal. Calcd.

(found) [%] for $C_{14}H_{17}N_5O_3$: C, 55.44 (55.10); H, 5.65 (5.63); N, 23.09 (22.96).

2.2.5 Stability in Iso-Sensitest Broth

On the day of the experiment, stock solutions of the quinolones were prepared in methanol with small amounts of DMSO ($\leq 2\%$) to ensure full dissolution. From these stock solutions, test solutions were prepared in Iso-Sensitest broth (5.9 g in 500 mL deionized water, autoclaved) via dilution to a final concentration of 0.1 mM quinolone. The amount of DMSO and the final concentration of the test solutions matched the conditions of the single-disk diffusion test (Section 2.2.6). UV-Vis spectra of each of the respective quinolone test solutions (in alphabetical order) were recorded at the following time points: 20 min, 1 h, 2 h, 4 h, 8 h, 18 h, 20 h, and 24 h. The UV-Vis spectrum of the Iso-Sensitest broth served as the blank. To avoid any contamination of the biological growth medium, which could alter the UV-Vis test results, the Iso-Sensitest test solutions were prepared in a biological safety cabinet in UBC's Biological Services Laboratory. Each test solution was transferred into a UV-Vis cuvette, and the cuvette opening was tightly covered with parafilm. All single-steps (dilution, mixing, transferring, parafilm wrapping, labelling, transport to UV-Vis spectrophotometer) took some time so that the first time point could only be measured approximately 20 min after the initial dilution step. On the following day, respective test solutions in water (0.1 mM) were made up from the same quinolone stock solutions, and the UV-Vis stability study was repeated with these quinolone-water solutions at time points of 0 min (immediately after dilution), 30 min, 1 h, 2 h, 4 h, 18 h, 20 h, and 24 h. Because the quinolone-water test solutions were not as sensitive to biological contamination as were the test solutions in Iso-Sensitest broth, these test solutions were diluted, mixed, and transferred into the cuvette directly next to the UV-Vis spectrometer, which allowed the measurement of an approximate 0 min time point.

2.2.6 Antimicrobial Susceptibility Single-Disk Test in Iso-Sensitest Medium

The antimicrobial activities of the selected quinolones were evaluated according to the agar diffusion single-disk testing method. The work was performed in UBC's Biological Services Laboratory, a biological level II facility, following respective operating and safety procedures. Iso-Sensitest media were prepared according to the manufacturer's specifications. Agar plates of 150 mm diameter were poured with an approximate height of 4 mm. Bacteria were grown in 5 mL broth (Falcon tube) at 37 $^{\circ}$ C on a shaker to an OD₆₀₀ of ≥ 1 . On the day of the experiment, the quinolone test solutions were prepared in methanol and DMSO to ensure complete solubility (max. 2% DMSO) alongside one pure methanol and one 2%-DMSO-methanol solution as controls. Agar plates were inspected for signs of degradation, and placement positions of the disks were marked at the bottom of the petri dish with a minimum distance between each disk (center to center) of at least 24 mm and with not more than 14 disk positions total. The following steps were done in triplicate. Paper filter disks were loaded with 20 μ L of each test and control solution and left to dry for about 5 min. While these were drying, the previously prepared agar plates were inoculated with 0.5 mL bacteria growth broth that was spread evenly across the plate. The loaded filter disks, including the two control disks, were placed on the marks and carefully pressed onto the agar. The lid was put back onto the petri dish, the sides of the petri dish were sealed with parafilm, and the petri dish was placed up-side-down in the incubator at 37 °C for 20 h. After this time, the plates were taken from the incubator, placed on a nonreflecting black surface, and the no-growth zone around each disk on each of the plates was measured with a ruler with the naked eye. As it is convention, the inhibition zone sizes were recorded as diameters rounded to the nearest millimeter with the diameter of each disk being included in the measurement. Growth up to the edge of a disk was evaluated

as 0 mm.

2.3 Results & Discussion

2.3.1 Chemical Characterization

All quinolones were dissolved in methanolic solutions with 20% DMSO content and diluted in methanol to appropriate UV-Vis concentrations. Depending on the nature of the aromatic core and the number of fluorine-substituents, a quinolone test concentration between $3 \cdot 10^{-5}$ M to $6 \cdot 10^{-5}$ M resulted in absorbance maxima between 1.3-0.5 AU over the studied range from 190 to 1100 nm wave numbers. The absorbance maxima of the selected nine quinolones are summarized in Table 2.1. All quinolones gave a broad absorbance band between 300-380 nm with a long tail. In addition, the quinolones with a 1,4-piperazinyl ring in C_{ar7} position on the condensed aromatic ring system showed a second sharp absorbance band of high intensity at lower wave numbers between 280-300 nm. These observations are not surprising, as from the molecular structure of the quinolones (Figure 2.1), one chromophore is expected for the condensed aromatic ring system with N_{ar1} (chromophore I), plus the 1,4-piperazinyl ring on C_{ar7} represents a second chromophore in respectively substituted quinolones (chromophore II).³¹¹

The first absorbance maximum (280–300 nm) is related to the energy absorption of the aromatic core, while the second absorbance maximum (300–380 nm) is composed of two sub-peaks and has been assigned to the $n \to \pi *$ (HOMO-LUMO) electronic transition.³¹² These two sub-peaks reflect two different types of hydrogen bonds forming, an intramolecular hydrogen bond between the carbonyl group in C_{ar4} position and the carboxylic acid group in C_{ar3} position as well as an intermolecular one between the quinolone molecule and residual water molecules in the organic solvent.^{312 313} The 1,4-piperazinyl ring on C_{ar7} has a strong effect on the electronics of the condensed aromatic ring system, as in quinolones

quinolone	A_{max1} [nm]	A_{max}	2 [nm]
Hcipro	289	317	331
Henox	287	*	345
Hflex	294	320	330
Hlevox	299	318	*
Hlomx	291	320	332
Hnxa	*	320	328
Hnofx	290	317	330
Hoxa	*	322	336
Hpia	288	325	342

Table 2.1: UV-Vis absorbance maxima (A_{max} [nm]) in methanol ($\leq 2\%$ DMSO) solution

*not observed. [Hquino] = $3-6 \ 10^{-5}$ M for intensity A_{max2} = 0.5-0.8 AU.

without this substituent, such as nalidixic acid and oxolinic acid, the strong first absorbance band was not observed. The second absorption maximum is highly affected by the chosen UV-Vis solvent,³¹⁴ because acetonitrile, methanol, water, or any mixtures thereof alter the requisite for hydrogen bond formation, which is as well influenced by pH. In Figure 2.2, the recorded UV-Vis spectra of ciprofloxacin over the pH range from 2 to 11 in an aqueous sodium chloride solution (0.16 M) are presented. Although hypso- and hyper-chromic effects can be observed for the first absorbance maximum between 260 to 290 nm wavelength from acidic to basic pH, the second absorbance band is the most affected by the pH changes. Here, bathochromic and hyperchromic effects are dominant when comparing acidic to basic pH; in addition, the two subpeaks become more defined and of equal value at a pH of 7 and higher. At 275 nm, 305 nm, and 346 nm, lie isobestic points. During the titration with sodium hydroxide, the deprotonation equilibria between pH 4.5 to 8 were slow. The acid-base equilibria of ciprofloxacin are drawn in Figure 2.3. Because the deprotonation of the N-atom in 4-position of the 1,4-piperazinyl ring in ciprofloxacin leads to a zwitterionic state in the neutral pH range (Figure 2.3),³¹⁵ this observation corresponds well to the major changes of the molecular structure occurring in this pH region, which



Figure 2.2: pH dependency of ciprofloxacin ([Hcipro] = $2 \cdot 10^{-5}$ M, pH 2–11, $I_{NaCl} = 0.16$ M).

are as well reflected in the changes dominating the 300 to 340 nm region in the recorded UV-Vis spectra (Figure 2.2). Previously reported additional protonation of the N_{ar1}-atom in the acidic pH range (pH \leq 3) was not observed.³¹⁶

The molecular structure of the quinolone drugs with the carboxyl functional group in C_{ar3} -position and the carbonyl functional group in C_{ar4} -position lends nice handles to spectroscopic analysis in the mid-infrared region (4000–400 cm⁻¹, Figure 2.4). The CO stretching vibration of the carboxyl group, ν_{COOH} , was observed around 1715 cm⁻¹ and the CO deformation vibration, δ_{COOH} , around 1350 cm⁻¹. As previously clarified,³¹⁷ ionic carboxylates, such as ciprofloxacin in its zwitterionic state (Figure 2.3), do not show a ν_{COOH} stretching vibration;³¹⁸ instead, two characteristic bands in the range of 1650–1510 cm⁻¹ and 1400–1280m⁻¹ were observed that were assigned as the asymmetric and symmetric ν_{OCO} stretching vibrations in agreement with the literature: 1587 cm⁻¹ and 1372 cm⁻¹



Figure 2.3: Protonation equilibria of ciprofloxacin.

for ciprofloxacin, 317 1577 cm⁻¹ and 1365 cm⁻¹ for enoxacin, 319 as well as 1577 cm⁻¹ and 1357 cm⁻¹ for pipemidic acid, 320 respectively.

Other highly characteristic IR features of these drug molecules are the C=C stretching vibration of the conjugated aromatic ring system, $\nu_{C=C}$, around 1620 cm⁻¹ as well as the stretching of the aromatic quinolone core, $\nu_{C=N}$, around 1400 cm⁻¹ and the C-H bending stretch, δ_{C-H} , in the range of 1440–1500 cm⁻¹. Oxolinic acid possesses with the pentacyclic ether (1,3-dioxolane) a unique structural feature, which gives a strong absorbance stretch of the C–O vibration at 1036 cm⁻¹. For a detailed discussion of FT-Raman spectroscopic characterizations of quinolones, the avid reader is referred to Neugebauer *et al.*³¹².

The ¹H, ¹³C, and ¹⁹F NMR spectral assignments of the selected nine quinolone antimic crobial agents are presented in Tables 2.2, 2.3, and 2.4 respectively. The resonances were allocated with confidence from recorded data at 300 or 600 MHz for the ¹H nucleus, 75 or 125 MHz for the ¹³C nucleus, and 128 MHz for the ¹⁹F nucleus as applicable, in addition to COSY, HSQC, and HMBC 2D-experiments. All samples were dissolved in deuterated



Figure 2.4: IR spectra of the nine quinolones.

dimethyl sulfoxide with the help of sonicating and heating, only for lomefloxacin hydrochloride deuterated water seemed to be the more appropriate solvent, in which it dissolved readily.

In ¹H NMR measurements, carboxylic protons protons were not observed in the standard range from 0 to 10 ppm, but could be detected in the spectra recorded in d_6 -DMSO at 600 MHz frequency in the lower field range as extremely broad singlets around 14.68 ppm (Hcipro), 14.77 ppm (Hflex), and 15.20 ppm (Hlevox); in contrast to literature reports, ³²¹ no tertiary nitrogen protons from the piperazinyl-substituent in C_{ar7} could be noted up to 23 ppm. In an earlier NMR study of selected gyrase inhibitors in acidic and basic solutions, Holzgrabe *et al.* showed that the deprotonation of the carboxyl group only affects the C_{ar3} atom and the carboxyl-C itself, while the protonation of the nitrogen atom of the piperazinyl-group only influences the C-atoms of the 1,4-piperazinyl-ring on C_{ar7}.³²²

From the ¹H NMR data summary in Table 2.2, three major observations can be made. Firstly, the introduction of a second nitrogen atom in C_{ar8} -position has only a small influence on the aromatic proton on C_{ar5} , as the comparison of norfloxacin (quinoline core, $C_{ar5}H$ at 7.85 ppm) vs. enoxacin (naphthyridine core, $C_{ar5}H$ at 7.98 ppm) reveals. Secondly, in the 6-fluoroquinolones, the fluorine couples not only with the vicinal aromatic proton on C_{ar5} ($J_{H,F}^3 = 11.9-13.8$ Hz) but as well with long-range $C_{ar8}H$ ($J_{H,F}^4 = 7.3-7.4$ Hz). Thirdly, cyclic alkyl-substituents on N_{ar1} rotate fast in solutions at room temperature, reflected in the more complex coupling patterns observed for ciprofloxacin and levofloxacin. The methylene groups in Hcipro are chemically not equivalent, as they give each one multiplet in the ¹H NMR measurements, resulting in the triplet of triplet pattern of the vicinal methine proton ($J_{H,H}^3 = 7.2, 3.7$ Hz). According to earlier NMR studies by Zieba *et al.*, the methylene protons were assigned as $H_{a,a'} = 1.20-1.17$ ppm and $H_{b,b'} = 1.34-1.31$ ppm.³¹⁰ In Hlevox, the methylene protons on the hexane ring, which connects to the quinoline
aromatic core through N_{ar1} and C_{ar8} , gave each a doublet of doublets with 4.58 ppm (dd, $J_{H,H}^3 = 11.5$, 1.7 Hz) and 4.36 (dd, $J_{H,H}^3 = 11.5$, 2.3 Hz). This indicates a rapid folding movement of the hexane ring in solution at room temperature; an observation that can as well be noted in the six-membered piperazinyl-ring on C_{ar7} , which gives doublet of doublets in ¹H NMR spectra of Henox and Hnofx ($J_{H,H}^3 = 5.9$, 3.8–4.1 Hz) or more complex coupling patterns resulting in multiplets in ¹H NMR spectra of Hlevox or Hlomx (Table 2.2). Opposite to the cyclic alkyl-substituents, the ethyl-chains on N_{ar1} move freely and were detected in the ¹H NMR measurements as characteristic quintet pattern of the methylene protons (q, $J_{H,H}^3 = 7.1$ Hz) and triplet pattern of the methyl protons (t, $J_{H,H}^3 = 7.1$ Hz) with matching coupling constants.³²³

The summarized results of the conducted ¹³C NMR measurements (Table 2.3) show clearly the difference in electronegativity of the condensed aromatic ring system depending on, if the aromatic core is a quinoline, a naphthyridine, or a [2,3-d]-pyrimidine, and the introduction of possibly one or two fluorine substituents on C_{ar6} and C_{ar8} . While the carboxylic-C and C_{ar2} to C_{ar4} are barely affected, the largest changes in chemical shifts were recorded for C_{ar6} and $C_{ar8'}$. The latter is shifted downfield in naphthyridines (>160 ppm) compared to quinolines (around 100 ppm). Although the effect of the 6-fluorine substituent is most strongly felt on C_{ar6} ($J_{C,F}^1$ around 207 ppm), its influence spreads over three bonds across the substituted ring and even into the piperazinyl-substituent on C_{ar7} in bi-fluorinated species (C_{ar6} , C_{ar8}), as the respective coupling constants ($J_{C,F}$) reveal. Overall, the determined $J_{C,F}$ coupling constants correspond well with reported literature values.^{322 324} Comparing the coupling of the fluorine atoms with the carbon atoms, the fluorine-substituted C_{ar6} -atom possesses a high carbon-fluorine coupling constant of $J_{C_{ar6},F}^1 = 206.3-207.9$ Hz (lit. 245.3 Hz)³²⁴ in mono-fluorinated species. The vicinal C_{ar5} atom couples to fluorine at $J_{C_{ar5},F}^2 = 19.1-23.1$ Hz (lit. 21.0 Hz)³²⁴, while the coupling with the other vicinal C_{ar7} -atom is slightly reduced in frequency probably due to the piperizylsubsituent in 7-position $J_{C_{ar7},F}^2 = 9.7-11.9$ Hz. Moreover, the $C_{ar4'}$ -atom couples to fluorine at $J_{C_{ar4'},F}^3 = 5.9-7.7$ Hz (lit. 7.7 Hz)³²⁴, while the fluorine coupling constants of the C_{ar8} atom with $J_{C_{ar8},F}^3 = 3.7-5.7$ Hz are slightly lower, possibly an influence of the N_{ar1} -atom close by. In cases where a coupling to fluorine could be detected for the carbonyl- C_{ar4} , its coupling constant was $J_{C_{ar4},F}^3 = 2.3-2.7$ Hz (lit. 3.3 Hz).³²⁴

Furthermore, it should be noted that the detection of the C_{ar3} signal even at high frequency (600 MHz) proved to be difficult for Heipro and Hflex. Unfortunately, even varying the NMR parameters did not lead to improved signal strengths. The standard pulse program configuration for ¹³C measurements at 600 MHz frequency was at ¹Dsequence with power gated decoupling with spin echo at a sleeve angle of 90° . Another data set recorded at ¹D-sequence with a larger spin-lattice relaxation time (t_1) of 30 seconds (normally in the range from 10^{-4} to 10^2 seconds)³²⁵ without spin echo and with a flip angle of 30° for 6.5 hours did not show any new signals either. The addition of chromium(III) acetylacetonate to the deuterated test solution, a standard relaxation agent in NMR spectroscopy, was redeemed unsuitable in this situation, because an earlier coordination chemistry experiment with Cr^{3+} had led to the formation of a green tris(quinolono)chromium(III) complex, which had been isolated in solid form; therefore, a reaction between Cr^{3+} and the quinolone seemed likely under the given conditions (d_6 -DMSO, ambient temperature). In addition to changing the ¹D-sequence parameters, three different sets of HMBC spectra at different coupling constants were recorded. The standard setting with a long-range coupling constant of J=8.0 Hz showed all correlations within the range of $J=8\pm 3$ Hz after recording data for 1 hour. Much more correlations could be detected in HMBC measurements with a coupling constant reduced by 50% (J = 4.0 Hz) for 2 hours. A third one-hour long run, with an even lower coupling constant of J = 2.0 Hz, however, did not reveal any further correlations between the ¹H and ¹³C nuclei. Finally, the reported signals for the C_{ar3} atoms in Hcipro and Hflex are based on estimated values resting upon weak interactions in the HMBC spectra that were recorded with set coupling constants at J=8.0 Hz and J=4.0 Hz. In case of Hcipro, the proton of the carboxylic acid substituent on C_{ar3} is coupling to a C-signal at 108 ppm, which corresponds to $J_{H,C}^3$; but, due to the extreme broadness of the hydroxyl proton signal, this gives only a faint signal in the corresponding HMBC spectra. In case of Hflex, the C_{ar3} signal could be estimated at 106 through a faint coupling signal with C_{ar2} ($J_{H,C}^2$) in the HMBC spectra. The signals for C_{ar3} extracted from the respective HMBC spectra correspond well with those of quinolones of similar chemical structure, such as Hlomx in case of Hflex as well as Hnofx in case of Hcipro (Table 2.3).

quinolone	solvent	$C_{ar2}H$	$C_{ar5}H$	$C_{ar6}H$	$C_{ar8}H$	$N_1 - R_1$	$C_{ar7} - R_2$		
Hcipro	d_6 -DMSO	8.67	7.93 (d, 13.0) ^{<i>a</i>}	n/a	7.60 (d, 7.4) ^{a}	$\begin{array}{ccccccc} 3.86 & (\text{tt}, & 7.2, & 3.7)^b, \\ 1.34{-}1.31 & (\text{m})^b, & 1.20{-}1.17 \\ (\text{m})^b \end{array}$	$3.54 (t, 5.0)^b, 3.27 (t, 5.0)^b$		
Henox	d_6 -DMSO	8.93	$7.98 (d, 13.8)^a$	n/a	n/a	4.45 (q, 7.1) ^b , 1.37 (t, 7.1) ^b	$3.73 (dd, 5.9, 4.1)^b, 2.84 (dd, 5.9, 4.1)^b$		
Hflex	d_6 -DMSO	8.84	$7.86 (11.9)^a$	n/a	n/a	$4.97 - 4.84 \ (m)^{a,b}$	3.34, 2.45, 2.23		
Hlevox	d_6 -DMSO	8.96	7.56 (d, 12.4) ^{a}	n/a	n/a	$\begin{array}{c} 4.91 \ (\mathrm{d}, \ 6.8)^b, \ 4.58 \ (\mathrm{dd}, \ 11.5, \\ 1.7)^b, \ 4.36 \ (\mathrm{dd}, \ 11.5, \ 2.3)^b, \\ 1.44 \ (\mathrm{d}, \ 3.3)^b \end{array}$	3.33–3.25 (m) ^b , 2.43 (br s) ^a , 2.23		
Hlomx	D_2O	8.55	7.46 (d, 11.4) ^{<i>a</i>}	n/a	n/a	4.48 $(6.0)^b$, 1.49 $(t, 7.1)^b$	$3.70-3.53 (m)^b$, $3.42-3.38 (m)^b$, $1.40 (d, 6.6)^b$		
Hnxa	d_6 -DMSO	9.18	$8.60 (d, 8.2)^b$	$7.59 (d, 8.2)^b$	n/a	4.64 (q, 7.1) ^b , 1.42 (t, 7.1) ^b	2.71		
Hnofx	d_6 -DMSO	8.92	$7.85 (d, 13.5)^a$	n/a	$7.12 (d, 7.3)^a$	$4.57 (q, 7.1)^{b}, 1.41 (t, 7.1)^{b}$	$3.22 (\mathrm{dd}, 5.9, 3.8)^b, 2.89 (\mathrm{dd}, 5.9, 3.8)^b$		
Hoxa	d_6 -DMSO	8.89	7.61	n/a	7.63	$4.53 (q, 7.1)^b, 1.38 (t, 7.1)^b$	6.29		
Hpia	d_6 -DMSO	8.93	9.15	n/a	n/a	4.36 (q, 7.1) ^b , 1.35 (t, 7.1) ^b	3.84 (d, 17.1) ^b , 2.78 (br s) ^b		

Table 2.2: ¹H NMR data in δ_H [ppm]

 ${}^{a}J_{H,F}$ [Hz], ${}^{b}J_{H,H}^{3}$ [Hz]

quinolone	solvent	COOH	C_{ar2}	C_{ar3}	C_{ar4}	$C_{ar4'}$	C_{ar5}	C_{ar6}	C_{ar7}	C_{ar8}	$C_{ar8'}$	$N_1 - R_1$	$C_{ar7} - R_2$
Hcipro	d_6 -DMSO	165.9	148.2	108	176.4	119.2 (5.9)	111.2 (19.1)	152.9 (207.9)	144.3 (7.9)	106.8	139.1	36.0, 7.6	46.8, 42.8
Henox	d_6 -DMSO	165.9	147.5	108.0	176.2 (2.3)	112.2 (3.6)	119.2 (22.0)	146.2 (204.2)	149.9 (9.0)	n/a	168.0	47.1, 14.6	$\begin{array}{l} 48.2 \\ 45.6 \end{array} (7.9),$
Hflex	d_6 -DMSO	165.5	152.1	106	175.6	120.1 (35.1)	107.0 (19.2)	154.5 (208.2)	$ \begin{array}{c} 133.7\\(13.9,11.7)\end{array} $	146.0 (212.2)	127.3 (9.1)	$\begin{array}{c} 82.1 \ (138.2), \\ 57.8 \ (15.8, \\ 12.2) \end{array}$	55.1, 50.3, 46.0
Hlevox	d_6 -DMSO	166.1	146.2	106.6	176.4	119.6 (7.7)	103.3 (20.4)	155.5 (206.3)	132.1 (11.9)	140.1 (5.7)	124.8	$ \begin{array}{ccc} 68.0, & 54.8, \\ 17.9 \end{array} $	55.3, 50.1, 46.1
Hlomx	D_2O	168.3	150.6	106.1	175.3	120.8 (7.3)	106.8 (19.1)	154.9 (208.6)	$ \begin{array}{c} 133.0\\(11.6,11.6)\end{array} $	146.1 (210.3)	127.0 (5.6)	53.4, 15.3	$\begin{array}{rrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrr$
Hnxa	d_6 -DMSO	164.7	149.7	108.6	178.2	118.3	135.6	123.6	148.3	n/a	165.6	46.8, 15.0	25.1
Hnofx	d_6 -DMSO	166.1	148.3	107.0	176.1 (2.7)	118.9 (7.7)	111.0 (23.1)	152.5 (207.5)	145.9 (9.7)	105.4 (3.7)	137.2	49.0, 14.3	50.8 $(4.8)^a$, 45.4
Hoxa Hpia	d_6 -DMSO d_6 -DMSO	$166.3 \\ 165.3$	$\begin{array}{c} 147.0\\ 150.6\end{array}$	$107.3 \\ 108.3$	176.0 177.1	$121.3 \\ 109.5$	101.8 160.1	147.1 n/a	$153.7 \\ 160.5$	97.2 n/a	$136.9 \\ 155.1$	$\begin{array}{c} 49.6, 14.6 \\ 45.8, 14.4 \end{array}$	103.3 45.3

Table 2.3: ¹³C NMR data in δ_C [ppm] (d, $J_{C,F}$ [Hz])

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quinolone	solvent	$C_{ar6}F$	$C_{ar8}F$	$N_1 - R_1$
Hcipro	d_6 -DMSO	-121.8	n/a	n/a
Henox	d_6 -DMSO	-127.4	n/a	n/a
Hflex	d_6 -DMSO	-127.6	-119.2	-224.1
Hlevox	d_6 -DMSO	-120.2	n/a	n/a
Hlomx	D_2O	-128.6	-118.6	n/a
Hnofx	d_6 -DMSO	-121.3	n/a	n/a

Table 2.4: ¹⁹F NMR data in δ_F [ppm] (referenced against $\delta(C_6F_6)$ = -164.9 ppm vs. $\delta(CFCl_3)$ = 0 ppm)

The ¹⁹F measurements support this chemical similarity argument (Table 2.4). For quinoline-based, mono-fluorinated (C_{ar6}) quinolones, such as Hcipro, Hlevox, Hnofx, the shift of the ¹⁹F appears at -121.8, -120.2, and -121.3 ppm, respectively. For enoxacin with its naphthyridine core, the chemical shift of ¹⁹F is shifted to high-field at -127.4 ppm due to the increased electronegativity in the condensed aromatic ring system accompanying the introduction of the second N-atom in 8-position. On the other hand, for quinolinebased, bi-fluorinated (C_{ar6} , C_{ar8}) lomefloxacin, the electronegativity seems to be slightly dispersed, which manifests itself in an upshifted $C_{ar6}F$ (-128.6 ppm) and a low-field shifted $C_{ar8}F$ (-118.6 ppm). The ¹⁹F NMR measurements of fleroxacin correspond to this with -127.6 ppm ($C_{ar6}F$) and -119.2 ppm ($C_{ar8}F$), in addition to -224.0 ppm for the fluorine atom at the end of the alkyl chain substituent on N_{ar1} .

Mass spectrometry revealed various re-combinations of single quinolone molecules (HL) with one sodium cation in the higher mass range, $[(HL)_{2-4} + Na^+]$, next to one single quinolone molecule plus either one proton, $[(HL) + H^+]$, or one sodium cation, $[(HL) + Na^+]$, as parent peak(s) in the lower mass range. No solvent influences were found in MS-spectra recorded in methanol, acetonitrile or aqueous mixtures of these solvents.

To complete the chemical characterization and to control the quality of the purchased chemicals, elemental analyses for the elements C, H, and N were performed as well. All analyzed quinolone drugs matched the calculated C, H, and N percentage values within an average difference of $\Delta = 0.21$. The largest differences between the analytically calculated and found values of C, H, and N were observed for levofloxacin (C, 0.39) and lomefloxacin hydrochloride (H, 0.28). These were the only two quinolone molecules out of nine that contain a stereocenter. In the levofloxacin molecule, the chiral center sits at the methyl-subsituted C-atom of the condensed hexane ring connecting to the quinoline core through N_{ar1} and C_{ar8}. In the lomefloxacin hydrochloride molecule, it is located in the 1,4-piperazinyl-ring at C_{pip3} attached to the condensed aromatic core in 7-position.

2.3.2 Stability in Iso-Sensitest Broth

Test solutions of the nine selected quinolones in Iso-Sensitest broth were monitored employing UV-Vis spectroscopy over 24 hours. In addition, test solutions in water were prepared from the same quinolone stock solutions and monitored with UV-Vis over 24 hours to allow for a direct comparison. In both experimental set-ups, the quinolone concentration in the final test solutions was 0.1 mM, the same concentration at which the quinolones entered the antimicrobial susceptibility disk test (Section 2.3.3). Sample spectra of all nine selected quinolones in Iso-Sensitest broth as well as water for comparison, including respective spectra of the solvent media themselves, are presented in Figure 2.5.

Before the UV-Vis spectra of the quinolones were recorded in alphabetical order at each time point, spectra of the initial blank sample of water and Iso-Sensitest broth were collected; these spectra have been drawn in subfigure (a) of Figure 2.5. Strikingly, these UV-Vis spectra showed an increase in absorbance by 0.02 AU with time in Iso-Sensitest broth as well as in water. The fact that such changes appeared not only in Iso-Sensitest broth but as well in pure water, resolved any immediate assumptions that these changes might reflect chemical alterations or biological degradations in the Iso-Sensitest broth. Because the trend in increasing absorbance from one UV-Vis measurement to the next immediately stopped after a new blank of these two solutions was recorded for all UV-Vis measurements to follow the 20 h time point, and the absorbance intensity fell back onto the initial absorbance curves recorded at the first time point, the observed trend of increasing absorbance can only be implicated in an effect of the UV-Vis machine related to the blank function of the instrument.³²⁶ Furthermore, the observed absorbance changes over time are not due to temperature or pH changes, as the room temperature as well as the pH values of the test solutions were monitored and provided constant values (data not shown) over the duration of both studies.

Comparing the recorded UV-Vis spectra for the nine selected quinolones in water and Iso-Sensitest medium over 24 hours (Figure 2.5 (b) to (d)), no changes between the freshly prepared test solutions and the 24 hour old test solutions are visible, neither in water nor in the Iso-Sensitest medium. Any alteration of the quinolone molecule through a chemical reaction with any of the ingredients of the iso-Sensitest broth would have resulted in detectable changes in the UV-Vis spectrum. As has been discussed in Section 2.3.1, the second absorbance maximum at higher wavelength (300-380 nm) is especially sensitive to pH changes, as these affect the intramolecular hydrogen bond formation between the carbonyl group in C_{ar4} position and the carboxylic acid group in C_{ar3} position. The quinolones are known to interact with metal ions through the same binding modality; ^{327 328} therefore, if the quinolones were to react with metals from the Iso-Sensitest medium, the respective molecular changes would be reflected in major changes in the second absorbance maximum. When Song *et al.* studied the effect of copper(II) and magnesium(II) ions on nalidixic acid in water (20 μ M), they saw a distinct hyperchrome shift in the second absorption maximum of Hnxa at a metal ion concentration of 0.4 mM and higher, which corresponded to an excess factor of 20x.³¹³

It must be mentioned that it was not possible to record the UV-Vis spectra in Iso-



Figure 2.5: UV-Vis study to monitor the stability of nine selected quinolones in Iso-Sensitest broth and in water. (A) Recorded blanks of water (top) and Iso-Sensitest broth (bottom) over time. (B) UV-Vis spectra of Hcipro, Henox, and Hflex in water (top) and Iso-Sensitest broth (bottom). (C) UV-Vis spectra of Hlevox, Hlomx, and Hnxa in water (top) and Iso-Sensitest broth (bottom). (D) UV-Vis spectra of Hnofx, Hoxa, and Hpia in water (top) and Iso-Sensitest broth (bottom).

Sensitives broth prepared according to the manufacturer's specification due to limitations of the UV-Vis technique. Instead it was necessary to dilute its concentration by a factor of two to limit the noise caused by the biological growth medium to an acceptable range and to ensure sufficient UV-Vis sensitivity towards the studied quinolones, as the medium itself absorbed UV light up to 340 nm wavelength, but any absorption above 320 nm was not larger than 0.02 AU (Figure 2.5a). According to the product data sheet of the Iso-Sensitest broth,³²⁹ the used product (OXOID CM0473) contains 0.2 g/L magnesium glycerophosphate and 0.1 g/L calcium gluconate, corresponding to a concentration of $c_{100\%} = 1.03 \cdot 10^{-3}$ M ($c_{50\%} = 0.515$ mM) and $c_{100\%} = 2.32 \cdot 10^{-4}$ M ($c_{50\%} = 0.116$ mM), respectively. Even at 50%- concentration, the concentration of metal cations in Iso-Sensitest broth exceeds the test concentration of the quinolones (0.1 mM), as the medium contains Mg^{2+} in 5.5x higher and Ca^{2+} in 1.2x higher concentration. Therewith, the concentration of Mg^{2+} in 50% Iso-Sensitest broth is with 0.515 mM as well already larger than in the study of Song et al. (0.4 mM);³¹³ therefore, any extra amounts of nutrients included in 100% Iso-Sensitest broth can be regarded as true excess, and it is highly unlikely that these should have any further effect on the quinolone molecules during the antimicrobial susceptibility test. Because no changes indicating a chemical modification of the quinolone molecule through interaction with metal ions present in Iso-Sensitest medium can be seen in the UV-Vis spectra, there is no evidence for chemical or biological degradation or decomposition of the selected nine quinolones in the tested solvent media over the monitored time frame of 24 hours.

2.3.3 Antimicrobial Susceptibility Disk Test

Quninolones demonstrate good *in vitro* activity against a range of Gram-positive and Gram-negative bacteria.^{284,285} The *in vitro* activities of the nine selected quinolones, at a test concentration of 0.1 mM, against strains of some of the most reported causative

pathogens³³⁰ are listed in Table 2.5. Their antimicrobial susceptibility was tested according to the single-disk method³³¹³³² on a selection of organisms comprising Grampositive, *Enterococcus faecalis* (*E. faecalis*) and *Staphylococcus aureus* (*S. aureus*), and Gram-negative bacteria, *Escherichia coli* (*E. coli*), *Klebsiella pneumonia* (*K. pneumonia*), and *Pseudomona aeruginosa* (*P. aeruginosa*).

The results of the antimicrobial susceptibility single-disk test show various resistances against the quinolones (0.1 mM), which partly can be related to the length of time that the drug has been in use and therewith the drug generations (Figure 2.1). Nalidixic acid, the oldest quinolone and the foundation of this drug class, has no efficacy anymore against any of the tested microbes. The same holds true for pipemidic acid, another member of the first generation of quinolone drugs. Oxolinic acid, on the other hand, is not potent against the tested Gram-positive bacteria strains, but it does inhibit the growth of *E. coli* and *K. pneumonia*, although it does show no efficacy against *P. aeruginosa*.

Enoxacin, an early member of the second generation of quinolone drugs, is not effective anymore against the Gram-positive bacteria included in the study. Norfloxacin and lomefloxacin hydrochloride do not inhibit the growth of *E. faecalis*, and Hlomx is as well not potent against *P. aeruginosa* at 0.1 mM concentration. The tested strain of *E. faecalis* appears to be resistant against the majority of quinolones included in this study, even against the third-generation fleroxacin, only ciprofloxacin (second generation) and levofloxacin (third generation) show efficacy against it. This comes as no surprise, as the (fluoro-)quinolones are known to exert a reduced rate of kill against enterococcal species.³³³ Overall, the results indicate that the selected quinolones are not as potent against the Gram-positive bacteria as they are against the Gram-negative organisms selected for this study. Once more, ciprofloxacin and levofloxacin proved to be the best all-round quinolone antimicrobial drugs available against a variety of pathogens with an activity relationship

Bacteria	Hcipro	Henox	Hflex	Hlevox	Hlomx	Hnxa	Hnofx	Hoxa	Hpia
E. faecalis	9(0)	0	0	8 (1)	0	0	0	0	0
S. aureus	12(1)	0	10(1)	14(1)	7(1)	0	7(1)	0	0
E. coli	22(1)	16(1)	19(1)	20(0)	17(2)	0	19(1)	14(1)	0
K. pneumonia	18(0)	14(1)	19(1)	18(0)	16(1)	0	15(1)	11(1)	0
P. aeruginosa	18(1)	7(1)	7(0)	9(0)	0	0	6(0)	0	0

Table 2.5: Results of antimicrobial susceptibility study of nine quinolones

Reported inhibition zones [mm] are averaged values from three plates (standard deviation). Disk diameter 0.6 mm. Loading volume 20 μ L. Concentration quinolone test solution 0.1 mM. Disks loaded with solutions of methanol and 2% DMSO in methanol served as controls, all of these showed no inhibition (0 mm).

that compares well to previous literature reports: Hlevox > Hcipro in *S. aureus*,³³⁴ and Hcipro > Hlevox in *P. aeruginosa*;³³⁵ however, this should not mask the fact that the overall bacterial susceptibility to quinolone antimicrobial agents continues to decrease,³³⁶ and Table 2.5 does identify patterns of resistance.

2.4 Conclusion

This chapter presented a comprehensive chemical characterization of nine selected quinolone antimicrobial drug molecules from three generations of quinolone drugs. Nalidixic acid, oxolinic acid, and pipemidic acid from the first generation; ciprofloxacin, enoxacin, lomefloxacin hydrochloric, and norfloxacin from the second generation, as well as fleroxacin and levofloxacin from the third generation of quinolone drugs. Melting point measurements, UV-Vis and IR spectroscopy data, ¹H NMR, ¹³C NMR, and ¹⁹F NMR measurements, have been reported and discussed, in addition to mass spectrometry and elemental analysis data. During a 24 hour-long UV-Vis study, the nine selected quinolones showed no signs of degradation or decomposition in Iso-Sensitest broth compared to test solutions in water.

Antimicrobial susceptibility single-disk test studies in this medium were performed

against the most commonly reported pathogens associated with nosocomical infections ³³⁰. The results proved once more the growing resistance of bacteria against commonly used antimicrobial drugs. ³³⁷ Nalidixic acid and pipemidic acid, both from the first generation of quinolones, were not effective against any of the tested Gram-positive and Gram-negative bacteria strains at the chosen test concentration of 0.1 mM. Only ciprofloxacin and lev-ofloxacin were successful in killing all of the five tested pathogens *in vitro*.

Chapter 3

Testing the "Trojan Horse Theory": Gallium(III) and Iron(III) Complexes of Quinolone Antimicrobials

In this chapter, the "Trojan Horse Theory" will be tested. Will the combination of gallium(III) ion with quinolone antimicrobial agents, which were introduced in the previous chapter, have a combinational, or maybe even a synergistic effect, leading to increased antimicrobial efficacy of such novel complexes?

3.1 A Bioinorganic Approach: Fighting the Growing Antimicrobial Resistance With Metallodrugs

"The world is on the brink of losing (...) miracle cures," with these words Director-General of the WHO, Dr. M. Chang, summarized the growing resistance of microbes to known antimicrobial drugs on World Health Day 2011.³³⁸ The WHO has rated these developments as one of the greatest threats to human health, because through antimicrobial resistance the control of infectious diseases is impeded, the achievements of modern medicine (e.g., surgeries) are jeopardized, and the costs of health care are rising globally.^{339,337} All of these development are only a weak foretaste of life in the post-antibiotic era that is near.³³⁷ For the trend of growing resistance of microbes, the annual antimicrobial resistance surveillance reports of the European Centre for Disease Prevention and Control (ECDC) provide evidence beyond doubt.³⁴⁰ According to the U.S. Centers for Disease Control and Prevention (CDC), each year at least 2 million patients in the US are infected with multi-drug resistant (MDR) bacteria, often in a hospital setting; the numbers of deaths vary between $23,000^{341}$ to 99,000.³⁴² This also costs the U.S. economy from 20 billion USD in excess direct health care costs to 35 billion USD including additional loss of productivity (2008).³⁴¹³⁴³ In the beginning of the 21st century, the glory days of antibacterial drug research seem to be coming to an end, as the Infectious Disease Society of America (IDSA) reports that only two new antibiotics have been approved, since their *trans*-Atlantic initiative with the EMA to develop 10 new antibiotic drugs by 2020 (titled "10x'20") was rolled out six years ago, ³⁴⁴ and the number of new antimicrobial agents approved by the FDA continues to decline.³⁴⁵ Scientists from academia and industry, health agencies, and policy makers need to take action to build a sustainable research and development structure for antimicrobial drugs, as well as global resistance surveillance systems to overcome the vawning innovation gap, manage the cost-benefit equation longterm, fight the imminent health crisis, and protect future generations against the ever-evolving resistance in microbes.^{343 346 347 348 349 350 351}

In the field of bioinorganic chemistry, the application of metal complexes to the therapy and diagnosis of developed drug resistance is an accepted concept, 264 which holds hope for novel parasitic 236 and antibacterial 352 drugs. In addition, a number of antimicrobial agents exact metal ions for their mechanism of action (see Chapter 2 for Mg²⁺ example), and an improved understanding of the structure, function, and actions of such "metalloantibiotics" 353 is essential to design metal complexes with new mechanisms of action to overcome growing antimicrobial resistance.

The transition metal iron is critical for the metabolism and growth of most organisms, with the possible exemption of some *lactobacilli*.³⁵⁴ About 0.3 to 4.0 μ M concentrations of iron are required for the growth of cells of animals (mouse), plants (algae), and microorganisms (fungi, Gram-positive/-negative bacteria).³⁵⁵ Hypoferraemia, the limitation of iron availability in vivo, is utilized by many species, including humans, as an autoimmune host defence.^{356,357} To be successful, such defence systems require extremely low levels of free Fe^{3+} ions of $<10^{-18}M$ in normal tissue fluids of the host; ³⁵⁸ however, microbial pathogens have learned to counteract this strategy and to scavenge iron from the host sources (ferritin, lactoferrin, transferrin, and heme compounds)³⁵⁹ by secreting siderophores, ^{360 361 362} a critical step in bacterial infections.^{363,364} The fact that bacterial virulence is highly enhanced. if free iron is widely available, was first recognized in the $clinic^{365}$ and has been further exemplified in medical practice.³⁶⁶ On the other hand, iron metabolism has been explored as a target for antimicrobial strategies³⁶⁷ and even for the treatment of cancer.³⁶⁸ Besides reducing the availability of free iron with chelating agents or inhibiting iron metabolism in the infected host, a third antimicrobial strategy utilizes the pathogen's own iron transport system for the delivery of bactericidal agents, and has been named the "Trojan Horse" strategy.³⁶⁷ The quintessence of this strategy lies in deceiving bacteria to take up antimicrobial agents that then kill them. The idea of linking antimicrobial agents to siderophores has been around for forty years, and various structures linking sulfonamides or β -lactam antibiotics to siderophores have been successfully realized, ³⁶⁹ e.g., a recent example of enterobactin-ampicillin conjugates showed a 1000-fold decrease in MIC against E. coli.³⁷⁰

In medicinal bioinorganic chemistry it is commonly accepted that biological systems cannot distinguish Ga^{3+} from the essential element iron in its tripositive ionic form due to their similarities in charge (both 3+), ionic radii (Fe³⁺= 0.65 Å, Ga³⁺= 0.62 Å), preferred

coordination number (CN= 6) and chemical behaviour (both hard Lewis acids). One important difference of both tripositive metals lies in their redox chemistry. In aqueous media, iron commonly exists in two stable oxidation states Fe^{2+} (d⁶) and Fe^{3+} (d⁵); *in vivo* the redox potential ($E^{\circ} = 0.771 \text{ V}, 25^{\circ}\text{C}$)³⁷¹ between these two states enables a wide range of metabolic activities that are carefully regulated to prevent cytotoxic reactions. As a group 13 metal, gallium lacks such interesting redox chemistry, which allows its use as a redox-inactive Fe^{3+} substitute *in vivo*. In biology this relationship is useful for studying metal complexation in proteins and bacterial populations;³⁷² however, the binding strength between biological (macro-)molecules and these two metals might differ slightly, e.g., gallium(III) binds to transferrin with a 300-fold less affinity than iron(III).³⁷³ The substitution of Ga³⁺ into metalloenzymes (at sufficient excess of Ga³⁺ over Fe³⁺) can result in a loss of enzymatic function, because the proteins are rendered inactive due to their inability to access the essential 3 + /2+ redox chemistry, with cellular toxicity as a result, which has stamped Ga³⁺ the "Trojan Horse" in biological systems.³⁷⁴

As described in Chapter 1, gallium(III) has been in therapeutic use for the past thirty years and is, in general, considered safe. $^{375\,376}$ Its coordination chemistry 377 is utilized in radioimaging, e.g., 68 Ga pasireotide tetraxetan (SOMscan) is a PET imaging agent for gastroentero-pancreatic neuroendocrine tumors in clinical phase I/II trials, 378 where 68 Ga seems a convenient PET alternative to 99m Tc. $^{39\,379}$ In therapy, intravenous gallium nitrate is already approved by the FDA for the treatment of cancer-associated hypercalcemia (Ganite), 170 the oral tris-(8-quinolinolato)gallium(III) (KP46) and tris(maltolato)gallium(III) are in clinical trials against cancer, 380 and recently, the antimicrobial effect of 69 Ga³⁺ has gained attention. Schlesinger and co-workers reported that gallium(III) nitrate and Gatransferrin inhibit the growth of *Mycobacterium tuberculosis* and *Mycobacterium avium* extracellularly and within human macrophages. $^{381\,382}$ A pharmacokinetic and safety study of Ganite in cystic fibrosis patients is underway,³⁸³ and a phase II study (IGNITE) has been scheduled.³⁸⁴ The use of gallium(III) salts at physiological pH, however, is considered a problem, because Ga³⁺ ions, similar to Fe³⁺ ions, are prone to hydrolysis, forming mono- and polynuclear oxo/hydroxo species of low solubility.^{385,386} This might as well be one of the reasons why Beraldo and co-workers realized that gallium(III) nitrate was not potent against the studied strain of *Pseudomona aeruginosa* but observed an increase in activity upon coordination to thiosemicarbazone ligands.³⁸⁷ Chelated to ligands, the gallium(III) at the center of the complex is sheltered from the otherwise inevitable hydrolysis in vivo. Other examples of gallium(III) coordination complexes with antimicrobial properties are: gallium-citrate,³⁸⁸ gallium-desferroxamine B,^{388,389} and gallium-maltol,³⁹⁰ which all have been primarily tested against *Pseudomona aeruqinosa*. Tris(maltolato)gallium(III) has gained special attention for the treatment of infections associated with bacterial biofilms.^{391 392 393 394} Besides complexation, co-administration of gallium(III) salts with known antimicrobial agents is another way to circumvent unwanted hydroxide formation in vivo, especially in smart formulations, such as gallium-gentamicin in liposomes.³⁹⁵ The combination of antimicrobial and non-antimicrobial agents has been described as a general concept to enhance antimicrobial potency.³⁹⁶

The quinolone antimicrobial agents were introduced in Chapter 2 (Figure 2.1), where we as well saw the developed bacterial resistance against some of the members of these drug class, although the resistance situation compared to other antimicrobial drug classes is still fortunate.³⁹⁷ Numerous metal complexes of main group and transition metals with a diversity of quinolone ligands have been reported,^{327 328} as have been the syntheses of the iron(III) complexes tris(nalidixido)iron(III),³⁹⁸ tris(norfloxacino)iron(III),³⁹⁹ tris(enrofloxacino)iron(III),⁴⁰⁰ tris(ciprofloxacino)iron(III),⁴⁰¹ bis(sparfloxacino)iron(III),⁴⁰² and tris(lomefloxacino)-

iron(III), ⁴⁰³ as well as some mixed complexes of iron(III) with quinolones and a second ligand, such as bispyrazolones ⁴⁰⁴ or the nitrilotriacetic anion. ⁴⁰⁵ Research in this area has been motivated by the clinical observation that bioavailability and bactericidal efficacy of quinolone antimicrobial agents are reduced through the interaction with cations in the human body, and has, therefore, focused on metals generally included in antacid preparations or vitamin supplements. ^{406 407 408} Bivalent or trivalent metal ions included in such preparations bind to one or up to three quinolone molecules through the ionized carboxylate on C_{ar3} and the adjacent keto group on C_{ar4} in vivo, ⁴⁰⁹ occupying the actual binding side to DNA gyrase or/and topoisomerase IV, therewith perturbing the mechanism of action (Section 2.1). Several studies support this observation, in *in vitro* tests the activity of ciprofloxacin was reduced upon complexation to Mg^{2+} , ³⁰⁴ while in *in vivo* tests (dogs) only a small extend of complexation of norfloxacin with Ca^{2+} , Mg^{2+} , Zn^{2+} , Fe^{2+} , Al^{3+} resulted in disproportionately large reductions (60–80%) in bioavailability.⁴¹⁰

In an attempt to develop novel antimicrobial agents, we have explored the "Trojan Horse" bactericidal concept with a coordination chemistry approach and synthesized tris(quinolono)gallium(III) complexes of nine selected quinolone antimicrobial drugs (Figure 3.1). In addition, we have prepared the analogous tris(quinolono)iron(III) complexes to be able to directly compare the effect of the Ga³⁺ versus the Fe³⁺ ion in these otherwise identical compounds. In first *in vitro* studies against pathogens associated with hospitalacquired diseases, we tested all tris(quinolono)metal(III) complexes (0.1 mM) along side the free quinolone ligands (0.1 mM and 0.3 mM) to determine if the combination of a quinolone antimicrobial agent with Ga³⁺ in a 3:1 ratio exhibits a combinational or even an anticipated synergistic effect. To further study the antimicrobial potency of Ga³⁺ versus Fe³⁺, complexes with maltol (Hma), a widely used food additive, have been synthesized and tested as well.



Figure 3.1: Overview of the synthesized and characterized tris(quinolono)metal(III) complexes ($M = Ga^{3+}, Fe^{3+}$).

3.2 Materials & Methods

3.2.1 Chemicals

The majority of chemicals used were purchased from Sigma-Aldrich: ciprofloxacin, enoxacin, levofloxacin, norfloxacin, oxolinic acid, pipemidic acid, and gallium(III)nitrate nonahydrate as well as iron(III)chloride hexahydrate. TCI America supplied the ciprofloxacin hydrochloride, nalidixic acid, and lomefloxacin hydrochloride. Organic solvents and sodium salts were obtained from Fisher Scientific. The 50 % sodium hydroxide solution came from ACROS. Atomic absorption standard (AAS) solutions of iron(III) and gallium(III) (1000 mg/L ± 4 mg/L) were obtained from Fluka. Potassium hydrogen phthalate from BDH Chemicals, Ltd. was recrystallized, and deionized water was purified using a ELGA MAXIMA ultra pure water system (resistivity 18 M Ω ·cm, 25°C); all other chemicals and solvents were used without further purification.

3.2.2 Instrumentation

During the synthetic preparation of the metal complexes, the pH of the reaction mixture was monitored with a Metrohm 6.0234.110 electrode connected to a Metrohm 713 pH meter. The electrode was filled with 3.0 M potassium chloride solution as the electrolyte and calibrated against reference buffer solutions (4.00, 7.00, 10.00) from the FisherScientific Buffer-Pac on a regular basis. Mp determinations were performed in triplicate on a DigiMelt SRS Stanford Research Systems melting point apparatus and are uncorrected. UV-Vis spectra were recorded on a Hewlett Packard 8453 instrument running UV-Vis ChemStation software (version B.04.01[61], Agilent Technologies, 2001–2010). IR spectra were obtained neat in the solid state on a Thermo Scientific Nicolet 6700 FT-IR spectrometer using OMNIC (version 7.4.127, Thermo Fisher Scientific Inc.) in the range of $4000-500 \text{ cm}^{-1}$. For the interpretation of bands, the following abbreviations were used: st, md, w, br, and sh. NMR spectroscopy was performed at the UBC NMR Facility. ¹H NMR and ¹³C spectra, as well as ¹⁹F NMR spectra as applicable, were recorded in addition to COSY, HSQC and HMBC experiments at ambient temperature on Bruker Avance 300 and 600 spectrometers running Topspin software (version 3.2, Bruker). The Bruker Avance 600 spectrometer was equipped with a Bruker TCI-Z-5mm cryoprobe, which allowed for the detection of ${}^{13}C$ and ${}^{1}H$ nuclei at a low signal-to-noise ratio of 6000/1 (${}^{1}H$) and 600/1 (¹³C). For the NMR experiments, the tris(quinolono)gallium complexes were dissolved in deuterated DMSO with the help of sonication and heating at a final concentration of approximately 6 mM. The residual solvent signal of d_6 -DMSO was used as the internal standard.³⁰⁹ Chemical shifts are referenced in ppm against tetramethylsilane $(\delta_{C,H}=0$ ppm) and trichlorofluoromethane ($\delta_F=0$ ppm), respectively. Multiplicities are described as: s, d, q, and m. Aromatic protons are abbreviated ar, while pip represents the 1,4-piperazinyl ring in C_7 position on the aromatic ring system and prop the propyl ring in N_1 position on the aromatic ring system. All NMR spectral analysis were performed with the software iNMR (version 5.1.2, Mestrelab Research). For low-resolution mass spectrometry, a Water ZQ spectrometer equipped with an electrospray and chemical ionization source was used. All samples showed acceptable solubility in methanol at varying pH values; MS experiments were conducted in methanol or nitromethane. Highresolution mass spectrometry was performed at the UBC Mass Spectrometry Centre on a Waters Micromass LCT employing electrospray-ionization. Characteristic signals have been listed as dimensionless mass-to-charge ratios with the intensity related to the base signal. The UBC Mass Spectrometry Centre as well determined the elemental composition of the synthesized compounds. Microanalyses for the elements C, H and N were prepared on a Carlo Erba Elemental Analyzer EA 1108.

3.2.3 Thermogravimetric Analysis

Combined thermogravimetric analysis (TGA) and differential thermal analysis (DTA) measurements were performed in minimum duplicate on a simultaneous thermal analyser Perkin Elmer STA 6000 running Pyris Manager (version 10.1.0.0411, Perkin Elmer) over the temperature range from 25–900°C under a stream of nitrogen gas (19.8 mL/min). The program started by stabilizing the sample at 25°C for 3 min, before it was heated up to 900°C in intervals of 5°C/min, held at 900°C for 3 min, and finally cooled down to 25°C at a rate of 50°C/min.

3.2.4 Potentiometry

Potentiometric titrations were carried out using a Metrohm 809 Titrando system with a Metrohm 800 Dosino unit and a Metrohm 801 Stirrer interfaced to a PC computer running Titrando PC Control (Version 5.0, Metrohm). The system was equipped with a Thermo Scientific ORION 8103BN combination electrode (precision: ± 0.1 mV). The reference compartment of the electrode was filled with 0.16 M aqueous sodium hydroxide solution as the electrolyte. All titrations were carried out under an inert atmosphere by bubbling nitrogen through the cell for at least five minutes prior to proceeding and also during the titration. To exclude any carbon dioxide the nitrogen was washed with 2.0 M aqueous sodium hydroxide solution prior to entering the cell. The titrations were performed at 25°C ($\pm 0.1^{\circ}$ C) in a 10 mL water-jacketed vessel. All solutions were prepared in ultra pure water having a constant ionic strength (I= 0.16 M) using sodium chloride.

Carbonate-free solutions of the titrant, sodium hydroxide (NaOH), were prepared by dilution of 50% solution with freshly boiled ultra pure water under a stream of nitrogen gas. The aqueous NaOH solution was standardized with potassium hydrogen phthalate. Prior to each potentiometric equilibrium study, electrode calibration was accomplished by titrating the sodium hydroxide stock solution into the standardized hydrochloric stock solution. Calibration data were analyzed by standard computer treatment provided within MacCalib⁴¹¹ to obtain the calibration parameter E° and pK_w . For the autoprotolysis constant of water at 25°C, the following diffusion correction terms were used: $E^{\circ} = 2.463$ V and $pK_w = 1.057$. Protonation constants of ciprofloxacin and iron(III) as well as gallium(III) complexation constants were obtained from titrations performed in triplicate with allowed equilibration times varying to 60 minutes maximum; data fitting was performed with the software Hyperquad2008 (Protonic Software).

3.2.5 Computational Details

With DFT, one of four possible isomer structures of gallium(III) coordinated to three ciprofloxacin anions was calculated at the B3LYP level utilizing the 6-31+G(d,p); LANL2DZ mixed basis set as implemented in Gaussian⁴¹² The optimized geometry is characterized as an energetic minimum, indicated by the absence of imaginary frequencies.

3.2.6 Antimicrobial Susceptibility Studies

The biological study was performed in UBC's Biological Service Laboratory, a biological safety level II facility, according to respective operating and safety protocols. Please see Appendix A for the detailed test procedure.

3.2.7 Synthesis & Characterization of Tris(quinolono)metal(III) Complexes

The tris(quinolino)metal(III) complexes were synthesized according to the following three general synthetic methods:

Method (a): To a solution of metal(III)nitrate nonahydrate (0.1 mmol) in water (2 mL), an acidified aqueous solution (8 mL) of the quinolone (0.3 mmol) was added dropwise. During the addition the pH was carefully monitored and kept below pH 5; finally, raising the pH to pH 7.5 with aqueous sodium hydroxide (1.0 M and 0.1 M) resulted in a characteristically colored solution (yellow for Ga^{3+} , red-brown for Fe^{3+}) that was stirred rigorously at room temperature for 20 min, before the vial was closed tightly and placed in the fridge at 4°C. After 3–5 days the desired product had precipitated. The solid was separated by filtration (glass frit size F), thoroughly washed with water (2 mL) and methanol (2 mL), and dried *in vacuo*.

Method (b) is a modification of the reported synthesis of tris(nalidixido)iron(III):³⁹⁸ The quinolone (0.3 mmol) was heated with sodium bicarbonate or sodium hydroxide (0.3 mmol) in water (10 mL) until the initially white suspension had turned into a clear solution, which was then added onto the solid metal(III) nitrate nonahydrate (0.1 mmol). Upon addition the pH was kept at pH \leq 5, the desired product started forming immediately and precipitated as solid (final pH \sim 7). The suspension was stirred rigorously until cooled to room temperature (for a minimum of 30 min, often overnight). The desired product, which precipitated often at room temperature or otherwise after placing the reaction vial in the fridge (4°C) was separated by filtration (glass grit, size F) as a solid, washed with water (2 mL) and methanol (2 mL), and dried *in vacuo*.

Method (c) is a modification of the reported synthesis of tris(ciprofloxacino)iron(III):⁴⁰¹ A suspension of quinolone (0.31 mmol) with sodium hydroxide or potassium hydroxide (0.33 mmol) in methanol (15 mL) was refluxed until it turned into a clear, colorless solution. The hot methanolic solution was added onto the solid metal(III)nitrate nonahydrate (0.1 mmol), and the resulting colored solution was refluxed further for thirty minutes. The reaction solution was left to cool in air. Evaporation of the solvent in air, or a reduction of the solvent by at least 50% volume, led to precipitation of a colored solid, which was separated by filtration (glass frit, size F), thoroughly washed with water (2 mL) and methanol

(2 mL), and dried in vacuo.

The method that gave the highest product purity, as determined by EA and highresolution electrospray ionization (HR-ESI) mass spectrometry, at a 0.1 mmol scale is reported.

3.2.7.1 Tris(ciprofloxacino)gallium(III), [Ga(cipro)₃]

Method (c) gave a pale vellow solid (99 mg, 0.094 mmol, 94%). Mp: $\geq 220^{\circ}$ C, decomposition to brown solid. IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3418 (md, br, water), 2846 (w, br) 1620 (st), 1545 (w), 1516 (sh), 1472 (st), 1451 (sh), 1373 (st, br), 1287 (sh), 1252 (st), 1182 (md) 1146 (md), 1106 (sh), 1025 (st), 949 (st), 893 (md), 810 (md), 787 (md), 765 (sh), 740 (st, br), 704 (md), 627 (st), 540 (md), 505 (st). NMR: δ_H (600 MHz, 298 K, d_6 -DMSO) $[\mathrm{ppm}] = 8.93 \text{ (s)}, 8.84 \text{ (s)}, 8.80 \text{ (s)}, (3 \text{ H}, \text{C}_{ar2}H); 7.65 \text{ (d}, J_{H,F}^3 = 13.8 \text{ Hz}), 7.57 - 7.53 \text{ (m)},$ 7.41 (d, $J_{H,F}^4$ = 7.2 Hz), (6 H, C_{ar5}H and C_{ar8}H); 3.95–3.81 (m, 3 H, C_{prop}H); 3.29–3.20 (m, 12 H overlaid with water, $C_{pip2,6}H_2$); 2.96–2.91 (m, 12 H, $C_{pip3,5}H_2$); 1.36–1.32 (m, 6 H, $C_{prop}H_{b,b'}$; 1.23–0.82 (m, 6 H, $C_{prop}H_{a,a'}$). δ_{C} (125 MHz, 298 K, d_{6} -DMSO) [ppm] = 173.7 (s), 173.6 (s), 173.2 (s), (C_{ar4}); 165.6 (s), 165.5 (s), 165.3 (s), (COOH); 153.1 (d, $J_{C,F}^1 = 206.1 \text{ Hz}), \, 153.0 \,\, (\mathrm{d}, \, J_{C,F}^1 = 207.1 \text{ Hz}), \, 152.9 \,\, (\mathrm{d}, \, J_{C,F}^1 = 207.3 \text{ Hz}), \, (C_{ar6}); \, 149.8 \,\, (\mathrm{s}), \, J_{C,F}^2 = 207.3 \,\, \mathrm{Hz}), \, (C_{ar6}); \, 149.8 \,\, (\mathrm{s}), \, J_{C,F}^2 = 207.3 \,\, \mathrm{Hz}), \, (C_{ar6}); \, 149.8 \,\, (\mathrm{s}), \, J_{C,F}^2 = 207.3 \,\, \mathrm{Hz}), \, (C_{ar6}); \, 149.8 \,\, (\mathrm{s}), \, J_{C,F}^2 = 207.3 \,\, \mathrm{Hz}), \, (C_{ar6}); \, (C_{ar6});$ 149.6 (s), 149.3 (s), 149.2 (s), (C_{ar2}); 145.4 (d, $J_{C,F}^2$ = 21.0 Hz), 145.3 (d, $J_{C,F}^2$ = 21.8 Hz), 145.2 (d, $J_{C,F}^2 = 19.5 \text{ Hz}$), (C_{ar7}); 139.0 (s), 138.8 (s), 138.7 (s), ($C_{ar8'}$); 118.7 (d, $J_{C,F}^3 = 6.9$ Hz), 118.6 (d, $J_{C,F}^3 = 7.0$ Hz), 118.4 (d, $J_{C,F}^3 = 7.8$ Hz), ($C_{ar4'}$); 111.8 (s), 111.7 (s), 111.5 (s), (C_{ar5}) ; 110.5 (s), 110.3 (s), 110.2 (s), (C_{ar3}) ; 106.3 (s), 105.7 (s), 105.3 (s), (C_{ar8}) ; 50.1 (s), 50.0 (s), 49.9 (s), $(C_{pip2,6})$; 45.0 (s), 44.9 (s), 44.8 (s), $(C_{pip3,5})$; 36.2 (s), 36.0 (s), 35.9 (s), 35.8 (s), ($C_{prop}{\rm H});$ 7.7 (s), 7.6 (s), 7.5 (s), ($C_{prop}{\rm H}_2).$ $\pmb{\delta_F}$ (282 MHz, 298 K, $d_6\text{-DMSO})$ $[ppm] = -121.1 \text{ (s)}, -121.15 \text{ (s)}, -121.21 \text{ (s)}, (3 \text{ F}, \text{C}_{ar6}F).$ MS (ES+, CH₃OH): m/z (%) = 1083 (40) [ML₃ + Na⁺], 730 (100) [ML₂]⁺. **HR-ESI-MS**: m/z for C₅₁H₅₁F₃ ⁶⁹GaN₉O₉ $+ H^+$ calcd. (found): 1060.3096 (1060.3073); for $C_{34}H_{34}F_2$ ⁶⁹GaN₆O₆⁺ calcd. (found): 729.1764 (729.1785). **EA**: Anal. Calcd. (found) [%] for $C_{51}H_{51}F_3GaN_9O_9 \cdot 8 H_2O$: C, 50.84 (50.61); H, 5.60 (4.80); N, 10.46 (10.79).

3.2.7.2 Tris(enoxacino)gallium(III), [Ga(enox)₃]

Method (b) gave a pale yellow solid (85 mg, 0.082 mmol, 82%). Mp: $\geq 200^{\circ}$ C, decomposition to light brown solid. IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3404 (md, br, water), 3045 (w, br), 2977 (w, br), 1625 (st), 1562 (md), 1519 (md), 1469 (sh), 1434 (st, br), 1369 (md), 1346 (st), 1323 (md), 1276 (st), 1253 (st, br), 1185 (md), 1153 (w), 1119 (md), 1092 (md), 1039 (md, br), 972 (md), 941 (md, br), 908 (sh), 812 (st), 789 (md), 766 (md), 746 (md), 677 (w), 651 (md), 626 (st), 563 (md), 516 (st), 453 (w). **NMR**: δ_H (300 MHz, 298 K, d_6 -DMSO) $[\text{ppm}] = 9.18 \text{ (s)}, 9.13 \text{ (s)}, 9.08 \text{ (s)}, 9.04 \text{ (s)}, 8.99 \text{ (s)} (3 \text{ H}, \text{C}_{ar2}H); 8.12 \text{ (d}, J_{H,F}^3 = 13.2 \text{ (d)})$ Hz), 7.75 (d, $J_{H,F}^3 =$ 13.2 Hz), 7.72 (d, $J_{H,F}^3 =$ 14.0 Hz), 7.69 (d, $J_{H,F}^3 =$ 13.8 Hz), (3 H, $C_{ar5}H$; 4.59–4.43 (m, 6 H, CH_2CH_3); 3.92–3.85 (m, 12 H, $C_{pip2,6}H_2$); 3.12–3.02 (m, 12 H) H, $C_{pip3.5}H_2$; 1.43–1.23 (m, 9 H, CH_2CH_3). δ_C (75 MHz, 298 K, d₆-DMSO) [ppm] = 173.9 (s), 173.5 (s), 173.4 (s), (C_{ar4}) ; 168.9 (s), 168.8 (s), 168.7 (s), $(C_{ar8'})$; 165.8 (s), 165.6 (s), 165.5 (s), 165.3 (s), (COOH); 149.6 (d, $J_{H,F}^2 = 90.3$ Hz), 149.4 (d, $J_{H,F}^2 = 85.1$ Hz), 149.0 (d, $J_{H,F}^2 = 89.4$ Hz), (C_{ar7}); 147.2 (d, $J_{H,F}^1 = 207.0$ Hz), 147.1 (d, $J_{H,F}^1 = 202.8$ Hz), 147.0 (d, $J_{H,F}^2 = 214.6$ Hz), 146.9 (d, $J_{H,F}^1 = 215.4$ Hz), (C_{ar6}); 144.7 (s), 144.3 (s), 144.1 (s), (C_{ar2}) ; 119.6 (d, $J_{C,F}^2 = 18.1$ Hz), 119.0 (d, $J_{C,F}^2 = 17.5$ Hz), 118.70 (d, $J_{C,F}^2 = 19.6$ Hz), (C_{ar5}) ; 113.2–112.6 (m, $C_{ar4'}$); 108.1 (s), 108.0 (s), 109.9 (s), (C_{ar3}) ; 47.5 (s), 47.3 (s), 47.2 (s), $(C_{pip2.6})$; 46.2 (s), 45.9 (s), 45.6 (s), (CH_2CH_3) ; 44.2 (s), 44.0 (s), 43. 7 (s), $(C_{pip3.5})$; 14.9 (s), 14.8 (s), 14.7 (s), (CH₂CH₃). δ_F (282 MHz, 298 K, d₆-DMSO) [ppm] = -126.4, -126.5, -126.6, -126.8 (3 F, $C_{ar6}F$). MS (ES+, CH₃OH): m/z (%) = 1050 (20) $[ML_3 + Na^+]$, 708 (100) $[ML_2]^+$. **HR-ESI-MS**: m/z for $C_{45}H_{48}F_3$ ⁶⁹GaN₁₂O₉ + Na⁺ calcd. (found): 1049.2773 (1049.2797).

3.2.7.3 Tris(fleroxacino)gallium(III), [Ga(flex)₃]

Method (b) gave a pale vellow solid (85 mg, 0.082 mmol, 82%). Mp: >230°C, decomposition to orange-brown solid. IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3392 (w, br, water), 3054 (md), 2943 (md), 2848 (w), 2796 (md), 1622 (st), 1556 (md), 1514 (md), 1475 (st, br), 1449 (sh), 1409 (w), 1391 (w), 1376 (md), 1360 (md), 1328 (md), 1280 (st), 1245 (md), 1230 (w), 1214 (w), 1206 (w), 1143 (st), 1123 (md), 1099 (w), 1077 (w), 1062 (md), 1037 (md), 1020 (st), 1010 (st), 970 (md), 942 (md), 926 (md), 869 (md), 853 (md), 807 (st), 783 (md), 754 (sh), 741 (st), 672 (w), 656 (st), 573 (st), 550 (md), 532 (w), 505 (w, br), 450 (md, br). **NMR**: δ_H (600 MHz, 298 K, d_6 -DMSO) [ppm] = 8.86 (s, 3 H, $C_{ar2}H$); 7.87 (d, $J_{H,F}^3$ = 11.4 Hz, 3 H, $C_{ar5}H$; 4.98–4.96 (m), 4.93–4.90 (m), 4.86–4.84 (m) (12 H, $(CH_2)_2$); 3.36 (br s, 12 H overlaid with water, $C_{pip2,6}H_2$; 2.44 (s, 12 H, $C_{pip3,5}H_2$); 2.23 (s, 9 H, CH_3). δ_C (125 MHz, 298 K, d_6 -DMSO) [ppm] = 175.8 (s, C_{ar4}); 165.5 (s, COOH); 154.6 (d, $J_{C,F}^1$ = 205.9 Hz), 154.5 (d, $J_{C,F}^1$ = 206.5 Hz), (C_{ar6}); 152.1 (s, C_{ar2}); 146.1 (d, $J_{C,F}^1$ = 205.9 Hz, C_{ar8}), 146.0 (d, $J_{C,F}^1$ = 205.9 Hz, (C_{ar8}); 133.9 (two overlapping d, $J_{C,F}^2$ = 11.3, 11.5 Hz, $C_{ar7});\,127.4~(\mathrm{d},\,J_{C,F}^2=\,9.1~\mathrm{Hz},\,C_{ar8'});\,120.1~(\mathrm{d},\,J_{C,F}^3=\,6.8~\mathrm{Hz},\,C_{ar4'});\,107.1~(\mathrm{d},\,J_{C,F}^2=\,18.9)$ Hz, C_{ar5}); 106.6 (s, C_{ar3}); 82.0 (d, $J_{C,F}^1 = 137.0$ Hz, CH_2CH_2F); 58.0 (d, $J_{C,F} = 11.9$ Hz), 57.8 (d, $J_{C,F}$ = 16.4 Hz), (CH₂CH₂F); 55.1 (s, $C_{pip2,6}$); 50.3 (s, $C_{pip3,5}$); 46.0 (s, CH₃). δ_F (282 MHz, 298 K, $d_6\text{-}\mathrm{DMSO})~[\mathrm{ppm}]$ = -119.2 (d, $J_{F,F}^4$ = 11.0 Hz, 3 F, $\mathrm{C}_{ar6}F);$ -127.6 (q, $J_{F,F} = 5.6 \text{ Hz}, 3 \text{ F}, \text{ C}_{ar8}F); -224.2 \text{ (d, } J_{C,F}^6 = 6.0 \text{ Hz}, 3 \text{ F}, (\text{CH}_2)_2F). \text{ MS (ES+, CH_3OH):}$ m/z (%) = 1197 (80) [ML₃ + Na⁺], 806 (100) [ML₂]⁺. HR-ESI-MS: m/z for C₅₁H₅₁F₉ $^{69}\mathrm{GaN_9O_9}$ + Na^+ calcd. (found): 1196.2820 (1196.2838); for $\mathrm{C_{34}H_{34}F_6}$ $^{69}\mathrm{GaN_6O_6}^+$ calcd. (found): 805.1700 (805.1719).

3.2.7.4 Tris(levofloxacino)gallium(III), $[Ga(levox)_3]$

Method (a) gave a yellow solid (47 mg, 0.041 mmol, 41%). Mp: >190°C, decomposition to orange-brown solid. IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3426 (md, br, water), 3033 (w, br), 2931 (w), 2848 (w), 2794 (w), 1616 (st), 1519 (st), 1447 (st), 1385 (md, sh), 1331 (st, br), 1261 (st), 1150 (sh), 1129 (md), 1093 (md), 1047 (st), 1003 (md), 978 (st), 898 (md), 866 (md), 844 (w), 810 (st), 763 (md), 744 (st), 696 (md), 638 (w), 554 (md), 510 (st), 463 (md, br). NMR: δ_H (600 MHz, 298 K, d₆-DMSO) [ppm] = 9.25 (s), 9.19 (s), 9.13 (s), 9.01 (s), 9.98 (s), 9.93 (s), 8.98 (s), (3 H, $C_{ar2}H$); 7.60 (d, $J_{H,F}^3 = 12.0$ Hz), 7.48–7.45 (m), 7.42 (d, $J_{H,F}^3 =$ 12.0 Hz), 7.29 (d, $J_{H,F}^3 =$ 12.0 Hz), 7.24–7.211, 7.14 (d, $J_{H,F}^3 =$ 12.6 Hz), (3 H, C_{ar5}H); 5.08–4.88 (m, 3 H, CH); 4.64–4.27 (m, 6 H, OCH₂CH); 3.50–3.26 (m, 12 H overlaid with water, $C_{pip2,6}H_2$; 2.76–2.61 (m, 12 H, $C_{pip3,5}H_2$); 2.46–2.38 (m, 9 H, NCH₃); 1.52–1.42 (m), 1.23 (br s), 1.14–1.08 (m), (9 H, CHCH₃). δ_C (125 MHz, 298 K, d_6 -DMSO) [ppm] = 176.4 (s), 173.6 (s), 173.4 (s), 173.2 (s), (C_{ar4}); 166.0 (s), 165.8 (s), 165.6 (s), 165.4 (s), (COOH); 155.5 (d, $J_{C,F}^1 = 204.8 \text{ Hz}$), 155.4 (d, $J_{C,F}^1 = 203.3 \text{ Hz}$), 155.2 $(d, J_{C,F}^1 = 200.0 \text{ Hz}), (C_{ar6}); 148.2 \text{ (s)}, 147.9 \text{ (s)}, 147.7 \text{ (s)}, 147.5 \text{ (s)}, 146.3 \text{ (s)}, (C_{ar2}); 140.4 \text{ (s)}, 147.7 \text{ (s)}, 147.5 \text{ (s)}, 147.5 \text{ (s)}, 146.3 \text{ (s)}, (C_{ar2}); 140.4 \text{ (s)}, 147.7 \text{ (s)}, 147.5 \text{ (s)}, 147.5 \text{ (s)}, 146.3 \text{ (s)}, (C_{ar2}); 140.4 \text{ (s)}, 147.5 \text{ (s)}, 147.$ (d, $J_{C,F}^3 = 5.8 \text{ Hz}$), 139.8 (d, $J_{C,F}^3 = 8.9 \text{ Hz}$), 139.2 (d, $J_{C,F}^3 = 10.4 \text{ Hz}$), (C_{ar8}); 131.1–130.8 (m, C_{ar7}); 124.9 (s), 124.6 (s), 124.5 (s), 124.2 (s), ($C_{ar8'}$); 120.0 (s), 119.8 (s), 119.5 (s), 119.4 (s) $(C_{ar4'})$; 112.6 (s), 112.5 (s), 111.8 (s), 111.6 (s), 106.7 (s), (C_{ar3}) ; 103.3 (d, $J_{C,F}^2 =$ 20.4 Hz), 102.9 (d, $J_{C,F}^2$ = 24.5 Hz), 102.3 (d, $J_{C,F}^2$ = 22.8 Hz), (C_{ar5}); 68.2 (s), 68.0 (s), 67.9 (s), (OCH₂CH); 55.2 (s), 55.0 (s), 54.8 (s), ($C_{pip2,6}$); 54.7 (s), 54.6 (s), 54.5 (s), (CH); 49.4–48.8 (m, $C_{pip3,5}$); 45.2–44.4 (m, NCH₃); 18.2–17.7 (m, CH(CH₃)). δ_F (282 MHz, 298 K, d_6 -DMSO) [ppm] = -119.4 (s), -119.5 (s), -119.7 (s), -119.8 (s), -119.86 (s), -119.93 (s), (3 F, $C_{ar6}F$). **MS** (ES+, CH₃OH): m/z (%) = 1173 (40) [ML₃ + Na⁺], 790 (100) $[ML_2]^+$. **HR-ESI-MS**: m/z for $C_{54}H_{57}F_3$ ⁶⁹GaN₉O₁₂ + Na⁺ calcd. (found): 1172.3232 (1172.3248); for $C_{36}H_{38}F_2$ ⁶⁹GaN₆O₈⁺ calcd. (found): 789.1975 (789.1989).

3.2.7.5 Tris(lomefloxacino)gallium(III), $[Ga(lomx)_3]$

Method (a) gave a pale yellow solid (40 mg, 0.035 mmol, 35%). Mp: >200°C, decomposition to brown solid. IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3419 (md, br, water), 2980 (w, br), 2848 (w, br), 2478 (w, br), 1619 (st), 1556 (w), 1523 (st), 1452 (st), 1354 (md, sh), 1323 (st, br), 1277 (md), 1247 (st), 1123 (md), 1090 (md), 1050 (st), 1001 (st), 932 (st), 886 (md), 811 (st), 776 (w), 742 (md), 651 (md), 542 (sh), 505 (md, br). NMR: δ_H (600 MHz, 298 K, D₂O) [ppm] = 9.18 (s), 9.15 (s), 9.01 (s), 9.05 (s), 8.94 (s), (3 H, $C_{ar2}H$); 7.87 (d, $J^3_{H,F} = \ 11.4 \ {\rm Hz}), \ 7.66 \ ({\rm d}, \ J^3_{H,F} = \ 11.4 \ {\rm Hz}), \ 7.61 \ ({\rm d}, \ J^3_{H,F} = \ 10.8 \ {\rm Hz}), \ 7.51 \ ({\rm d}, \ J^3_{H,F} = \ 10.2 \ {\rm Hz}), \ 7.51 \ ({\rm d}, \ J^3_{H,F} = \ 10.2 \ {\rm Hz}), \ 7.51 \ ({\rm d}, \ J^3_{H,F} = \ 10.2 \ {\rm Hz}), \ 7.51 \ ({\rm d}, \ J^3_{H,F} = \ 10.2 \ {\rm Hz}), \ 7.51 \ {\rm Hz}), \ 7.51 \ {\rm Hz}$ Hz), (3 H, $C_{ar5}H$); 4.68–4.58 (m, 6 H, CH_2CH_3); 3.43–3.28 (m, 12 H, $C_{pip2,6}H_2$, and 3 H, $C_{pip3}H$; 3.10–2.91 (m, 12 H, $C_{pip4}H_2$); 1.48–1.34 (m, 9 H, CH_2CH_3); 1.10–1.07 (m, 9 H, CH₃). δ_{C} (125 MHz, 298 K, d₆-DMSO) [ppm] = 175.7 (s), 173.1 (s), 172.8 (s), (C_{ar4}); 165.7 (s), 165.3 (s), 165.0 (s), 164.1 (s), (COOH); 154.8 (d, $J_{C,F}^1 = 207.1$ Hz), 154.6 (d, $J_{C,F}^{1} = 205.8 \text{ Hz}$, 154.6 (d, $J_{C,F}^{1} = 207.8 \text{ Hz}$), (C_{ar6}); 153.4 (s), 153.1 (s), 152.8 (s), 151.4 (s), (C_{ar2}) ; 146.2 (d, $J_{C,F}^1 = 207.0$ Hz); 146.1 (d, $J_{C,F}^1 = 207.4$ Hz); 145.5 (d, $J_{C,F}^1 = 212.2$ Hz), (C_{ar8}) ; 133.7 (s), 133.6 (s), 133.5 (s), 133.4 (s), (C_{ar7}) ; 127.4 (d, $J_{C,F}^2 = 5.5$ Hz), 127.2 $(\mathbf{d}, J_{C,F}^2 = 5.0 \text{ Hz}), (C_{ar8'}); 120.8 \ (\mathbf{d}, J_{C,F}^3 = 6.3 \text{ Hz}), 120.4 \ (\mathbf{d}, J_{C,F}^3 = 6.0 \text{ Hz}), (C_{ar4'}); 112.3 \text{ Hz}), (C_{ar4'}); 112.3 \text{ Hz}) = 0.0 \text{ Hz}$ (s), 112.2 (s), 112.1 (s), (C_{ar5}) ; 107.2 (s), 107.1 (s), 106.6 (s), (C_{ar3}) ; 56.5 (s), 56.4 (s), 56.3 (s), (C_{pip2}) ; 54.2 (s), 54.1 (s), 53.9 (s), 53.8 (s), (CH_2CH_3) ; 51.0 (s), 50.9 (s), 49.5 (s), (C_{pip6}) ; 49.5 (br s, C_{pip3}); 44.9 (br, s, C_{pip5}); 17.9 (s), 17.7 (br s) (CH₂CH₃); 16.3 (s), 16.2 (s), 16.1 (s), (CH₃). δ_F (282 MHz, 298 K, d₆-DMSO) [ppm] = (-118.8)-(-118.9) (m), -119.4 (d, $J_{F,F}^4$ = 10.7 Hz), (3 F, C_{ar6}F); -129.4 (d, $J_{F,F}^4$ = 10.4 Hz), (-129.9)-(-130.4) (m), (3 F, $C_{ar8}F$). **MS** (ES+, CH₃OH): m/z (%) = 1143 (30) [ML₃ + Na⁺], 770 (100) $[ML_2]^+$. **HR-ESI-MS**: m/z for $C_{51}H_{54}F_6$ ⁶⁹GaN₉O₉ + Na⁺ calcd. (found): 1142.3102 (1142.3093).

3.2.7.6 Tris(nalidixo)gallium(III), [Ga(nxa)₃]

Method (b) gave an off-white solid (37 mg, 0.046 mmol, 46%). Mp: $\geq 190^{\circ}$ C, decomposition to beige-brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3443 (md, br, water), 3025 (md, br), 2985 (w), 1676 (sh), 1608 (st, br), 1557 (st), 1518 (sh), 1490 (st), 1440 (st), 1380 (w), 1365 (md), 1348 (md), 1320 (md), 1293 (st), 1256 (st), 1227 (sh), 1168 (w), 1130 (st), 1109 (w), 1089 (md), 991 (w, br), 944 (w), 898 (md), 843 (w), 807 (st), 776 (st), 702 (w), 663 (md), 639 (md), 561 (sh), 543 (md), 506 (st), 452 (st). NMR: δ_H (600 MHz, 298 K, d_6 -DMSO) [ppm] = 9.45 (s), 9.35 (s), 9.27 (s), 9.19 (s), (3 H, C_{ar2}H); 8.62 (d, J^3_{H,H} = 8.2) Hz), 8.43–8.31 (m) (3 H, $C_{ar5}H$); 7.61 (d, $J_{H,H}^3 = 8.2$ Hz), 7.56 (d, $J_{H,H}^3 = 8.4$ Hz), 7.51 (d, $J_{H,H}^3 = 7.8$ Hz), (3 H, $C_{ar6}H$); 4.76–4.57 (m, 6 H, CH_2CH_3); 2.69 (d, $J_{H,H}^4 = 30.0$ Hz, 9 H, C_{ar7}CH₃); 1.45 (t, $J_{H,H}^3 = 6.9$ Hz), 1.42 (t, $J_{H,H}^3 = 7.1$ Hz), 1.31 (t, $J_{H,H}^3 = 7.1$ Hz), (9 H, CH₂CH₃). δ_{C} (125 MHz, 298 K, d₆-DMSO) [ppm] = 178.1 (s), 175.6 (s), 175.4 (s), 175.2 (s), (C_{ar4}) ; 165.6 (s), 165.3 (s), 165.1 (s), 165.0 (s), $(C_{ar8'})$ 164.9 (s), 164.8 (s), 164.7 (s), (COOH) 151.7 (s), 151.4 (s), 151.3 (s), 151.1 (s), (C_{ar2}) ; 149.7 (s), 148.4 (s), 147.7 (s), 147.4 (s), (C_{ar7}) ; 135.9 (s), 135.7 (s), 135.4 (s), 135.3 (s), (C_{ar5}) ; 123.0 (s), 122.9 (s), 122.6 (s), (C_{ar6}) ; 118.4 (s), 118.2 (s), 118.1 (s), 118.0 (s), $(C_{ar4'})$; 113.7 (s), 113.6 (s), 113.3 (s), (C_{ar3}) ; 47.1 (s), 47.0 (s), 46.9 (s), 46.8 (s), (CH_2CH_3) ; 25.1 (br s) (CH_3) ; 15.2 (s), 15.1 (s), 15.0 (s), (CH₂CH₃). **MS** (ES+, CH₃OH): m/z (%) = 1296 (10) [M₂L₅]⁺, 785 (100) $[ML_3 + Na^+]$, 531 (20) $[ML_2]^+$. **HR-ESI-MS**: m/z for $C_{36}H_{33}$ ⁶⁹GaN₆O₉ + Na⁺ calcd. (found): 785.1463 (785.1479). **EA**: Anal. Calcd. (found) [%] for $C_{36}H_{33}GaN_6O_9 \cdot 1.5 H_2O$: C, 54.70 (54.59); H, 4.59 (4.42); N, 10.63 (10.24).

3.2.7.7 Tris(norfloxacino)gallium(III), [Ga(nofx)₃]

Method (a) gave a pale yellow solid (38 mg, 0.036 mmol, 36%). **Mp**: $\geq 200^{\circ}$ C, decomposition to orange-brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3391 (md, br, water), 2839 (w, br),

1615 (st, br), 1551 (w), 1519 (md), 1471 (st, br), 1377 (md), 1320 (md), 1255 (st, br), 1188 (md), 1129 (md), 1089 (w), 1037 (md), 971 (w), 930 (st), 889 (sh), 812 (st), 788 (md), 769 (md), 747 (st), 697 (w), 628 (st), 561 (md), 513 (st). **NMR**: δ_H (600 MHz, 298 K, d_6 -DMSO) [ppm] = 9.19 (s), 9.09 (s), 9.05 (s), 8.97 (s), (3 H, C_{ar2}H); 7.94 (d, J_{H,F}^3 = 13.2) Hz), 7.75 (d, $J_{H,F}^3 = 13.2$ Hz), 7.57 (d, $J_{H,F}^3 = 13.8$ Hz), 7.53 (d, $J_{H,F}^3 = 13.2$ Hz), (3 H, $C_{ar5}H$); 7.26 (d, $J_{H,F}^4$ = 6.6 Hz), 7.21 (d, $J_{H,F}^4$ = 7.8 Hz), 7.19 (d, $J_{H,F}^4$ = 7.2 Hz), 7.13 (d, $J_{H,F}^4 = 7.2 \text{ Hz}), \, (3 \text{ H}, \text{ C}_{ar8}H); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 3.44 - 3.31 \ (\text{m}, \, 12 \text{ H overlaid}); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 4.70 - 4.48 \ (\text{m}, \, 6 \text{ H}, \, \text{C}H_2\text{CH}_3); \, 4.70 - 4.48 \ (\text{m}, \, 12 \text{ H}, \, 12 \text{ H$ with water, $C_{pip2,6}H_2$; 3.09–3.02 (m, 12 H, $C_{pip3,5}H_2$); 1.46–1.40 (m), 1.30 (t, $J_{H,H}^3 =$ 7.2 Hz), (9 H, CH₂CH₃). δ_{C} (125 MHz, 298 K, d₆-DMSO) [ppm] = 173.5 (s), 172.9 (s), 172.8 (s), (C_{ar4}) ; 165.7 (s), 165.6 (s), 165.4 (s), (COOH); 152.9 (d, $J_{C,F}^1 = 208.4$ Hz), 152.7 (d, $J_{C,F}^1 = 206.9 \text{ Hz}$), (C_{ar6}) ; 150.5 (s), 150.0 (s), 149.9 (s), (C_{ar2}) ; 145.5–145.0 (m, C_{ar7}); 136.9, 136.8, 136.6 ($C_{ar8'}$); 119.3 (d, $J_{C,F}^3 =$ 7.8 Hz), 119.2 (d, $J_{C,F}^3 =$ 8.2 Hz), 119.0 (d, $J_{C,F}^3 =$ 7.4 Hz), $(C_{ar4'})$; 112.0–110.4 (m, C_{ar5} and C_{ar3}); 105.5 (s), 105.3 (s), 105.0 (s), (C_{ar8}) ; 49.4–48.5 (m, $C_{pip2,6}$ and CH_2CH_3); 44.4 (s), 44.2 (s), 44.1 (s), 44.0 (s), $(C_{pip3,5})$; 14.62 (s), 14.60 (s), 14.5 (s), 14.4 (s), (CH₂CH₃). δ_F (282 MHz, 298 K, d₆-DMSO) [ppm] = -120.9 (s), -121.0 (s), -121.1 (s), -121.2 (s), (3 F, $C_{ar6}F$). **MS** (ES+, CH₃OH): m/z (%) = 1047 (10) [ML₃ + Na⁺], 706 (100) [ML₂]⁺. **HR-ESI-MS**: m/z for C₄₈H₅₁F₃ ⁶⁹GaN₉O₉ + Na⁺ calcd. (found): 1046.2915 (1046.2925).

3.2.7.8 Tris(oxalino)gallium(III), $[Ga(oxa)_3]$

Method (b) gave an off-white solid (69 mg, 0.082 mmol, 82%). **Mp**: \geq 240°C, decomposition to beige-brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3402 (md, br), 3060 (w, br), 2979 (w, br), 2918 (w,br), 1637 (st), 1599 (st), 1567 (md), 1539 (st), 1463 (st, br), 1412 (sh), 1387 (md), 1329 (md), 1258 (st, br), 1193 (md), 1158 (w), 1126 (w), 1087 (w), 1029 (st, br), 933 (md), 904 (md), 846 (w), 812 (st), 777 (st, br), 656 (md), 618 (md), 563 (w). **NMR**: δ_H (600 MHz, 298 K, d_6 -DMSO) [ppm] = 8.90 (s, 3 H, C_{ar2}H); 7.64 (s, 3 H, C_{ar8}H); 7.63 (s, 3 H, $C_{ar5}H$); 6.30 (s, 6 H, OCH₂O); 4.53 (q, $J_{H,H}^3 = 7.1$ Hz, 6 H, CH₂CH₃); 1.37 (t, $J_{H,H}^3 = 7.1$ Hz, 9 H, CH₂CH₃). δ_C (125 MHz, 298 K, d₆-DMSO) [ppm] = 176.0 (s, C_{ar4}); 166.3 (s, COOH); 153.7 (s, C_{ar7}); 147.1 (s, C_{ar6}); 147.0 (s, C_{ar2}); 136.9 (s, $C_{ar8'}$); 121.3 (s, $C_{ar4'}$); 107.4 (s, C_{ar3}); 103.3 (s, OCH₂O); 101.9 (s, C_{ar5}); 97.3 (s, C_{ar8}); 49.6 (s, CH₂CH₃); 14.6 (s, CH₂CH₃). **MS** (ES+, CH₃OH): m/z (%) = 873 (100) [ML₃ + Na⁺], 589 (80) [ML₂]⁺. **HR-ESI-MS**: m/z for C₃₉H₃₀ ⁶⁹GaN₃O₁₅ + Na⁺ calcd. (found): 872.0830 (872.0822). **EA**: Anal. Calcd. (found) [%] for C₃₉H₃₀GaN₃O₁₅·3.5 H₂O: C, 51.28 (51.37); H, 4.08 (3.97); N, 4.60 (4.70).

3.2.7.9 Tris(pipemido)gallium(III), $[Ga(pia)_3]$

Method (b) gave an off-white solid (64 mg, 0.066 mmol, 66%). Mp: $>190^{\circ}$ C, decomposition to beige-brown solid. IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3373 (st, br, water), 3029 (w), 2980 (w), 1616 (st), 1578 (st), 1536 (md), 1510 (md), 1471 (st), 1430 (st), 1378 (sh), 1358 (st, br), 1310 (md), 1280 (md), 1249 (st, br), 1159 (w), 1148 (w), 1128 (st), 1092 (md), 1079 (md), 1045 (md), 1024 (st), 976 (md), 940 (md), 915 (st), 903 (md) 868 (md), 832 (st), 802 (md), 744 (st), 715 (md), 703 (md), 657 (w), 609 (w), 541 (md), 489 (md), 463 (st). NMR: δ_H (600 MHz, 298 K, d_6 -DMSO) [ppm] = 9.17 (s, 3 H, $C_{ar5}H$); 8.94 (s, 3 H, $C_{ar2}H$); 4.37 (q, $J^3_{H,H} = 7.1 \text{ Hz}, 6 \text{ H}, \text{ } \text{C}H_2\text{C}\text{H}_3); 3.85 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ H}, \text{ } \text{C}_{pip2,6}H_2); 2.78 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ H}, \text{ } \text{C}_{pip2,6}H_2); 2.78 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ H}, \text{ } \text{C}_{pip2,6}H_2); 2.78 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ H}, \text{ } \text{C}_{pip2,6}H_2); 2.78 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ H}, \text{ } \text{C}_{pip2,6}H_2); 2.78 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ H}, \text{ } \text{C}_{pip2,6}H_2); 2.78 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ H}, \text{ } \text{C}_{pip2,6}H_2); 2.78 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ H}, \text{ } \text{C}_{pip2,6}H_2); 2.78 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ H}, \text{ } \text{C}_{pip2,6}H_2); 2.78 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ H}, \text{ } \text{C}_{pip2,6}H_2); 2.78 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ H}, \text{ } \text{C}_{pip2,6}H_2); 2.78 \text{ (d}, J_{H,H} = 40.1 \text{ Hz}, 12 \text{ Hz}, 12$ 16.2 Hz, 12 H, $C_{pip3,5}H_2$); 1.35 (t, $J^3_{H,H}$ = 6.9 Hz, 9 H, CH_2CH_3). δ_C (125 MHz, 298 K, d_6 -DMSO) [ppm] = 177.1 (s, C_{ar4}); 165.4 (s, COOH); 160.6 (s, C_{ar7}); 160.1 (s, C_{ar5}); 155.1 (s, $C_{ar8'}$); 150.6 (s, C_{ar2}); 109.7 (s, $C_{ar4'}$); 108.3 (s, C_{ar3}); 45.9 (s, CH_2CH_3); 45.6 (s), 45.3 (s), $(C_{pip2,3,5,6})$; 14.4 (s, CH₂CH₃). **MS** (ES+, CH₃OH): m/z (%) = 999 (100) $[ML_3 + Na^+], 673 (80) [ML_2]^+.$ **HR-ESI-MS**: m/z for $C_{42}H_{48}$ ⁶⁹GaN₁₅O₉ + Na⁺ calcd. (found): 998.2913 (998.2939); for $C_{28}H_{32}$ ⁶⁹GaN₁₀O₆⁺ calcd. (found): 673.1762 (673.1776). **EA**: Anal. Calcd. (found) [%] for $C_{42}H_{48}GaN_{15}O_9 \cdot 12.5 H_2O$: C, 41.97 (41.70); H, 6.12 (5.85); N, 17.48 (17.26).

3.2.7.10 Tris(ciprofloxacino)iron(III), $[Fe(cipro)_3]$

Method (c) gave a red-brown solid (51 mg, 0.049 mmol, 49%). **Mp**: $\geq 220^{\circ}$ C, decomposition to black-brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3411 (w, br, water), 2846 (w, br), 1610 (st), 1543 (w), 1513 (sh), 1450 (st, br), 1371 (md, br), 1285 (sh), 1252 (st), 1182 (md), 1129 (md), 1108 (sh), 1026 (st), 949 (st), 890 (md), 809 (md), 788 (md), 761 (sh), 738 (st), 702 (md), 627 (st), 577 (md), 556 (w), 538 (w), 506 (st). **MS** (ES+, CH₃OH): m/z (%) = 1763 (≤ 10) [M₂L₅]⁺, 716 (100) [ML₂]⁺. **HR-ESI-MS**: m/z for C₅₁H₅₁F₃ ⁵⁶FeN₉O₉ + Na⁺ calcd. (found): 1069.3009 (1069.3007). **EA**: Anal. Calcd. (found) [%] for C₅₁H₅₁F₃FeN₉O₉·2.5 H₂O: C, 56.10 (56.04); H, 5.17 (4.80); N, 11.55 (11.27).

3.2.7.11 Tris(enoxacino)iron(III), $[Fe(enox)_3]$

Method (b) gave a red-orange solid (59 mg, 0.058 mmol, 58%) **Mp**: $\geq 200^{\circ}$ C, decomposition to black brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3403 (md, br, water), 3039 (w, br), 2976 (w, br), 1626 (st), 1565 (md), 1516 (md), 1441 (st, br), 1368 (md), 1347 (md), 1323 (md), 1275 (st), 1251 (st, br), 1184 (md), 1153 (w), 1119 (md), 1093 (md), 1039 (md, br), 971 (md), 943 (md, br), 907 (sh), 812 (st), 788 (md), 763 (md), 744 (md), 677 (w), 650 (md), 625 (st), 562 (md), 518 (st). **MS** (ES+, CH₃NO₂): m/z (%) = 694 (100) [ML₂]⁺ **HR-ESI-MS**: m/z for C₄₅H₄₈F₃ ⁵⁶FeN₁₂O₉ + Na⁺ calcd. (found): 1036.2866 (1036.2866). **EA**: Anal. Calcd. (found) [%] for C₄₅H₄₈F₃FeN₁₂O₉·3 H₂O: C, 50.62 (50.48); H, 5.10 (4.82); N, 15.74 (16.07).

3.2.7.12 Tris(fleroxacino)iron(III), $[Fe(flex)_3]$

Method (b) gave a red-brown solid (91 mg, 0.078 mmol, 78%). **Mp**: $\geq 230^{\circ}$ C, decomposition to brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3054 (w), 2930 (md), 2848 (md), 2792 (md), 1625 (st), 1548 (w), 1526 (md), 1471 (sh), 1447 (st, br), 1413 (md), 1389 (w), 1377 (md), 1358 (md), 1325 (md), 1295 (st), 1245 (md), 1236 (sh), 1203 (md), 1160 (md), 1144 (st),

1121 (md), 1097 (w), 1075 (w), 1045 (md), 1026 (st), 1002 (st), 963 (md), 949 (md, br), 896 (md), 861 (md), 803 (st), 779 (md), 752 (md), 736 (md), 714 (md), 648 (st), 599 (md), 568 (st), 536 (w, br), 517 (w), 491 (w), 474 (w). **MS** (ES+, CH₃OH): m/z (%) = 1184 (20) [ML₃ + Na⁺], 793 (100) [ML₂]⁺. **HR-ESI-MS**: m/z for C₅₁H₅₁F₉ ⁵⁶FeN₉O₉ + Na⁺ calcd. (found): 1183.2913 (1183.2938); for C₃₄H₃₄F₆ ⁵⁶FeN₆O₆⁺: 792.1793 (792.1785). **EA**: Anal. Calcd. (found) [%] for C₅₁H₅₁F₉FeN₉O₉: C, 52.77 (52.78); H, 4.43 (4.53); N, 10.86 (10.49).

3.2.7.13 Tris(levofloxacino)iron(III), $[Fe(levox)_3]$

Method (a) gave a red-brown solid (102 mg, 0.090 mmol, 90%). **Mp**: \geq 190°C, decomposition to black-brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3427 (water), 3041 (w, br), 2935 (w), 2846 (w), 2797 (w), 1615 (st), 1518 (st), 1443 (st), 1382 (md, br), 1330 (st, br), 1291 (w), 1256 (st, br), 1150 (sh), 1129 (md), 1094 (md), 1048 (st), 1003 (md), 978 (st), 896 (md), 864 (w), 843 (w), 826 (sh), 810 (st), 759 (md), 742 (md), 693 (md), 637 (w), 553 (md, br), 508 (st), 443 (st, br). **MS** (ES+, CH₃OH): m/z (%) = 1160 (40) [ML₃ + Na⁺], 777 (100) [ML₂]⁺. **HR-ESI-MS**: m/z for C₅₄H₅₇F₃ ⁵⁶FeN₉O₁₂ + Na⁺ calcd. (found): 1159.3326 (1159.3328); for C₃₆H₃₈F₂ ⁵⁶FeN₆O₈ calcd. (found): 776.2069 (776.2065).

3.2.7.14 Tris(lomefloxacino)iron(III), $[Fe(lomx)_3]$

Method (b) gave a red-brown solid (80 mg, 0.072 mmol, 72%). **Mp**: $\geq 210^{\circ}$ C, decomposition to black-brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3423 (w, br), 2979 (w, br), 2844 (w, br), 2725 (w, br), 2467 (w, br), 1616 (st), 1552 (w), 1520 (st), 1446 (st), 1358 (md, sh), 1321 (st, br), 1275 (w), 1244 (st), 1122 (md), 1089 (md), 1049 (st), 1002 (st), 932 (st), 880 (md), 810 (st), 760 (md, br), 738 (md), 654 (md), 541 (sh), 502 (md), 449 (md). **MS** (ES+, CH₃OH): m/z (%) = 1130 (10) [ML₃ + Na⁺], 756 (100) [ML₂]⁺. **HR-ESI-MS**: m/z for C₅₁H₅₄F₆ ⁵⁶FeN₉O₉ + Na⁺ calcd. (found): 1129.3196 (1129.3193).

3.2.7.15 Tris(nalidixo)iron(III), $[Fe(nxa)_3]$

Method (b) gave a red-brown solid (66 mg, 0.089 mmol, 89%). **Mp**: $\geq 180^{\circ}$ C, decomposition to black-brown solid. **IR** (neat): $\tilde{\nu} \text{ [cm}^{-1]} = 3436$ (water), 3023 (md, br), 2983 (w), 1668 (sh), 1606 (st, br), 1556 (st), 1515 (sh), 1486 (st), 1440 (st), 1366 (md), 1347 (md), 1313 (md), 1289 (st), 1255 (st), 1226 (sh), 1168 (w), 1129 (st), 1089 (md), 990 (w, br), 942 (w), 895 (md), 842 (w), 806 (st), 769 (st), 702 (w), 663 (md), 639 (md), 559 (w), 545 (md), 503 (st), 442 (st). **MS** (ES+, CH₃OH): m/z (%) = 1268 (≤ 10) [M₂L₅]⁺, 772 (100) [ML₃ + Na⁺], 518 (40) [ML₂]⁺. **HR-ESI-MS**: m/z for C₃₆H₃₃ ⁵⁶FeN₆O₉ + Na⁺ calcd. (found): 772.1556 (772.1562). **EA**: Anal. Calcd. (found) [%] for C₃₆H₃₃FeN₆O₉·1 H₂O: C, 56.33 (56.62); 4.60 (4.80); N, 10.95 (11.05).

3.2.7.16 Tris(norfloxacino)iron(III), $[Fe(nofx)_3]$

Method (b) gave a red-brown solid (68 mg, 0.067 mmol, 67%). **Mp**: ≥190°C, decomposition to black-brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3371 (md, br, water), 2838 (w, br), 1610 (st, br), 1547 (w), 1516 (md), 1453 (st, br), 1380 (st), 1324 (md), 1282 (sh), 1252 (st, br), 1186 (st), 1124 (md, br), 1024 (md), 923 (st, br), 891 (sh), 875 (w), 810 (md), 786 (md), 760 (md), 738 (st), 694 (w), 624 (md), 558 (md), 512 (md), 443 (md, br). **MS** (ES+, CH₃OH): m/z (%) = 692 (100) [ML₂]⁺, 1033 (20) [ML₃ + Na⁺]. **HR-ESI-MS**: m/z for C₄₈H₅₁F₃ ⁵⁶FeN₉O₉ + Na⁺ calcd. (found): 1033.3009 (1033.3002). **EA**: Anal. Calcd. (found) [%] for C₄₈H₅₁F₃FeN₉O₉·6 H₂O: C, 51.52 (51.42); H, 5.67 (5.72); N, 11.27 (10.89).

3.2.7.17 Tris(oxalino)iron(III), $[Fe(oxa)_3]$

Method (b) gave a golden-brown solid solid (70 mg, 0.084 mmol, 84%). **Mp**: \geq 240°C, decmposition to brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3030 (w, br), 2979 (w, br), 2913 (w, br), 1633 (st), 1604 (st), 1563 (md), 1536 (md), 1493 (sh), 1460 (st, br), 1410 (sh), 1385
(md), 1319 (md), 1288 (md), 1259 (st, br), 1198 (st), 1155 (w), 1130 (w), 1079 (w), 1041 (st, br), 978 (w), 934 (md), 899 (w), 886 (w), 875 (w), 849 (w), 813 (st), 775 (st), 759 (sh), 714 (w), 656 (md), 621 (md), 570 (md), 524 (st), 504 (sh), 455 (st). **MS** (ES+, CH₃OH): m/z (%) = 860 (\leq 10) [ML₃ + Na⁺], 576 (100) [ML₂]⁺. **HR-ESI-MS**: m/z for C₃₉H₃₀ ⁵⁶FeN₃O₁₅ + Na⁺ calcd. (found): 859.0924 (859.0914). **EA**: Anal. Calcd. (found) [%] for C₃₉H₃₀FeN₃O₁₅·1.5 H₂O: C, 54.24 (54.24); H, 3.85 (3.72); N, 4.87 (4.74).

3.2.7.18 Tris(pipemido)iron(III), $[Fe(pia)_3]$

Method (b) gave a brown solid (78 mg, 0.081 mmol, 81%). **Mp**: $\geq 200^{\circ}$ C, decomposition to brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3406 (md, br), 3028 (w), 2979 (w), 1615 (st), 1578 (st) 1534 (md), 1510 (md), 1471 (st), 1429 (st), 1377 (sh), 1357 (st, br), 1310 (md), 1280 (md), 1248 (st, br), 1158 (w), 1147 (w), 1128 (st), 1092 (md), 1078 (md), 1045 (md), 1023 (st), 975 (md), 940 (md), 914 (st), 903 (md), 867 (w), 832 (st), 802 (md), 784 (md), 744 (st), 715 (md), 704 (sh), 656 (w), 609 (w), 540 (md), 489 (md), 454 (st). **MS** (ES+, CH₃OH): m/z(%) = 986 (20) [ML₃ + Na⁺], 715 (100) [ML₂·3H₂O]⁺, 661 [ML₂]⁺ (10). **HR-ESI-MS**: m/z for C₄₂H₄₈ ⁵⁶FeN₁₅O₉ (M + Na⁺) calcd. (found): 985.3007 (985.3017). **EA**: Anal. Calcd. (found) [%] for C₄₂H₄₈FeN₁₅O₉·4 H₂O: C, 48.75 (48.94); H, 5.45 (6.23); N, 20.30 (20.33).

3.2.8 Synthesis & Characterization of Tris(maltolato)metal(III) Complexes

3.2.8.1 Tris(maltolato)gallium(III), [Ga(ma)₃]

The synthesis followed the literature procedure.⁴¹³ Scale: 3-hydroxy-2-methyl-4*H*-pyran-4-one (2.60 g, 20.55 mmol), gallium(III) nitrate nonahydrate (2.86 g, 6.85 mmol), water (40 mL). Yield: off-white solid (1.957 g, 4.40 mmol, 64%). **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3027 (w), 1611 (sh), 1568 (st), 1514 (st), 1456 (st), 1295 (sh), 1277 (st), 1241 (md), 1192 (st), 1087 (w), 1043 (md), 921 (md), 850 (st), 830 (st), 745 (st), 720 (st), 664 (md), 617 (md), 557 (sh), 528 (sh), 511 (w), 493 (md). **MS** (ES+, CH₃OH): m/z (%) = 765 (100) [M₂L₅]⁺, 445 (10) [ML₃H⁺], 319 (40) [ML₂]⁺. **HR-ESI-MS**: m/z for C₁₈H₁₅ ⁶⁹GaO₉ (M + Na⁺) calcd. (found): 466.9870 (466.9866). **EA**: Anal. Calcd. (found) [%] for C₁₈H₁₅GaO₉·1 H₂O: C, 46.69 (46.77); H, 3.70 (3.34).

3.2.8.2 Tris(maltolato)iron(III), $[Fe(ma)_3]$

The synthesis followed the literature procedure. ⁴¹⁴ Scale: 3-hydroxy-2-methyl-4*H*-pyran-4-one (3.792 g, 3.0 mmol), iron(III) nonahydrate (4.037 g, 1.0 mmol), water and ethanol (100 mL each). Yield: ruby-red solid (0.354 g, 0.82 mmol, 82%). **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3024 (w), 1605 (sh), 1564 (st), 1505 (st), 1455 (st), 1293 (sh), 1272 (st), 1238 (md), 1190 (st), 1084 (w), 1038 (md), 920 (md), 849 (st), 829 (st), 745 (st), 719 (st), 664 (md), 607 (md), 536 (st), 470 (st). **MS** (ES+, CH₃OH): m/z (%) = 737 (20) [M₂L₅]⁺, 454 (70) [ML₃Na⁺], 306 (100) [ML₂]⁺. **HR-ESI-MS**: m/z for C₁₈H₁₅ ⁵⁶FeO₉ (M + Na⁺) calcd. (found): 453.9963 (453.9963). **EA**: Anal. Calcd. (found) [%] for C₁₈H₁₅FeO₉·2 H₂O: C, 46.28 (46.19); H, 4.10 (3.86).

3.3 Results & Discussion

3.3.1 Synthesis

The tris(quinolono)metal(III) complexes were derived through the reaction of quinolone and gallium(III) or iron(III) nitrate in a 3:1 ratio (Figure 3.2). Similar to the reaction pathways reported to give a diversity of metal-quinolone complexes,^{398 401 415} the synthesis can be carried out in three principle steps. First, the respective quinolone was dissolved in water or methanol. Most quinolones exist in a zwitterionic state of neutral charge in the neutral pH-range (Chapter 2), which makes their dissolution difficult. Upon proto-

$$3 \xrightarrow[R_{7}]{} \begin{array}{c} O \\ R_{7} \\ R_{7} \\ R_{8} \\ R_{1} \end{array} \xrightarrow{P} O \\ R_{8} \\ R_{1} \end{array} + M(NO_{3})_{3} \cdot 9 H_{2}O \xrightarrow{PH 5-7} r.t. \text{ or } \Delta \\ H_{2}O/CH_{3}OH \end{array}$$
 [ML_3]

Figure 3.2: Synthetic pathway to tris(quinolono)metal(III) complexes, $M = Ga^{3+}$, Fe^{3+} .

nation (HCl) or deprotonation (NaHCO₃, NaOH, KOH) at room temperature or in heat $(60-100^{\circ}C)$, depending on the chemical nature of the quinolone, it dissolves more easily; Hcipro·HCl and Hlomx·HCl were readily dissolved in the solvent of choice at room temperature. The dissolved quinolone was then added dropwise to the respective metal(III) nitrate, which was either solid or previously had been dissolved in water or methanol as well. Throughout the addition, the pH was carefully monitored and kept below pH 3 to avoid the formation of insoluble metal(III) hydroxides (Ga(OH)_{3(s)}, Fe(OH)_{3(s)}). Finally, the pH of the reaction solution was adjusted to pH 5-7 and stirred rigorously. The desired complex of the general form $[M(quino)_3]$ with $M = Ga^{3+}$, Fe^{3+} was obtained as a colored solid, which in most cases readily precipitated from the aqueous reaction mixture at room temperature and was isolated on a fine glass frit; in some reaction mixtures with a high methanol content, the precipitate started to form after reduction of the initial reaction volume by at least 50% (*in vacuo* or evaporation in air). The precipitate was washed thoroughly with water and methanol and dried *in vacuo*. In general, the yields were between 40-90% but varied with the employed method and the respective quinolone ligand. Analyses of the obtained compounds via HR-ESI mass spectrometry were consistent with the formation of tris(quinolono)metal(III) formulations. Elemental analyses for the three elements C, H, and N supported these findings successfully for the majority of compounds.

Besides the nine novel tris(quinolono)gallium(III) complexes, $[Ga(quino)_3]$, and their

nine iron(III) analogs, [Fe(quino)₃], tris(maltolato)gallium(III) and tris(maltolato)iron(III) were synthesized following the published procedures.^{413,414} In addition, several synthetic attempts were made to prepare mixed ligand complexes of the general form $[\text{GaL}_{x}^{a}L_{y}^{b}]$ $(x+y=3 \text{ and } x, y \neq 0)$ with $L^{a,b}$ being potentially bidentate ligands such as anions of the quinolones, maltol, acetylacetonate,⁴⁰⁵ or other heterochelates.⁴⁰⁴ Today, a combination of different bactericidal drugs is often administered to the patient in severe cases of infections, and such treatment showed potential to overcome bacterial resistance; ³⁹⁶ likewise, the complexation of different quinolones to Ga^{3+} might lead to a deadly combination cocktail for bacteria, which could be conveniently administered in a single dose. Moreover, introducing maltol, a widely used food-additive, into such a mixed ligand complex, the pharmacological properties could be altered further. Unfortunately, such mixed ligand complexes were difficult to realize because the ligand exchange rates of Ga^{3+} as well as Fe^{3+} are fast $(k_{H2O} = 10^3 - 10^2 \text{ s}^{-1})$,⁴¹⁶ and only a cocktail of respective metal(III)-complexes with various ratios of 2:1 ligands coordinated to the central metal ion could be detected in the mass spectra. Compared to Ga³⁺ and Fe³⁺, the ligand exchange rate of Cr³⁺ is slow (k_{H2O} = 10^{-6} s^{-1} ;⁴¹⁶ however, not even in the attempted chromium(III) mixed-ligand complexes one single species formed dominantly (data not shown).

3.3.2 Characterization

Tris(quinolono)gallium(III) complexes are off-white to yellow in color, while tris(quinolono)-iron(III) complexes come in various shades of red-brown; the intensity in color is directly related to the electronegativity present at the core aromatic ring system, as highly fluorinated quinolone-ligands, such as lomefloxacin or fleroxacin, give gallium(III) and iron(III) complexes of more intense color. All synthesized complexes are nonvolatile and stable. When stored in a desiccator at ambient temperature in darkness (cupboard), the complexes did not degrade over the course of four years; however, melting point measurements and TGA revealed that they start to decompose when heated to 250°C or higher. The solubility of these complexes is low and highly pH dependent. Upon heating and sonicating, they dissolve in DMSO, which was therefore the chosen solvent for structure analyses by NMR spectroscopy at 600 MHz, while MS spectra were recorded at much lower compound concentration in methanol or acetonitrile, if necessary under pH adjustments. Of course, the lack of solubility as well affected the growth of crystals suitable for X-ray analysis (Section 3.3.3).

The recorded mass spectra were diagnostic of the complex formulations being 3:1 quino:metal. In all cases, loss of one ligand from a $[ML_3]$ unit was observed as the $[ML_2]^+$ fragment with a mass-to-charge ratio of 100%. With the exception of $[Fe(enox)_3]$, all iron(III) and gallium(III) complexes gave $[NaML_3]^+$ or $[KML_3]^+$ as the parent peak. In addition, $[M_2L_5]^+$ peaks of low intensity ($\leq 10\%$) were observed, which occur through the cationization of the molecular unit by recombination of one $[ML_2]^+$ fragment with one $[ML_3]$ unit and have been previously reported as a characteristic MS feature of tris(maltolato)gallium(III).⁴¹³ Due to limitations of the mass spectrometer, these recombination peaks could be only observed for complexes of nalidixic acid, a low-mass quinolone ligand (232.24 g/mol). Figure 3.3 shows the ES+ spectra of the novel $[Ga(nxa)_3]$ complex and its iron(III) derivative.

Elemental analyses have been performed on all eighteen tris(quinolono)metal(III) complexes. Similar to literature reports of other tris(quinolono)iron(III) complexes, $^{398\,417}$ they contain several water molecules in their elemental formulae. This holds especially true for the gallium(III) derivatives, of which only [Ga(nxa)₃], [Ga(oxa)₃] and [Ga(pia)₃] match the calculated results for C,H, and N with 1.5, 3.5, and 12.5 molecules of water included in the elemental formula, respectively. Theses three quinolone ligands all belong to the first generation of quinolone antimicrobial agents (Chapter 2) and their molecular structures do



Figure 3.3: Low-resolution MS spectra (ES+) of $[GaL_3]$ and $[FeL_3]$ (L = nxa).

not contain any bulky substituents, fluorine atoms, or stereocenters. Numerous attempts from many different compound batches have been made for the tris(quinolono)gallium(III) complexes to pass EA, but over and over only the same three representatives matched their calculated result. For the tris(quinolono)iron(III) complexes, the situation is different. With the exception of $[Fe(lomx)_3]$ and $[Fe(levox)_3]$, the iron(III) analogs match the calculated values for C, H, and N. Although various different batches of $[Fe(lomx)_3]$ and $[Fe(levox)_3]$ compounds were submitted for elemental analysis, the analytical result for the amount of carbon in in elemental formula did never match, which is probably related to the fact that the ligands, levofloxacin as well as lomefloxacin, contain a stereocenter. Already in Chapter 2, these two quinolones showed the largest differences between the analytical and found values of C, H, and N.

Next to mass spectrometry and elemental analysis confirming the total mass of the compound and therewith the metal-to-ligand ratio, infrared spectroscopy was employed to study the metal-ligand coordination. Although the IR spectra of the quinolones are quite complex, due to the varied C-H and C-N vibrations as well as various functional sub-

stituents on the condensed aromatic ring system, the stretching frequencies of the carboxylate on C_{ar3} and the carbonyl on C_{ar4} -position are strong and lend themselves as suitable IR-handles to characterize the chelation of the metal (Figure 3.4). The IR spectrum of the free ligand (HL) shows the intense stretching vibration of the dimeric carboxylate group (ν_{COOH}) between 1722–1707 cm⁻¹ with the exception of quinolones in their ionic form, in which the carboxylate group is deprotonated, because ionic carboxylates show no carbonyl stretching.^{317 318} When the quinolone ligand is fully coordinated to the metal, this strong band disappears completely, indicating that no free HL is present in the sample and that the quinolone anion binds to the metal through one carboxylato-O. Furthermore, the two distinct bands in the range of 1637–1606 cm⁻¹ and 1400–1300 cm⁻¹ are assigned as ν_{CO2} asymmetric and symmetric stretching vibrations, being characteristic for the complexation of a quinolone ligand to a metal.³²⁸ In the vast amount of IR data on metal-quinolone complexes described in the literature, there is almost complete agreement about the assignment of the asymmetric stretching frequency ($\nu_{asym}(CO_2)$), however, the frequency of the symmetric stretch $(\nu_{sym}(CO_2))$ has been assigned quite ambiguously. From our own experience, we know that the difficulty in identifying $\nu_{sym}(CO_2)$ with certainty lies with the number of bands appearing in the IR spectrum between 1400 $\rm cm^{-1}$ and 1300 $\rm cm^{-1}$, in addition stretches in this region are often quite broad. In the case of the tris(levofloxacino)metal(III) and tris(lomefloxacino)metal(III) complexes, only a single broad band with several weak shoulder stretches is observed in this specific 100 $\rm cm^{-1}$ range. After carefully considering the IR stretches of the nine free ligands in this region (Chapter 2) as well as comparing the IR spectra of the eighteen synthesized $[M(quino)_3]$ complexes $(M = Ga^{3+}, Fe^{3+})$, we have decided to include an overview of the single carboxylato stretching frequencies in our discussion despite the previously mentioned controversy and described challenges. The difference $\Delta = \nu_{asym}(CO_2) - \nu_{sym}(CO_2)$ varies within values from minimum 230 cm⁻¹ for [Fe(nofx)₃] to maximum 265 cm⁻¹ for [Ga(lomx)₃] with an average value of $\Delta_{Ga,average} = 248$ cm⁻¹ for all nine tris(quinolono)gallium(III) complexes and $\Delta_{Fe,average} = 246$ cm⁻¹ for all nine tris(quinolono)iron(III) complexes (Table 3.1). The similarities between the Δ values for complexes with the same ligand, which is further reflected in the conformity of the $\Delta_{average}$ values, demonstrate the uniformity of the studied [Ga(quino)₃] and [Fe(quino)₃] originating from the chemical similarity of the central metals. According to the Δ values, the central metal is chelated in a monodentate mode, ^{318 418} chemically bound through the deprotonated carboxylate group on C_{ar3} and coordinated through the carbonyl group on C_{ar4}, which is the most common coordination mode in quinolone chelates.³²⁷ The cooperation of the carbonyl group in the chelation of the metal is further supported by a shift to lower wave numbers for the C_{ar4}=O band upon complexation, a critical observation that has been previously made for tris(norfloxacino)aluminium(III).⁴¹⁹ Furthermore, the coordination of the metal(III) ion via the carbonyl-O and the carboxylato-O is reflected in the ν (M-O) stretching vibrations dominant between 600–500 cm⁻¹.⁴²⁰

Figure 3.4 shows the recorded IR spectra of $[Ga(nofx)_3]$ and $[Fe(nofx)_3]$ together with

Table 3.1: Carboxylate stretching frequencies $[cm^{-1}]$ of the synthesizedtris(quinolino) gallium(III) and iron(III) complexes. IR spectra were recordedneat in solid state.

complex	$\nu_{asym}(CO_2)$	$\nu_{sym}(CO_2)$	$\Delta^{(a)}$	complex	$ u_{asym}(CO_2) $	$\nu_{sym}(CO_2)$	$\Delta^{(a)}$
$Ga(cipro)_3$	1620	1373	247	Fe(cipro) ₃	1610	1371	239
$Ga(enox)_3$	1625	1369	256	$Fe(enox)_3$	1626	1368	258
$Ga(flex)_3$	1622	1376	246	$Fe(flex)_3$	1625	1377	248
$Ga(levox)_3$	1616	1385	231	$Fe(levox)_3$	1615	1382	233
$Ga(lomx)_3$	1619	1354	265	$Fe(lomx)_3$	1616	1358	258
$Ga(nxa)_3$	1608	1365	243	$Fe(nxa)_3$	1606	1366	240
$Ga(nofx)_3$	1615	1377	238	$Fe(nofx)_3$	1610	1380	230
$Ga(oxa)_3$	1637	1387	250	$Fe(oxa)_3$	1633	1385	248
$Ga(pia)_3$	1616	1358	258	$Fe(pia)_3$	1615	1357	258

$$^{(a)}\Delta = \nu_{asym}(CO_2) - \nu_{sym}(CO_2).$$



Figure 3.4: IR spectra of norfloxacin as free ligand (HL, black), $[Ga(nofx)_3]$ (green), and $[Fe(nofx)_3]$ (red).

that of the free ligand norfloxacin. Characteristic is the intense band at 1722 cm⁻¹ of the aromatic carbonyl functionality in the spectrum of the free ligand, which disappears upon coordination to the metal. Other major differences in the spectrum of Hnofx compared to its metal(III) complexes are the single broad stretch in the range from 1400–1300 cm⁻¹ for the metal complexes, in addition to the shift of the $C_{ar4}=O$ band from 1614 cm⁻¹ (Hnofx) to 1615 cm⁻¹ ([Ga(nofx)₃]) and 1610 cm⁻¹ ([Fe(nofx)₃]), in addition to new bands occurring

in the low finger-print region (800–400 cm⁻¹) that are related to the ν (M–O) stretching vibrations. The broad absorption around 3400 cm⁻¹ in both tris(norfloxacino)metal(III) complexes once more shows the presence of water in the compound, as discussed in connection with the EA and TGA results (Section 3.3.4).

To record nuclear magnetic resonance spectra of the ¹H and ¹³C nuclei, as well as of the 19 F nucleus where applicable, the isolated complexes were dissolved in d_6 -DMSO, heated with a heat gun, and sonicated until the test solutions turned clear ($\sim 4 \text{ mg/mL}$). In the tris(quinolono)iron(III) complexes, the central Fe^{3+} ion retains a paramagnetic highspin state upon complexation, therefore, NMR signals for all three nuclei are substantially broadened and impossible to assign with certainty. While MS and EA data proved the formation of ML₃ complexes, and IR data showed that the central metal(III) ion was coordinated from three quinolone bidentate anions, the NMR data offered a first impression into the stereochemistry and solution chemistry of the diamagnetic tris(quinolono)gallium(III) complexes. Four stereoisomers are possible for the coordination of three bidentate ligands in an octahedral fashion: Δ -fac, Λ -fac, Δ -mer, and Λ -mer. In the ¹H and ¹³C spectra recorded at 298 K, the stereoisomers gave a multitude of signals.ⁱ Figure 3.5 shows the recorded ¹⁹F spectra for tris(enoxacino)gallium(III). Enoxacin contains one fluorine atom on C_{ar6} that gives one single peak at -127.3 ppm in the ¹⁹F spectrum (Section (2.2.4.2), which upon complexation of the metal multiplies to four signals for the respective $[Ga(enox_3]$ complex with an integration of 1:1:0.8:0.2 adding up to a total of 3 ¹⁹F. Interestingly, $[Ga(oxa)_3]$ as well as $[Ga(pia)_3]$ do not show multiple signals in the ¹H and ¹³C NMR measurements; either these complexes possess a unique stereochemistry, because of the condensed cyclic ether or the pyrido [2,3] pyrimidine aromatic core, respectively, or they simply cannot stand the rather rough conditions necessary to dissolve them in the NMR

ⁱPlease see Appendix B for a temperature dependent ¹H NMR study of tris(vosaroxacino)gallium(III), where the interchange happened rapidly on the NMR time scale at 393 K.



Figure 3.5: ¹⁹F NMR spectrum of [Ga(enox)₃].

solvent, however, both complexes were successfully analyzed by HR-ESI mass spectrometry and elemental analysis.

3.3.3 Solid State Structure

To further characterize the coordination complexes, attempts were made to grow a single crystal of a tris(quinolono)metal(III) complex suitable for X-ray diffraction. Numerous crystallization experiments were performed with all nine quinolones and their respective gallium(III) and iron(III) complexes trying various organic solvents (acetone, acetonitrile, chloroform, DMSO, dimethyl formamide, ethanol, ethyl acetate, methanol) and aqueous solvents as well as mixtures thereof following many different crystallization procedures (concentration gradients, layering of solvents, reactive crystallization, diffusion in solution and in air with different glass ware set-ups, open to air or tightly/partly capped) in a multitude of environmental settings (window sill at all four seasons, fume hood, shelf, dark cupboard,

fridge, freezer); unfortunately, these only yielded crystals of tris(quinolono)metal(III) complexes that were not suitable for X-ray diffraction, such as very fragile needles, or crystals of the free quinolone ligand that were suitable for X-ray diffraction, but had already been reported. Although reactive crystallization attempts were not successful with Ga³⁺, Fe³⁺, or Al³⁺, crystals were grown by layering lanthanum(III) nitrate (44 mg, 0.1 mml) with water (1 mL) and a solution of cipro⁻ (deprotonated with NaOH) in methanol/DMSO/diethyl ether, giving the solid-state structure depicted in Figure 3.6. Unfortunately, the counter cation Na⁺ could not be detected with certainty in this solid state structure, therefore, further reactive crystallization attempts were made employing bulky cations, such as tetrabutyl- or tetraethyl-ammonium; however, these attempts only resulted in structures further complicated through the coordination of multiple molecules of methanol solvent and nitrate (data not shown). In the $[La(cipro)_4]^-$ complex (Figure 3.6), the lanthanum ion at the center is coordinated by four ciprofloxacin ligands via the carboxylate-O on C_{ar3} and the carbonyl-O on C_{ar4} . Bond lengths between the carboxylate-O and La(1) vary from 2.26(2) Å (O(8)) to 2.480(14) Å (O(2)) and are therewith shorter than the bond contacts between the carbonyl-O and La^{3+} , which lie in the range from 2.538(12) Å (O(12)) to 2.564 (12) Å (O(6)).

As crystals of a tris(quinolono)gallium(III) could not be obtained, DFT calculations were run on one of the four possible stereoisomers of $[Ga(cipro)_3]$ to propose a possible 3D structure model of the complex. The result of the DFT calculation is graphically presented in Figure 3.7. Earlier, Psomas had demonstrated that the average energies of all four stereoisomers of tris(ciprofloxacino)iron(III) were almost equal (~150 kcal mol⁻¹) and that the difference in minimum energy between the *fac* and *mer* isomers of $[Fe(cipro)_3]$ was 0.7 kcal mol⁻¹, essentially negligible;⁴⁰¹ therefore, it was deemed sufficient to calculate only one of the possible four stereoisomers of the gallium(III) analog. The three carbonyl-O



Figure 3.6: Solid state structure of $[La(cipro)_4]^-$. Hydrogen atoms omitted for clarity. The La³⁺ ion at the center is coordinated by four ciprofloxacin ligands through one O-atom of the carboxylate group on C_{ar3} and the carbonyl-O on C_{ar4} with the following respective bond contacts [Å]: O(2)-La(1) 2.480(14), O(3)-La(1) 2.470(10), O(5)-La(1) 2.449(14), O(6)-La(1) 2.564(12), O(8)-La(1) 2.26(2), O(9)-La(1) 2.540(13), O(11)-La(1) 2.471(11), O(12)-La(1) 2.538(12).

atoms and the three carboxylate-O atoms are arranged around the central Ga^{3+} atom in an octahedral fashion. The calculated bond angles reveal a slight distortion that constitutes the difference in bond lengths between the Ga^{3+} atom the O-atoms of the carbonyl and carboxylate groups. The calculated bond lengths of the Ga–O(carbonyls) and the Ga–O(carboxylates) in the equatorial plane are 2.167 Å and 1.679 Å, respectively. A similar trend can be observed along the vertical axis of the distorted octahedron, where the



Figure 3.7: Result of the DFT calculation of (fac, Δ) -[Ga(cipro)₃]. Graphical presentation with *Avogadro* as stick-model (hydrogen atoms omitted for clarity). Calculated bond lengths [Å] and bond angles [°]: Ga-O(1) 1.671, Ga-O(2) 2.167, Ga-O(3) 2.167, Ga-O(4) 1.679, Ga-O(5) 1.679, Ga-O(6) 2.165, O(1)-Ga-O(2) 87.4, O(1)-Ga-O(4) 107.9, O(2)-Ga-O(5) 85.3, O(5)-Ga-O(4) 107.3, O(4)-Ga-O(3) 87.2, O(3)-Ga-O(2) 74.4, O(1)-Ga-O(6) 155.3.

Ga–O(carbonyl) bond was 2.165 Å longer than the Ga–O(carboxylate) bond of 1.67 Å, proving a very slight compression along the vertical axis. This difference in bond lengths between the O-atoms of the carbonyl and the carboxylate groups corresponds well with the experimental result of $[La(cipro)_4]^-$ (Figure 3.6).

3.3.4 Thermal Stability

TGA/DTA measurements were carried out for tris(ciprofloxacino)gallium(III), its iron(III) analog, and free ciprofloxacin ligand to investigate and compare the thermal stability of these three compounds (Figure 3.8). Around 250°C, ciprofloxacin starts to show weight loss that finishes around 450°C, resulting in a total weight reduction of about 76%. Two endothermic peaks appear during the single weight loss step, the first one at 255°C matches



Figure 3.8: TGA/DTA results of [Ga(cipro)₃] (green), [Fe(cipro)₃] (red), and ciprofloxacin (Hcipro) (gray). Measurements were taken in the temperature range of 25-900°C with a heating rate of 5°C/min under N₂-flow (19.8 mL/min).

well with the previously determined melting point of ciprofloxacin $(252-255^{\circ}C, Section 2.2.4.1)$, while the second one at 415°C can be interpreted as the boiling point, above which only the bare aromatic core remains as a graphite fragment $[C_9H_3N]$ corresponding to 37.8% (calc.) of the total molecular mass. The observed weight loss curves of tris(ciprofloxacino)gallium(III) and its iron(III) complex analog proceed similarly up to about 600°C, when the iron(III) complex begins to decline at a faster rate than the corresponding gallium(III) complex. In the initial heating step from up to 100°C, both complexes shed water molecules, $[Ga(cipro)_3]$ one and $[Fe(cipro)_3]$ four. This again further supports the observation that quinolone-metal complexes have numerous water molecules in their lattice, 421422423 as was previously discussed for FT-IR spectra and EA results. Corresponding well with the behaviour of ciprofloxacin, the respective metal complexes are thermally stable from 25°C to approximately 260°C. At 263°C, $[Ga(cipro)_3]$ starts de-

composing in one single step resulting in a total weight loss of 62.2% (expected weight loss 64.5%), corresponding to the remaining fragment $[GaC_9H_3N]$. At 258°C, $[Fe(cipro)_3]$ starts decomposing in one single step, however, here the total weight loss is almost complete to 4.22%, which corresponds well with the formation of FeO at temperatures above 575°C. Both tris(ciprofloxacino)metal(III) complexes possess similar thermal stability, which ultimately arises from the thermal stability of the free ligand itself; possible degradation products (metal oxides) depend on the accessible chemistry of the metal ion.

3.3.5 Stability in Solution

Solution thermodynamic investigations of ciprofloxacin in aqueous sodium chloride solution (0.16 M) have provided pK_a values of 6.40 (1) and 8.65 (1), obtained by potentiometric titration. These are in good accordance with values reported for similar systems (Table 3.2); as discussed in Chapter 2 (see Figure 2.3), in the neutral pH-range the zwitterionic species is dominant.

Although potentiometric titration curves, starting at pH 2 and ending at pH 12, were obtained in triplicate for the Ga³⁺:Hcipro as well as the Fe³⁺:Hcipro system in ratios of 1:1, 1:2, 1:3, and 1.4:1, the fitting of the obtained data with the Hyperquad software remains a challenge. All three potentiometric curves obtained under the same conditions match beautifully. Throughout the titration process, the solution remains clear, but after

Table 3.2: Comparison of the determined pK_a values of ciprofloxacin with literature values.

conditions	(a)	$(b)^{424}$	$(c)^{424}$	$(d)^{315}$	
pK_{a1}	6.40(1)	6.18(1)	6.17(2)	6.17(1)	

⁽a) [Hcipro]= $8 \cdot 10^{-4}$ M, 298 K, $I_{NaCl} = 0.16$ M. (b) [Hcipro]= 10^{-3} M, 298 K, $I_{NaCl} = 0.15$ M. (c) [Hcipro]= 10^{-3} M, 310 K, $I_{NaCl} = 0.15$ M. (d) [Hcipro]= $8 \cdot 10^{-4}$ M, 298 K, $I_{KCl} = 0.20$ M.

reaching the end point (pH 12) and standing overnight, insoluble hydroxides precipitated from the test solution of the Fe^{3+} :Hcipro system (Fe(OH)₃, red). Equilibria from pH 5 to pH 8 were slow and the maximum wait time of 1 h was reached in some cases. In this pH range, the obtained titration curve is not as smooth as in the lower and higher pH ranges, which could indicate some small amounts of insoluble hydroxides forming, however, not enough to be detected by human eye. Numerous attempts have been made to slow down the titration. The concentration of ciprofloxacin ligand in the system was reduced from $8 \cdot 10^{-4}$ M to $7 \cdot 10^{-4}$ M and $5 \cdot 10^{-4}$ M. Furthermore, the concentration of sodium hydroxide base was reduced from 0.1597 M to 0.1006 M, while the volume of the titration vessel was increased from 5 mL to 10 mL to ensure that with the smallest increment of base addition $(2 \ \mu L)$ less hydroxide ions entered the test solution. The tardiness of the equilibria between Hquino and metal ions is a known fact; a previous study of the Fe³⁺:Hcipro system reported waiting times of seven days until the pH of the test solution was stable (within ± 0.01 pH unit).⁴²⁵ Apart from the slow equilibria, the representation of zwitterionic species in the fitting model is a complex issue, in addition to the myriad of possible hydroxide species, which need to be included in the solution as well. Previously reported calculated $\log\beta$ values for the Fe³⁺:Hcipro system did not lead to a mathematical fit or a chemically sensible solution of the potentiometric data either.⁴²⁶ My colleague Dr. Jacqueline F. Cawthray, who is highly experienced in the field of potentiometric titrations, who has trained me in this technique, and who has attempted to fit the data herself, contacted Dr. Peter Gans about this, a renowned analytical chemist and the software engineer of the Hyperquad program; however, even insights from these discussions did not guide us to a good fit. Dr. Cawthray repeated some of the titrations of the Ga^{3+} :Hcipro system herself and got the same results than I had previously obtained, therefore, potential operator errors can be excluded with confidence as well.



Figure 3.9: Initial spectrophotometric study of the Ga³⁺:Hcipro system. [Hcipro]= $8.0 \cdot 10^{-4}$ M, [Ga³⁺]= $6.7 \cdot 10^{-4}$ M, I_{NaCl}= 0.16 M, ambient temperature.

Some of my preliminary spectrophotometric studies proved that UV-Vis titrations might be a suitable avenue to determine the stability constants (Figure 3.9), which has led to success in case of the iron(III)-vosaroxin system discussed in Chapter 5. Spectrophotometric measurements were made on solutions of ciprofloxacin ($8 \cdot 10^{-4}$ M) with varying amounts of Ga³⁺ to give ratios of Ga³⁺:Hcipro= 1:1, 1:2, 1:3. For each ratio, different test solutions were prepared with a pH value from pH 2 to pH 12. Similar to the UV-Vis spectra of the free ligand (Hcipro) discussed in Section 2.3.1, both absorbance maxima show a strong pH dependence; isobestic points exist at 268 nm, 317 nm, 345 nm.

3.3.6 Antimicrobial Susceptibility Testing

The antimicrobial activities of the synthesized tris(quinolono)metal(III) complexes were evaluated according to the single-disk method against pathogens associated with nosocomial diseases. *E. faecalis* and methicillin-susceptible *S. aureus* (both Gram-positive), as well as *E. coli*, *K. pneumonia*, and *P. aeruginosa* (all Gram-negative).³³⁰ The metal complexes were tested at a concentration of 0.1 mM against the respective free quinolone ligand at 0.1 mM as well as at 0.3 mM corresponding to the ratio of ligand to metal (3:1) in the complexes.

The susceptibility of pathogens against various quinolone-metal and various gallium(III) complexes has been previously evaluated using the single-disk method in a various growth media, such as Bactec, ³⁸¹ Bacto, ³⁷² Iso-Sensitest, ^{427 428 429} Lauria³⁰⁴, Mueller-Hinton,⁴³⁰ and modified personal recipes.⁴³¹ In their antimicrobial susceptibility single-disk test procedure, the CLSI recommends Mueller-Hinton medium,⁴³² but Iso-Sensitest is a synthetic and chemically reliable medium, which has been widely used in Europe. As elucidated further in Chapter 2, Iso-Sensitest is our medium of choice for these tests; however, to be able to exclude potential effects of the test medium on our metal complexes, e.g., crossmetallation, we examined the antimicrobial potency of all metal complexes in Iso-Sensitest media (Table 3.3) and additionally chose to test ciprofloxacin, levofloxacin, nalidixic acid, and their respective metal compounds in Mueller-Hinton media as well (Figure 3.10). These three quinolones were selected, as they represent three different generations of quinolone antimicrobial agents (Chapter 2). Their respective gallium(III) and iron(III) complexes performed comparably well in both media giving similar inhibition zone sizes against the pathogens, with K. pneumonia being an exception to the rule. This microorganism had already developed a resistance against nalidixic acid and was only susceptible to ciprofloxacin and levoxfloxacin and their respective gallium(III) and iron(III) complexes. All four complexes seemed to perform slightly better against K. pneumonia in Mueller-Hinton than in Iso-Sensitest medium with recorded inhibition zone sizes of >25 mm over 20 mm, respectively (Figure 3.10). Because the pathogen grew on both agar medium plates in an off-white, pale yellow color, the evaluation was not influenced by any differences in bacterium growth color but, measuring the inhibition zone sizes of K. pneumonia growth was challenging due to the frayed edges of the inhibition zones and several different inhibition rings of weakening intensity around each disk, and both effects have potentially led to a larger divergence of the measured inhibition zone size values in this organism. ATCC recommends to grow the chosen strain of K. pneumonia (ATCC-13883) on a nutrient agar composition of 3.0 g beef extract, 5.0 g peptone, and 15.0 g agar, which is neither the exact recipe of Mueller-Hinton nor Iso-Sensitest medium (Appendix A);⁴³³ therefore, any further differences in growth related to the chosen biological growth medium, which further could have affected the size of the inhibition zone, can be ruled out.

To further study the stability of tris(ciprofloxacino)gallium(III) and tris(ciprofloxacino)-iron(III), solutions of both compounds (0.1 mM) in 50% Iso-Sensitest broth were monitored via UV-Vis spectroscopy over the course of 24 hours (experimental set-up as described in Chapter 2, data not shown). No changes in the UV-Vis spectra were detected, which would have indicated potential chemical alterations or degradation, and the complexes are therewith considered stable in Iso-Sensitest medium. In summary, we are confident that the tris(quinolono)metal(III) complexes stay intact over the course of the antimicrobial single-disk test and are not affected by ingredients of the Iso-Sensitest medium.

The results of the antimicrobial susceptibility study of the tris(quinolono)gallium(III) and -iron(III) complexes (0.1 mM) in direct comparison to the respective free quinolone ligand at single (0.1 mM) and triple concentration (0.3 mM) are summarized as average values of recorded inhibition zone sizes from three independent plates in Table 3.3. Three conclusions can be drawn from these results. First, bacteria that have developed a resistance against quinolone antimicrobial agents do not become susceptible to these again



Figure 3.10: Comparison of inhibition zone sizes measured for six different tris(quinolono)metal(III) complexes in Iso-Sensitest medium (a) and Mueller-Hinton medium (b).

upon complexation of the quinolone to Ga^{3+} or Fe^{3+} . Second, the recorded inhibition zone sizes of the tris(quinolono)metal complexes (0.1 mM) are in the same range than those of the respective free quinolone ligands at 0.3 mM. Third, there are no differences in recorded inhibition zone sizes between the gallium(III) and the iron(III) complexes of the same quinolone ligand. Hence, complexation of Ga³⁺ to a quinolone does not have a synergistic effect, or even only a combinational effect, that would lead to increased antimicrobial potency compared to the quinolone on its own. Moreover, the observed increase in measured inhibition zone sizes of the tris(quinolono)metal(III) complexes is solely related to the fact that there are three quinolone molecules coordinated as ligands to the metal, whether Ga³⁺ or Fe^{3+} is at the center of the complex does not fortify or weaken the antimicrobial effect. According to the "Trojan Horse Theory", the tris(quinolono)gallium(III) complexes should have had superior antimicrobial powers compared to the respective tris(quinolono)iron(III) complexes, however, such an effect was not observed (Table 3.3). Control disks loaded with gallium(III) nitrate (0.1 mM) were placed on each test plate. These Ga^{3+} controls never showed any inhibition, which might be due to the formation of gallium(III) hydroxides (Section 3.1); therefore, we included tris(maltolato)gallium(III) and its iron(III) analog in this study. Because the maltol ligand does not possess any antimicrobial properties, any antimicrobial effect of these complexes could be solely associated with the respective metal ion; however, no growth inhibition was observed (Table 3.3).

Although the single-disk test has been successfully used to evaluate the performance of metal-quinolone complexes many times before, $^{304\,372\,381\,427\,428\,429\,430\,431}$ additional MIC studies were performed to rule out any doubts regarding the suitability of the evaluation method. In a growth assay, varying ratios of ciprofloxacin and gallium(III) (Ga³⁺:Hcipro = 0:1, 1:1, 1:2, 1:3, 1:4) were tested against *P. aeruginosa* in cation-adjusted Mueller-Hinton broth, however, these test results did not show a combinational effect for the interaction of quinolone and Ga^{3+} cation (MIC) either.⁴³⁴

Although no combinational effect for the coordination of one Ga^{3+} cation with three free quinolone ligands was observed, neither did the complexation to Ga^{3+} or Fe^{3+} lead to a reduction in quinolone antimicrobial activity, as it had been previously reported for some synthesized Mg^{2+} -quinolone complexes.³⁰⁴

bacteria	Hcipro	Hcipro	$[Ga(cipro)_3$] [Fe(cipro) ₃]	Henox	Henox	$[Ga(enox)_3]$] [Fe(enox) ₃]
	(0.3 mM)	(0.1 mM)	(0.1 mM)	(0.1 mM)	(0.3 mM)	(0.1 mM)	(0.1 mM)	(0.1 mM)
E. faecalis	13~(0)	9(0)	12(1)	12(1)	8 (1)	0 (0)	7(0)	7(0)
S. aureus	16(1)	12(1)	16(2)	16(1)	12 (0)	0 (0)	11 (1)	10 (1)
E. coli	24(1)	22(1)	24(1)	24(1)	20 (0)	16(1)	19(1)	19(1)
K. pneumonia	20 (0)	18(0)	20(0)	20(0)	19(1)	14(1)	18 (1)	18(0)
P. aeruginosa	25(1)	18 (1)	24(1)	24(1)	15(3)	7(1)	12(2)	12 (2)
hacteria	Hfley	Hfley	$[G_2(flex)_2]$	[Fe(flex)a]	Hlevoy	Hlevoy	[Ga(levox)	$\left[\operatorname{Fe}(\operatorname{levox})_{n} \right]$
bacteria	$\begin{array}{c} \text{Hflex} \\ (0.3 \text{ mM}) \end{array}$	Hflex (0.1 mM)	[Ga(flex) ₃] (0.1 mM)	[Fe(flex) ₃] (0.1 mM)	Hlevox (0.3 mM)	Hlevox (0.1 mM)	$[Ga(levox)_3]$ $(0.1 mM)$	[Fe(levox) ₃] (0.1 mM)
bacteria <i>E. faecalis</i>	Hflex (0.3 mM) 9 (1)	Hflex (0.1 mM) 0 (0)	[Ga(flex) ₃] (0.1 mM) 14 (1)	[Fe(flex) ₃] (0.1 mM) 8 (1)	Hlevox (0.3 mM) 13 (1)	Hlevox (0.1 mM) 8 (1)	[Ga(levox) ₃ (0.1 mM) 13 (1)	$\frac{[\text{Fe}(\text{levox})_3]}{(0.1 \text{ mM})}$ 13 (1)
bacteria E. faecalis S. aureus	Hflex (0.3 mM) 9 (1) 18 (1)	Hflex (0.1 mM) 0 (0) 10 (1)	[Ga(flex) ₃] (0.1 mM) 14 (1) 19 (2)	[Fe(flex) ₃] (0.1 mM) 8 (1) 16 (1)	Hlevox (0.3 mM) 13 (1) 19 (1)	Hlevox (0.1 mM) 8 (1) 14 (1)	[Ga(levox) ₃ (0.1 mM) 13 (1) 18 (1)	$[Fe(levox)_3] = (0.1 \text{ mM}) = 13 (1) = 18 (1)$
bacteria <i>E. faecalis</i> <i>S. aureus</i> <i>E. coli</i>	Hflex (0.3 mM) 9 (1) 18 (1) 23 (1)	Hflex (0.1 mM) 0 (0) 10 (1) 19 (1)	[Ga(flex) ₃] (0.1 mM) 14 (1) 19 (2) 24 (1)	[Fe(flex) ₃] (0.1 mM) 8 (1) 16 (1) 23 (1)	Hlevox (0.3 mM) 13 (1) 19 (1) 22 (1)	Hlevox (0.1 mM) 8 (1) 14 (1) 20 (0)	[Ga(levox) ₃ (0.1 mM) 13 (1) 18 (1) 22 (0)	$\begin{array}{c} & [\text{Fe}(\text{levox})_3] \\ (0.1 \text{ mM}) \\ \hline 13 (1) \\ \hline 18 (1) \\ \hline 23 (0) \end{array}$
bacteria <i>E. faecalis</i> <i>S. aureus</i> <i>E. coli</i> <i>K. pneumonia</i>	Hflex (0.3 mM) 9 (1) 18 (1) 23 (1) 24 (1)	Hflex (0.1 mM) 0 (0) 10 (1) 19 (1) 19 (1)	[Ga(flex) ₃] (0.1 mM) 14 (1) 19 (2) 24 (1) 25 (1)	[Fe(flex) ₃] (0.1 mM) 8 (1) 16 (1) 23 (1) 22 (1)	Hlevox (0.3 mM) 13 (1) 19 (1) 22 (1) 20 (1)	Hlevox (0.1 mM) 8 (1) 14 (1) 20 (0) 18 (0)	[Ga(levox) ₃ (0.1 mM) 13 (1) 18 (1) 22 (0) 20 (1)	$[Fe(levox)_3] = (0.1 \text{ mM}) = (0.$

Table 3.3: Results of antimicrobial susceptibility study in Iso-Sensitest medium of tris(quinolono)- and
tris(maltolato)gallium(III) and -iron(III) complexes in comparison to free ligands at two concentrations.

bacteria	Hlomx	Hlomx	[Ga(lomx) ₃]	[Fe(lomx) ₃]	Hnxa	Hnxa	[Ga(nxa) ₃]	$[Fe(nxa)_3]$
	(0.3 mM)	(0.1 mM)	(0.1 mM)	(0.1 mM)	(0.3 mM)	(0.1 mM)	(0.1 mM)	(0.1 mM)
E. faecalis	8 (0)	0 (0)	7(0)	7(0)	0 (0)	0 (0)	0 (0)	0 (0)
S. aureus	15(1)	7(1)	12(1)	12(1)	0 (0)	0 (0)	0 (0)	0 (0)
E. coli	20(1)	17(2)	20(1)	19 (1)	0 (0)	0 (0)	0 (0)	0 (0)
K. pneumonia	20(1)	16(1)	18 (1)	19 (1)	0 (0)	0 (0)	0 (0)	0 (0)
P. aeruginosa	14(2)	0	10 (1)	10 (1)	0 (0)	0 (0)	0 (0)	0 (0)
bacteria	Hnofx	Hnofx	$[Ga(nofx)_3]$	$[Fe(nofx)_3]$	Hoxa	Hoxa	$[Ga(oxa)_3]$	$[Fe(oxa)_3]$
	(0.3 mM)	(0.1 mM)	(0.1 mM)	(0.1 mM)	(0.3 mM)	(0.1 mM)	(0.1 mM)	(0.1 mM)
E. faecalis	9(1)	0 (0)	9(1)	9(1)	0 (0)	0 (0)	0 (0)	0 (0)
S. aureus	15(1)	7(1)	12(1)	13(1)	0 (0)	0 (0)	0 (0)	0 (0)
E. coli	21 (1)	19 (1)	21 (1)	21 (0)	19 (1)	14 (1)	19(1)	15(1)
K. pneumonia	16(2)	15(1)	15 (0)	15(1)	15(1)	11 (1)	15(1)	10 (1)
P. aeruginosa	16(1)	6(0)	12(1)	14 (1)	0 (0)	0 (0)	0 (0)	0 (0)

bacteria	Hpia	Hpia	[Ga(pia) ₃]	$[Fe(pia)_3]$	Hma	Hma	[Ga(ma) ₃]	$[Fe(ma)_3]$
	(0.3 mM)	(0.1 mM)	(0.1 mM)	(0.1 mM)	(0.3 mM)	(0.1 mM)	(0.1 mM)	(0.1 mM)
E. faecalis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
S. aureus	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
E. coli	11 (1)	0 (0)	11 (1)	11 (1)	0 (0)	0 (0)	0 (0)	0 (0)
K. pneumonia	6(0)	0 (0)	6(0)	6(0)	0 (0)	0 (0)	0 (0)	0 (0)
P. aeruginosa	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Reported inhibition zones [mm] are averaged values from three plates (standard deviation). Disk diameter 0.6 mm. Loading volume 20 μ L. Disks loaded with solutions of methanol and 2% DMSO in methanol served as controls, all of these showed no inhibition (0 mm).

3.4 Conclusion

In this chapter, the potential of a combinational or even synergistic effect between Ga^{3+} and quinolone antimicrobial agents was investigated. According to the "Trojan Horse Theory", the Ga^{3+} ion does kill bacteria by tricking them into uptake in that they get the Fe³⁺ ion necessary for their own growth. Nine tris(quinolono)gallium(III) coordination complexes and their respective iron(III) analogs were synthesized and characterized (Figure 3.1). A comparison of their stability in solution and in heat did reveal slight differences between the gallium(III) and the iron(III) complexes, however, their antimicrobial efficacy was similar against five of the most common nosocomial pathogens, following the single-disk test procedure. Further competing antimicrobial studies with tris(maltolato)gallium(III) and its iron(III) analog were not able to confirm the claimed antimicrobial efficacy of Ga^{3+} either, and neither did MIC studies in solution; however, the quinolones did not lose their antimicrobial activity upon complexation to Ga³⁺, as it had been reported earlier for Mg²⁺quinolone complexes.³⁰⁴. The antimicrobial effect of the eighteen tris(quinolono)metal(III) $[ML_3, M = Ga^{3+}, Fe^{3+}]$ complexes correspond well with the effect of the free quinolone ligand (HL) at triple concentrations. In conclusion, the "Trojan Horse Theory" and the "hype" around the antimicrobial properties of Ga^{3+} do not hold any truth according to the results of this study.

Chapter 4

Syntheses, Characterization, and Evaluation of the Antimicrobial Potential of Copper(II) Coordination Complexes with Quinolone and Xylenyl-Linked Quinolone Ligands

In this chapter, the focus of the antimicrobial project will be broadened. On the metal side, the antimicrobial properties of copper(II) will be explored. In addition, modified quinolone ligands will be introduced, in which the secondary amine of the piperazinyl rings of two quinolone drug molecules will be joined with a α, α' -xylenyl-linker. The antimicrobial susceptibility of these α, α' -xylenyl-linked quinolone copper(II) sandwich complexes will be compared to the activity of the bis(quinolono)copper(II) complexes.

4.1 Mixing Things Up: Another Metal, a Modified Ligand

The growing resistance of bacteria to commonly used antimicrobial drugs is a global health concern that threatens the effective prevention and therapy of bacterial and fungal infections, possibly leading to a post-antibiotic era (see as well Section 3.1).^{337,339} For the past 50 years, quinolone antimicrobial agents have been widely used in the clinic for the treatment of bacterial infections, but bacterial resistance against this drug class is rising.^{435,436,437} For example, pipemidic acid (Figure 4.1), one of the first generation quinolone agents, is not effective against many bacterial strains anymore, as data presented in Table 2.5 and 3.3 show. The development of novel quinolone agents is ongoing with the aim to modify the aromatic core structure of the quinolones in a way that makes bacteria susceptible again to new members of this drug class and that increases the ability of the agents to kill bacteria cells.^{299 438 439 440 441 442} Previous research in the groups of Drlica and Kerns has focused on crosslinking two molecules of ciprofloxacin, norfloxacin, or pipemidic acid via the 1,4-piperazinyl-group in C_{ar7} -position with crosslinking moieties, such as trans-butenyl, 2,6-pyridinyl, meta-xylenyl, and para-xylenyl (Figure 4.1). 443 444 445 446 447 Depending on the linker, these crosslinked dimers displayed a higher antimicrobial activity than the single quinolones.^{446 447} In the case of virtually inactive pipemidic acid, the crosslinkage led to increased antimicrobial susceptibility against the tested strains of *Staphylococcus aureus*, indicating a unique, non-equivalent interaction of this crosslinked dimer with the bacterial target site (topoisomerase/gyrase, DNA).⁴⁴⁷

These findings suggest that novel mechanisms of action are needed to regain a lead over bacteria. Because many fundamental processes in the pathogen, as well as the host, are regulated by metal-requiring cofactors,⁴⁴⁸ the targeting of metal transport pathways opens up diverse mechanisms of action. In the area of tropical parasitic diseases, metallodrugs have shown potential to overcome drug resistance by novel modes of action.²³⁶ ²⁶⁴ Moreover, as



Figure 4.1: Pipemidic acid (Hpia, 1st generation), ciprofloxacin (Hcipro, 2nd generation), and their respective α, α' -xylenyl-linked dimers: H₂piaXpia and H₂ciproXcipro.

discussed in Chapter 3, iron metabolism *in vivo* has been the target of antibacterial strategies for many years³⁶⁷ and can be seen as the starting point for the growing discussion of nutrient metal homeostasis at the pathogen-host interface. ⁴⁴⁹ The ancient Egyptians valued the antimicrobial properties of copper, using copper formulations in personal hygiene and for the treatment of wounds. ⁴⁵⁰ Over the past years, there has been a trend in chemistry to use copper for a variety of antimicrobial applications: antifouling coatings for marine environments, ^{451 452} antimicrobial alloys for use in healthcare settings, ^{453 454} surface treatments for joint replacement implants, ⁴⁵⁵ antimicrobial nanomaterials, ⁴⁵⁶, and medical uses, ^{457 458} have been reported. Into the latter category fall a multitude of copper-quinolone complexes, ^{459 327} such as $[Cu(Hcipro)_2]Cl_2 \cdot 6H_2O^{460}$ and $[Cu(pia)_2(H_2O)] \cdot 2H_2O$. ⁴⁶¹ Recently, Wolschendorf *et al.* observed that guinea pigs increased their levels of copper(II) in the lung tissue upon infection with *Mycobacterium tuberculosis* in an autoimmune response, 462 similar to the lowering of iron(III) levels at the site of infection widely observed in mammals. ³⁵⁸ Although the mechanism of copper-dependent bacterial killing remains unclear, the Cu(II)/Cu(I) redox couple, which could catalyze the formation of toxic radical oxygen species as well as the disruption of iron-sulfur cluster proteins through the formation of Cu(I)-thiolate bonds, appears to play a key role in the bactericidal action. ⁴⁶³ In dependence on Greek methology, copper has been named the "Achilles Heel" of bacteria. ^{462 464}

In an attempt to combine the antimicrobial properties of copper(II) with the increased antimicrobial potency of α, α' -xylenyl-linked quinolones, this chapter describes the synthesis and characterization of copper(II)-sandwich complexes with α, α' -xylenyllinked quinolone dimers of ciprofloxacin and pipemidic acid. The antimicrobial efficacy of these complexes was evaluated in direct comparison to bis(ciprofloxacino)copper(II) and bis(pipemido)copper(II), as well as copper(II) chloride and bis(maltolato)copper(II), following the single-disk test procedure in Iso-Sensitest medium.

4.2 Materials & Methods

4.2.1 Chemicals

Chemicals and materials were purchased from commercial suppliers (Alfa-Aesar, BD BBL-Difco, Cambridge Isotope Laboratories, Inc., Fischer-Scientific, Sigma-Aldrich) and used without further purification. Water was purified through a Elga Purelab Pure Water System to 18 M Ω ·cm.

4.2.2 Instrumentation

Reversed-phase high performance liquid chromatography (HPLC) was conducted on Phenomenex Synergi Hydro-RP 80 Å columns (250 mm x 4.6 mm analytical or 250 mm x 21.2 mm semipreparative) on a Waters WE 600 HPLC system consisting of a Waters 600 controller running Empower Pro software (version 5.00.00, 2002), a Waters 2478 dual wavelength absorbance detector, and a Waters delta 600 pump. Melting points were taken on a Stanford Research Systems DigiMelt SRS melting point apparatus and are uncorrected. UV-Vis spectra were recorded on a Hewlett Packard 8453 instrument operated by ChemStation Software (version B.04.01[61], Agilent Technologies, 2001-2010). IR spectra were recorded in solid state on a PerkinElmer Frontier FT-IR spectrometer (4000-650 cm⁻¹) running PerkinElmer Spectrum Software (version 10.03.02, 2011); characteristic bands were interpreted using the abbreviations: st, md, w, sh. At the UBC Mass Spectrometry Centre, low-resolution mass spectra were recorded on a Water ZQ spectrometer equipped with an electrospray and chemical ionization source, while high-resolution mass spectra were obtained on a Waters Micromass LCT (electrospray-ionization), and elemental analyses (C, H, N) were performed on a Carlo Erba Elemental Analyzer EA 1108.

4.2.3 Antimicrobial Susceptibility Studies

The following bacteria strains were used in the antimicrobial susceptibility single-disk test: Gram-positive *E. faecalis* (ATCC-51575) and *S. aureus* (MSSA-476, ATTC-BAA-1721); Gram negative *E. coli* (ATCC-25922), *K. pneumonia* (ATCC-13883), and *P. aeruginosa* (ATCC-27853). Filter disks ($^{1}/_{4}$ inch, approx. 0.6 mm, diameter) were purchased from Schleicher & Schüll, Germany. The study was performed in UBC's Biological Services Laboratory (biological safety level II), according to the procedure presented in Appendix A.

4.2.4 Synthesis & Characterization

4.2.4.1 α, α' -Xylenyl-Linked Ciprofloxacin Dimer, H₂ciproXcipro

The synthesis was based on the reported synthetic scheme by Kerns and co-workers.⁴⁴⁷ Ciprofloxacin (0.663 g, 2.0 mmol), α, α' -chloro-xylene (0.175 g, 1.0 mmol) and sodium bicarbonate (0.401 g, 4.8 mmol) were transferred into a 100 mL round-bottom flask, suspended in dimethylformamide (60 mL) and heated to 80° C for 24 hours. An off-white solid was isolated from the reaction mixture after cooling on a fine glass frit, and washed with water and methanol (yield: 0.686 g, 89%). HPLC (linear gradient of acetonitrile in 0.1% TFA/water) afforded separation of the desired product as a single peak at 220 nm and 254 nm. Removal of solvents and drying in vacuo yielded an off-white solid. Mp: $\geq 230^{\circ}$ C, decomposition to pale brown solid. IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3047 (w), 2937 (w), 2810 (md), 2772 (w), 1721 (st), 1627 (st), 1607 (sh), 1545 (md), 1495 (st), 1450 (st), 1437 (sh), 1384 (md), 1334 (md), 1293 (w), 1255 (st), 1203 (sh), 1110 (md), 1047 (w), 1030 (w), 1005 (st), 945 (st), 892 (st), 831 (st), 807 (st), 781 (w), 747 (md), 709 (md), 667 (md). **NMR**: δ_H (300 MHz, 298 K, d₆-DMSO) [ppm] = 8.64 (s, 2 H, C_{ar2}H); 7.88 (d, $J_{H,F}^3$ = 13.5 Hz, 2 H, $C_{ar5}H$; 7.55 (d, $J_{H,F}^4$ = 7.5 Hz, 2 H, $C_{ar8}H$); 7.33 (s, 4 H, $C_{ar,linker}H$); 3.81-3.76 (m, 2 H, $C_{prop}H$); 3.57 (s, 4 H, $C_{linker}H_2$); 3.33 (br s, 8 H overlaid with water, $\mathbf{C}_{pip2,6}H_2);\,2.89-2.60\;(\mathbf{m},\,8\;\mathbf{H},\,\mathbf{C}_{pip3,5}H_2);\,1.32-1.15\;(\mathbf{m},\,8\;\mathbf{H},\,\mathbf{C}_{prop}H_2).~\boldsymbol{\delta_F}\;(282\;\mathrm{MHz},\,298)$ K, d_6 -DMSO) [ppm] = -121.8 (s, $C_{ar6}F$). MS (ES+, CH₃OH): m/z (%) = 765 (100) [M + H⁺], 787 (40). **HR-ESI-MS**: m/z for $C_{42}H_{42}F_2N_6O_6$ + H⁺ calcd. (found): 765.3212 (765.3210).

4.2.4.2 α, α '-Xylenyl-Linked Pipemidic Acid Dimer, H₂piaXpia

The synthesis followed the procedure described in Section 4.2.4.1 using pipemidic acid (0.608 g, 2.0 mmol), α , α '-chloro-xylene (0.177 g, 1.0 mmol), sodium bicarbonate (0.349 g,

4.0 mmol), and 60 mL dimethylformamide. Yield: off-white solid (0.570 g, 80%). The desired product eluted from HPLC column (linear gradient of acetonitrile in 0.1% TFA/water) as a single peak at 220 nm and 254 nm. Mp: \geq 245°C, decomposition to pale brown solid. IR (neat): $\tilde{\nu} \ [\text{cm}^{-1}] = 3047$ (w), 2982 (w), 2934 (w), 2826 (w), 1725 (st), 1637 (st), 1569 (w), 1549 (md), 1518 (md), 1483 (st), 1452 (md), 1376 (md), 1359 (md), 1305 (w), 1264 (md), 1226 (w), 1200 (w), 1127 (md), 1114 (md), 1092 (w), 1053 (w), 1007 (st), 971 (md), 854 (w), 830 (w), 812 (st), 793 (w), 763 (w), 737 (w), 718 (md), 698 (w), 667 (w). NMR: δ_H (300 MHz, 298 K, d_6 -DMSO) [ppm] = 9.02 (s, 2 H, $C_{ar5}H$); 8.48 (s, 2 H, $C_{ar2}H$); 7.34 (s, 4 H, $C_{ar,linker}H$); 4.23–4.20 (m, 4 H, CH_2CH_3); 3.90–3.86 (m, 4 H, $C_{linker}H_2$); 3.81–3.78 (m, 8 H, $C_{pip2,6}H_2$); 2.77–2.71 (m, 8 H, $C_{pip3,5}H_2$); 1.32–1.23 (m, 6 H, CH₂CH₃). MS (ES+, CH₃OH): m/z (%) = 710 (100) [M + H⁺], 731 (80) [M + Na⁺]. HR-ESI-MS: m/z for $C_{36}H_{40}N_{10}O_6 + H⁺$ calcd. (found): 709.3211 (709.3214).

4.2.4.3 $[Cu_2(ciproXcipro)_2]$

In a warm solution (50°C) of methanol (15 mL), H₂ciproXcipro (0.077 g, 0.1 mmol) was dissolved with two drops of triethylamine under sonication and rigorous stirring. The warm solution was filtered and added dropwise to the blue solution of copper(II) chloride (0.017 g, 0.1 mmol) in 2 mL methanol, which resulted in a color change via green to turquoise. The reaction mixture was warmed up to 50°C once more and stirred over night with the flask open to air. After removal of half of the remaining solvent *in vacuo*, the flask was left standing for further evaporation, which resulted in a light turquoise solid that was collected on a glass frit (size F), washed with cold water and diethyl ether, and dried *in vacuo* (0.042 g, 25%, first crop). **Mp**: \geq 234°C, black-brown decomposition melt. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3042 (w), 2944 (w), 2821 (md), 2791 (w), 1625 (st), 1592 (sh), 1545 (md), 1520 (sh), 1471 (st), 1451 (md), 1397 (w), 1372 (md), 1352 (w), 1285 (st), 1256 (st) 1224 (sh), 1180 (md), 1144 (w), 1089 (w), 1030 (w), 1001 (st), 948 (st), 890 (st), 836 (md), 811 (st), 788 (w), 765 (md), 743 (md), 708 (md). **MS** (ES+): m/z (%) = 1715 $[Cu_3(LXL)_2]^{++}$, 1654 $[Cu_2(LXL)_2 + H^+]$, 826 $[Cu(LXL)]^+$.

4.2.4.4 $[Cu_2(piaXpia)_2]$

H₂piaXpia (0.071 g, 0.1 mmol) was dissolved in a warm solution (50°C) of methanol:water (70:30, 20 mL) with two drops of triethylamine and the help of sonication and rigorous stirring. The warm solution was filtered and added dropwise to a solution of copper(II) chloride (0.017 g, 0.1 mmol) in 2 mL methanol. After an immediate color change to green, the solution turned blue-turquoise. Re-heating the solution again to 50°C, stirring it overnight in open air, and leaving the flask standing for further evaporation, resulted in a turquoise solid, which was washed with cold water and diethyl ether, and dried *in vacuo* (0.055 g, 36%, first crop). **Mp**: \geq 246°C, black decomposition melt. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3414 (br, water), 3046 (w), 2987 (w), 2937 (w), 2811 (w), 1608 (st), 1540 (md), 1476 (st), 1446 (st), 1386 (sh), 1355 (st), 1312 (md), 1254 (st), 1128 (st), 1093 (sh), 1054 (w), 1002 (st), 926 (md), 854 (w), 817 (st), 785 (md), 769 (md), 742 (w), 718 (st), 699 (w). **MS** (ES+): m/z (%) = 1604 [Cu₃(LXL)₂]⁺⁺, 1542 [Cu₂(LXL)₂ + H⁺], 771 [Cu(LXL)]⁺.

4.2.4.5 $Bis(ciprofloxacino)copper(II), [Cu(cipro)_2]$

Ciprofloxacin (0.077 g, 0.2 mmol) was suspended in methanol (15 mL), and one drop of triethylamine was added. The reaction mixture was refluxed at 80°C for 30 min, before the still warm, clear solution was added dropwise to a solution of copper(II) chloride (0.017 g, 0.1 mmol) in methanol (2 mL). A color change to grass-green and then to blue was observed. Once the addition was completed, the reaction mixture was refluxed at 80°C for 30 min. Upon heating it turned into a clear, turquoise solution, from which a light turquoise solid precipitated. The solid was separated from the cold reaction mixture by filtration (glass frit, size F), washed with water and diethyl ether, and dried *in vacuo* (0.0461 g, 64%).

Mp: ≥237°C, black decomposition melt. IR (neat): $\tilde{\nu}$ [cm⁻¹] = 3396 (br, water), 3198 (w), 2935 (w), 2844 (w), 1622 (st), 1571 (st), 1538 (w), 1491 (st), 1465 (sh), 1447 (sh), 1433 (sh), 1372 (md), 1335 (md), 1297 (st), 1280 (w), 1262 (st), 1193 (w), 1172 (w), 1116 (w), 1060 (w), 1019 (st), 946 (st), 889 (md), 830 (st), 812 (st), 788 (md), 749 (st), 702 (w), 670 (w). MS (ES+, CH₃OH): m/z (%) = 724 (100) [ML₂ + H⁺], 747 (40) [ML₂ + Na⁺]; 1119 (10) [M₂L₃]⁺. HR-ESI-MS m/z for C₃₄H₃₄ ⁶³CuF₂N₆O₆ + Na⁺ calcd. (found): 746.1702 (746.1702). EA: Anal. Calcd. (found) [%] for C₃₄H₃₄CuF₂N₆O₆·2 H₂O [%]: C, 53.71 (53.62); H, 5.04 (5.14); N, 11.05 (11.14).

4.2.4.6 $Bis(pipemido)copper(II), [Cu(pia)_2]$

A solution of pipemidic acid trihydrate (0.752 g, 2.1 mmol) and sodium hydroxide (0.096 g, 2.4 mmol) in water (75 mL) was added dropwise to a solution of copper(II) chloride (0.171 g, 1.0 mmol) dissolved in water (5 mL), while the pH was kept at pH~4 (HCl_(aq)). Once the addition was completed, the pH was adjusted to 7.4 (NaOH_(aq)). While stirring the reaction solution rigorously overnight, a deep turquoise solid began to form, which was isolated by filtration (glass frit, size F), washed with methanol, and dried *in vacuo* (0.229 g, 34%). **Mp**: ≥209°C, black-brown decomposition melt. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3283 (w), 2812 (w), 1609 (st), 1559 (w), 1537 (md), 1476 (st), 1444 (sh), 1380 (md), 1359 (st), 1308 (md), 1286 (w), 1245 (st), 1147 (w), 1114 (md), 1060 (md), 987 (md), 921 (md), 814 (st), 787 (st), 770 (st), 741 (w), 717 (md), 618 (md). **MS** (ES+, CH₃OH): m/z (%) = 668 (100) [ML₂ + H⁺]; 690 (40) [ML₂ + Na⁺]. **HR-ESI-MS** m/z for C₂₈H₃₂ ⁶³CuN₁₀O₆ + H⁺ calcd. (found): 668.1881 (668.1882). **EA**: Anal. Calcd. (found) [%] for C₂₈H₃₂CuN₁₀O₆·2 H₂O: C, 47.76 (48.17); H, 5.15 (5.15); N, 19.89 (19.49).
4.2.4.7 $Bis(maltolato)copper(II), [Cu(ma)_2]$

The reaction has been previously reported. ⁴⁶⁵ Maltol (0.689 g, 5.5 mmol), copper(II) sulphate (0.662 g, 2.65 mmol) in 40 mL water gave a green solid (0.569 g, 68%). **Mp**: \geq 250°C, decomposition to olive-brown solid. **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3118 (w), 3087 (w), 2951 (w), 2911 (w), 1906 (w), 1604 (md), 1563 (st), 1507 (st), 1470 (st), 1361 (w), 1275 (st), 1240 (md), 1198 (st), 1085 (w), 1039 (md), 955 (w), 923 (md), 849 (st), 826 (st), 764 (w), 680 (w), 719 (st), 626 (md). **MS** (ES+, CH₃OH): m/z (%) = 651 (100) [M₂L₄ + Na⁺], 336 (80) [ML₂ + Na⁺]. **HR-ESI-MS** m/z for C₁₂H₁₀ ⁶³CuO₆ + H⁺ calcd. (found): 313.9852 (313.9863). **EA**: Anal. Calcd. (found) [%] for C₁₂H₁₀CuO₆: C, 45.94 (45.92); H, 3.21 (3.24).

4.3 Results & Discussion

4.3.1 Synthesis & Characterization

The copper(II) complexes were synthesized by combining the copper(II) salt (chloride, sulphate) with the respective ligand that was deprotonated with sodium hydroxide or triethylamine. Water and methanol were the solvents; the higher the amount of water in the solvent mixture, the easier the desired product formed as a solid precipitating from solution. The resulting copper(II) complexes of the general formula $[Cu(L)_2]$ for ciprofloxacin, pipemidic acid, and maltol were neutral of charge and challenging to dissolve, preferring polar-aprotic solvents such as DMSO; however, the copper(II) sandwich complexes, $[Cu_2(ciproXcipro)_2]$ and $[Cu_2(piaXpia)_2]$, were even harder to bring into and especially to keep in solution. Because of the paramagnetic nature of the copper(II) center, respective NMR spectra of the complexes were extremely noisy. The isolated products were characterized through melting point determination, IR spectroscopy, mass spectrometry (lowand high-resolution), and elemental analysis. Mass spectrometry confirmed the general composition of the complexes to be 2:1 and 1:1 ligand:metal ratios for $[Cu(L)_2]$ and $[Cu_2(LXL)_2]$, with $[M + H^+]$ or $[M + Na^+]$ as parent peaks of exact mass. In the low-resolution MS spectra, the complexes displayed the characteristic copper isotope distribution of ${}^{63}Cu/{}^{65}Cu$ with the peak of 100% intensity corresponding to the ${}^{63}Cu$ isotope of higher abundance, and the [M + 2] peak of approximately 50% intensity corresponding to the ${}^{65}Cu$ isotope. For the $[Cu(L)_2]$ complexes, $[Cu_2L_3]^+$ peaks of lower intensity were detected, formed via the recombination of a $[CuL_2]$ with a $[CuL]^+$ fragment. The $[Cu_2(LXL)_2]$ complexes, on the other hand, showed the doubly-charged recombination peak of $[Cu_3(LXL)_2]^{++}$.

Spectroscopic analysis in the mid-infrared region $(4000-600 \text{ cm}^{-1})$ of the copperquinolone complexes confirmed the complete coordination of the copper(II) ion through the carboxylate-O on C_{ar3} and the carbonyl-O on C_{ar4} . Figure 4.2 shows the recorded IR spectra of ciprofloxacin, bis(ciprofloxacino)copper(II), the xylenyl-linked ciprofloxacin dimer, and the respective copper(II) complex $[Cu_2(ciproXcipro)_2]$. In the spectrum of H_2 ciproXcipro, the intense stretching vibration at 1721 cm⁻¹ stems from the dimeric carboxylate group (ν_{COOH}) ; upon coordination to copper(II) this band disappears, and the spectrum of $[Cu_2(ciproXcipro)_2]$ reveals full coordination of the ligand to the copper(II) ions. The spectrum of ciprofloxacin does not show the dominant stretch of the hydroxide group, because its proton from the carboxyl group on C_{ar3} is free to move to the secondary amine on the piperazinyl group on C_{ar7} (Figure 4.1) resulting in the zwitterionic state (Figure 2.3) for which no OH-stretch can be expected.^{317,318} Following previous literature reports, $^{327\,328}$ the two distinct bands in the range of 1650-1600 cm⁻¹ and 1400-1300 cm⁻¹ were assigned as $\nu_{\rm CO_2}$ asymmetric and symmetric stretching vibrations characteristic for the metal complexation of deprotonated quinolone ligands. In addition, new peaks were observed in the low fingerprint region $(800-700 \text{ cm}^{-1})$ and assigned to metal-oxygen vibra-

complex	$\nu_{asym}(CO_2)$	$\nu_{sym}(CO_2)$	$\Delta^{(a)}$	$\nu(CuO)$
$[Cu(cipro)_2]$	1622	1372	250	749
$[Cu(pia)_2]$	1609	1359	250	770
[Cu ₂ (ciproXcipro) ₂]	1625	1372	253	765
$[Cu_2(piaXpia)_2]$	1608	1355	253	769
$^{(a)}\Delta = \nu_{asym}(CO_2) - \nu_s$	$_{um}(CO_2).$			

Table 4.1: Selected IR stretching frequencies $[cm^{-1}]$ and their assignments.

asym(-2) = sym(-2)

tions in the formed complexes. Selected IR bands and their assignments are summarized in Table 4.1.

To evaluate the behaviour of the complexes under bioreductive conditions, attempts were made to record cyclic voltammograms of $[Cu_2(ciproXcipro)_2]$, $[Cu_2(piaXpia)_2]$, and the respective free ligands (H₂ciproXcipro, H₂piaXpia). Unfortunately, due to lack of solubility of the linked-quinolone dimer ligands at suitable concentration in suitable solvents for CV studies (acetonitrile, DMSO, methanol, tetrahydrofuran), these planned studies could not be completed. Moreover, many attempts were made to grow single crystals of the α, α' -xylenyl-linked quinolones and their copper(II) complexes, however, the low solubility of the free ligands and metal complexes hampered these attempts due to DMSO being the only solvent in which a certain amount of solubility could be reached. The crystallization attempts ranged from slow diffusion in DMSO with acetone, acetonitrile, chloroform, diethyl ether, and methanol in various environmental settings (window sill, fume hood, dark cupboard, fridge, freezer) to reactive crystallization experiments, in which the xylenyl-linked quinolone dimer, dissolved in heat in DMSO and filtered, at the bottom of the vial was layered with 1 mL of copper(II) chloride in methanol. The latter resulted in crystals, but, unfortunately, these were unsuitable for X-ray diffraction.



4.3.2 Antimicrobial Susceptibility Testing

The antimicrobial activity of the synthesized copper(II) complexes and their free ligands was evaluated against the following pathogens associated with nosocomial diseases: ³³⁰ *E. faecalis* and methicillin-susceptible *S. aureus* (both Gram-positive); *E. coli, K. pneumonia*, and *P. aeruginosa* (all Gram-negative). Copper(II) complexes were tested at 0.1 mM concentration, while the respective free ligands were investigated at 0.1 mM and 0.2 mM concentrations to preclude any effects arising from the double concentration of quinolones in the copper-complexes. Furthermore, solutions of copper(II) chloride as well as bis(maltolato)copper(II) over the concentration range 10 mM-0.1 mM were included to allow the assessment of the antibacterial properties of copper(II) ions. Maltol is widely used as a flavour enhancer in the food industry and considered non-toxic and safe, ⁴⁶⁵ therefore, any antimicrobial effect of this complex would be directly arising from Cu²⁺.

The results of the antimicrobial study, presented in Table 4.2, are averaged values of the recorded inhibition zone sizes from three independent test plates. Three conclusions can be drawn from the data. Firstly, complexing pipemidic acid with copper(II) did not overcome the developed resistance against this first generation quinolone. Secondly, the antimicrobial potency of the bis(quinolono)copper(II) complexes is solely determined by the concentration of quinolone, as the recorded inhibition zone sizes for 0.2 mM ciprofloxacin and pipemidic acid correspond well with those recorded for 0.1 mM bis(ciprofloxacin)copper(II) and bis(pipemido)copper(II), respectively.

bacteria	Hcipro	Hcipro	$[\mathrm{Cu}(\mathrm{cipro})_2]$	Hpia (0.2 mM)	Hpia (0.1 mM)	$[\mathrm{Cu}(\mathrm{pia})_2]$
	(0.2 mM)	(0.1 mM)	(0.1 mM)			(0.1 mM)
E. faecalis	13~(0)	9(0)	11 (1)	0 (0)	0 (0)	0 (0)
S. aureus	17(1)	12(1)	15(1)	0 (0)	0 (0)	0 (0)
E. coli	25~(1)	22(1)	24(1)	10(1)	0 (0)	7(0)
K. pneumonia	22(1)	18 (0)	21 (1)	5(4)	0 (0)	0 (0)
P. aeruginosa	12 (2)	8 (1)	9 (1)	0 (0)	0 (0)	0 (0)

Table 4.2: Inhibition zone sizes [mm] of copper(II) complexes.

bacteria	H_2 ciproXcipro	H_2 ciproXcipro	[Cu ₂ (ciproXcipro) ₂]H ₂ piaXpia		H_2 piaXpia	$[Cu_2(piaXpia)_2]$
	(0.2 mM)	(0.1 mM)	(0.1 mM)	(0.2 mM)	(0.1 mM)	(0.1 mM)
E. faecalis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
S. aureus	5(4)	0 (0)	8 (1)	3(5)	0 (0)	8 (1)
E. coli	8 (1)	7(0)	0 (0)	0 (0)	0 (0)	0 (0)
K. pneumonia	5(4)	5(4)	0 (0)	0 (0)	0 (0)	0 (0)
P. aeruginosa	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Reported inhibition zones [mm] are averaged values from three plates (standard deviation). Disk diameter 0.6 mm. Loading volume 20 μ L. Disks loaded with solutions of methanol and 2% DMSO in methanol served as controls, all of these showed no inhibition (0 mm).



Figure 4.3: Photo of a growth plate of *P. aeruginosa* after 20 h of incubation at 37°C during antimicrobial single-disk test. Disk 1, 2% DMSO in methanol control; disk 2–7, copper(II) chloride (20 μ L) in decreasing concentrations (10 mM-0.5 mM); disk 8–14, bis(maltolato)copper(II) (20 μ L) in decreasing concentrations (10 mM-0.1 mM). No test compound showed inhibition of growth, recorded inhibition zone size: 0 mm.

Thirdly, the xylenyl-linked quinolone dimers are not as potent as the quinolone monomers, which could suggest that due to the difference in size the quinolone-dimers are not diffusing as well into the agar medium as are the smaller free quinolone ligands. This observation is supported by the large differences between the recorded inhibition zone sizes from one plate to another, as reflected in the high values for the standard deviations for H₂ciproXcipro and H₂piaXpia. The diffusion effect is even more prominent for the copper(II) sandwich complexes [Cu₂(ciproXcipro)₂] and [Cu₂(piaXpia)₂], which do not seem to move far on the plate. Both complexes appear to be only potent against the tested strain of methicillinsusceptible *S. aureus*, as depicted in the small inhibition zones of 8 (1) mm. This means that, although *S. aureus* showed resistance against pipemidic acid (0.1 and 0.2 mM) and bis(pipemido)copper(II) (0.1 mM), linking two pipemidic acid molecules together via the xylene-linker makes the test strain susceptible again. This effect has been first reported and studied by Kerns and co-workers.^{446 447} Further complexation of these xylenyl-linked quinolones with copper(II) in a sandwich fashion seems to increase this effect (Table 4.2), however, we were unable to measure the antimicrobial effect of copper(II) alone. An extensive single-disk study of solutions of copper(II) chloride and bis(maltolato)copper(II) ranging from 10 mM to 0.1 mM (loading volume 20 μ L) was performed on all five bacteria in triplicate. Figure 4.3 shows a photo of one growth plate of *P. aeruginosa* as a representative example for the entire study. Neither the disks loaded with copper(II) chloride nor the ones loaded with bis(maltolato)copper(II) showed any growth inhibition against the five tested pathogens, which grew onto the edge of each disk seemingly unaffected by the presence of copper(II).

4.4 Conclusion

In this chapter, the syntheses of bis(ciprofloxacino)copper(II) and $[Cu_2(ciproXcipro)_2]$ as well as bis(pipemido)copper(II) and $[Cu_2(piaXpia)_2]$ were reported. The complexes were characterized by elemental analysis, IR spectroscopy, mass spectrometry, and melting point determination; due to the paramagnetic nature of the copper(II) core, NMR spectroscopy measurements were not possible. In a single-disk test procedure, the antimicrobial efficacy of these complexes was tested against five pathogens that are commonly associated with nosocomial diseases (*E. faecalis, S aureus, E. coli, K. pneumonia, P. aeruginosa*) and directly compared to the antimicrobial effect of the respective free ligands, as well as copper(II) chloride, and bis(maltolato)copper(II). Maltol, a food additive does not posses any antimicrobial properties, but coordinates the copper(II) tightly,⁴⁶⁶ preventing the formation of insoluble metal hydroxides. The test results did not show a combinational antimicrobial effect between Cu^{2+} and the respective quinolone ligand, but the antimicrobial potency of the synthesized copper(II) complexes rather depends solely on the concentration of quinolone alone. Further studies of copper(II) chloride and bis(maltolato)copper(II) over the concentration range from 10 mM to 0.1 mM did not show any growth inhibition of any of the five tested bacteria strains either.

In conclusion, the "Achilles Heel Theory" may be questioned, as the combination of Cu^{2+} with quinolone antimicrobial agents, and derivatives thereof, based on this theory, as well as high concentrations (10 mM) of copper(II) chloride alone or $[Cu(ma)_2]$ did not show an improved antimicrobial effect, or any bacterial growth inhibitory effect at all.

Chapter 5

Iron(III)-Binding of the Anticancer Agents Doxorubicin and Vosaroxin

The importance of kinetic and thermodynamic stability *in vivo* has already been introduced in Section 1.4.3 and will be exemplified through the discussion of the interaction of iron(III) with two anticancer agents *in vivo* in this chapter.

5.1 The Two Anticancer Agents: Doxorubicin & Vosaroxin

Anthracycline anticancer drugs, such as doxorubicin (Hdox) (Figure 5.1), are in broad use clinically but are associated with cumulative-dose cardiomyopathy. $^{467\,468\,469\,470\,471\,472}$ Molecular mechanisms for doxorubicin induced cardiomyopathy remain controversial, $^{469\,473}$ despite decades of investigations that have been recently reviewed. $^{471\,474}$ These include interaction of doxorubicin with topoisomerase II β and induction of DNA damage, $^{475\,476}$ accumulation in normal myocardium, 477 metabolic conversion including the formation of damaging species, $^{478\,479\,480\,481\,482\,483}$ and the generation of oxidative stress resulting from the interaction of doxorubicin with oxygen catalyzed by iron. $^{484\,485}$ Through iron-mediated interactions, doxorubicin causes the formation of reactive oxygen species such as hydrogen



Figure 5.1: Chemical structures of doxorubicin, vosaroxin, and dexrazoxane.

peroxide (H_2O_2) and superoxide radical anion (O_2^{--}) . As depicted in Figure 5.2, the univalent reduction (e^-) of the aromatic core of the doxorubicin molecule gives the unstable semiquinone free-radical, doxoquinone that in the presence of oxygen can auto-oxidize back to its parent quinone. The reduction of molecular oxygen leads to the formation of ROS which then can react further with free iron following the well-travelled pathways of Fenton chemistry, leading to an accumulation of iron in the mitochondria, increased levels of ROS, and overall impaired mitochondrial respiration.⁴⁷⁴ The bivalent reduction $(2 e^-)$ of the side-chain carbonyl group of the doxorubicin molecule converts Hdox irreversibly into its secondary alcohol metabolite, doxol, which is slightly less redox active than the unstable semiquinone free-radical, therefore able to accumulate; doxol's disruptive effect on human Ca^{2+} and Fe^{3+} homeostasis appears stronger than the parent compound.⁴⁷³

Reduction of Fe(III) to Fe(II) is an essential biological step that occurs widely in Nature. ⁴⁸⁶ On the molecular level, *in vivo* iron homeostasis is heavily regulated because living organisms carefully sequester iron(II) in stable complexes with biomolecules such as transferrin to prevent any toxicity arising from free iron overload. Reactive oxygen species, such as O_2^{--} , H_2O_2 as well as the hydroxyl radical (OH[•]), dramatically affect iron homeostasis in Nature. While ROS are involved in various essential biological functions, they can become harmful at higher concentrations, when their oxidation reaction of biomolecules increases physiological stress.⁴⁸⁷ ROS species directly interact with ferrous and ferric ions *in vivo*



Figure 5.2: Doxorubicin affects Fe³⁺ homeostasis *in vivo*: univalent reduction to semiquinone (left), bivalent reduction to secondary alcohol (right).

according to Fenton oxidation chemistry.⁴⁸⁸ Superoxide radical anions reduce Fe(III) that is coordinated to biological ligands to Fe(II) and dioxygen. In an inner-sphere electrontransfer mechanism, H_2O_2 oxidizes biologically-ligated Fe(II) to Fe(III) with concomitant formation of hydroxyl radical and hydroxide (HO⁻) as reductive side-products, which leads to increased oxidative stress.

Oxidative stress is directly involved in the pathogenesis of heart failure; it damages the mitochondria through excess formation of $O_2^{\cdot-}$, reduction of adenosine triphosphate (ADP), and transcriptional alteration of genes associated with heart failure.⁴⁸⁹⁴⁹⁰ To circumvent induction of oxidative stress and cardiomyopathy, doxorubicin is administered with radical scavenger drugs, such as dexrazoxane (5.1), which reduce mitochondrial ironlevels.⁴⁹¹⁴⁹²⁴⁹³

Vosaroxin (Hvox), presented in Figure 5.1, is a first-in-class anticancer quinolone derivative (AQD) that induces DNA damage and inhibits topoisomerase II, inducing site-selective DNA damage, G2 arrest and apoptosis.^{295 297 494 495} Vosaroxin induces DNA double/strand breaks (DSB) in cancer cells in guanine/cytosine rich sequences analogous to those caused by quinolone antimicrobials in bacteria.^{494 495} In contrast to doxorubicin, vosaroxin is not a substrate for the multidrug resistance protein P-glycoprotein⁴⁹⁶ and evades resistance mechanisms associated with p53 deficiencies.^{497 498} Vosaroxin has been studied in both solid tumor cancers,^{499 500} as well as hematologic malignancies,⁵⁰¹ and it is currently completing a phase III clinical trial in patients with relapsed or refractory acute myeloid leukemia.⁵⁰² In contrast to doxorubicin, vosaroxin's anticancer activity appears to result exclusively from intercalation of DNA and inhibition of topoisomerase II. Unlike doxorubicin, vosaroxin is minimally metabolized,^{480 483 503} and thereby produces limited free radicals ROS via intrinsic metabolic activation.⁴⁹⁴ As mentioned above, cardiomyopathy is a serious side-effect of treatment with doxorubicin, which has been associated with the formation of ROS and other toxic metabolites partly catalyzed by iron.

The fact that quinolones coordinate metals in various oxidation states in different coordination geometries³²⁷ is a known side-effect for antimicrobial therapy, as such coordination leads to a reduction in quinolone bioavailability.⁵⁰⁴ Iron(III) complexes with various commercially available quinolone antimicrobial drugs in which the iron coordinates the drug ligands in a stable octahedral 1:3 fashion have been reported and studied.^{398 399 400 401 403 505 506} Iron(III) forms some of the most stable complexes with quinolones compared to other bivalent and trivalent metals, ⁴²⁶ and the determined stability constants range from $\log \beta_{FeL3} = 25.16$ (1) for enoxacinⁱ to $\log \beta_{FeL3} = 46.94$ for ciprofloxacin.ⁱⁱ

Antimicrobial treatment with quinolone drugs is, in general, considered safe, as they are known to be commonly well tolerated and have safety profiles that compare to those of other antimicrobial drug classes.^{282,507,508} Frequently reported mild adverse reactions affect the gastrointestinal (GI) tract (e.g., nausea, vomiting, diarrhea) and the central nervous system (e.g., dizziness, headache, drowsiness), while tendinitis and tendon rupture as well as phototoxicity⁵⁰⁹ are more severe side-effects for which certain quinolone antimicrobial agents are widely known.^{282 295 297 507 508 510}

The molecular mechanisms of actions of vosaroxin, a quinolone derivative, and doxorubicin, an anthracycline, are differentiable as a result of their distinct chemical scaffolds (Figure 5.1). In order to further understand the properties of these two compounds, the interaction of vosaroxin with iron(III) has been characterized, and solution spectrophotometric studies of iron(III) coordination chemistry with vosaroxin and doxorubicin have been conducted. In addition, the novel tris(vosaroxino)iron(III) complex has been synthesized and characterized in order to examine the iron(III) coordination properties of vosaroxin in direct comparison to doxorubicin.

ⁱHL= enoxacin, determined potentiometrically: 22°C, $I_{NaCl} = 0.1$ M, inert gas N₂.⁴²⁶ ⁱⁱHL= ciprofloxacin, extrapolated from potentiometric data: 25°C, $I_{KCl} = 0.2$ M.³¹⁵

Because the Ga^{3+} ion possesses many chemical similarities with the Fe³⁺ ion (see Chapter 3), their chemical binding properties are similar, especially in regard to ligand chelation or protein binding.³⁷⁶ Biological systems cannot distinguish between Fe³⁺ and Ga^{3+} , a fact exploited in Fe³⁺ transport studies *in vivo*³⁷² or imaging.³⁷⁷ Therefore, the diamagnetic tris(vosaroxino)gallium(III) analog has been synthesized as well, which allowed thorough NMR-studies of the isolated complex.

5.2 Materials & Methods

5.2.1 Chemicals

Doxorubicin and iron(III) nitrate nonahydrate were purchased from Sigma-Aldrich, gallium(III) nitrate nonahydrate was obtained from Alfa-Aesar, and dimethyl sulfoxide was from Fisher Scientific. Sunesis Pharmaceuticals, Inc. provided the vosaroxin (reference standard quality, lot# 12AK0025B). Deuterated dimethyl sulfoxide was purchased from Cambridge Isotope Laboratories, Inc., and Sigma-Aldrich delivered the deuterium oxide as well as the phosphoric acid- d_3 solution and the sodium deuteroxide. The atomic iron(III) standard solution for AAS (1000 mg/L ± 4 mg/L) was obtained from Fluka. In the preparation of all aqueous solutions for spectrophotometric measurements and syntheses, only deionized water, purified through a ELGA PURELAB ultrapure water system with a resistivity of 18 MΩ·cm (25°C), was used.

5.2.2 Instrumentation

Melting points were determined using a Stanford Research Systems DigiMelt SRS melting point apparatus and are uncorrected. Ultraviolet-Visible spectrophotometry was performed on a Hewlett Packard 8453 instrument. Spectra were recorded using the UV-Vis Chem-Station Software (version B.04.01[61], Agilent Technologies, 2001–2010), and all maximum absorption bands and extinction coefficients (ϵ) are listed. Infrared spectra were recorded in the solid state on a PerkinElmer Frontier FT-IR spectrometer in the range 4000-650 cm⁻¹ using the software PerkinElmer Spectrum (version 10.03.02, 2011). Only the most characteristic bands were interpreted using the following abbreviations: s, strong; m, moderate; w, weak; br, broad; sh, shoulder. Nuclear magnetic resonance data (1D, 2D) were collected on a BRUKER AV600 spectrometer (600 MHz). The residual solvent signal of the deuterated solvent was used as the internal standard.³⁰⁹ Chemical shifts δ are referenced in ppm against tetramethylsilane (δ = 0). Multiplicities are abbreviated as: s, singlet; m, multiplet; br, broad. Ar represents aromatic protons. All spectra were analyzed with the software inmr (version 5.3.4, Mestrelab Research). Low-resolution mass spectral analysis was performed on a Water ZQ spectrometer equipped with ESCI sources. High-resolution mass spectra were obtained at the UBC Mass Spectrometry Centre on a Waters Micromass LCT employing electrospray-ionization. Only characteristic signals have been listed as the dimensionless mass-to-charge ratio, with intensity related to the base signal.

5.2.3 Spectrophotometry

The instrumental set-up comprised a Corning Hot Plate Stirrer PC-351, a Fisher Scientific Accumet Basic pH-meter and a ThermoFischer ORION 8103BN ROSS Semi-Micro Combinational pH-electrode, which was calibrated before each titration using reference solutions (pH 4.00, 7.00, 10.00) from the FisherScientific Buffer-Pac. All solutions were prepared in aqueous sodium chloride ($I_{NaCl} = 0.15$ M). Stock solutions of vosaroxin and doxorubicin were prepared as follows: about 1 mg of Hvox, or about 1.5 mg of Hdox, respectively, was dissolved in 5.00 mL sodium chloride solution, and after 30 min of sonication the suspension turned to a clear, colorless solution; these stock solutions were stable and used for two to three days. Solutions of ligand (Hvox, Hdox) only (~2.0·10⁻⁵ M) or ligand (Hvox, Hdox) (~2.0·10⁻⁵ M) mixed with iron(III) standard solutions in Fe³⁺:HL ratios of 1:1, 1:2, or 1:3 in a total volume of 10.00 mL were freshly prepared on the day of the experiment. To ensure that the titration started with the fully protonated species of ligand, the ligand solutions were prepared in aqueous hydrochloric acid (0.2 M). The freshly prepared solution was sonicated for 15 min and transferred into a glass vial before the electrode was submerged in the test solution. The solution was stirred and the electrode remained submerged in the solution throughout the titration. The data were collected as pH vs. volume of titrant (2–20 μ L). For each determination of ligand protonation constant or Fe(III) complexation constant, titrations were conducted in triplicate. Equilibration times between additions ranged from 3–10 min. All titration data were manipulated in MS Excel and plotted using Plot2 (version 2.0, Michael Wesemann). HypSpec (Protonic Software, Leeds) was used to fit the obtained UV-Vis curves to obtain the stability constants.

5.2.4 Computational Details

Calculations of vosaroxin with iron(III) were performed using DFT at the B3LYP level utilizing the 6-31+g(d,p) basis set as implemented in Gaussian.⁴¹² All optimised geometries are characterised as minima as indicated by the absence of imaginary frequencies. Geometry optimisations were performed on the systems and were evaluated to obtain stable Fe^{3+} :vosaroxin structures. Computational modelling was used to verify the binding site of the iron(III) ion to Hvox. The initial geometry of vosaroxin was optimised and was used in a series of subsequent calculations, whereby the Fe³⁺ was placed at various regions around the vosaroxin ligand but at distances greater than ~3.5 Å so as not to bias the interaction, if any, between the Fe³⁺ metal ion and the potential binding partner.

5.2.5 Synthesis & Characterization

5.2.5.1 Vosaroxin, Hvox

Appearance: Amorphous, off-white solid. **Mp**: $\geq 240^{\circ}$ C, decomposition to dark yellow solid. UV-Vis (DMSO): λ [nm] (ϵ) [M⁻¹cm⁻¹] = 275 (80000), 250 (50000). IR (neat): $\tilde{\nu} \text{ [cm}^{-1]} = 3316 \text{ (w)}, 3088 \text{ (md)}, 3047 \text{ (sh)}, 2988 \text{ (w)}, 2940 \text{ (md)}, 2885 \text{ (md)}, 2819 \text{ (w)},$ 1728 (st), 1619 (st), 1548 (st), 1510 (sh), 1491 (st), 1439 (md), 1417 (md); 1386 (md), 1327 (md), 1299 (md), 1261 (sh), 1251 (md), 1220 (w), 1185 (w), 1161 (w), 1113 (st), 1092 (sh), 1016 (w), 958 (st), 871 (w), 854 (w), 830 (md), 799 (st), 764 (md), 736 (md), 698 (w), 681 (md), 657 (md). **NMR**: δ_H (600 MHz, 298 K, d_6 -DMSO) [ppm] = 9.72 (s, 1 H, $C_{ar2}H$); 8.23 (d, $J_{HH}^3 = 9.1$ Hz, 1 H, $C_{ar5}H$); 7.81–7.80 (m, 1 H, $C_{taz4}H$); 7.78–7.76 (m, $1 \text{ H}, \text{C}_{ar6}H$; $6.85-6.83 \text{ (m, 1 H, C}_{taz5}H$; $3.96 \text{ (s, 1 H, C}_{azo3}H$); 3.88-3.80 (m, 1 H, NH); $3.78-3.71 \text{ (m, 2 H, } C_{azo5}H_2\text{)}; 3.66-3.63 \text{ (m, 1 H, } C_{azo4}H\text{)}; 3.52-3.43 \text{ (m, 2 H, } C_{azo2}H_2\text{)};$ 3.31–3.28 (m, 3 H, OCH₃); 2.37 (d, $J_{HH}^3 = 2.6$ Hz, 3 H, NH(CH₃)). δ_C (150 MHz, 298 K, d_6 -DMSO) [ppm] = 176.8 (C_{ar4}); 165.3 (COOH); 157.3 (br, C_{ar7}); 155.2 (br, C_{taz2}); 147.7, 147.6 (C_{ar8}) ; 141.9 (br, C_{ar2}); 137.8, 137.7 (C_{taz4}) ; 135.4, 135.3 (C_{ar5}) ; 121.6, 121.5 (C_{ar6}) ; 110.0 (br, C_{ar4}); 109.3 (br, C_{taz5}); 109.0, 108.9 (C_{ar3}); 82.0, 81.6 (C_{azo3}); 62.4, 62.1 (OCH_3) ; 56.3, 56.3 (C_{azo2}) ; 53.4, 53.1 (C_{azo5}) ; 51.2, 50.8 (C_{azo4}) ; 34.2, 34.1 (NCH_3) . MS $(\text{ES}+, \text{CH}_3\text{OH}): m/z \ (\%) = 402 \ (100) \ [\text{HL} + \text{H}^+].$ **HR-ESI-MS**: $m/z \ \text{for } \text{C}_{18}\text{H}_{19}\text{N}_5\text{O}_4\text{S} +$ H⁺ calcd. (found): 402.1236 (402.1242). **EA**: Anal. Calcd. (found) [%] for $C_{18}H_{19}N_5O_4S$: C, 53.86 (53.97); H, 4.77 (4.74); N, 17.45 (17.24); S, 7.99 (7.64).

5.2.5.2 Tris(vosaroxino)iron(III), $[Fe(vox)_3]$

Dissolving vosaroxin (125 mg, 0.31 mmol) in deionized water (20 mL) and stirring for 15 min at ambient temperature gave a clear, colorless solution of neutral pH. This solution was added dropwise into a previously prepared solution of iron(III) nitrate nonahydrate (40 mg, 0.1 mmol) in deionized water (5 mL). During the addition, the pH was adjusted to <3, if necessary, with aqueous hydrochloride solution (0.1 M). Upon completion of the addition, the pH was raised to pH 5 with aqueous sodium hydroxide (1.0 M), changing the color of the reaction mixture to red-brown. The reaction mixture was stirred at ambient temperature overnight, before the solvent was removed *in vacuo* to result an amorphous, lustrous, dark red-brown solid, which was washed repeatedly with deionized water and methanol, and then thoroughly dried *in vacuo* (108 mg, 0.086 mmol, 86%). **Mp**: $\geq 200^{\circ}$ C, decomposition to black-brown solid. **UV-Vis** (DMSO): λ [nm] (ϵ) [M⁻¹cm⁻¹] = 275 (80000), 250 (50000). **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3433 (w, br), 3083 (w), 2997 (w), 2941 (w), 2881 (w), 2832 (w), 2732 (w), 2462 (w, br), 1621 (st), 1562 (sh), 1494 (st), 1443 (md), 1419 (md), 1317 (w), 1293 (w), 1276 (w), 1253 (st), 1178 (w), 1097 (st), 1038 (md), 968 (md), 921 (md), 854 (w), 825 (w), 803 (st), 758 (st), 722 (w), 701 (w), 675 (md). **MS** (ES+): m/z (%) = 1280 (< 10) [ML₃ + Na⁺], 857 (100) [ML₂]⁺. **HR-ESI-MS**: m/z for C₅₄H₅₄ ⁵⁶FeN₁₅O₁₂S₃ ³⁹K⁺ calcd. (found): 1295.2225 (1295.2233).

5.2.5.3 Tris(vosaroxino)gallium(III), [Ga(vox)₃]

Vosaroxin (125 mg, 0.31 mmol) was dissolved in deionized water (12 mL) and stirred at ambient temperature for 10 min. The clear colorless vosaroxin solution was added drop-wise into a solution of gallium(III) nitrate nonahydrate in deionized water (5 mL). The reaction mixture turned pale yellow during the addition, and its pH increased to pH 5. Without further adjustments, the reaction solution was stirred at ambient temperature overnight. Removal of the solvent *in vacuo* gave an amorphous, lustrous, pale yellow solid that was washed repeatedly with water and methanol, and then thoroughly dried *in vacuo* (69 mg, 0.082 mmol, 82%). **Mp**: $\geq 190^{\circ}$ C, decomposition to brown solid. **UV-Vis** (DMSO): λ [nm] (ϵ) [M⁻¹cm⁻¹] = 275 (80000), 250 (50000). **IR** (neat): $\tilde{\nu}$ [cm⁻¹] = 3421 (md, br), 3086 (w), 3001 (w), 2937 (w), 2874 (w), 2832 (w), 2733 (w), 2473 (w, br), 1615 (st), 1564 (sh), 1494 (st), 1446 (md), 1423 (md), 1318 (w), 1277 (w), 1256 (st), 1179 (w), 1098 (st), 1040 (md), 970 (md), 923 (st), 858 (w), 826 (w), 803 (st), 760 (st), 728 (w), 702 (w), 680 (md). **NMR**: δ_H (400 MHz, 363 K, d_6 -DMSO) [ppm] = 10.05 (s), 9.96 (s), 9.79 (s), (3) H, $C_{ar2}H$); 8.42 (d, $J_{HH}^3 = 8.9$ Hz), 8.33 (d, $J_{HH}^3 = 9.1$ Hz), 8.21 (d, $J_{HH}^3 = 7.8$ Hz), (3 H, $C_{ar5}H$); 7.91-7.80 (m, 6 H, $C_{taz4}H$ and $C_{ar6}H$); 7.00 (d, $J_{HH}^3 = 9.1$ Hz), 6.90 (d, $J_{HH}^3 = 7.2$ Hz), (3 H, C_{taz5}H); 4.30-4.26 (m, 3 H, C_{azo3}H); 4.12-3.78 (m, 15 H, C_{azo5}H₂, C_{azo4}H, 3.43 $C_{azo2}H_2$; 3.43 (s, 9 H, OCH₃); 2.68 (d, $J_{HH}^3 = 17.4$ Hz, 9 H, NH(CH₃)). δ_C (150 MHz, 298 K, d_6 -DMSO) [ppm] = 178.4, 176.1, 174.5 (C_{ar4}); 165.0, 164.9, 164.8 (COOH); 157.3, 157.1, 157.0 (C_{ar7}) ; 155.6, 155.5, 155.4 (C_{taz2}) ; 147.9, 147.7, 146.5 (C_{ar8}) ; 144.4, 144.1, 143.4 (C_{ar2}) ; 138.4, 138.1, 137.9 (C_{taz4}) ; 136.4, 136.2, 135.8 (C_{ar5}) ; 122.3, 122.1, 121.8 (C_{ar6}) ; 110.7, 110.6, 110.4 (C_{ar4}) ; 110.0, 109.8, 109.6 (C_{taz5}) ; 109.9, 109.0, 108.8 (C_{ar3}) ; 79.5, 79.4, 79.3 (C_{azo3}) ; 60.7, 60.6, 60.5 (OCH_3) ; 57.2, 57.1, 57.0 (C_{azo2}) ; 53.8, 53.7, 53.6 (C_{azo5}) ; 50.7, 50.6, 50.5 (C_{azo4}) ; 31.9, 31.8, 31.7 (NCH_3) . **MS** (ES+): m/z (%) = 1272 (50) $[ML_3 + H^+]$, 869 (100) $[ML_2]^+$. **HR-ESI-MS**: m/z for $C_{54}H_{54}$ ⁶⁹GaN₁₅O₁₂S₃ ²³Na⁺ calcd. (found): 1292.2392 (1292.2397); m/z for $C_{36}H_{36}$ ⁶⁹GaN₁₀O₈S₂⁺ calcd. (found): 869.1415 (869.1414).

5.2.6 Electrochemistry

Cyclic voltammetry studies were performed with Hvox, $[Fe(vox)_3]$ and $[Ga(vox)_3]$ in a dimethyl sulfoxide solution (V = 10.0 mL) containing 0.1 M of tetra(n-butyl)ammonium perchlorate as the supporting electrolyte in a three electrode system composed of a platinum-disk electrode as the working electrode, a platinum-mesh electrode as the counter electrode and a silver electrode as the pseudo reference electrode. A potentiostat (Pine AFCBP1, ID 23051890) was integrated into the electric circuit. The software AfterMath, Inc. (version 1.2.4532) was used for controlling the potentiostat and recording the data. CV measurements followed standard procedures. All glassware was dried in the oven at 100°C for 24 hours before use. Tetra(n-butyl)ammonium perchlorate (0.342 g, 0.001 mol) was transferred into the electrochemical cell and dried *in vacuo* for 15 min. In parallel, dimethyl sulfoxide was degassed with N_2 gas. Using Schlenk techniques, 10.0 mL of the degassed dimethyl sulfoxide was transferred onto the tetra(n-butyl)ammonium perchlorate inside the electrochemical cell. The mixture was stirred vigorously until the electrolyte salt had completely dissolved. Throughout the experiment the electrochemical cell was kept under N_2 gas at all times. Firstly, a blank voltammogram of the electrolyte solution was recorded. Secondly, the compound to be measured was added as a solid and dissolved in the electrolyte solution (0.01 mmol, respectively: Hvox, 41 mg; $[Fe(vox)_3]$, 12.6 mg; $[Ga(vox)_3]$ 12.0 mg). Measurements on these compounds were performed in the general voltage range between +1.3 V and -2.3 V, starting from an initial voltage of 0 V and ending at a final voltage of 0 V. The sweep rate was $100 \text{ mV} \cdot \text{s}^{-1}$, the electrode range was varied between 5–10 μ A, and the number of segments was set to 5 as default. The experiment concluded with a reference measurement of ferrocene. All electrodes were cleaned accordingly. The platinum-mesh electrode and the silver pseudo-reference electrode were submerged in methanol (separate vials) and sonicated for 30 min. The platinum-disk electrode was polished using Buehler MicroPolish II Alumina Powder (0.3 μ m and 0.05 μ m); polish residues were rinsed off with deionized water and the electrode was dried.

5.3 Results & Discussion

5.3.1 Stability Constants

Vosaroxin and doxorubicin each contain ionizable protons. The pK_a values for each ligand, as determined by spectrophotometric titration, are given in Table 5.1. In acidic solutions, doxorubicin exists as the singly charged species $[H_2 dox]^+$ with the positive charge at the sugar amino group. Initial dissociation (pK_a 7.67) is assigned to the amino sugar group

K_{FeLH}	doxorubicin	vosaroxin
$\log K_{110}$	17.985^{a}	16.31(3)
$\log K_{120}$	11.049	8.70(2)
$\log K_{130}$	4.379	7.80(3)
$\log \beta_{130}$	33.413	32.80(3)
pM^b	17.0	15.9
pK_{a1}	10.96(1)	9.97(2)
pK_{a2}	9.46(1)	7.091(4)
pK_{a3}	7.67(2)	5.125(4)
pK_{a4}	n/a	2.779(4)

Table 5.1: Protonation and Fe³⁺ formation constants for doxorubicin and vosaroxin.

^{*a*}37°C, $I_{NaCl} = 0.15$ M. ⁵¹¹ ^{*b*} pM = $-\log[Fe]$, pH 7.4, $[L]_T = 10 \ \mu$ M, $[Fe]_T = 1 \ \mu$ M.

followed by the dissociation of the phenolic hydrogens (p K_a 9.46 and 10.96). These assignments are in agreement with those in the literature.⁵¹¹ It is interesting to note that there is some variation in literature data on the amino group p K_a value 6.8–8.99,⁵¹² and p K_a of 9.01–11.2 for the phenolic group. These differences can be attributed, in part, to self-association and decomposition of the drug at higher concentrations (>30 μ M).⁵¹³ The concentrations of doxorubicin used in the present studies were purposefully low enough to avoid self-association (~20 μ M). Vosaroxin has four ionizable protons, existing as the triply charged species [H₄vox]³⁺ under acidic conditions. The ionization process and p K_a values determined in this study are in close agreement with those, which have been extrapolated from a co-solvent system earlier.⁵¹⁴ Small differences can be attributed to the use of sodium chloride at biologically relevant concentrations (I_{NaCl}= 0.15 M) as background electrolyte.

The formation of the Fe(III)-doxorubicin and Fe(III)-vosaroxin complexes as a function of pH and $[Fe^{3+}]$:[HL] molar ratios was investigated by spectrophotometric titration. The formation of the Fe³⁺-doxorubicin complexes elicited broad absorption bands centered at 600 nm (data presented in Appendix B), as previously reported. ^{512 515 516} Experiments with Fe(III)-doxorubicin proved problematic due to drifting electrode measurements, formation and precipitation of $Fe(OH)_3$ as well as low concentrations of complex formed under the experimental conditions utilized. Despite the apparent distance between the amino group and the iron binding site of doxorubicin, the charge on the amino group is known to influence the strength of interaction with the metal ion.⁵¹⁷ These problems interfered with convergence within HypSpec software when fitting the data, and there were large uncertainties in the logK values that could be obtained. Consequently, the logK values for doxorubicin presented in Table 5.1 have been taken from the literature.⁵¹¹

The visible absorption spectra of solutions containing Fe(III) and vosaroxin as a function of pH (Appendix B) are characterized by a new, broad absorption band centered around 400 nm as shown in Figures 5.3 and 5.4. In order to fit the iron(III)-vosaroxin titration data, it was necessary to select a model incorporating all possible species. Computational modelling was utilized in order to identify the preferred binding site of the Fe³⁺ ion to vosaroxin.⁵¹⁸ For this, geometry optimisation (energy minimization) calculations were performed on different states, whereby the Fe^{3+} ion was placed at various locations around the vosaroxin ligand; however, not to bias the interaction, the Fe^{3+} ion was never placed in a distance closer than ~ 3.5 Å to vosaroxin, the potential binding partner. The most stable conformer involves the ferric ion being chelated by the ketone oxygen and the deprotonated carboxylate moiety (Figure 5.5). It is interesting to note that energy minimization of vosaroxin alone or in the presence of iron results in rotation of the thiazole ring such that the orientation of this group differs between bound and unbound species. For unbound vosaroxin, the thiazole group is almost co-planar with the naphthyridine ring and the sulfur atom nearest the ring nitrogen atoms. Following complexation of Fe^{3+} at the diketone, the thiazole group rotates so it is now almost at a right angle to the naphthyridine ring. This was confirmed by scanning the potential energy surface for rotation around the N-C bond of the thiazole, and is consistent with the ¹H NMR data collected



Figure 5.3: Comparison of the changes in absorbance at 400 nm for a solution of Fe^{3+} -vosaroxin ([Fe³⁺] = 5.8·10⁻⁶ M and [Hvox] = 2.24·10⁻⁵ M) (\blacksquare) and vosaroxin only (\square) with varying pH in aqueous electrolyte solution (I_{NaCl}= 0.15 M).

following titration of Fe^{3+} with vosaroxin. These data show that addition of Fe^{3+} influences the chemical shift of neighboring hydrogens including those around the thiazole and naphtyridine rings, not just those expected to be influenced by chelation (data shown in Appendix B).

Speciation plots for solutions of doxorubicin and vosaroxin with Fe(III) as a function of pH are shown in Figure 5.6 and Figure 5.7, respectively. At a ratio of 3:1 ligand to metal, the predominant species in the Fe(III)-doxorubicin system at pH 7.4 is the noncoordinated, singly charged ligand (H₃dox⁺), in contrast to the Fe(III)-vosaroxin system where [Fe(vox)₃] is the predominant species. This reflects the differences in both stability of the metal-ligand and metal-protonated ligand complexes, and also the ionization state of the ligands at physiological pH. For vosaroxin, the [Fe(vox)₃] complex is the single,



Figure 5.4: Representative fit at 400 nm using obtained stability constants for a solution of Fe(III)-vosaroxin in 1:3 molar ratio: \circ experimental data, fitted absorbance.

dominant species from pH 6.5 onwards into the basic pH range; however, the interaction between Fe³⁺ and doxorubicin appears to be more complex as various species of iron coordinated and protonated doxorubicin are observed in the distribution. [Fe(vox)₃] only slowly starts to form at pH 8 and higher, while at physiological pH free ligand in various protonation states (H₂dox, H₃dox⁺) exist next to the hydroxide adduct [Fe(dox)(OH)]. The latter is a minor species (\leq 30%) between pH 6.5–9. In their study of the stability and iron coordination in DNA adducts of anthracycline based anticancer drugs, Eriksson and coworkers⁵¹⁹ found that the Fe³⁺ in the [dox-DNA]Fe³⁺ system was coordinated to four O-atoms belonging to the [dox-DNA] adduct, and that it was in addition coordinating five water molecules as well. They suggested that the lower number of O-atoms and the higher number of H₂O molecules bound to the Fe³⁺ were related to a lower binding energy of the



Figure 5.5: Interaction of the Fe^{3+} ion (lavender) with vosaroxin, showing the most stable diketone-coordinated conformation obtained following energy minimization using B3LYP/6-31+g(d,p).

metal ion possibly resulting in an increased production of hydroxyl radicals *in vivo*. This suggests that the $[Fe(vox)_3]$ species is potentially more thermodynamically stable, because the central Fe³⁺ is coordinated to a total of six O-atoms of the three vox-ligands, versus the [Fe(dox)(OH)] species, in which the metal is only coordinated to two ligand O atoms and a hydroxide. The coordination number of iron(III) is six. In the $[Fe(vox)_3]$ complex, all six iron coordination positions are occupied leaving no access for further hydroxyl coordination to the unoccupied iron orbitals.

5.3.2 Synthesis & Characterization of Tris(vosaroxino)iron(III) and -gallium(III) Complexes

Aqueous solutions of vosaroxin were mixed with aqueous solutions of iron(III) nitrate nonahydrate, and gallium(III) nitrate nonahydrate respectively, in a 3:1 ratio (Figure 5.8).



Figure 5.6: Species distribution curves for the iron(III)-doxorubicin system. $[Fe^{3+}]_T = 3.3 \cdot 10^{-4} \text{ M}, [L]_T = 1 \cdot 10^{-3} \text{ M}.$



Figure 5.7: Species distribution curves for the iron(III)-vosaroxin system. $[Fe^{3+}]_T = 3.3 \cdot 10^{-4} \text{ M}, [L]_T = 1 \cdot 10^{-3} \text{ M}.$

The coordination of vosaroxin was favoured over the formation of metal-hydroxide species around pH 5, as indicated by the lack of precipitation due to hydroxide formation, and by MS-samples taken out of the reaction solution with a peak for $[ML_2]^+$ (100%). Allowing the reaction solution to stand at ambient temperature, or in the fridge at 4° C, for several days did not promote precipitation, and the MS signal continued to show that characteristic $[ML_2]^+$ peak (100%). Upon removal of the solvent *in vacuo*, and thorough washing of the obtained solid with small amounts of water and methanol, the respective metal-vosaroxin complexes were isolated and characterized by HR-ESI mass spectrometry. The data were consistent with the formation of tris(vosaroxino)metal(III). The compounds are nonvolatile and stable, decomposing at approximately 200°C. The solubility of the obtained $[M(vox)_3]$ (M= Fe³⁺, Ga³⁺) complexes is generally low and shows a high pH dependence; therefore, solution characterization of these complexes by MS was carried out with solutions in methanol, acetonitrile and nitromethane. Structure analysis by ${}^{1}\mathrm{H}$ and ¹³C NMR spectroscopy was only possible in d_6 -DMSO due to the extremely low solubility of both complexes in D_2O and other standard NMR-solvents. This low solubility, in addition to the various stereoisomers, negatively impacted both elemental analyses and attempts to grow single crystals suitable for X-ray analysis of either complex. It has been reported previously that it is challenging to grow single-crystals of quinolone-metal complexes.³²⁸ Over the duration of eighteen months, a multitude of attempts were undertaken to grow single crystals according to various crystallization methods and personal tricks of fellow researchers in the department. Diffusion methods in varying volumes, concentration and glass ware set-ups were used, employing mainly acetonitrile, chloroform, diethyl ether, DMSO, methanol and water, according to solubility. Respective crystallization vials were placed in the freezer, the fridge, on the window sill and in a dark cupboard at room temperature, but crystals suitable for single x-ray diffraction did not grow. In addition, reactive



Figure 5.8: Synthetic route to tris(vosaroxino)metal(III) complexes $[M(vox)_3]$, $M = Fe^{3+}$, Ga^{3+} .

crystallization experiments were conducted, in which the starting materials were dissolved in different solvents over a range of pH conditions and layered on top of each other in one vial according to the density of the solvent. Unfortunately, the diffusion reaction did not lead to crystals but instead to an amorphous powder in some cases (systems of DMSO, methanol, water), or to no reaction at all.

The mass spectra, however, were diagnostic of the complex formulations at a 3:1 ratio of vosaroxin:metal. With both metal ions (Fe³⁺, Ga³⁺), loss of one ligand from a [ML₃] unit was observed giving the [ML₂]⁺ fragment with 100% intensity. In addition, the tris(vosaroxino)iron(III) complex in methanol cationized in the high-resolution ES+ by attachment of one sodium or potassium cation to form [NaML₃]⁺ or [KML₃]⁺ as the parent peak. [Ga(vox)₃] was dissolved in low concentrations in various solvents (methanol, acetonitrile, nitromethane, DMSO) for further characterization with low- and high-resolution ES+ techniques. The spectra clearly reflected the effect of the different solvents on the mass pattern. In addition to the [NaML₃]⁺ parent peak, recombination signals corresponding to [M₂L₅]⁺ were observed for [Ga(vox)₃] in nitromethane, which we have previously reported as characteristic for tris(ligand)metal(III) complexes.⁴¹³

Spectroscopic analysis in the mid-infrared region $(4000-650 \text{ cm}^{-1})$ supported the complete coordination of the respective metal through the carboxylate-O-atom. Although the IR spectra of the quinolones are in general quite complex because of the numerous functional groups in the molecule, the stretching frequencies of the carbonyl and carboxyl group are strong and can be identified as prominent absorption bands among the many and varied C_{aryl} -H and C-N vibrations in the same IR region.^{327 328} The IR spectrum of the free ligand showed a strong characteristic band at 1728 cm⁻¹ attributed to the stretching frequency of the carboxyl-OH-group in C_{ar3} -position on the aromatic ring system; upon coordination of Fe³⁺ or Ga³⁺ it disappeared completely, as the IR-spectra of the respective metal-vosaroxin complexes show (Figure 5.9). In the IR spectra of both vosaroxin-metal complexes, two distinct bands in the range 1620-1315 cm⁻¹ could be assigned to the ν_{CO_2} asymmetric and symmetric stretching vibrations. The difference $\Delta[cm^{-1}] = \nu_{asym(CO_2)} - \nu_{sym(CO_2)}$ is quite large with $\Delta= 304 \text{ cm}^{-1}$ for [Fe(vox)₃], and $\Delta= 297 \text{ cm}^{-1}$ for [Ga(vox)₃], likely characteristic for a monodentate coordination mode of the carboxyl group.^{318 418}

¹H NMR spectra of vosaroxin were recorded in D₂O as well as in d_6 -DMSO, showing a negligible solvent effect. Vosaroxin can form four different stereoisomers around the metal(III) center upon coordination of three bidentate anions in an octahedral fashion; the four possible stereoisomers of $[M^{3+}(vox)_3]$ are: $\Delta - fac$; $\Lambda - fac$; $\Delta - mer$; $\Lambda - mer$. In the case of the diamagnetic $[Ga(vox)_3]$, the different stereoisomers gave a multitude of signals in a ¹H NMR spectrum recorded at 298 K, but at 393 K the interchange happened so rapidly on the NMR time scale that a separation of signals occurred and a clear assignment was possible (T dependent NMR study presented in Appendix B). In the case of $[Fe(vox)_3]$, the Fe³⁺ ion retains a paramagnetic high-spin state upon complexation. As a result, the NMR signals are broadened considerably and impossible to assign with certainty. In an attempt to support the DFT calculations, which favoured coordination through the carboxyl-substituent and the carbonyl-group on the aromatic ring system, and to rule



Figure 5.9: IR spectra of vosaroxin (Hvox, black, top), $[Fe(vox)_3]$ (red, middle) and $[Ga(vox)_3]$ (green, bottom); the spectrum of Hvox shows the peak at 1728 cm⁻¹ disappearing upon coordination to Fe³⁺ or Ga³⁺.

out experimentally that the metal was not also coordinated to vosaroxin via the nitrogen atoms at the substituent on N_{ar1} and C_{ar6} , complexes were characterized by ¹H NMR after incremental addition of AAS-standard iron(III) solution. Vosaroxin (c= $5 \cdot 10^{-4}$ M, V= 5 mL) was dissolved in deuterated phosphate buffer pH 7.20 (pD 7.0), because the ligand was insoluble in deuterated phosphate buffer at pH 2.15. Small increments (V= 2 μ L) of AAS-standard iron(III) solution were added to the titration solution, which was then stirred rigorously for three minutes, before a sample of the solution (V = 0.5 mL) was transferred into a NMR tube. The titration was monitored via ¹H NMR at 600 MHz (data presented in Appendix B). Unfortunately, upon addition of iron(III), all NMR signals broadened significantly; therefore, it was impossible to detect a measurable increased broadening in the aromatic region, which would have supported coordination through the carboxyl-group on C_{ar3} and the carbonyl-group on C_{ar4} , over the aliphatic region. This would indicate coordination via the substituent ring systems on N_{ar1} and C_{ar6} . The experiment was further complicated by the precipitation of a dark red solid, (presumably $[Fe(vox)_3]$) from the solution, although the chemical identity of the precipitate could not be determined to our full satisfaction.

5.3.3 Cyclic Voltammetry Studies

To evaluate redox/decomplexation of $[Fe(vox)_3]$ and $[Ga(vox)_3]$, the complexes were studied via cyclic voltammetry (CV curves of Hvox and $[Ga(vox)_3]$ are presented in Appendix B). For $[Fe(vox)_3]$ (Figure 5.10), the Fe(III)/Fe(II) couple at 0.771 V vs. NHE³⁷¹ could not be clearly identified in the recorded cyclic voltammogram. The cyclic voltammogram, while irreversible, was found to be reproducible over multiple cycles without a large decrease of intensities of either peak. For the iron(III) as well as for the gallium(III) complex of vosaroxin, a dissimilar peak shape was observed, which indicates a reorganization in the coordination sphere, and therewith the coordination symmetry, around the metal center upon



Figure 5.10: Cyclic voltammograms, 0.001 M $[Fe(vox)_3]$ in DMSO (red), 0.1 M tetra(n-butyl)ammonium perchlorate, scan rate 100 mV·s⁻¹ (background, grey).

reduction of the metal, as had been previously observed for tris(ciprofloxacino)iron(III).⁴⁰¹

5.4 Conclusion

The Fe(III)-binding constant of vosaroxin, an anticancer quinolone derivative, has been determined spectrophotometrically and compared with the analogous iron(III) complex formed with doxorubicin, an anticancer agent widely used in the clinic. These spectrophotometric titrations in 0.15 M NaCl, at ambient temperature, in the pH range from pH 2–12, showed that the two anticancer agents doxorubicin and vosaroxin bind Fe³⁺ with similar strength: Hdox (log β_{FeL3} = 33.41, pM= 17.0) and Hvox (log β_{FeL3} = 33.80(3), pM= 15.9). At physiological pH, however, [Fe(vox)₃] is the predominant species in contrast to the mixture of protonated ligand species observed for the Fe³⁺:doxorubicin system. Here, H₂dox (~30%) and H₃dox⁺ (~40%), in addition to the minor (~30%) [Fe(dox)(OH)] species, are observed, indicating a more labile interaction between Fe³⁺ and doxorubicin than between Fe³⁺ and vosaroxin at physiological pH. Furthermore, two novel vosaroxin-metal(III) complexes were successfully synthesized from iron(III) nitrate and gallium(III) nitrate at a 1:3 ratio. In tris(vosaroxino)iron(III) as well as in tris(vosaroxino)gallium(III), the metal ion is coordinated through the deprotonated carboxylate oxygen on the C_{ar3} -atom of the naphthyridine ring system in a monodentate coordination mode leading to the formation of four stereoisomers. Their redox behavior was studied by CV, and the stereochemistry of the gallium(III) analog was further explored in temperature dependent ¹H NMR studies. For the $[Fe(vox)_3]$ complex, the iron redox couple was observed in the recorded CV spectrum. Both complexes were fully characterized. The obtained results are consistent with the well-studied clinical and chemical interaction between iron preparations (ferrous gluconate/sulfate, various multivitamin preparations) and quinolone-based drug molecules.⁵²⁰ When co-administered, the ferrous iron is oxidized to its ferric form, which rapidly forms quite stable tris(quinolono)iron(III) complexes. In vivo, the quinolones are very likely stable in the presence of $iron^{521}$ in contrast to the anthracyclines whose interaction with iron presumably leads to the formation of free radicals and lipoperoxidation.^{484,485} The stable $[Fe(vox)_3]$, dominant at physiological pH, seems unlikely to produce toxic metabolites and ROS associated with the more labile interaction from doxorubicin and Fe^{3+} . The data presented here suggest that the molecular pharmacology of their interaction with iron(III) may be one possible differentiation in the safety profile of quinolones compared to anthracyclines in relation to cardiotoxicity.

<u>Please see Appendix B for:</u> UV-Vis spectra of one titration run of vosaroxin to determine the pK_a s of the test solution, of Hvox:Fe³⁺ in the ratio of 1:1, of Hvox:Fe³⁺ in the ratio of 2:1, and of Hvox:Fe³⁺ in the ratio of 3:1; as well as UV-Vis spectra of one titration run of doxorubicin to determine the pK_a s of the test solution, of Hdox:Fe³⁺ in the ratio of 1:1, of Hdox:Fe³⁺ in the ratio of 2:1, and of Hdox:Fe³⁺ in the ratio of 3:1; cyclic voltammetric curves for 0.001 M of vosaroxin and $[Ga(vox)_3]$ in DMSO solution containing tetra(n-butyl)ammonium perchlorate 0.1 M at a scan rate of 100 mV·s⁻¹; ¹H NMR spectra of the titration of Hvox with Fe³⁺ at pD 7 (deuterated phosphate buffer) at 298 K; as well as ¹H NMR spectra of the temperature dependence study conducted on $[Ga(vox)_3]$ in the range from 298 K to 363 K.
Chapter 6

Conclusion & Outlook

The field of metallodrugs in medicinal inorganic chemistry has grown constantly during the past 50 years; however, despite the tremendous advancement of a few metallodrugs, the discipline is still less fully developed compared to the traditional medicinal chemistry areas of small organic or biological drug molecules. Twelve metals 522 are essential for the human body. For these metals, the human body has developed a sophisticated and sensitive system of pathways for their transport as different and diverse as the essential metals themselves; consequently, this diversity amounts to a core challenge for the systematic development of metallodrugs. In addition, other nonessential metals can be used for therapy as well. Of course, many great discoveries in science have been made by accident, and the serendipitous discovery of the anticancer activity of platinum or the antiarthritis activity of gold or the antidiabetic activity of vanadium are good examples. It might be surprising to some readers that many metallodrugs on the market today are being used in patients without a thorough understanding of the active structure, behaviour in the biological environment or indeed the exact molecular mechanisms of action; the beneficial therapeutic effect of these metallodrugs is the sole sanction of their continuing use in the clinic. As debated in Chapter 1, the majority of approved metallodrugs are either quite old (e.g., Pepto-Bismol, aurothioglucose) or are, despite their toxic side effects, still in use for the treatment of a neglected disease occurring in a developing country (e.g., melarsoprol against human African sleeping sickness) for which advanced treatment options with less side effects have not yet been developed.

To exploit fully the potential of metallodrugs, it is absolutely essential to understand what happens to the coordination complex and its components, the metal and the ligand(s), once the metal-ligand-complex enters the body. To what extent can the active metabolite be defined for drugs that are essentially delivery vehicles for metal ions to undergo dissociation and ligand exchange once administered? What role does the design of the ligand itself play in this? Are the pharmacological and toxicological properties of novel metallodrugs predictable based on an improved understanding of metal ion speciation in vivo? In what way does the oxidation state of the metal influence this? How important are the thermodynamic versus kinetic considerations for metallodrugs in the body? What is there still to learn from the biochemistry of essential metals and metal ion distribution in the human body? These are questions that were raised years ago^{18} and the answers are slow in coming. Funding from research councils across the world, some of which seem to recognize the tremendous therapeutic potential of metallodrugs, brings the field of medicinal inorganic chemistry closer to these answers. The European Cooperation in Science and Technology (COST) has been funding research actions in the area of medicinal inorganic chemistry and metallodrugs for many years. Action "CM1105 Functional Metal Complexes that Bind to Biomolecules", currently a four year long project from 2012-2016, aims at a structure-targeted approach to develop and evaluate new metal-based compounds that exert their function as therapeutic metallodrugs, as research tools, or as diagnostic metallodrugs by binding to biomolecules, and to understand their modes of action.⁵²³ The U.S. National Institutes of Health (NIH) program "Metals in Medicine" pursued a similar aim. This thesis has presented FDA and EMA approved diagnostic and therapeutic metallodrugs together with biological challenges of metallodrug research and development as well as potential strategies to overcome these in the Introduction. In Chapter 2, nine commercially available quinolone antimicrobials of different drug generations – ciprofloxacin, enoxacin, fleroxacin, levofloxacin, lomefloxacin hydrochloride, nalidixic acid, norfloxacin, oxolinic acid, and pipemidic acid – were introduced. For the first time, comprehensive chemical characterization data comprising infrared spectroscopy, mass spectrometry, melting point determination, nuclear magnetic resonance spectroscopy (1D and 2D NMR spectra were recorded of ¹H, ¹³C, ¹⁹F nuclei as applicable), and elemental analyses (C, H, N) was recorded and summarized. Furthermore, the long-time question of their stability in metal ion containing biological medium (Iso-Sensitest) was addressed in a UV-Vis monitoring study over 24 hours. No signs of decomposition or degradation of the nine quinolone agents were observed. The antimicrobial susceptibility of these nine quinolones was tested against five of the most causative pathogens (*E. faecalis, S. aureus, E. coli, K. pneumonia, P. aeruginosa*), and revealed various patterns of resistance.

Chapter 3 and 4 focused on the development of novel antimicrobial agents based on a coordination chemistry approach with gallium(III), iron(III), and copper(II) ions. Iron is an essential nutrient for many microbes. According to the "Trojan Horse Theory," biological systems cannot distinguish between Fe^{3+} and Ga^{3+} , which constitutes the antimicrobial efficacy of the gallium(III) ion. Based on the quinolone agents introduced in Chapter 2, nine novel tris(quinolono)gallium(III) complexes and their corresponding iron(III) analogs were synthesized and fully characterized, because a synergistic effect between the antimicrobial potency of Ga^{3+} and the antimicrobial effect of three quinolone ligands combined in one coordination complex was anticipated. The antimicrobial efficacy of these tris(quinolono)gallium(III) complexes was studied against *E. faecalis* and *S. aureus* (both Gram-positive), as well as *E. coli*, *K. pneumonia*, and *P. aeruginosa* (all Gram-negative) in direct comparison to the tris(quinolono)iron(III) complexes and the corresponding free quinolone ligands at various concentrations. For the tris(quinolono)gallium(III) complexes, no synergistic or even only combinational antimicrobial effects between Ga^{3+} and the quinolone antimicrobial agents were observed.

The antimicrobial properties of copper have been known to mankind since the ancient times. In a coordination chemistry approach to develop novel antimicrobial agents, the antimicrobial properties of ciprofloxacin and pipemidic acid, as well as the xylenyl-linked dimers thereof, were combined with copper(II) following the "Achilles Heel Theory" in Chapter 4. The preparation and antimicrobial evaluation of bis(ciprofloxacino)copper(II) [Cu(cipro)₂], bis(pipemido)copper(II) [Cu(pia)₂], and the corresponding dimer complexes [Cu₂(ciproXcipro)₂] and [Cu₂(piaXpia)₂] were reported. Again, no combinational antimicrobial effect between Cu²⁺ and the respective quinolone ligands was observed.

A pressing problem in the pharmacology of the anticancer agents doxorubicin and vosaroxin was tackled in Chapter 5 in a coordination chemistry approach. Although anthracycline drugs, such as doxorubicin, are widely used in cancer therapy, the cumulative-dose cardiomyopathy associated with these anticancer agents remains a challenge during oncological treatment. Through iron-mediated interactions, doxorubicin causes the formation of ROS *in vivo*, which leads to oxidative stress affecting the heart. Vosaroxin is a first-class anticancer quinolone derivative that is currently in clinical trials. The Fe(III)-binding constant of vosaroxin was determined spectrophotometrically and compared with the analogous Fe(III) complex formed with doxorubicin. The *in vivo* metabolic stability and iron coordination properties of the quinolones compared to the anthracylines could provide significant benefit to cardiovascular safety. Both doxorubicin (Hdox, $\log\beta_{FeL3}= 33.41$, pM= 17.0) and vosaroxin (Hvox, $\log\beta_{FeL3}= 33.80(3)$, pM= 15.9) bind iron(III) with com-

parable strength; at physiological pH however, $[Fe(vox)_3]$ is the predominant species in contrast to a mixture of species observed for the Fe:dox system. Iron(III) nitrate and gallium(III) nitrate at a 1:3 ratio with vosaroxin formed stable tris(vosaroxino)iron(III) and tris(vosaroxino)gallium(III) complexes that were isolated and characterized. Their redox behavior was studied by CV, and their stereochemistry was further explored in temperature dependent ¹H NMR studies. The molecular pharmacology of their interaction with iron(III) could be one possible differentiation in the safety profile of quinolones compared to anthracyclines in relation to cardiotoxicity.

All of the discussed challenges so far have been scientific; however, another critical aspect of metallodrugs is in perception. Although metallodrugs have been used for many years successfully in medical therapy, and self-medication with metal-containing dietary supplements is widely accepted, it seems that there is still a lack of public acceptance for the use of metal ions in the clinic. One of the greatest commonly espoused counterarguments for metallodrugs is the "toxicity associated with metals". The general public has only a basic understanding of chemistry and may know metals only from jewelry or have read in the press about the harm of metals, such as aluminium(III) salts in antiperspirants might be linked to Alzheimer Disease. The public often judges chemistry in a negative way. Chemists of all backgrounds must acknowledge and overcome this. One important aspect is to communicate to the public that, whatever we put into our bodies, the dosage determines if it harms or benefits us, or if it has any effect at all. This idea goes back to the Middle Ages in Europe, when Paracelsus first described the concept of dose-dependency of medical potions in his *Defensiones*.⁵²⁴ In the 20th century, Bertrand followed up on this concept with his work on the connection between the pharmaceutical dose and the beneficial therapeutic effect or detrimental toxic effect.⁵²⁵ Figure 6.1 is a novel presentation of the Bertrand diagram including the time component next to the dosage and



Figure 6.1: Effect of metal ion intake on overall health. Concentration of metal ions in the body, represented on the y-axis, varies widely for different metal ions. Following the traffic light principle, the optimal provision with metal ions according to the guidelines of the medical community is shaded in green, while deficiencies or overload of metal ions can be harmful (yellow) to lethal (red). Another important factor in the dose-response scenario is the time during which the body is exposed to conditions of metal ion deficiency or overload, shown on the x-axis.

its effects. In dose-dependence, the beneficial versus the detrimental effect applies equally to essential and non-essential metal ions. For the field of medicinal inorganic chemistry and metallodrugs to expand further, the medicinal inorganic chemistry community must address public misapprehension.

If the scientific community succeeds to communicate the benefit of metallodrugs to the public, in addition to answering the questions raised above and gaining an increased understanding of the metal homeostasis in the body, the chances that the public and thereby as well "Big Pharma" will become more receptive to medicinal inorganic chemistry approaches will improve. The revenue from such successful metallodrugs as imaging agents, anticancer drugs, and metal supplements ought to be a persuasive argument to invest in this interdisciplinary area of medicinal chemistry. Particularly, metal coordination compounds in therapy open an array of possibilities, which traditional organic or biological molecules cannot fulfill any longer due to growing drug resistance. Metallodrugs hold still tremendous potential to help mankind overcome drug resistance and to find new cures in medicine.

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Appendix A

Procedure: Antimicrobial Susceptibility Testing By Single-Disk Method

A.1 Scope

To test the antimicrobial activity of coordination complexes of gallium(III), iron(III) and copper(II) with (fluoro-)quinolones, quinolone-dimers and maltol, and to compare their bactericidal activity directly against commercially available (fluoro-)quinolone drugs.

A.2 Introduction

Since the first antimicrobials have been developed, their potency to inhibit the growth of bacteria has been evaluated. The specific susceptibility of an antimicrobial agent to a microorganism was originally tested using broth dilution methods. Because such broth dilution tests are time consuming to perform and pipetting errors occur easily in this type of experiment, the disk diffusion procedure for the determination of susceptibility of bacteria to antimicrobials was developed as a quick test alternative for the clinic.⁵²⁶ By the early 1950s, the majority of U.S. clinical microbiological laboratories had assumed the so-called disk diffusion method, and exactly therein lay the problem. As soon as different laboratories started using the disk diffusion method, they as well started adapting the method to their own needs, using different test media, inoculum concentrations, incubation times, or incubation temperatures. In addition, many academic researchers published their own variations for the disk test procedure. It does not come as a surprise that these developments resulted in multiple protocols, most of them public, and therewith widespread confusion.

The lack of standardization for the determination of bacterial susceptibility continued to be a problem throughout the 1950s, until William M. M. Kirby and Alfred W. Bauer extensively reviewed the susceptibility testing literature, consolidated and updated all the previous descriptions of the disk diffusion method.^{331,332} Around the same time, the World Health Organization stressed the importance for the "Standardization of Methods for Conducting Microbic Sensitivity Tests" in regard to reliably measure and compare bacterial resistance to antimicrobials across the world.⁵²⁷ The report⁵²⁷ concluded that the method in which single filter paper discs are placed on the surface of an inoculated culture medium on a plate is suitable and recommended for general clinical use. Based on the WHO recommendation, standardized procedures for single antimicrobial disk testing, often referred to as "Kirby-Bauer Single-Disk Diffusion Test", were developed across the world on the national level. In North America, the Clinical and Laboratory Standards Institute (CLSI) is responsible for updating and modifying the original procedure of Kirby and Bauer through a global consensus process.^{432 528} In Europe, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has developed a common method calibrated to European MIC breakpoints over the past five years and with that harmonized an array of national procedures across the European Union in 2014.³⁰⁶

This procedure describes the antimicrobial susceptibility testing by single-disk method following a modified version of the original procedure by Kirby and Bauer^{331,332} taking into account recommendations from the CLSI^{432,528} and EUCAST³⁰⁶ test procedures. One major deviation from the CLSI and EUCAST test procedures is the use of Iso-Sensitest medium instead of Mueller-Hinton medium. Please see Chapter 2 for an overview of the arguments used in the debate of Iso-Sensitest vs. Mueller-Hinton medium. Furthermore, several reports of potential metalloantimicrobials being tested in Iso-Sensitest medium without any comments regarding cross-metalation can be found in the literature, including coordination complexes of gallium(III),^{388,529} iron(III),⁴²⁹ and copper(II).^{427,428}

It should be clarified that both biological growth media, Iso-Sensitest³²⁹ and Mueller-Hinton⁵³⁰, contain relatively large amounts of metal salts compared to the concentrations of test compounds (0.1 mM), after all it is a nutrient rich medium to grow cultures. The fact, however, that Mueller-Hinton medium is less defined than the purely synthetic Iso-Sensitest medium (media recipes are described in Tables A.1 and A.2) and that the composition of Mueller-Hinton, especially in regard to cations, such as Ca^{2+} and Mg^{2+} , has been known to vary widely across manufacturers and even across different batches from the same manufacturer, ³⁰³ made Iso-Sensitest medium occur as the better choice of the necessary evil, as at least the ingredients of Iso-Sensitest medium are clearly defined.

ingredients	nutrient class	amount $[g/L]$
agar	agar	8.0
glucose	sugar	2.0
starch	sugar	1.0
casein (hydrolized)	protein	11.0
peptones	peptides, amino acids	3.0
cystein	amino acid	0.02
tryptophan	amino acid	0.02
adenine	nucleotide	0.01
guanine	nucleotide	0.01
uracil	nucleotide	0.01
xanthine	nucleotide	0.01
sodium chloride	metal salt	3
disodium hydrogen phosphate	metal salt	2.0
sodium acetate	metal salt	1.0
magnesium glycerophosphate	metal salt	0.2
calcium gluconate	metal salt	0.1
maganese(II) chloride	metal salt	0.002
cobalt(II) sulfate	metal salt	0.001
copper(II) sulfate	metal salt	0.001
ferrous sulfate	metal salt	0.001
zinc sulfate	metal salt	0.001
cyanocobalamine	vitamin	0.001
menadione	vitamin	0.001
nicotinamide	vitamin	0.003
panthothenate	vitamin	0.003
pyridoxin	vitamin	0.003
biotin	vitamin	0.0003
thiamine	vitamin	0.00004

Table A.1: Synthetic formula of Iso-Sensitest agar (Oxoid, pH 7.4±0.2, $25^{\circ}\mathrm{C})^{329}$

ingredients	nutrient class	amount $[g/L]$
ogor	ogor	17.0
agai	agai	11.0
starch	sugar	1.5
case in (hydrolized)	protein	17.5
beef extract	various	2.0

Table A.2: Approximate formula of Mueller-Hinton II (cation-adjusted) agar $(BBL)^{530}$

The microorganisms selected for this study are summarized in Table A.3.⁵³¹ Selection criteria were to include (a) a mix of Gram-positive and Gram-negative bacteria, (b) only microbes that are pathogenic for humans, (c) only such pathogens that are commonly associated with hospital-acquired infections, so-called nosocomial diseases. Please refer to section A.4 for the safe handling of the selected pathogens.

 Table A.3:
 Selected pathogenic bacteria

bacterium	strain	nosocomial
Gram-positive		
Enterococcus faecalis	ATCC-51575	Yes
$Staphylococcus \ aureus$	MSSA-476, ATTC-BAA-1721	Yes
Gram-negative		
Escherichia coli	ATCC-25922	Yes
Klebsiella pneumonia	ATCC-13883	Yes
$Pseudomonas\ aeruginos a$	ATCC-27853	Yes

A.3 Equipment

A.3.1 Purchased Items

Purchased from manufacturer:

- Mueller-Hinton II (Cation-Adjusted) Agar, BD BBL, product# 211441
- Mueller-Hinton Broth, BD Difco, product# 272530

- Iso-Sensitest Agar, Fisher Scientific-Thermo Scientific Oxoid, product# OXCM0471B
- Iso-Sensitest Broth, Fisher Scientific-Thermo Scientific Oxoid, product# OXCM0473B
- Filter disks (1/4 inch diameter), Schleicher & Schüll, product
10328171

Purchased from UBC-Chem Stores:

- Disposable petri dishes (large 150 x 15 mm), UBC-Chem stores product# GL281005
 (25 dishes per sleeve)
- Disposable petri dishes (small 60 x 15 mm), UBC-Chem stores product# GL28137F
 (25 dishes per sleeve)
- Capped tubes (V = 1.5 mL), UBC stores product# EQ95515M
- Falcon tubes (V = 15 mL), UBC stores product # GL95397R
- Forceps, UBC stores product# EQ067761
- Serological pipets (V = 10 mL), sterile, UBC-Chem stores product# GL68551J
- Micro-Pipet filter tips, 20–200 and 100–1000 $\mu \rm L,$ UBC-Chem stores product #EQ67897Y and EQ67767U
- Parafilm, UBC stores product# EQ072201

Purchased from UBC-Biological Services (Jessie):

• Inoculation loops (disposable, pack of 25), sterile packaged, UBC-Biological Services product

- Swabs (disposable), sterile packaged, UBC-Biological Services product
- Syringe (V = 3 mL), sterile packaged, UBC-Biological Services product
- Syringe filter (0.22 μ m) -GP-, sterile packaged, UBC-Biological Services product
- UV-cuvettes, UBC-Biological Services product

Obtained from various sources:

- Black folder cover as photo background
- Tooth picks (grocery store)
- Ruler

A.3.2 Instruments

- Autoclave
- Balance
- Biological safety cabinet (II)
- Heatgun
- Incubator $(37^{\circ}C, \text{non-CO}_2)$
- Shaker
- UV-Vis spectrometer

A.4 Safety

A.4.1 Personal Safety

A laboratory coat with both hand openings taped tightly should be worn at all times.⁵³² All infectious material should be handled wearing nitrile gloves,⁵³² wearing two pairs of gloves is recommended when handling bacteria. The use of safety-googles is recommended to avoid splashes into the eye even when working inside the biological safety cabinet.

A.4.2 Biosafety

organism	strain	biosafety level
Gram-positive		
Enterococcus faecalis	ATCC-51575	II
$Staphylococcus \ aureus$	MSSA-476, ATTC-BAA-1721	II
Gram-negative		
Escherichia coli	ATCC-25922	Ι
Klebsiella pneumonia	ATCC-13883	II
Pseudomonas aeruginosa	ATCC-27853	II

Table A.4: List of Gram-positive and Gram-negative bacteria tested

A.4.3 Important Pathogen Safety Information by the Public Health Agency of Canada

For detailed information, confer the Public Health Agency of Canada's pathogen safety data sheets.⁵³¹

A.4.3.1 Bacterium Enterococcus faecalis

Characteristics: streptococci, facultatively anaerobic, arranged in pairs and short chains.Host: normal human flora (intestinal tract, female genital tract, oral cavity), humans, pets,livestock. Pathogenicity: urinary tract, wound and soft tissue infection, bacteremia.

Susceptibility to disinfectants: susceptible to ethyl alcohol (70%). Physical inactivation: heat treatment >80°C. Autoclave at standard solid program.

A.4.3.2 Bacterium Escherichia coli

Characteristics: rod-shaped, strain ATCC 25922 is a recommended reference strain for antibiotic susceptibility testing. **Host:** humans, animals, lifestock. **Pathogenicity:** food poisoning, wound infection. **Susceptibility to disinfectants:** susceptible to ethyl alcohol (70%, 20°C, 30 seconds contact time). **Physical inactivation:** Heat treatment >80°C. Autoclave at standard solid program.

A.4.3.3 Bacterium Klebsiella pneumonia

Characteristics: rod-shaped **Host:** humans, animals, plants (flora). **Pathogenicity:** pneumonia, septicaemia, urinary tract infection, wound infection, intensive care unit infections, neonatal septicaemias. **Susceptibility to disinfectants:** susceptible to ethyl alcohol (70%). **Physical inactivation:** heat treatment, autoclave at standard solid program.

A.4.3.4 Bacterium Pseudomona aeruginosa

Characteristics: pseudomonadaceae, non-spore forming, pigmented. Host: humans, wild and domestic animals, plants (flora, fungi). Pathogenicity: infection of respiratory and urinary tract, deep disseminated infections leading to pneumonia and bacteremia, eye infections; increasingly associated with bacterial meningitis, abscesses, endocarditis. Susceptibility to disinfectants: susceptible to ethyl alcohol (70%); few reports of this bacteria growing in disinfectant solutions, alcohol-containing disinfectants recommended for resistant strains. Physical inactivation: inactivated by moist heat (121°C for >15 min), autoclave at standard solid program.

A.4.3.5 Bacterium Staphylococcus aureus

Characteristics: cocci, usually in clusters. Host: humans, wild and domestic animals. Pathogenicity: normal human flora (nose, skin), food intoxication, localized surface infections (from animal bites, impetigo, folliculitis, abscesses, boils, infected lacerations), deep infections include endocarditis, meningitis, septic arthritis, pneumonia, osteomyelitis. Susceptibility to disinfectants: susceptible to ethyl alcohol (70%). Physical inactivation: inactivated by moist heat (121°C for >15 min), autoclave at standard solid program.

A.5 Test Protocol

A.5.1 Marking of Petri Dishes

No more than fourteen disks should be placed on a 150 mm diameter plate. The distance between each disk (center to center) should be at least 24 mm. All petri-dishes are to be marked on the bottom according to the respective template shown in Figure A.1.

A.5.2 Preparation of Agar Plates

The dehydrated culture medium is prepared according to the manufacturer's specifications. A volume of 1 L agar medium fills about twenty-two petri-dishes $(100 \times 15 \text{ mm})$ to a uniform depth of 4 mm. After sterilization by autoclaving the medium is poured immediately into the dishes to an approximate height of 4 mm on a level surface.ⁱ (Careful: The medium is very hot, wear proper gloves!) The agar cools and solidifies in 30 minutes. A lid is placed on each dish and all dishes are stored in the original storage bag, which is closed tightly with a rubber band or clip; single plates can be wrapped with parafilm for storage. The plates are stored at ambient temperature and should be used within 14 days of preparation.

ⁱAgar deeper than 4 mm may cause false resistance results (excessively small zones), while agar less than 4 mm deep may be associated with excessively large zones and false susceptibility.



Figure A.1: Template for plate marking (numerical values in [cm]).

A.5.3 Preparation of Broth Storage

The dehydrated culture medium is prepared according to the manufacturer's directions. After sterilization by autoclaving (liquid program), the medium is left to cool and transferred into a storage bottle with a tightly closed cap that is wrapped in parafilm. After each opening, the broth storage bottle must be autoclaved again.

A.5.4 Transferring of Bacteria Culture

The bacteria cultures are kept growing on an agar plate for a maximum of seven days before a few single colonies are transferred onto a fresh agar plate. To ensure a smooth work flow, a list of items needed for this process has been compiled in Table A.5. With an inoculation loop or a swab, bacteria samples from the old petri dish are collected and streaked onto a fresh agar plate. The freshly inoculated plates are incubated at 37° C for 24 h, after which growth can be observed (Figure A.2). The growth culture plates are then kept at room temperature. In addition to keeping the bacteria culture alive by growing them on agar plates, it is as well advised to keep each culture growing in broth as a back-up as well: 5 mL fresh broth + 1 mL bacteria growth broth culture.

Table A.5: List of items needed for the transfer of bacterial of	cultures
--	----------

cultures on agar plates fresh agar plates inoculation loops parafilm (stripes cut for wrapping) broth
fresh agar plates inoculation loops parafilm (stripes cut for wrapping) broth
inoculation loops parafilm (stripes cut for wrapping) broth
parafilm (stripes cut for wrapping) broth
broth
Falcon tubes (for grow up)
serological pipets (to transfer broth into Falcon tubes)



Figure A.2: Bacteria culture plates.

A.5.5 Preparations for the Actual Test Day (Day 1)

On the day before the test, the items listed in Table A.6 need to be autoclaved. In addition, the agar plates should be counted and checked for first signs of contamination to ensure a sufficient supply of intact plates for the following day.

A.5.6 Growing Bacteria in Broth (Day 1)

Four to five bacteria colonies are picked from the growth culture plates and inoculated into 5-10 mL of broth in a Falcon tube. The inoculated broth tube is placed on a shaker at 37° C for about 16–24 hours prior to inoculation.

item	checkmark
forceps (1)	
tin foil (several pieces about 15 x 10 cm in size)	
filter disks in glas bottle (unloaded)	
PCR tubes (1.5 mL)	
PCR tube rack (1)	
tips (1000 μ L, 1 box)	
tips (200 μ L, 2 boxes)	
tooth picks in glass bottle	

Table A.6: Items to incubate on day 1

A.5.7 Setting up the Biosafety Cabinet (Day 2)

To ensure a smooth work flow, a list of items needed on the test day has been compiled in Table A.7.

A.5.8 Preparation and Standardization of Inoculum Suspension (Day 2)

The bacteria concentration in the growth broth is measured by UV (optical density, OD). (<u>Reminder</u>: The UV-lamp needs to warm up 15 minutes before use.) One single use cuvette is filled with broth solution as the blank. Another single use cuvette is filled with the bacteria growth broth and its opening is tightly closed with parafilm. The UV reading is taken at 600 nm wave length. OD readings between 0.7-1.5 are acceptable. If the OD is lower, the culture needs to grow longer; if the OD is higher, the culture needs to be diluted with fresh broth solution, and the UV-reading needs to be taken again until the OD is in the acceptable range.

A.5.9 Preparation of Test Solutions (Day 2)

The test solutions are prepared in organic solvents that evaporate easily. One of the preferred solvents is methanol, but often dimethyl sulfoxide has to be added to overcome

item	checkmark
previously incubated items:	
forceps	
tin foil pieces	
filter disks in glas bottle (unloaded)	
PCR tubes and rack	
micro-pipet tips (1000 μ L)	
micro-pipet tips (200 μ L)	
solvents:	
dimethyl sulfoxide	
methanol	
single use items:	
agar plates	
swabs (sterile)	
Falcon tubes for preparation of test solutions	
serological pipets	
syringe and syringe filter for DMSO	
UV-cuvettes	
items to wipe with 70% ethyl alcohol:	
micro-pipet (1000 μ L volume)	
micro-pipet (200 μ L volume)	
pipet helper	
lighter	

Table A.7: Items needed in biosafety cabinet on day 2

solubility issues. Falcon tubes are used for the stock solutions, PCR tubes are used for all dilutions and the final test solution (c= 0.1 mM). Due to the toxicity of DMSO on living organisms, the concentration of DMSO in all test solutions should be $\leq 2\%$. To ensure that the DMSO is not toxic for the test organisms and therewith introducing an error into the test results, a methanol solution of 2% DMSO as well as a pure methanol solution are included into the test as controls.

A.5.10 Inspection of Agar Plates (Day 2)

Before usage, all agar plates have to be inspected for cracks, biological contamination, and other irregularities. Only plates without detected abnormalities are used for the test, others are discarded.

A.5.11 Loading of Filter Disks with Test Compound (Day 2)

The empty disks are carefully spread out on sterile tin foil. Each disk is loaded with 20 μ L of test compound. About 5 minutes after loading, the methanol has evaporated; the dried and loaded disks are then ready for placement on the agar.

A.5.12 Inoculation of Plates (Day 2)

At the time the agar is inoculated, no droplets of moisture should be visible on its surface or on the petri dish cover.

<u>Method A:</u> A sterile cotton swab is dipped into the growth broth solution, rotated several times, and gently pressed onto the inside wall of the Falcon tube above the fluid level to remove excessive inoculum from the swab. The swab is then streaked over the entire surface of the agar plate three times, while the plate is being rotated 60° each time to ensure even distribution of the inoculum. A final sweep of the swab is made around the agar rim. If necessary, the lid may be left ajar for 3 minutes to allow excess surface moisture to be absorbed before the impregnated disks are applied.

<u>Method B:</u> About 0.5 mL of growth broth solution are pipetted onto a fresh agar plate. The liquid is evenly distributed over the plate with a spatula or a cotton swab. The advantage of this dilution method is the even growth pattern compared to the visible streaks that remain from the swabbing method, however, this method bears the risk of contamination with accidental splashes of the broth solution.

A.5.13 Placement of Loaded Disks (Day 2)

Within 10 minutes after the plates have been inoculated, the impregnated disks are placed onto the surface in the previously marked positions (Section A.5.1). Each impregnated disk is positioned with iso-prop flamed forceps and pressed down firmly on the agar to ensure complete, level contact. Once a disc has touched the agar surface, it is not to be relocated. When all disks have been placed, the petri dish is closed with the lid and wrapped in parafilm.

A.5.14 Incubation of Test Plates (Day 2)

Within 20 minutes of disk placement, all plates are inverted and placed in the air incubator with the agar side up. Incubation conditions: 37°C for 20 hours.

A.5.15 Interpretation and Measurement of Zone Sizes (Day 3)

Immediately following the incubation, the zone sizes are measured using a ruler. All measurements are made with the unaided eye on the backside of the petri dish on a black, nonreflecting surface illuminated with reflected light. The plate is viewed directly in a vertical line of sight to avoid any parallax. The zone margin is considered to be the area showing no obvious, visible growth that can be detected with the unaided eye. The diameter of the disk is included in the measurement, and the measurement is rounded to the nearest millimeter. All zone sizes are recorded on the zone size recording sheet (Figure A.4), and a photo of each plate is taken for documentation (Figure A.3). Further clarification:

- Growth up to the edge of a disk is reported as a zone of 0 mm.
- If the placement of disk does not allow a direct reading of the zone diameter, the distance from the centre of the disk to a point on the circumference with a distinct edge is measured; this radius measurement is then multiplied by factor x^2 to indirectly determine the diameter.
- Distinct, discrete colonies within an obvious zone of inhibition should not be considered swarming. Should the repeated testing show the same growth pattern, the



Figure A.3: Example photo for test plate documentation.

organism must be considered resistant to the antimicrobial agent loaded on the disk.

A.5.16 Reporting of Measured Zone Sizes

All tests are done in triplicate, and the inhibition zone sizes of one test compound against the same bacteria are measured on three different plates. From these three independent measurements, the average and the respective standard deviation are calculated and reported in tabular format.

Plate co	ding: EF1-HL	Organism: E.	faecalis	Incubated from (date/time): DD.MM.YYY / HH:MM h
Date - Mi	Date - MHA plates inoculated: DD.MM.YYY		Incubated to (date/time): DD.MM.YYY / HH:MM h	
Date - Inhibition zones measured: DD.MM.YYY			Total incubation time = 20 h	
Disk #	Compound coding		Inhibition zone [mm]	Comments
1	DMSO control (2% in methanol)			
2	methanoi control			
3	HL (0.3 mM)			
4	HL (0.2 mM)			
5	HL (0.1 mM)			
6	GaL ₃ (0.1 mM)			
7	FeL ₃ (0.1 mM)			
8	CuL ₂ (0.1 mM)			
9				
10				
11				
12				
13	Ga(NO ₃) ₃ · 9 H ₂ O (0.1 mM)			
14	Fe(NO ₃) ₃ · 9 H ₂ O (0.1 mM)			

KD-01 Rev.01: Antimicrobial Susceptibility Testing By Single Disk Method - Inhibition Zone Size Recording Sheet

Figure A.4: Example of inhibition zone size recording sheet.

A.5.17 Waste Management

All waste from the Biosafety Cabinet is handled in closed autoclavable plastic waste bags. These are autoclaved for 121°C at 25 minutes, which decontaminates all organisms handled in this procedure. The autoclaved box content is then double-packaged: first in an orange UBC Biohazard RG2 bag, then once more in a clear bag. Each waste bag is labelled with a red biological waste "UBC Autoclaved Risk Group II" tag.⁵³²

Appendix B

Supplementary Information to Chapter 5



B.1 UV-Vis Titration of the Vosaroxin-Iron(III) System

Figure B.1: UV-Vis spectra of one titration run of Hvox to determine the pK_as of the test solution.



Figure B.2: UV-Vis spectra of one titration run of $Hvox:Fe^{3+}$ in the ratio of 1:1.



Figure B.3: UV-Vis spectra of one titration run of Hvox:Fe³⁺ in the ratio of 2:1.



Figure B.4: UV-Vis spectra of one titration run of Hvox:Fe³⁺ in the ratio of 3:1.

B.2 UV-Vis Titration of the Doxorubicin-Iron(III) System



Figure B.5: UV-Vis spectra of one titration run of Hdox to determine the pK_as of the test solution.



Figure B.6: UV-Vis spectra of one titration run of $Hdox:Fe^{3+}$ in the ratio of 1:1.



Figure B.7: UV-Vis spectra of one titration run of Hdox:Fe³⁺ in the ratio of 2:1.



Figure B.8: UV-Vis spectra of one titration run of Hdox:Fe³⁺ in the ratio of 3:1.



B.3 Cyclic Voltammograms of Hvox and [Ga(vox)₃]

Figure B.9: Cyclic voltammogram of vosaroxin (0.001 M, solid line) in DMSO solution; also shown is the blank voltammogram containing tetra(n-butyl)ammonium perchlorate 0.1 M (dotted line). Scan rate was 100 mV/s. Potential values are given with reference electrode Ag/AgCl(sat) and against the ferrocene couple $Fc^+/Fc = +0.64$ V vs. SHE³⁷¹.



Figure B.10: Cyclic voltammogram of $[Ga(vox)_3]$ (0.001 M, green) in DMSO solution; also shown is the blank voltammogram containing tetra(n-butyl)ammonium perchlorate 0.1 M (dotted line). Scan rate was 100 mV/s. Potential values are given with reference electrode Ag/AgCl(sat) and against the ferrocene couple $Fc^+/Fc = +0.64$ V vs. SHE³⁷¹.

B.4 NMR Studies



Figure B.11: Titration of vosaroxin $(5 \cdot 10^{-4} \text{ M})$ in deuterated phosphate buffer $(5 \cdot 10^{-2} \text{ M})$ at pD 7.0 with increasing amounts of iron(III) nitrate in D₂O monitored via ¹H NMR (600 MHz, D_2O , 298 K) with a total increase in volume throughout the titration of 0.6%. The ¹H NMR spectra with different ratios of Fe³⁺:Hvox are shown with the same intensities for better comparison. From bottom to top: Hvox (dark blue), Fe^{3+} :Hvox = 1:50 (light blue), Fe^{3+} :Hvox = 1:25 (intense blue), Fe^{3+} :Hvox = 1:12.5 (teal), Fe^{3+} :Hvox = 1:6.25 (purple), Fe^{3+} : Hvox = 1:6.25 after 60 min wait time (grey), Fe^{3+} : Hvox = 1:3.33 (black). The NMR signals broaden with increasing amounts of Fe³⁺. At a ratio of Fe^{3+} : Hvox = 1:6.25, one NMR spectrum was recorded after the standard time of 3 min (purple), and a second spectrum was recorded after the sample had been stirred for 60 min at ambient temperature upon which a precipitate had formed (grey). The second spectra showed a clear narrowing of signals again, which indicated that the amount of Fe^{3+} ions in solution was reduced and supported the assumption that the $Fe[(vox)_3]$ complex, a complex not soluble in aqueous media, formed over the course of the titration. This assumption is further supported by the fact that the precipitate did not have the characteristic orange color of insoluble $Fe(OH)_{3(s)}$.



Figure B.12: Temperature dependent NMR study (400 MHz, d_6 -DMSO) of $[Ga(vox)_3]$. The sample was heated inside the spectrometer from ambient temperature (298 K, bottom, dark blue) to 363 K (red). To conclude the experiment, the sample was cooled down again to ambient temperature (top, black). As it could be expected, the heat accelerated the interchanging of the various stereoisomers in solution, which was reflected in more defined signals in the NMR spectra recorded at higher temperature. This phenomenon was solely temperature dependent and fully reversible, as a comparison of the first spectrum (bottom, dark blue) and the final spectrum (top, black) show.