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DEPARTMENT OF CHEMISTRY

RESEARCH REPORT NO. 150

ONDŘEJ JURČEK

STEROID CONJUGATES FOR APPLICATIONS IN PHARMACOLOGY AND BIOLOGY

Academic Dissertation for
the Degree of Doctor of Philosophy

UNIVERSITY OF JYVÄSKYLÄ
2011

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Doctor of Philosophy

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INSTITUTE OF CHEMICAL TECHNOLOGY, PRAGUE

Faculty of Food and Biochemical Technology

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and the

INSTITUTE OF EXPERIMENTAL BOTANY

Academy of Sciences of the Czech Republic

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ABSTRACT

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The scope of present study is rather broad and may seem to be non-uniform, covering subjects such as the treatment of insect pest species and atherosclerosis. Nevertheless, the magic word "steroid" connects the presented projects together. Steroid compounds possessing various unique physico-chemical and biological properties have been utilized as carriers of biologically active compounds. In this work novel conjugates of steroids with insecticidal agents (I. part), and anti-atherosclerotic drug (II. part) are presented.

The first introductory part deals with the history, development and importance of novel insecticides. In the second part the metabolism of cholesterol, development of atherosclerosis and its treatment are discussed. The importance of polymorphism in drug discovery is stated in the end.

Juvenoids, juvenile hormone analogues, environmentally safe insecticides, non-persistent, non-toxic for warm-blooded animals and fish, can become advanced compounds in regulating many aspects in insect physiology. Structure modification of juvenoids via juvenogen derivatives (hormonogenic substances) plays an important role in controlling the juvenoid liberation rate in the insect digestive system and can also play an important role in the mode of action toward different arthropod groups, with the focus on insect pest species. For that reason, racemic mixtures of juvenoid were esterified with hexadecanoic, butanoic, 3-methylbut-2-enoic and 3α,7α,12α-triformyloxy-5β-cholan-24-oic acid. Cholic acid-based juvenogens were further subjected to dimerization resulting in agents possessing two molecules of juvenoid in their structure, which was expected to lead to higher biological activity. A total of 20 new compounds with potential insecticidal activity were synthesized, characterized, and tested for their inhibitory activity on the reproduction of blowflies *Neobellieria (Sarcophaga) bullata*.

Succinobucol, a hypolipidemic drug that possesses significant antioxidant and anti-inflammatory properties, was studied for its polymorphism with the result of four discovered polymorphs. With an idea to alter its pharmacological profile and carry the drug more efficiently through intestinal membranes its conjugates with plant stanol and sterols, as well as with cholesterol, were prepared, described and studied for their toxicity, antioxidant activity and availability.

Keywords: insecticide, juvenoid, juvenogen, steroid, bile acid, phytosterol, cholesterol, succinobucol, probucol, atherosclerosis.

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FOREWORD

The present work was carried out in a fruitful collaboration between three different institutes: University of Jyväskylä, Department of Chemistry; Institute of Chemical Technology, Prague, Department of Chemistry of Natural Compounds; and Academy of Sciences of the Czech Republic, Institute of Experimental Botany, Isotope Laboratory, within the period 2006-2011.

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With respect, friendship and deep gratitude

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P.S.: Wishing you enjoyable reading of this thesis!

LIST OF ORIGINAL PUBLICATIONS

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I Jurček O., Wimmer Z., Bennettová B., Moravcová J., Drašar P., Šaman D.: Novel Juvenogens (Insect Hormonogenic Agents): Preparation and Biological Tests on *Neobellieria bullata*, *J. Agr. Food Chem.* **2009**, 57, 10852.
https://doi.org/10.1021/jf902290s

II Jurček O., Wimmer Z., Svobodová H., Bennettová B., Kolehmainen E., Drašar P.: Preparation and Preliminary Biological Screening of Cholic Acid - Juvenoid Conjugates, *Steroids* **2009**, 74, 779.
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III Jurček O., Lahtinen M., Wimmer Z., Drašar P., Kolehmainen E.: Crystallization, Spectral, Crystallographical and Thermoanalytical Studies of Succinobucol Polymorphism, *J. Pharm. Sci.*, under revision, **2011**.
https://doi.org/10.1002/jps.23068

IV Jurček O., Ikonen S., Buřičová L., Wimmerová M., Wimmer Z., Drašar P., Horníček J., Galandáková A., Ulrichová J., Kolehmainen E.: Succinobucol's new coat – conjugation with steroids to alter drug effect and bioavailability, *Mole-cules*, **2011**, 16, 9404.
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Author's contribution

The author carried out the syntheses and most of the analytical work as presented in the papers. The author has written all parts of the papers, excluding the biological part in paper I, and the X-ray crystallography and thermoanalytical parts in paper III. The author is also the corresponding author of publications II-IV. The content of article I was submitted on 9.6.2009 as a patent application: Jurček O., Wimmer Z.: Estery mastných kyselin a juvenoidních alkoholů a způsob jejich výroby a použití. (Fatty acid esters of juvenoid alcohols, their preparation and utilization), patent description CR 2009-369 (language Czech). Content of articles III and IV is part of registered inventions at the University of Jyväskylä: Jurček O., Kolehmainen E.: Two polymorphs of succinobucol – succinobucol I and succinobucol II (2009); Jurček O., Kolehmainen E., Wimmer Z.: Novel probucol conjugates with steroidal compounds, new approach to enhance its bioavailability and positive biological impact (2010).

OTHER PUBLICATIONS AND MATERIAL

Related to the topic but not included in the thesis.

Wimmer Z., Jurček O., Jedlička P., Hanus R., Kuldová J., Hrdý I., Bennettová B., Šaman D.: Insect pest management agents: Hormonogen Esters (Juvenogens), *J. Agric. Food Chem.* **2007**, *55*, 7387.

Ryšavá H., Štursa P., Kurzawová V., Jurček O., Pavlík M., Macková M., Ryšánek P., Wimmer Z.: Diversity of intestinal microflora by the red firebug *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) and its impact on the efficiency of juvenoid insecticides, *J. Insect Physiol.* submitted, **2011**.

Jurček O., Wimmerová M., Wimmer Z.: Selected chiral alcohols: Enzymic resolution and reduction of convenient substrates, *Coord. Chem. Rev.* **2008**, *252*, 767.

Ikonen S., Jurček O., Wimmer Z., Drašar P., Kolehmainen E.: Pharmaceutically significant succinobucol-steroid conjugates; crystal structures and pseudosymmetry in the crystals, *J. Mol. Struct.*, accepted, **2011**.

Buřičová L., Andjelkovic M., Čermáková A., Réblová Z., Jurček O., Kolehmainen E., Verhé R., Kvasnička F.: Antioxidant capacity and antioxidants of Strawberry, Blackberry, and Raspberry Leaves, *Czech J. Food Chem.* **2011**, *29*, 181.

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ABBREVIATIONS

ACN	Acetonitrile
AO	Antioxidant
APT	Atomic polar tensor
ARP	Antiradical power
ATR-IR	Attenuated total reflection infrared spectroscopy
BA	Bile acid
B3LYP	Becke 3-Parameter, Lee, Yang and Parr
E	Diethyl ether
EA	Ethyl acetate
CP MAS	$^{13}C\{^{1}H\}$Cross Polarization Magic Angle Spinning
DCC	*N,N′*-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DDT	2,2-Di(4-chlorophenyl)-1,1,1-trichloroethane
DMAP	4-*N,N′*-Dimethylaminopyridine
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DSC	Differential scanning calorimetry
EtOH	Ethanol
HDL	High density lipoproteins
Hex	*n*-Hexane
HMG-CoA	3-methyl-3-methylglutaryl coenzyme A
IDL	Intermediate density lipoprotein
IGR	Insect growth regulator
JH	Juvenile hormone
JHA	Juvenile hormone analogues
LDL	Low density lipoproteins
MeOH	Methanol
MS	Mass spectrometry
nAChR	Nicotinic acetylcholine receptor
NMR	Nuclear magnetic resonance
PE	Petroleum ether (40-60 °C)
PXRD	Powder X-ray diffraction
TAG	Triacylglycerol
TG	Thermogravimetry
THF	Tetrahydrofuran
VLDL	Very low density lipoprotein
W	Water
Xy	*p*-Xylene

1 INTRODUCTION

1.1 Steroid compound based prodrugs

Several potential biologically active molecules fail in the developmental phase, since they lack features that enable them to overcome biological barriers (epithelial, endothelial, elimination barriers, etc.), or their catabolism is too fast to induce desired changes. Many strategies have been developed for efficient delivery and controlled release of a biologically active agent from its site of administration to its site of action. It can be incorporated into a delivery system, equipped with structural features necessary for interacting with the target and/or for crossing the barriers, as well as conjugated with a non-toxic and rapidly excreted moiety, which once past the barrier is enzymatically or chemically cleaved from the parent biologically active compound (prodrug strategies). The prodrug approach may involve coupling of an agent to a natural substrate for a transporter or so-called "substrate mimicry", wherein the three-dimensional drug structure resembles that of a natural substrate. Prodrugs have become an established tool for improving physicochemical, biopharmaceutical or pharmacokinetic properties of pharmacologically active agents. In some cases, a prodrug may consist of two pharmacologically active drugs that are coupled together in a single molecule so that each drug acts as a promoiety for the other; such derivatives are called co-drugs.[1] These approaches are usually utilized in the design of novel drugs for humans, but they can be also generalized to a development of application forms of biologically active compounds for different organisms (e.g. insecticides).

There is a history in the utilization of steroidal compounds in prodrug design (e.g. bile acids and cholesterol for targeting a drug to liver or to etherohepatic circulation).[2,3] Steroid compounds are a broad family of compounds derived from the cholestane skeleton (Fig. 1). In nature steroid compounds are present in animals, plants and fungi and maintain many various biological functions. In this thesis the

main focus is on animal sterols (bile acids and cholesterol) and plant stanols and sterols.

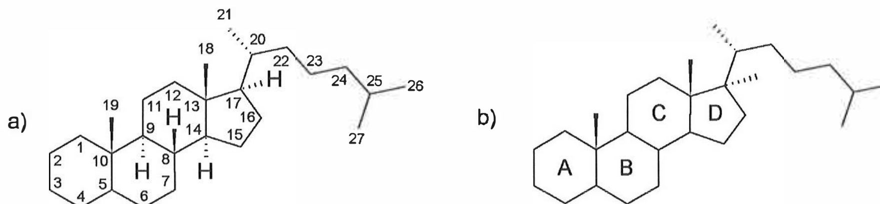


Figure 1. Atom numbering (a) and ring lettering (b) of the steroid skeleton of cholestane. Rings A-D form the gonane (cyclopentanoperhydrophenanthrene) nucleus of the steroid.

The first goal of the present investigation is to prepare novel types of insecticides with prolonged and selective release of the active compound in the digestive system of insects (part I). The second goal is to design novel anti-atherosclerotic drugs where plant stanols/sterols act as carriers of succinobucol, and may extend a range of drug pharmacological action or, in case of cholesterol conjugate, increase the absorption of the drug (part II).

I

JUVENILE HORMONE ANALOGUES AND NOVEL STEROIDAL JUVENOGENS

1.2 History of insecticides: inorganics, botanics and synthetic organics

Human life is influenced by a great number of various insect species. No wonder, arthropods existed already 550 million years ago and evolved into the animal class with the highest number of species and individuals. Some of these we can beneficially utilize, while some of them bring a negative impact to our lives and for that reason their population has to be regulated. Insect pest species (e.g. flies, mosquitoes, etc.) can be vectors of various diseases, competitors (e.g. aphids, locusts, etc.) with humans in utilization of natural food sources, or causing other damage in public and/or private domains (e.g. termites, etc.).

Until the mid 1800s insect pest control depended largely on picking or washing off. The few chemical agents were inorganic compounds such as sulfur (since 1000 BC), arsenic (900 AD), and later lead arsenate, cryolite, and boric acid, some of which (e.g. sulfur) are still marginally used, despite their high toxicity for both the targeted insects and non-targeted species including vertebrates.[4]

The next generation of agents came from the nature - botanicals (e.g. pyrethrum, nicotine, etc.). This was later a great inspiration and challenge for chemists and biologists, who prepared and tested their different analogues possessing greater

stability and availability. Moreover, derivatives could be prepared in a greater amount and were cheaper than parental natural products isolated from plant tissues.[4]

The introduction of organochlorine, organophosphorus and carbamate insecticides meant a real revolution in the agrochemical sector, as these compounds have allowed an important minimization of crop losses caused by insect activity. Also mosquito-transmitted diseases could be controlled to a certain extent. In this context, the introduction of DDT (Fig. 2) by the Swiss chemist Paul Hermann Müller in 1939 (1948 Nobel Prize in Physiology or Medicine), as one of the first organochlorine insecticides, was remarkable for its wide spectrum of action and a long residual activity.[5,6]

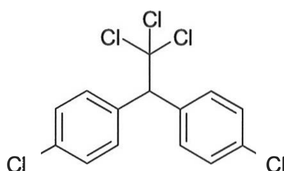


Figure 2. Organochlorine insecticide, 2,2-di(4-chlorophenyl)-1,1,1-trichloroethane (dichlorodiphenyltrichloroethan), DDT.

DDT's quick mode of action consists of opening sodium ion channels in neurons, which leads to spasms and eventual death of insects. Nevertheless, these effective and persistent lipophilic organochlorine insecticides were later found to be harmful also to mammals. Organochlorines are accumulated in the fat of animals throughout the food chain. Recent studies suggest that chronic exposure to DDT is associated with many different health problems related to its genotoxicity and endocrine disruption.[7] DDT possesses the ability to mimic estrogens and so can cause problems as premature birth,[8] harm of ability to breast feed,[9] disruption in semen quality,[10] breast cancer,[11] and some studies also suggests that children exposed while in the womb have a greater chance of developmental problems (neurotoxicity).[12,13] There is evidence from epidemiological studies indicating that DDT also causes cancer of the liver and pancreas.[12,14] Moreover, the connection of DDT to diabetes has also been considered.[15-17] This resulted in the banning of the majority of these organochlorine insecticides in many countries, although DDT is still in use in some countries where malaria is endemic.

At almost the same time (1937), German chemist Gerhard Schrader discovered organophosphorous insecticides (organophosphates, e.g. malathion, Fig. 3). These proved to be reliable and effective pest control agents. Organophosphorous insecticides affect the nervous system by phosphorylation of acetylcholinesterase (irreversible inactivation of the enzyme),[18] inducing respiratory muscle weakness and neuromuscular dysfunction in insects as well as in humans and animals.[19,20] These chemicals have a broad spectrum of activity against insect pests and showed only moderate stability in the environment, although they are considered the most toxic among insecticides.[5]

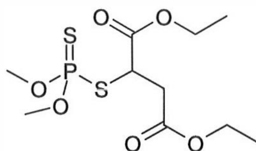


Figure 3. Organophosphorous insecticide 2-(dimethoxyphosphinothioylthio)butandioic acid diethyl ester, malathion.

Carbamates (e.g. fenoxycarb, Fig. 4) were developed in the 1950s. These insecticides are rapidly detoxified and excreted in warm-blooded animals, but also their persistence in the environment is low, which means that their application is needed several times over a season.[21] In general, they show selectivity against targeted insect pests. Nevertheless, carbamates can also be toxic to some useful insects, such as honeybees. Carbamate insecticides are comparable to organophosphorous insecticides in that they both suppress the activity of acetylcholinesterase, but compare to them reversibly. Carbamates are not regarded as mutagenic, carcinogenic or teratogenic substances.[5,22]

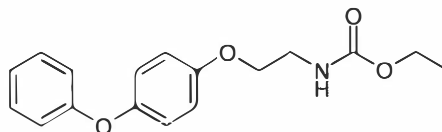


Figure 4. Carbamate insecticide ethyl N-[2-(phenoxyphenoxy)ethyl]carbamate, fenoxycarb.

Pyrethroids are other synthetic insecticides structurally derived from natural, photosensitive pyrethrins (Fig. 5 a),[23] compounds isolated from flowers of pyrethrum (*Chrysanthemum cinerariaefolium* and *C. coccineum*). The first generation of pyrethroids was developed by English chemist Michael Elliott in the 1960s (e.g. tetramethrin in Fig. 5 b).[24] Their activity was significantly higher than of the original pyrethrins, but these compounds were still relatively unstable in direct sunlight.

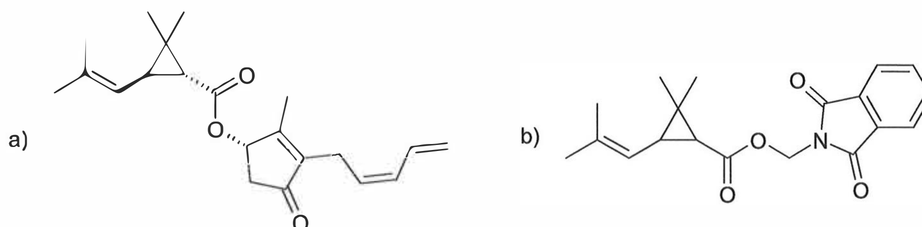


Figure 5. Natural pyrethrin I (a) and its synthetic derivative pyrethroid, tetramethrin (b).

A variety of structural modifications allowed the preparation of the second generation of pyrethroids with improved photostability (e.g. permethrin in Fig. 6), but also higher mammalian toxicity.[25,26]

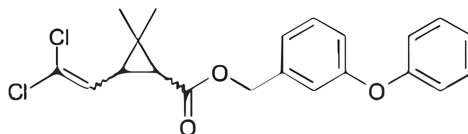


Figure 6. 2nd generation pyrethroid, permethrin.

Similarly to DDT, pyrethroids open insect neuronal sodium ion channels, causing neuronal hyperexcitability and paralysis. Their activity against insects, together with a good degree of biodegradability, selectivity and relatively low mammalian toxicity, put these insecticides among the most effective with a good environmental profile.[21,23]

Nicotine (Fig. 7, a) was used for centuries as an aphicide in the control of sucking insects, in spite of of its low effectivity and high toxicity to mammals. Further, in the search for compounds with a higher potency and lower mammalian toxicity, nicotinoids were developed, but they have never reached the degree of commercialization.[27] Commercial success has been reached with the next generation of derivatives – neonicotinoids (e.g. imidacloprid, Fig. 7, b).[28,29] All these nicotinic compounds are agonists at the nicotinic acetylcholine receptors (nAChRs), but the nicotinoids are selective to mammalian nAchRs and the neonicotinoids are selective for the insect nAChRs.[27] Neonicotinoids represent a new generation of synthetic insecticides with a broad spectrum of insecticidal activity, low application rates, a novel mode of action and favorable safety profile, as well as lacking cross-resistance to other insecticides.[30] Nevertheless, to an account of the activity of neonicotinoids were also assigned bee colony collapse disorders, which led to a limitation of their use in several countries.[31]

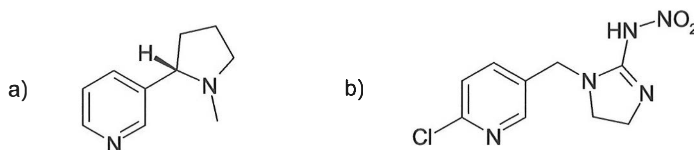


Figure 7. (-)-Nicotine (a) and the principal example of neonicotinoid insecticide, imidacloprid (b).

Apart from negative environmental aspects, pest control subjects the insect population to a Darwinian selection and survival of the fittest. Attempts to kill the tolerant individuals lead to ever increasing doses and eventually resistant pest populations. This is an inevitable limitation in the use of any class of insecticides. Nowadays there are populations of insects which are resistant to organochlorine, organophosphorus and carbamate insecticides.[32] For example, certain mutations in sodium channel gene or up-regulation of genes expressing cytochrome P450 (catalyze oxidation of organic compounds, metabolize endogenous and exogenous substances) can in some insect species lead to a resistance.[33,34] In order to overcome this problem it would be interesting to have new pesticides with different mechanisms of action. Thus, the main goal of pesticide research is the development of new, selective and highly effective substances that cause no harm to human health and the envi-

ronment. In the search for greener insecticides we decided to study novel insect growth regulators and their derivatives.

Insect growth regulators (IGRs) were at the time of their discovery considered to be the third generation of insecticides (after inorganics and synthetic organics). The term IGR was introduced by G. B. Staal (1975) to describe a class of bio-rational insecticides. These compounds alter normal growth processes of insects and interfere with insect metamorphosis, embryogenesis and reproduction. Three major groups can be recognized: i) substances that inhibit chitin synthesis or moult; ii) ecdysone agonists and iii) substances that mimic or antagonize insect juvenile hormone activity (this group is introduced in detail in chapter 1.3). The main advantage of these compounds over other insecticidal substances is their low mammalian toxicity and often quite high insect species specifity.[35-37]

i) Chitin is an important compound providing rigidity and mechanical stability to the exoskeleton of insects and crustaceans. Several substances have shown strong inhibition of the biosynthesis of chitin, e.g. diflubenzuron (Fig. 8), which can lead to weakness of the cuticle and disruption in the moulting process.[38] Diflubenzuron possesses a high selectivity and efficiency against lepidopterans and is considered safe from the view point of its acute, chronic and genotoxicity in mammal tests,[39] but it can be harmful to aquatic animals.[40]

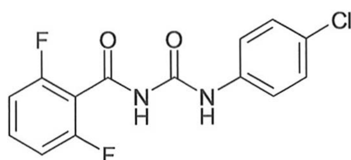


Figure 8. *N*-acyl urea derivative, diflubenzuron, as the chitin synthesis inhibitor.

ii) Ecdysone (Fig. 9, a) acts as a trigger in several systems required to coordinate the numerous events of the moult cycle. The application of ecdysone agonists (e.g. RH-5849 in Fig. 9, b) leads to premature cuticle synthesis and/or reduction of oviposition and feeding. These compounds are also poisonous for several insect species.[37]

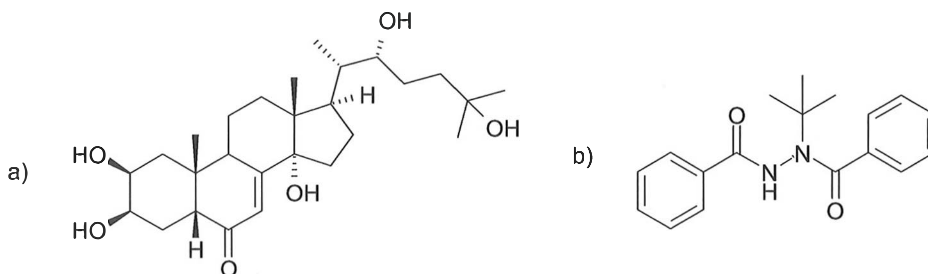
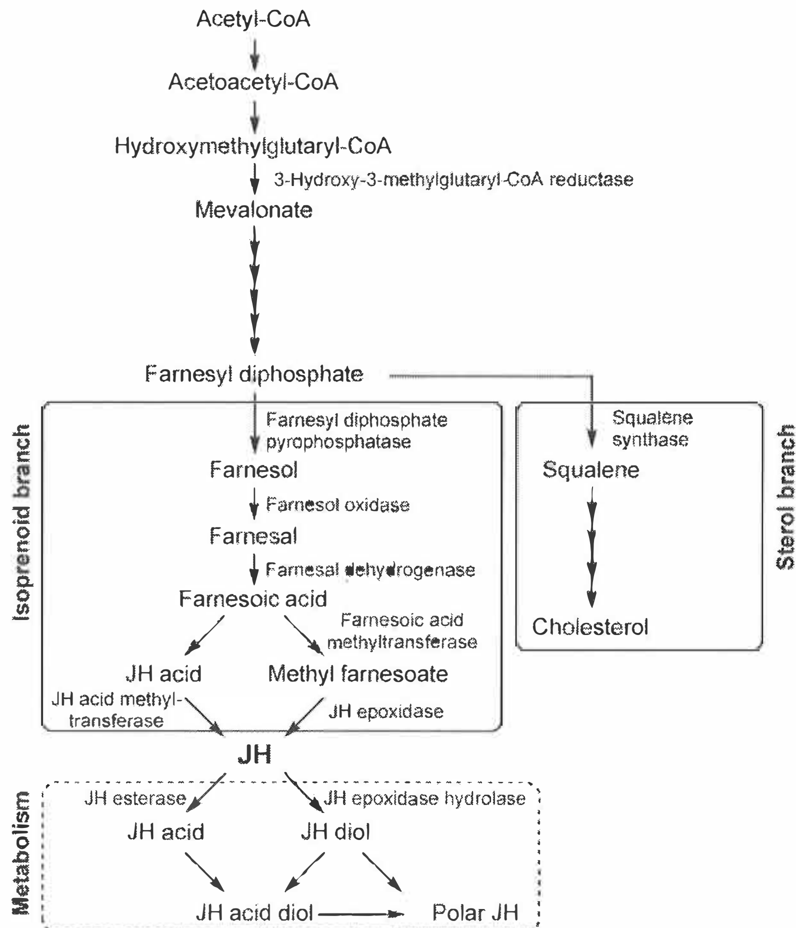


Figure 9. Ecdysone (a) and ecdysone agonist, RH-5849 (b).

1.3 Juvenile hormones and their analogues

The growth and development of insects are regulated by hormones including peptide hormones, the molting hormone ecdysone and the juvenile hormones (JHs). JHs are a family of naturally occurring sesquiterpenes biosynthesized in the paired endocrine gland *corpora allata* from acetyl-CoA *via* the mevalonate pathway (Scheme 1). Interestingly, insects and other arthropods do not synthesize cholesterol *de novo* but have to acquire it from dietary sources, because they lack the genes encoding squalene synthase and other subsequent enzymes of the sterol branch.[41]



Scheme 1. Metabolic pathway of juvenile hormones and sterols.[41]

Up to the present, six major JH homologs have been discovered and are known to regulate a wide spectrum of critical biological events in insects including development, metamorphosis, reproduction, polyphenisms, and alteration in behavior. JH has two distinct biochemical effects: one during the larval stage and the other in the adult stage. During the larval stage JH suppresses metamorphic change during

moulting; in the adult it induces vitellogenin synthesis during ovarian development.[42] The concentration of JHs varies according to the developmental stage of an individual insect; and the addition of externally applied JH or juvenile hormone analogue (JHAs) to the insect during the stage in which concentration of the natural JH is low affects development and usually results in fatal morphological changes.[43,44] Contrariwise, prolongation of the pupal stage by JHA for certain kinds of economically important insects may have a beneficial effect (e.g. silkworm).[45] JHs possess an α,β-unsaturated methyl ester group at one end of the molecule and an epoxide at the other. Since the deduction of the structure of the first JH by German scientist Herbert Röller in 1967 (Fig. 10, a),[46,47] hundreds of biologically and environmentally more stable but not persistent JHAs have been synthesized. The most successful ones became important tools in insect pest control, since they fulfill the basic requirements for environmentally safe, efficient and biorational insecticides (e.g. hydroprene (b),[48] methoprene (c),[49] fenoxycarb (d),[50] pyriproxyfen (e)[51]) (Fig. 10). JHAs can function as agonists or antagonists or a mixture of both with natural JHs.[42] Our effort in this field is aimed towards the synthesis of biodegradable, non-toxic JHAs.[43,52] Our attention has been focused on the derivatives of 2-(4-hydroxybenzyl)-1-cyclohexanone, where a ketone functional group enables variable modifications. The diversity in modifications and substituents led to detailed studies of the relationship between chemical structure and biological activity, out of which two important and interesting JHAs (also called juvenoids) (Fig. 10, f and g) were selected and studied further.[53-55] A study of juvenoid (f) is a part of this thesis.

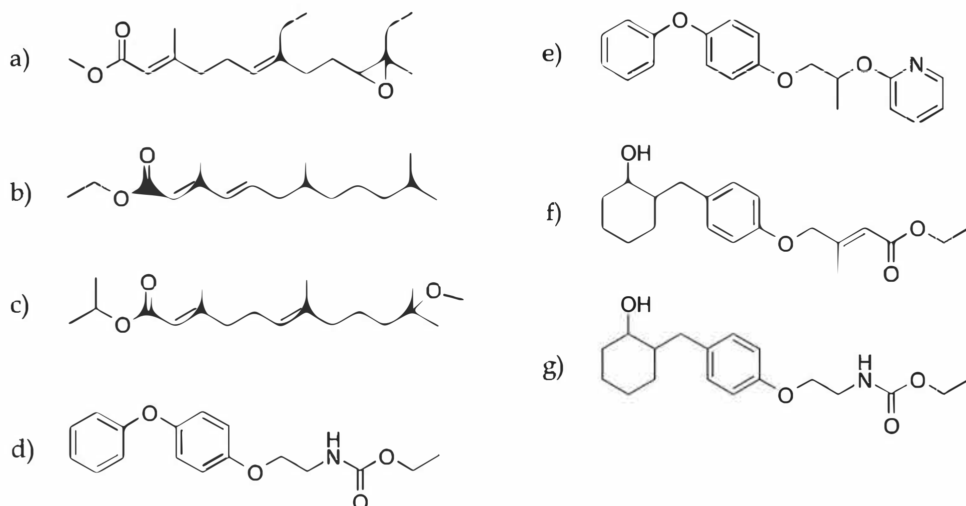


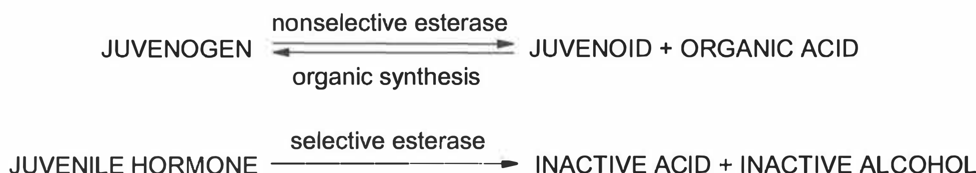
Figure 10. Structures of JH and JHAs: JH I (a); hydroprene (b); methoprene (c); fenoxycarb (d); pyriproxyfen (e); juvenoid (f) and juvenoid (g).

As the chirality of natural products plays an important role in their activity,[56] also the absolute configuration at the chiral centers of the JHs and JHAs can be the key for effective binding to a receptor site. Indeed, the naturally occurring (+)-juvenile hormone I is about 12000 times more bioactive than its enantiomer,[57] and

the naturally occurring (+)-juvenile hormone III is about 5000 times more bioactive than its enantiomer.[58,59] Therefore our effort has been also focused on the preparation of enantiomerically pure juvenoids by an enzymatic pathway, either by lipases or oxido-reductases.[60-64]

1.4 Application forms of juvenoids

Juvenogens (projuvenoids) were discovered by Sláma and Romaňuk in 1976.[65] They are complex chemical substances, usually displaying low or no biological activity toward insect species, but capable of liberating the biologically active ingredients under the effect of abiotic or biotic conditions. The basic idea of the juvenogen concept arose from the understanding of the metabolism of lipids in an insect's digestive tract and from the steps of biodegradation of the juvenile hormone molecule. Lipids (esters) are generally cleaved into corresponding alcohols and acids by non-selective lipases within the digestive tract and are further transported into hemolymph. This similarly applies to juvenogens (Scheme 2). Released juvenoids then take the action inside the insect body, which eventually needs not to be specific as for JHs (see below), thus causing more harm to the treated insect. JHs, after their release from *corpora allata* into hemolymph, are bound to juvenile hormone binding proteins (JHBP), which have functions of protection from the degradation by non-selective hydrolytic enzymes in the hemolymph, and target the JH to the place of its action. JHs are later degraded by selective enzymes, JH esterases, by which the ester is hydrolyzed with the liberation of the corresponding juvenile hormone acid and a small alcohol (Scheme 2).



Scheme 2. Degradation of juvenile hormones and juvenogens.

Thus, juvenogen is fed together with feed to the insect, where digestive enzymes metabolize the compound into a biologically active JHA and an inactive or active organic acid. This discovery led to a boom in the synthesis of a variety of juvenogen esters or ethers with different physico-chemical properties and thus different ways of application, different biological activity and different insect group selectivity based on the feed received by insects (e.g. esters with different fatty acids,[66,67] short organic acids,[68,69] dicarboxylic acids,[43] sugars,[55,70] bile acids,[71] phytosterols,[72] and several successful groups of juvenogens were also prepared as enantiomerically pure[69,73]). In some cases the acquired biological activity was even higher than the corresponding values for the parental JHA. This phenomenon is explained by the slow liberation of the JHA from the juvenogen molecule that supplies a biologically active analog to target cells in smaller quantities during a longer peri-

od of time.[55] Contrarily, application of the total quantity of juvenoid topically in one portion results in rapid enzymatic deactivation of juvenoid in the insect body. Juvenogens and juvenoids are not toxic, thus they do not directly influence the treated generation of pests. Their power lies in the influence on the next generation of insects by interrupting the reproduction cycle or the larval stage of the upcoming generation, which may lead to the eradication of the whole local population.[43,55,74] Their lack of toxicity may even bring benefits in a case of social species of insect (e.g. termites). Termites have developed a system of signaling a toxic hazard. If a worker carrying a feed to a nest dies, the feed is not further distributed to the nest population. This does not happen in our case, when, while feeding, the active compound is disseminated among nestmates or even to the queen and no toxic hazards can be observed by the insects. Some juvenogens also showed their power as systemic insecticides. The systemic application of the isomeric glycosidic juvenogens (Fig. 11, a) was investigated using pea plants grown hydroponically, on which sucking insects (fire bug, *Pyrrhocoris apterus*) were studied. The insects ingest water from the stems or leaves of the treated plants. The effect observed in the insects proved the absorption of the JHA from the hydroponic medium through the plant root system into its stem and leaves. Sequentially, stems and leaves of the plants were collected and extracted, and analysis of this extract showed the presence of juvenoid alcohol (Fig. 11, b).[43,55,75,76]

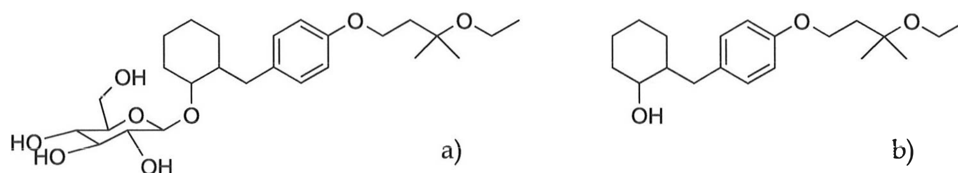


Figure 11. Systemic insecticides, juvenogen (a) and its active component juvenoid (b).

From the results of comparative screening tests of JHAs prepared in our laboratories with natural JH I and JH II, together with commercially available agents such as fenoxycarb, methoprene and pyriproxyfen, we can conclude the following: i) all tested compounds have higher biological activity than natural JH; ii) biological activities of JHAs of Czech origin are comparable to, or in some cases even higher than, those of commercially available JHAs; and iii) in case of our JHAs we have additionally a possibility to modify their physico-chemical and biological properties by esterification of the hydroxyl functionality.

1.5 Steroids and design of novel juvenogens

Ecdysteroids are present in animals (zooecdysteroids), plants (phytoecdysteroids) and fungi (mycoecdysteroids). Arthropods cannot synthesize cholesterol *de novo* from acetate and are thus dependent on ingested cholesterol or several other side-chain alkylated plant sterols as precursors for their ecdysteroid

synthesis. Phytoecdysteroids, in addition to being a source of precursors for hormone synthesis, participate in the defence of plants against non-adapted phytophagous invertebrates.[77,78] Ecdysteroids affect arthropod life from early embryogenesis to reproduction and adult life, and so it is not surprising that practically every organ is a target organ for ecdysteroids. Ecdysteroids is a generic name for a class of steroid hormones involved mainly in moulting and metamorphosis and in a variety of other processes in arthropods. Juvenile hormones and ecdysones together regulate the morphogenetic changes during metamorphosis. Ecdysones are secreted for varying periods in the larval stadium. Withdrawal of JHs and ecdysones induces the secretion of moulting hormones, and the insects ecdyse.[36,37] These ecdysteroid-controlled processes are successfully used as a target for the development of insecticides with low vertebrate toxicity (e.g. antagonist diacylhydrazines).[36]

Different steroid dimers were discovered in natural products. There is also an interest in their synthetic preparation for pharmacological and dietetic purposes, and for designing of new agrochemicals.[79-83] Inspired by the nature and publications dealing with the synthesis of steroidal dimers,[79,80,84] some of them also from our laboratories,[85-87] we utilized commercially easily accessible cholic acid (Fig. 12) as the building block for the preparation of potential functional carriers of juvenoids. For our purpose, cholic acid is considered as the most potent, due to its topology and a number of hydroxyl and carboxyl groups offering a broad spectrum of modifications and possibilities to form different types of interconnections with juvenoid via easily biodegradable ester bonds. Also cholic acid polarity profile may be the closest approximation, out of accessible bile acids, to the profile of ecdysteroids.

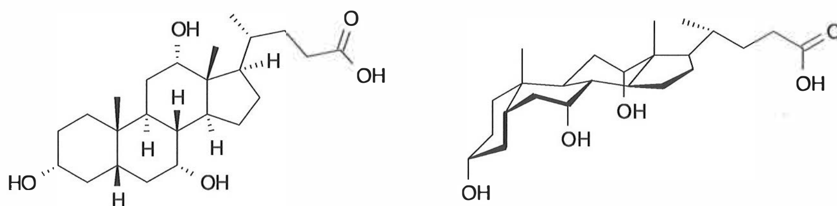


Figure 12. Unique structure of cholic acid.

By binding more than one juvenoid in a juvenogen structure we expected even higher biological activity in insect pest treatment than with a conjugate binding only one molecule of the juvenoid. Racemic mixtures of juvenoids were used for the synthesis for their ease of chemical preparation and lower cost. Also, enantiomerically pure compounds are not necessary for preliminary screening biological studies. As also noted, ecdysteroids are biosynthetized from externally uptaken steroidal compounds, which suggests this novel family of steroid based juvenogens to be potential systemic insecticides.

II

NOVEL SUCCINOBUCOL-STEROL CONJUGATES IN THE TREATMENT OF ATHEROSCLEROSIS

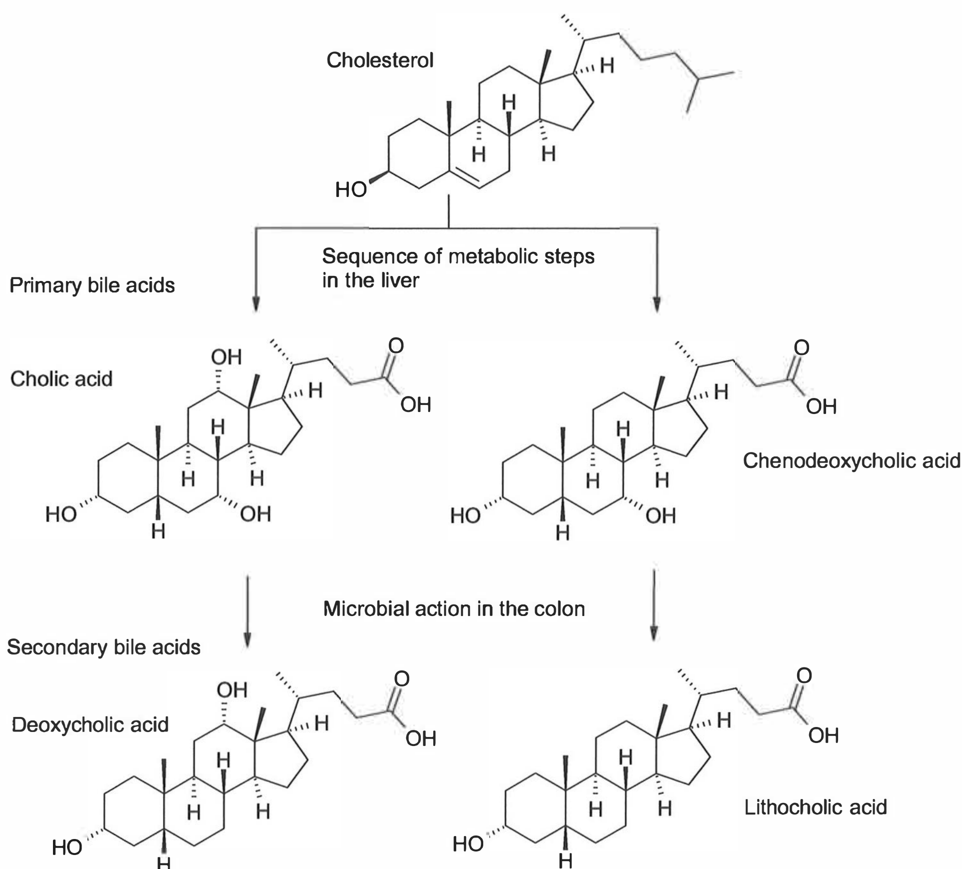
1.6 Metabolism of lipids, cholesterol and bile acids

Lipids (triacylglycerols, TAGs) are important components of biological membranes and sources of energy. Ingested lipids are cleaved within the digestive system by nonselective lipases into free fatty acids and mono- and diacylglycerols, which are further absorbed on the surface of intestinal mucous membrane in the form of mixed micelles with bile acids. The absorbed products of lipid digestion are throughout this membrane transformed back into TAGs and together with phospholipids, cholesterol and proteins are assembled into lipoprotein particles – chylomicrons. Chylomicrons are released into the blood through the lymphatic system and further distributed to tissues, where TAGs are again hydrolyzed and products are uptaken and utilized by skeletal muscles and adipose tissues. Lipoprotein lipase hydrolyzes the TAGs of chylomicrons, which, as a consequence, become smaller cholesterol rich chylomicron remnants. The remnant particles are then taken to the liver. Similarly TAGs biosynthetized in the liver are assembled together with phospholipids, cholesterol, cholesterol esters and proteins forming very low density lipoproteins (VLDLs). VLDLs are further released into the blood, where they undergo a sequence of events similar to chylomicrons, namely the progressive removal of TAGs from its core by lipoprotein lipase, first to intermediate density lipoproteins (IDLs) and further to low density lipoproteins (LDLs). LDLs transport cholesterol to peripheral tissues for the synthesis of cellular membranes and steroidal hormones.[88,89] LDLs may be subclassed to small dense LDLs and large LDLs based on their size, density, and lipid components. Small dense LDLs contains relatively abundant amounts of TAGs compared to large LDLs, which contain an abundant amount of cholesteryl esters.[90,91]

Within the transformation of VLDL to LDL almost all apolipoproteins* (apo-) are removed except a few and most of the cholesterol is esterified.[89] LDLs are bound to specific LDL receptors recognizing apoB-100 or apoE and are transported inside the cells by endocytosis. The expression of the LDL receptor is negatively regulated by intracellular cholesterol. Increased levels of LDL (hyperlipidemia) and accelerated development of atherosclerosis were found in humans and animals with a deficiency or mutation of the LDL receptor.[91] Around half of LDLs together with HDLs are absorbed by liver cells through LDL and HDL receptor endocytosis. Liver cells uptake also chylomicron remnants by the same mechanism. The function of HDLs is to transport endogenous cholesterol from the tissues to the liver.[89] HDL is considered as a factor participating in the regression of atherosclerosis.[91,92]

* apolipoprotein is a lipid binding protein, a constituent of plasma lipoproteins

Both the liver and the intestine are essential in the regulation of the cholesterol metabolism. Cholesterol is an important component of cell membranes, where it occupies the space between the polar head groups of the phospholipid molecular bilayer, reducing its fluidity. Cholesterol is also the precursor molecule for the synthesis of steroid hormones, vitamin D and bile salts.[88] Cholesterol that is transported to the liver via lipoproteins becomes partly a substrate for bile acid synthesis in hepatocytes, and partly it can be secreted into the bile together with bile acids. Cholesterol can be lost from the body as fractions of non-absorbed intestinal cholesterol, as bile salts and through sebum. Approximately 50 % of cholesterol secreted in the form of bile into the gastrointestinal tract is not absorbed back and is excreted. The enterohepatic pathway provides the major route for cholesterol removal from the body.[88,93] The conversion of cholesterol into bile acids involves the modification of the steroid moiety (characterized by isomerization of the 3β-hydroxyl group, addition of one or two hydroxyl groups, and saturation of the double bond) and the shortening of the side chain to yield a molecule containing 24 carbons.[93]



Scheme 3. Biosynthesis of primary and secondary bile acids from cholesterol.

Bile acids (BAs) are a group of, to some variable extent, water-soluble steroids possessing a unique orientation of the hydroxyl groups on one face of the molecule

imparting an amphipathic character to them. This underlies their most commonly regarded biological function of BAs as a detergent aiding in the absorption of lipids and lipid-soluble nutrients in the small intestine. BAs are essential in maintaining the solubility of cholesterol in the bile. It is now recognized that BAs have a much wider repertoire of biological activities than originally thought. BAs can modulate cellular signaling cascades that control a variety of ongoing cellular processes, such as cell viability and the metabolic fate of hepatic cholesterol. By serving as ligands for several nuclear receptors, BAs also have the capacity to regulate the expression of many genes and thereby control metabolic pathways and processes to attain BA synthesis and cholesterol homeostasis.[2,94]

The products of cholesterol catabolism, cholic acid (CA) and chenodeoxycholic acid (CDCA), are called primary bile acids (Scheme 3). These primary BAs are then conjugated, mainly to either glycine or taurine. The conjugated BAs are secreted from gallbladder through the bile duct into the duodenum as bile-acid anions, which mix with ingested food as it passes by. The conjugated BAs play a pivotal role in the digestion and absorption of lipids, products of their digestion and lipid soluble vitamins. The association of these lipid components, cholesterol, and phospholipids with BAs forms mixed micelles. The micellar mixture continues down the small intestine to the jejunum, where the contained lipids may diffuse through the epithelium to the portal vein. The micelles continue to the distal ileum, where about 95 % of the BAs are reabsorbed, and transported to the liver via the portal vein. This recycling of BAs is known as the enterohepatic circulation and can occur 10 times every day (from the gallbladder to the ileum, to the portal vein, and back to the liver). This cycling conserves BAs, thus avoiding the need to synthesize new BAs from cholesterol. The remaining 5 % of BAs enter the colon, where deconjugation of the conjugated primary BAs occurs via the action of bacterial enzymes, producing free BAs. The bacterial flora converts them into secondary BAs by removing the hydroxyl group from the C7 carbon atom of the steroidal skeleton. The responsible enzyme 7α-dehydroxylase forms deoxycholic acid (DCA) from cholic acid and lithocholic acid (LCA) from chenodeoxycholic acid (Scheme 3). These secondary bile acids, deoxycholic acid in particular, then pass into the portal vein and reach the liver, where they are conjugated to glycine or taurine and join new primary BAs in the liver. The conjugates are stored in the gallbladder together with other components forming bile, from where they again access the duodenum. A part of the bile acids returning to the liver is further metabolized to glucuronidated and sulfated derivatives that are eventually excreted out of the body.[2,94]

1.7 Atherosclerosis and its treatment

Coronary artery diseases (CADs) still remain one of the major causes of morbidity and mortality in the developed world. There are a number of disorders affecting the cardiovascular system; insufficient function of the heart caused by low contractility of myocardium or arrhythmia, atherosclerosis, thrombosis, hypertension, or their combinations. We focused our attention on atherosclerosis. Atherosclerosis

comes from the Greek words "athero" meaning gruel or paste and "sclerosis" meaning hardness. Atherosclerosis is a form of arterosclerosis† characterized by the deposition of the atheromatous plaques containing cholesterol and lipids on the innermost layer of the walls of large and medium-sized arteries. Atherosclerosis begins with damage to the artery caused by high blood pressure or oxidative stress, as well as elevated levels of cholesterol and triglycerides in the blood. Subsequently, the accumulation of lipids within arteries continues the pathogenesis of atherosclerosis, where both inflammation and oxidative stress are considered to play crucial roles. The formation of lesions and their transformation into fibrous tissues, calcified plaques, leads to narrowing and blocking of blood vessels. The rough surface of arteries can gather blood clots (thrombi), which may in the worst scenario lead to a closing of the artery and necrosis of the unnourished tissue (infarct).

Several other factors are also associated with the onset and progression of atherosclerosis, i.e. hyperglycemia, hyperhomocysteinemia, disruption of the immune system, glycation end products, and infectious agents.[89] Some of these factors are connected with diabetes.‡ Hyperlipidemia (especially elevated levels of small dense LDL) is closely associated with the incidences of ischemic heart diseases and diabetes. Basically, hyperlipidemia is subdivided into primary hyperlipidemia caused by genetic disorders in lipid metabolism, or secondary hyperlipidemia as the symptom and result of a disease (e.g. diabetes, hypothyreosis, diseases of kidneys or liver), or being induced by pharmaceuticals (diuretics, hormonal contraceptives).[91]

Physical or biological factors may oxidatively modify LDL. Oxidized LDL is considered a high risk factor for atherothrombosis and atherosclerosis. Increased levels of LDL and oxidative stress often coexist with other risks factors such as diabetes, hypercholesterolemia, hyperglycemia, hypertriglyceridemia, and low HDL-cholesterol. Increased peroxidation products are especially detected in glycated or cell-modified LDL.[91] Glycation and glycoxidation reactions appear to play a critical role in the development and progression of diabetes-associated atherosclerosis.[89]

Hyperlipidemia leads to an inflammatory response within the microvasculature, reflected by endothelial cell activation, leukocyte recruitment, rolling and adherence, as well as platelet activation and adhesion.[95] Chronic inflammation has been postulated to play a role in the development and propagation of atherosclerosis.[96] Inflammation is a major driving force underlying the initiation of coronary plaques, their unstable progression, and eventual disruption.[97]

Clinical trials have demonstrated the benefit of LDL reduction and, with less robust evidence, reduction of TAG and increased HDL, in the prevention of atherosclerotic cardiovascular disease. The treatment of atherosclerosis is usually complex, based on a diet supported by doses of hypolipidemic (or a combination of several hypolipidemics - combination therapy). Hypolipidemics are a group of pharmaceuticals that lower the level of lipoproteins in the plasma; nevertheless, they do not cure the disorders which originally cause hyperlipidemia. Some of these substances, besides influencing lipoprotein metabolism, have also additional effects on other risk

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† Arterosclerosis is a chronic disease in which thickening, hardening, and loss of elasticity of the arterial walls result in impaired blood circulation.

‡ People with diabetes have a 2–4-fold increased risk of developing cardiovascular diseases which is responsible for ca. 50 % of deaths among people with diabetes.

factors, such as anti-inflammatory and antioxidant processes (e.g. succinobucol). According to their mechanism of action hypolipidemics can be divided into several groups: bile acid sequestrants (chapter 1.7.1); pharmaceuticals altering the synthesis of lipoproteins (1.7.2); pharmaceuticals supporting the excretion of lipoproteins – fibrates (1.7.3); inhibitors of HMG-CoA reductase – statins (1.7.4); cholesterol absorption inhibitors – ezetimibe (1.7.5), and plant stanols and sterols (1.8); or probucol and succinobucol (1.9).[88,91]

1.7.1 Bile acid sequestrants (resins)

Bile acid sequestrants (e.g. cholestyramine, Fig. 13) lower the level of cholesteryl esters in LDL by blocking the absorption of BAs from the intestines. Strongly basic annexes insoluble in water absorb anions of BAs in the gastrointestinal tract and are excreted together from the body. Failure of reabsorption of BAs is compensated by their enhanced hepatic synthesis from cholesterol, which is connected with increased LDL receptor expression and, subsequently, with lowering of circulating LDL.

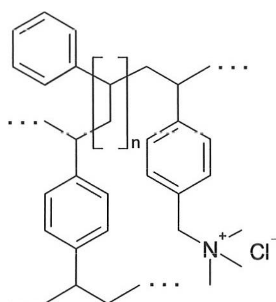

Figure 13. Structure of cholestyramine.

Patients assigned to cholestyramine have an average 8.5 % decrease in plasma cholesterol and 19 % reduction in coronary events. Two potentially adverse effects of these agents are on the metabolism of other drugs (since the sequestrants bind nonselectively all anions, e.g. statins) and on TAG metabolism (they increase TAG level, which may be a contraindication for patients with hypertriglyceridemia). A disadvantage of bile acid resins is also their potential to block the absorption of fat-soluble vitamins. Their greatest current use is in a combination with statins when the response to statins is inadequate.[72,98,99]

1.7.2 Cholesterol absorption inhibitors - ezetimibe

Ezetimibe (Fig. 14) interrupts the absorption of dietary cholesterol and biliary cholesterol from the gut by blocking the uptake of cholesterol into jejunal enterocytes[100] without affecting the absorption of fat-soluble vitamins or drugs, such as bile acid sequestrants do. Furthemore, ezetimibe does not increase serum TAGs. Ezetimibe has a limited LDL cholesterol-lowering effect of around 15-20 %, either

alone or in the presence of statins.[101,102] Ezetimibe is rapidly absorbed and is recycled enterohepatically multiple times.[103]

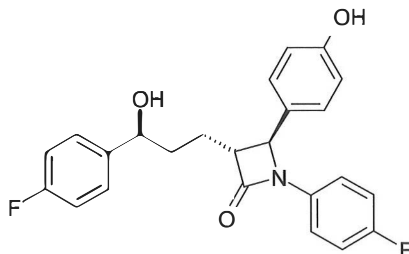

Figure 14. Ezetimibe

Nevertheless, adverse effects of ezetimibe compared to a cheaper medication, niacin, bring doubt about improvement of clinically significant outcomes. Paradoxically, greater reduction in the LDL cholesterol level in association with ezetimibe was significantly associated with an increase in carotid intima-media thickness,[§,104] and did not affect cardiovascular events, although a significant reduction of ischemic events was reported. It has been recently suggested, that ezetimibe should remain a second-line therapy.[105] Still, its use in the treatment of sitosterolemia is important.[106]

1.7.3 Pharmaceuticals altering the synthesis of lipoproteins

Nicotinic acid (niacin, vitamin B_3) (Fig. 15, a) as well as its derivatives (e.g. acipimox, Fig. 15, b) inhibit the mobilization of free fatty acids from peripheral tissues, hepatic synthesis of TAG and secretion of VLDL. It may also decrease the conversion of VLDL to LDL, alter small dense LDL, and lead to increase in HDL concentration.[90,107,108]

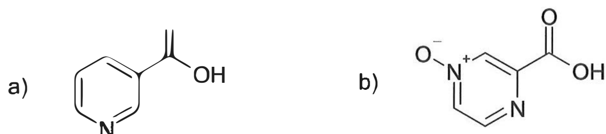

Figure 15. Structure of (a) nicotinic acid and (b) acipimox.

Many of the effects of niacin are considered to result from its action on adipose tissue.[109] It has been found during the Coronary Drug Project that niacin treatment showed a modest benefit in decreasing myocardial infarction and decreased the mortality by 11 %.[110]

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§ Measurement of the thickness of artery walls to detect and to track the progression of atherosclerosis.

1.7.4 Fibrates

Derivatives of α-aryloxyalkane acids, fibrates (e.g. bezafibrate, gemfibrozil, Fig. 16), are nowadays the most used hypolipidemics. Their major mode of action lies in the increase of the activity of lipoprotein lipase and decrease of hepatic synthesis of VLDL, by which they lower serum total cholesterol, small dense LDL, and TAG level by stimulation of β-oxidation. Further, the drugs are responsible for increase in HDL level, inhibition of biosynthesis of different proinflammatory molecules, or regulation of oxidative stress.[91-93] Fibrate treatment directed at markers of atherogenic dyslipidemia substantially reduces subsequent vascular event risk. In treatment with gemfibrozil there is a risk of myopathy and gallstone formation, but otherwise the fibrates are well tolerated.[90,108,111-113]

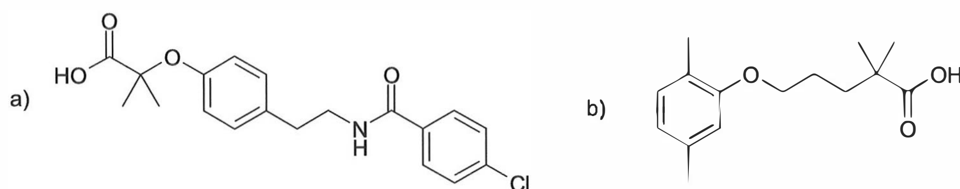


Figure 16. Structure of bezafibrate (a) and gemfibrozil (b).

1.7.5 Statins

The first member of the statin class – compactin (later renamed mevastatin, Fig. 17, a),[114] was isolated by Japanese biochemist Akira Endo from *Penicillinum citricum* in the 1970s.[115] However, this never underwent full clinical trials in man. Mevinolin (later renamed lovastatin, Fig. 17, b), a derivative of compactin with similar properties, was isolated from *Aspergillus terreus* later, and has become the first statin which received extensive clinical use.[116,117] This class of compounds inhibits 3-methyl-3-methylglutaryl-coenzyme A (HMG-CoA, Fig. 17, c), a reductase (enzyme of the mevalonate pathway), which plays a key role as the catalyst of the rate-controlling step in the biosynthesis of cholesterol (Schemes 1 and 4).[89] This inhibition induces the expression of LDL receptors in the liver, which in turn increases the catabolism of plasma LDL and lowers the plasma concentration of cholesterol. Statins have also shown potent anti-inflammatory and anti-thrombotic effects.[118]

Scheme 4. Reduction of 3-methyl-3-methylglutaryl-CoA to mevalonic acid by HMG-CoA reductase.

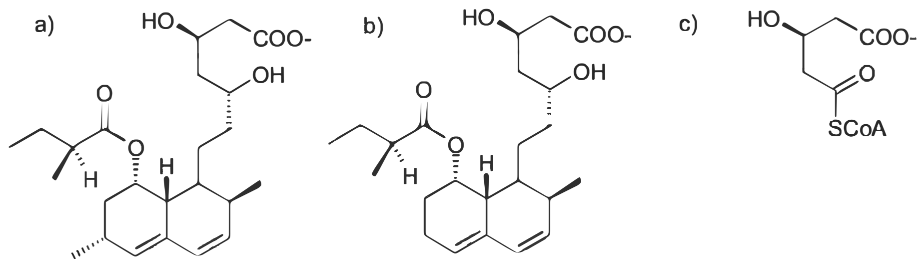


Figure 17. Statins mevastatin (a) and lovastatin (b) compared to HMG-CoA (c).

It was shown that these compounds significantly decrease the level of cholesterol in blood serum. Lovastatin generally decreases LDL cholesterol by 30-40 % together with a small decrease in serum TAGs and a tendency of HDL cholesterol to increase.[88] The effect of decreasing intrahepatic cholesterol is to induce the expression of sterol regulatory element binding proteins (SREBP),[119] which indirectly participate in cholesterol and fatty acid biosynthesis and cholesterol uptake. The increased expression of SREBP is the main pharmacological limitation to the use of statins: it increases the biosynthesis of HMG-CoA reductase, which thus opposes the competitive inhibition with statin treatment.[88]

1.8 Plant stanols and sterols

Plant sterols, also called phytosterols, have been reported to include over 250 different sterols and related compounds in various plant and marine materials.[120] Sitosterol, campesterol, and stigmasterol are the most abundant. Stanols are saturated sterols. The major plant stanols are sitostanol and campestanol, which are much less abundant in nature than sterols.[120-122] Whereas about 50 % of cholesterol is absorbed in the intestinal tract, plant stanols and sterols are absorbed much less: the absorption is about 10 % to 15 % for campesterol and campestanol, 4 % to 7 % for sitosterol, and 1 % for sitostanol.[121-123] Plant stanols are absorbed much less than plant sterols, and they even inhibit plant sterol absorption to some extent.[124] Nevertheless, both have a similar ability to inhibit cholesterol uptake.[125] Assuming that modified fat-soluble plant sterols and stanols would inhibit cholesterol absorption more markedly than the crystalline ones, the idea of using plant sterol and stanol fatty acid esters (Fig. 18) was applied, e.g. in fatty foods such as margarine (Benecol®). Their efficiency was supported by an extensive clinical study known as the North Karelia Project demonstrating that substituting sitostanol-ester margarine for a part of the daily fat intake in subjects with mild hypercholesterolemia lowers serum total and LDL cholesterol. In 1995 plant stanol fatty acid esters were introduced to the market as cholesterol lowering agents in hypercholesterolemic patients.[120-122,124,126]

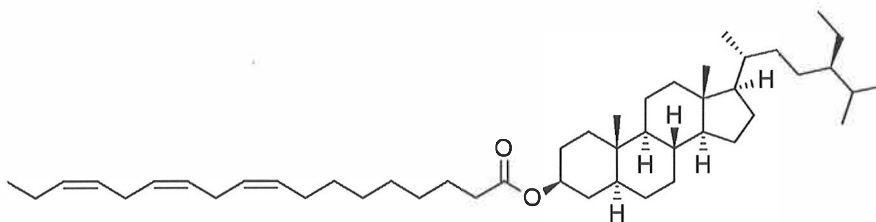


Figure 18. An example of fatty acid ester of plant stanol (α-linoleic acid ester of sitostanol).

The addition of plant stanols/sterols to a diet in doses of about 1.5–2 g/day lowers serum total and LDL cholesterol by up to 20 %, but cholesterol malabsorption in general does not consistently affect HDL cholesterol or triglyceride levels. A lifelong consumption of plant stanol ester spread has been predicted to reduce coronary events by 20 %, and even more if combined with small doses of statin. Adding sterols or stanols to statin medication is more effective than doubling the statin dose.[120-122,124,126-128] The stanol esters have been shown to be effective in reducing LDL cholesterol in subjects with type 1[129] and type 2 diabetes.[130] In contrast, the sterol esters only seem to have a small short term ability to reduce LDL cholesterol in subjects with type 2 diabetes.[131,132]

Sterols other than cholesterol entering the enterocytes via NPC1L1 are prevented from entering the body to a significant extent, because they are transported back into the intestinal lumen by the ATP binding cassette G5/G8. This transporter comprises the two half-transporters ABCG5 and ABCG8. Mutations of these transporters lead to the excessive accumulation of plant sterols, a rare disorder called sitosterolemia (phytosterolemia), where phytosterols accumulate in tendons and arteries, which can subsequently lead to atherosclerosis.[88,132,133] Ezetimibe is uniquely effective in suppressing both cholesterol and sitosterol absorption from the gut,[106] presumably acting at the NPC1L1 transporter. The bile-acid sequestrants also lower sitosterol absorption, but ezetimibe appears to be more effective in improving symptoms with few, if any, side-effects.[88]

After ingestion, esterified dietary plant sterols/stanols are hydrolyzed in the upper small intestine and are incorporated into the biological system as free dietary ones.[121,122,124] Multiple theories have been advanced concerning the mechanism by which phytosterols function as plasma cholesterol depressants. The potential mechanisms by which phytosterols inhibit cholesterol absorption include inhibition of mixed micelle formation, changing micellar solubilization, competition with the brush border for cholesterol uptake, altering intracellular esterification, and/or influencing incorporation into chylomicron. Inhibition of cholesterol absorption leads to reduced feed-back regulation of enterohepatic cholesterol circulation, and produces a compensatory increase in cholesterol synthesis. Nevertheless, administration of phytosterol intraperitoneally or subcutaneously to animals has been reported to lead to depressed levels of plasma cholesterol. These studies suggest that phytosterols may have also an intrinsic hypocholesterolemic effect via mechanisms other than this involving cholesterol absorption.[122,128]

Besides antihyperlipidemic properties, phytosterols have also been found to have anti-inflammatory, anti-bacterial, anti-ulcerative and anti-tumor activities and therefore their contribution to medicinally active substances is significant.[122]

1.9 Probucol and succinobucol

Probucol (Fig. 19, a) potentially impacts on atherosclerosis and related disorders via a range of biological activities, including its ability to affect lipid metabolism, exert anti-inflammatory and antioxidant activities, and maintain endothelial cell function.[134,135] Probucol can effect only a modest reduction in the LDL level, apparently by an increase of nonreceptor-mediated catabolism of LDL. The drug does not exert a consistent effect on plasma triglycerides, while it reduces the average baseline values of HDL.[136] Despite its unimpressive influence on atherogenic and cardioprotective plasma lipoprotein, probucol happens to be a particularly efficient antioxidant. Experimental studies show the drug may exert its antiatherogenic effect through inhibition of LDL oxidation,[137-139] with a related suppression of macrophage apoptosis present in advanced atherosclerotic lesions,[140] and thus may serve as a potent chemopreventive agent to suppress oxidant induced tissue injury[141] and carcinogenesis.[142] Clinical and laboratory studies showed probucol to induce the regression of tendon and cutaneous xanthomata[**] in patients with hypercholesterolemia, and the stabilization and regression of coronary arterial lesions under conditions that do not lower plasma cholesterol levels to a significant degree.[111] Moreover, probucol has been also studied as a potential antidiabetic therapy with promising results.[135]

Unfortunately, during clinical trials it was found in several patients that probucol prolongs cardiac cellular repolarization, resulting in a prolongation of the QT interval and an attendant risk of potentially fatal cardiac arrhythmias.[143] The main reason for continuation of probucol research is its broad spectrum of activities. Thus, compounds that share the antiatherosclerotic acitivities but not the deleterious effects of probucol are potentially interesting antiatherosclerotic agents. In search of such agents, succinobucol (AGI-1067), the monosuccinate ester of probucol that does not significantly change the QT interval, was developed (Fig. 19, b).[135,144]

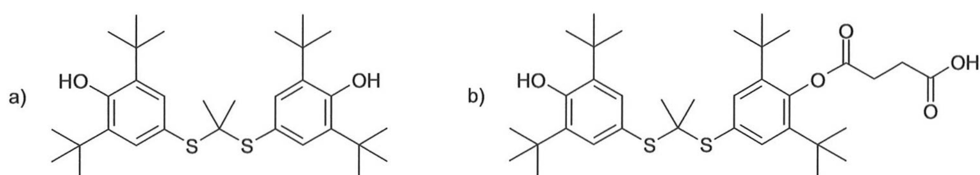


Figure 19. Structure of probucol (a) and succinobucol (b).

[**] A yellowish-orange, lipid-filled nodule or papule in the skin, often on an eyelid or over a joint.

Succinobucol increases luminal volume after percutaneous coronary intervention and tends to regress coronary atherosclerosis in patients already treated with statins, and this may translate into improved cardiovascular outcomes and decrease in new-onset diabetes.[135,145] Succinobucol has undergone phase III clinical trials to determine its effect on atherosclerotic endpoints; nevertheless, these results have not provided consistent data supporting strong cardioprotective effects.[146,147] Still, during clinical trials interesting antihyperglycemic effects were found. An additional phase III clinical study is evaluating its effects on type 2 diabetes.[148]

1.10 Polymorphism of active pharmaceutical ingredients (APIs)

The importance of polymorphism, the ability of a solid substance to exist in at least two different crystal lattices, is due to the correlation between the structure and the properties of solids.[149,150] In the crystalline state (polymorphs, co-crystals and solvates/hydrates), the constituent molecules are arranged into a fixed repeating array built of unit cells, which is known as lattice, where the molecules can have different arrangements (packing polymorphism) and/or conformations (conformational polymorphism). Polymorphs contain molecules of only one chemical species in their unit cells.[151] In many cases the appearance of polymorphism is very sensitive to the precise conditions involved and there are well-authenticated cases of "disappearing polymorphism" where reproducible production appears to be very difficult.[152-154] Moreover, when two conformers are very close energetically and structurally, the nucleation rates are of the same order of magnitude, and both structures are formed simultaneously, which is known as concomitant polymorphism.[151,155-158]

The identification and characterization of the desired polymorphic form of active pharmaceutical ingredients (APIs) are necessary in order to ensure reliable and robust manufacturing processes of a substance. The phenomenon is important for pharmaceutical companies since polymorphs differ in their physicochemical properties like rate of dissolution, equilibrium solubility, rate of reaction (hydrolysis and decomposition), photosensitivity, dispersibility and recrystallization behavior – and hence in their bioavailability and stability during transport and storage. Discovery of a novel polymorph of a drug is often a subject of protection and patent establishment.[150,159]

Up to the present time, a great number of patents and articles has been published describing probucol/succinobucol preparation, isolation, biological studies and applications.[135,136,144,148,160-163] Still, only two conformational polymorphs of probucol were described in 1993 by means of thermal analysis, PXRD, and single crystal X-ray analyses.[164] To the best knowledge of the author, there is no report describing polymorphism of succinobucol. Hence, a detailed study on the preparation, isolation and structural characterization of the succinobucol's polymorphs using $^{13}C\{^{1}H\}$Cross Polarization Magic Angle Spinning (CPMAS), ATR-IR, X-ray crystallography (single crystal and powder), DSC and TG was carried out and is reported here.

2 EXPERIMENTAL

I

JUVENILE HORMONE ANALOGUES AND NOVEL STEROIDAL JUVENOGENS

2.1 Preparation and studies of novel juvenogens

The invention of novel effective insecticides with good selectivity towards insect pest species only has been the goal of this study. In particular, our interest has been drawn to a group of juvenile hormone analogues (juvenoids) and their application forms (projuvenoids, juvenogens), which has a more than 30 years long history in Prague. A total of 20 new insecticides with potential insecticidal activity was prepared and characterized by means of NMR, IR spectroscopy, mass spectrometry and elemental analysis. As determinants of physico-chemical properties and biological activity of juvenogens hexadecanoic, butanoic, and 3-methylbut-2-enoic acids were selected as representatives of linear organic acids, and cholic acid as a representative of steroidal compounds. In the case of cholic acid based juvenogens we also prepared dimeric structures having double amount of biologically active juvenoid in the single molecule, thus expecting them to possess enhanced activity. All prepared compounds were tested on their ovicidal activity on *Neobellieria (Sarcophaga) bullata*. All results, observations and conclusions are presented either herein in the experimental part or in the articles I and II.

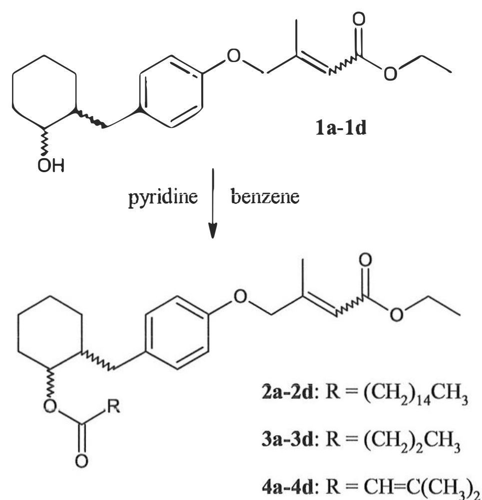
2.2 Syntheses and characterization

2.2.1 Juvenoids

The synthetic procedure for the preparation of the racemic mixtures of juvenoids **1a-1d** has already been described more than three decades ago.[165] The compounds are described by the following lettering system: **a** for racemic mixture (*E*, *cis*), **b** for (*E*, *trans*), **c** for (*Z*, *cis*), and **d** for (*Z*, *trans*) (Scheme 3).

2.2.2 Non-steroidal juvenogens

The racemic alcohols **1a-1d** were dissolved in benzene, pyridine was added and the mixture was cooled down in an external ice-water bath. The corresponding acyl chloride was added within 10 min under vigorous stirring. The reaction mixtures were stirred at room temperature overnight, processed and the crude products were purified by column chromatography resulting in racemic juvenogens **2a-2d**, **3a-3d** and **4a-4d** in high yields (Scheme 5).



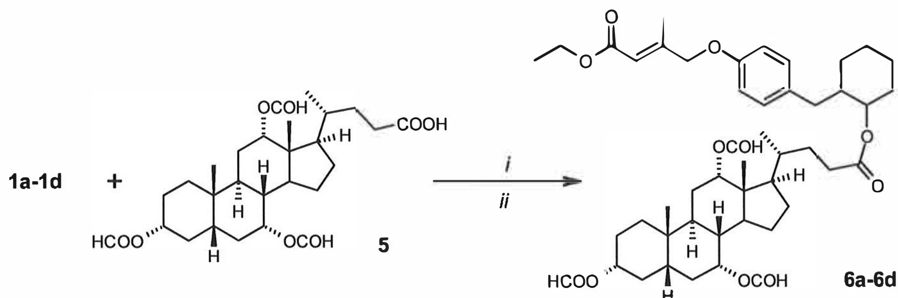
Scheme 5. Preparation of juvenogens **2a-2d**, **3a-3d** and **4a-4d**.

The obtained products were fully characterized and submitted for biological studies.[I]

2.2.3 Steroidal juvenogens

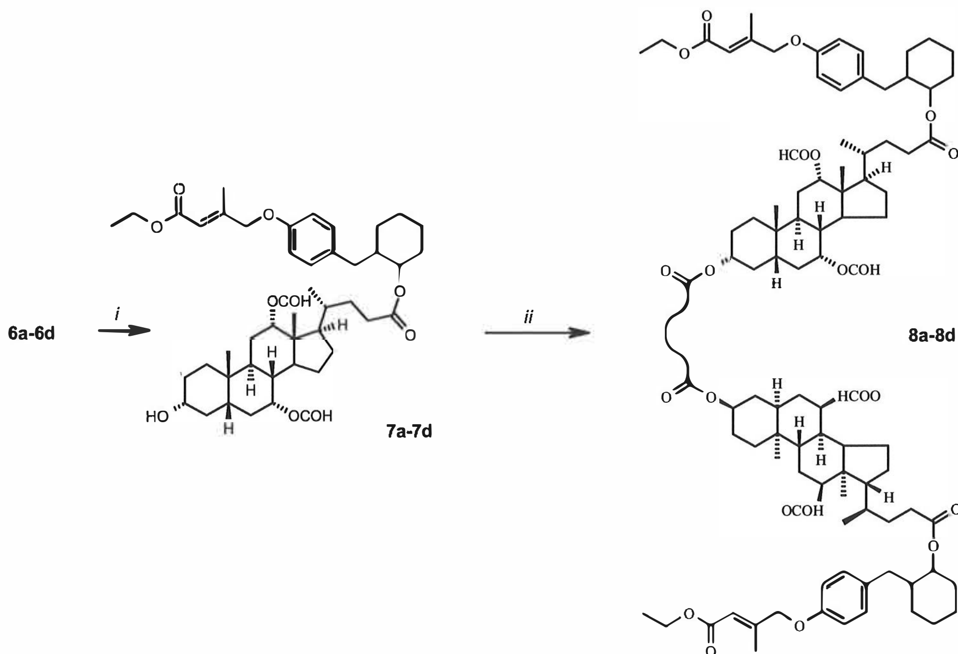
Formic acid was used to protect all three hydroxyl groups of cholic acid.[166] 3α,7α,12α-Triformyloxy-5β-cholan-24-oic acid (**5**) was subsequently dissolved in benzene, cooled in an external ice-water bath and oxalyl chloride was added

dropwise. The formed acyl chloride was immediately used in the next step, where racemic mixtures of alcohols **1a-1d** dissolved in benzene with pyridine were added. The reaction mixtures were stirred overnight, processed and the crude products were purified to result in steroidal juvenogens **6a-6d** in high yields (Scheme 6).



Scheme 6. Preparation of juvenogens **6a-6d**: i) oxalyl chloride, benzene; ii) racemic alcohols **1a-1d**, pyridine, benzene.

To deprotect the hydroxyl group at the C-3 position for the next reaction, the juvenogens **6a-6d** were dissolved in a methanolic solution of sodium bicarbonate (Scheme 7). The reaction mixture was stirred overnight. The solvent was evaporated, reaction mixtures were processed and crude products were purified by column chromatography yielding the compounds **7a-7d** in high yields.



Scheme 7. Preparation of juvenogens **8a-8d**: i) $NaHCO_3$, MeOH; ii) adipoyl chloride, pyridine, benzene.

In the last step, the compounds **7a-7d** were dissolved in benzene with pyridine, cooled in an external ice-water bath under nitrogen atmosphere and adipoyl chloride was added. The reaction mixture was stirred overnight, processed and the crude products were purified by column chromatography to give dimeric juvenogens **8a-8d** in satisfactory yields (Scheme 7).

The obtained products were fully characterized and submitted for biological studies.[II]

2.3 Biological activity on blowflies *Neobellieria (Sarcophaga) bullata*

Blowflies are serious household pests. In the screening tests the solutions of the compounds in acetone (concentrations 10 mg mL^{-1}) were applied to the upper part of the thorax of 40 freshly emerged blowfly females (5 μL $specimen^{-1}$). The blowfly females were kept in nylon net covered cages together with intact (untreated) males. Eight females each were dissected at regular intervals (6, 9, 12, 15, and 21 d after treatment) and the morphology of their ovaries (Fig. 20) was studied. The morphologically changed ovaries were subjected to a histological investigation. The shapes of each of the developing eggs and yolk deposition, both in the first and the second egg chambers, as well as signs of resorption, were observed. The tested compounds reduce egg hatchability due to lethal disorders in embryonic development. Larval hatching of eggs was calculated in dissected uteri from the number of developed larvae and those in which development had not taken place.

The ripe eggs descend into the uterus via a common oviduct, where insemination takes place to undergo embryogenesis. Developed larvae together with empty chorions are deposited onto a nutritive medium. During the second gonotrophic cycle the morphological and physiological changes are usually more striking.

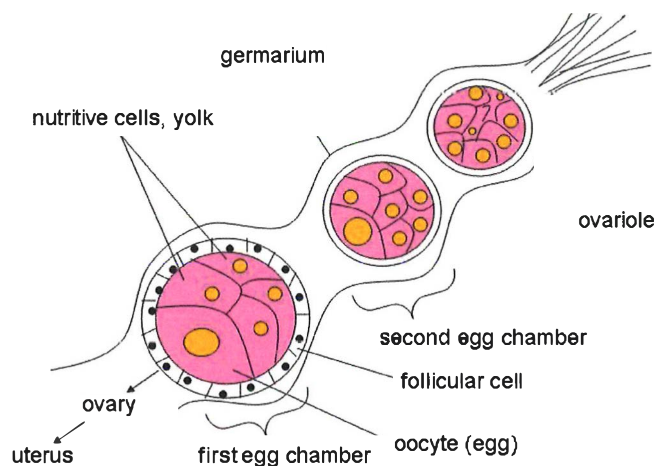


Figure 20. Sketch of a blowfly ovariole, one of many tubes of which the ovaries of the insect are composed.

The effect of the tested compounds on larval hatching is presented in Table 1 in comparison to methoprene, often used as a reference juvenile hormone analogue. Besides presenting the values of hatchability, the effective dose (ED_{50}) and effective concentration (EC_{50}) were calculated. ED_{50} shows the average amount of the tested compound applied on the tested insect species that resulted in 50 % reduction of hatchability. EC_{50} shows the concentration of a solution of the tested compound, the application of which in the given volume (5 µL per individual) resulted in a 50 % reduction of hatchability.

Table 1. The effect of methoprene and the juvenogens on blowfly *N. bullata*.

Compound	ED_{50} (µg individual^{-1})	EC_{50} (µg µL^{-1})	Hatchability (%)
Methoprene	>9	>1.8	>90
2a	6	1.2	60
2b	5	1.0	50
2c	>9	>1.8	>90
2d	>9	>1.8	>90
3a	6	1.2	60
3b	6	1.2	60
3c	7	1.4	70
3d	7	1.4	70
4a	>9	>1.8	>90
4b	2	0.4	20
4c	4	0.8	40
4d	6	1.2	60
6a	8	1.6	80
6b	8	1.6	80
6c	>9	>1.8	>90
6d	8	1.6	80

The development of the first batch of eggs was not morphologically affected, but the hatchability of the eggs was lowered. Significant differences within/among juvenogen groups were observed. To compare, methoprene decreased larval hatching to 90 %, a value in the range of hatching rates for untreated females (85-95 %). Important results were gained after the application of juvenogens with a 3-methylbut-2-enoic acid moiety (**4a-4d**). While compound **4a** showed no effect, the application of juvenogen **4b** led to a decreased hatchability of 20 %, **4c** was even toxic for the treated insect, and **4d** displayed only a mild effect (60 % hatchability). From the group of hexadecanoic acid derivatives, only compound **2b** displayed considerable ovicidal activity, 50 % hatchability. The effect of the juvenogens **3a-3d** was lower (60-70 % hatchability). The effect of steroidal juvenogens **6a-6d** was low (80-90 % hatchability) and juvenogens **8a-8d** showed no effect.

A histological observation of the ovaries revealed pathological changes in all structures of the egg chamber. Histological pictures of eggs are presented in Figure 21 (a-d). Histological changes in the development of the second egg chamber are manifested by a disruption of the structure of follicular cells in a low number of ovarioles, often about 10-20 %; in the case of compounds **2b**, **4b**, and **4d** even more, 25-65 %. Small deviations of eggs from normal (Fig. 21, a) were found in the case of **8d**, with results similar to those of **6a-6d**. They start as a division of nucleoli in the nuclei of follicular cells (Fig. 21, b) and subsequent proliferation of the nuclei of fol-

licular cells (Fig. 21, c). Reduced proliferation of follicular cells follows (Fig. 21, d). Such an ovariole does not produce viable eggs, which decreases offspring production.

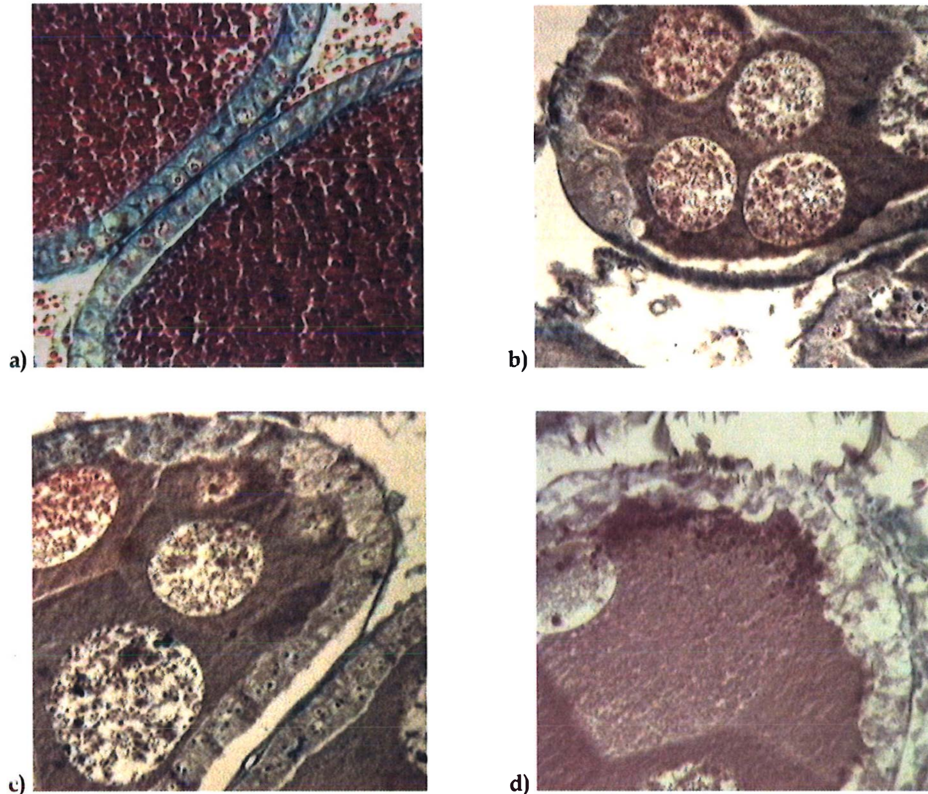


Figure 21. a) normal development of ovaries of *N. bullata* (a reference picture), b) division of nucleoli in follicular cell nucleus of the second egg chamber (first stadium of the juvenogens effect), c) nucleoli of the follicular epithelium cells divide and the cell layer adjoining the oocyte is thickened, and d) proliferation of folicular cells into the region of oocyte.

II

NOVEL SUCCINOBUCOL-STEROL CONJUGATES IN THE TREATMENT OF ATHEROSCLEROSIS

2.4 Goal of studies of succinobucol and its steroidal conjugates

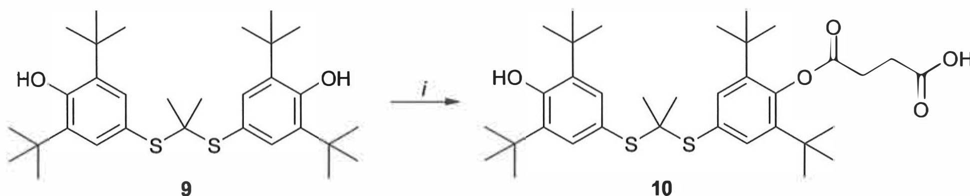
New therapies for the management of cardiovascular diseases remain highly desirable. The development of conjugates of succinobucol on one side, mainly targeting anti-inflammatory and antioxidant processes, with plant stanols/sterols on the

other side that significantly decrease LDL-cholesterol level, coupled with enhancing the bioavailability and targeting of the drug into enterohepatic circulation (cholesterol), may add to the armamentarium of current agents used in the treatment of atherosclerotic diseases and type 2 diabetes. During this research succinobucol additionally showed interesting polymorphic behavior which was studied in detail. Prepared compounds were extensively characterized by NMR (solid and liquid state), mass spectrometry, elemental analysis, ATR-IR spectroscopy, X-ray crystallography (single crystal and powder), differential scanning calorimetry and thermogravimetry. The antioxidant properties, toxicity and bioavailability were also studied to obtain more information about the medicinal potential of the conjugates.

2.5 Synthesis and characterization

2.5.1 Succinobucol

Succinobucol (**10**) was prepared from probucol (**9**) according to a procedure described in a patent (Scheme 8).[167]



Scheme 8. Preparation of succinobucol: i) potassium *t*-butoxide, succinic acid anhydride, THF.

2.5.2 Studies of succinobucol polymorphism

First observations about the polymorphic behavior of succinobucol were made during the crystallization of a crude product. Two different crystalline forms were observed. This started further studies of succinobucol polymorphism resulting in the discovery of four different crystal forms and a phase mixture of two characterized polymorphs. The recrystallization procedure and particular results of polymorphism studies obtained by various analytical methods are presented below.

2.5.2.1 Succinobucol recrystallization

Succinobucol was recrystallized by the method of slow evaporation of solvent(s) using different solvents and their mixtures (Table 2). As can be seen from the table, polymorphs A and C were exclusively prepared by crystallization from non-polar solvents and their mixtures. Crystalline solid B can be obtained by recrystallization

in non-polar or polar solvents or a mixture of both. Polymorphs D and E were obtained from polar solvent mixtures.

Table 2. Recrystallization of succinobucol. The relation of used solvents or their mixtures to succinobucol polymorphism leading to polymorphs A, C, D, E or the phase mixture B of polymorphs C and D.

A	B	C	D	E
Hex/THF	Hex/THF	Hex/THF	MeOH/ACN/W	$CHCl_3$/ACN
	ACN	Hex/PE/E		W/MeOH
	E/W/EtOH	Xy		DCM/ACN
	EtOH	E/Hex		
	PE/E			
	EA/Hex			

Solvent abbreviations: ACN (acetonitrile), DCM (dichloromethane), E (diethyl ether), EA (ethyl acetate), EtOH (ethanol), Hex (hexane), MeOH (methanol), PE (light petroleum ether), THF (tetrahydrofuran), W (water), Xy (*p*-xylene).

2.5.2.2 Nuclear magnetic resonance spectroscopy in solid state (^{13}C CPMAS NMR)

Different crystal forms can be mainly distinguished by carbon chemical shift differences of carbonyls (suggesting the importance of hydrogen bonding on polymorphism) and of the central carbon atom C-11 (suggesting differences in angle S-C-S as in the case of probucol polymorphism[164] and studied crystals of succinobucol-steroid conjugates[168]). The number of resonances in the ^{13}C CPMAS spectra of crystals exceeded the number of carbon atoms in the molecule (which itself indicates the presence of polymorphism), and a slight deviation in chemical shift was also observed (Table 3). In the case of poorly resolved carbon signals the shape of the peaks was taken into consideration in lieu of the actual chemical shifts.

Table 3. ^{13}C CPMAS NMR shifts (referenced to glycine C=O at 176.03 ppm) of samples A – E.

Carbon	A	B	C	D	E
1	179.47	179.28	180.38	180.65	181.06
4	171.77	170.52	171.61	171.79	171.65
17	154.12	152.99	154.26	153.98	154.87
5	149.84	146.84	147.87	148.39	149.19
6,10	143.03 142.54	141.30 139.98	141.09	142.51 141.26	143.57 142.86

Table 3. ^{13}C CPMAS NMR shifts (referenced to glycine C=O at 176.03 ppm) of samples A – E (continued from previous page).

Carbon	A	B	C	D	E
14	123.91	122.16	123.48	123.19	124.49
11	57.46	60.66 57.98	61.76	61.56 59.24	63.57

2.5.2.3 Powder X-ray diffraction (PXRD)

A comparison of PXRD patterns of the fresh samples reveals that they clearly originate from different polymorphic structure forms. The samples A, C, D and E are most likely comprised of a single polymorphic structure form, whereas a comparison of the PXRD patterns strongly suggests that sample B is a phase mixture of polymorphs C and D (Fig. 22) which was also verified by the SS NMR data.

The PXRD patterns of long-term stored (aged) samples show that only the polymorph A appears to remain unchanged during storage (1- 1½ years), whereas all the other polymorphic forms are either partially or completely transformed to form A, which can also be observed from the differences found in DSC scans. Observed behavior indicates polymorphs C, D and E to be kinetically, but not thermodynamically, stable as their structural properties can change either by aging and/or thermal annealing.

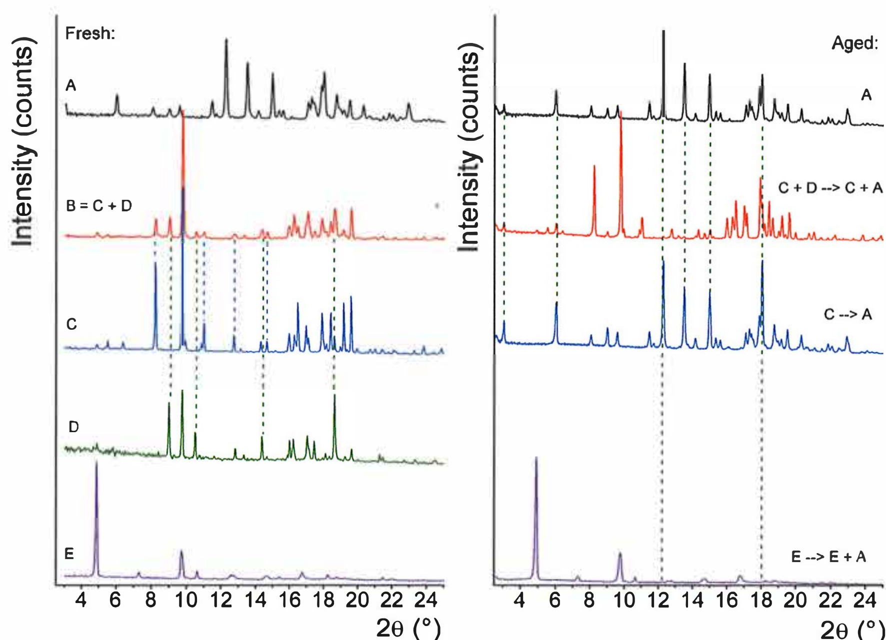


Figure 22. PXRD patterns of samples A – E measured from fresh and long-term stored (aged) samples.

In the case of phase mixture B, the polymorph D is almost completely transformed to the polymorphs C and A as overall intensity gain of C is increased from that found on fresh samples, and diffraction peaks of polymorph A appeared in the pattern. Similarly the neat polymorph C has been transformed completely to form A, and in the case of polymorph E, peak pattern of form A can be observed along with those characteristic for the form E. Furthermore, based on the observation of the fate of polymorph D in the phase mixture B, it can be assumed that similar process is expected to occur with the neat polymorph D, although there was no longer sample available to carry out the PXRD measurement from an aged sample.

2.5.2.4 X-ray single crystal analysis

Persistent crystallization attempts resulted in crystals suitable for X-ray single crystal analysis affording a crystal structure of polymorph C (crystallized from a mixture hexane/THF). The succinobucol polymorph C crystallizes in monoclinic space group $P2_1/n$ (No. 14) having a single molecule in the asymmetric unit. The succinyl groups of two adjacent succinobucol molecules are connected via hydrogen bonding, forming infinite rows of succinobucol acid dimers along *a*- and *b*-axes (Fig. 23). By sharing the carboxylic acid groups in the dimeric packing, the outer surface of the formed dimer is left hydrophobic due to the bulky *t*-butyl groups. The terminal hydroxyl group (O33a) is only weakly interacting with nearby methylene group of adjacent molecule, as the *t*-butyl groups on both sides of OH-group clearly hinder its ability to form classical hydrogen bonding by blocking potential directions for the proper hydrogen bond formation.

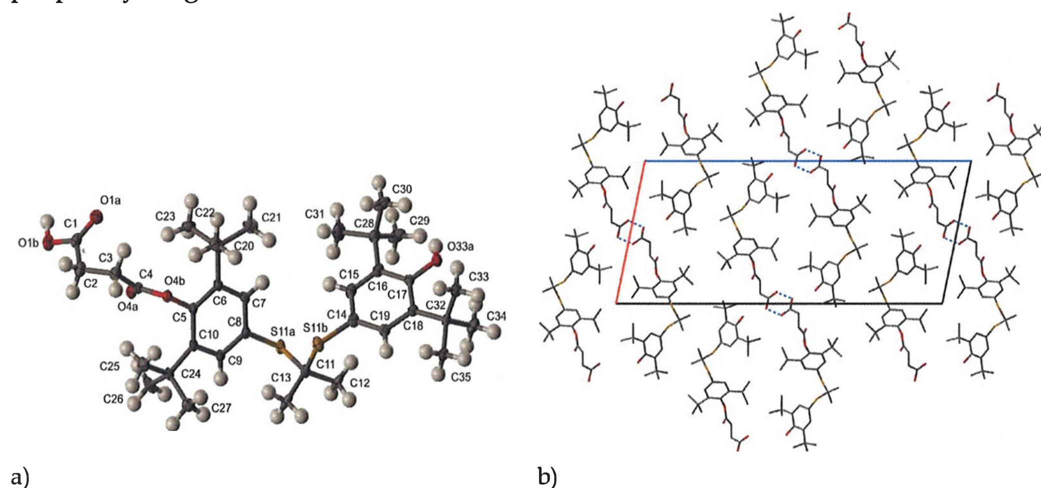


Figure 23. a) Asymmetric unit and atom labeling of polymorph C; b) molecular packing of polymorph C along b-axis. Hydrogen bonds between carboxylic groups of adjacent succinobucol molecules are shown by blue dashed lines. Hydrogen atoms are omitted for clarity.

2.5.2.5 *Thermal analyses: Differential scanning calorimetry (DSC) and thermogravimetry (TG)*

To further elucidate the thermodynamic behavior of the succinobucol polymorphs, DSC and TG analyses were made. The DSC scans are depicted in Figure 24 and the thermal transitions are summarized in Table 4. In fresh samples, the polymorph A shows a single melting transition at 157 °C. For the phase mixture B broadened melting peak at about 144 °C was observed, having a shoulder on the low temperature end of the transition which indicates the melting of both coexisting polymorphs C and D. The major component of the phase mixture B is form C, which melting transition forms the higher end and larger proportion of the transition peak and is closer to the melting temperature of the neat form of C (148 °C). Similarly the broad and rather weak shoulder corresponding to polymorph D in the phase mixture B is consistent to that of observed for the neat form D (140 °C). All four polymorphs exhibit only glass transition on the second heating scan indicating poor recrystallization properties from a melt. Typically the melting enthalpy of a polymorph ranges from 40 to 45 kJ/mol, of which the polymorph D seems to be less crystalline, because its melting enthalpy is less than a half of that observed for instance for the polymorph A (Tab. 4).

Table 4. Thermal properties of samples A – E.

Sample	1st heating	2nd heating	Decomp.
	T_m, ΔH	T_g, ΔC_p	T_d
	°C, (kJ mol⁻¹)	°C, [kJ mol⁻¹ °C⁻¹]	(°C)
A	157.3 (42.01)	43.7 [0.165]	191
B	144.1 (42.12)	41.7 [0.114]	198
C	147.7 (45.51)	43.2 [0.201]	186
D	140.2 (20.47)	49.1 [0.226]	186
E	135.6 (36.56)	46.7 [0.237]	187

The DSC scans of the aged samples (dashed lines in Figure 24) support the observations made by the X-ray powder diffraction, as the DSC scans of polymorph A do not change. The phase mixture of B shows melting transitions which is shifted closer to the melting temperature of the fresh neat polymorph C and the broad shoulder indicating the melting of polymorph D is nearly extinct. Similarly for aged neat polymorph C, its original melting transition is changed to that of corresponding to the melting temperature of polymorph A at about 157 °C. A major fraction of polymorph D is transformed to C as well. The same effect was also induced in a fresh sample of D by its thermal annealing for several hours at the temperature about 120 °C before the final heating to a melt. Also the polymorph E showed similar transformation to form A but in lesser extent. Thermal decomposition of the polymorphs starts within a close temperature range of 182 - 196 °C.

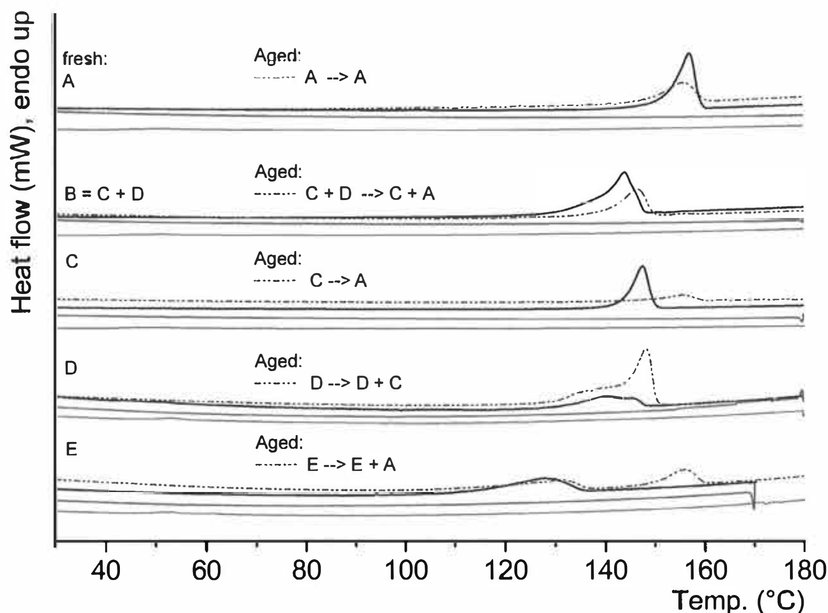
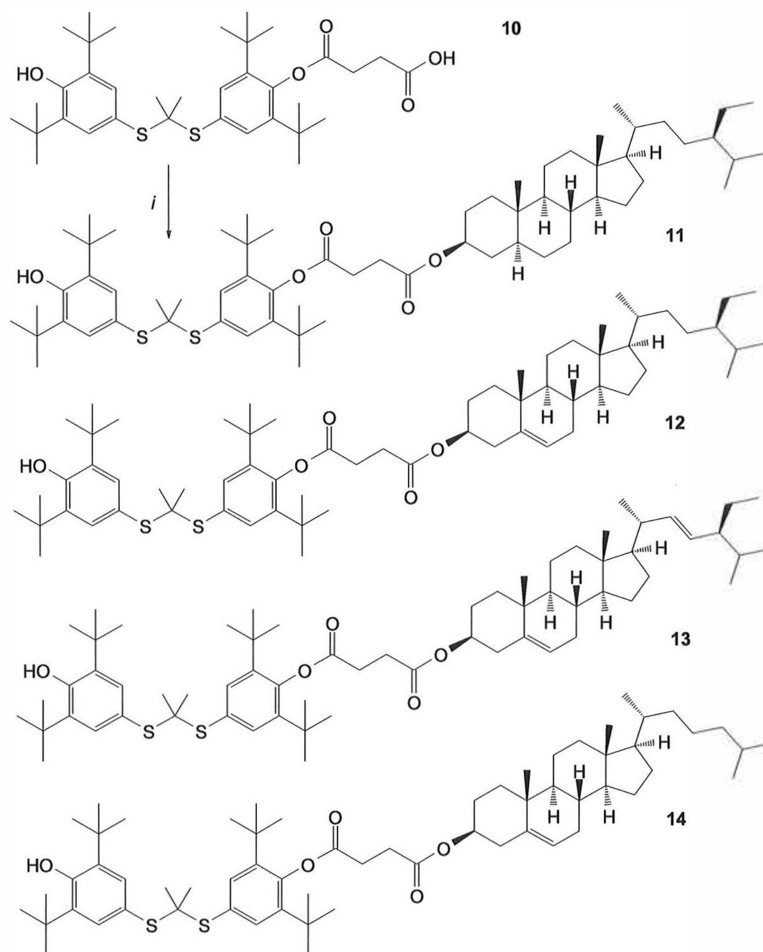


Figure 24. DSC scans of samples A – E. Order of the scans on each sample: 1st heating on the top, cooling in the middle and 2nd heating at the lowest. The 1st heating scans of aged samples are shown by dashed lines.

2.5.3 Succinobucol-steroid conjugate

Succinobucol ester conjugates of stigmastanol (**11**), β-sitosterol (**12**), stigmasterol (**13**) and cholesterol (**14**) were prepared following the procedure of Steglich esterification (Scheme 9).[169] To a stirred solution of **10** in anhydrous THF, DMAP and steroid alcohol were added. DCC was added under cooling in an external ice-water bath. The reaction mixture was stirred overnight at room temperature under nitrogen atmosphere. The reaction mixture was processed and pure products **11-14** were isolated by column chromatography in reasonable yields.



Scheme 9. The preparation of stigmastanol (**11**), β-sitosterol (**12**), stigmasterol (**13**) and cholesterol (**14**) conjugates with succinobucol (**10**): i) corresponding steroidal alcohol, DCC, DMAP, THF.

2.5.4 X-ray crystallography of succinobucol conjugates

Compounds **11-14** were crystallized from the mixture of diethyl ether and acetonitrile at ambient temperature as colorless single crystals of X-ray quality. All the compounds **11-14** crystallized in the triclinic spacegroup *P*1 (No. 1) with either two (compounds **11, 12** and **14**) or four (compound **13**) crystallographically independent molecules in the asymmetric unit. Molecular structures of one of the crystallographically independent molecule of each compound in crystalline state are shown in Figure 25.

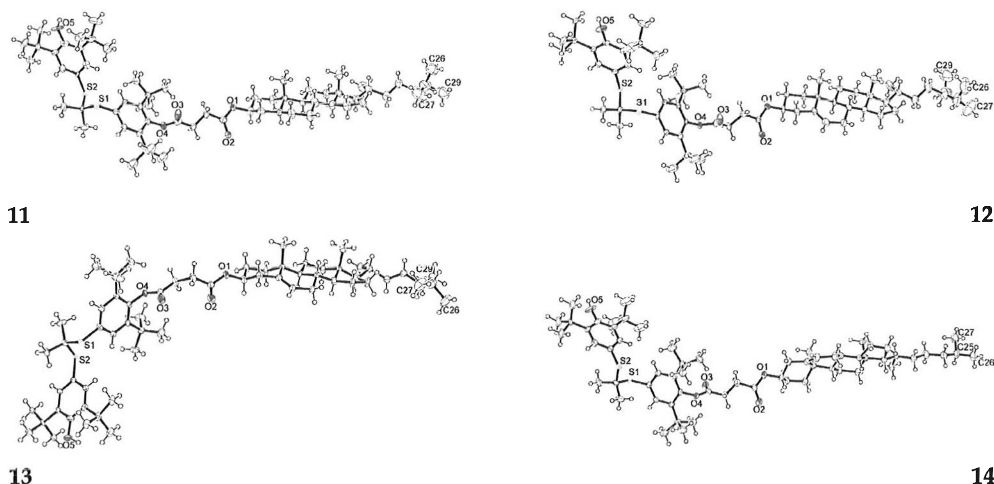


Figure 25. Molecular structures of **11-14** in crystalline state. Only one molecule from each asymmetric unit is shown for clarity.

2.6 Activity studies of compounds 9-14

2.6.1 Toxicity studies

Compounds **9-14** were tested for their toxicity towards mouse fibroblasts Balb/c 3T3 cell line. Of all the studied compounds only succinobucol (**10**) showed a toxic effect on the cells with $IC_{50} = 4 \pm 1$ µmol L^{-1}. Probucol (**9**) and conjugates **12** and **13** were not toxic at the highest soluble concentration of 19 µmol L^{-1} and 5 µmol L^{-1}, respectively. Conjugates **11** and **14** showed slight cytotoxic effect, the cell viability at the highest soluble concentration of 5 µmol L^{-1} being 70 % for both compounds.

2.6.2 Antioxidant activity: DPPH radical scavenging and mechanism of action

The EC_{50} value (Efficient Concentration = ((mol/L)AO/(mol/L)DPPH) means the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50 %. For reasons of clarity we speak in terms of $1/EC_{50}$ or the antiradical power (ARP): the larger the ARP, the more efficient is the antioxidant.[170] It can be concluded from Table 5 that conjugates **13** and **14** have an ARP similar to that of ascorbic acid. The remaining studied compounds, parental compounds **9** and **10** and conjugates **11** and **12,** have a higher ability to scavenge DPPH radical and thus a higher ARP than ascorbic acid.

Table 5. Radical scavenging activity of test compounds **9-14**

Compounds	Ascorbic acid	**9**	**10**	**11**	**12**	**13**	**14**
EC_{50}	0.27	0.20	0.17	0.20	0.14	0.27	0.26
ARP	3.7	5.0	5.9	4.9	7.1	3.7	3.8

From the comparison of results of probucol measurements with other compounds from the studied group one can deduce that the activity is not dependent on the number of available phenolic groups, but some other feature should be considered. Sulfur atoms in organic compounds are known to be active in the quenching of radicals.[171,172] It was also observed by the group of Stocker that sulfur atoms, rather than phenolic moieties of probucol (or succinobucol), may play the key role in antioxidant activity and thus may be responsible for the antiatherogenic and antirestenotic protection. [173] We assume that in our case also the sulfur atoms are responsible for the antioxidant activity. This was also suggested by the computed electrostatic potential mapped on the electron density surface of the succinobucol molecule in methanol. The negative atomic polar tensor (APT) charge for oxygen of free phenolic hydroxyl group is -0.64 (reduced by the value of hydrogen charge) compared to sulfur atoms, -0.03 and -0.09, respectively. Even though the oxygen carries a higher negative charge, it may be less susceptible for electron/radical donation, because of shielding by bulky *t*-butyl groups, contrary to the relatively unshielded sulfur atoms (Fig. 26).[IV]

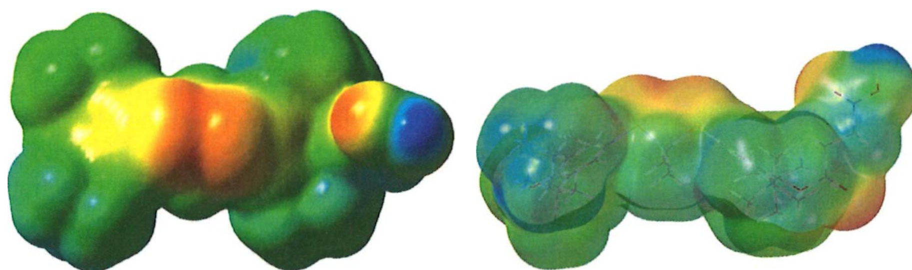


Figure 26. Electrostatic potential on the molecule surface of a succinobucol molecule in methanol (red indicates negative, blue indicates positive and green indicates neutral electrostatical potential).

In addition to this experimental section more details can be found within publications I-IV.

3 SUMMARY AND CONCLUSIONS

A total of 20 new juvenogens derived from the racemic juvenoids were synthesized, identified, and tested on the blowfly *N. bullata* for their influence on reproduction. The most remarkable effect of a non-steroidal acyl was observed with juvenogens **4a-4d**. Generally, only the EC_{50} values not exceeding 1 µg µL^{-1} indicated the potential of juvenogens **2b**, **4b** and **4c** for practical applications. In the case of steroidal juvenogens the biological activity on blowflies was not satisfactory. We observe a dependence of the biological activity on the size of the used moiety. This can be due to a capability of non-selective lipases for enzymatic cleavage of bulkier molecules, where juvenoids cannot be released. Substances **6a–6d** may be limiting for utilization as insect pest management agents in the size of their molecule, which should not be exceeded in the insecticide design. The synthetic approach represents a pathway for the preparation of conjugates of bile acids with biologically active compounds possessing primary or secondary hydroxyl group(s) in general.

Four different succinobucol-steroid conjugates **11-14**, derived from plant stanol (stigmastanol), plant sterols (β-sitosterol and stigmasterol) and animal sterol (cholesterol), were prepared and fully characterized on their molecular and submolecular level. Toxicity, antioxidant and bioavailability studies were performed and summarized, suggesting that these compounds are interesting for further studies of their biological activity. Furthermore, four different polymorphs of succinobucol A, C, D and E, and the phase mixture B of polymorphs C and D were characterized by spectral (^{13}C CPMAS NMR and ATR-IR), X-ray crystallographical (single crystal and powder) and thermoanalytical (TG and DSC) methods. Based on the analysis of both fresh and aged polymorph samples it was possible to deduce coarse estimate of the thermodynamic stability of the polymorphs; for the polymorph A being the most stable. Polymorphs C, D and E are kinetically metastable polymorphs which change monotropically to the form A either by long-term storage or by thermal annealing. We believe that succinobucol possesses some undiscovered potential as drug and therefore further research is desirable.

Steroids form a unique system of compounds: they are readily available, inexpensive, efficient carriers of unique biological and physico-chemical properties, with

a number of ready-made chiral centers in a rigid skeleton, offering a broad variety of possibilities for their modifications and thus for tuning their properties, or offering a broad variety for conjugation with biologically active compounds. Our interest in this field is perpetual.

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ORIGINAL PAPERS

I

Novel Juvenogens (Insect Hormonogenic Agents): Preparation and Biological Tests on *Neobellieria bullata*

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by

Ondřej Jurček, Zdeněk Wimmer, Blanka Bennettová, Jitka Moravcová, Pavel Drašar and David Šaman

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II

Preparation and Preliminary Biological Screening of Cholic Acid-Juvenoid Conjugates

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Ondřej Jurček, Zdeněk Wimmer, Hana Svobodová, Blanka Bennettová, Erkki Kolehmainen, Pavel Drašar

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III

Crystallization, Spectral, Crystallographical and Thermoanalytical Studies of Succinobucol Polymorphism

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Ondřej Jurček, Manu Lahtinen, Zdeněk Wimmer, Pavel Drašar, Erkki Kolehmainen

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IV

Succinobucol's new coat – conjugation with steroids to alter its drug effect and bioavailability

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Ondřej Jurček, Satu Ikonen, Lucie Buřičová, Martina Wimmerová, Zdeněk Wimmer, Jan Horníček, Pavel Drašar, Adéla Galandáková, Jitka Ulrichová, Erkki Kolehmainen

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