KUOPION YLIOPISTON JULKAISUJA A. FARMASEUTTISET TIETEET 104
KUOPIO UNIVERSITY PUBLICATIONS A. PHARMACEUTICAL SCIENCES 104

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Novel Prodrug Structures for Improved Drug Delivery

Doctoral dissertation

To be presented by permission of the Faculty of Pharmacy of the University of Kuopio for public examination in Auditorium, Mediteknia building, University of Kuopio, on Saturday 27th October 2007, at 12 noon

Department of Pharmaceutical Chemistry
Faculty of Pharmacy
University of Kuopio

KUOPION YLIOPISTO

KUOPIO 2007

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ISBN 978-951-27-0842-0
ISBN 978-951-27-0634-1 (PDF)
ISSN 1235-0478

Kopijyvä
Kuopio 2007
Finland

Kumpulainen, Hanna-Maarit S. Novel Prodrug Structures for Improved Drug Delivery. Kuopio University Publications A. Pharmaceutical Sciences 104. 2007. 129 p.
ISBN 978-951-27-0842-0
ISBN 978-951-27-0634-1 (PDF)
ISSN 1235-0478

ABSTRACT

The requirements demanded with respect to the potency and safety of novel chemical entities are increasing and correspondingly the financial investments needed for developing a new drug are exploding. Finding ways to overcome the poor drug-like properties, such as solubility, permeability and toxicity are essential in diminishing the numbers of failures, and thus reducing the costs of drug development. In many cases prodrugs may offer a way to overcome the poor drug-like properties of a very potent lead and provide the opportunity to convert a non-developable molecule into a potent candidate for clinical use.

Prodrugs are pharmacologically inactive molecules that require an enzymatic and/or chemical transformation before release of a pharmacologically active parent drug *in vivo*. The prodrug approach can be applied to a variety of drug molecules, administration routes and formulations, and is one way to improve the physicochemical, pharmaceutical and biopharmaceutical properties of pharmacologically potent structures and to overcome barriers to a drug's usefulness.

In the present study, several different prodrug structures were synthesized to achieve improved drug delivery, including hydroxyimines, ethylidene phosphates and monomethyl phosphate esters. The model drugs used in this study were nabumetone, ketoprofen and propofol. The physicochemical and pharmaceutical properties of novel prodrug structures, including aqueous solubility, lipophilicity, chemical stability and enzymatic release of the parent drug were evaluated *in vitro* and the bioconversion of the prodrug to the parent drug was further confirmed *in vivo* in rats.

Hydroxyimine was shown to be bioconverted to the corresponding ketone by microsomal cytochrome P450-enzymes both *in vitro* and *in vivo* and the released nitric oxide evoked no acute liver toxicity after peroral administration in rats. Hydroxyimine is thus a potential intermediate prodrug structure, especially for ketone drugs. However, the chemical and the enzymatic hydrolysis rate of different hydroxyimines to the parent ketone drug varied extensively between the two different model compounds, suggesting that the chemical environment around the hydroxyimine structure affects to the applicability of hydroxyimine prodrug structure.

A method to synthesize of 1-chloroethyl phosphates and phosphoramidates was developed. This method is a versatile and simple way to prepare 1-chloroethyl phosphates and phosphoramidates either under normal temperature and pressure conditions or using microwave-assisted synthesis. 1-Chloroethyl phosphates could not be used as starting materials of ethylidene phosphate prodrugs as such. However, the synthesis method developed for 1-chloroethyl phosphates could be modified to the synthesis of ethylidene phosphate prodrug of propofol.

Ethylidene phosphate propofol increased the aqueous solubility of propofol and enzymatically released propofol *in vitro* and *in vivo* in rats, thus proving to be a suitable water-soluble prodrug of propofol for *i.v.* administration. Bioconversion of the ethylidene phosphate prodrug releases acetaldehyde, which is less toxic than the formaldehyde released from traditionally used phosphonooxymethyl prodrugs.

The monomethyl phosphate and phosphonooxymethyl ester prodrugs of propofol did not release the parent drug *in vitro* or *in vivo*, probably due to inability of propofol to act as a substrate for the phosphodiesterases. Thus, the monoalkyl esters of phosphates do not seem to be suitable as versatile prodrug structures, but more studies with nucleotide-type model compound are needed to confirm this speculation.

In conclusion, the present study provided useful information of several novel prodrug structures. In particular, the ethylidene phosphate structure holds a great promise for further development of phosphate prodrugs.

National Library of Medicine Classification: QV 744, QV 785, QV 38
Medical Subject Headings: Drug Design; Prodrugs / chemical synthesis; Molecular Structure; Drug Delivery Systems; Ketoprofen; Propofol; Solubility; Drug Stability; Pharmacokinetics; Phosphates; Microwaves

"Prodrug research needs more imagination and less dependency on what has been tried in the past".

Valentino J. Stella in Advanced Drug Delivery Reviews, 1996

ACKNOWLEDGEMENTS

The present study was carried out in the Department of Pharmaceutical Chemistry, University of Kuopio during the years 2003-2007. The study was financially supported by the Graduate School of Organic Chemistry and Chemical Biology (former the Graduate School Bioorganic and Medicinal Chemistry), the Finnish Funding Agency for Technology and Innovation (Tekes), the Research Foundation of Orion Corporation, the Association of Finnish Chemical Societies and the Alfred Kordelin Foundation (the Gustav Komppa fund), which are all appreciated.

I owe my deepest gratitude to my principal supervisor Professor Tomi Järvinen for all support, advices and encouragement you have given me during these years. You were always present in both good days as well as the bad ones and you had always time for a little conversation or depate. Above of all, your have always shown continuous confidence in my cababilities and for that I am forever grateful. I am also very thankful for my other supervisor Docent Jarkko Rautio for your support, advices, discussions spiced with humour and especially for your tireless reviewing my written "outputs". I would also like to thank my third supervisor Professor Jouko Vepsäläinen for advices and help in synthetic chemistry and NMR spectroscopy.

I acknowledge all the people who have contributed to this work: Dr. Tapio Nevalainen, Dr. Jukka Leppänen, Professor Risto Juvonen, Professor Hannu Raunio, Dr. Antti Mäntylä, M.Sc. Niina Mähönen, B.Sc. Raimo Saari, M.Sc. Marko Lehtonen, Lic.Ph. Anne Mannila, Dr. Marja-Leena Laitinen, M.Sc. Marja Jaurakkajärvi, M.Sc. Katariina Huttunen, Mr. Jan Boier Pedersen and M.Sc. Kristiina Huttunen. Especially I want to thank B.Sc. Raimo Saari for being my hands in the laboratory during my pregnancy. I owe special thanks also to M.Sc. Niina Mähönen for excellent collaboration in our shared publication (publication I in this thesis). I am sincerely grateful to all our laboratory assistants, especially Ms. Miia Reponen, Ms. Tiina Koivunen, Ms. Helly Rissanen and Ms. Anne Riekkinen for their support, friendship and technical assistance in the laboratory.

I am extremely grateful to Professor Valentino J. Stella, who kindly accepted the invitation to serve as an opponent in the public defence of my dissertation. You are

unquestionably the most recognized prodrug researcher in the world and I really appreciate to have you as my opponent. I also want to thank Dr. Jeffrey Krise and Dr. Juhani Huuskonen for reviewing this thesis and for many valuable comments and critisms and Professor Ewen McDonald for revising the language of the dissertation.

I want to thank the former and current deans of the Faculty of Pharmacy, Professor Jukka Gynther and Professor Jukka Mönkkönen and the former and current heads of the Department of Pharmaceutical Chemistry, Professors Seppo Lapinjoki, Tomi Järvinen and Antti Poso for providing excellent facilities to work. Many thanks for the office personnel for their great work with the bureaucracy. Especially I want to thank the personnel working in the Pharmaceutical and Medicinal Chemistry group. This group forms such a great environment to work and it has been privilege to work with you! I want also thank the "laatutiimi" of PMC-group, especially Dr. Kirsi Luoto for your skillful guidance to the strict world of quality assurance.

My sincere gratitude belongs to all my close friends and relatives in and outside of the university. Tarja "The Hurja Poronainen", you have been my tower of strength so many times! You understand me with such an unique way and we have shared so many great moments. I am extremely grateful of your friendship. Päivi, I can not imagine better roommate than you were and mainly because of that I have been lucky to have you as my close friend. Hanna-Riikka and Janne, time goes by, but I can rely that you are always there. Sanna-Riikka, thank you for your friendship and interest in this project. Especially I want to thank my parents Asta and Juhani for their endless love and support.

Finally, my deepest gratitude belongs to my dear husband Teemu and our beloved daughter Inka. How many times you have heard me to say: I will became a postman! I am trying to learn from you not to worry so much beforehand and to have more relaxed way of life. Inka, you have shown me, what is important in life!

Kuopio, September 2007

Hanna Kumpulainen

ABBREVIATIONS

ADEPT	Antibody-directed enzyme prodrug therapy
ADME/Tox	Absorption, distribution, metabolism, excretion, toxicology
ALAT	Alanine aminotransferase
BA	Bioavailability
BBB	Blood-brain barrier
BCS	Biopharmaceutics classification system
CES	Carboxylesterases
CNS	Central nervous system
CRP	C-reactive protein
CYPs	Cytochrome P450-enzymes
DCM	Dichloromethane
DMAE	Desglymidodrine
DMSO	Dimethylsulfoxide
ESI-MS	Electron spray ionization- mass spectrometry
FDA	U.S. Food and Drug Administration
GDEPT	Gene-directed enzyme prodrug therapy
GIT	Gastrointestinal tract
GLC	Gas-liquid chromatography
hPEPT	Human intestinal peptide transporter
HPLC	High-performance liquid chromatography
HSV	Herpes simplex virus
HTS	High-throughput screening
i.v.	Intravenous
LAT	Neutral amino acid transporter
log D	Distribution coefficient
log P	Partition coefficient
m/z	Mass-charge ratio
MS	Mass spectrometry
MW	Molecular weight

NADPH	Nicotinamide adenine dinucleotide phosphate
NCE	New chemical entity
NME	New molecular entity
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NTP	Normal temperature and pressure
PD	Pharmacodynamics
P-gp	P-glycoprotein
PK	Pharmacokinetics
pK_a	Acid constant
ppm	Parts per million
QIDSM	Quantitative integrated drug selection method
R&D	Research and development
RP	Reversed phase
r.t.	Room temperature
$t_{1/2}$	Half-life
t_{max}	The time taken to reach the maximum concentration of drug in plasma
TEA	Triethylamine
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TSP	3-(Trimethylsilyl)propionate
UDP	Uridine diphosphate
UV	Ultraviolet

LIST OF ORIGINAL PUBLICATIONS

The present doctoral dissertation is based on the following original publications:

I Kumpulainen H,[#] Mähönen N,[#] Laitinen M-L, Jaurakkajärvi M, Raunio H, Juvonen R, Vepsäläinen J, Järvinen T, Rautio J. Evaluation of hydroxyimine as Cytochrome P450- Selective Prodrug Structure.
Journal of Medicinal Chemistry 49: 1207-1211, 2006.
[#] Authors contributed equally to this work

II Kumpulainen H, Järvinen T, Saari R, Lehtonen M, Vepsäläinen J. An efficient strategy for the synthesis of 1-chloroethyl phosphates and phosphoramidates.
The Journal of Organic Chemistry 70: 9056-8, 2005.

III Kumpulainen H, Saari R, Lehtonen M, Rautio J, Järvinen T, Vepsäläinen J. Convenient microwave-assisted synthesis of 1-chloroethyl phosphates.
Tetrahedron Letters 47: 2003-2004, 2006.

IV Kumpulainen H, Järvinen T, Mannila A, Leppänen J, Nevalainen T, Mäntylä A, Vepsäläinen J, Rautio J. Synthesis, *in vitro* and *in vivo* characterization of novel ethylide phosphate prodrug of propofol.
Manuscript, submitted for publication to the Journal of Medicinal Chemistry.

In addition, this thesis contains previously unpublished data, presented in chapter 8.

CONTENTS

1 INTRODUCTION

The drug development process from the target identification to the final product being marketed is a time and money consuming process, with the total research and development (R&D) costs reported as being up to 800 million dollars and an average of 10 years (DiMasi *et al.* 2003). While the dynamic combinatorial chemistry and high-throughput screening (HTS) technologies are identifying thousands and thousands of active compounds, the numbers of launched new drugs are declining; only 17-31 new molecular entities (NMEs) per year was released for marketing by U.S. Food and Drug Administration (FDA) during 2002-2006 (Owens 2007, Traynor 2003-2007).

The discovery and development of a new drug starts from the identification and validation of the target protein or receptor (Figure 1.1). A large set of compounds, of which only approximately 0.1 % will show any activity, is tested against the target to find possible lead or hit structures for further studies. These lead structures are optimized in terms of their potency, physicochemical, pharmaceutical and pharmacokinetic properties *in vitro* and the activity of the most promising compounds is tested *in vivo* yielding to 10 drug candidates for human clinical trials. Finally perhaps only one out of ten clinically studied drug candidates may reach the market, and as a whole, the probability for each synthesized compound to reach the market is thus only one in a million (Balbach and Korn 2004, Hageman 2006, Oprea 2002).

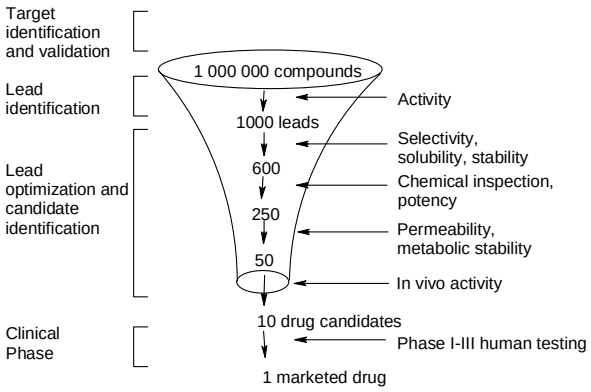


Figure 1.1. The drug development funnel (modified from Balbach and Korn 2004, Hageman 2006 and Oprea 2002).

What are the reasons for this poor percentage of success in the drug development process? Often the structures of the potent HTS leads means that they may be difficult or even impossible to develop or to act as medicines. The poor drug-like properties, such as solubility and permeability, in addition to toxicity, are among the main causes for failures during the drug development process (Frantz 2007, Kola and Landis 2004). Yet, these barriers need not be insurmountable, as they can be often bypassed by using prodrugs.

Prodrugs are pharmacologically inactive molecules of an active drug molecule that, prior to exerting a pharmacological effect, require an enzymatic and/or chemical transformation to release the active parent drug *in vivo*. Prodrugs can be used to bypass physicochemical, pharmaceutical, pharmacokinetic and pharmacodynamic barriers to drug formulation and delivery, such as poor aqueous solubility, chemical instability, insufficient oral absorption, rapid presystemic metabolism, inadequate tissue penetration, toxicity and local irritation (Stella 2006, Stella *et al.* 1985). Currently, 5-7 % of all approved drugs worldwide can be classified as prodrugs and in the years 2001 and 2002 approximately 15 % of all new launched drugs were prodrugs (Stella 2004, 2006).

Prodrug structures have been mostly developed using hydroxyl, amine and carboxyl functionalities, but the possibilities to use prodrugs are not restricted to a few groups of structures. Furthermore, as the diversity and data of possible prodrug structures increase, the threshold to use prodrugs becomes reduced. The objective of this study was to design and synthesize novel prodrug structures including a hydroxyimine and several phosphate prodrugs, as well as to evaluate their usefulness as potential prodrug structures *in vitro* and *in vivo*.

2 REVIEW OF LITERATURE

2.1 Drug-like properties of compounds

For a drug candidate to be sufficiently bioavailable and ultimately a commercial success, its pharmacodynamic activity needs to be in consistent with its structural, physicochemical and biological properties (Li 2001, Lipinski 2000, Sugiyama 2005). Drug-like properties are characteristics of compounds, which need to be in balance to make compounds suitable to act as drugs. Structural properties, such as molecular size and hydrogen bonding capacity affect the physicochemical properties, such as solubility and lipophilicity and are contributers to the unique biological properties of each compound (Table 2.1) (Chan and Stewart 1996, Kerns and Di 2003). Due to this complex set of drug properties, no strict line for each characteristic can be drawn. However, classifications and proposals for the optimal properties of the majority of drug-like compounds have been frequently described in the literature (Chan and Stewart 1996, Lipinski 2000, Lipinski *et al.* 1997, Oprea 2002, Walters and Murcko 2002, Wenlock *et al.* 2003). In a simplified form, it can be said that "druggable compounds should be such that they can be delivered effectively to the target tissues and cells" (Sugiyama 2005).

Table 2.1. The key properties of drug-like compounds (Kerns and Di 2003, Sugiyama 2005).

- Structural or molecular properties:
  - Molecular weight
  - Hydrogen bonding
  - Polarity
- Physicochemical properties:
  - Solubility and dissolution
  - Permeability and lipophilicity
  - Stability
  - pK_a
- Biological properties:
  - Absorption, distribution, metabolism, excretion (ADME)
  - Transporters, plasma protein binding, CYP inhibition
  - Toxicity and safety

Poor drug-like properties are probably the most common reason for the failures in the drug development. In 2000, approximately 40 % of attritions could be traced to poor drug-like properties (mainly pharmacokinetic and safety reasons) (Kola and Landis 2004). Instead of developing methods for screening the drug-like properties of lead compounds, much more investments are focused on screening for new biologically active drug molecules which often possess poor drug-like properties (Li 2001). The *in vitro* testing of leads in HTS is no longer restricted due to poor solubility in the aqueous test media. Nowadays even extremely insoluble compounds can be tested and profiled (Di and Kerns 2006, Lipinski *et al.* 1997). Thus, it is worth asking, whether the early-stage "druggability" screening should precede the biological activity screening (Lipinski 2000, Waterbeemd *et al.* 2001).

The screening against the drug-like properties has been named as druggability screening (Lipinski 2000), as property-based drug design (Waterbeemd *et al.* 2001), as a quantitative integrated drug selection method (QIDSM) (Sugiyama 2005) or as pharmaceutical profiling (Di and Kerns 2005, Kerns and Di 2003). All together, these terms include the *in vitro*, *in vivo* and *in silico* screening against physicochemical, molecular, ADME-, PK- and safety properties of drug compounds (Kerns and Di 2003, Sugiyama 2005). The aim of these screens is not to find a compound superior with all criteria, but to serve as filters when selecting the best candidate for further studies. These screens could also shorten the time for a drug candidate to reach the market by combining the discovery and development sections. An appreciation that drug-like properties of structural hits, leads and drug candidates are intrinsic properties of the molecules (Borchardt *et al.* 2006) might bring the "development-thinking" also to the lead discovery stage.

2.1.1 ADME/Tox

Many factors contribute to the therapeutic efficacy and usability of drugs e.g. ADME-Tox or ADMET (absorption, distribution, metabolism, excretion and toxicity). A compound has sufficient ADME-Tox properties, if it is soluble and chemically stable in the gastrointestinal tract or in its dosage form, it can be absorbed from its site of administration, it is lipophilic enough to permit membrane penetration and distribution

to the target tissues, it is not metabolized too rapidly in the liver or in the other extrahepatic tissues, it is not excreted too rapidly from the body via renal or extrahepatic clearance mechanisms and its toxicity profile is acceptable for the disease in question (Borchardt *et al.* 2006, Stella 2006).

Often the ADME properties and suitability of drug compounds for oral administration are defined by the term bioavailability. Bioavailability is defined as that fraction of an orally administered drug that reaches the systemic circulation (Agoram *et al.* 2001), but it may as well be expanded to parenteral administration routes, if also the distribution, metabolism and excretion of drug compounds are considered. Bioavailability also involves intestinal absorption and distribution, which are dependent on the drug stability, dissolution, solubility, permeability and dosage forms, first-pass metabolism and various clearance mechanisms (Agoram *et al.* 2001, Amidon *et al.* 1995, Chan and Stewart 1996).

2.1.1.1 Absorption

The most important segments of the GI-tract for drug absorption are the stomach and small intestine. The stomach is mainly responsible for breaking down large molecules, which are then absorbed more easily from the small intestine. The small intestine consists of three different parts: duodenum, jejunum and ileum (Waterbeemd *et al.* 2001). The duodenum connects the stomach to jejunum, in which the absorption mainly takes place (Avdeef 2003). The wall of jejunum is covered with fingerlike projections called villi, which are populated with millions of microvilli. These structures increase the absorptive surface area of the intestine over 600-fold (Avdeef 2003, Chan and Stewart 1996, Waterbeemd *et al.* 2001). The pH of the GI-tract varies from the acidic pH of the stomach to the neutral pH of the small intestine. The gastrointestinal pH is dependent on whether the subject is in the fed or fasted states, *i.e.* pH changes in response to food consumption. The pH is also affected by age, pathophysiological conditions and concurrent drug therapy (Hörter and Dressman 1997).

Absorption across the biological membranes can occur via four different pathways as illustrated in Figure 2.1: passive transcellular and paracellular absorption, carrier-mediated absorption and receptor-mediated endocytosis (Chan and Stewart 1996,

Lehninger *et al.* 1993). Passive transcellular absorption is the most common route for absorption of drug molecules through the intestinal mucosal membranes (Chan and Stewart 1996). It occurs by diffusion through the lipid bilayer and since it is driven by a concentration gradient, it does not require energy (Lehninger *et al.* 1993, Li 2001). Paracellular absorption occurs by diffusion through the tight-junctions between cells and is a route for absorption of very polar compounds (Taylor 1996). Carrier-mediated and receptor-mediated endocytosis are active or facilitated transport mechanisms that require energy and a specific carrier receptor or protein on the cell surface (Lehninger *et al.* 1993).

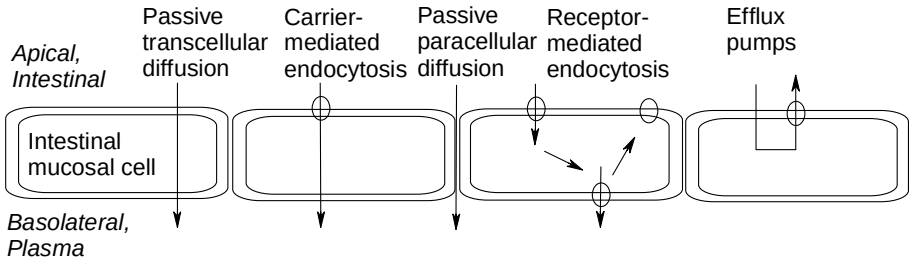


Figure 2.1. Pathways of transport of drugs across biological membranes (modified from Chan and Stewart 1996, Lehninger *et al.* 1993, Taylor 1996).

Passive diffusion requires that a drug molecule is dissolved in the aqueous media of the GI content, since only the dissolved molecule can diffuse across the membranes. The compound needs to be stable at the site of absorption and also to be able to partition into the lipid bilayer of the gut wall as well. Absorption is thus dependent on the lipophilicity, intrinsic aqueous solubility, surface area of a gut and molecular weight of the drug molecule (Navia and Chaturvedi 1996, Taylor 1996). The most important physicochemical properties for passive absorption of drugs from GI-tract are aqueous solubility and lipophilicity, which will be discussed in more detail in chapter 2.1.3.

In addition to the receptors and proteins responsible for the active transport of drug molecules across the intestinal membrane, the human intestinal epithelium also expresses efflux proteins that limit drug absorption and secrete intracellularly formed metabolites back into the intestinal lumen (Figure 2.1). One well known example of this kind of efflux pump is P-glycoprotein (P-gp) (Lennernäs 2003, Waterbeemd and Smith

2001, Waterbeemd *et al.* 2001). The expression of P-gp is localized to the apical surfaces of the gut wall epithelium, where the pump acts by preventing the absorption of drug molecules and other xenobiotics into the systemic circulation. Carrier-mediated intestinal efflux may be one of the major reasons for incomplete absorption and the variable bioavailability of drugs (Lennernäs 2003, Waterbeemd and Smith 2001).

2.1.1.2 Distribution

After a drug has reached the circulation by either being absorbed from the GI-tract or via some other administration route, it will pass through the endothelium of the capillary and distribute into tissues (Thakker 2006). The distribution of the compound into the body is described by the term "volume of distribution", which is defined as the entire dose of the drug in the body (mg) divided by the concentration of the drug in plasma (mg/l). The distribution is dependent on several structural and physicochemical properties (molecular size, logP, H-bonding, charge state etc.) of a drug molecule, its ability to serve as a substrate for influx and efflux transporters and the binding of drug molecule to plasma proteins (Thakker 2006, Waterbeemd and Smith 2001). In general, the volume of distribution increases with increasing lipophilicity, but the charge state can also influence the volume of distribution of drug molecules; basic compounds, which have the largest volume of distribution, are mainly found in the tissues, while the acidic molecules with the smallest volume of distribution are mainly located in plasma and are more susceptible to elimination via liver and kidneys (Waterbeemd *et al.* 2001).

Drug distribution is an important factor when targeting a drug to a specific tissue, such as the brain which is separated from the blood by the complex blood-brain barrier (BBB). The BBB is formed of the capillary endothelial cells packed with epithelial-like high-resistance tight junctions, which prevent the transport of hydrophilic and large molecular weight compounds from the circulation to the brain tissue (de Boer and Gaillard 2007, Waterbeemd and Smith 2001). If a drug is to penetrate the BBB, it needs to be either sufficiently lipophilic to undergo passive diffusion across lipid-membranes or alternatively it has to be a substrate of some of the active transport systems present in the BBB (de Boer and Gaillard 2007, Pajouhesh and Lenz 2005). In contrast, drugs

designed for effects on peripheral organs and tissues should not be able to cross the BBB to avoid CNS side-effects (Alavijeh *et al.* 2005).

2.1.1.3 Metabolism

The liver is the major organ for drug metabolism, but metabolism occurs also in GI-tract and to some extent in lungs, skin and kidneys (Li 2001). The hepatic liver metabolism can be divided into two main groups, phase I oxidation reactions catalyzed by cytochrome P450-enzymes (first-pass metabolism) and phase II conjugation reactions catalyzed by several enzymes e.g. UDP-glucuronosyl transferases, sulfotransferases and glutathione S-transferases (Li 2001, Testa and Cruciani 2001). In addition, the widespread expression of CYP3A4 in the gut wall epithelium makes it an effective barrier preventing the absorption of drug molecules. It is believed that CYP3A4 and P-glycoprotein may act together and limit oral absorption since the CYP3A4 can metabolize those substrates effluxed back to the gut lumen by P-gp (Lennernäs 2003, Waterbeemd and Smith 2001).

The consequences of drug metabolism can be both pharmacodynamic (the production of active, inactive or toxic metabolites) and pharmacokinetic (effects on the duration of action of the drug, inhibition or induction of the drug metabolism or effects on the different pharmacokinetic properties of the metabolites) (Testa and Cruciani 2001). Drug metabolism is involved in many important properties of drug molecules, such as metabolic stability and clearance, drug-drug interactions, clearance mechanisms and drug toxicity (Li 2001, Yengi *et al.* 2007). Metabolism and metabolic enzymes play also crucial role in the activation of prodrugs, this will be discussed in chapter 2.2.

2.1.1.4 Excretion

Excretion, elimination or clearance of a drug molecule from the body starts immediately after the drug has entered the circulation. The term "clearance" consists of metabolism (hepatic metabolic clearance, see above) (Liu and Pang 2006, Obach 2001) and the elimination of the unchanged drug molecule (renal clearance) (Shitara *et al.* 2006, Waterbeemd *et al.* 2001). Thus, two organs are mainly responsible for clearance i.e. the liver and the kidney. Renal clearance occurs via filtration of the blood in the glomerulus

of the kidneys and the further reabsorption of the compounds from the kidney tubule back to the systemic circulation. Compounds may also be secreted from the circulation to the proximal tubule by active transport mechanisms via carrier-mediated transport receptors (Shitara *et al.* 2006).

The molecular weight of a drug molecule determines whether it can be filtered through the glomerulus. Molecules with MW greater than 50 000 cannot pass the glomerulus (e.g. drug bounded to the plasma proteins) (Janku 1993). Unlike glomerular filtration, the tubular reabsorption occurs via lipid membranes and is thus limited by the lipophilicity of the compounds (Janku 1993, Waterbeemd *et al.* 2001). Compounds with low lipophilicity (log D < 0) are not able to reabsorb across the tubular membrane and are eliminated in the urine (Waterbeemd *et al.* 2001). Thus, hydrophilic compounds with a molecular weight below 50 000 and log D < 0 are often eliminated more easily intact whereas more lipophilic compounds tend to undergo metabolic conversion to a more easily excreted form (Waterbeemd *et al.* 2001).

2.1.1.5 Toxicity

Drug toxicity issues were the reason for 30 % of failures in the pharmaceutical industry in 2000 (Kola and Landis 2004) and the withdrawals of marketed or investigated drugs, such as torcetrapib, ximelagatran (Exanta®) and rofecoxib (Vioxx®) due to safety concerns have been common occurences in recent years (Frantz 2007). The toxic effects can be divided into genotoxicity, carcinogenicity, reproductive toxicity, target organ toxicity and local toxicity (Bentley 2001). Toxicity may be specie-specific, organ-specific and it can arise, when the drug has been used for the long period of time (Li 2001). Toxic effects may be a result of the chemical structure of the drug or its metabolites or they can be attributable to exaggeration of the desired pharmacological activity (Bentley 2001).

The toxicity of the drug molecule is difficult to predict, but there are several known substructures that are prone to form reactive metabolites as reviewed by Nassar and coworkers (Nassar *et al.* 2004). The reactive metabolites formed from these substructures are usually unstable and undergo rapid reaction to more stable metabolites (Nassar *et al.* 2004). In addition to the structural properties, also many *in vitro*

experimental assays can be used to evaluate possible toxicity of a drug (Bentley 2001, Nassar *et al.* 2004).

2.1.2 Methods for predicting the drug-likeness

Several methods to identify the drug-like properties of drugs have been described in the literature (Chan and Stewart 1996, Lipinski 2000, Lipinski *et al.* 1997, Oprea 2002, Walters and Murcko 2002, Wenlock *et al.* 2003), of which the Lipinski's rule of 5 and the biopharmaceutics classification system (BCS) are presented below as examples. Both the rule of 5 and the BCS are methods or guidances for the indicative screening of the sufficient drug-like properties and they can be helpful during the early-stage discovery and development.

The rule of 5 was published in 1997 by Dr Christopher Lipinski (Lipinski *et al.* 1997). It states four characteristics, H-bond donors and acceptors, molecular weight and logP (Table 2.2), that are usually needed to make molecules more drug-like or orally active. It also helps to predict oral absorption or permeation of a drug molecule (Lipinski *et al.* 1997). The idea behind the rule of 5 is the ability to identify possible drug candidates from the ADME point of view, which is at least as important as their biological receptor activity when considering orally administered drugs with real therapeutic potential (Lipinski 2000).

The results from the rule of 5 -calculations are only indicative, meaning that a drug molecule with values outside the rule of 5 does not automatically have poor bioavailability. It should be viewed more as a quantitative predictor, it gives a warning that poor absorption or permeability is possible, and thus, helps to highlight potential pitfalls in drug properties (Lipinski *et al.* 1997, Waterbeemd *et al.* 2001).

Table 2.2. The rule of 5 (Lipinski *et al.* 1997).

Poor absorption or permeation of a drug is more likely when
o there are more than 5 H-bond donors
o there are more than 10 H-bond acceptors
o MW is greater than 500
o calc. logP is greater than 5 (or measured logP is greater than 4.15)

The biopharmaceutics classification system (BCS) is a scientific framework for the classification of drug substances based on their aqueous solubility and intestinal permeability (Amidon *et al.* 1995). It was provided by FDA in 1995 with the objective of helping predict, whether there is a correlation between *in vitro* dissolution and *in vivo* bioavailability and whether some of the bioequivalence studies could be replaced by *in vitro* tests (Amidon *et al.* 1995, FDA 2006).

BCS takes into account three major factors, dissolution, solubility and intestinal permeability that influence the rate and extent of drug absorption from immediately release (IR) solid dosage forms (Table 2.3). The solubility of a drug substance is determined under physiological pH conditions (pH 1-7.5) by either a shake-flask or titration method and the permeability is determined in human subjects using pharmacokinetic studies (mass balance, absolute BA), intestinal permeability methods and instability-studies in the GI tract (FDA 2000). After the measurement of these three properties, the drug can be classified into one of four groups (Table 2.4) (Amidon *et al.* 1995, FDA 2000).

Table 2.3. Physicochemical / biopharmaceutical properties affecting to the rate and extent of drug absorption from immediately release (IR) solid dosage forms according to BCS (FDA 2000).

- Dissolution
  - Rapidly dissolving drug = no less than 85 % of the labelled amount of the drug substance dissolves within 30 min
- Solubility
  - Highly soluble drug = highest dose strength is soluble in 250 ml or less of aqueous media (pH 1-7.5)
- Intestinal permeability
  - Highly permeable drug = the extent of absorption in humans is 90 % or more of an administered dose on a mass balance determination or in comparison to an intravenous reference dose

The classification of drugs by the BCS offers an opportunity to predict the absorption of the drug from the GI-tract. A drug in class I having high solubility and high permeability is most likely to have a sufficient absorption, while a drug in class IV having both low solubility and low permeability will require much more studies before

it can be demonstrated that the absorption will be adequate for oral administration (Amidon *et al.* 1995).

Table 2.4. The Biopharmaceutics drug classification system.

Class	Solubility / Permeability	Properties
I	High / High	- The drug is well absorbed - Dissolution or gastric emptying is rate-limiting step of absorption
II	Low / High	- Dissolution is rate-limiting step of absorption
III	High /Low	- Permeability is rate-limiting step of absorption
IV	Low / Low	- Significant problems for effective oral drug delivery

2.1.3 The crucial physicochemical properties of drugs

Passive transcellular absorption requires a drug molecule to be able to dissolve into the aqueous media of the GI content, to be soluble and stable at the site of absorption and to be able to partition into the lipid membrane of the gut wall. Absorption is thus dependent on the lipophilicity, intrinsic aqueous solubility, surface area and molecular weight of the drug molecule (Taylor 1996, Navia et al 1996). In addition to the situation for oral absorption, adequate solubility is also necessary for the intravenous administration, since the therapeutic dose needs to be dissolved before administration (Fahr *et al.* 2005). An inadequate balance between solubility and lipophilicity also leads to poor permeation across the stratum corneum of the skin (Sloan and Wasdo 2003, Sloan *et al.* 2006), the corneal barrier in the eye (Järvinen and Järvinen 1996) and the BBB in the central nervous system (Anderson 1996). Aqueous solubility and lipophilicity are interrelated with each other such that often the lipophilicity of a highly soluble compound is low and vice versa. Thus, finding a suitable balance between these characteristics is essential if one wishes to obtain drug-like compounds.

The relationship between solubility, lipophilicity and absorption of drugs across the biological membranes can be explained by Fick's first law (equation 1) (Amidon *et al.* 1995).

$$J_w = \frac{dm}{dt} = P_w \times C_w \qquad \text{Equation 1.}$$

J_w is the drug flux through the membrane (mass/area/time), P_w is the permeability coefficient of the intestinal wall and C_w is the drug concentration at the intestinal surface. Fick's first law shows that as the solubility (and thus concentration) and the permeability of the drug increases, also the total flux across the biological membrane will increase. These two important properties, solubility and permeability have been discussed in more detail below.

2.1.3.1 Dissolution and aqueous solubility

Drug dissolution is the prerequisite and often the rate-limiting step for the effective drug absorption and clinical response, since only dissolved drug can be absorbed through the biological membrane and be transported to its site of action (Amidon *et al.* 1995, Hörter and Dressman 1997, Waterbeemd *et al.* 2001). A slow dissolution rate may lead to the situation in which the time available for a drug to transit to the absorption site in the GI-tract is too short for complete dissolution, thus diminishing the bioavailability of the drug (Hörter and Dressman 1997). The dissolution rate is affected by the solubility of the drug in the GI content, the pH of the solvent and the structural and physicochemical properties of drug molecules (e.g. molecular weight, the shape of a molecule, number of H-bonds). Also drug diffusivity, the surface area of the solid wetted by the luminal fluids and GI hydrodynamics can influence on the drug dissolution from a solid dosage form (Hörter and Dressman 1997).

The dissolution rate can be calculated from the Noyes-Whitney-equation (equation 2),

$$DR = \frac{dX}{dt} = \frac{A \times D}{h} \times \left(Cs - \frac{X_d}{V} \right) \qquad \text{Equation 2.}$$

in which DR is the dissolution rate, A is the surface area available for the dissolution, D is the diffusion coefficient of the drug, h is the diffusion layer thickness, Cs is the

saturation solubility of the drug, X_d is the amount of the dissolved drug and V is the volume of the dissolution media (Hörter and Dressman 1997). The Noyes-Whitney equation indicates that the saturation solubility, the concentration of the dissolved drug and the thickness of the diffusion layer are the factors determining the concentration gradient across the diffusion layer, and thus have a great influence on the dissolution rate of the drug (Hörter and Dressman 1997).

Saturation solubility Cs describes the concentration of a drug molecule that can be dissolved in a solvent to reach the equilibrium between the solid phase and the solute (Martin 1993). Solubility is affected by the physicochemical and structural properties of a drug molecule and the surrounding solvent, including molecular weight, the shape of the molecule, the melting point, lipophilicity, the molecule's H-bonding with the solvent, intra- and intermolecular H-bonding, ionic charge, pK_a and counter ion, the pH of the surrounding GI fluids, polymorphism and the surfactants of the drug formulation (Amidon *et al.* 1974, Avdeef 2003, Chan and Stewart 1996, Hörter and Dressman 1997, Lipinski 2000). A high molecular weight, lipophilicity and tight crystal packing usually indicates poor solubility (Amidon *et al.* 1974, Hörter and Dressman 1997) but increasing the H-bonding capacity often leads to better solubility (Chan and Stewart 1996). The charge state and pK_a have a major impact on the solubility of acids and bases, since their solubility changes as a function of the pH: the solubility of a weak acid is enhanced at a pH above its pK_a-value and the solubility of weak base is enhanced at a pH below its pK_a. The intrinsic solubility of the ionized form is greatly increased when compared to unionized form at pH of $pK_a + 1$ for weak acids and $pK_a - 1$ for weak bases (Hörter and Dressman 1997).

For parenteral administration, a solubility of at least 10 mg/ml is usually needed for adequate formulation of drugs (Stella 2006). The case of oral administration is more complex since the absorption, and thus, the bioavailability is the sum of solubility and permeability of the drug. The limited volume of the gastric fluids to dissolve the drug dose quantifies the required solubility with the correlation termed as the dose-solubility ratio. This term describes the volume of GI fluid that is needed to dissolve the administered drug (Hörter and Dressman 1997). The relevant solubility is also limited by the permeability of the drug, meaning that solubility required for sufficient

absorption and bioavailability is greater for a drug with low permeability and vice versa (Lipinski 2000). This correlation of the minimum acceptable solubility between the permeability and the dose is well illustrated in Figure 2.2 (Lipinski 2000). Figure 2.3 describes the solubility needed for low, medium and high permeability drugs at doses 0.1, 1.0 and 10 mg/kg. For example, if the solubility of a poorly permeable orally administered drug at a dose of 1 mg/kg is greater than 207 μg/ml, then solubility will not limit the absorption. At a similar dose, a drug with high permeability requires a solubility of only 10 μg/ml (Lipinski 2000).

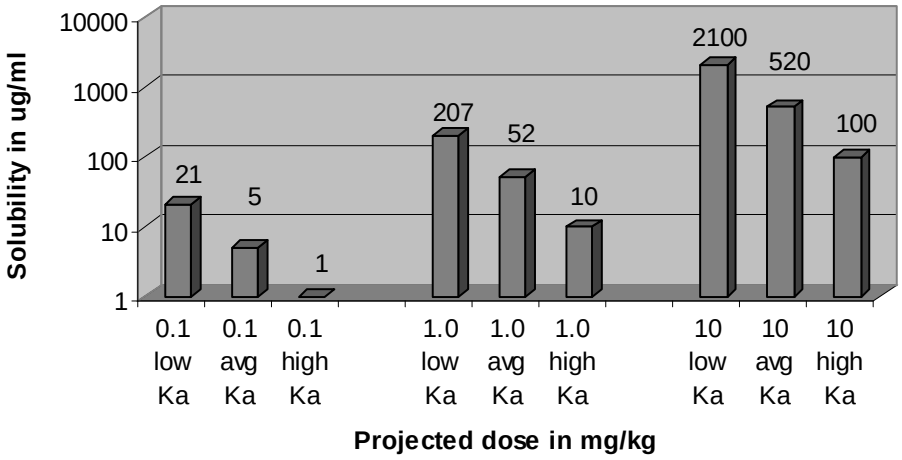


Figure 2.2. Minimum acceptable solubility in μg/ml. Bars show the minimum solubility for low, medium, and high permeability (Ka) at different clinical doses (Lipinski 2000).

A drug with poor solubility and dissolution representing the limiting step of absorption does not mean that it is not developable (Huang and Tong 2004). Poor aqueous solubility can be enhanced by several approaches. Changes to the formulation of the drug can improve the solubility by altering the physical factors, such as changing the particle size and the crystal structure, by adding cosolvents and surfactants to the formulation and by using amorphous material or salt formulations (Davis 2005, Huang and Tong 2004, Strickley 2004). Cyclodextrins can be used to enhance the aqueous solubility by complexation of the drug molecules inside cyclodextrin inclusion complexes. The outside of the oligosaccharide molecule of cyclodextrin is polar and

inside is nonpolar, forming a central cavity. The poorly soluble drug molecule can be complexed to the central cavity, which makes it soluble in aqueous media (Loftsson and Brewster 1996, Loftsson and Duchene 2007). However, probably the most promising approach for the enhancement of the aqueous solubility is prodrug technology which will be discussed in chapter 2.2.

2.1.3.2 Lipophilicity and permeability

The ability of a drug to passively diffuse across the cell membrane by the transcellular route requires the drug molecule to enter into the phospholipid bilayer and then travel from the apical to the basolateral side of the cell, either through the cytoplasmic aqueous phase or along the lipid membranes of the cell (Krämer 1999). The permeability of drugs across biological membranes is dependent on the lipophilicity and diffusion coefficient, as can be seen from equation 3:

$$P_{ptc} = \frac{D \times PC}{h} \qquad \text{Equation 3.}$$

P_{ptc} is passive transcellular permeability, D is the diffusion coefficient, PC is the partition coefficient between the membrane and the luminal fluid and h is the thickness of the membrane. This equation when combined with Fick's first law (equation 1) shows that as the lipophilicity, and thus, the partition coefficient of the drug increases, also the permeability and finally the total flux of the drug across the membrane increases (Taylor 1996).

Lipophilicity refers to the ability of the drug molecule to partition between two immiscible solutions, such as water and lipid-like solutions (such as 1-octanol). In a similar manner to solubility, lipophilicity is the sum of different physicochemical characteristics, such as molecular weight, intra- and intermolecular H-bonding, polar surface area, ionic charge, pK_a and counter ion (Kramer 1999, Waterbeemd *et al.* 2001). Lipophilicity increases with the elevation in the molecular weight and decreases with the increase of H-bonding capacity and polar surface area (Chan and Stewart 1996, Kramer 1999). Also lipophilicity, again similar to solubility, is dependent on the pH in

an inverse manner; ionization of the compound usually leads to decreased lipophilicity (Comer and Tam 2001).

Lipophilicity of drug molecules is most often estimated by 1-octanol/water partition (logP) and distribution (logD) coefficients (Waterbeemd et al 2001). The term logP is used when all the solute is in the neutral state and the logD is used when pH causes part or all of the solute to be ionized (Kerns et al 2003). To achieve sufficient membrane permeation by a passive transcellular mechanism, the logD values are generally between 0-3 and molecular weight < 550. On the other hand, good permeation of the drug molecule with a negative logP value points to the involvement of carrier-mediated transport (Navia et al 1996). However, the permeability of a drug across the biological membrane cannot be predicted exclusively by lipophilicity, instead the specific permeation assays using the calculation models (Waterbeemd 2001) and biological models, such as Caco-2 cell lines (Kretz and Probst 2001), artificial membrane permeability assays (PAMPA) (Kansy *et al.* 2001) and biological BBB-models (Krämer *et al.* 2001) are needed to predict the permeability into the tissue in question.

Poor lipophilicity can be enhanced by expanding the time that the drug resides in the GI-tract by dietary components (Singh 1999), by adding bioadhesive polymers such as chitosans (Takishima *et al.* 2002) or by prolonging gastroretention using microparticles (Burton *et al.* 1995) or swelling systems (Chen *et al.* 2000). However, many of these systems are still being investigated and problems, such as safety and effectiveness require clarification (Davis 2005). A more interesting and perhaps also a more straightforward approach for the enhancement of lipophilicity, and thus, better membrane permeation is to resort to the prodrug technology, which is discussed in more detail in chapter 2.2.

2.2 Prodrugs

Prodrugs have become an established concept and a powerful tool in optimizing the pharmacologically potent structures and overcoming physicochemical, pharmaceutical and biopharmaceutical barriers to a drug's usefulness. Prodrugs can be used for several reasons e.g. to enhance poor aqueous solubility, permeability or chemical stability, to prolong the duration of drug action, to improve drug targeting, to reduce side-effects and too rapid elimination and to extend the patent protection of the parent drug (Stella 1996, Stella 2004, Testa 2004).

Prodrugs are pharmacologically inactive molecules that require an enzymatic and/or chemical transformation to release an active parent drug *in vivo* to exert a pharmacological effect (Figure 2.3) (Stella *et al.* 1985). The release of the active drug molecule and the promoiety may occur prior to, during or after absorption or in the specific target tissue. An ideal prodrug has optimal physicochemical properties, such as lipophilicity and solubility, it is stable in the GI-tract or in its desired dosage form, the promoiety is non-toxic and it releases the active drug at an appropriate rate *in vivo* (Stella 1996, Stella 2004, Testa 2004).

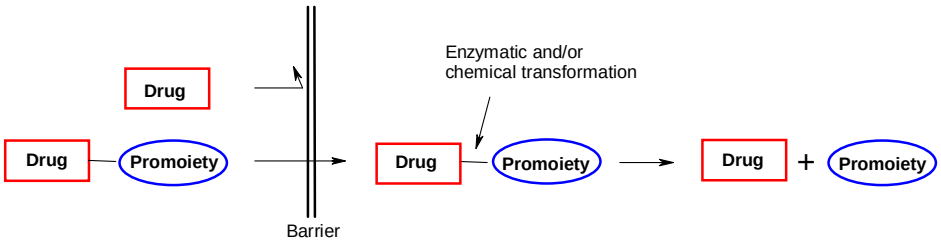
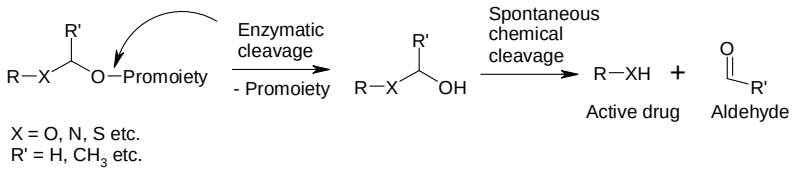


Figure 2.3. The illustration of the prodrug concept (prodrug = drug + promoiety)

The bioactivation mechanism for most prodrug structures is enzymatic or at least requires enzymes to initialize the bioactivation process, which can then further continue chemically. More rapid bioactivation has often been achieved by a double prodrug by linking a short alkyloxy (e.g. methyloxy and ethyloxy) spacer between a promoiety and parent drug. These spacers are used to enhance the chemical space around the enzymatically cleavable bond and undergo a spontaneous chemical hydrolysis after the

enzymatic hydrolysis of the promoiety (Scheme 2.1) (Safadi *et al.* 1993, Varia, Schuller and Stella 1984).



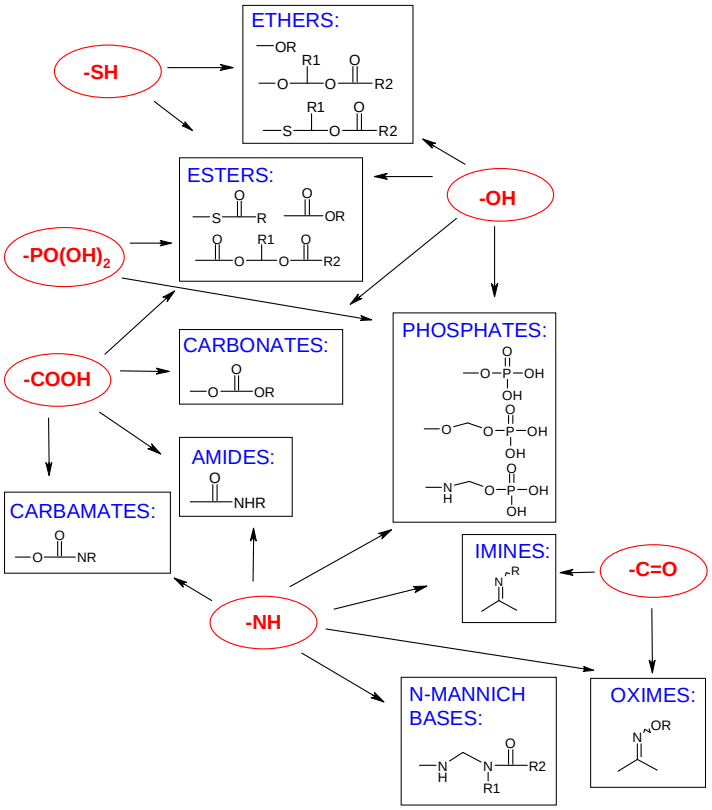
Scheme 2.1. The two-step hydrolysis of alkyloxy-linked prodrugs

One prerequisite for prodrugs is that the promoiety should be nontoxic. However, there are cases in which a questionable structure provides properties superior to other structures and thus the risk of the toxic promoiety may be worthwhile. A methyloxy spacer is an example of a marketed prodrug structure (e.g. fosphenytoin, tenofovir disoproxil fumarate), where a toxic linker or promoiety is released in the body during the bioactivation process. This is because the bioconversion of methyloxy linked prodrugs leads to the liberation of toxic formaldehyde within the body (Heck *et al.* 1990). Other examples of possible toxicity caused by prodrugs are pivaloyl derivatives (e.g. adefovir dipivoxil) that release the pivalate-group (trimethylacetic group). Pivalates have been reported to interfere with carnitine homeostasis in humans (Brass 2002). However, the risk of dose-dependent formaldehyde-mediated toxicity (Slikker *et al.* 2004) of prodrugs can be managed by measuring the levels of formaldehyde with and without the prodrug administration, and the risk of changes in carnitine homeostasis can be reduced by carnitine supplementation coadministered simultaneously with a pivalate-generating prodrug (Brass 2002).

2.2.1 Functional group considerations for prodrug derivatization

Prodrug structures for the most common functionalities, such as carboxylic, hydroxyl, amine, phosphate/phosphonate and carbonyl groups, include esters, carbonates, carbamates, amides, phosphates and oximes. However, various other, more uncommon, functional groups have been investigated as potentially useful structures in prodrug design. Thiols, for example, react in a similar manner as alcohols and can be derivatized

to thioethers (Majumdar and Sloan 2006) and thioesters (Peyrottes *et al.* 2004). Amines may be derivatized into imines (Fozard 2000, Rouleau *et al.* 1997) and *N*-Mannich bases (Simplicio *et al.* 2007). Some typical prodrug structures for the most common functionalities are illustrated in Scheme 2.2.



Scheme 2.2. Functional groups amenable to prodrug design

Ester prodrugs are most often used if one wishes to enhance the lipophilicity, and thus, the passive membrane permeation of water-soluble drugs by masking charged groups (e.g. carboxylic acids and phosphates) (Beaumont *et al.* 2003, Taylor 1996). Once in the body, the ester bond is readily hydrolyzed by the many esterases (carboxylesterases, acetylcholinesterases, butyrylcholinesterases, paraoxonases and arylesterases) found in the blood, liver, and other organs and tissues (Liederer and

Borchardt 2006). Esters are the most common prodrugs used, and it has been estimated that approximately 50 % of all marketed prodrugs are activated by hydrolysis (Ettmayer *et al.* 2004). There are a number of ester prodrugs in clinical use and they have many applications (Beaumont *et al.* 2003). Some representative examples are oseltamivir, famciclovir, valacyclovir, adefovir dipivoxil, latanoprost, tazarotene and pradefovir and these are all described more in greater details below with a summary of their uses.

Carbonates and carbamates differ from esters by having an oxygen or nitrogen on both sides of the carbonyl carbon. Carbonates are carboxylic acid and alcohol derivatives, and carbamates are carboxylic acid and amine derivatives. Carbonates and carbamates often are enzymatically more stable than the corresponding esters but more susceptible to hydrolysis than amides. The bioconversion of many carbonate and carbamate prodrugs require esterases for the formation of the parent drug (Potter and Wadkins 2006). Representative examples are tenofovir disoproxil fumarate of the carbonate prodrugs and irinotecan and cabecitabine of the carbamate prodrugs.

Amides are derivatives of amine and carboxyl functionalities of a molecule. In prodrug design, amides have been used to only a limited extent due to their relatively high enzymatical stability *in vivo*. An amide bond is usually hydrolyzed by ubiquitous carboxylesterases (Potter and Wadkins 2006), peptidases or proteases (Yang *et al.* 1999). Amides are often designed for enhanced oral absorption by synthesizing substrates of some specific intestinal uptake transporter (Steffansen *et al.* 2004, Yang *et al.* 1999). The orally administered amide prodrug, midodrine, is a representative example of the amide prodrugs.

Phosphate ester prodrugs are typically designed for hydroxyl and amine functionalities of poorly water-soluble drugs with the intention of enhancing their aqueous solubility to achieve improved oral and parenteral administration. The dianionic phosphate promoiety usually clearly elevates the aqueous solubility (Heimbach *et al.* 2007, Heimbach *et al.* 2003). Phosphate prodrugs typically show reasonable chemical stability and rapid bioconversion back to the parent drug by the alkaline phosphatases present at the intestinal brush border or in the liver (Heimbach *et al.* 2007, Heimbach *et al.* 2003). Fosphenytoin and phosphonooxymethyl propofol are

examples of parenteral phosphate prodrugs, and fosamprenavir and estramustine phosphate are examples of orally active phosphate prodrugs.

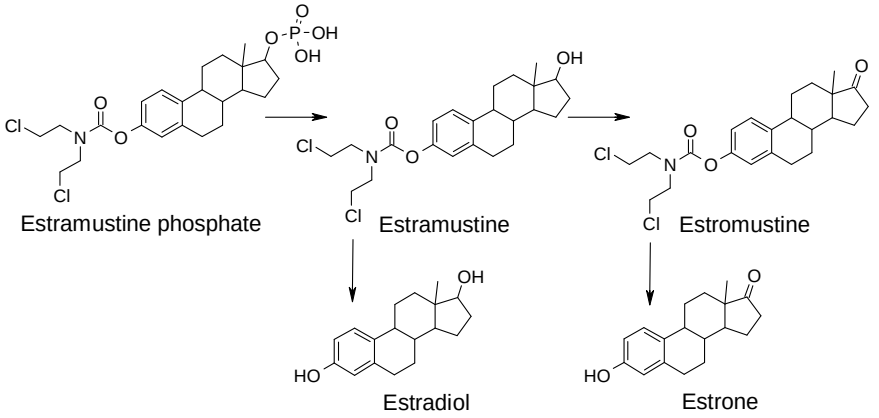
Oximes (ketoximes, amidoximes, and guanidoximes) are derivatives of ketones, amidines and guanidines, and they thus provide an opportunity to modify molecules that lack hydroxyl, amine or carboxyl functionalities. Oximes are hydrolyzed by microsomal cytochrome P450-enzymes (CYP 450) (Jousserandot *et al.* 1998, Kumpulainen *et al.* 2006), which are versatile xenobiotic metabolizing enzymes (Guengerich 2001, Meunier *et al.* 2004). Oximes, especially strongly basic amidines and guanidoximes, may be used in enhancing the membrane permeability and absorption of a drug (Clement 2002). An example of an amidine prodrug is ximelagatran.

2.2.2 Prodrugs for improved oral absorption

2.2.2.1 Improved aqueous solubility

Approximately 40 % of NCEs produced from combinatorial screening programs suffer from poor aqueous solubility having an aqueous solubility less than 10 μM (Lipinski 2002, 2004). Many of the water-soluble prodrugs for enhanced oral drug delivery include the addition of an ionizable progroup to the parent compound (such as phosphate group), but enhanced water-solubility and thus better oral bioavailability may also be achieved by decreasing the crystal packing or by affecting to the melting point of the parent drug (Stella and Nti-Addae 2007). Oral phosphate prodrugs are cleaved at the intestinal brush border by membrane-bound alkaline phosphatase just prior to the passive absorption of the active drug through the intestinal membrane, thus providing a great concentrational driving force for absorption in the intestinal lumen (Fleisher *et al.* 1996, Heimbach *et al.* 2007). Phosphate esters offer one way to increase the oral bioavailability of many sparingly water-soluble drugs, but only a few phosphate prodrugs for oral administration have been marketed to date (Heimbach *et al.* 2003). The challenges to the development of oral phosphate prodrug, are related to poor enzymatic bioconversion (usually the aqueous solubility of the phosphate prodrug is enhanced to such an extent that passive diffusion through the intestinal membrane is prevented), the precipitation of the parent drug after enzymatic cleavage in the intestinal lumen and poor permeability of the parent drug (Heimbach *et al.* 2003).

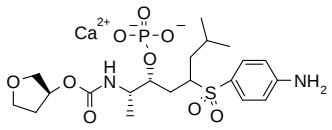
Estramustine phosphate is a phosphate ester prodrug of estramustine (Emcyt®), which was launched in the mid 1970s for the treatment of prostate carcinoma and is marketed in both injectable and oral formulations (Bergenheim and Henriksson 1998, Perry and McTavish 1995). After the dephosphorylation of the water-soluble estramustine phosphate to estramustine, it is metabolized to its ketone derivative, estromustine (Scheme 2.3). Further metabolism of estramustine and estromustine produces two active metabolites, estradiol and estrone. Thus estramustine phosphate has a dual mechanism of action, i.e. the estramustine and estromustine exert cytotoxic (antimicrotubule) effects and estradiol and estrone are responsible for antigonadotropic activity (Bergenheim and Henriksson 1998, Perry and McTavish 1995). After oral administration, estramustine phosphate is rapidly converted to estramustine in the GI-tract, and approximately 75 % of the drug is absorbed. The bioconversion of intravenously administered estramustine phosphate to estramustine is slower with the half-life of 1.3 hours in plasma (Perry and McTavish 1995).



Scheme 2.3. The metabolism of estramustine phosphate (Bergenheim and Henriksson 1998, Perry and McTavish 1995)

Another orally administered phosphate ester prodrug is fosamprenavir (Telzir®, Lexiva™), a phosphate ester of amprenavir (Agenerase®). The ester prodrug shows 10-fold higher water-solubility and equivalent or higher oral bioavailability compared to amprenavir, which is marginally water-soluble (0.04 mg/ml) and requires a high dose

(2400 mg or 16 capsules required per day) (Chapman *et al.* 2004, Furfine *et al.* 2004, Wire *et al.* 2006). Fosamprenavir is rapidly hydrolyzed by gut epithelial alkaline phosphatase to amprenavir during absorption with only a minimal concentration of fosamprenavir reaching the circulation (Chapman *et al.* 2004, Wire *et al.* 2006). The absolute bioavailability of amprenavir after oral administration of fosamprenavir in humans has not been established, but the maximum plasma concentration of amprenavir is reached in 1.5-2 hours. A substantial reduction in the capsule size and number has been achieved (4 capsules daily, 700 mg each) thus enhancing the patient compliance and decreasing the incidence of side effects (Wire *et al.* 2006). Fosamprenavir is also often coadministered with ritonafir, which inhibits amprenavir metabolism, leading to the increased plasma amprenavir concentrations (Wire *et al.* 2006).

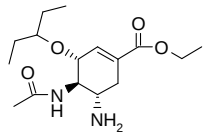


Fosamprenavir

2.2.2.2 Improved lipophilicity

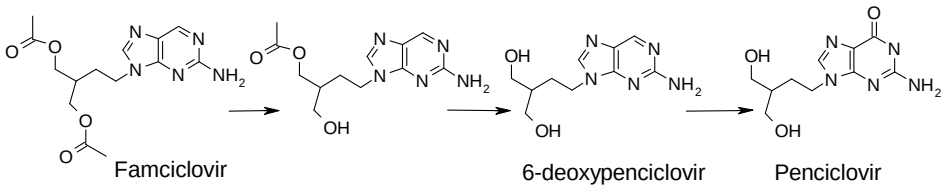
Prodrugs are most frequently applied to mask polar and ionizable groups of a drug molecule with the aim to improve the membrane permeability and oral absorption. Oseltamivir (Tamiflu®) is an orally active prodrug of oseltamivir carboxylate (GS4071, Ro 64-0802), a selective inhibitor of viral neuramidase glycoprotein of influenza A and B (Bardsley-Elliot and Noble 1999, Doucette and Aoki 2001, McClellan and Perry 2001) and it has been proposed to have antiviral activity also against avian H5N1 virus, the virus responsible for the bird flu (O'Malley 2006). As an ethyl ester, oseltamivir is rapidly and well absorbed from the GI-tract and it increases the oral bioavailability of oseltamivir carboxylate from 5 % to 79 % (McClellan and Perry 2001, Shi *et al.* 2006). Oseltamivir undergoes rapid bioconversion to oseltamivir carboxylate mostly by human carboxylesterase 1 and the maximum plasma concentration of oseltamivir carboxylate is reached within 3 to 4 hours after oral administration of oseltamivir (elimination half-life

= 6-10 hours) (Bardsley-Elliot and Noble 1999, Massarella *et al.* 2000, Schmidt 2004, Shi *et al.* 2006).



Oseltamivir ethyl ester

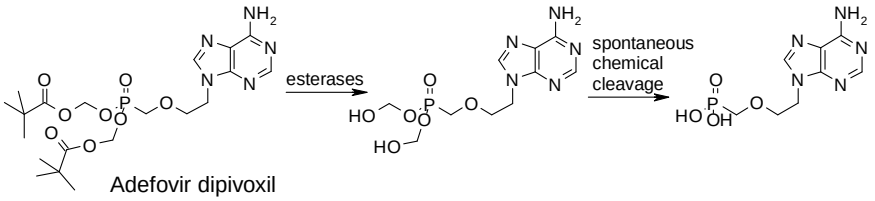
Famciclovir (Famvir®) is an oral diester prodrug of penciclovir, an antiviral agent used against herpes simplex viruses, type 1 (HSV 1) and type 2 (HSV 2) and varicella zoster virus (VZV) (Simpson and Lyseng-Williamson 2006). The bioconversion of famciclovir to penciclovir is two-pronged and requires two enzymes: the acetyl groups are first hydrolyzed by esterases followed by 6-oxidation of the purine ring by xanthine or aldehyde oxidases (De Clercq and Field 2006, Harnden *et al.* 1989, Rashidi *et al.* 1997). The bioconversion takes places mostly in the liver after rapid absorption of the prodrug from the GI-tract (Hodge *et al.* 1989, Simpson and Lyseng-Williamson 2006), though hydrolysis of one of the ester groups may occur to some extent also in the intestine before or during absorption (Hodge *et al.* 1989). After the hydrolysis of both ester groups, 6-deoxypenciclovir is oxidized to penciclovir by aldehyde oxidase (Scheme 2.4) (Rashidi *et al.* 1997). The poor bioavailability of penciclovir is enhanced up to 77 % and the maximum plasma concentration of penciclovir is achieved within 0.75 hours after oral administration of famciclovir (Hodge *et al.* 1989, Simpson and Lyseng-Williamson 2006).



Famciclovir 6-deoxypenciclovir Penciclovir

Scheme 2.4. The bioactivation of famciclovir to penciclovir (Rashidi *et al.* 1997)

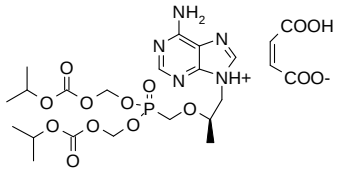
Adefovir dipivoxil (Hepsera®) is an orally active diester prodrug of adefovir, a nucleoside reverse transcriptase inhibitor against hepatitis B virus (Noble and Goa 1999). Pivaloyloxymethyl promoiety is attached to both hydroxyl groups of the phosphonate group of adefovir, which enhances its lipophilicity sufficiently to allow passive oral absorption (Dando and Plosker 2003, Noble and Goa 1999). Ester groups of adefovir dipivoxil are cleaved by hepatic extracellular esterases, following the rapid chemical hydrolysis of oxymethyl groups to free phosphonate (Scheme 2.5). Further phosphorylation to the active monophosphate is catalyzed by cellular adenylate kinases (Dando and Plosker 2003, Noble and Goa 1999). Absorption of adefovir dipivoxil is rapid and the maximum plasma concentration of adefovir is reached within 0.75 hours after oral administration of adefovir dipivoxil with oral bioavailability of 59 % (elimination half-life = 6-7 hours) (Dando and Plosker 2003, Sun *et al.* 2007).



Scheme 2.5. The enzymatical and chemical bioactivation of adefovir dipivoxil

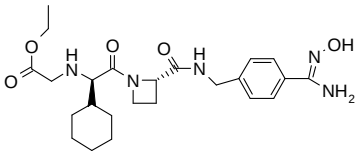
Tenofovir disoproxil fumarate (Viread®) is a carbonate prodrug of tenofovir, a nucleotide transcriptase inhibitor against HIV (Chapman *et al.* 2003) and hepatitis B virus (van Bommel *et al.* 2006). Diisopropyl carbonate groups are attached to both hydroxyl functionalities of tenofovir phosphonate group via an oxymethyl spacer to enhance the lipophilicity, and thus, to improve the oral bioavailability of tenofovir. The carbonate structure was found to be chemically more stable compared to the corresponding esters and the prodrug is rapidly converted to tenofovir in the liver by esterases and subsequently phoshorylated by cellular enzymes to form tenofovir diphosphate (Shaw *et al.* 1997, van Gelder *et al.* 2002). The enzymatic reaction followed with rapid spontaneous cleavage of formaldehyde is similar to that of adefovir dipivoxil. The bioavailability of tenofovir after oral administration of tenofovir

disoproxil fumarate is 25 % and the maximum plasma concentration of tenofovir is reached within 2.3 hours (Chapman *et al.* 2003, Lyseng-Williamson *et al.* 2005).



Tenofovir disoproxil fumarate

A more recent example of an ethyl ester prodrug is ximelagatran (Exanta®), a prodrug of melagatran, which is the first member of orally administered direct thrombin inhibitors (Eriksson *et al.* 2003). As a zwitterion, melagatran has poor oral bioavailability, only 3-7 %. Ximelagatran is a double prodrug, as in addition to an ethyl ester group in the carboxylic acid end, it contains an *N*-hydroxyamidine group in the amidine end of melagatran. Therefore, the formation of melagatran requires two metabolic reactions. The *N*-hydroxy group is reduced to an amidine in the liver, and to some extent also in the intestine, by CYP-enzymes. The ethyl ester is then hydrolyzed to free carboxylic acid in the liver by carboxyl esterases (Clement and Lopian 2003, Eriksson *et al.* 2003). The poor oral bioavailability of melagatran is improved to 20 % by using ximelagatran and the maximum plasma concentration of melagatran is reached within 1.9 hours (Eriksson *et al.* 2003). Ximelagatran was marketed between 2003-2004 in the US and in Europe as the first oral treatment categorized under the World Health Organization class of direct thrombin inhibitors. However, in February 2006, it was withdrawn from the market after an extended clinical trial confirmed the initial concerns that it caused severe liver toxicity (Frantz 2007).

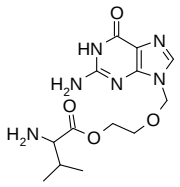


Ximelagatran

2.2.2.3 Carrier-mediated absorption

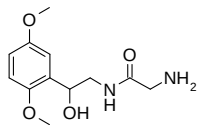
Recent progress in molecular biology has allowed the identification and cloning of nutrient transporters. Prodrugs targeted towards specific membrane transporters are designed to have structural features that mimic the natural substrates of the endogenous uptake transporters present on the intestinal epithelium (Han and Amidon 2000, Majumdar *et al.* 2004, Sai and Tsuji 2004, Yang *et al.* 1999). Targeting specific transporters is particularly important for drugs that are either so polar or so charged that they have negligible passive absorption.

Peptide transporters appear to be attractive targets in prodrug design, as they are widely distributed throughout the small intestine and show sufficiently high transport capacity and broad substrate specificity (Han and Amidon 2000, Steffansen *et al.* 2004, Yang *et al.* 2001). Valacyclovir (Valtrex®) is a representative example of a prodrug that exploits carrier-mediated transport. Valacyclovir is an oral L-valine amino acid ester prodrug of acyclovir, an antiviral agent used against herpes zoster virus (Acosta and Fletcher 1997, Ormrod and Goa 2000, Wu *et al.* 2003). Valacyclovir is a substrate of human intestinal peptide transporter (hPEPT1) (Balimane *et al.* 1998, de Vrueh *et al.* 1998, Guo *et al.* 1999), and it is rapidly transported across the intestinal membranes. Bioconversion of valacyclovir to acyclovir by valacyclovir hydrolase (VACVase) is efficient as over 99 % of valacyclovir is converted to acyclovir (Kim *et al.* 2003, Ormrod and Goa 2000). The bioavailability of acyclovir after oral administration of valacyclovir is enhanced from 20 % to 54 % with the t_{max} of approximately 2 hours (Acosta and Fletcher 1997, Ormrod and Goa 2000).



Valacyclovir

Midodrine (ProAmatine®) is an oral prodrug of desglymidodrine (DMAE), a selective α1-adrenoceptor agonist for the treatment of orthostatic hypotension (Tsuda *et al.* 2006). The glycine promoiety is attached to the amine functionality of DMAE and the prodrug is converted into its active drug DMAE, mainly in the liver and in the systemic circulation by unknown peptidases (Cruz 2000). Midodrine is a substrate of the H^+-coupled human peptide transporter 1 (hPEPT 1) expressed in the intestinal epithelial cells and this active carrier-mediated transport system elevates the bioavailability of midodrine to 93 % compared to only 50 % for DMAE (Cruz 2000, Tsuda *et al.* 2006). DMAE crosses the BBB poorly and thus it exerts some unnecessary effects on the central nervous system. The maximum plasma level of DMAE after oral administration of midodrine is reached in 1-2 hours and it has a $t_{1/2}$ of approximately 25 minutes (Stewart 2006).



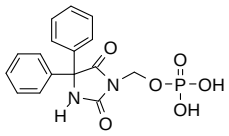
Midodrine

2.2.3 Prodrugs for improved intravenous administration

While examples of marketed water-soluble oral prodrugs are rare, there are several successful prodrugs with improved solubility properties for i.v. administration. The most commonly used approach to increase the water-solubility by prodrugs is to introduce an ionizable/polar promoiety into the parent drug. A number of phosphate esters have been developed as potential water-soluble prodrugs for i.v. administration and less commonly for oral administration.

Fosphenytoin (Cerebyx®) is a phosphate ester prodrug of poorly water-soluble phenytoin for the acute treatment of seizures, and can be used for either i.v. or intramuscular administration (Boucher 1996, Browne *et al.* 1996). In fosphenytoin, a phosphate ester is attached to the weakly acidic (pK_a = 8.3) amine functionality of phenytoin via the oxymethyl spacer group leading to a remarkable increase in its aqueous solubility (from 20-25 μg/ml of phenytoin to 140 mg/ml of fosphenytoin) (Varia, Schuller, Sloan *et al.* 1984). Following i.v. administration, fosphenytoin is

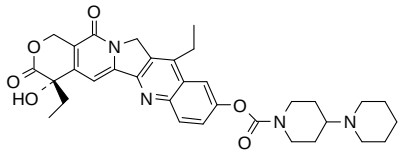
completely converted back to phenytoin by hepatic alkaline phosphatases, with half-lives ranging from 7 to 15 minutes in patients (Browne *et al.* 1996, Varia, Schuller, Sloan *et al.* 1984). The recovery of phenytoin is almost quantitative with only 1-5 % of the fosphenytoin dose being recovered in urine. Thus, the bioavailability of fosphenytoin is almost 100 % and comparable with that of the intravenously delivered phenytoin (Boucher 1996). After intramuscular administration, the maximum plasma concentration of phenytoin is reached within 2-4 hours (Fischer *et al.* 2003). Fosphenytoin has been reported to be safer than phenytoin in terms of its ability to evoke cardiac toxicity (DeToledo and Ramsay 2000, Ramsay and DeToledo 1996), but recent studies have indicated that the occurence of these side effects may be more common than previously thought (Adams *et al.* 2006).



Fosphenytoin

Irinotecan (CPT-11, Camptosar®) is a parenteral water-soluble carbamate prodrug of a lipophilic antineoplastic topoisomerase I inhibitor, camptothecin (SN-38) which is used in the treatment of recurrent, metastatic colorectal cancer. It represents an ionizable promoiety to improve the aqueous solubility (Bencharit *et al.* 2002, Rothenberg 2001, Sanghani *et al.* 2004, Slatter *et al.* 1997). In the irinotecan molecule, a dipiperidino promoiety is attached to the phenol moiety of camptothecin via a carbamate bond. The bioconversion back to camptothecin occurs primarily in the liver, and to a minor extent inside the tumors (Guichard *et al.* 1999), by human carboxylesterases (Sanghani *et al.* 2004). Both irinotecan and camptothecin exist in pH-dependent equilibrium between lactone and carboxylate forms, of which the lactone is the pharmacologically active form (Slatter *et al.* 2000). The bioconversion of CPT-11 to camptothecin is not very effective, as only 10 % of CPT-11 is converted to camptothecin with an elimination half-life ($t_{1/2}$) of approximately 14 hours (Rivory *et al.* 1997). In addition, the intestinal bioconversion of the prodrug to the cytotoxic drug leads to severe diarrhoea after the

treatment and limits its use. Thus recent studies have focused more on finding tumor-sensitive bioconversion mechanisms of CPT-11 by exploiting hypoxia- and transgene-mediated over-expression of the carboxylesterases in tumors (Matzow *et al.* 2007).

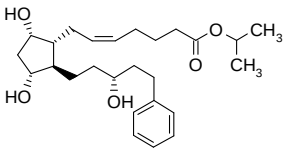


Irinotecan, CPT-11

2.2.4 Prodrugs for improved topical administration

Topical administration of drugs encompasses all external membranes, though ocular and dermal drug delivery are the most widely used forms of topical prodrug applications. The unfavourable physicochemical properties of many drug molecules lead to poor permeation across the stratum corneum of the skin or the corneal barrier of the intraocular tissues (Brown *et al.* 2006, Urtti 2006). These features can often be achieved by the prodrug approach (Fang and Leu 2006, Järvinen and Järvinen 1996, Sloan and Wasdo 2003).

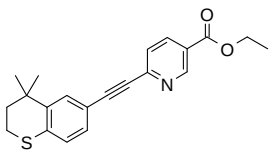
A prostaglandin analog latanoprost (Xalatan®) represents a new class of active ocular hypotensive agents for the treatment of glaucoma. It is a lipophilic isopropyl ester prodrug that is rapidly hydrolyzed inside the ocular tissue to biologically active prostaglandins. In its carboxylic acid form, the active drug is poorly permeable and causes irritation while the lipophilic prodrug achieves improved ocular absorption and better safety (Hellberg *et al.* 2003, Netland *et al.* 2001, Sharif *et al.* 2003, Susanna *et al.* 2002).



Latanoprost

Latanoprost has been shown to be more effective when compared to the traditionally used β-adrenergic receptor antagonist timolol in reducing intraocular pressure (Chew *et al.* 2004, Harasymowycz *et al.* 2007); it enables once-daily dosing and maintains efficacy even after long-term use and causes no severe systemic and ocular side effects (Thelen *et al.* 2007). The reduced systemic side-effects are a result of the lowered concentrations of latanoprost in the systemic circulation and rapid elimination of latanoprost in the plasma ($t_{1/2}$ = 17 minutes) (Alm 1998).

Tazarotene (Tazorac®) is a lipophilic ethyl ester of tazarotenic acid used for the treatment of psoriasis and acne (Dando and Wellington 2005, Guenther 2003). It is rapidly converted to an active drug after percutaneous absorption by esterases and only 5 % of tazarotene is systemically absorbed (Foster *et al.* 1998). Since it masks the free carboxylic acid functionality of tazarotenic acid, it evokes less skin irritation (Marks 1997). There is very little systemic absorption of tazarotene because of its limited percutaneous penetration. Principally due to the short systemic half-life and less extensive lipophilicity of the released tazarotenic acid, this compound displayed no accumulation in adipose tissues (Tang-Liu *et al.* 1999). Tazarotene is rapidly metabolized to tazarotenic acid and to its metabolites (sulfoxide and sulfone) in the skin and the plasma, thus resulting in minimal systemic adverse effects (Chandraratna 1996, Dando and Wellington 2005, Marks 1998). Thus, tazarotene is not only a carboxylic acid prodrug with enhanced skin permeability, but it also acts as a soft-drug with enhanced systemic metabolism. Soft-drugs, unlike prodrugs, are active drugs that are designed to undergo a predictable and controllable deactivation or metabolism *in vivo* after achieving their therapeutic effect (Bodor and Buchwald 1997, 2000, Thorsteinsson *et al.* 2003). In addition, much smaller doses of topically administered tazarotene are required compared to oral retinoids and the drug is delivered directly into the target skin tissues (Tang-Liu *et al.* 1999).



Tazarotene

2.2.5 Prodrugs for site-selective drug delivery

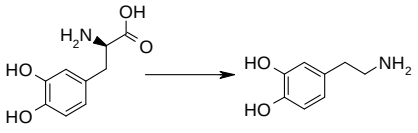
Site-selective drug delivery is the ultimate goal in drug therapy. Site-selectivity may be achieved by passive drug enrichment in the organ, via transporter mediated delivery, by selective metabolic activation through enzymes, and by antigen targeting (Ettmayer *et al.* 2004, Petrak 2005). Some examples of the more widely studied applications, e.g. CNS-, tumor- and liver-targeting are discussed in more detail below.

2.2.5.1 CNS-targeted drug delivery

As described in chapter 2.1.1.2, the BBB is a challenge to the transport of drugs to the CNS. A traditional approach to increase the drug concentration in CNS has been to increase of the lipophilicity of the parent drug. This passive enrichment requires the prodrug to have ready access to the brain tissue, the site-selective bioconversion of the prodrug back to the parent drug and the prolonged retention of the parent drug within the brain tissue (de Boer and Gaillard 2007, Siegal and Zylber-Katz 2002). Once the lipophilicity of the drug is increased via a prodrug, the prodrug will exhibit improved penetration into the CNS. Increased lipophilicity alone, however, does not ensure that a higher concentration of parent drug will be presented in the target tissue. The target tissue bioconversion needs to be rapid and selective enough to compete with the elimination from the target tissue, and also to ensure that the premature bioconversion of the prodrug is sufficiently low. By understanding the specific transport mechanisms and enzymatic activity at the CNS and BBB, it is often possible to achieve substantially enhanced CNS delivery and to prevent possible adverse effects in other organs / tissues (Anderson 1996, de Boer and Gaillard 2007).

Levodopa (Dopar®) can be considered as a prodrug which achieves targeted delivery into central dopaminergic neurones. Levodopa is a substrate for the neutral amino acid transporter (LAT1) expressed at the BBB (Sampaio-Maia *et al.* 2001). This uptake transporter carries levodopa to the brain tissue, where levopoda is rapidly converted to dopamine by enzymes only present in nerves (Scheme 2.6). After the bioconversion, a very hydrophilic dopamine is trapped into the CNS, enabling its pharmacodynamic effects selectively in brain tissue (Nutt and Woodward 1986, Nutt *et al.* 1984). Levodopa is usually administered along with peripheral dopa-decarboxylase inhibitors

(DCI), such as carbidopa or benserazide. Thus the peripheral decarboxylation to dopamine, norepinephrine, and epinephrine by dopa-decarboxylase can be avoided leading to diminished risk of undesired cardiovascular and gastrointestinal side effects (Kadieva *et al.* 2005, Lledó 2001).



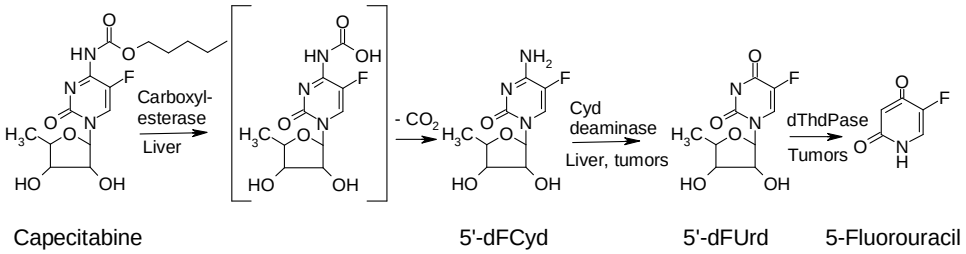
Scheme 2.6. The bioconversion of levodopa to dopamine

2.2.5.2 Tumor-targeted drug delivery

The aim of cancer therapy is to target an inactive prodrug selectively to tumor cells, where the active drug is released without being toxic to normal, healthy tissue (Aisner 2007, de Groot *et al.* 2001). Due to the high proliferation rates of tumor cells, in addition to the bioreductive activity, certain enzyme levels are often elevated in these cells and this property has been exploited in targeted prodrug-tumor delivery (Aisner 2007). The development pipelines of the pharmaceutical companies also reflect the importance and benefits of targeting of antitumor agents, i.e. in 2006 half of the total 380 oncology products in late-stage development were targeted therapies (Longwell 2007).

Capecitabine (Xeloda®) is an orally administered carbamate prodrug of 5-FU, requiring a cascade of three enzymes for its bioconversion to the active drug. Capecitabine is absorbed intact from the intestine and undergoes the final step of the bioconversion pathway within the tumors, thus avoiding systemic toxicity (Doyle and Engelking 2007, Walko and Lindley 2005). The enzymatic bioconversion pathway is initiated in the liver, where human carboxylesterases 1 and 2 cleave the ester bond of the carbamate (Scheme 2.7) (Quinney *et al.* 2005). This is followed by a rapid, spontaneous decarboxylation reaction resulting in the formation of 5'-deoxy-5-fluorocytidine (5'-dFCyd) (Walko and Lindley 2005). The reaction continues in the liver, and to some extent in tumors, by cytidine deaminase which converts 5'-dFCyd to

5'-deoxyuridine (5'-dFUrd). The final activation step takes place in the tumors, where thymidine phosphorylase (dThdPase) liberates the active drug 5-FU (Miwa *et al.* 1998, Walko and Lindley 2005). The bioavailability of 5-fluorouracil after oral administration of cabecitabine is almost 100 % (Doyle and Engelking 2007, Walko and Lindley 2005) and the safety profile of cabecitabine is much more favorable compared to that of intravenous 5-fluorouracil (Cassidy *et al.* 2002).

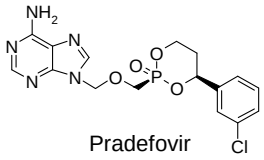


Scheme 2.7. The bioconversion of cabecitabine to 5-fluorouracil (Miwa *et al.* 1998, Walko and Lindley 2005).

To expand the range of tumors susceptible to enzyme-prodrug cancer therapy, prodrug-activating exogenous enzymes can be delivered to tumor cells by using antibodies and genes. The most common approaches are antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT). ADEPT is a two-step therapy, in which the enzyme-antibody conjugate first binds to a tumor-specific antigen on the malignant cell membrane (Bagshawe *et al.* 2004, Damen *et al.* 2001, de Groot *et al.* 2001, Sharma *et al.* 2005). The inactive prodrug is then administered and activated to an active cytotoxic drug by the localized enzyme. The principle of GDEPT is similar, but the enzyme is localized within tumor cells using a targeting vector to deliver the gene encoding the enzyme into the tumor cells (Dachs *et al.* 2005, Niculescu-Duvaz *et al.* 1998). While few ADEPT systems have so far progressed to the clinical phase I studies (Chester *et al.* 2004, Francis *et al.* 2002, Napier *et al.* 2000), one GDEPT has reached multicenter phase III clinical trials (Dachs *et al.* 2005).

2.2.5.3 Liver-targeted drug delivery

Since the liver is the most important metabolizing organ, it possesses a wide variety of liver-specific metabolizing enzymes capable of prodrug activation (van Montfoort *et al.* 2003). Pradefovir mesylate is a cyclic 1,3-propanyl ester prodrug of a nucleoside monophosphate (NMP), adefovir, which is being tested for the use in the treatment of hepatitis B (Erion *et al.* 2006, Erion *et al.* 2004). Pradefovir undergoes a cytochrome P450 (CYP) 3A4 catalyzed oxidation predominantly in liver hepatocytes (Erion *et al.* 2004, Lin *et al.* 2006). The oxidation results in ring opening and β-elimination of an aryl vinyl ketone. The released monophosphate adefovir is further converted by nucleotide kinases to the active nucleoside triphosphate (NTP) (Erion *et al.* 2004). In phase II clinical trials in hepatitis B patients, pradefovir has demonstrated good efficacy with low systemic adefovir levels, which is indirect evidence for liver targeting (Erion *et al.* 2006).



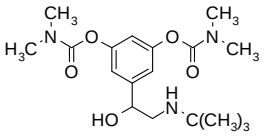
Pradefovir

2.2.6 Prodrugs for prolonged duration of drug action

Although various pharmaceutical formulations are frequently used to prolong the duration of drug action, there are a few examples where prodrugs have been used for this purpose. Very lipophilic prodrugs of several steroids (e.g. testosterone nandrolone) and neuroleptics (e.g. fluphenazine, flupenthixol, haloperidol) are slowly released from the site of intramuscular injection and result in a prolonged duration of action (Altamura *et al.* 2003, Minto *et al.* 1997). Once released from the injection site, the prodrugs are usually rapidly bioconverted, in most cases with no attenuation of their therapeutic action. As an example, the onset of action of fluphenazine generally appears between 24 to 72 hours after injection of its lipophilic decanoate ester prodrug, and thus gradual release continues for 1 to 8 weeks with an average duration of 3 to 4 weeks (Marder *et al.* 2002).

Bambuterol (Bambec®, Oxeol®) is a biscarbamate ester prodrug of the beta$_2$-agonist terbutaline for use in the treatment of asthma. It is slowly bioconverted to terbutaline

predominantly outside the lungs via hydrolysis by butyrylcholinesterases and oxidation reactions to products which can further be hydrolysed to terbutaline (Sitar 1996, Svensson 1987, 1991). Bambuterol is stable to presystemic elimination and is concentrated by lung tissue after absorption from the GI tract. The maximum plasma concentrations of terbutaline after bambuterol administration occur approximately at 4 to 7 hours and the efficacy of bambuterol has been shown to last for 24 hours (Sitar 1996). As a result of its prolonged duration of action, once-daily bambuterol treatment provides relief of asthma with a lower incidence of side-effects than terbutaline which has to be taken three times a day (Persson *et al.* 1995).



Bambuterol

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3 AIMS OF THE STUDY

The general objective of the present study was to design, synthesize and evaluate novel prodrug structures, which could improve the physicochemical, pharmaceutical or biopharmaceutical properties of drug molecules. The model compounds used in this study were nabumetone, ketoprofen and propofol. The specific aims were:

1. To synthesize and fundamentally characterize the usefulness of the hydroxyimine as a prodrug structure of a ketone drug *in vitro* and *in vivo*.

2. To develop a synthesis route for 1-chloroethyl phosphates and to evaluate their usefulness as building blocks of ethylidene linked prodrug structures.

3. To synthesize an ethylidene linked phosphate prodrug of propofol and to evaluate its usefulness as a prodrug structure for improved aqueous solubility *in vitro* and *in vivo*.

4. To synthesize monomethyl phosphate esters of propofol and to evaluate their usefulness as biodegradable prodrug structures with improved membrane permeation abilities *in vitro* and *in vivo*.

4 GENERAL EXPERIMENTAL PROCEDURES

4.1 General synthetic procedures

This chapter describes the general synthetic procedures of the compounds evaluated in this thesis. Detailed descriptions of the synthesis procedures are given in chapters 5-8.

All reagents obtained from commercial suppliers were used without further purification. The described reactions were monitored by thin-layer chromatography using aluminum sheets precoated with Merck silica gel 60 F_{254}. Samples were visualized by UV-light and staining using anisaldehyde, $KMnO_4$ or ninhydrine. Column chromatography was performed with silica gel (0.063-0.200 mm mesh). ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 500.13, 125.78 and 200.46 MHz at 25ºC, respectively. TMS or TSP was used as an internal reference for ^{1}H and ^{13}C measurements and H_3PO_4 as an external reference for ^{31}P measurements. The splitting pattern abbreviations are as follows: s = singlet, d = doublet, t = triplet, q = quartet, sep = septet, m = multiplet. Electron impact or positive chemical ionization mass spectra were acquired by an Agilent Technologies gas chromatograph- mass spectrometry system (part numbers 6890N G 1530N and 5973 G 2589A). Electronspray ionization mass spectra were acquired by an LCQ quadrupole ion trap mass spectrometer with an electronspray ionization source (Finnigan MAT, San Jose, CA). Elemental analyses were carried out on a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer.

4.2 Determination of the aqueous solubility

The aqueous solubilities were determined at room temperature in an appropriate buffer solution (0.16 M phosphate buffer or 0.05 mM Tris-HCl-buffer at pH 7.4, 0.05 M acetate buffer at pH 5.0). The pH of the mixtures was held constant during the study. Excess amounts or a known amount (due to the small amount of compound available) of each component were added to 1 or 0.5 ml of buffer solution and the mixtures were stirred at room temperature for 48 hours or less, filtered (0.45 μm Millipore) and analyzed by HPLC.

4.3 Determination of the distribution coefficient

The distribution coefficients (log D) were determined at room temperature with a 1-octanol- aqueous buffer system at pH 7.4 and at pH 5.0 (0.16 M phosphate buffer or 0.05 mM Tris-HCl-buffer at pH 7.4, 0.05 M acetate buffer at pH 5.0). Before use, the 1-octanol was saturated with buffer by stirring vigorously for 24 h. A known concentration of each compound was dissolved in buffer and pH was adjusted to either 7.4 or 5.0. The solution was shaken with a suitable volume of 1-octanol for 60 min. After shaking, the phases were separated by centrifugation at 10 000 rpm for 10 min. The concentrations of the compounds in the buffer phase before and after the partitioning were determined by HPLC.

4.4 Determination of the chemical stability

The rate of the chemical degradation was determined at 37ºC in an appropriate buffer (0.16 M phosphate buffer, 0.05 mM Tris-HCl-buffer or 0.18 M borate buffer at pH 7.4, 0.05 M acetate buffer at pH 5.0). An appropriate amount of compound was dissolved in preheated buffer solution and the solutions were placed in a thermostatically controlled water bath at 37ºC. Samples were taken at predetermined time intervals and analyzed by HPLC. Pseudo-first-order half-lives ($t_{1/2}$) for the degradation of prodrugs were calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug versus time.

4.5 Determination of the degradation in human serum

The rate of enzymatic degradation of prodrugs was determined in human serum at 37°C. One volume of 100 µM solution of prodrug in 0.05 mM phosphate buffer (pH 7.4) was diluted with four volumes of preheated human serum and vortex mixed. The solutions were kept in an incubator at 37°C and 200 µl aliquots of serum/buffer solution were taken at appropriate time intervals followed by addition of 200 µl of ice-cold acetonitrile to precipitate protein from serum. After immediate mixing and centrifugation for 15 min at 11000 rpm, the supernatant was analyzed for remaining prodrug and released parent drug by the HPLC. Pseudo-first-order half-life ($t_{1/2}$) for the

degradation of prodrug was calculated from the slope of the linear portion of the plotted logarithm of the remaining prodrug versus time.

4.6 Determination of the enzymatic hydrolysis in alkaline phosphatase or in phosphodiesterase solution

The unit amounts of alkaline phosphatase type VII-S from bovine intestinal mucosa and phosphodiesterase I from Western Diamondback Rattlesnake venom (*Crotalux atrox*) were used as defined by a supplier (Sigma-Aldrich). An appropriate amount of prodrug (final concentration typically 50 µM) was dissolved in preheated buffer solution (pH 7.4) and the solutions were placed in a thermostatically controlled water bath at 37ºC. The enzymatic reaction was started by adding enzyme to the solution. In blank solutions, enzyme was replaced with the same volume of water to ensure that the hydrolysis was clearly enzymatic. At predetermined time intervals, 200 µl samples were removed and 200 µl ice-cold acetonitrile was added to each sample to stop the enzymatic hydrolysis. The samples were kept on ice, centrifuged for 10 min at 14000 rpm, and the supernatant was analyzed by the HPLC. Pseudo-first-order half-lives ($t_{1/2}$) for the hydrolysis of prodrugs were calculated from the slope of the linear portion of the plotted logarithm of the remaining prodrug versus time.

4.7 Determination of the enzymatic hydrolysis in rat liver homogenate

The 20 % rat liver homogenate was prepared by homogenizing rat liver with four equivalents of isotonic 50 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged for 90 min at 9000 × g and the supernatant was stored at -80 ºC before use. One volume of prodrug solution in 50 mM Tris-HCl buffer (pH 7.4, 37°C, final concentration typically 100 µM) was added into one volume of rat liver homogenate. In blank solutions, liver homogenate was replaced with the same volume of water to ensure that the hydrolysis was clearly enzymatic. At predetermined time intervals, 150 µl samples were removed and 150 µl ice-cold acetonitrile was added to each sample to stop the enzymatic hydrolysis. The samples were kept on ice, centrifuged for 10 min at 14000 rpm, and the supernatant was analyzed by the HPLC. Pseudo-first-order half-

lives ($t_{1/2}$) for the hydrolysis of prodrugs were calculated from the slope of the linear portion of the plotted logarithm of the remaining prodrug versus time.

4.8 Animal treatments

Adult male Wistar rats weighing approximately 250 g were used in all animal studies and were purchased from the National Laboratory Animal Centre (Kuopio, Finland). Rats were housed on a 12 hours light/dark cycle. All experiments were carried out during the light phase. The rats had free access to tap water and food pellets. All procedures were reviewed and approved by the Animal Ethics Committee at the University of Kuopio. Detailed experimental procedures are described in chapters 5, 7 and 8.

5 EVALUATION OF HYDROXYIMINE AS CYTOCHROME P450-SELECTIVE PRODRUG STRUCTURE*

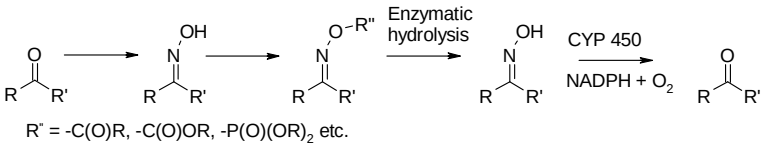
Abstract: Hydroxyimine derivatives of ketoprofen (**1**) and nabumetone (**2**) were synthesized and evaluated *in vitro* and *in vivo* as cytochrome P450-selective intermediate prodrug structures of ketones. While **1** released ketoprofen extremely slowly *in vitro*, **2** released nabumetone at an appropriate rate *in vitro* in the presence of isolated rat and human liver microsomes and different recombinant human CYP isoforms. Bioconversion of **2** to both nabumetone and its active metabolite, 6-methoxy-2-naphtylacetic acid (6-MNA), was further confirmed in rats *in vivo*. Results indicate that hydroxyimine is a useful intermediate prodrug structure for ketone drugs.

* Adapted with permission from: Kumpulainen H,[#] Mähönen N,[#] Laitinen M-L, Jaurakkajärvi M, Raunio, H, Juvonen, R, Vepsäläinen, J, Järvinen, T, Rautio J. Evaluation of hydroxyimine as Cytochrome P450-Selective Prodrug Structure: Journal of Medicinal Chemistry 49: 1207-11, 2006. Copyright 2007 American Chemical Society. [#] Authors contributed equally to this work

5.1 Introduction

Prodrug structures have mainly been developed for amine, hydroxyl and carboxyl functionalities of drug molecules (Fleisher *et al.* 1996). Only a few prodrug structures have been developed for molecules containing ketone functionality, although ketone group is a common functional group in drug molecules. One promising strategy to apply prodrug approach to a ketone compound is to synthesize an oxime structure via a hydroxyimine, which is oxidized to the corresponding ketone by microsomal cytochrome P450 (CYP) enzymes (Jousserandot *et al.* 1998). CYP enzymes are versatile xenobiotic metabolizing enzymes (Meunier *et al.* 2004) and particularly abundant in the liver and intestinal tract, although they are expressed practically in every tissue of the body (Ding and Kaminsky 2003). This wide substrate specificity of CYP enzymes can be exploited in prodrug technology. CYP enzyme mediated reactions have been utilized in targeting drugs into the liver (Erion *et al.* 2005), in CYP based gene therapy (Baldwin *et al.* 2003) and in activating a prodrug to an active drug during first-pass metabolism in the liver (Ikeda *et al.* 2000).

In the prodrug approach, hydroxyimine is especially suitable as an intermediate structure since it can be further derivatized, for example, to an ester, phosphate or carbamate. Thus, oxime prodrugs are actually double-prodrugs requiring a two-step bioactivation process (Scheme 5.1). The oxime structure is first hydrolyzed enzymatically to hydroxyimine, which is further oxidized to the corresponding active ketone drug by CYP enzymes. The bioconversion of hydroxyimines to corresponding ketones also leads to the liberation of nitric oxide (NO) in biological systems (Jousserandot *et al.* 1995, Jousserandot *et al.* 1998, Mansuy *et al.* 1995, Shaik *et al.* 2005). Because the CYP catalyzed oxidation reaction of hydroxyimine occurs *in vivo* mainly in liver, oximes can be used to improve site-specificity, especially liver-targeting, of a parent drug. Previously hydroxyimine structure has been used in a very few prodrug molecules (Mäntylä *et al.* 2004, Prokai *et al.* 1995, Venhuis *et al.* 2003) and their fundamental *in vitro/in vivo*-characterization has been lacking.

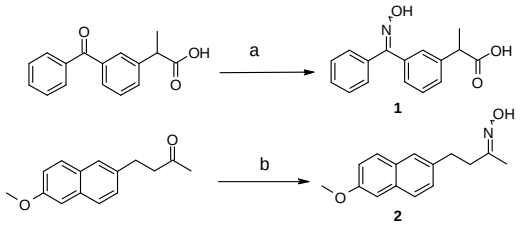


Scheme 5.1. The two-step process of the synthesis of an oxime prodrug and enzymatic activation of an oxime prodrug to the corresponding ketone.

To evaluate the *in vitro* and *in vivo* usefulness of the hydroxyimine moiety as an intermediate prodrug structure for ketones, hydroxyimine derivatives of ketoprofen and nabumetone, were synthesized. These the ketone functionality containing compounds were chosen as model compounds because of their suitable detection properties and their low quantity of other functional groups. In addition, ketoprofen and nabumetone are both non-steroidal anti-inflammatory drugs (NSAID) and the hydroxyimine can act as a NO-donating group. Some NSAIDs, e.g. aspirin, naproxen and diclofenac have been coupled to a NO-donating moiety which may have either protective (e.g. GI-sparing effect or protective role in some liver injuries) (Whittle 2004) or harmful effects (in conditions such as liver ischemia (Chen *et al.* 2003), asthma (Barnes 1995) or rheumatoid arthritis (van't Hof *et al.* 2000)) in the body. In this study, these two hydroxyimine intermediate prodrug structures are extensively characterized with respect to their physicochemical properties, chemical and enzymatic stability, formation of nitric oxide from the hydroxyimine structure and the release of their representative parent drug *in vitro* and *in vivo*.

5.2 Results and Discussion

Synthesis. The syntheses of ketoprofen and nabumetone hydroxyimines (**1** and **2**, respectively) were obtained in good yields by treating the parent drugs, ketoprofen and nabumetone with hydroxylamine hydrochloride in the presence of pyridine (Scheme 5.2). The E- and Z-isomers of **2** could be isolated by column chromatography. However, all the studies described below were carried out by using the mixture of E- and Z-isomers.



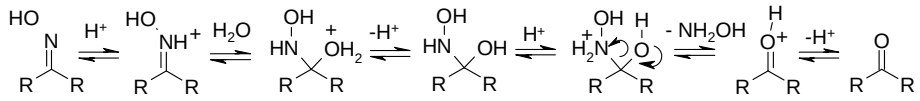
Scheme 5.2. Reagents a) NH_2OH*HCl, Pyr, dioxane, 75 %, b) NH_2OH*HCl, Pyr, EtOH, 94 %

Aqueous solubility and lipophilicity. Since hydroxyimines are designed to be intermediate structures, not final prodrugs, and will be further derivatized, it may be desired that their physicochemical properties do not differ much from parent molecules. However, it is important to find out the effects of hydroxyimine structure on physicochemical properties of a parent drug. Therefore, the aqueous solubilities and distribution coefficients (log D) of **1** and **2** and their parent compounds at pH 7.4 and 5.0 were determined (Table 5.1). The aqueous solubilities of both hydroxyimines were lower than their parent compounds at pH 7.4. At pH 5.0 the hydroxyimine structure enhanced the aqueous solubility of ketoprofen but diminished the aqueous solubility of nabumetone. The distribution coefficients of **1** and **2** were similar compared to their parent compounds at both pH 7.4 and 5.0. In conclusion, the differences in aqueous solubility and lipophilicity between both **1** and **2** compared to their parent drugs were not significant.

Table 5.1. Aqueous solubilities and distribution coefficients (log D) of ketoprofen, nabumetone and their hydroxyimines (**1**, **2**) in buffer solutions (mean ± SD, n = 3 unless otherwise mentioned)

Compd	Aqueous solubility (µg/ml)		log D	
	pH 5.0	**pH 7.4**	**pH 5.0**	**pH 7.4**
Ketoprofen	740 ± 30 mg/ml	465 ± 76 mg/ml	2.1 ± 0.01	0.008 ± 0.02
1	1800 ± 300 mg/ml	116 ± 9.2 mg/ml $^{n=2}$	2.0 ± 0.01	-0.04 ± 0.01
Nabumetone	6.4 ± 0.2	4.3 ± 0.4	3.2 ± 0.04	3.3 ± 0.04
2	3.0 ± 0.1	2.0 ± 0.1	3.6 ± 0.04	3.6 ± 0.04

Stability in buffer solutions and in 80 % human serum. Chemical degradation of both **1** and **2** followed first-order kinetics at pH 1.0-9.0 and the half-lives are presented in Table 5.2. The hydrolysis of **2** was faster than that of **1** at all pH values, probably due to the aromatic nature around the hydroxyimine structure of ketoprofen, which makes the hydroxyimine nitrogen less basic than that of **2**. Also the bulk aromatic rings around the carbonyl carbon of **1** increase the steric hindrance, thus slowing down the nucleophilic attack of the water molecule, which can be understood by examining the mechanism of hydrolysis illustrated in Scheme 5.3. The nitrogen of hydroxyimine is first protonated, which leads to the nucleophilic attack of the water molecule to the carbonyl carbon. Second protonation of the nitrogen makes NH_2OH a good leaving group, the C-N bond breaks and a double bond of the corresponding ketone is formed. The chemical stability of **2** increases with pH (Table 5.2) and in the pH-range of pH 5.0 to 9.0 the half-lives of **2** are over 16 hours.



Scheme 5.3. The mechanism of the chemical hydrolysis of hydroxyimine to ketone in acidic conditions.

Both **1** and **2** showed high stability towards enzymatic hydrolysis in 80% human serum (pH 7.4) with the half-life of approximately 33 and 25 days, respectively (Table 5.2).

Table 5.2. Rates of hydrolysis of nabumetone hydroxyimine (**1**) and ketoprofen hydroxyimine (**2**) in buffer solutions and human plasma at 37ºC (mean ± SD, n = at least 3, unless otherwise mentioned)

	$t_{1/2}$ **min pH 1.0**	$t_{1/2}$ **min pH 3.0**	$t_{1/2}$ **h pH 5.0**	$t_{1/2}$ **days pH 7.4**	$t_{1/2}$ **days pH 9.0**	$t_{1/2}$ **days 80 % human serum**
1	$360.0 \pm 1.8^{n=2}$	n.d.	- [a]	- [a]	- [a]	33.2 ± 2.9
2	6.0 ± 0.15	11.0 ± 0.6	16.3 ± 2.6	12.0 ± 5.0	- [a]	24.6 ± 4.6

[a] no degradation was observed during the four weeks' incubation, n.d. = not determined

Enzymatic degradation. The oxidation of hydroxyimines was determined in liver microsomes from humans, untreated rats and rats treated with CYP inducing agents (dexamethasone (CYP3A), phenobarbital (CYP2B), 3-methylcholantrene (CYP1A)) (Pelkonen *et al.* 1998). The half-lives of the oxidation reactions were determined for both **1** and **2** and the formation of ketoprofen also for **1**. During the oxidation of **2** two major metabolites, nabumetone and 6-methoxy-2-naphthylacetic acid (6-MNA) were formed but were not quantified due to their fast metabolism to several other metabolites.

The oxidation of **2** occurred more than 1000-fold faster than that of **1** with half-lives of 2 to 58 minutes and 72 to 167 hours, respectively (Table 5.3). The degradation of **2** was the fastest with 3-methylcholanthrene-treated microsomes with the half-life of 2 min followed by phenobarbital- and dexamethasone-treated rat microsomes, respectively. For both compounds **1** and **2** the reaction was the slowest in control rat microsomes ($t_{1/2}$ values were 10020 and 58 min, respectively).

Table 5.3. Half-lives of the oxidation of **1** and **2** in liver microsomes from untreated (CTRL), dexamethasone (DEX)-, 3-methylcholanthrene (3-MC)- and phenobarbital (PB)-treated rats and from humans (mean ± SD, n = 3, unless otherwise mentioned).

Microsomes	$t_{1/2}$ **of 1 min** [n=1]	$t_{1/2}$ **of 2 min**
Without microsomes	-[a]	-[a]
CTRL	10020	58 ± 3.8
DEX	4320	46 ± 5.0
PB	5040	28 ± 2.7
3-MC	6000	2.0 ± 0.16
Human	5016	51± 3.6

[a] No oxidation was detected during incubation

The formation of ketoprofen from **1** was also affected by enzyme induction, the most efficient reaction being mediated by dexamethasone pretreated rat liver microsomes (Table 5.4). These oxidation reactions did not occur without microsomes or NADPH, proving that the oxidation reactions are catalyzed by microsomal oxidative enzymes.

Table 5.4. Formation of ketoprofen in liver microsomes from untreated (CTRL), dexamethasone (DEX)-, 3-methylcholanthrene (3-MC)- and phenobarbital (PB)-treated rats and from humans.[n=1]

Incubation conditions	**1**[a]
CTRL	0.12
- NADPH	0.02
DEX	6.72
- NADPH	0.48
+ ketoconazole	2.50
3-MC	1.46
- NADPH	0.14
PB	1.39
- NADPH	0.10
Human	0.80 [b]
Without microsomes	-[c]

[a] Results are expressed as nmol of metabolite (mg of protein)$^{-1}$ (24h)$^{-1}$. [b] Results are expressed as nmol of metabolite (mg of protein)$^{-1}$ (4h)$^{-1}$ [c] No formation of ketoprofen was observed.

The difference in degradation rates between **1** and **2** is significant (Table 5.3). This is likely due to CYP substrate specificity of the two compounds. Since the active site of CYP enzymes is negatively charged, **1** bearing a negative charge is suggested to be poor substrate of CYPs. Also the aromatic rings enhance the steric hindrance around the hydroxyimine structure and may also explain the slow oxidation rate. Since the oxidation of **2** was much more effective compared to **1**, the metabolism of **2** was investigated in detail using human recombinant CYP enzymes *in vitro*.

Formation of nabumetone in human recombinant CYP enzymes. Incubation of 100 µM **2** was performed with eight different human CYP enzymes. All tested CYP forms (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) metabolized **2** to nabumetone (Table 5.5). The nabumetone formation rate was most rapid with CYP3A4 and CYP2A6 with rates of 5.6 and 5.4 nmol of metabolite (mg of protein)$^{-1}$ (60 min)$^{-1}$, respectively. According to these results it is obvious that the enzymatic oxidation of hydroxyimine is catalyzed by several human CYP enzymes *in vitro*.

All but one CYP form (CYP2C9) followed Michaelis-Menten kinetics and the parameters K_m and V_{max} could be determined. Kinetic studies indicated that CYP3A4 possessed the highest V_{max} (1600 min^{-1}) and CYP2B6 the lowest (290 min^{-1}) (Table 5.5). K_m values of **2** varied between 40 μM (CYP2E1) and 200 μM (CYP3A4). According to the specificity constant (V_{max}/K_m), the most efficient enzymes were CYP2A6 and CYP2E1 with specificity constants of 11.9 μM^{-1} min^{-1} and 11.0 μM^{-1} min^{-1}, respectively.

Table 5.5. Formation of nabumetone from nabumetone hydroxyimine (**2**) in human recombinant enzymes (mean ± SD, n = 2) and enzyme kinetic parameters for nabumetone hydroxyimine (mean, n = 3)

Recombinant enzyme	**Formation of nabumetone from 2**[a]	**K_m [μM] of 2**	**95% confidence intervals**	**V_{max}**[b]	**95% confidence intervals**	**V_{max}/K_m**
CYP1A2	2.71 ± 0.78	180	0-430	670	140-1190	3.7
CYP2A6	5.42 ± 0.20	90	60-120	1070	900-1240	11.9
CYP2B6	4.65 ± 0.09	70	40-110	290	30-350	4.14
CYP2C9	1.43 ± 0.04	-[c]		-[c]		-[c]
CYP2C19	4.5 ± 0.52	60	30-90	380	300-450	6.3
CYP2D6	3.96 ± 0.06	90	30-140	870	630-1110	9.7
CYP2E1	4.02 ± 0.01	40	30-60	440	390-480	11
CYP3A4	5.62 ± 0.14	200	30-360	1600	780-2400	8

[a] Results are expressed as nmol of metabolite (mg of protein)$^{-1}$ (60 min)$^{-1}$. [b] pmol nabumetone (nmol CYP)$^{-1}$ (min)$^{-1}$, [c] kinetics does not follow Michaelis-Menten kinetics

Determination of nitrite. The release of nitric oxide from **2** during the incubation in microsomes from dexamethasone induced rats was determined as nitrite via nitrate reductase by the standard Griess reaction (Jousserandot *et al.* 1998). Nitric oxide was formed during the 4 h incubations at almost equimolar quantities, as nabumetone was formed (11.0 ± 0.8 and 13.0 ± 0.24 $nmol^{n=2}$, respectively) and it was not formed without microsomes or without **2**. Although small quantities of other unidentified metabolites were formed during the incubation, this study does demonstrate that nitric oxide is formed simultaneously with nabumetone during incubation.

Conversion of nabumetone hydroxyimine to nabumetone *in vivo*. The conversion of **2** to nabumetone and 6-MNA, the main metabolite of nabumetone, was demonstrated *in vivo* in rats after per os administration. Other minor metabolites formed were not assayed. Since 6-MNA is partially conjugated with glucuronic acid in its metabolism (Davies 1997, Haddock *et al.* 1984), the amount of 6-MNA was determined after the alkaline hydrolysis (Mikami *et al.* 2000) to break the ether bond of the glucuronide. The amounts of **2** and nabumetone were determined without hydrolysis, since **2** hydrolyzes to nabumetone under alkaline hydrolysis conditions.

The recovery of the extractions from urine, determined by using naproxen as an internal reference, was high with an average of 97 % (from 70 to 100 %). The variance of urine volumes between rats was substantial with an average of 17.9 ± 8.5 ml (from 8.9 to 39.9 ml). In general, the variation between rats was quite high, whether assessed as nmol/total urine sample or as nmol/ml of urine.

The amount of 6-MNA in Group 1 administered with **2** was 23 % compared to that of in Group 2, which were administered with nabumetone (Table 5.6). The combined quantity of the nabumetone and 6-MNA in the total urine samples of Group 1 was 37 % of the quantity of 6-MNA in Group 2 (8.4 ± 2.9 and 23.0 ± 8.3 nmol, respectively) revealing that oxidation of **2** to nabumetone occurs also *in vivo*. This bioconversion is thought to be enzymatic after absorption, not chemical in GI-tract. Since the pH of the fed rat stomach is approximately 4.5 (Davies and Morris 1993) and the relatively large volume of oil administered can increase the gastric pH, **2** is suggested to be stable enough to be absorbed intact ($t_{1/2}$ approx. 16 h at pH 5.0). The reason for unchanged **2** in the rat urine samples of Group 1 is probably due to the high dose of **2**, which may have caused saturation of the CYP enzymes in the liver. Thus, a part of the prodrug is secreted as intact prodrug. To some extent, the situation is the same in samples of Group 2 rats, in which the unchanged nabumetone was also presented in urine. **2** was also found to be stable enough in rat urine at 37ºC ($t_{1/2}$ = 69 ± 14 h (n = 4)). No 6-MNA and only small amounts of nabumetone was formed during the incubation of **2** in rat urine at 37ºC. In summary, the data shows that **2** is converted to both nabumetone and 6-MNA in rats *in vivo*.

Table 5.6. Formation of nabumetone and 6-MNA from nabumetone hydroxyimine (**2**) *in vivo* in rat (mean ± SD, n = 3)

Animal group	**6-MNA nmol/total urine / nmol/ml of urine**	**2 nmol/total urine / nmol/ml of urine**	**Nabumetone nmol/total urine / nmol/ml of urine**
Group 1[a]	5.2 ± 1.7 / 0.23 ± 0.06	46.7 ± 32.3 / 1.8 ± 1.0	3.3 ± 1.6 / 0.15 ± 0.08
Group 2[b]	23.0 ± 8.3 / 1.48 ± 0.75	n.d.[d]	9.3 ± 4.9 / 0.59 ± 0.46
Group 3[c]	n.d.[d]	n.d.[d]	n.d.[d]

[a] Rats received 105 mg /kg of **2**, [b] Rats received 100 mg / kg of nabumetone, [c] CTRL rats received 2 ml of turnip rape oil, [d] not detected

Determination of ALAT and CRP. NO plays an important physiological role in the liver, the effect being either protective or toxic depending on the source of NO (Baumann and Gauldie 1994). The levels of alanine aminotransferase (ALAT) and C-reactive protein (CRP) in rat serum samples were assayed to determine whether administration of **2** and the released NO could evoke tissue damage in liver. Damage to liver cells leads to the release of acute phase protein ALAT and inflammation, reflected by elevated CRP levels in serum (Baumann and Gauldie 1994).

The levels of ALAT and CRP were similar in groups 1-3 and no differences between rats administered with nabumetone or **2** and control rats were detected. ALAT values were 70 ± 11, 70 ± 13 and 71 ± 11 U/l for groups 1, 2 and 3, respectively. CRP values were 4.2 ± 0.4 mg/l for each group. This result suggests that no overt acute toxic reaction occurred in rats fed with **2**.

5.3 Conclusions

Hydroxyimine derivatives of ketones offer a feasible tool to modify properties of drugs containing the ketone functionality. The results show that nabumetone hydroxyimine (**2**) was oxidized to nabumetone *in vitro* by microsomal CYP enzymes. Activation of **2** to nabumetone and other metabolites occurred also *in vivo* in rats. Urine samples of **2** treated rats contained nabumetone, 6-MNA and also **2** probably due to treatment with

high dose of prodrug causing the saturation of liver enzymes. The present study shows that the hydroxyimine structure is activated to the corresponding ketone both *in vitro* and *in vivo* and the released nitric oxide causes no acute liver toxicity after peroral administration in rats. Detailed studies on each oxime derivative of hydroxyimine are needed for the full characterization of the physicochemical properties, especially chemical stability and the activation of prodrugs. The present study reveals that the hydroxyimine is a potential intermediate prodrug structure especially for ketone drugs, and can easily been further derivatized using appropriate pro-moieties depending upon the purpose of prodrug application.

5.4 Methods

Synthesis. The ^{1}H NMR parameters have been solved precisely with the PERCHit iterator (Laatikainen *et al.* 1996) under PERCH software version 2004 (PERCH Solutions Ltd, Kuopio, Finland).

2-{3-[(*E,Z*)-(hydroxyimino)(phenyl)methyl]phenyl}propionic acid (1). 2-(3-benzoylphenyl)propanoic acid (2.0 g, 7.86 mmol), hydroxylamine hydrochloride (1.52 g, 21.9 mmol) and pyridine (1.76 ml, 21.9 mmol) were dissolved in dioxane (50 ml) and refluxed for 24 h. Solvent was evaporated and the residue was diluted with 40 ml saturated Na_2CO_3. The obtained solution was washed twice with diethylether (40 ml). The water phase was acidified with concentrated HCl and extracted with diethylether (3 × 30 ml). The organic phase was dried over Na_2SO_4 and evaporated to dryness to give a mixture of **1a** 2-{3-[(*E*)-(hydroxyimino) (phenyl)methyl]phenyl}propanoic acid and **1b** 2-{3-[(*Z*)-(hydroxyimino)(phenyl)methyl] phenyl}propanoic acid as a white solid (1.59 g, 75 %, E:Z ratio 50:50). **1a**: ^{1}H NMR ($CDCl_3$) δ 7.79 (1H, t, $^{4}J_{HH}$=1.79 and 1.76), 7.43 (5H, bs), 7.35 (1H, dt, J=7.71 and 1.79), 7.24 (1H, t, J=7.71, 7.81), 7.02 (1H, dt, J= 7.81 and 1.76), 3.83 (1H, q, J=7.18), 1.60 (3H, d)); ^{13}C NMR: δ 178.84 s, 157.26 s, 140.48 s, 136.17 s, 132.38 s, 129.45 d (2C), 128.95 d, 128.56 d, 128.21 d (2C), 128.13 d 126.98 d, 45.49 d, 17.60 q. **1b**: ^{1}H NMR ($CDCl_3$) δ 7.46-7.30 (9H, m), 3.80 (1H, q, J=7.2), 1.53 (3H, d); ^{13}C NMR: δ 179.64 s, 157.39 s, 140.03 s, 135.91 s, 132.76 s, 129.69 d, 129.50 d, 129.07 d, 128.50 d, 128.44 d (2C), 128.03 d (2C), 45.20 d, 17.94 q. ESI-MS: 268.0 (M – 1). Anal. ($C_{16}H_{15}NO_3$) C, H, N.

(2*E*)-4-(6-methoxy-2-naphthyl)butan-2-one oxime and (2*Z*)-4-(6-methoxy-2-naphthyl) butan-2-one oxime (2). 4-(6-methoxy-2-naphthyl)butan-2-one (1 g, 4.38 mmol), hydroxylamine hydrochloride (609 mg, 8.76 mmol) and pyridine (707 µl, 8.76 mmol) were dissolved in ethanol (22 ml) and refluxed for 24 h. Solvent was evaporated and the residue was diluted with Et_2O. The obtained solution was washed with saturated Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Flash chromatography on SiO_2 (petrol ether:ethyl acetate 4:1) gave a mixture of **2a**, (2*E*)-4-(6-methoxy-2-naphthyl)butan-2-one oxime and **2b**, (2*Z*)-4-(6-methoxy-2-naphthyl)butan-2-one oxime as a white solid (total yield 1 g, 4.12 mmol, 94 %). **2a**: ^{1}H NMR ($CDCl_3$) δ 8.20 (1H, bs), 7.664 (1H, d, J=8.56), 7.662 (1H, d, J=8.82), 7.55 (1H, d, $^4J_{HH}$=1.80), 7.29 (1H, dd, J=8.56 and 1.80), 7.12 (1H, dd, J=8.82 and 2.56), 7.10 (1H, d, $^4J_{HH}$=2.56), 3.90 (3H, s), 2.96 (2H, dd*, J=10.36 and 5.83), 2.58 (2H, dd*), 1.93 (3H, s); ^{13}C NMR: δ 158.03 s, 157.30 s, 136.26 s, 133.15 s, 129.12 s, 128.96 d, 127.54 d, 126.93 d, 126.28 d, 118.77 d, 105.71 d, 55.30 q, 37.73 t, 32.59 t, 13.76 q. **2b**: ^{1}H NMR (CDCl3) δ 8.01 (1H, bs), 7.674 (1H, d, J= 8.37), 7.674 (1H, d, J= 8.96), 7.59 (1H, d, $^4J_{HH}$=1.70), 7.34 (1H, dd, J=8.37 and 1.70), 7.12 (1H, dd, J=8.96 and 2.60), 7.11 (1H, d, $^4J_{HH}$=2.60), 3.91 (3H, s), 2.97 (2H, dd*, J=9.85 and 6.25), 2.75 (2H, dd*), 1.83 (3H, s); ^{13}C NMR: δ 158.54 s, 157.31 s, 136.40 s, 131.17 s, 129.11 s, 128.99 d, 127.57 d, 126.90 d, 126.23 d, 118.76 d, 105.71 d, 55.30 q, 31.51 t, 30.62 t, 20.36 q. *as typically, this Z-CH_2CH_2-Y is analysed as AA'BB'-spinsystem due to strong trans orientation (here the system is insensitive to geminal coupling and is not analysed). ESI-MS: 244.0 (M+1). Anal. $C_{15}H_{17}NO_2$: C, H, N.

HPLC analysis. HPLC analysis was performed using a Beckman HPLC system, which consisted of a Beckman System Gold Programmable Solvent Module 126, a Beckman System Gold Detector Module 166 (wavelength 254 nm for ketoprofen and **1**; 225 nm for nabumetone and **2**) and Beckman System Gold Autosampler 507e and a Zorbax Eclipse SB-C18 (4.6 mm × 150 mm, 5 µm) analytical column (Agilent Technologies Inc., Little Falls, Wilmington, DE, USA). Separations were performed with isocratic elution by using a mobile phase consisting of either 90 % (v/v) acetonitrile and 2.2 % (v/v) acetic acid with a ratio of 50:50 (ketoprofen and **1**) or 90 % (v/v) acetonitrile and 20 mM phosphate buffer (pH 2.5) with a ratio of 40:60 (nabumetone and **2**) at a flow rate of 1.0 ml/min at 25ºC.

Preparation of human hepatic microsomes. Human liver samples were obtained from the University Hospital of Oulu as surplus from kidney transplantation donors (approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Oulu, Finland). The livers were transferred to ice immediately after surgical excision, cut into pieces, snap-frozen in liquid nitrogen and stored at -80°C until the microsomes were prepared by standard differential ultracentrifugation. The metabolic characteristics of these microsomes have been published earlier (Turpeinen *et al.* 2005). A weight-balanced microsomal pool of seven liver microsomal preparations which have been extensively characterized for primary metabolic screening was employed. Baculovirus-insect cells expressing human CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 were purchased from Gentest Corp (Woburn, MA, USA).

Preparation of rat hepatic microsomes. Male wistar rats (150-200 g) were treated i.p. for four days with either phenobarbital (80 mg/kg/day in 0.9% NaCl solution), 3-methylcholanthrene (20 mg/kg/day in olive oil), dexamethasone (50mg/kg/day in olive oil) or 0.5 ml olive oil (control rats) (Jousserandot *et al.* 1998). Microsomes were prepared as previously reported (Pearce *et al.* 1996) and stored at -80°C until use. Protein concentrations were determined by using the Biorad Protein Assay (Bio-Rad, Hercules, CA, USA), and cytochrome P450 contents were determined as reported earlier (Omura and Sato 1964).

***In vitro* metabolism and enzyme kinetic analyses of hydroxyimines.** The *in vitro* metabolism of **1** and **2** was evaluated in isolated liver microsomes from humans, from untreated rats and from rats treated with inducing agents. A typical incubation mixture, in a final volume of 150 µl-1500 µl, contained 20 µM prodrugs in phosphate buffer for **1** and in ethanol for **2**, 50 mM sodium phosphate buffer (pH 7.4) and 100µl liver microsomes. Ethanol concentration was 1 % or less. The reaction was started by the addition of a NADPH-regenerating system (1.15 mM NADP, 12.5 mM isocitric acid, 56.25 mM KCl, 187.5 mM Tris-HCl, pH 7.4 12.5 mM $MgCl_2$, 0.0125 mM $MnCl_2$ and 1.2 U isocitric acid dehydrogenase) following 2 minutes of pre-incubation at 37°C. In blank solutions liver microsomes and in samples without NADPH, the NADPH solution was replaced with the same volume of water. The reactions were terminated by the addition of

the same amount of ice-cold acetonitrile as the sample. The samples were kept on ice, centrifuged for 15 min at 11000 rpm, and the supernatant was analyzed by the HPLC.

Incubations of **2** with human recombinant P450s were performed using conditions described before for liver microsomes, except the mixture contained 5 pmol of P450 (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) instead of microsomes. Enzyme kinetic studies of oxidation of **2** to nabumetone by CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 were also conducted in the same conditions using 10 µM-200 µM of **2** and incubating for 60 min.

Pseudo-first-order half-lives ($t_{1/2}$) for the enzymatic degradation of prodrug at different liver microsomes were calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug versus time.

Determination of nitrite. For the determination of nitrite (NO_2^-), enzymatic incubations in microsomes were performed similarly as described above, except that 100 µM solution of **2** was used and nitrate reductase (60mU from *Aspergillus Niger*) was added after three hours to incubation medium to reduce available nitrate (NO_3^- to nitrite NO_2^-) and the incubation was continued for 1 hour. After four hours of incubation, the reaction was stopped by heating samples for 5 min at 100°C to precipitate proteins and to eliminate the bleaching effect of NADPH on the Griess reaction. The samples were centrifuged for 15 min at 11000 rpm. The concentration of nitrite in the supernatants were determined by the Griess reaction, where 210 µl of sulfanilamide (1.8% w/v) solution in 1M HCl solution and 140 µl of N-(1-naphthyl)ethylenediamide dihydrochloride (0.3% w/v) solution in 1M HCl solution were added to 700 µl of the supernatants before analysis at an absorbance of 548 nm. For the standard curve, solutions of known nitrite concentrations (0 - 40 µM sodium nitrite solution) were prepared into the mixture of 50 mM phosphate buffer pH 7.4 and 5 mM $MgCl_2$ solution, and the absorbance was measured after treatment with Griess reagents. Quantitations of total NO_2^- were obtained from the resulting standard curve.

Animal treatments. Nabumetone and **2** were dissolved in 10.0 mg/ml and 10.5 mg/ml turnip rape oil, respectively. Wistar male rats (aged, weight 221-339 g) were randomly assigned into three groups of five rats. Rats were adapted for 48 h to metabolic cages.

After 24 h, urine and feces were collected and rats were treated by administering either **2** (100 mg/kg, Group 1), nabumetone (105 mg/kg, Group 2) or oil only (2 ml / rat, Group 3) by gavage. After 24 h of drug administration, the rats were decapitated and urine, blood and liver samples were collected and frozen at -78ºC.

Rat urine sample preparations. Rat urine samples were frozen and stored at -78ºC until analysis. **2**, nabumetone and 6-MNA were analyzed by diluting 250 µl of urine sample with 210 µl of water and vortex mixed. 40 µl of internal standard solution (naproxen) was added. **2** and nabumetone were applied to the C18 solid-phase extraction cartridges (Discovery DSC-18; Supelco, Bellefonte, PA, USA), dried by aspiration of air, eluted with 3 ml of 40 % hexane in ethyl acetate and evaporated to dryness under a nitrogen stream at 40 ºC. 250 µl of 1 M NaOH-solution was added to the sample containing 6-MNA, vortex mixed and allowed to stand for 1 h at room temperature. 250 µl of 1 M HCl-solution was added, vortex mixed and applied to the C18 solid-phase extraction cartridges. After drying the analytes were eluted with 3 ml of 40 % hexane in ethyl acetate and evaporated to dryness under a nitrogen stream at 40 ºC. After evaporation, the samples were dissolved to 250 µl of 50 % acetonitrile in water and measured by HPLC. The quantities of analytes were determined using external, rat urine spiked standards (nabumetone, **2** and 6-MNA) and the recovery of the extractions was determined with internal standard (naproxen).

Stability of 2 in rat urine. The rate of hydrolysis of **2** was determined in rat urine at 37°C. 160 µL of the solution of **2** in 50 % acetonitrile (0.75 µg/ml) was placed to the test tube and evaporated to dryness under a nitrogen stream at 40ºC. 2 ml of preheated rat urine was added to the tubes and the solutions were placed in a thermostatically controlled water bath at 37ºC. 230 µl samples of solution were taken at appropriate time intervals. 250 µl of water and 20 µl of naproxen solution (0.51 µg/ml in 50 % acetonitrile) was added to the samples, vortex mixed and applied to the preconditioned and equilibrated C18 solid-phase extraction cartridges (Discovery DSC-18; Supelco, Bellefonte, PA, USA). The test tube was washed with 500 µl of water, added to the cartridge and dried by aspiration of air. The analytes were eluted with 3 ml of 40 % hexane in ethyl acetate and evaporated to dryness under a nitrogen stream at 40 ºC.

After evaporation, the samples were dissolved to 250 µl of 50 % acetonitrile in water and measured by HPLC as previously described with rat urine samples.

Determination of ALAT and CRP from rat liver samples. The analysis of amounts of alanineaminotransferase (ALAT) and C-reactive protein (CRP) was performed at the Laboratory Centre of Kuopio University Hospital. The ALAT assay was performed by a kinetic method (by ECCLS/IFCC guideline) and CRP assay by immunoturbidometrics.

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6 1-CHLOROETHYL PHOSPHATES AND PHOSPHORAMIDATES AS BUILDING BLOCKS OF PRODRUGS *

Abstract: A versatile, efficient and simple method for the preparation of various 1-chloroethyl phosphates and phosphoramidates was developed. The protected chlorophosphate or phosphoramidate was synthesized to the vinyl derivative under mild conditions, followed by the conversion to the 1-chloroethyl phosphate or phosphoramidate by dry HCl-gas resulting in good to excellent yields. This method was further improved by the use of microwaves. The protected vinyl chlorophosphates underwent fast microwave-assisted conversion to the 1-chloroethyl phosphates in the presence of HCl with excellent yields up to 900-times faster than at standard NTP conditions. 1-Chloroethyl phosphates and phosphoramidates could be used as building blocks for the synthesis of novel ethylidene linked phosphate prodrugs.

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Kumpulainen H, Järvinen T, Saari R, Lehtonen M, Vepsäläinen, J. An efficient strategy for the synthesis of 1-chloroethyl phosphates and phosphoramidates: The Journal of Organic Chemistry 70: 9056-8, 2005. Copyright 2007 American Chemical Society

and

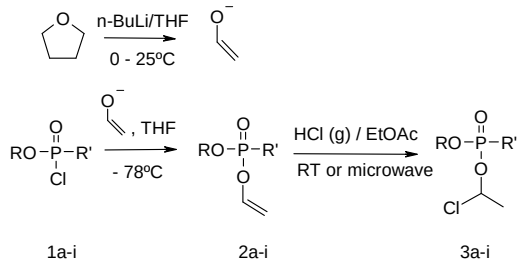
Kumpulainen H, Saari R, Lehtonen M, Rautio J, Järvinen, T, Vepsäläinen, J. Convenient microwave-assisted synthesis of 1-chloroethyl phosphates: Tetrahedron Letters 47: 2003-2004, 2006. Copyright 2007 Elsevier Science.

6.1 Introduction

Phosphates and phosphoramidates are widely used as prodrug structures to enhance water-solubility (Fleisher *et al.* 1996) or to increase the therapeutic potential of a parent drug (Chang *et al.* 2001, Egron *et al.* 2003, Freel Meyers and Borch 2000). The phosphoramidates are also used to synthesize phosphate esters with different protecting groups by the replacement of an amide with an ester group (Alberg *et al.* 1992, Mathé *et al.* 1998). In addition, they can be used to synthesize a monoester phosphate prodrug by hydrolysing the phosphoramide bond (Garrison and Boozer 1968). Phosphate promoieties are often attached to the parent drug via an oxymethyl spacer group (Krise *et al.* 1999, Mäntylä *et al.* 2004, Ueda *et al.* 2003), but this can give rise to a problem since the highly toxic formaldehyde (Slikker *et al.* 2004) is released systemically during metabolism of the oxymethyl group in the body (Nudelman *et al.* 2001, Nudelman *et al.* 2005). In order to overcome this drawback, we were interested in developing ethylidene linked prodrugs, which are metabolized to the less toxic acetaldehyde. Previously, an ethylidene spacer group has been used in (acyloxy)alkyl prodrugs (Alexander *et al.* 1991, Sum *et al.* 1999), but no descriptions of ethylidene linked phosphate prodrugs to increase the water-solubility of drugs have been published. The compounds described in the present study could be used as starting materials for the preparation of ethylidene linked phosphate prodrugs.

The ethylidene phosphate prodrugs could be prepared via addition of 1-chloroethyl phosphates or phosphoramidates to an appropriate functional group (e.g. hydroxyl or amine group) of the drug molecule. However, there is no efficient and practical method to synthesize 1-chloroethyl phosphates or phosphoramidates. The 1-chloroethyl derivatives of carboxylic acids are traditionally prepared from acetaldehyde and the corresponding acid chloride using $ZnCl_2$ as the catalyst (Bodor *et al.* 1980). However, this method was not successful when applied for the synthesis of 1-chloroethyl phosphates. The only reported approach to synthesize 1-chloroethyl phosphates is a method in which diethyl 1-chloroethyl phosphate is synthesized from ethyl phosphorodichloridate using hazardous chemicals and methods (chlorine gas, mercury vapour lamp) (Allen 1960). In this present study, an efficient and practical synthetic approach for the preparation of 1-chloroethyl phosphates and phosphoramidates was

developed. Protected chlorophosphates or phosphoramidates are synthesized to the vinyl derivative under mild conditions, followed by the conversion to the 1-chloroethyl phosphate or phosphoramidate by dry HCl-gas either at NTP conditions or in a microwave-oven.

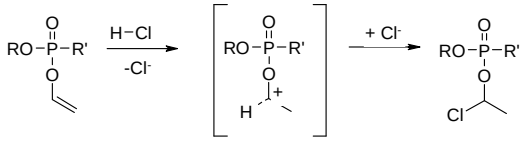


Scheme 6.1. Synthesis of vinyl (2a-i) and 1-chloroethyl phosphates and phosphoramidates (3a-i).

6.2 Results and Discussion

The desired vinyl intermediates were prepared from the corresponding chlorophosphates **1**, which are commercially available (**1b-c**, **e**, and **g**) or prepared by the known method from phosphorous trichloride (Mark and van Wazer 1964, Steinberg 1949) (**1a**, **d**, **f** and **h**) or from diethyl phosphorodichloridate (Quin and Jankowski 1994) (**1i**). Nine different vinyl phosphates or phosphoramidates were prepared by treating **1** with the enolate of acetaldehyde (Stowell and Widlanski 1994) with reasonably good yields (Scheme 6.1, Table 6.1). The vinylation occurred under mild conditions, which makes it a suitable method for a wide variety of molecules.

The electrophilic addition reaction of HCl to the vinyl double-bond was initially obtained by bubbling dry HCl gas through a solution of **2** in dry ethyl acetate. The reaction occurs via an attack of H^+ to yield an intermediate carbo-cation, which quickly undergoes a reaction with a negative halide ion to yield an alkyl halide (Scheme 6.2).



Scheme 6.2. The electrophilic addition reaction of HCl to vinyl phosphate

The results listed in Table 6.1 show that there are major differences in the reaction times and conditions depending on the chemical nature of the phosphate substituents. The compounds **3h** and **3i** were readily obtained under mild conditions, but on the other hand, the diphenyl derivative **2g** did not react at all, even though several common catalysts and over pressure (5 atm) were tested. In order to determine, whether the microwave-assisted synthesis would provide some benefits compared to the method at NTP conditions, the addition reaction of HCl to vinyl ester of phosphates (Scheme 6.1) was studied in a microwave oven.

Table 6.1. Synthesis of vinyl and chloroethylidene phosphates and phosphoramidates[a]

				NTP conditions:		**Microwave-assisted:**	
R		**R'**	**2**: yield %	**3**: yield %	Time (h) [b]	**3**: yield %	Time (h)
1a:	Me	OMe	**2a**: 52	**3a**: 86	29 (12) [c]	**3a**: - [f]	- [f]
1b:	Et	OEt	**2b**: 64	**3b**: 91	24 (7) [c]	**3b**:99	0.25
1c:	Cl_3Et	OCl_3Et	**2c**: 68	**3c**: 66	912 (195) [c]	**3c**:95	1
1d:	Me	OPh	**2d**: 54	**3d**: 87	500 (50) [c]	**3d**:95	0.75
1e:	(2,6-Me_2)Ph	O-(2,6-Me_2)Ph	**2e**: 35	**3e**: 92	480 (90) [c]	**3e**:96	0.5
1f:	-$CH_2CH_2CH_2O$-		**2f:** 76	**3f**: 98	30 (12)[c]	**3f**: 99	0.125
1g:	Ph	OPh	**2g**: 70	**3g**: - [d]	- [d]	**3g**:98	1
1h:	Bz	OBz	**2h**: 30	**3h**: 68	48[e]	**3h**: - [f]	- [f]
1i:	Me	NEt_2	**2i**: 88	**3i**: 91	21[e]	**3i**: - [f]	- [f]

[a] Yields without purification. [b] The reaction times are the total reaction times of step 2, with the times of HCl-bubbling in parenthesis. [c] Reaction conditions: HCl-bubbling, R.T. [d] Did not react under any kind of conditions. [e] Reaction conditions: EtOAc saturated with HCl, 4ºC. [f] Not examined

The microwave-assisted synthesis of compounds **3b-g** was initiated using trichloroethylvinyl phosphate, which had the longest reaction time under NTP conditions. Trichloroethylvinyl phosphate **2c** was dissolved in ethyl acetate saturated with dry HCl-gas. The mixture was heated at 176 ºC and 20 bars in a microwave oven for 60 minutes, after which 95 % of **2c** was converted to 1-chloroethyl phosphate **3c**. By comparison, the synthesis under NTP conditions resulted in a yield of 68 % after 195 hours of bubbling (Table 6.1). After these promising results, the microwave-assisted

reaction was carried out with diphenylvinyl phosphate **2g**, which did not react at all using the previously described method, even if catalysts or higher pressure were used. With microwave-assisted heating for 60 min at 173 ºC and 20 bar, 98% of diphenylvinyl phosphate **2g** was converted to the corresponding 1-chloroethyl phosphate **3g**. Also, all of the other tested vinyl phosphates reacted under microwave conditions (Table 6.1) to give excellent yields (>95%) with significantly shorter reaction times.

All 1-chloroethyl products, except for **3h**, could be purified and isolated with silica gel column chromatography, but some of the products partially decomposed during the purification procedure. Since the crude products from the synthesis of **3** did not contain any major impurities according to ^{1}H-, ^{13}C- and ^{31}P-NMR, these building blocks should be able to be used without further purification. Thus, the yields of **3a-i** presented in Table 6.1 are reported without purification and are estimated from the actual chemical yields according to purity of ^{1}H- and ^{31}P-NMR spectra, but the NMR data, GC-MS analyses and elemental analyses (CHNS) were obtained from the purified products.

6.3 Conclusions

A novel method for the synthesis of 1-chloroethyl phosphates and phosphoramidates was developed. The synthetic route was started with vinylation of chlorophosphate or phosphoramidate as the starting material, followed by addition of hydrogen chloride to the vinyl double bond to yield 1-chloroethyl phosphate or phosphoramidate. This method was found to be versatile and a simple way to synthesize phosphates and phosphoramidates with various protecting groups and is thus applicable for a wide range of molecules. In addition, increased yields, shortened reaction times and the successful synthesis of otherwise unavailable reaction products can be achieved using microwave-assisted synthesis of 1-chloroethyl phosphates from vinyl phosphates. 1-Chloroethyl phosphates and phosphoramidates may further be used as building blocks for the synthesis of ethylidene linked prodrugs.

6.4 Methods

Synthesis. The ^{1}H NMR parameters have been solved precisely with the PERCHit iterator (Laatikainen *et al.* 1996) under PERCH software version 2004 (see Figure 6.1) (PERCH Solutions Ltd, Kuopio, Finland). Electron impact or positive chemical ionization mass spectra were acquired by a gas chromatograph- mass spectrometry system. The yields of the reactions are illustrated in Table 6.1.

A general method for the preparation of vinyl phosphates 2. To a 100 ml round-bottom flask dry THF (50 ml) was added under argon and cooled to 0ºC followed by addition of 1.6 M n-butyl lithium (15 ml, 24 mmol) in hexane at 0ºC. The reaction mixture was stirred for 0.5 h at 0ºC and then left overnight at 25ºC. After 18 h without cooling, this enolate of acetaldehyde was added dropwise to the chlorophosphate or phosphoramidate **1** (25.2 mmol, 1.05 equiv) in THF (5 ml) during 15 minutes at -78ºC. After the addition was complete, the reaction mixture was allowed to reach room temperature over 45 minutes and the solvents were evaporated. The residue was dissolved in CH_2Cl_2 (50 ml), washed with 10 % sodium phosphate buffer (pH 7.0, 5 × 15 ml), dried with Na_2SO_4 and evaporated to dryness. The residue was purified by column chromatography to yield vinyl derivative **2**. The chromatography purification conditions are described in detail along with a description of each molecule.

A general method for the synthesis of chloroethylidene phosphates 3 at RT conditions. To a 10 ml round-bottomed flask vinyl phosphate **2** (5.6 mmol) and EtOAc (2 ml) were added under argon. Dry gaseous HCl was bubbled through the solution (**2a-f**). In the case of **3h-i**, the solution of **2** in EtOAc was cooled to 4ºC followed by addition of EtOAc saturated with HCl (2 ml). The progress of the HCl-addition was monitored by measuring ^{1}H and ^{31}P NMR spectra from the reaction mixture. After the appropriate reaction time, the solvents were evaporated and the residue was purified by column chromatography. The reaction time and conditions varied between the reactions and are illustrated in detail in Table 6.1.

A general method for the synthesis of chloroethylidene phosphates in microwave oven. Ethyl acetate was saturated with dry HCl-gas by bubbling HCl through the solution for 15 min. Trichloroethylvinyl phosphate **2c** (100 mg, 0.26 mmol) was dissolved in 4 ml of saturated HCl-ethyl acetate solution. The mixture was heated at 176

ºC at 20 bar for 60 min in a Biotage Initiator microwave reactor. The progress of the HCl-addition was monitored by measuring ^{1}H and ^{31}P NMR spectra from the reaction mixture during the course of the reaction. After the appropriate reaction time, the solvents were evaporated to dryness. The reaction time and conditions varied between the reactions and are described in detail along with a description of each molecule.

Phosphoric acid dimethyl ester vinyl ester 2a: Colorless oil. Chromatography eluent EtOAc:petrol ether 2:3. ^{1}H NMR ($CDCl_3$) δ 6.579 (1H, ddd, J=13.52 Hz, 5.86 Hz, $^{3}J_{HP}$=6.52 Hz), 4.933 (1H, ddd, J=13.52 Hz, 2.17 Hz, $^{4}J_{HP}$=1.19 Hz), 4.601 (1H, ddd, J=5.86 Hz, 2.17 Hz, $^{4}J_{HP}$=2.67 Hz), 3.825 (6H, d, $^{3}J_{HP}$=11.24 Hz Hz); ^{13}C NMR ($CDCl_3$) δ 142.14 (d, $^{2}J_{CP}$ = 5.7 Hz), 100.11 (d, $^{3}J_{CP}$ = 10.2 Hz), 54.69 (d, $^{2}J_{CP}$ = 6.0 Hz); ^{31}P NMR ($CDCl_3$) δ -2.10. GC-MS *m/z* 151 (M-1).

Phosphoric acid diethyl ester vinyl ester 2b: Colorless oil. Chromatography eluent EtOAc:petrol ether 1:3. ^{1}H NMR ($CDCl_3$) δ 6.584 (1H, ddd, J=13.54 Hz, 5.87 Hz, $^{3}J_{HP}$=6.50 Hz), 4.901 (1H, ddd, J=13.54 Hz, 2.05 Hz, $^{4}J_{HP}$=1.19 Hz), 4.568 (1H, ddd, J=5.87 Hz, 2.05 Hz, $^{4}J_{HP}$=2.71 Hz), 4.174 (4H, dq, J=7.08 Hz, $^{3}J_{HP}$=8.28 Hz), 1.363 (6H, td, J=7.08 Hz, $^{3}J_{HP}$=1.01 Hz); ^{13}C NMR ($CDCl_3$) δ 142.17 (d, $^{2}J_{CP}$ = 5.6 Hz), 99.63 (d, $^{3}J_{CP}$ = 10.4 Hz), 64.33 (d, $^{2}J_{CP}$ = 5.8 Hz), 16.00 (d, $^{3}J_{CP}$ = 6.6 Hz); ^{31}P NMR ($CDCl_3$) δ -4.28. GC-MS *m/z* 180 (M).

Phosphoric acid bis-(2,2,2-trichloro-ethyl) ester vinyl ester 2c: Colorless oil. Chromatography eluent EtOAc:petrol ether 1:2. ^{1}H NMR ($CDCl_3$) δ 6.630 (1H, ddd, J=13.44 Hz, 5.78 Hz, $^{3}J_{HP}$=6.71 Hz), 5.082 (1H, ddd, J=13.44 Hz, 2.59 Hz, $^{4}J_{HP}$=1.30 Hz), 4.758 (1H, ddd, J=5.78 Hz, 2.59 Hz, $^{4}J_{HP}$=2.66 Hz), 4.680 (4H, d, $^{3}J_{HP}$=6.90 Hz); ^{13}C NMR ($CDCl_3$) δ 141.34 (d, $^{2}J_{CP}$ = 6.2 Hz), 102.17 (d, $^{3}J_{CP}$ = 10.6 Hz), 94.34 (d, $^{3}J_{CP}$ = 10.6 Hz), 77.42 (d, $^{2}J_{CP}$ = 4.5 Hz); ^{31}P NMR ($CDCl_3$) δ -7.59. GC-MS *m/z* 387 (M).

Phosphoric acid methyl ester phenyl ester vinyl ester 2d: Colorless oil. Chromatography eluent EtOAc:petrol ether 1:4. ^{1}H NMR ($CDCl_3$) δ 7.346 (2H, m, J=8.05 Hz, 7.40 Hz, $^{4}J_{HH}$=-1.74 Hz, $^{5}J_{HH}$= 0.77 Hz) , 7.218 (2H, m, J=8.05 Hz, $^{4}J_{HH}$=-1.20 Hz, -1.17 Hz, $^{5}J_{HH}$= 0.77 Hz, $^{4}J_{HP}$=1.19 Hz), 7.195 (1H, m, J=7.40, $^{4}J_{HH}$=-1.17), 6.634 (1H, ddd, J=13.50 Hz, 5.82 Hz and $^{3}J_{HP}$=6.59 Hz), 4.966 (1H, ddd, J=13.50 Hz, 2.27 Hz and $^{4}J_{HP}$=1.19 Hz), 4.825 (1H, ddd, J=5.82 Hz, 2.27 Hz and $^{4}J_{HP}$=2.80 Hz), 3.908 (3H, d, $^{3}J_{HP}$=11.51 Hz); ^{13}C NMR ($CDCl_3$) δ 150.36 (d, $^{2}J_{CP}$ = 6.9 Hz), 141.99 (d,

$^{2}J_{CP}$ = 5.9 Hz), 129.85, 125.47, 120.01 (d, $^{3}J_{CP}$ = 4.8 Hz), 100.70 (d, $^{3}J_{CP}$ = 10.5 Hz), 55.23 (d, $^{2}J_{CP}$ = 6.2 Hz); ^{31}P NMR ($CDCl_3$) δ -8.79. GC-MS *m/z* 215 (M + 1).

Phosphoric acid bis-(2,6-dimethyl-phenyl) ester vinyl ester 2e: Colorless oil. Chromatography eluent EtOAc:petrol ether 1:20. ^{1}H NMR ($CDCl_3$) δ 7.044-6.977 (6H, m), 6.661 (1H, ddd, J=13.49 Hz, 5.77 Hz and $^{3}J_{HP}$=5.78 Hz), 4.840 (1H, ddd, J=13.49 Hz, 2.19 Hz and $^{4}J_{HP}$=1.01 Hz), 4.525 (1H, ddd, J=5.77 Hz, 2.19 Hz and $^{4}J_{HP}$=3.91 Hz), 2.343 (s, 12H); ^{13}C NMR ($CDCl_3$) δ 147.99 (d, $^{2}J_{CP}$ = 8.6 Hz), 142.45 (d, $^{2}J_{CP}$ = 5.4 Hz), 130.29 (d, $^{3}J_{CP}$ = 3.4 Hz), 129.16 (d, $^{4}J_{CP}$ = 1.5 Hz), 125.54 (d, $^{5}J_{CP}$ = 1.7 Hz), 99.57 (d, $^{3}J_{CP}$ = 11.3 Hz), 17.00; ^{31}P NMR ($CDCl_3$) δ -14.69. GC-MS *m/z* 333 (M + 1).

2-vinyloxy-[1,3,2]dioxaphosphinane 2-oxide 2f: Colorless oil. Chromatography eluent EtOAc:petrol ether 1:1. ^{1}H NMR ($CDCl_3$) δ 6.611 (1H, ddd, J=13.56 Hz, 5.87 Hz and $^{3}J_{HP}$=7.47 Hz), 4.962 (1H, ddd, J=13.56 Hz, 2.28 Hz and $^{4}J_{HP}$=1.21 Hz), 4.637 (1H, ddd, J=5.87 Hz, 2.28 Hz and $^{4}J_{HP}$=2.12 Hz), 4.520-4.400 (4H, m), 2.390-2.287 (1H, m), 1.834-1.779 (1H, m); ^{13}C NMR ($CDCl_3$) δ 141.64 (d, $^{2}J_{CP}$ = 5.7 Hz), 100.22 (d, $^{3}J_{CP}$ = 9.9 Hz), 69.23 (d, $^{2}J_{CP}$ = 6.3 Hz), 25.42 (d, $^{3}J_{CP}$ = 7.3 Hz); ^{31}P NMR ($CDCl_3$) δ -11.15. GC-MS *m/z* 165 (M + 1).

Phosphoric acid diphenyl ester vinyl ester 2g: Light-yellow oil. Chromatography eluent EtOAc:petrol ether 1:4. ^{1}H NMR ($CDCl_3$) δ 7.451 (4H, m, J=8.20 Hz, 7.44 Hz, $^{4}J_{HH}$=-2.35 Hz, $^{5}J_{HH}$= 0.55 Hz), 7.290 (2H, m, J=7.44, $^{4}J_{HH}$=-1.04), 7.265 (4H, m, J=8.20 Hz, $^{4}J_{HH}$=-2.06 Hz, -1.04 Hz, $^{5}J_{HH}$= 0.55 Hz, $^{4}J_{HP}$=1.22 Hz), 6.845 (1H, ddd, J=13.34 Hz, 5.76 Hz and $^{3}J_{HP}$=6.82 Hz), 5.056 (1H, ddd, J=13.34 Hz, 2.38 Hz and $^{4}J_{HP}$=1.23 Hz), 4.825 (1H, ddd, J=5.76 Hz, 2.38 Hz and $^{4}J_{HP}$=2.79 Hz).

Phosphoric acid dibenzyl ester vinyl ester 2h: Colorless oil. Chromatography eluent EtOAc:petrol ether 1:4. ^{1}H NMR ($CDCl_3$) δ 7.386-7.314 (12H, m), 6.529 (1H, ddd, J=13.51 Hz, 5.85 Hz and $^{3}J_{HP}$=6.51 Hz), 5.083 (4H, d, $^{3}J_{HP}$=8.23), 4.862 (1H, ddd, J=13.51 Hz, 2.16 Hz and $^{4}J_{HP}$=1.23 Hz), 4.544 (1H, ddd, J=5.85 Hz, 2.16 Hz and $^{4}J_{HP}$=2.70 Hz); ^{13}C NMR ($CDCl_3$) δ 141.96 (d, $^{2}J_{CP}$ = 5.9 Hz), 135.40 (d, $^{3}J_{CP}$ = 6.9 Hz), 128.69, 128.62, 128.04, 100.23 (d, $^{3}J_{CP}$ = 10.4 Hz), 69.77 (d, $^{2}J_{CP}$ = 5.6 Hz); ^{31}P NMR ($CDCl_3$) δ 3.93. GC-MS *m/z* 305 (M + 1).

N,N-diethylphosphoramidic acid methyl ester vinyl ester 2i: Colorless oil. Chromatography eluent EtOAc:petrol ether 1:1. ^{1}H NMR ($CDCl_3$) δ 6.576 (1H, ddd,

J=13.60 Hz, 5.93 Hz and $^{3}J_{HP}$=6.73 Hz), 4.827 (1H, ddd, J=13.60 Hz, 1.76 Hz and $^{4}J_{HP}$=1.33 Hz), 4.505 (1H, ddd, J=5.93 Hz, 1.76 Hz and $^{4}J_{HP}$=2.21 Hz), 3.722 (3H, d, $^{3}J_{HP}$=11.32 Hz), 3.104 (4H, dq, J=7.11 Hz, $^{3}J_{HP}$=11.87 Hz), 1.122 (6H, t, J=7.11 Hz; ^{13}C NMR ($CDCl_3$) δ 142.39 (d, $^{2}J_{CP}$ = 5.3 Hz), 98.88 (d, $^{3}J_{CP}$ = 10.2 Hz), 53.05 (d, $^{2}J_{CP}$ = 5.7 Hz), 39.79 (d, $^{2}J_{CP}$ = 4.5 Hz), 14.17 (d, $^{3}J_{CP}$ = 1.5 Hz); ^{31}P NMR ($CDCl_3$) δ -9.14. GC-MS *m/z* 193 (M).

Phosphoric acid 1-chloro-ethyl ester dimethyl ester 3a: Colorless viscose oil. Chromatography eluent hexane:EtOAc 1:1. ^{1}H NMR ($CDCl_3$) δ 6.242 (1H, dq, J=5.62 Hz, $^{3}J_{HP}$=7.62 Hz), 3.837* (3H, d, $^{3}J_{HP}$=11.44 Hz), 3.802* (3H, d, $^{3}J_{HP}$=11.31), 1.856 (3H, dd, J=5.62 Hz, $^{4}J_{HP}$= 1.09 Hz); ^{13}C NMR ($CDCl_3$) δ 85.78 (d, $^{2}J_{CP}$ = 6.4 Hz), 54.82* (d, $^{2}J_{CP}$ = 6.1 Hz), 54.55* (d, $^{2}J_{CP}$ = 6.0 Hz), 27.54 (d, $^{3}J_{CP}$ = 8.4 Hz); ^{31}P NMR ($CDCl_3$) δ -0.76. GC-MS *m/z* 189 (M + 1), 153 (M - Cl). Anal. Calcd for $C_4H_{10}ClO_4P \cdot 0.7$ HCl: C, 22.44; H, 5.04. Found: C, 22.53; H, 4.76. *Due to their chiral center, OMe-signals have different chemical shifts.

Phosphoric acid 1-chloro-ethyl ester diethyl ester 3b: MW-conditions: 0.25 hours, 100 ºC, 6 bars. Yellow viscose oil. Chromatography eluent hexane:EtOAc 1:1. ^{1}H NMR ($CDCl_3$) δ 6.247 (1H, dq, J=5.61 Hz, $^{3}J_{HP}$=7.79 Hz), 4.201* (1H, ddq, $^{2}J_{HH}$= -10.07 Hz, J=7.10 Hz, $^{3}J_{HP}$= 7.79 Hz), 4.184* (1H, ddq, $^{2}J_{HH}$= -10.07 Hz, J=7.05 Hz $^{3}J_{HP}$=8.05 Hz), 4.159* (1H, ddq, $^{2}J_{HH}$= -10.13 Hz, J=7.08 Hz, $^{3}J_{HP}$= 8.08 Hz), 4.136* (1H, ddq, $^{2}J_{HH}$= -10.13 Hz, J=7.06 Hz, $^{3}J_{HP}$= 7.99 Hz), 1.845 (3H, dd, J=5.61 Hz, $^{4}J_{HP}$= 1.03 Hz), 1.368 (3H, ddd, J=7.10 Hz, 7.05 Hz, $^{4}J_{HP}$=1.13 Hz), 1.358 (3H, ddd, J=7.08 Hz, 7.06 Hz, $^{4}J_{HP}$=1.09 Hz); ^{13}C NMR ($CDCl_3$) δ 85.72 (d, $^{2}J_{CP}$ = 6.3 Hz), 64.58* (d, $^{2}J_{CP}$ = 6.1 Hz), 64.33* (d, $^{2}J_{CP}$ = 5.8 Hz), 27.65 (d, $^{3}J_{CP}$ = 8.2 Hz), 16.02 (d, $^{3}J_{CP}$ = 6.9 Hz); ^{31}P NMR ($CDCl_3$) δ -3.11. GC-MS *m/z* 217 (M + 1), 181 (M - Cl). Anal. Calcd for $C_6H_{14}ClO_4P \cdot 0.6$ HCl: C, 30.22; H, 6.17. Found: C, 29.98; H, 5.94.

*Due to the chiral center and prochirality there are four ^{1}H and two ^{13}C chemical shifts for OCH_2-groups

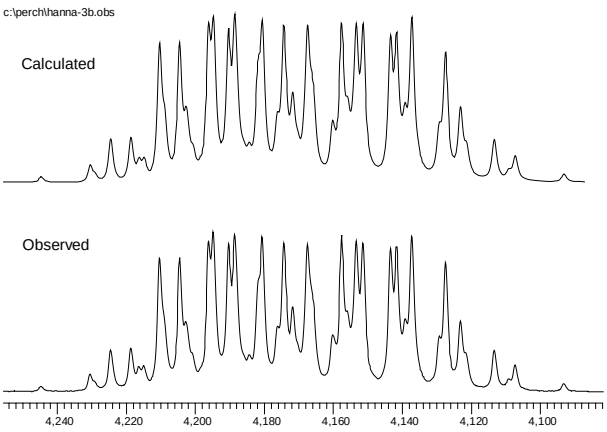


Figure 6.1. As an example of the complicated structure of the spectra with the calculated and observed ^{1}H NMR spectrum for OCH_2-protons signals of **3b**

Phosphoric acid 1-chloro-ethyl ester bis-(2,2,2-trichloro-ethyl) ester 3c: MW-conditions: 1 hour, 176 ºC, 20 bars. Colorless viscose oil. Chromatography eluent CH_2Cl_2:hexane:EtOAc (gradient). ^{1}H NMR ($CDCl_3$) ^{1}H NMR ($CDCl_3$) δ 6.332 (1H, dq, J=5.63 Hz, $^{3}J_{HP}$=7.40 Hz), 4.716* (1H, dd, $^{2}J_{HH}$= -10.97 Hz, $^{3}J_{HP}$=6.82 Hz), 4.667* (1H, dd, $^{2}J_{HH}$= -7.03 Hz, $^{3}J_{HP}$=6.88 Hz), 4.666* (1H, dd, $^{2}J_{HH}$= -10.97 Hz, $^{3}J_{HP}$=6.59 Hz), 4.661* (1H, dd, $^{2}J_{HH}$= -7.03 Hz, $^{3}J_{HP}$=6.44 Hz), 1.919 (3H, dd, J=5.63 Hz, $^{4}J_{HP}$= 1.35 Hz); ^{13}C NMR ($CDCl_3$) δ 94.28, 86.27 (d, $^{2}J_{CP}$ = 6.8 Hz), 77.44* (d, $^{2}J_{CP}$ = 4.4 Hz), 77.27* (d, $^{2}J_{CP}$ = 4.2 Hz), 27.35 (d, $^{3}J_{CP}$ = 8.8 Hz); ^{31}P NMR ($CDCl_3$) δ -6.18. GC-MS *m/z* 423 (M), 389 (M - Cl). Anal. Calcd for $C_6H_8Cl_7O_4P \cdot 0.3$ EtOAc: C, 19.23; H, 2.33. Found: C, 19.15; H, 2.21.

*Due to the chiral center and prochirality there are four ^{1}H and two ^{13}C chemical shifts for OCH_2-groups

Phosphoric acid 1-chloro-ethyl ester methyl ester phenyl ester 3d: MW-conditions: 0.75 hours, 100 ºC, 6 bars. White solid. Chromatography eluent CH_2Cl_2. Major isomer ca. 80%: ^{1}H NMR ($CDCl_3$) δ 7.380-7.325 (2H, m), 7.260-7.179 (3H, m), 6.328-6.271 (1H,m), 3.929 (3H, d, $^{3}J_{HP}$=11.69 Hz), 1.796 (3H, d, J=5.60 Hz); ^{13}C NMR ($CDCl_3$) δ 150.13 (d, $^{2}J_{CP}$ = 7.0 Hz), 129.82, 125.54, 120.06 (d, $^{3}J_{CP}$ = 4.7 Hz), 86.08 (d, $^{2}J_{CP}$ = 6.2 Hz), 55.30 (d, $^{2}J_{CP}$ = 6.3 Hz), 27.34 (d, $^{3}J_{CP}$ = 8.6 Hz); ^{31}P NMR ($CDCl_3$) δ -6.94; Minor isomer ca 20%: δ 7.380-7.325 (2H, m), 7.260.7.179 (3H, m), 6.342-6.280

(1H,m), 3.887 (3H, d, $^{3}J_{HP}$=11.59 Hz), 1.888 (3H, d, J=5.64 Hz). ^{13}C NMR ($CDCl_3$) δ 150.13 (d, $^{2}J_{CP}$ = 7.0 Hz), 129.77, 125.46, 120.06 (d, $^{3}J_{CP}$ = 4.7 Hz), 85.99 (d, $^{2}J_{CP}$ = 7.2 Hz), 55.30 (d, $^{2}J_{CP}$ = 6.3 Hz), 27.55 (d, $^{3}J_{CP}$ = 8.6 Hz); ^{31}P NMR ($CDCl_3$) δ -7.46. GC-MS *m/z* 251 (M + 1), 215 (M - Cl). Anal. Calcd for $C_9H_{12}ClO_4P$: C, 43.13; H, 4.83. Found: C, 43.21; H, 5.05.

Phosphoric acid 1-chloro-ethyl ester bis-(2,6-dimethyl-phenyl) ester 3e: MW-conditions: 0.5 hours, 170 ºC, 20 bars. Colorless viscose oil. Chromatography eluent CH_2Cl_2. ^{1}H NMR ($CDCl_3$) ^{1}H NMR ($CDCl_3$) δ 7.050-6.969 (6H, m), 6.285 (1H, dq, J=5.52 Hz, $^{3}J_{HP}$=7.62 Hz), 2.403* (6H, s), 2.303* (6H, s), 1.694 (3H, dd, J=5.52 Hz); ^{13}C NMR ($CDCl_3$) δ 147.95 (d, $^{3}J_{CP}$ = 8.7 Hz), 147.76 (d, $^{3}J_{CP}$ = 8.7 Hz), 130.42 (d, $^{2}J_{CP}$ = 3.4 Hz), 130.34 (d, $^{2}J_{CP}$ = 3.4 Hz), 129.17 (d, $^{4}J_{CP}$ = 1.7 Hz), 129.09 (d, $^{4}J_{CP}$ = 1.7 Hz), 125.60 (d, $^{5}J_{CP}$ = 2.0 Hz), 125.55 (d, $^{5}J_{CP}$ = 2.0 Hz), 86.66 (d, $^{2}J_{CP}$ = 6.4 Hz), 27.22 (d, $^{3}J_{CP}$ = 8.1 Hz), 17.22, 16.99; ^{31}P NMR ($CDCl_3$) δ -12.99. GC-MS *m/z* 369 (M + 1), 333 (M - Cl). Anal. Calcd for $C_{18}H_{22}ClO_4P$: C, 58.62; H, 6.01. Found: C, 59.02; H, 6.27.

2-(1-Chloro-ethoxy)-[1,3,2]dioxaphosphinane 2-oxide 3f: MW-conditions: 0.125 hours, 120 ºC, 6 bars. Colorless oil. Chromatography eluent hexane:EtOAc 1:4. ^{1}H NMR ($CDCl_3$) ^{1}H NMR ($CDCl_3$) δ 6.309 (1H, dq, J=5.59 Hz, $^{3}J_{HP}$=8.05 Hz), 4.560-4.399 (4H, m), 2.390-2.810 (1H, m), 1.879 (3H, dd, J=5.59 Hz, $^{4}J_{HP}$= 0.85 Hz), 1.833-1.773 (1H, m); ^{13}C NMR ($CDCl_3$) δ 85.44 (d, $^{2}J_{CP}$ = 6.2 Hz), 69.63 (d, $^{2}J_{CP}$ = 7.4 Hz), 69.00 (d, $^{2}J_{CP}$ = 7.2 Hz), 27.74 (d, $^{3}J_{CP}$ = 7.7 Hz), 25.75 (d, $^{3}J_{CP}$ = 7.4 Hz); ^{31}P NMR ($CDCl_3$) δ -9.64. GC-MS *m/z* 201 (M + 1), 165 (M - Cl). Anal. Calcd for $C_5H_{10}ClO_4P$: C, 29.94; H, 5.03. Found: C, 30.06; H, 5.15.

Phosphoric acid 1-chloro-ethyl ester diphenyl ester 3g: MW-conditions: 1 hour, 173 ºC, 20 bars. Colorless, viscose oil. Chromatography eluent hexane:EtOAc 1:1. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.38-7.16 (10H, m), 6.39 (1H, dq, $^{3}J_{HP}$ = 7.63, $^{3}J_{HH}$ = 5.61 Hz), 1.75 (3H, dd, $^{3}J_{HH}$ = 5.61, $^{4}J_{HP}$ = 1.22 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 150.24¶ (d, $^{2}J_{CP}$ = 7.4 Hz), 150.07¶ (d, $^{2}J_{CP}$ = 7.4 Hz), 129.85 (d, $^{2}J_{CP}$ = 7.9 Hz), 125.74 (d, $^{2}J_{CP}$ = 8.6 Hz), 120.26¶ (d, $^{3}J_{CP}$ = 4.8 Hz), 120.17¶ (d, $^{3}J_{CP}$ = 4.4 Hz), 86.41 (d, $^{2}J_{CP}$ = 6.5 Hz), 27.41 (d, $^{3}J_{CP}$ = 8.4 Hz); ^{31}P NMR ($CDCl_3$) δ -13.81. GC-MS *m/z* 312 (M^{+}), 277 (M - Cl). Anal. Calcd for $C_{14}H_{14}ClO_4P$ * 0.1 Hexane: C, 54.58; H, 4.83%. Found: C,

54.45; H, 4.58%. ¶ Due to the chiral center and prochirality there are two ^{13}C chemical shifts for ipso- and ortho-carbons.

Phosphoric acid 1-chloro-ethyl ester dibenzyl ester 3h: Colorless viscose oil. ^{1}H NMR ($CDCl_3$) ^{1}H NMR ($CDCl_3$) δ 7.397-7.298 (10H, m), 6.150 (1H, dq, J=5.49 Hz, $^{3}J_{HP}$=7.58 Hz), 5.120-5.051* (4H, m), 1.782 (3H, dd, J=5.49 Hz); ^{13}C NMR ($CDCl_3$) δ 135.18, 128.75, 128.59, 128.28, 85.84, 69.91, 27.54; ^{31}P NMR ($CDCl_3$) δ -1.55.

*Due to the chiral center and prochirality, all four OCH_2-signals have different chemical shifts. Due to decomposition of the product, peaks are broad and the J_{CP}-coupling constants can not be calculated.

Diethyl-phosphoramidic acid 1-chloro-ethyl ester methyl ester 3i: Slightly yellow solid. Chromatography eluent 0.5 % MeOH in CH_2Cl_2. Major isomer ca. 60%: ^{1}H NMR ($CDCl_3$) δ 6.247-6.189 (1H, m), 3.682 (3H, d, $^{3}J_{HP}$=11.38 Hz), 3.178-3.021 (4H, m), 1.828 (3H, dd, J=5.69 Hz, $^{4}J_{HP}$=0.72 Hz), 1.141 (6H, t, J=7.14 Hz); ^{13}C NMR ($CDCl_3$) δ 85.04 (d, $^{2}J_{CP}$ = 5.7 Hz), 52.92 (d, $^{2}J_{CP}$ = 5.8 Hz), 39.90 (d, $^{2}J_{CP}$ = 4.8 Hz), 27.87 (d, $^{3}J_{CP}$ = 7.6 Hz), 14.04 (d, $^{3}J_{CP}$ = 2.2 Hz); ^{31}P NMR ($CDCl_3$) δ 10.14. Minor isomer ca. 40%: ^{1}H NMR ($CDCl_3$) δ 6.247-6.189 (1H, m), 3.737 (3H, d, $^{3}J_{HP}$=11.45 Hz), 3. 178-3.021 (4H, m), 1.819 (3H, dd, J=5.49 Hz, $^{4}J_{HP}$=0.71 Hz), 1.116 (6H, t, J=7.08 Hz); ^{13}C NMR ($CDCl_3$) δ 85.30 (d, $^{2}J_{CP}$ = 7.0 Hz), 53.12 (d, $^{2}J_{CP}$ = 5.8 Hz), 39.70 (d, $^{2}J_{CP}$ = 4.4 Hz), 28.01 (d, $^{3}J_{CP}$ = 7.2 Hz), 14.06 (d, $^{3}J_{CP}$ = 2.2 Hz); ^{31}P NMR ($CDCl_3$) δ 10.34. GC-MS *m/z* 230 (M + 1), 194 (M - Cl). Anal. Calcd for $C_7H_{17}ClNO_3P$: C, 29.94; H, 5.03. Found: C, 30.06; H, 5.15.

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7 SYNTHESIS, *IN VITRO* AND *IN VIVO* CHARACTERIZATION OF ETHYLIDENE LINKED PHOSPHATE PRODRUG OF PROPOFOL

Abstract. An ethylidene phosphate prodrug of propofol (**3**) was synthesized and characterized as a novel phosphate prodrug structure that would represent a safer alternative for phosphonooxymethyl prodrugs. **3** greatly enhanced the aqueous solubility of propofol, having solubility over 10 mg/ml. The stability of **3** in buffer solution ($t_{1/2}$ = 5.2 ± 0.2 days at pH 7.4, r.t.) was sufficient for i.v. administration. The enzymatic hydrolysis of **3** to propofol in alkaline phosphatase solution was extremely rapid ($t_{1/2}$ = 21 ± 3 sec) and the prodrug was readily converted to propofol *in vivo* in rats. The maximum plasma concentration (C_{max}) of propofol, 3.0 ± 0.2 μg/ml, was reached within 2.1 ± 0.8 minutes (T_{max}) after i.v. administration of **3**. During bioconversion, **3** releases acetaldehyde, a less toxic compound than the formaldehyde released from the phosphonooxymethyl prodrug of propofol (Aquavan®), currently undergoing clinical trials. The present study indicates that ethylidene phosphate represents a potentially useful water-soluble prodrug structure suitable for i.v. administration.

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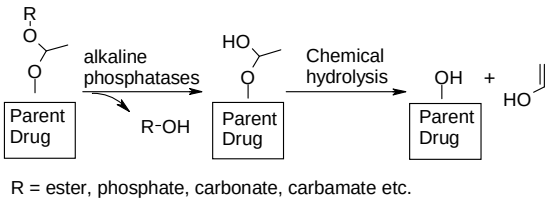
7.1 Introduction

Phosphates are probably the most extensively used water-soluble prodrugs for i.v. administration. They have been synthesized to hydroxyl or amine functionalities of the parent drug, either directly or via an oxymethyl spacer group. Oxymethyl spacers are generally used to increase the space around the enzymatically cleavable bond (phosphates, esters, carbonates) (Safadi *et al.* 1993, Varia *et al.* 1984) and they undergo rapid spontaneous chemical hydrolysis after enzymatic hydrolysis of the promoiety. One possible drawback of the oxymethyl linker structure is the systemic liberation of the highly toxic compound, formaldehyde, in the body, which in addition to its toxicity, may alter homeostasis within cells (such as induction of enzymes, metabolic switching and cell proliferation) (Heck *et al.* 1990). An alternative strategy to achieve sufficiently fast enzymatic hydrolysis rate and a more favourable safety profile of prodrugs is to use an ethyloxy-spacer structure (which will be referred to as an ethylidene structure) that liberates the much less toxic compound, acetaldehyde, from the prodrug structure (Scheme 7.1). Although acetaldehyde is not fully harmless, since it can cause gastrointestinal tract cancer particularly after long-term chronic alcohol consumption (Seitz *et al.* 2005), it is substantially less toxic than formaldehyde.



Scheme 7.1. The hydrolysis of ethylidene-linked phosphate prodrug of propofol to the parent drug, promoiety and acetaldehyde.

The (acyloxy)alkyl group has been widely used to link an ester, a carbonate or a carbamate promoiety to parent drugs containing a carboxylic acid functionality (Beaumont *et al.* 2003), in fact many such prodrugs are even marketed (e.g. candesartan cilexitil (Easthope and Jarvis 2002), cefuroxime axetil (Scott *et al.* 2001), bacmecillinam (Josefsson *et al.* 1982)). By comparison, no ethylidene linked phosphate prodrugs have been described in the literature. One possible reason is the challenge involved in the synthesis of these compounds, in fact there are no techniques for the

synthesis of ethylidene phosphates described in the literature (SciFinder Scholar, Beilstein Crossfire). This represented our starting point; we were interested in determining whether an ethylidene linked phosphate could be synthesized and whether the ethylidene spacer could serve as a safe and biodegradable linker between the parent drug and the phosphate promoiety.

For this study, we chose an anesthetic drug propofol as our model drug. Propofol (Figure 7.1) is a widely used anesthetic with a rapid onset, a short duration of action and minimal side-effects (Baker and Naguib 2005). However, the extremely low water-solubility, high lipophilicity, inherent emulsion instability, pain on injection and hyperlipidemia on prolonged administration (Baker and Naguib 2005) are major drawbacks, and thus, have lead to the development of water-soluble prodrugs. At least two phosphate prodrugs of propofol have been described in the literature (Figure 7.1), one with the phosphate group attached directly to the hydroxyl functionality of propofol (Propofol Phosphate, PP) (Banaszczyk *et al.* 2002) and another with an oxymethyl spacer between the parent drug and the phosphate promoiety (GPI 15715, Aquavan®) (Gibiansky *et al.* 2005, Schywalsky *et al.* 2003, Struys *et al.* 2005). Both prodrugs significantly enhance the water-solubility of propofol by many-fold, as would be predicted from their structures. The enzymatic hydrolysis of GPI 15715 (Schywalsky *et al.* 2003) is faster than that of PP (Banaszczyk *et al.* 2002), but on the other hand, PP does not liberate the toxic formaldehyde as a breakdown product to the body. We hoped to combine the properties of these two prodrugs and therefore we synthesized an ethylidene linked phosphate prodrug **3** of propofol. Furthermore, we have extensively characterized the ethylidene structure as a novel water-soluble phosphate prodrug structure *in vitro* and *in vivo* in rats.

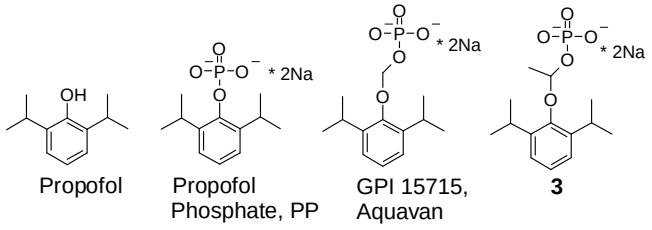
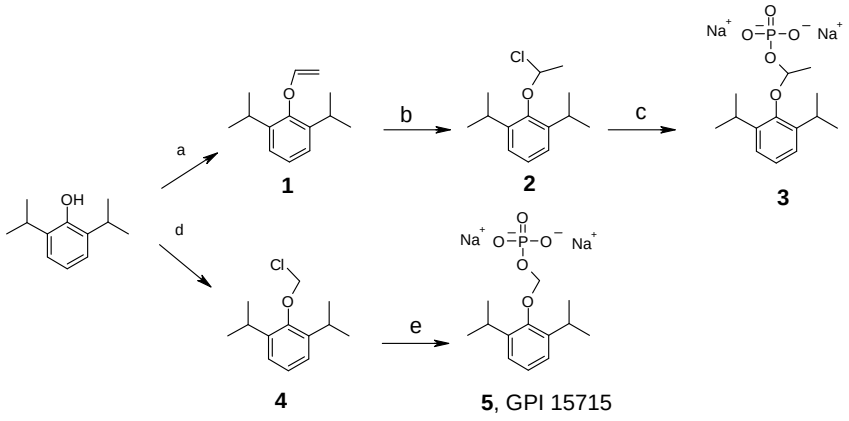


Figure 7.1. The structures of propofol and its phosphate-, phosphonooxymethyl- (GPI 15715, Aquavan®) and ethylidene phosphate- (**3**) prodrugs.

7.2 Results and Discussion

Synthesis. The phosphonooxymethyl derivative of propofol (**5**, GPI 15715) was synthesized as previously described (Bonneville *et al.* 2003) from propofol with bromochloromethane and phosphoric acid (Scheme 7.2) so that chemical stability studies could be undertaken. The synthesis of ethylidene linked prodrug **3** was more challenging. Our first attempt to develop a suitable synthesis route for ethylidene linked prodrugs involved the method for the synthesis of 1-chloroethyl phosphates (see chapter 6). It was thought that 1-chloroethyl phosphates might possibly be used as starting materials for the synthesis of ethylidene linked phosphate prodrugs. However, probably because of the steric hindrance and the presence of a phenolic hydroxyl group which is a good leaving group, the 1-chloroethyl phosphates did not react with propofol. The second attempt was to use the same synthesis route to synthesize 1-chloroethyl ether of propofol via vinyl ether, which could be further derivatized to phosphate. We found that the only way to make vinyl ether of propofol was the use of tetravinyl tin and cuprous acetate in an oxygen atmosphere (Blouin and Frenette 2001). The vinyl ether underwent fast microwave-assisted conversion to 1-chloroethyl ether in the presence of HCl-gas at 100ºC within 10 minutes. This method was similar to that used to prepare vinyl phosphates described previously (see chapter 6).



Scheme 7.2. Reagents a) tetravinyltin, $Cu(OAc)_2$, ACN b) HCl (g) / EtOAc, c) i. tetrabutylammonium phosphate, TEA, ACN ii. NaOH, ACN d) bromochloromethane, NaOH, THF, e) 85% phosphoric acid, TEA, ACN.

Since the ethylidene structure was found to be chemically acid-labile and since many protecting groups of phosphates require acidic conditions for their removal, the synthesis of an ethylidene phosphate was carried out without any protecting groups being present. The synthesis was achieved by using tetrabutylammonium phosphate in acetonitrile with an excess of triethylamine under dry and basic conditions in organic solvent. The sodium salt **3** was obtained with the treatment of the product with sodium hydroxide and the final product was purified with preparative HPLC. Since most of the marketed (acyloxy) alkyl prodrugs are racemic mixtures, the enantiomers were not separated but the racemic mixture was used in all assays which will be described.

Aqueous solubility. The aqueous solubility of propofol (130 ± 2 μg/ml) was greatly enhanced by the prodrug **3** since this compound has a solubility over 10 mg/ml (Table 7.1). The maximum solubility of **3** was not determined due to the small amount of prodrug available. However, this limit was chosen since it has been estimated that in general a drug needs to have a solubility of at least 10 mg/ml to be suitable for i.v. administration (Stella 2006). Due to the fast and complete dissolution and limited stability of **3**, the shaking time was reduced to 1 hour from the conventional 72 hours.

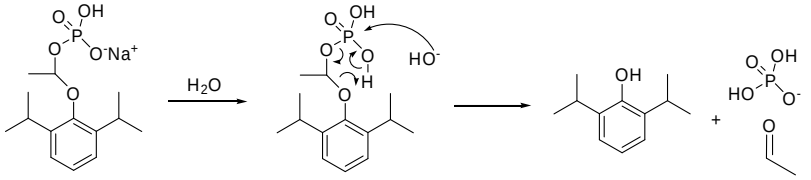
Table 7.1. Half-lives of **3** and **5** in a buffer solution, half-life of **3** in alkaline phosphatase solution and aqueous solubility of **3** and propofol in a buffer solution, all at pH 7.4 (mean ± S.D.; n = at least 3).

Cmpd	**Solubility mg ml^{-1} Tris buffer r.t.[c],**	**Chemical stability, $t_{1/2}$ Borate buffer 37°C**	**Chemical stability, $t_{1/2}$ Borate buffer r.t.[c]**	**Enzymatic hydrolysis, $t_{1/2}$ Alkaline phosphatase, 37°C**
3	> 10	21.5 ± 0.8 h	5.2 ± 0.2 days	21 ± 3 sec
5	-[a]	Stable[b]	-[a]	-[a]
Propofol	0.13 ± 0.002	-[a]	-[a]	-[a]

[a] Not determined, [b] No degradation was observed after three weeks [c] Room temperature

Chemical stability. The chemical degradation rates of **3** and **5** were determined in 180 mM borate buffers at pH 7.4 at 37°C and at room temperature. The chemical degradation of both **3** and **5** followed first-order kinetics and the half-lives are presented

in Table 7.1. The stability of **3** in buffer solution ($t_{1/2}$ = 5.2 ± 0.2 days at r.t. and $t_{1/2}$ = 21.5 ± 0.8 h at 37ºC, pH 7.4) was reasonably good if the drug were to be used for i.v. administration, although the chemical degradation was significantly faster than that of **5** (no degradation was observed after three weeks). The faster chemical degradation of **3** compared to that of **5** is probably due to lower stability of the ethylidene acetal compared to the methylene acetal. It is known that acetals with a good leaving group (such as phenol) and a neighbouring carboxylic acid group undergo intramolecular facilitated hydrolysis (Anderson and Fife 1973, Fife *et al.* 1996). Therefore, it can be postulated that the acidic groups of phosphate facilitate the hydrolysis of **3** by forming an oxocarbonium ion, which is internally greatly resonance-stabilized, leading to the breaking of the C-O bond and the release of propofol (Scheme 7.3). Nonetheless, the chemical stability of **3** is reasonable for i.v. use, though it may require the reconstitution of the preparation prior to use.



Scheme 7.3. The chemical hydrolysis of the ethylidene phosphate prodrug of propofol (**3**).

Enzymatic degradation *in vitro*. The enzymatic hydrolysis of **3** was determined in alkaline phosphatase solution. Only the half-life of **3** but not the exact amount of formed propofol was determined due to the low solubility of propofol. The enzymatic hydrolysis of **3** to propofol was extremely rapid with a half-life of approximately 20 seconds. This fast enzymatic hydrolysis of **3** suggests that ethylidene linked prodrugs act in a similar manner as conventional phosphate and phosphonooxymethyl prodrugs and release the parent drug rapidly via the action of alkaline phosphatases.

Bioconversion of ethylidene phosphate to propofol *in vivo*. The conversion of **3** to propofol was demonstrated *in viv*o in rats after a single i.v. bolus administration. One group of three adult male Wistar rats received a single bolus dose of 10 mg/kg of

propofol (0.056 mmol/kg) and another group of three rats received 19.4 mg/kg of **3** (0.056 mmol/kg). The plasma concentration of propofol was determined from whole blood samples using thymol as an internal standard with the method described previously in the literature (Yeganeh and Ramzan 1997). Only the amount of propofol, not the amount of prodrug, was determined and compared between the groups. The concentration curves of propofol after administration of **3** and propofol are presented in Figure 7.2, in which each line shows the values of individual rats. After administration of prodrug or propofol the pharmacokinetics revealed a fast initial decline and a slower elimination phase and these were best described by a two-compartmental model. The levels of propofol in whole blood (μg/ml) and the pharmacokinetic parameters (T_{max}, C_{max}) were determined using the above mentioned simulation and are shown in Table 7.2. The pharmacokinetic parameters of GPI 15715 and propofol phosphate are gathered from the publications of Schywalski *et al.* 2003 and Banaszczyk *et al.* 2002, respectively.

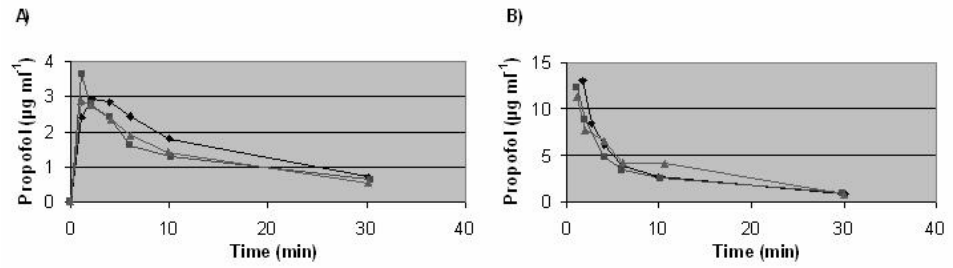


Figure 7.2. Propofol levels after single i.v. bolus dose of A) **3** (19.4 mg/kg) and B) propofol (10 mg/kg) into rats (n = 3). Each line shows the measured values of one rat.

Prodrug **3** was readily converted to propofol in each rat after i.v. administration. The maximum plasma concentration (C_{max}) of propofol, 3.0 ± 0.2 μg/ml, was reached within 2.1 ± 0.8 minutes (T_{max}) after the administration of **3**. Rats that received **3** were uncoordinated and had difficulties in maintaining their balance. In contrast, the dose of 10 mg/kg of propofol was sufficient to evoke sedation which occurred at approximately 13 seconds after the i.v. bolus. The difference between the concentrations of propofol after equimolar administration of **3** and propofol is similar to the results reported for GPI 15715 (Schywalsky *et al.* 2003) and is probably due to formulation-dependent

pharmacokinetics of propofol. A lipid-free formulation of propofol has a greater volume of distribution and a greater elimination clearance, but a similar terminal half-life when compared to the oil-in-water formulation (Dutta and Ebling 1998b). The lipid-free formulation of propofol has also been shown to have a larger distribution in the lungs compared to the oil-in-water formulation and the slow release of propofol from the lungs leads to decreased concentrations in the circulation (Dutta and Ebling 1998a). **3**, like GPI 15715 (Schywalsky *et al.* 2003), probably acts like the lipid-free formulation, releasing the lower concentrations of propofol to the circulation than is seen after equimolar administration of propofol.

Table 7.2. Estimated pharmacokinetic parameters (mean ± SD) of propofol after single i.v. bolus dose of **3** (19.4 mg/kg, 0.056 mmol/kg) and propofol (10 mg/kg, 0.056 mmol/kg) into rats (n = 3) and pharmacokinetic parameters of GPI 15715 and PP from the literature.

Cmpd	**3**	**Propofol**	**GPI 15715**[a]	**Propofol phosphate**[b]
Dose	19.4 mg /kg (0.056 mmol/kg)	10 mg / kg (0.056 mmol/kg)	40 mg /kg (0.120 mmol/kg)	130 mg /kg (0.499 mmol/kg)
C_{max} (μg/ml)	3.0 ± 0.2	14.3 ± 1.8	7.1 ± 1.6	9.6 ± 2.1
T_{max} (min)	2.1 ± 0.8	-[c]	3.7 ± 0.2	7.3 ± 3.8

C_{max} = maximum concentration of propofol, T_{max} = time to maximum concentration of propofol
[a] From Schywalsky et al 2003, [b] From Banaszczyk et al 2002, [c] After i.v. bolus Tmax = 0 min

When compared to GPI 15715, the maximum concentration of propofol, 3.0 ± 0.2 μg/ml after administration of **3** (19.4 mg/kg, 0.056 mmol/kg), is approximately half of the concentration achieved after administration of GPI 15715 with over double the dose (7.1 ± 1.6 μg/ml after 40 mg/kg, 0.120 mmol/kg) (Schywalsky *et al.* 2003). Since the maximum concentration of propofol was also reached at approximately the same time (2.1 ± 0.8 and 3.7 ± 0.2 min for **3** and GPI 15715, respectively), we conclude that the bioconversion of **3** to propofol is as effective as that of GPI 15715. The bioconversion of **3** to propofol was much faster and more effective, when compared to PP (Banaszczyk *et al.* 2002) which has a T_{max} of 7.3 ± 3.8 minutes and a C_{max} of 9.6 ± 2.1 μg/ml after i.v. administration of PP with a dose as great as 130 mg/kg (0.499 mmol/kg).

The purpose of this study was to confirm the bioconversion of **3** to propofol *in vivo*, not to evoke complete sedation in the rats. The sedated behaviour of rats demonstrated that propofol had been released from **3**, but larger doses would be needed to achieve complete sedation. This effect is similar to that reported earlier in case of GPI 15715 (Schywalsky *et al.* 2003). Even though the pharmacokinetic parameters are not truly comparable between different studies due to the dose-dependent onset of the action and recovery of propofol from prodrugs, this study indicates that **3** is bioconverted to propofol in a similar manner as the phosphonooxymethyl prodrug GPI 15715.

7.3 Conclusions

A novel ethylidene-linked phosphate prodrug of propofol (**3**) has been shown to increase the water-solubility of the poorly water-soluble drug propofol. The chemical stability of **3** is sufficient for i.v. administration, but is limited due to the presence of the more labile ethylidene acetal. The enzymatic release of propofol from **3** is rapid both *in vitro* with alkaline phosphatase solution and *in vivo* in rats. The bioconversion of **3** to propofol is similar to that of GPI 15715, the phosphonooxymethyl prodrug of propofol, and much faster when compared to propofol phosphate. In this study, no evidence was observed of any abnormal pharmacokinetics that could be explained by chirality. However, since enantiomers often have different pharmaceutical and pharmacokinetic profiles (Hutt and Tan 1996) and also differences in binding to serum albumin (Chuang and Otagiri 2006), in the future it might be worthwhile to separate the enantiomers and to evaluate their individual pharmaceutical and pharmacokinetic behaviours. This is not a unique problem of **3**, it applies to all ethyloxy prodrugs, in fact most of the (acyloxy) alkyl prodrugs are marketed as racemic mixtures.

The results of the present study provide clear evidence that the ethylidene linker is suitable for preparing phosphate prodrugs designed for i.v. administration. This novel prodrug structure combines the properties of previously described phosphate and phosphonooxymethyl prodrugs i.e. there is as efficient bioconversion to propofol as can be attained with the phosphonooxymethyl prodrug, but there is no systemic release of formaldehyde after administration of the phosphate prodrug. Further studies with

different model drugs containing hydroxyl and amine functionalities are ongoing in order to explore the overall versatility of this novel phosphate prodrug structure.

7.4 Methods

2,6-Diisopropyl-2-vinyloxy-benzene 1. Anhydrous copper (II) acetate (3.67 g, 20.19 mmol) was added to a solution of 2,6-diisopropyl phenol (3 g, 16.83 mmol) in dry acetonitrile. The reaction flask was rinsed with O_2. Tetravinyltin (3.74 g, 16.48 mmol) was added to the reaction mixture via a syringe and the reaction was stirred for 1 hour at room temperature. O_2-bubbling was replaced by a O_2-balloon and reaction was stirred at 60ºC for 16 h. The resulting brown mixture was poured into a 25 % NH_4OAc-solution (30 ml) and stirred for 15 min. The reaction was extracted with Et_2O (150 ml). The organic layer was washed with saturated brine (2 × 40 ml), dried over Na_2SO_4 and evaporated to dryness. Flash chromatography on SiO_2 (2 % ethylacetate in petrol ether) gave **1** as a colorless oil (1.34 g, 6.56 mmol, 39 %). ^{1}H NMR ($CDCl_3$) δ 7.18-7.10 (3H, m), 6.60 (1H, dd, J = 14.0 Hz and 6.5 Hz), 4.15-4.08 (2H, m), 3.18 (2H, h, J = 6.9 Hz), 1.19 (12 H, d, J = 6.9 Hz).

2-(1-Chloro-ethoxy)-1,3-diisopropyl-benzene 2. Ethyl acetate was saturated with dry HCl-gas by bubbling gaseous HCl through the solution for 10 min. **2** (1.04 g, 5.1 mmol) was dissolved in 10 ml of HCl-saturated ethyl acetate. The mixture was heated in a microwave-oven at 100ºC at 5 bar for 10 min and evaporated to dryness. The next reaction was continued immediately without any further purification.

Phosphoric acid mono-[1-(2,6-diisopropyl-phenoxy)-ethyl] ester disodium salt 3. To a solution of tetrabutylammonium phosphate (0.4 M in acetonitrile, 30 ml, 12 mmol) was added **2** (5.1 mmol, theoretical maximum yield) in triethylamine (5 ml, 36 mmol) under argon. The reaction mixture was stirred for 18 hours and the solvents were evaporated. 30 ml of water was added and the residue was extracted with diethyl ether (3 × 50 ml). Solvents were evaporated, 10 ml of acetonitrile was added and the pH of the mixture was adjusted to 11 with saturated NaOH. After stirring for 10 minutes, the solvents were evaporated, the residue was purified with preparative HPLC on a reversed phase Kromasil 100 Å (C8) column by gradient elution using water and acetonitrile (40-80 % ACN) as the eluent and lyophilized to obtain **3** as a white solid (494 mg, 1,43

mmol, 28 %). ^{1}H NMR (D_2O) δ 7.29-7.22 (3H, M), 5.54 (1H, q, J = 5.1 Hz), 3.42 (2H, m), 1.66 (3H, d, J = 5.1 Hz), 1.21 (12 H, t, J = 6.9 Hz). ^{13}C NMR (D_2O) δ 152.30, 146.45, 128.57, 127.08, 103.04, 29.28, 26.03 (d, J = 15.2 Hz), 24.97. ^{31}P NMR (D_2O) δ 0.03. ESI-MS: 301.3 (M - 2*Na + 1). Anal. Calcd for $C_{14}H_{21}Na_2O_5P \cdot 0.7\ H_2O$: C, 46.85; H, 6.29. Found: C, 46.79; H, 6.29.

2-Chloromethoxy-1,3-diisopropyl-benzene 4. Sodium hydroxide pellets (1.2 g, 30.0 mmol) and bromochloromethane (28.7 ml, 57.1 g, 441 mmol) were added to a solution of 2,6-diisopropyl phenol (2.7 g, 15.2 mmol) in dry tetrahydrofuran under nitrogen. The reaction mixture was heated at 64ºC for two hours, allowed to cool to room temperature and filtered. The filtrate was evaporated to dryness to obtain **4** as a yellowish oil (3.2 g, 14.1 mmol, 93 %). **4** was used in the next reaction without any further purification. ^{1}H NMR ($CDCl_3$) δ 7.20-7.09 (3H, m), 5.75 (2H, s), 3.35 (2H, m), 1.22 (12 H, d, J = 6.9 Hz).

Phosphoric acid mono-(2,6-diisopropyl-phenoxymethyl) ester disodium salt 5. To a solution of triethylamine (13.03 ml, 9.46 g, 93.5 mmol) and 85 % phosphoric acid (5.86 ml, 9.87 g, 101 mmol) was added **4** (3.2 g, 14.1 mmol) and the reaction mixture was heated at 64ºC for 2.5 hours. The solvents were evaporated, 100 ml of water was added to the residue and the pH of the mixture was adjusted to 1.5 with 6 M HCl. The aqueous solution was extracted with diethyl ether (3 × 50 ml), washed with brine and evaporated to dryness. 50 ml of water was added to the residue and pH was adjusted to 9 with saturated NaOH-solution. The mixture was washed with toluene (2 × 30 ml) and concentrated under reduced pressure to half of the volume. 100 ml of isopropanol was added, the mixture was frozen, allowed to warm to 0ºC, filtered and dried in a vacuum oven to obtain **5** as a white solid (0.87 g, 3.0 mmol, 21 %) ^{1}H NMR (D_2O) δ 7.32-7.24 (3H, m), 5.27 (2H, d, J = 7.5 Hz), 3.46 (2H, m), 1.22 (12 H, d, J = 6.9 Hz). ^{13}C NMR (D_2O) 152.96, 145.90, 128.63, 127.03, 96.11, 28.97, 25.95. ^{31}P NMR (D_2O) δ 2.02. ESI-MS: 287.1 (M - 2×Na + 1). Anal. Calcd for $C_{13}H_{19}Na_2O_5P \cdot 0.2\ H_2O$: C, 46.49; H, 5.82. Found: C, 46.12; H, 5.82.

HPLC analysis. HPLC analysis of **3**, **5** and propofol was performed using an Agilent Technologies 1100 series gradient RP-HPLC system with UV detection (220 nm) and

for rat whole blood samples the fluorescence detection (Ex = 276 nm, Em = 310). The HPLC system consisted of Agilent 1100 Series Binary Pump, 1100 Series Autosampler, 1100 Series Micro Vacuum Degasser, 1100 Series Thermostated Column Compartment, 1100 Series Fluorescence Detector, HP 1050 Variable Wavelenght Detector, 1100 Series Control Module and a Zorbax Eclipse XDB-C8 (4.6 mm × 150 mm, 5 μm) analytical column (Agilent Technologies Inc., Little Falls, Wilmington, DE, USA). Loop injection volume was 20 μl. The isocratic elution was performed by using a mobile phase consisting of 90 % (v/v) acetonitrile and 10 mM tetrabutylammonium dihydrogen phosphate at a ratio of 65:35 at a flow rate of 1.0 ml/min at 30ºC.

Animal treatments. Adult male Wistar rats weighing 250 ± 5 g were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). The right jugular vein was exposed aseptically as described by Waynforth and Flecnell (Waynforth and Fleckell 1994), and cannulated with a PE-50 catheter filled with 100 IE/ml heparin. The catheter was exteriorised through a small incision made in the neck. The rats were allowed to recover until the following day.

The drug solutions were administered to the conscious rats as 30-second i.v. injections (including the rinsing of the catheter with 0.9 % NaCl). For the determination of propofol, venous blood samples of 200 μl were collected immediately before and 1, 2, 4, 6, 10 and 30 min after the i.v. bolus administration, mixed with 20 μl of 3 % EDTA in 0.7 % NaCl-solution and frozen immediately. The drawn blood volume was replaced with 0.9 % NaCl.

Rat blood sample preparations. For the assay of propofol in rat whole blood samples, the red blood cells were lysed with a total of three freeze/thaw cycles. 10 μl of 90 μg/ml thymol as internal standard was added to 100 μl of lysed whole blood, then 250 μl of acetonitrile was added to precipitate proteins and the samples were centrifuged for 3 min at 12 000 rpm. The supernatant was injected directly into the HPLC column. The quantity of propofol was determined using external, spiked standards of lysed whole blood and the concentration was estimated based on peak-area ratio of propofol to the internal standard (thymol). All concentration curves and pharmacokinetic parameters (T_{max}, C_{max}) after i.v. administration for each animal were calculated by standard pharmacokinetic methods

using WinNonlin Nonlinear Estimation Program, Professional version 5.0.1 (Pharsight Corporation).

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8 SYNTHESIS, *IN VITRO* AND *IN VIVO* CHARACTERIZATION OF DIESTER PHOSPHATE PRODRUGS*

Abstract: Monomethyl ester derivatives of phosphate and phosphonooxymethyl prodrugs of propofol were synthesized and evaluated *in vitro* and *in vivo* as possible prodrug structures for enhanced lipophilicity and permeability compared to the corresponding phosphate prodrug and for enhanced water-solubility compared to the parent drug. However, neither of the monomethylphosphates was enzymatically cleaved *in vitro* in solutions of alkaline phosphatase, phosphodiesterase or rat liver homogenate *in vivo* in rat. Thus, the monoalkyl esters of phosphates do not seem to be suitable for use as potential bioreversible prodrug structures.

* Unpublished results

8.1 Introduction

The enzymatic hydrolysis of orally administered phosphate prodrugs to the parent drug takes place at the intestinal brush border by alkaline phosphatases (Heimbach *et al.* 2007). The bioconversion occurs just prior to the absorption, after which the more lipophilic parent drug is absorbed through the intestinal membrane. Due to the presence of two negative charges, the phosphate prodrug itself is too hydrophilic to pass through the biological membrane by passive diffusion. If the bioconversion of the prodrug to the parent drug is not complete or if the bioconversion rate is not fast enough, the absorption, and thus, the bioavailability of the parent drug will limited. In addition, poor permeability or precipitation of the cleaved parent drug at the surface of the intestinal membranes may limit the absorption of the parent drug (Heimbach *et al.* 2003).

Phosphate diesters with one free negative charge in the phosphate structure might serve as prodrugs with enhanced aqueous solubility compared to the parent drug but greater lipophilicity and thus better permeability compared to the corresponding phosphate prodrug. These prodrugs may not be substrates of alkaline phosphatases themselves (McComb *et al.* 1979), but they may be hydrolyzed first to phosphates by a group of enzymes called phosphodiesterases (Jeon *et al.* 2005, Khan *et al.* 2005), followed by the enzymatic activation by alkaline phosphatases. Phosphodiesterases (PDE) are enzymes that hydrolyze cyclic nucleotides to monophosphates in the body (Jeon *et al.* 2005). Inhibitors of PDEs are well-known (e.g. sildenafil (Viagra®), an inhibitor of PDE5 (Goldstein *et al.* 2002)), but the use of phosphodiesterases in the design of prodrug bioactivation has been studied to a lesser extent. The literature contains only a few descriptions of diester phosphate prodrugs (Hadimani *et al.* 2003, Rosowsky *et al.* 1982) cleaved by phosphodiesterases to improve the passive uptake through membranes, but there has been no evaluation of diester phosphates as prodrugs with the exception of some nucleotide-type structures.

In order to find out if diester phosphates would be useful prodrug structures, we chose propofol to represent a model drug. In this study we synthesized phosphate (**1**, Figure 8.1) and phosphonooxymethyl (**4**) prodrugs of propofol as reference compounds and monomethyl esters of these phosphates (**2** and **7**). We evaluated the suitability of these structures as prodrug structures *in vitro* and *in vivo*. The enzymatic release of the active

drug from the prodrugs was studied *in vitro* in solutions of alkaline phosphatase, phosphodiesterase and rat liver homogenate and finally *in vivo* in rats.

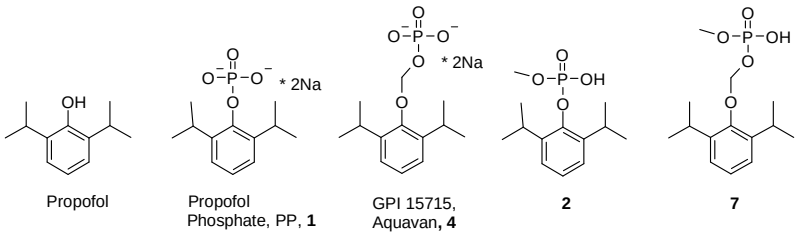


Figure 8.1. The structures of propofol and its phosphate (PP, **1**)-, phosphonooxymethyl- (GPI 15715, Aquavan®, **4**) and their monomethyl ester derivatives (**2** and **7**).

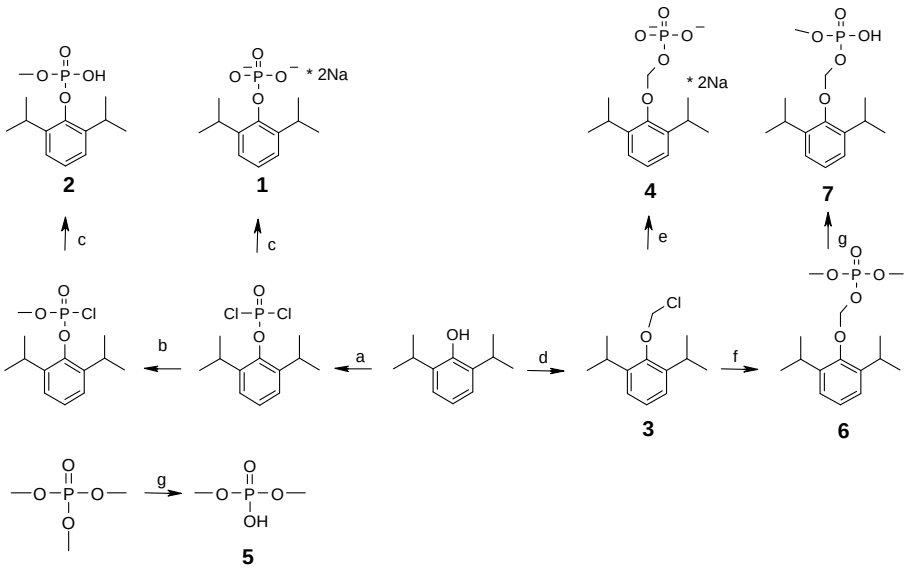
8.2 Results and Discussion

Synthesis. The propofol phosphate **3** was synthesized by a standard method from the propofol and $POCl_3$. The monomethyl phosphate **2** was synthesized from the dichlorophosphoryl intermediate by adding an equivalent of methanol, following the addition of water to hydrolyze the remaining chloride (Scheme 8.1). Due to the mixture of byproducts (including dimethyl phosphate and free phosphates), which required several HPLC-purification steps to obtain analytically pure products, the yield of this synthesis was not very high. However, due to the low costs of the reagents and small number of reaction steps, it was felt worthwhile to use this reaction route instead of synthesizing dimethyl phosphate and then attempting to selectively remove one of the methyl groups.

The phosphonooxymethyl derivative of propofol (**4**) was synthesized as previously described from propofol with bromochloromethane and phosphoric acid (Bonneville *et al.* 2003). A similar synthesis route was used for the synthesis of the monomethyl ester **7**. The chloromethoxy derivative of propofol (**3**) was coupled with dimethyl phosphate **5** to obtain dimethyl phosphonooxymethyl propofol (**6**) with reasonably good yields, followed by the selective removal of one of the methyl groups. This synthesis, as well as the synthesis of dimethyl phosphate **5** from trimethyl phosphate, was carried out in a microwave oven using sodium iodide in acetone for 10-20 min at 100ºC. In particular, the synthesis of **5** proved to be a convenient way to make dimethyl phosphates, when compared to the previously described method with tetra-*n*-butylammonium hydroxide

and 24 hours of refluxing (Phan and Poulter 2001). The method described now also lacks the tetra-*n*-butylammonium counter ion, which is often difficult to remove.

The synthesis product **7** contained some impurities that made the crude product extremely difficult to purify. Nonetheless, three consecutive purifications by preparative HPLC yielded approximately 80 % pure product, which was then used for the *in vitro* and *in vivo* assays described below. Thus, the elemental analysis and MS-spectras were not obtained.



Scheme 8.1. Reagents: a)$POCl_3$, n-BuLi, Et_2O, b) MeOH, TEA, c) H_2O, d) $BrCH_2Cl$, NaOH, THF, e) 85% phosphoric acid, TEA, ACN, NaOH, f) **5**, TEA g) NaI, acetone.

Enzymatic degradation. The enzymatic degradation of prodrugs was studied in solutions of alkaline phosphatase, phosphodiesterase I and in rat liver homogenate, in which both of the monomethyl ester prodrugs **2** and **7** were stable. The stability in alkaline phosphatase solution was anticipated since it is known that diesters do not serve as substrates for alkaline phosphatase (McComb *et al.* 1979). However, the stability in phosphodiesterase and liver homogenate solutions was somewhat surprising, since phosphodiesterase is known to be able to break O-C bonds of diester phosphates. One possible reason for the stability might be the small sizes of both the methyl group and

propofol. Endogenous PDEs degrade cyclic adenosine and guanosine monophosphates, which are much larger in their molecular size and also have a larger size of the ester group. The PDEs may also require the presence of a nucleotide structure in its substrate before they are able to degrade the ester bond.

Conversion of monomethyl phosphate prodrugs to propofol *in vivo*. In order to confirm that the results obtained from the *in vitro* tests would reflect the *in vivo* situation, the bioconversion of prodrugs to propofol was studied in rats after a single i.v. bolus administration by the method described in chapter 7. One male Wistar rat received a single bolus dose of 21.8 mg/kg of **2** (0.056 mmol/kg) and another rat received 16.9 mg/kg of **7** (0.056 mmol/kg). Propofol was determined from the rat whole blood samples.

The results from the *in vivo* experiment confirmed the results from *in vitro* studies, and no propofol was obtained in the rat whole blood samples during 30 min experiment. In addition, no changes in the behaviour of the rat were observed during experiment. This seemed to be convincing proof that propofol was not released from the prodrug structure.

Chemical stability, aqueous solubility and lipophilicity. Due to the unpromising results of the *in vitro* and *in vivo* enzymatic cleavability studies, the chemical stability, lipophilicity and aqueous solubility experiments were not clarified. However, using the retention times in HPLC method as a rough indicator, it can be estimated that these diesters are more water-soluble than propofol, but more lipophilic than the free phosphates (retention times for free phosphates around 3 min, for monomethyl phosphates around 4 min and for propofol 9.1 min). The chemical stability of the diesters seems to be limited, as the free phosphates were formed during storage of the stock solutions.

8.3 Conclusions

The monomethyl ester derivatives of propofol phosphate were studied as possible prodrug structures with the aim of creating compounds with enhanced lipophilicity and permeability compared to the corresponding phosphate prodrugs and better water-solubility compared to the parent drug. However, neither monomethylphosphate of propofol (**2**) nor monomethyl phosphonooxymethyl propofol (**7**) was enzymatically cleaved *in vitro* in solutions of alkaline phosphatase, phosphodiesterase or rat liver

homogenate or *in vivo* in rat. The probable reason for high enzymatic stability is the structure of propofol. The small sizes of both the methyl group and propofol differ from the natural substrates of PDEs (nucleotides such as cyclic adenosine and guanosine monophosphates). However, these prodrug structures may be potentially useful for compounds with a nucleotide-type structure and larger ester groups but this speculation will need to be studied in future experiments.

8.4 Methods

Phosphoric acid mono-(2,6-diisopropyl-phenyl) ester 1. To a solution of 2,6-diisopropylphenol (2.0 g, 11.2 mmol) in diethylether (20 ml) was added n-butyl lithium (6.9 mL, 11.3 mmol) at -30 °C under argon over 10 min. The mixture was stirred for 30 min at -30 °C and then for 30 min at r.t. The reaction mixture was then slowly added to a solution of phosphorous oxychloride (1.1 ml, 12.0 mmol) in 20 ml of diethylether at -30 °C. The reaction mixture was allowed to reach the room temperature and stirred at r.t. for 21 h. 10 ml of distilled water was added to the reaction mixture and the mixture was stirred for an additional 1 hour. Sodium hydroxide (70 ml, 1 M) was added and the reaction mixture washed three times with hexane. The pH of the aqueous phase was adjusted to 2 with hydrochloric acid (3 M) and washed with diethyl ether (3 × 50 ml). The combined organic phases were dried over Na_2SO_4 and evaporated to dryness. The residue was purified with Shimadzu preparative HPLC on reversed phase Supelco PLC-8 column using a 60:40 mixture of acetonitrile and trifluoroacetic acid (0.1 % in water) as eluent and lyophilized to dryness to yield **1** as a white solid (0.41 g, 1.6 mmol, 14 %). ^{1}H NMR (D_2O) δ 7.14 (1H, t, 7.5 Hz), 7.09 (2H, d, 7.5 Hz), 3.32 (2H, sep, J = 6.8 Hz), 1.13 (12H, d, J = 6.8 Hz). ^{13}C NMR ($CDCl_3$) 145.7 (d, J = 8.9 Hz), 140.54 (d, J = 3.4 Hz), 126.17 (d, J = 1.8 Hz), 124.40, 27.04, 23.15. ^{31}P NMR (D_2O) δ -1.96. ESI-MS: 257.4 (M - 1). Anal. Calcd for $C_{12}H_{19}O_4P \cdot 0.1\ H_2O$: C, 55.42; H, 7.44. Found: C, 55.31; H, 7.36.

Phosphoric acid 2,6-diisopropyl-phenyl ester methyl ester 2. To a solution of 2,6-diisopropylphenol (2.0 g, 11.2 mmol) in diethylether (20 ml) was added n-butyl lithium (6.9 mL, 11.3 mmol) at -30 °C under argon over 10 min. The mixture was stirred for 30 min at -30 °C and then for 30 min at r.t. The reaction mixture was then slowly added to

a solution of phosphorous oxychloride (1.1 ml, 12.0 mmol) in 20 ml of diethylether at -30 °C. The reaction mixture was allowed to reach the room temperature and stirred at rt. After 27 h, methanol (0.5 ml, 12.3 mmol), triethylamine (1.7 ml, 12.2 mmol) and diethylether (20 ml) were added to the reaction mixture. The reaction mixture was stirred at r.t. for 70 h, after which distilled water (10 ml) was added. The mixture was stirred for an additional 1.5 hours, washed three times with hydrochloric acid (0.5 M) and extracted with diethylether (3 × 50 ml). The combined organic phases were dried over Na_2SO_4 and evaporated to dryness. The residue was purified with Shimadzu preparative HPLC on reversed phase Supelco PLC-8 column using a 60:40 mixture of acetonitrile and trifluoroacetic acid (0.1 % in water) as eluent and lyophilized to dryness to yield **2** as a white solid (1.11 g, 4.0 mmol, 36 %). ^{1}H NMR (D_2O) δ 7.16-7.03 (3H, m), 3.74 (3H, d, J = 11.5 Hz), 3.42 (2H, sep, J = 6.8 Hz), 1.18 (12H, d, J = 6.8 Hz). ^{13}C NMR (D_2O) 147.08 (d, J = 8.5 Hz), 141.31 (d, J = 2.4 Hz), 125.17, 124.08, 153.22 (d, J = 6.2 Hz), 26.61, 23.01. ^{31}P NMR (D_2O) δ -2.49. ESI-MS: 271.5 (M - 1). Anal. Calcd for $C_{13}H_{21}O_4P \cdot 0.1\ H_2O$: C, 56.97; H, 7.80. Found: C, 56.90; H, 7.71.

2-Chloromethoxy-1,3-diisopropyl-benzene 3. To a solution of 2,6-diisopropyl phenol (2.7 g, 15.2 mmol) in dry tetrahydrofuran sodium hydroxide pellets (1.2 g, 30.0 mmol) and bromochloromethane (28.7 ml, 57.1 g, 441 mmol) were added under nitrogen. The reaction mixture was heated at 64ºC for two hours, allowed to cool to room temperature and filtered. The filtrate was evaporated to dryness to obtain **3** as a yellowish oil (3.2 g, 14.1 mmol, 93 %). **3** was used in the next reaction without any further purification. ^{1}H NMR ($CDCl_3$) δ 7.20-7.09 (3H, m), 5.75 (2H, s), 3.35 (2H, sep, J = 6.9 Hz), 1.22 (12 H, d, J = 6.9 Hz).

Phosphoric acid mono-(2,6-diisopropyl-phenoxymethyl) ester disodium salt 4. To a solution of triethylamine (13.03 ml, 9.46 g, 93.5 mmol) and 85 % phosphoric acid (5.86 ml, 9.87 g, 101 mmol) was added **4** (3.2 g, 14.1 mmol) and the reaction mixture was heated at 64ºC for 2.5 hours. Solvents were evaporated, 100 ml of water was added to the residue and the pH of the mixture was adjusted to 1.5 with 6 M HCl. The aqueous solution was extracted with diethylether (3 × 50 ml), washed with brine and evaporated to dryness. 50 ml of water was added to the residue and the pH was adjusted to 9 with saturated NaOH-solution. The mixture was washed with toluene (2 × 30 ml) and

concentrated under reduced pressure to half of the volume. 100 ml of isopropanol was added, the mixture was frozen, allowed to warm to 0ºC, filtered and dried in vacuum oven to obtain **4** as a white solid (0.87 g, 3.0 mmol, 21 %) ^{1}H NMR (D_2O) δ 7.32-7.24 (3H, m), 5.27 (2H, d, J = 7.5 Hz), 3.46 (2H, sep, J = 6.9 Hz), 1.22 (12 H, d, J = 6.9 Hz). ^{13}C NMR ($CDCl_3$) 151.61, 141.99, 125.54, 124.16, 94.07 (J = 2.9 Hz), 26.72, 24.06. ^{31}P NMR (D_2O) δ 2.02. ESI-MS: 287.1 (M - 2×Na + 1). Anal. Calcd for $C_{13}H_{19}Na_2O_5P \cdot 0.2\ H_2O$: C, 46.49; H, 5.82. Found: C, 46.12; H, 5.82.

Phosphoric acid dimethyl ester 5. To a solution of trimethyl phosphate (1.7 ml, 14.6 mmol) in acetone (15 ml) NaI (2.18 g, 14.6 mmol) was added. The reaction was heated for 20 min at 100°C in a microwave oven. A white precipitate was collected and dried in a vacuum oven to obtain **5** as a white solid (2.03 g, contains solvent). **5** was used to the next reaction without any further purification.

Phosphoric acid 2,6-diisopropyl-phenoxymethyl ester dimethyl ester 6. To a solution of **3** (1.04 g, 4.6 mmol) in acetonitrile (60 ml) **5** (1.96 g, 13.2 mmol) and triethylamine (4 ml, 28.6 mmol) were added. After stirring at 50-55 °C for 17 hours, the reaction mixture was evaporated to dryness and dissolved in water. The pH of the aqueous mixture was adjusted to 1.5 with HCl (3 M), extracted with diethyl ether (3 × 50 ml), dried over Na_2SO_4 and evaporated to dryness to yield **6** as white solid (1.20 g, 3.79 mmol, 82 %, unpurified). **6** was used in the next reaction without any further purification.

Phosphoric acid 2,6-diisopropyl-phenoxymethyl ester methyl ester 7. To a solution of **6** (1.05 g, 3.3 mmol) in acetone (20 ml) NaI (0.28 g, 1.86 mmol) was added. The reaction was heated for 10 min at 100°C in a microwave oven and the solvents were evaporated. The residue was dissolved in water. The pH of the aqueous mixture was adjusted to 1 with HCl (3 M), extracted with diethyl ether (3 × 50 ml), dried over Na_2SO_4 and evaporated to dryness. The residue was purified with a Shimadzu preparative HPLC on a reversed phase Supelco PLC-8 column using a 60:40 mixture of acetonitrile and trifluoroacetic acid (0.1 % in water) as eluent and lyophilized to dryness to yield **2** as á white solid (0.2 g, 0.7 mmol, 21 %). ^{1}H NMR ($CDCl_3$) δ 7.14-7.06 (3H, m), 5.43 (2H, d, J = 9.95 Hz), 3.68 (3H, d, J = 11.45), 3.34 (2H, sep, J = 6.85 Hz), 1.20

(12H, d, J = 6.85 Hz). ^{13}C NMR ($CDCl_3$) 151.37, 141.80, 125.84, 124.29, 94.35 (J = 4.0 Hz), 54.29 (J = 5.7 Hz), 26.81, 24.02. ^{31}P NMR ($CDCl_3$) δ 0.18.

HPLC analysis. HPLC analysis of prodrugs and propofol from the *in vitro* experiments was performed using an analytical HPLC system, which consisted of a Merck Hitachi (Hitachi Ltd., Tokyo, Japan) L-7100 pump, D-7000 interface module, L-7455 diode-array UV detector (190-800 nm, set at 220 nm), and L-7250 programmable autosampler. Rat whole blood samples were analyzed using an analytical Agilent Technologies 1100 series gradient RP-HPLC system consisted of Agilent 1100 Series Binary Pump, 1100 Series Autosampler, 1100 Series Micro Vacuum Degasser, 1100 Series Thermostated Column Compartment, 1100 Series Fluorescence Detector (Ex = 276 nm, Em = 310), HP 1050 Variable Wavelength Detector, 1100 Series Control Module and a Zorbax Eclipse XDB-C8 (4.6 mm × 150 mm, 5 μm) analytical column (Agilent Technologies Inc., Little Falls, Wilmington, DE, USA). The loop injection volume was 20 μl. The isocratic elution was performed by using a mobile phase consisting of 90 % (v/v) acetonitrile and 10 mM tetrabutylammonium dihydrogen phosphate at a ratio of 65:35 at a flow rate of 1.0 ml/min at 30ºC.

Animal treatments. Rats were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). The right jugular vein was exposed aseptically as described by Waynforth and Flecnell, (Waynforth and Fleckell 1994) and cannulated with a PE-50 catheter filled with 100 IE/ml heparin. The catheter was exteriorised through a small incision made in the neck. The rats were allowed to recover till the following day.

The drug solutions were administered to conscious rats as 30-second intravenous injections (including the rinsing of the catheter with 0.9 % NaCl). For the determination of propofol, venous blood samples of 200 μl of each were collected immediately before and 1, 2, 4, 6, 10 and 30 min after the i.v. bolus administration, mixed with 20 μl of 3 % EDTA in 0.7 % NaCl-solution and frozen immediately. The drawn blood volume was substituted by 0.9 % NaCl.

Rat blood sample preparations. For the assay of propofol in rat whole blood samples, the red blood cells were lysed by a total of three freeze/thaw cycles. 10 μl of 90 μg/ml thymol as internal standard was added to 100 μl of lysed whole blood. 250 μl

of acetonitrile was added to precipitate proteins and the samples were centrifuged for 3 min at 12 000 rpm. The supernatant was injected directly into the HPLC column.

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9 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Prodrug technology has become an integral part of the drug discovery and development process and at present a significant proportion of marketed drugs are prodrugs. However, systematic basic research focusing on the discovery of novel promoieties for different functional groups and pharmaceutical / clinical applications is still needed in order to expand the applicability of prodrugs. In addition, as the information about numerous enzymes, transporters and their substrates is expanding, the possibilities are increasing to utilize these proteins as activators or as uptake transporters of prodrugs through the intestinal membranes or into the specific tissues / organs.

A successful prodrug project requires intensive collaboration between medicinal chemists, pharmacokinetists, pharmacologists, toxicologists and formulation scientists in the drug design and development process. If a potential lead compound has some characteristic that limits its use as a drug, these teams should collaborate on the selection of an appropriate prodrug structure. The most important step is to identify the property of the parent drug that needs improvement. These properties may include the poor oral bioavailability resulting from poor aqueous solubility, poor lipophilicity, extensive first-pass metabolism or acting as a substrate for the efflux proteins on the intestinal membranes or in other organs (such as the brain), limiting the bioavailability and transport of the parent drug. Other important factors affecting the selection of the prodrug structure are the presence of functional groups amenable to prodrug derivatization, the desired mechanism and location of the bioactivation pathway, the route of administration, the chemical environment of the functional group in question and the nontoxicity of the promoiety. In addition, this study also highlighted the significance of the selection of an appropriate model compound for the studies of novel prodrug structures.

Academic research aims at producing reliable and objective basic studies that can be exploited by other academic and industrial research groups. However, academic projects may also lead to results that are patentable or even commercially exploitable. From the commercialization point of view, the most promising prodrug structure presented in this thesis work is the ethylidene phosphate prodrug of propofol, for which a patent

application was submitted in 2007. Future studies focusing on this structure are needed in order to confirm the efficacy of the prodrug. This thesis work confirmed the bioconversion of propofol ethylidene phosphate to propofol *in vivo*, but to achieve the complete sedation of rats, the study would need to be repeated with larger doses. It might also be worthwhile to separate the enantiomers and to evaluate the pharmaceutical and pharmacokinetic behaviour of the enantiomers separately. Furthermore, certain tests were not conducted due to small quantities of products available (e.g. maximum aqueous solubility) and these do need to be performed.

Further studies with different model drugs containing hydroxyl and amine functionalities would also expand the usefulness of this novel ethylidene phosphate prodrug structure. The hydroxyl group of propofol is phenolic, and thus, differs from primary, secondary and tertiary hydroxyl groups. The evaluation of ethylidene phosphates of these structures would be interesting. Another interesting application would be the ethylidene-linked phosphate prodrug of phenytoin, comparing to its properties to the marketed prodrug, fosphenytoin, in which the phosphonooxymethyl promoiety is attached to a weakly acidic amine functionality of phenytoin. In addition, a more detailed evaluation of the monoalkyl phosphates with enhanced membrane permeation properties would require more studies with a variety of model compounds (e.g. those with a nucleotide-type structure) to clarify the applicability of these structures as prodrugs.

Prodrugs have been applied to many of the most common functionalities in drug molecules, and a large number of novel and potential prodrug structures are described in the literature. Nonetheless, pharmaceutical companies often rely on old and familiar structures, such as esters, when making prodrugs. If there was a large and detailed database about the applicability of different promoieties to various functionalities, this might encourage the researchers to select a more appropriate prodrug structure for the desired purpose. This data could be in the form of an on-line database, e.g. where one could quickly find all prodrug structures of a secondary hydroxyl group that enhance aqueous solubility of a parent drug. The availability of this kind of database could possibly make prodrugs more easily accessible to scientists less familiar with this versatile approach to improve drug delivery.

Prodrugs are now an established part of drug discovery and development. Several successful prodrugs have been created with enhanced solubility and permeability characteristics, but much work is still needed, especially in the areas of site-selective drug delivery. Surprisingly few examples of prodrugs for cancer therapy are marketed, even though the side-effects of the traditional anticancer agents are distressing, often requiring premature termination of the therapy and poor compliance. Similarly, there are relatively few marketed prodrugs for CNS-targeted drug delivery, despite the benefits of achieving selective drug concentration into the brain in preference to other tissues in the body. Perhaps it can be concluded that the easiest prodrugs have now been examined and the more complex applications of prodrugs are still waiting investigation. I believe that especially CNS and tumor targeted prodrugs will be fruitful research areas in the future as the benefits compared to traditional therapy are so obvious.

Prodrug approach is undoubtedly a useful and versatile method in improving the poor drug-like properties of an active drug. Nonetheless, the optimal situation in the drug discovery and development process is, when directly active drug molecule can be used as is and prodrugs are not needed.

10 SUMMARY AND CONCLUSIONS

The present study described the synthesis, *in vitro* and *in vivo* evaluation of several prodrug structures including hydroxyimine and phosphate prodrugs. The aim of this study was to develop synthesis routes for the novel prodrug structures and to evaluate their capabilities to improve drug delivery. The following conclusions can be made based on the present study:

1. The nabumetone hydroxyimine underwent microsomal CYP 450-mediated oxidation to nabumetone *in vitro* and *in vivo*. The hydroxyimine prodrug structure is a potential intermediate prodrug structure for ketone drugs that can easily be derivatized to oximes using the appropriate promoieties depending on the prodrug application. However, insufficient chemical stability and an inadequate enzymatic bioconversion rate of the prodrugs to the active drug may limit the usefulness of hydroxyimine or oxime structures as prodrugs.

2. A novel synthesis route to prepare 1-chloroethyl phosphates and phosphoramidates as possible building blocks of novel ethylidene linked phosphate prodrugs was developed. This versatile and simple route can be conducted under NTP conditions, but increased yields, shortened reaction times and the possibility to synthesize otherwise unavailable reaction products were achieved with a microwave-assisted synthesis.

3. 1-Chloroethyl phosphates could not be utilized in the synthesis of ethylidene phosphate propofol probably due to steric reasons. Instead, a similar synthesis route using tetrabutylammonium phosphate was successfully exploited in the synthesis of an ethylidene phosphate prodrug of propofol.

4. The ethylidene phosphate prodrug of propofol increased the aqueous solubility of propofol and its chemical stability was sufficient to permit i.v. administration. The enzymatic release of propofol from the prodrug was rapid both *in vitro* in

alkaline phosphatase solution and *in vivo* in rats. These results show that ethylidene linked phosphates are suitable water-soluble prodrugs for i.v. administration. In general, ethylidene linked phosphates appear to be promising water-soluble prodrug structures for achieving enhanced drug delivery.

5. The monomethylester phosphates of propofol were not enzymatically cleaved either *in vitro* in solutions of alkaline phosphatase, phosphodiesterase or liver homogenate, or *in vivo* in rats, probably due to the inability of propofol to act as a substrate for phosphodiesterases. Thus, the diesters of phosphates do not seem to be suitable as potential prodrug structures, but more studies will be needed to confirm this conclusion.

Taken together, the results obtained in this dissertation project provide useful information of several novel prodrug structures for future prodrug design and development. As many new questions were raised during this work as were answered; this reflects the challenges that exist in this fascinating area of prodrug research.

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