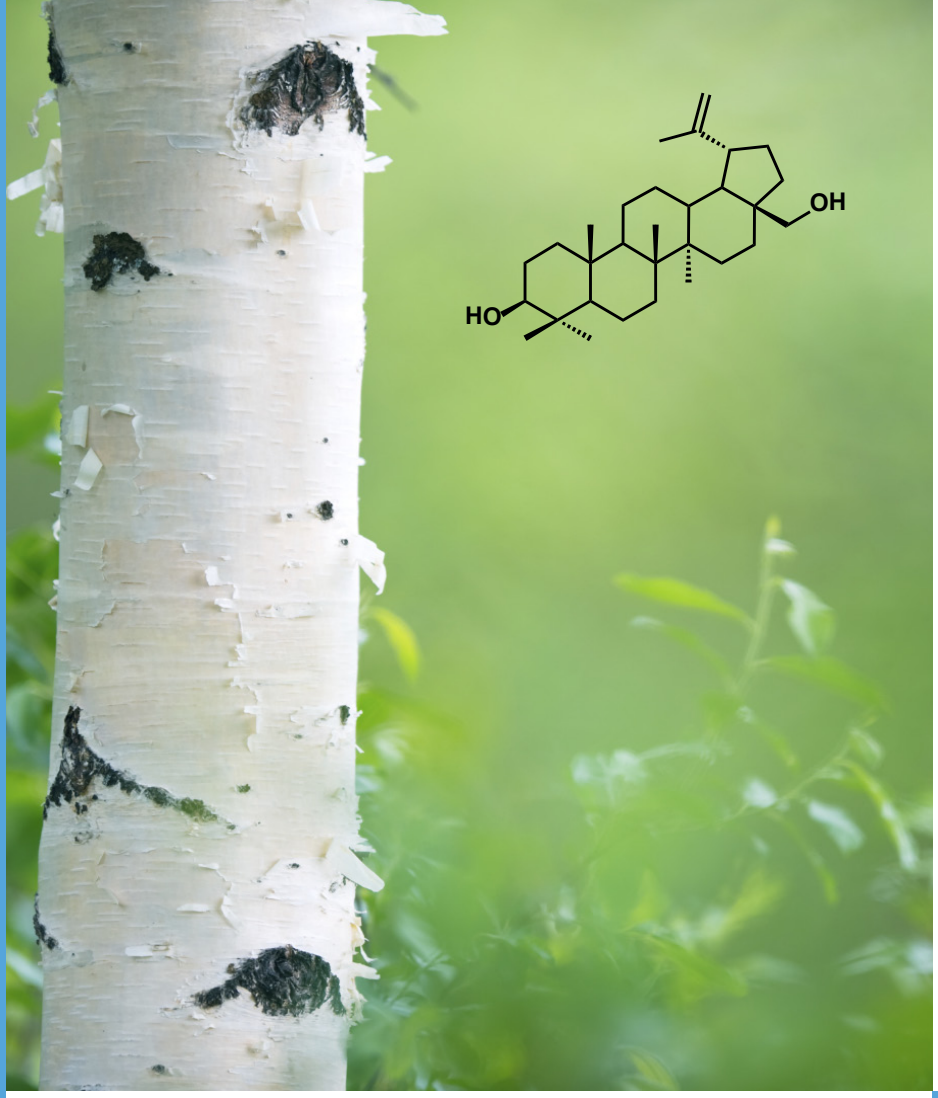

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Dissertation
39

Synthesis of betulin derivatives against intracellular pathogens

Sami Alakurtti

VTT

VTT SCIENCE 39

Synthesis of betulin derivatives against intracellular pathogens

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VTT Technical Research Centre of Finland

Division of Pharmaceutical Chemistry, Faculty of Pharmacy,
University of Helsinki, Finland

ACADEMIC DISSERTATION

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Synthesis of betulin derivatives against intracellular pathogens

Betuliinijohdannaisten syntetisointi solunsisäisiä patogeenejä vastaan. **Sami Alakurtti.** Espoo 2013. VTT Science 39. 99 p. + app. 43 p.

Abstract

Birch (*Betula* spp.) is utilized in huge quantities in the forest industry throughout the Northern Hemisphere, and low-value side-stream birch bark is burnt for energy. Outer birch bark is rich in (up to 30% dry weight) triterpene betulin, which is readily isolable by solvent extraction. Betulin can be used both in its raw form and as a starting material for more valuable products and fine chemicals.

The increasing drug resistance of numerous microbes and viruses is an issue of global concern, and new inexpensive therapeutic agents are urgently needed. In this study two sets of betulin derivatives were synthesized and screened as antiviral, antileishmanial and antibacterial agents. The first set includes relatively easily synthesizable betulin derivatives, such as esters and various oxidation products. The second set includes novel heterocyclic betulin derivatives, where the triazole ring is fused by the Diels-Alder reaction to the lupane skeleton of betulin.

Alphavirus *Semliki Forest virus* (SFV) is distributed by mosquitoes and infects avian and mammalian hosts. Some alphaviruses may cause fatal encephalitis in humans, although the number of cases is small. On the other hand, some alphaviruses have caused millions of cases of serious illnesses characterized by fever, rash and painful arthralgia. There is currently no efficient medical treatment against alphaviruses. In the antiviral assay, 18 betulin-derived compounds displayed good activity against SFV with low-micromolar 50% inhibitory concentration values combined with low cytotoxicity. In addition, three assayed potent and representative compounds displayed synergistic effect with modified nucleoside analogue against SFV, and similar good antiviral efficacy against another alphavirus, *Sindbis virus*.

The neglected tropical disease leishmaniasis is caused by protozoan parasites belonging to the genus *Leishmania*, and is transmitted to mammalian hosts by sandflies. It is estimated that around 12 million people are currently infected, mostly in developing countries. The most severe form, visceral leishmaniasis, is fatal if not treated. There are currently several drugs marketed for the treatment of leishmaniasis. However, none of these are fully effective against *Leishmania*, and severe side effects, often requiring hospitalization, are common. In addition, parasite resistance to drugs is a serious growing problem. In the present study, the most potent betulin derivatives displayed low-micromolar 50% growth inhibition values against *L. donovani* amastigotes. Good inhibition activity was well retained against *L. donovani* amastigotes growing inside macrophages. However, in some cases betulin derivatives also showed cytotoxicity to host macrophage cell line.

Chlamydia pneumoniae is a common Gram-negative human pathogen mainly causing mild respiratory infections, which can lead to pneumonia or bronchitis. There is also strong evidence that associates *C. pneumoniae* with other severe diseases, such as atherosclerotic cardiovascular diseases as well as some neurodegenerative diseases, such as Alzheimer's disease and multiple sclerosis. *C. pneumoniae* is susceptible to antibiotics that interfere with DNA and protein synthesis. However, its complex life cycle and its chlamydial persistence, which can last for years, as well as, importantly, the lack of specific diagnostic tests for detection of the organism in clinical samples, make the current treatment regimens unsatisfactory. Out of 32 betulin derivatives, five betulin derivatives showed high (>70% growth inhibition) antichlamydial activity against *C. pneumoniae* at 1 µM concentration. The most potent derivative displayed a remarkable 50% inhibition at nanomolar concentration.

Keywords Betulin, betulinic acid, terpene, organic synthesis, derivative, bioactivity, *Semliki Forest virus*, *Leishmania* sp., *Chlamydia pneumoniae*

Betuliinijohdannaisten syntetisointi solunsisäisiä patogeenejä vastaan

Synthesis of betulin derivatives against intracellular pathogens. **Sami Alakurtti.** Espoo 2013. VTT Science 39. 99 s. + liitt. 43 s.

Tiivistelmä

Metsäteollisuus käyttää koivua raaka-aineenaan suunnattomia määriä. Sivuotteena syntyvä koivunkuori poltetaan energian tuotantoon. Koivun ulkokuori sisältää runsaasti betuliini-nimistä triterpeeniä jopa 30 % kuivapainostaan. Betuliini voidaan helposti eristää kuoresta liuotinuutolla. Betuliinia voidaan käyttää sellaisenaan tai lähtöaineena muille tuotteille ja hienokemikaaleille.

Useiden pieneliöiden ja virusten kasvava lääkeresistenssi on maailmanlaajuinen ongelma, minkä takia on ilmennyt suuri tarve kehittää uusia lääkeaineita niitä vastaan. Tässä väitöskirjatyössä syntetisoitiin kaksi betuliinijohdannaisryhmää ja yhdisteiden tehokkuutta testattiin alfavirusten, Leishmania-suvun alkueläinten ja keuhkoklamydiabakteerin vastaisina yhdisteinä. Ensimmäinen ryhmä sisältää melko helposti valmistettavissa olevia johdannaisia, kuten betuliinin estereitä ja erilaisia hapetustuotteita. Toinen ryhmä sisältää uusia heterosyklisiä betuliinijohdannaisia, joissa triatsoli-rengas on fuusioitu betuliinin lupaanirakenteiseen hiilivetyrankaan.

Alfaviruksiin kuuluva *Semliki Forest virus* (SFV) leviää moskiittojen välityksellä, ja se infektoi lintuja ja nisäkkäitä. Jotkin alfavirukset voivat aiheuttaa tappavaa aivotulehdusta, mutta nämä tapaukset ovat hyvin harvinaisia. Useimmiten alfavirukset aiheuttavat sairauskohtauksia, joiden oireet ilmenevät usein kuumeena, allergisena ihottumana ja kivuliaana niveltulehduksena. Tällä hetkellä alfaviruksia vastaan ei ole tehokasta ja turvallista lääkitystä. Kaikkiaan 18 betuliinijohdannaista osoitti alfavirusten vastaista aktiivisuutta mikromolaarisella konsentraatiolla. Lisäksi kolmella potentiaalisella ja kemialliselta rakenteeltaan erilaisella betuliinijohdannaisella oli synergistisiä alfaviruksen vastaisia vaikutuksia muokatun nukleosidijohdannaisen kanssa. Lisäksi valitut kolme johdannaista olivat aktiivisia myös toista alfavirusta, *Sinbis virusta*, vastaan.

Leishmaniaasi-tautia aiheuttavat *Leishmania*-sukuun kuuluvat alkueläimet. Tauti esiintyy nisäkkäissä etenkin tropiikissa, ja sitä levittävät perhossääsket. Arvioiden mukaan 12 miljoonaa ihmistä sairastaa tällä hetkellä leishmaniaasia. Taudin vakavin muoto on sisäelinleishmaniaasi, joka on hoitamattomana tappava. Leishmaniaasia vastaan on käytössä useita lääkeaineita, mutta niiden tehokkuudessa ja turvallisuudessa on toivomisen varaa. Ne aiheuttavat usein vakavia sivuvaikutuksia, ja niiden käyttö edellyttää sairaalahoitoa. Lisäksi lääkeresistenssi on vakava ja kasvava ongelma. Tässä tutkimuksessa lupaavimmat betuliinijohdannaiset osoittivat *Leishmania donovanin* vastaisia vaikutuksia mikromolaarisilla konsentraatioilla. Johdannaisten estovaikutus pysyi hyvänä myös selvitettäessä niiden vaikutuksia syöjäsolujen sisällä kasvavia *L. donovani* -alkueläimiä vastaan. Valitet-

tavasti osa betuliinijohdannaisista osoitti sytotoksisuutta myös itse syöjäsoluja vastaan.

Keuhkoklamydia (*Chlamydia pneumoniae*) on yleinen Gram-negatiivinen bakteeri, joka aiheuttaa lieviä hengitystieinfektioita. Ne voivat pahimmillaan johtaa keuhkokuumeeseen tai keuhkoputkentulehdukseen. *C. pneumoniaen* aiheuttamilla infektiolla on myös osoitettu olevan yhteys useisiin muihin vakaviin sairauksiin, kuten sydän- ja verisuonisairauksiin, ja hermostoperäisiin sairauksiin, kuten Alzheimerin tautiin ja MS-tautiin. *C. pneumoniaen* aiheuttamia infektioita voidaan hoitaa antibiooteilla, jotka vaikuttavat bakteerin DNA- tai proteiinisynteesiin. Valitettavasti bakteerin monimutkainen elämänkierto, taudin vaikea diagnosointi ja bakteerille tyypillinen oireeton, jopa vuosia kestävä piilevänä sairautena pysyminen tekevät taudin hoidosta ongelmallista. Testisarjasta viisi betuliinijohdannaista esti huomattavasti *C. pneumoniae* -bakteerin kasvua 1 µM konsentraatiolla. Parhaalla johdannaisella 50 %:n inhibitioarvo saavutettiin nanomolaarisella konsentraatiolla.

Avainsanat Betulin, betulinic acid, terpene, organic synthesis, derivative, bioactivity, *Semliki Forest virus*, *Leishmania* sp., *Chlamydia pneumoniae*

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Academic dissertation

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Custos	Professor Jari Yli-Kauhaluoma

List of publications

This thesis is based on the following original publications which are referred to in the text as I–IV. The publications are reproduced with kind permission from the publishers.

I Pohjala, L.*, Alakurtti, S.*, Ahola, T., Yli-Kauhaluoma, J. and Tammela, P. Betulin-derived compounds as inhibitors of alphavirus replication, *J. Nat. Prod.* **2009**, *72*, 1917.

II Alakurtti, S., Heiska T., Kiriazis, A., Sacerdoti-Sierra, N., Jaffe, C., Yli-Kauhaluoma, J. Synthesis and anti-leishmanial activity of heterocyclic betulin derivatives. *Bioorg. Med. Chem.* **2010**, *8*, 1573.

III Alakurtti, S., Bergström, P., Sacerdoti-Sierra, N., Jaffe, C., Yli-Kauhaluoma, J. Anti-leishmanial activity of betulin derivatives. *J. Antibiot.* **2010**, *63*, 123.

IV Salin, O.*, Alakurtti, S.*, Pohjala, L., Siiskonen, A., Maass, V., Maass, M., Yli-Kauhaluoma, J., Vuorela P. Inhibitory effect of the natural product betulin and its derivatives against the intracellular bacterium *Chlamydia pneumoniae*. *Biochem. Pharmacol.* **2010**, *80*, 1141.

* Equal contribution

Related publications, not included in this thesis:

Alakurtti, S., Mäkelä, T., Koskimies, S., Yli-Kauhaluoma, J. Pharmacological properties of the ubiquitous natural product betulin. *Eur. J. Pharm. Sci.* **2006**, *29*, 1.

Šilhár P., Alakurtti, S., Capková, K., Xiaochuan, F., Shoemaker, C. B., Yli-Kauhaluoma, J., Janda, K. Synthesis and evaluation of library of betulin derivatives against the botulinum neurotoxin A protease. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 2229.

Wert, L., Alakurtti, S., Corral, M., Sánchez-Fortún S., Yli-Kauhaluoma, J., Alunda, J. Toxicity of betulin derivatives and *in vitro* effect on promastigote and amastigotes of *Leishmania infantum* and *L. donovani*. *J. Antibiotics.* **2011**, *64*, 475.

Author's contributions in the original publications

I The author synthesized and characterized almost all of the betulin derivatives (few derivatives were synthetized by Mrs. P. Bergström, A. Salakari, senior laboratory technician and undergraduate students T. Heiska and E. Metsälä under author's supervision). The author wrote the manuscript together with Dr. L. Pohjala with the aid of other co-authors. This publication is included as one of the required publications in Dr. L. Pohjala's academic dissertation as well.

II The author synthesized and characterized almost all of the betulin derivatives (few urazole intermediates were obtained from Dr. A. Kiriazis and few betulin derivatives were synthetized by undergraduate student T. Heiska under author's supervision). Author wrote the article with the aid of other co-authors.

III The author synthesized and characterized almost all of the betulin derivatives (few derivatives were synthetized by Mrs. P. Bergström, A. Salakari, senior laboratory technician and undergraduate student E. Metsälä under author's supervision). The author wrote the article with aid of other co-authors.

IV The author synthesized and characterized almost all of the betulin derivatives (few derivatives were synthetized by Mrs. P. Bergström and undergraduate students T. Heiska and E. Metsälä under author's supervision). The author wrote the manuscript together with Dr. O. Salin with the aid of other co-authors. This publication is included as one of the required publications in Dr. O. Salin's academic dissertation as well.

Contents

Publications II and IV of this publication are not included in the PDF version.

Abbreviations

^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{1}H NMR	Hydrogen-1 nuclear magnetic resonance
AB	Aberrant body
AIDS	Acquired immunodeficiency syndrome
AT	Adenine, thymine
ATP	Adenosine triphosphate
AV	Antiviral effect
BALB/c	Bagg albino, laboratory-bred, genotype c/c
BHK	Baby hamster kidney fibroblast cell line
BPQ	Buparvaquone
BVM	Bevirimat
CHIKV	Chikungunya virus
CL	Cutaneous leishmaniasis
CTP	Cytidine triphosphate
CV	Cell viability
DCC	*N,N'*-Dicyclohexylcarbodiimide
DEAD	Diethyl acetylenedicarboxylate
DHP	3,4-Dihydro-2*H*-pyran
DMAP	4-(Dimethylamino)pyridine
DMC	2',6'-dihydroxy-4'-methoxychalcone
DMSO-d_6	Deuterated dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DSB	Dimethylsuccinyl betulinic acid
EB	Elementary body
EC_{50}	Half maximal effective concentration
ECHO	Enteric cytopathic human orphan virus
ED_{50}	50% effective dose
EEEV	Eastern equine encephalitis virus
FPV	Fowl plague virus
FTIR	Fourier transform infrared spectroscopy
GETV	Getah virus
GI_{50}	50% growth inhibition
HeLa	Cancer cell line taken from Henrietta Lacks
Hep	Human epithelial cervix carcinoma cell line
HIV	Human immunodeficiency virus
HL	Human promyelocytic leukemia cell line
HPLC	High-performance liquid chromatography
HPLC-MS	High-performance liquid chromatography – mass spectrometry
HSV-1	Herpes simplex type 1
Huh-7	Human hepatocellular carcinoma cell line
I	Interaction index
IC_{50}	50% inhibitory concentration
ICTV	International Committee on Taxonomy of Viruses
IMPDH	Inosine-5′-monophosphate dehydrogenase
kDNA	Kinetoplast DNA
LP	Liposome
MB-III	Maesabalide III
MCL	Mucocutaneous leishmaniasis
mCPBA	*m*-Chloroperbenzoic acid
MIC	Minimum inhibitory concentration
NC	Nucleocapsid
NSV	Neuroadapted Sinbis virus

p-TSA	*p*-Toluenesulfonic acid monohydrate
PCC	Pyridinium chlorochromate
Pd/C	Palladium on carbon
PLA	Polylactic acid
PPTS	Pyridinium *p*-toluenesulfonate
PS	Phosphatidylserine
RB	Reticulate body
RNA	Ribonucleic acid
SAR	Structure–activity relationships
SARS	Severe acute respiratory syndrome
SFV	Semliki Forest virus
SI	Selectivity index
SINV	Sindbis virus
spp	Species
t-BuOK	Potassium *tert*-butoxide
TEA	Triethylamine
THF	Tetrahydrofuran
THP	Tetrahydropyran
THP-1	Human leukaemia monocyte cell line
TLC	Thin layer chromatography
TMS	Trimethylsilyl
tRNA	Transfer RNA
UV	Ultra violet
VEEV	Venezuelan equine encephalitis virus
VL	Visceral leishmaniasis
WEEV	Western equine encephalitis virus

1. Introduction

1.1 Birch bark

Birch is (*Betula* spp.) widespread throughout the Northern Hemisphere and harvested in huge volumes. Birch bark is produced in considerable quantities as a by-product of the forest industry and its upgrading is almost totally neglected. Currently, this low-value side stream is burnt for combined heat and power production. Birch bark could, however, find more valuable uses, for example as an additive in plastic composite materials.[1] Birch bark consists of brown inner bark ~75% and white outer bark ~25%.[2] The inner bark consists mainly of wood-like material such as lignin, pentosans and hexosans. The outer bark contains, by dry weight, up to 40% fats, fatty acids, resins and triterpenes, in particular betulin, at up to 30%. In addition, the outer bark contains up to 35% suberin. Valorization and upgrading of these compounds by using modern chemical technology opens up entirely new opportunities to produce new speciality chemicals from this low-value biomass stream. It has been estimated theoretically that a pulp mill with an annual production capacity of 200,000 tonnes of birch kraft pulp produces enough bark to produce around 2,500 tonnes of betulin of around 95% purity and 4,000 tonnes of suberin acids *per annum*.[3,4] Suberin polyester can be hydrolyzed by base treatment to multifunctional suberin acids, which are potential raw materials for paints, adhesives, lubricants and surface-active agents.[5] New potential applications for betulin or betulin derivatives include pharmaceuticals and cosmetic products as well as agrochemicals.[6]

1.2 Alphaviruses

The *Semliki Forest virus* (SFV) belongs to the alphaviruses, which are small enveloped viruses containing a single-stranded positive-sense RNA genome.[7] Viruses belonging to this genus are predominantly arthropod-borne viruses using mosquitoes as vectors and have a very wide geographic distribution, with isolates having been reported from all continents except Antarctica and from many islands.[8] Alphaviruses infect avian and mammalian hosts and are a serious or potential threat to human health. In North and South America some alphaviruses are known to cause fatal encephalitis in humans, although the number of recorded

fatalities is small.[9] Alphaviruses have, however, caused millions of cases of serious illness characterized by fever, rash and painful arthralgia.[10] There is currently no efficient pharmacotherapy for alphavirus-borne diseases.

1.3 *Leishmania* spp.

Leishmaniasis is a disease caused by protozoan parasites belonging to the genus *Leishmania*. The disease is transmitted by sandflies and is present in all inhabited continents.[11] It is estimated that around 12 million people are currently infected. More than 350 million people live in risk areas for the disease, and 2 million new cases occur every year, especially in the developing countries. Leishmaniasis has been designated as a neglected tropical disease by the World Health Organization. The most severe form, visceral leishmaniasis, in which parasites invade the liver, spleen and bone marrow, is fatal if not treated.[12] Current treatment includes pentavalent antimony compounds, pentamidine and amphotericin B.[13] However, there are risks of severe side effects and all of these current drugs are administered by intravenous injection. Parasite resistance to pentavalent antimony drugs has become a serious problem and is present in approximately 65% of patients with visceral leishmaniasis in India.[14] New drugs, such as primaquine, allopurinol, imipramine, are being developed, however none of them are fully effective against *Leishmania*.[12] Recently, orally administrable miltefosine has shown promising antileishmanial activity.[15]

1.4 Chlamydia pneumoniae

Chlamydia (Chlamydophila) pneumoniae is an important Gram-negative human pathogen, mainly causing respiratory infections. It has been proposed that almost all humans will become infected with *C. pneumoniae* during their life.[16] *C. pneumoniae* frequently causes community-acquired pneumonia in adults and children. Often infections are asymptomatic and frequently of long duration, up to several years. There is also strong evidence that associates *C. pneumoniae* with other severe diseases, such as atherosclerotic cardiovascular diseases,[17] as well as some neurodegenerative diseases, such as Alzheimer's disease and multiple sclerosis.[18] Interestingly, *C. pneumoniae* infection has also been reported in a wide range of animals, including other mammals such as horses and koalas, and frogs and other reptiles.[19] All chlamydial species are intracellular bacteria that infect and replicate inside a variety of human cells, including epithelial, endothelial, macrophages, and smooth muscle cells.[20] *C. pneumoniae* is classified as an obligate intracellular pathogen, as it has to infect another cell to reproduce. *C. pneumoniae* is susceptible to antibiotics that interfere with DNA and protein synthesis, including tetracyclines, macrolides and quinolones.[16] However, its complex life cycle and its chlamydial persistence, which can last for years, as well as, importantly, the lack of specific diagnostic tests for detection of the organism in clinical samples, make the current treatment regimens unsatisfactory.

1.5 Origin of drugs during the last 25 years

A total of 1,184 new chemical entities were approved for the market between 1981 and 2006 for treatment of various infective diseases caused by bacterial, fungal, viral and parasitic infections and for the treatment of cancer.[21] The new drugs can be classified according to their source into six major categories and one subcategory (Table 1).

Table 1. Classification of new drugs according to source.[21]

Category	Source
B	Biological; usually large peptide or protein
N	Natural product
ND	Derived from a natural product, usually semisynthetic
S	Totally synthetic
S*	Made by total synthesis, but the pharmacophore is from a natural product
V	Vaccine
Subcategory NM	Natural product mimic

A breakdown of the 1,184 new drugs based on the above categories is illustrated in Figure 1. Only 30% of the drugs are completely synthetic; the rest have their origins in the natural products.

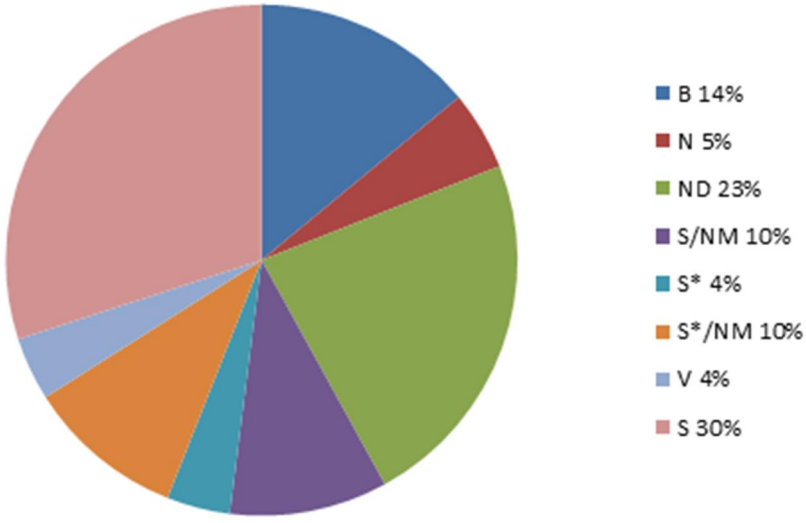


Figure 1. New chemical entities during 1981–2006, according to source (N = 1,184) (original from ref. 21).

2. Review of the literature

2.1 Betulin

Betulin **1**, lup-20(29)-ene-3β,28-diol, also known as betulinol, betuline and betulinic alcohol (Figure 2), is a pentacyclic triterpene alcohol with a lupane skeleton. Common structural features of the lupane skeleton are its five-membered ring E and isopropylidene group. Betulin **1** can be isolated (up to 30% dry weight) from the outer birch bark by extraction with high boiling hydrocarbon solvents or with water azeotropes of alcohols.[4]

Betulin **1** can be used as a starting compound for other useful compounds that possess various interesting pharmacological properties. Betulin **1** has three positions in its structure, namely a secondary hydroxy group at position C-3, a primary hydroxy group at position C-28, and an alkene moiety at position C-20, where chemical modifications can be easily performed to yield derivatives for structure–activity relationship (SAR) studies. It is clear from the chemical structure of betulin that most of the derivatives presented in the experimental part of this thesis are lipophilic compounds and thus poorly soluble in water if no organic co-solvent is used. This may have important implications for the interpretation of the results of the bioactivity assays, which have been carried out predominantly in cell cultures. Observed differences in bioactivity between different modified betulin derivatives may be explained, at least partly, by different water/lipid distribution constants between these analogues.

2.1.1 Pharmacological properties of betulin derivatives

Betulin **1** is biologically a relatively inactive compound. However, betulin **1** can be oxidized to pharmacologically more active betulinic acid **2**.[22] Betulinic acid **2** and its derivatives possess a wide spectrum of biological and pharmacological activity.[6] Most notably, betulinic acid **2** and its derivatives have shown activity against human immunodeficiency virus (HIV) as well as cytotoxicity against a variety of tumour cell lines comparable to some clinically used drugs.

A new mechanism of action has been confirmed for some of the most promising anti-HIV derivatives, which makes them potentially useful additives to current anti-HIV therapy. A43D[23] **3** and statine-derived IC9564[24] **4** act as entry inhibitors

and block HIV adsorption or membrane fusion. Furthermore, the dimethylsuccinyl derivative of betulin, bevirimat **5** (DSB, BVM), acts as a virus maturation inhibitor.[25] Bevirimat **5** was demonstrated to have dose-dependent anti-HIV potency in phase I and phase II clinical studies.[26] However, mutations in HIV cause resistance to BVM **5**, in addition to which some patients have this polymorphism present, resulting in lower BVM **5** anti-HIV efficacy.[27] Development of BVM **5** has thus been recently halted.

Betulinic acid **2** is specifically cytotoxic to several tumour cell lines by directly triggering mitochondrial membrane permeabilization and inducing apoptosis in cells.[28,29] Moreover, it is non-toxic up to 500 mg/kg body weight in mice.[28] Currently, betulinic acid **2** is undergoing anti-cancer development with assistance from the Rapid Access to Intervention Development Program of the National Cancer Institute.[30]

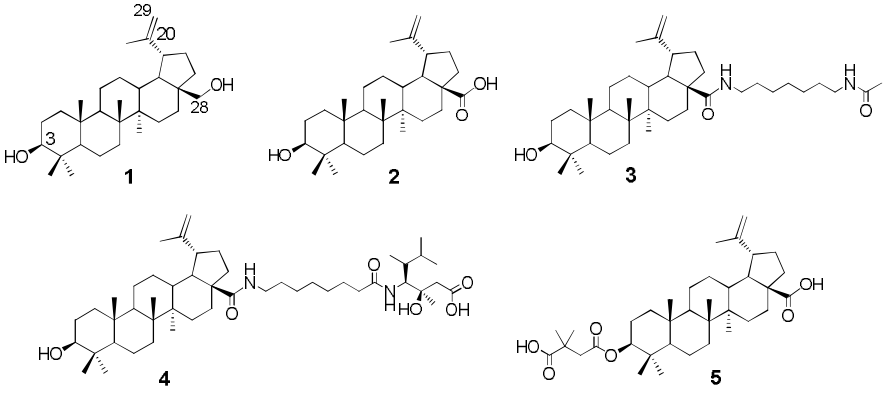


Figure 2. Betulin **1** and potential anticancer agent betulinic acid **2**, as well as potential anti-HIV agents A43D **3**, IC9564 **4** and bevirimat **5**.

2.2 Alphaviruses

2.2.1 Species, taxonomy and lifecycle

Currently there are two main schemes used for the classification of viruses: the International Committee on Taxonomy of Viruses (ICTV) system and the Baltimore classification system. A universal system for classifying viruses, and a unified taxonomy, is being established by the International Committee on Taxonomy of Viruses (ICTV). [31] The system makes use of a series of ranked taxons:

Table 2. Taxonomy of *Semliki Forest virus*.

Order (-virales)	Virus
Family (-viridae)	*Togaviridae*
Subfamily (-virinae)	-
Genus (-virus)	*Alphavirus*
Species	*Semliki Forest virus*

In the ICTV 2012 taxonomy classification, the *Semliki Forest virus* (SFV) (which was used as a target pathogen for betulin inhibition activity in publication I) species is classified under the family *Togaviridae* and the genus *Alphavirus*, which contains a total of 30 viruses, including human pathogens such as *Rubella, Sindbis* and *Chikungunya* viruses (Table 2).[31]

In the Baltimore classification system, viruses are divided into one of seven groups depending on a combination of their nucleic acid (DNA or RNA), strandedness (single- or double-stranded), sense, and method of replication (Table 3).[32]

Table 3. Virus groups according to the Baltimore classification system.

Group	Classification
I	Double-stranded DNA (dsDNA) viruses
II	Single-stranded DNA (ssDNA) viruses (+)sense DNA
III	Double-stranded RNA (dsRNA) viruses
IV	Single-stranded RNA [(+)ssRNA] viruses (+)sense RNA
V	Single-stranded RNA [(-)ssRNA] viruses (−)sense RNA
VI	Single-stranded RNA (ssRNA-RT) viruses (+)sense RNA with replication through a DNA intermediate
VII	Double-stranded DNA (dsDNA-RT) viruses with replication through a single-stranded RNA intermediate

Alphaviruses have a single-stranded, positive-sense RNA genome and are classified under group IV according to the Baltimore classification.[8] Group IV includes viruses from several ICTV orders: 1. *Nidovirales* including significant pathogens such as *Coronavirus* and *Severe acute respiratory syndrome virus* (SARS); 2. *Picornavirales* including significant pathogens such as Norwalk, Polio, the common cold and Hepatitis A viruses; 3. *Tymovirales*; and unassigned virus families including significant human pathogens such as Yellow fever, West Nile, Hepatitis C and Dengue fever.[33]

Entry of alphavirus into cells is initiated by receptor-binding, followed by endocytosis (Figure 3).[7] Fusion to endosomal membranes transports nucleocapsid (NC) into the cytoplasm, where ribonucleic acid (RNA) is released after disassembly. Genomic RNA is used for transcription of nascent (+)RNA via a (−)RNA template

and translation of proteins from genomic and subgenomic (26S) RNA. The structural proteins translated from 26S RNA encapsidate nascent genomic RNA before budding from cells, and eventual release.

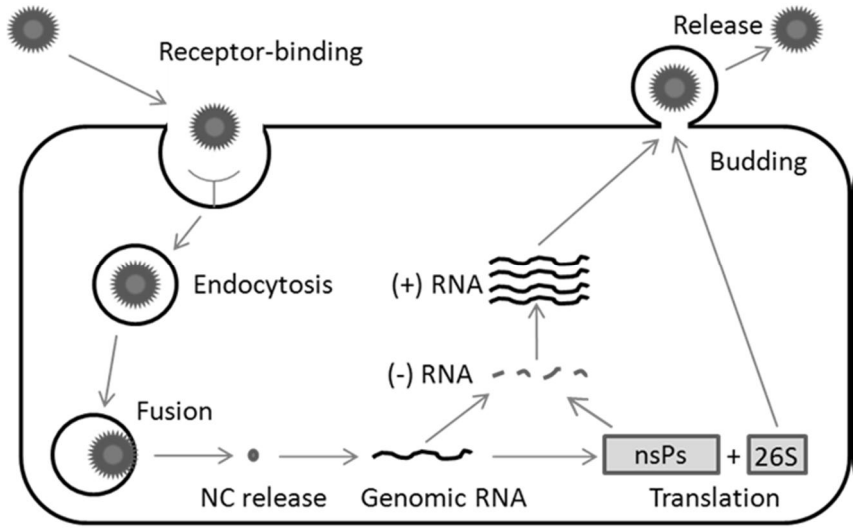


Figure 3. Life cycle of alphavirus (original from ref. 7).

2.2.2 Chemotherapy against infections caused by alphaviruses

This section focuses on small-molecule antiviral inhibitors found to be effective against viruses in the genus Alphavirus. The alphaviral species discussed in this section include the *Chikungunya virus* (CHIKV), *Eastern equine encephalitis virus* (EEEV), *Getah virus* (GETV), *Semliki Forest virus* (SFV), *Sindbis virus* (SINV), *Venezuelan equine encephalitis virus* (VEEV) and *Western equine encephalitis virus* (WEEV). As there are only very few reported *in vivo* alphavirus experiments, *in vitro* experiments are also discussed here.

Nucleoside or nucleotide analogues

Synthetic nucleoside or nucleotide analogues are prodrugs that are phosphorylated to active triphosphate drugs by viral enzymes and used as normal building blocks for the DNA polymerase-catalyzed replication of viral DNA.[34] However, as a consequence of the modifications in the nucleobase or in the sugar moiety, viral DNA polymerase enzyme is deactivated or DNA chain formation is terminated.

In an early study by Huffman and co-workers, the synthetic nucleoside guanosine analogue ribavirin **6** (Figure 4) was screened against a wide panel of viruses, including SFV.[35] Ribavirin **6** showed broad antiviral activity, with moderate anti-

alphaviral activity and a minimum inhibitory concentration (MIC) value of 131 µM. Ribavirin **6** has since been used as a positive control in several assays.

Nucleotide analogue ribavirin 5'-sulphamate **7** displayed several-fold improved antiviral activity against SFV with an IC_{50} value of 10 µM. The IC_{50} value represents the concentration of drug required for 50% inhibition *in vitro* (50% inhibitory concentration). Ribavirin **6** displayed an IC_{50} value of <1 mm.[36] In *in vivo* testing with lethally SFV infected mice, ribavirin 5'-sulphamate **7** showed a clear protective effect with survival rates of 92% and 83% when intraperitoneally administered at doses of 20 and 40 mg/kg/day for seven days.

Selenazofurin **8** and tiazofurin were assayed alone or in combination with ribavirin **6** against several viruses, including VEEV. Selenazofurin **8** displayed good activity against VEEV with a 50% effective dose (ED_{50}) value of 0.5 µg/mL, whereas tiazofurin was inactive.[37] Combination of ribavirin and **6** selenazofurin **8** displayed synergistic efficacy with an ED_{50} value of 0.1 µg/mL.

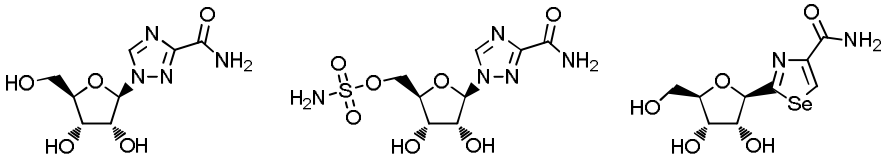


Figure 4. Nucleoside guanosine analogues with antiviral activity against alphavirus.

Nucleoside adenosine-derived neplanocins A **9** and C **10** (Figure 5) displayed broad antiviral activity and significant anti-alphaviral activities against SFV with IC_{50} values of 4 and 7 µM, respectively.[38]

Five 3'-fluorinated ribonucleosides were synthesized and screened against a panel of viruses, including SFV and SINV.[39] 3'-Fluoro-3'-deoxyadenosine **11** displayed the best activity against several viruses, with IC_{50} values of 7 µM and 15 µM against SINV and SFV, respectively. General cytotoxic effects were observed at 150 µM concentration.

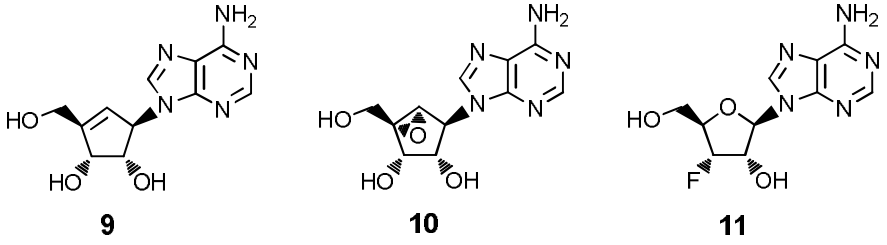


Figure 5. Nucleoside adenosine analogues with antiviral activity against alphavirus.

In a study by de Clercq et al., antiviral activity of racemic cytidine analogue carbodine **12** and **13** (Figure 6) was compared to known antiviral agents such as carbocyclic 3-deazaadenosine (C-c^3 Ado) and ribavirin **6**.[40] The racemic mixture of carbodine enantiomers **12** and **13** showed broad activity against most of the viral species tested by inhibition of cellular cytidine triphosphate (CTP) synthetase. Especially against SINV, carbodine showed an IC_{50} value of 3 µM, which was almost 60 times more potent than C-c^3 Ado and over 200 times more potent than ribavirin **6**. In addition, the carbodine racemate displayed good inhibition against SFV with an IC_{50} value of 12 µM.

In a study by Julander et al., activity of the D-(–)- and L-(+)-enantiomers of carbodine was determined against VEEV in cell culture and in an *in vivo* mouse model. (–)-Carbodine **13** showed good inhibition activity with an EC_{50} value of 0.8 µM, while (+)-carbodine **12** was not active (EC_{50} > 100 µM). Post-virus exposure treatment with (–)-carbodine **13** was effective in significantly improving disease parameters in mice infected with VEEV when treatment was initiated as late as 4 days post-virus installation, with a mouse survival rate of 90% (placebo 0%).

In another study by De Clercq et al., cyclopentenylcytosine (Ce-Cyd) **14** displayed broad-spectrum antiviral activity against several viruses, including SFV and SINV with IC_{50} values of 0.4 and 0.2 µg/mL, respectively.[41]

Antiviral activity of eight commercially available compounds was estimated against CHIKV and SFV.[42] When comparing antiviral activities against CHIKV, positive control ribavirin **6** had an EC_{50} value of 83 µM and a selectivity index (SI) of 24. The two best test compounds, 6-azauridine **15** and sulfated polysaccharide iota-carrageenan, displayed significantly better EC_{50} values of 0.2 and 3.8 µM and SI values of 204 and >133, respectively. Against SFV, these compounds showed similar activities: 6-azauridine **15** and iota-carrageenan EC_{50} values of 0.4 and 0.7 µM and SI values of 85 and >714, respectively, while ribavirin **6** showed a moderate EC_{50} value of 47 µM and an SI value of 109.

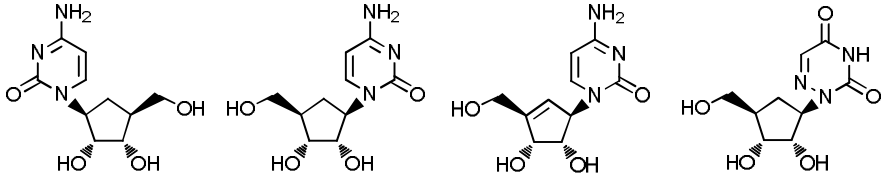


Figure 6. Nucleoside cytidine analogues with antiviral activity against alphavirus.

Non-nucleoside analogues

In a study by Pohjala et al.,[43] a library of 356 compounds was screened *in vitro* against CHIKV and SFV. The library consisted of natural compounds (mainly flavonoids, coumarins and other phenolic compounds) as well as clinically approved drugs and their metabolites. Four natural 5,7-dihydroxyflavones, **16**–**19**,

were found to be effective against CHIKV with IC_{50} values of around 20–70 µM (Figure 7). In the assay against SFV, in addition to the same four flavones (naringenin **18** as the most potent compound with an IC_{50} value of 2.2 µM, SI 43), several other natural compounds such as a synthetic coumarin derivative, coumarin-30 **20**, (IC_{50} value of 0.4 µM, SI 231) was found to be effective.

In addition, several pharmaceutical compounds were found to be effective as SFV entry inhibitors. A phenothiazine core was identified in six out of twelve pharmaceutical compound hits, with antipsychotic prothipendyl **21** and antihistamine methdilazine **22** (Figure 8) showing the best IC_{50} values of 8 and 11 µM, respectively. The reference compounds 3'-amino-3'-deoxyadenosine **23** and chloroquine **24** showed good activity with IC_{50} values of 13.4 and 16.2 µM and SI values of 14.9 and 10.7, respectively. Mycophenolic acid **25** was relatively inactive (IC_{50} = 121 µM).

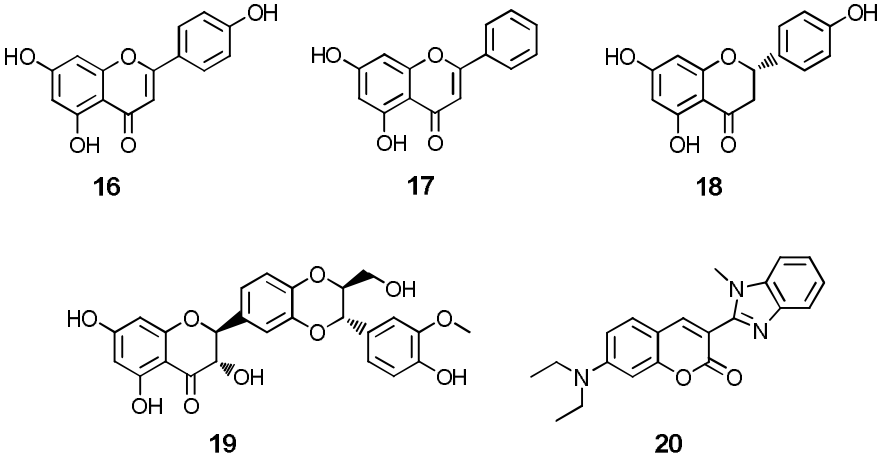


Figure 7. Natural compounds or their derivatives with good SFV inhibition activity.

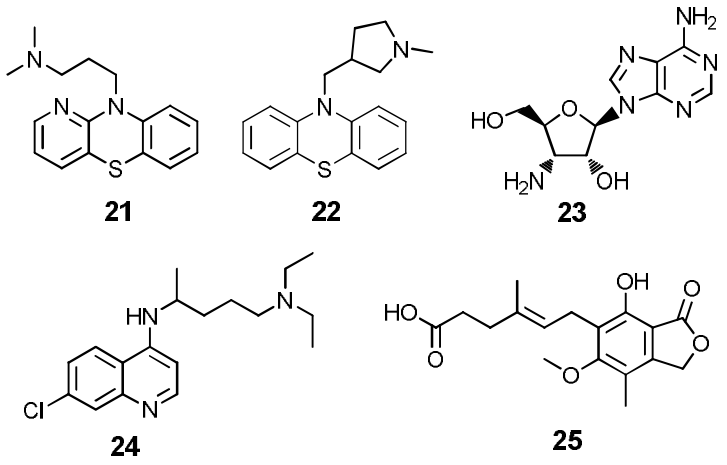


Figure 8. Pharmaceutical and selected reference compounds with good SFV inhibition activity.

In a study by Peng at al., a library of over 50,000 compounds was assayed in a high-throughput screening against WEEV RNA replication.[44] The most promising lead compound, CCG-32091 **26** (Figure 9), displayed an IC_{50} value of 24 µM with low cytotoxicity against BSR-T7/5 cells, which are BHK cells that constitutively express bacteriophage T7 RNA polymerase. Several derivatives of CCG-32091 **26** were synthesized for SAR studies, and in a follow up study[45] the most promising compound, **27** (IC_{50} value of 6.5 µM), was subjected to *in vivo* NSV (neuroadapted Sindbis virus) infected mouse testing. After 12 h post treatment, **27** (30 mg/kg twice a day) displayed a clear protective effect with a mouse survival rate of 80%, compared to the control group with a survival rate of 20%.

Arbidol **28**, an antiviral drug originally licensed in Russia for treatment of influenza, showed potent CHIKV inhibition activity with an IC_{50} value of 12.2 µM.[46] However, a single amino acid substitution in a mutant CHIKV envelope protein caused resistance to arbidol **28**.

In another HTS study, 61,600 commercial small molecular weight compounds were screened against influenza virus.[47] The most promising lead compound, **29**, was further evaluated against a broad spectrum of viruses representing several different families, including alphavirus SINV. Compound **29** showed a broad spectrum of viral inhibition and a degree of inhibition of 2 logs against SINV.

VX-497 **30** is a potent, reversible uncompetitive inosine-5′-monophosphate dehydrogenase (IMPDH) inhibitor, which was investigated in a comparison study with ribavirin **6** in terms of their cytotoxicities and their efficacies against a variety of viruses including alphavirus VEEV.[48] VX-497 **30** displayed superior anti-alphaviral activity (IC_{50} = 19.2 µM) compared to non-active ribavirin **6** (IC_{50} > 500).

The effect of four lysosomotropic weak bases (chloroquine **24**, amantadine, tributylamine and methylamine) and NH_4Cl against SFV infection has been studied

in BHK cells.[49] All substances prevent the transfer of the virus nucleocapsid into the cytoplasm by increasing the lysosomal pH from 4–5 to approximately 6, which is above the critical value needed to trigger a low pH-dependent fusion reaction between the membranes of the lysosome and the virus. Antimalarial drug chloroquine **24** displayed the best IC_{50} value of 50 µM. However, in a double-blind placebo-controlled randomized human trial with chloroquine **24** doses of 600 mg at days 1–3 and 300 mg at days 4–5, no significant difference between the treated and placebo groups could be identified.[50]

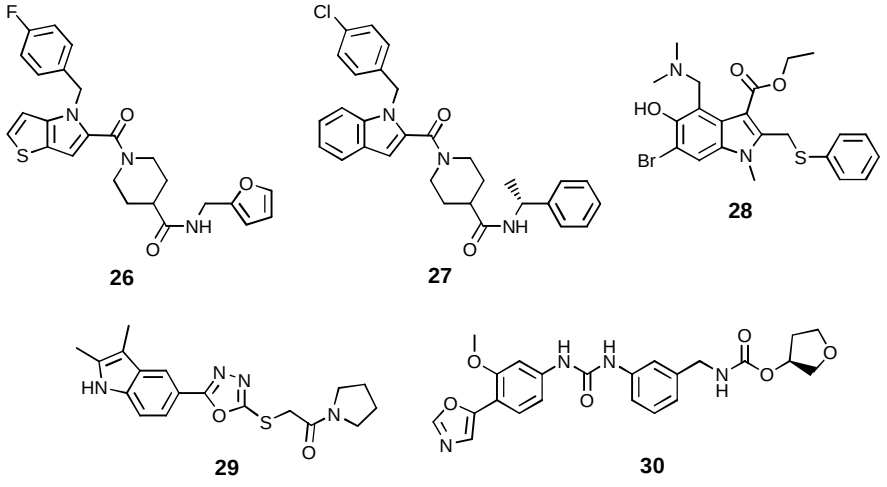


Figure 9. N-heterocyclic compounds possessing anti-alphaviral activity.

Molecular modelling of the hydrophobic binding pocket of SINV capsid protein was used to design 1,4-dioxane-based antiviral agents.[51] Surprisingly, the best target compound according to the model with a three-carbon linker chain connecting two 1,4-dioxane **31** (Figure 10) moieties was significantly less active (EC_{50} = 40 µM) than the synthetic intermediate **32** (EC_{50} = 1 µM). Both compounds were not cytotoxic in uninfected BHK cells at concentrations of 1 mm.

Seco-pregnane steroid glaucogenin C **33** and its glycosides isolated from *Strobilanthes cusia* (Nees) Kuntze and *Cynanchum paniculatum* (Bunge) Kitagawa displayed selective inhibitory activity against several members of the alphavirus genus.[52] The EC_{50} value of the most potent glaucogenin C derivative, pentasugar-glycoside paniculatumoside C **34**, was remarkable. Paniculatumoside C **34** showed EC_{50} values of 1.5, 1 and 2 nM against SINV, GETV and EEEV, respectively. In addition, it proved effective in reducing the mortality rate (18%, 54%, and 100%) of SINV infected mice when administered at a dose of 5, 50, or 100 mg/kg body weight before inoculation. However, when paniculatumoside C **34** was administered 1 or 4 h after SINV infection, no mice survived, although delayed mortality was observed.

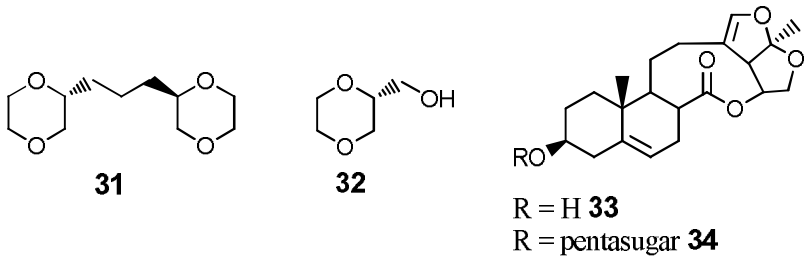


Figure 10. 1,4-Dioxane-based compounds **31** and **32**, and s*eco*-pregnane-derived compounds **33** and **34**, R = [(*O*-*β*-D-glucopyranosyl-(1→4)-*O*-*β*-D-glucopyranosyl-(1→4)-*O*-2,6-dideoxy-3-*O*-methyl-*α*-D-arabino-hexopyranosyl-(1→4)-*O*-2,6-dideoxy-*β*-D-ribo-hexopyranosyl-(1→4)-2,6-dideoxy-3-*O*-methyl-*β*-D-arabino-hexopyranosyl)oxy] with anti-alphaviral activity.

Betulin-derived compounds

Bevirimat **5** (Figure 2), a semisynthetic dimethylsuccinic acid derivative of natural product betulinic acid[53], blocks HIV maturation by inhibiting the final stage of HIV Gag protein processing.[54] It was initially considered as a possible first member of the HIV maturation inhibitors, and it successfully demonstrated potency in phase I and phase II clinical studies.[26] However, mutations in HIV cause resistance to BVM **5**, in addition to which some patients also have this polymorphism present, resulting in lower anti-HIV efficacy.[27] Thus, development of BVM **5** has been currently halted. Bevirimat **5** was found to be inactive against herpes simplex type 1 (HSV-1) and influenza virus.[25]

The antiviral activity of betulin **1** and betulinic acid **2** and their derivatives have also been studied against influenza A, herpes simplex type 1 (HSV-1), influenza FPV/Rostock and ECHO-6 enterovirus, however their antiviral activities were weak. [55,56,57]

2.3 *Leishmania* spp.

2.3.1 Species, taxonomy and lifecycle

Protozoan parasites belonging to the genus *Leishmania* are transmitted to mammalian hosts by female sandflies of the genera *Phlebotomus* and *Lutzomyia* in the Old and New World, respectively.[58] The life cycle of the leishmanial parasite consists of a flagellated promastigote phase in the sandfly gut and a non-flagellated intracellular amastigote phase in mammalian macrophages (Figure 11).[59] After a blood meal from the infected host, amastigotes are released from the macrophages into the sandfly gut; these then transform through a multiplying non-infectious procyclic promastigote phase to infectious metacyclic promastigotes and migrate to the pharyngeal valve of the sandfly.[60] The metacyclic promastigotes are transmitted during feeding to the mammalian host, where the promastigotes successfully

infect and invade the host macrophages. Inside the macrophages, the parasites transform into amastigotes and start to multiply. The amastigotes are released into the blood stream from the infected macrophages to infect new macrophages, which are finally transferred during feeding back to the vector sandfly.

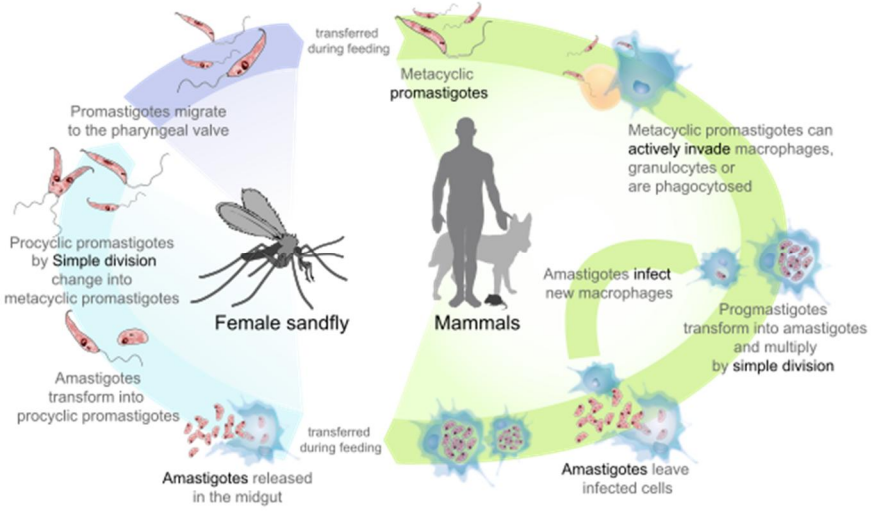


Figure 11. Life cycle of leishmania parasite.[59]

In taxonomy, the species *Leishmania donovani* (used as a target microbe for betulin inhibition activities in publications II and III), is classified under the subkingdom Protozoa, order Kinetoplastida, family Trypanosomatidae, genus *Leishmania*, subgenus *Leishmania* and complex *L. donovani* (Table 4). Other complexes under the subgenus *Leishmania* are *L. tropica, L. major, L. aethiopica* and *L. mexicana*. In addition, the genus *Leishmania* also contains the subgenus *Viannia*, which includes the complexes *L. braziliensis, L. guyanensis, L. naiffi* and *L. lainsoni*. These complexes include a total of 30 species, of which 20 are infectious to humans.[61,62]

Table 4. Taxonomy of *L. donovani*.

Subkingdom	**Protozoa**
Order	Kinetoplastida
Family	Trypanosomatidae
Genus	*Leishmania*
Subgenus	*Leishmania*
Complex	*L. donovani*
Species	*L. donovani*

Leishmaniasis is a disease caused by leishmania parasites and it has been divided into three major clinical manifestations:[12]

1) Cutaneous leishmaniasis (CL): produces skin lesions.
2) Mucocutaneous leishmaniasis (MCL): ulcerates oro-naso-pharyngeal region.
3) Visceral leishmaniasis (VL): devastates internal organs, especially liver, spleen and bone marrow and the untreated disease is usually fatal.

2.3.2 Leishmania and HIV co-infection

Interaction between leishmaniasis (VL) and HIV has been well established, making VL-HIV co-infection a serious worldwide concern.[63] HIV-infected people are particularly vulnerable to VL infection and the risk of developing active VL is increased dramatically. VL accelerates HIV replication and progression to AIDS. In areas endemic for VL, many people have asymptomatic infection and patients should be considered as potential reservoirs of infection. In addition, all antileishmanial therapies are less effective with HIV-positive patients and the risk of treatment failure or relapse of VL is increased. There is a high mortality rate due to concurrent illness, complications, and drug toxicity. VL-HIV co-infection decreases host humoral and cellular responses (specific antibodies), which limits the diagnostic value of simple serological tests for co-infected patients.

2.3.3 Chemotherapy against infections caused by *Leishmania* spp.

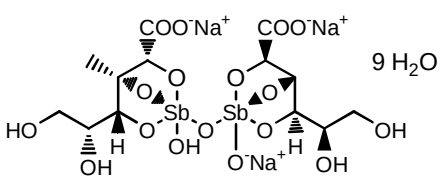
This section focuses on small molecule antileishmanial drugs currently in use as well as on compounds in clinical or preclinical trials, and experimental inhibitors found promising in *in vivo* activity in mouse trials. The results of preclinical human trials are collected in Table 5.

Vaccines

The ideal antileishmanial treatment would be an effective vaccine. The *Leishmania* parasite has a relatively uncomplicated life cycle and patients that recover from infection have resistance to subsequent infection. This indicates that a successful vaccine could be produced. For example, vaccines comprising killed parasites, subunits such as parasite proteins, DNA, poly-protein, and peptides derived from leishmanial antigens have shown promising results on animal models. However, these vaccines have been disappointing when tested in field trials and currently there is no effective vaccine available.[64,65]

Currently approved drugs

The first-line drugs for treatment of leishmaniasis include pentavalent antimony compounds such as stibogluconate (Pentostam®) **35** and meglumine antimonate (Glucantime®) **36** (Figure 12),[66] which have been in use for over 70 years. However, there are several drawbacks with these drugs. As pentavalent antimony drugs are highly water soluble, they are not absorbed through the lipophilic intestinal barrier and must be administered by intravenous injection.[67] In addition, drugs have severe toxic side effects, such as cardiotoxicity and hepatotoxicity, and their use requires clinical supervision or hospitalization. Moreover, resistance to antimony-based drugs is increasing, for example in India.[68] Although antimonials have been used for decades for the treatment of leishmaniasis, their mode of action is not fully known. Pentavalent antimony compounds are accumulated to promastigotes and amastigotes and, in the most accepted model for the mechanism of action, Sb(V) acts as a prodrug and is reduced to the more biologically active and toxic Sb(III).[69] There is evidence that Sb(III) inhibits trypanothione reductase and glutathione reductase[70] or induces apoptosis[71] of the parasite.



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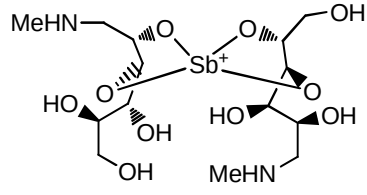

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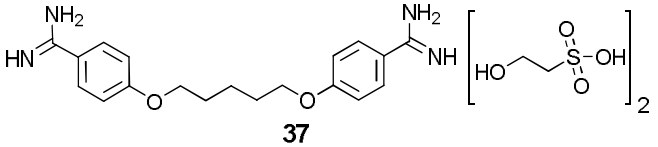
Figure 12. Traditional antimony-based drugs stibogluconate **35** and meglumine antimonate **36** used for treatment of leishmaniasis.

The second-line drugs for treatment of leishmaniasis include aromatic diamine pentamidine **37** and amphotericin B **38** (Figure 13). Pentamidine **37** binds to the adenine and thymine (AT) sequences of leishmanial kinetoplast DNA, leading to mitochondrial destruction and parasite death.[72] Pentamidine **37** has severe toxic side effects leading to renal toxicity[73] and cardiotoxicity[74], in addition to which resistance to pentamidine **37** has developed.[75] The polyene macrolide compound amphotericin B **38** increases parasite membrane permeability by acting with membrane sterols.[76] This leads to release of cellular components, mainly potassium, thus killing the parasite. Using relatively expensive lipid-based formulations of the drug,[77] the occurrence of severe side effects, such as nephrotoxicity, is reduced.[78]



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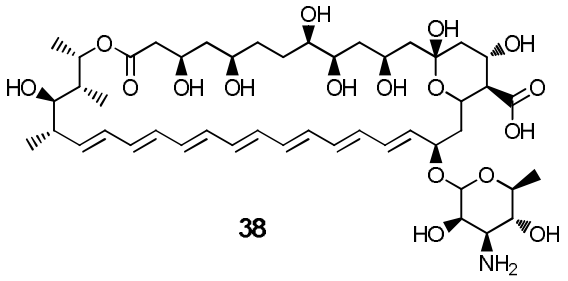

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Figure 13. Second-line drugs, pentamidine **37** and amphotericin B **38**, used for treatment of leishmaniasis.

The aminoglycoside antibiotic paromomycin **39** (Figure 14) was approved in 2006 for treatment of leishmaniasis in India. Paromomycin administered intramuscularly for 21 days showed an initial cure rate of 96% of patients and a definitive cure rate of 92% after 6 months.[79] It has a reasonable safety profile with only a minimal proportion of patients suffering possible side effects such as ototoxicity.[80] However, the drug must be administered by injection. Paromomycin **39** affects mitochondrial activity[81] and ribosomes.[82]

8-Aminoquinoline sitamaquine **40**[83] has been approved for treatment of leishmaniasis in India. This orally administered drug has shown good cure rates of up to 87%, and side effects seem to be relatively mild, such as vomiting, dyspepsia and methemoglobinemia.[84] The drug induces collapse of mitochondrial membrane potential[85] as well as alkalization of acidocalcisomes, specific organelles present only in parasites.[86]

Alkylphosphocholine miltefosine **41**[87] was the first approved orally administered antileishmanial drug on the market. Miltefosine has displayed an initial cure rate of up to 97% and a final cure rate of 82% after 6 months.[88] It has a generally safe toxicology profile, the main side effects being related to toxicity to the gastrointestinal tract. However, due to possible teratogenic effects of miltefosine **41**,[89] it is not suitable for treatment of pregnant women. Suggested targets for miltefosine **41** include inhibition of glycosomal acyltransferase,[90] mitochondrial enzyme cytochrome c oxidase[91] and effects on lipid metabolism.[92]

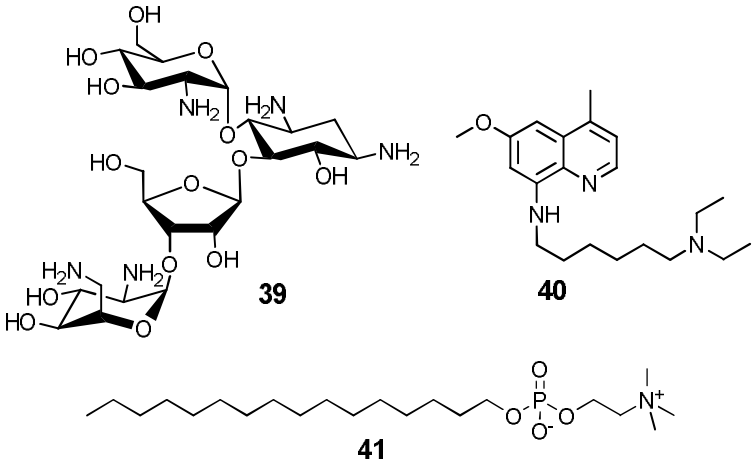


Figure 14. New drugs for treatment of leishmanial infections.

Lead compounds in clinical and preclinical trials

In this section antileishmanial agents showing promising *in vivo* inhibition activities in human clinical or preclinical studies are presented. Some of the following lead compounds are currently in further development.

Antifungal heterocyclic imidazole and 1,2,4-triazole-derived compounds have been tested in several clinical trials for the treatment of leishmaniasis. The effectiveness of itraconazole **42**, ketoconazole **43** and fluconazole **44** (Figure 15) in *in vivo* human studies are promising but still inconclusive. The performance of itraconazole **42** (400 mg dosage for a minimum of 3 months) was studied in an uncontrolled study with 13 Ecuadorian patients with mucocutaneous leishmaniasis.[93] All 13 subjects responded to itraconazole **42** during the first month of treatment, but by 12 months after treatment complete resolution of mucocutaneous leishmaniasis lesions was observed in only three (23%) patients. No adverse effects of treatment were reported.

The efficacy of ketoconazole **43** (600 mg/day for 28 days) was compared to reference drug pentostam **35** (20 mg antimony/kg for 20 days) against Panamanian cutaneous leishmaniasis due to *L. braziliensis panamensis*.[94] Ketoconazole **43** clinically cured 16 out of 21 (76%) patients. Side effects were limited to a 27% incidence of mild, reversible hepatocellular enzyme elevation and an asymptomatic, reversible, approximately 70% decrease in serum testosterone in all patients. Pentostam **35** cured 13 of 19 (68%) patients and a placebo group of 11 patients had a 0% cure rate. The authors conclude that oral ketoconazole **43** is comparable in efficacy to this parenteral pentostam regimen and can be recommended as initial treatment for this disease.

Fluconazole **44** (200 mg daily for six weeks) was studied for the treatment of cutaneous leishmaniasis caused by *L. major*.[95] A total of 106 patients received

fluconazole **44** and 103 patients received a placebo. 79% of patients in the fluconazole **44** group and 34% of the placebo group showed complete recovery during the trial. Side effects were mild, and oral fluconazole **44** thus seems to be a safe and useful treatment for cutaneous leishmaniasis caused by *L. major*.

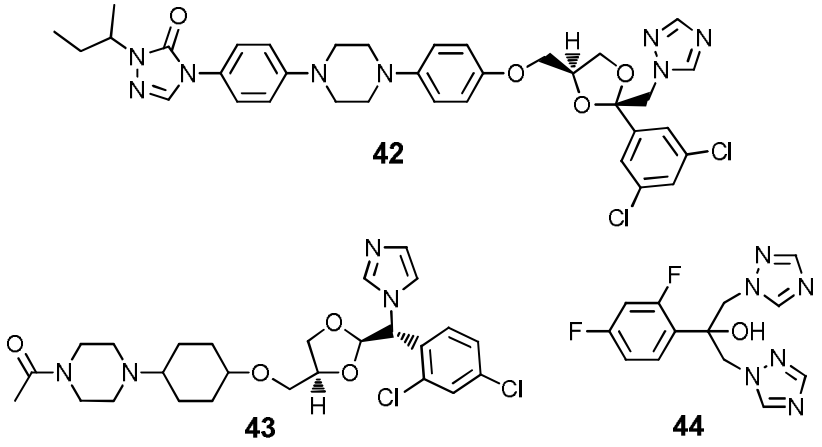


Figure 15. Antifungal heterocyclic imidazole and 1,2,4-triazole drugs displaying antileishmanial activity.

Purine analogue allopurinol **45** (drug used to treat hyperuricaemia, including chronic gout; see Figure 16) has been tested in a clinical trial (20 mg/kg for 15 days) for treatment of American cutaneous leishmaniasis in an area where *L. b. panamensis* is endemic.[96] Reference treatment with meglumine antimonate **36** (20 mg antimony/kg for 15 days) showed a moderate 36% cure rate, a combination treatment of allopurinol **45** and meglumine antimonate **36** displayed a 74% cure rate, and allopurinol **45** alone showed a good, 80%, cure rate. There were no complete recoveries among the untreated patients. In addition, no major toxic effects were observed. However, in another study contradictory results were observed.[97] A group of 187 patients with cutaneous leishmaniasis caused by *L. panamensis* (84% of patients) or *L. braziliensis* (16% of patients) were treated with allopurinol **45** (20 mg/kg of body weight per day for 28 days), a placebo, or meglumine antimonate **36** (20 mg of intramuscular antimony/kg per day for 20 days). After the treatment period, the allopurinol **45** group showed 33%, the placebo group 37% and the meglumine antimonate **36** group 93% cure rates. The authors of that study concluded that allopurinol **45** monotherapy has no effect on Colombian cutaneous disease primarily caused by *L. panamensis* and therefore is unlikely to be effective against cutaneous leishmaniasis in other endemic regions.

Topical treatment with antiviral drug imiquimod **46** (immune response modifier) in combination with meglumine antimonate **36** has shown very good efficacy against meglumine antimonate **36** resistant cutaneous leishmaniasis.[98] The study

was performed in Peru, where *L. peruviana* is endemic. All of the patients responded well to this combination therapy, and 90% of the patients were found to be cured after a 6-month follow up period.

The affectivity of the antibiotic/anti-leprosy drug dapsone **47** was studied with 120 patients infected with cutaneous leishmaniasis.[99] Two patient groups were treated with tablets of dapsone **47** (100 mg) or placebo tablets every 12 h for 6 weeks. 82% of the dapsone treated group were cured. In addition, dapsone **47** is economical, widely available and well-tolerated.

Azalide antibiotic azithromycin **48** has demonstrated very good efficacy (85%) for the treatment of patients infected with cutaneous leishmaniasis caused by *L. (Viannia) braziliensis.*[100] However, unsatisfactory results were obtained from trials conducted against *L. major* in Iran, with only 10% of patients displaying fully cured lesions.[101] A reference group treated with meglumine antimonate **36** showed a better, though still moderate, cure rate of 34%.

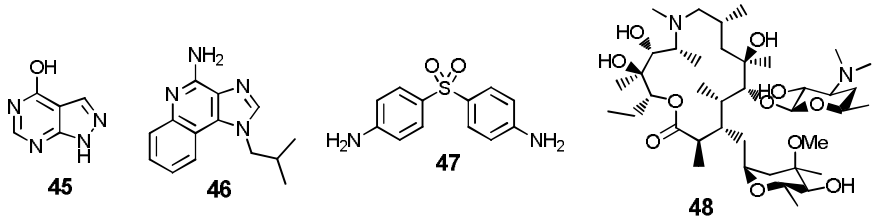


Figure 16. N-heterocyclic drugs **45** and **46** and antibiotics **47** and **48** showing potency as antileishmanial drugs in humans.

Table 5. Antileishmanial cure rates in human preclinical trials.

Compound	Regimen	Species	Cure %	Ref.
42	400 mg/day/3 months	*L. viannia braziliensis / L. viannia*	23	93
43	600 mg/day/28 days	*L. b. panamensis*	76	94
44	200 mg/day/6 weeks	*L. major*	79	95
45	20 mg/kg/15 days	*L. b. panamensis*	80	96
	20 mg/kg/28 days	*L. panamensis*	33	97
45 + **36**	20 mg/kg/15 days + 20 mg antimony/kg/15 days	*L. b. panamensis*	74	96
46 + 36	topically 250 mg/every other day/20 days + 20 mg antimony/kg/20 days	*L. peruviana*	90	98
47	100 mg tablets/every 12 h/6 weeks	*L. major / L. tropica*	82	99
48	500 mg/3 days, 500 mg/5 days, 500 mg/10 days and 1000 mg/2 days	*L. viannia braziliensis*	85	100
	500 mg/day/5 days/month	*L. major*	10	101

	Controls			
35	20 mg antimony/kg/20 days	*L. b. panamensis*	68	[94]
36	20 mg antimony/kg/15 days	*L. b. panamensis*	36	[96]
	20 mg of antimony/kg/20 days	*L. panamensis*	93	[97]
	60 mg/kg meglumine antimonate/20 days	*L. major*	34	[101]
Placebo		*L. b. panamensis*	0	[94]
		L. major	34	[95]
		L. b. panamensis	0	[96]
		L. panamensis	37	[97]
		L. major / *L. tropica*	0	[99]

Hit compounds in *in vivo* animal testing

In this section, antileishmanial agents showing promising *in vivo* inhibition activities in animal studies are presented. Some of these early lead compounds are currently in further development.

Investigational triazole antifungal agent posaconazole (SCH 56592) **49** (Figure 17) showed very good *in vivo* activity with BALB/c mice against cutaneous leishmaniasis due to *L. amazonensis* infection.[102] At a dose of 60 mg/kg/day posaconazole **49** was superior to amphotericin B **38** at a dose of 1 mg/kg/day. However, activity against visceral leishmaniasis due to *L. donovani* infection was clearly lower when compared to positive control amphotericin B.

In a mouse model of *L. major* leishmaniasis, administration of indole-based imidazole antifungal agent **50** led to a clear parasite burden reduction: 99% in the spleen, 79% in the liver and 50% in the cutaneous lesion.[103] Compound **50** decreases ergosterol biosynthesis, leading to membrane fungal cell alterations. In addition, **50** induces a parasite burden-correlated decrease in interleukine-4 production both in the splenocyte and the popliteal node of the mouse.

Based on *in vitro* studies of 16 synthetic oximino benzocycloalkyl azoles against *L. donovani*, five compounds showing best activity profile (SI) were tested further *in vivo* in a hamster model.[104] Of these derivatives, imidazole **51** showed significant 70% reduction of parasite burden in spleen. However, **51** was still clearly less active than the reference drug miltefosine **41** (96% inhibition) *in vivo*.

A library of 19 synthetic aryloxy alkyl/aryl alkyl imidazoles displayed very good 94–100% inhibition at 10 µg/mL *in vitro* against *L. donovani* promastigotes.[105] In addition, 12 compounds exhibited high inhibition with IC_{50} values in the range of 0.47–4.85 µg/mL against amastigotes (compound **52** had the best IC_{50} activity at 0.47 µg/mL). However, *in vivo* assays with the most promising four compounds showed only moderate activities with 58–60% inhibition.

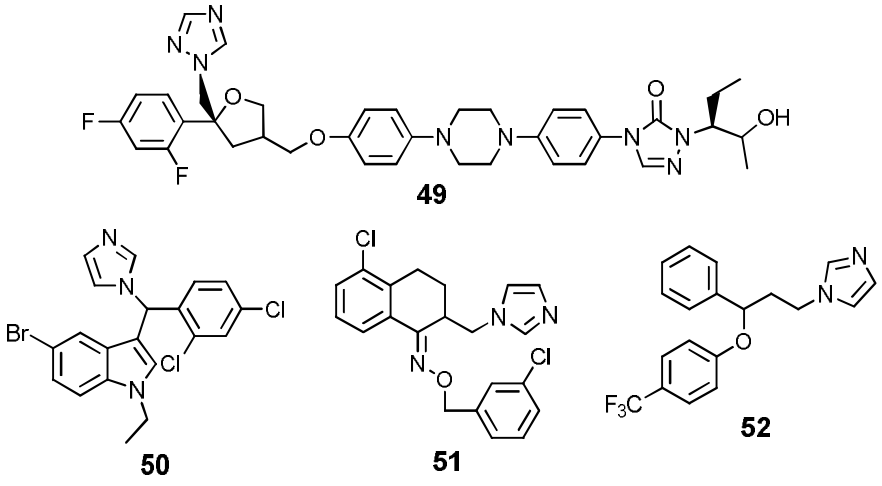


Figure 17. Triazole and imidazole-based antileishmanial compounds.

Pyrazinamide **53** (Figure 18), which is used in tuberculosis chemotherapy, showed good efficacy in the treatment of *L. major*-infected mice with a 100-fold reduction in parasite burden, when compared to the control.[106] In addition, mechanistic studies suggest that pyrazinamide **53** enhances effective immune responses against the parasite and has an immunostimulatory effect. Treatment was non-toxic and did not affect the growth of the experimental animals.

Two novel arylimidamide class antileishmanial drug candidates, DB745 and DB766 **54** showed exceptional activity against intracellular *L. donovani*, *L. amazonensis,* and *L. major in vitro*.[107] *In vivo* orally given, DB766 **54** produced a dose-dependent inhibition of liver parasitemia in two efficacy models, *L. donovani*-infected mice and hamsters, (71% and 89%, respectively). A marked reduction in parasitemia in the spleen (79%) and bone marrow (92%) of hamsters was also observed. Furthermore, the compounds were well distributed in the liver and spleen target tissues, showed moderate oral bioavailability (up to 25%), and had a suitable elimination half-life ranging from 1 to 2 days in mice. No toxic side effects to liver or kidney were observed, although mild hepatic cell eosinophilia, hypertrophy, and fatty changes were noted. The results demonstrated that arylimidamides are a promising class of compounds for preclinical development as an orally administered drug.

The conventional dihydropyridine antihypertensive drugs amlodipine **55** and lacidipine **56** inhibited *L. donovani* infection *in vitro* and in mice when administered orally.[108] Amlodipine **55** and lacidipine **56** therapies led to significant reductions in splenic (85% and 75%) and liver (86% and 72%) parasite burdens, when compared to controls. The compounds functioned through dose-dependent inhibition of oxygen consumption, triggering caspase 3-like activation-mediated programmed cell death of the parasites.

A series of 2,4,6-trisubstituted pyrimidines and 1,3,5-triazines were synthesized and screened for *in vitro* and *in vivo* antileishmanial activity against *L. donovani*.[109] Three compounds, **57**, **58** and **59**, showed a good selectivity index (SI) *in vitro*, and these were screened for *in vivo* activity in golden hamsters infected with *L. donovani*. The compounds showed decent *in vivo* inhibition of 48–56% at a dose of 50 mg/kg when administered intraperitoneally.

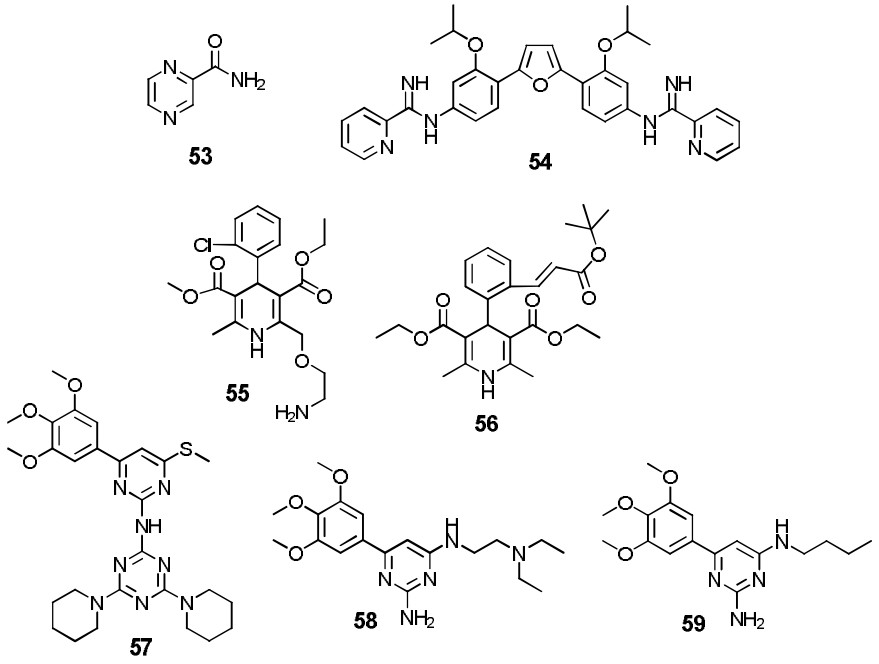


Figure 18. Six-membered nitrogen-containing heterocycles with *in vivo* antileishmanial activity.

A sitamaquine derivative of 8-aminoquinoline (±)NPC1161B **60** (Figure 19) and pure (–)- and (+)-enantiomers were studied *in vivo* with *L. donovani*-infected mice.[110] All three components (dose 10 mg/kg/day) showed very good and comparable activity to the parent compound sitamaquine, and cleared the parasites after a 5-day course of treatment. (–)-Enantiomer was better tolerated and had an increased therapeutic window when compared to the racemate or (+)-enantiomer of the compound.

Oral administration of natural quinoline-based compound chimanine B **61** (twice a day 50 mg/kg) to mice infected with *L. amazonensis* or *L. venezuelensis* resulted in a decrease in lesion weight by 70% and parasite loads by 95% when compared to the group of untreated mice.[111] Injections of chimanine B **61** (five injections at four-day intervals) and subcutaneous administration or intralesional injections of reference drug meglumine antimonate **36** displayed similar and very good efficacy. In the follow up study, the efficacy of nine chimanine B derivatives was determined

in a murine model.[112] Activity was further improved by the hydroxy derivative **62**, showing antileishmanial activity up to 90% in *in vivo* rat models.

A series of synthetic bis-quinolines showed excellent antileishmanial efficacy against *L. donovani* in both *in vitro* and *in vivo* studies.[113] Compound **63** exhibited the most significant activity against visceral leishmaniasis in a mouse model without showing any toxic manifestation. Intraperitoneal treatment with compound **63** at 12.5 mg/kg body weight led to significant reduction of parasite burden in spleen (95%) and liver (98%) compared to untreated controls. Ultrastructural studies of treated promastigotes demonstrated membrane blebbing, chromatin condensation and vacuolization in the parasites, and flagellated parasites became round-shaped after treatment.

The quinazoline derivative peganine hydrochloride dihydrate **64** isolated from *Peganum harmala* L. seeds showed only moderate *in vitro* activity against both extracellular promastigotes as well as intracellular *L. donovani* amastigotes growing inside macrophages.[114] However, the alkaloid **64** administered by oral route exhibited good *in vivo* activity with 80% reduction of *L. donovani* parasites in hamster spleen at a dose of 100 mg/kg. The reference drug miltefosine **41** resulted in 96% inhibition of parasites at a dose of 40 mg/kg.

The quaternary isoquinoline alkaloid berberine **65** and several of its derivatives were tested for efficacy against *L. donovani* and *L. braziliensis panamensis* in golden hamsters.[115] Tetrahydroberberine **66** was the most potent derivative against *L. donovani* with 50% suppression of parasite burden, but was not as potent as the reference drug meglumine antimonate **36**. Only berberine **65** and 8-cyanodihydroberberine **67** showed significant activity (>50% suppression of lesion size) against *L. braziliensis panamensis.*

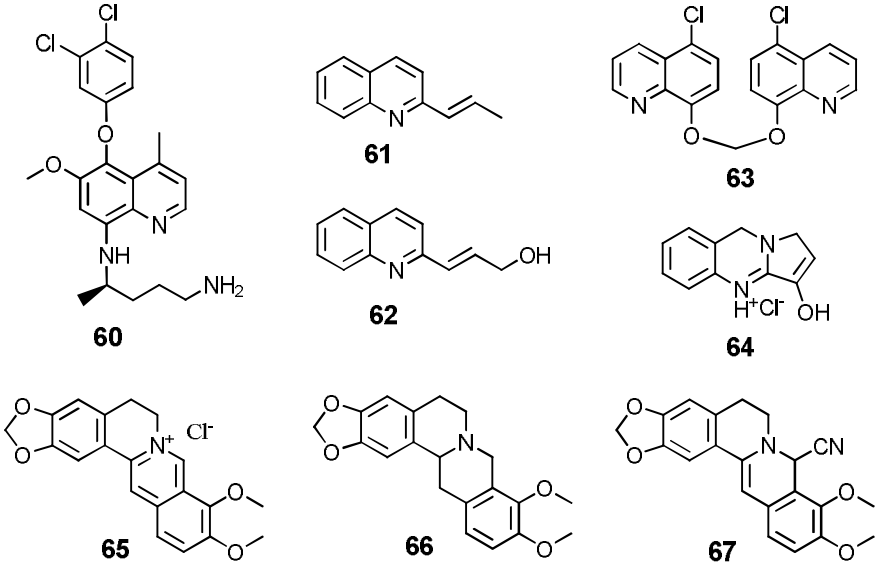
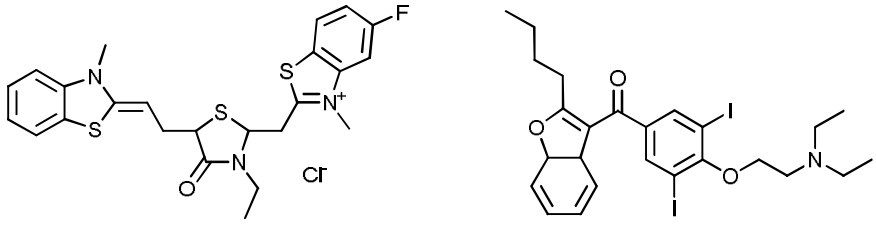


Figure 19. Quinoline-derived compounds showing antileishmanial activity *in vivo*.

Various synthetic rhodacyanine derivatives were studied for their antileishmanial *in vitro* and *in vivo* activities.[116] Among the derivatives, the fluorinated variant SJL-01 **68** (Figure 20) showed an excellent *in vitro* selectivity index of >15,000 and an IC_{50} value of 0.011 µM against *L. donovani.* The fluorinated compound **68** displayed an exceptional 95% inhibition against *L. donovani* parasites in female mice by 1.3 mg/kg intravenous administrations. Preliminary studies showed that no bioavailability was obtained by subcutaneous administration.

In vitro and *in vivo* (mice) activities of antiarrhythmic amiodarone **69** and miltefosine **41** were investigated alone or in combination on *L. mexicana.*[117] It was found that whereas both drugs given individually failed to cure lesions, a combination of amiodarone **69** and miltefosine **41** had synergistic effects on the proliferation of intracellular amastigotes growing inside macrophages and led to 90% parasitological cures in a murine model. Amiodarone **69** disrupts intracellular Ca^{2+} homeostasis and inhibits the *de novo* sterol biosynthesis of the parasite.



68 **69**

Figure 20. Structures of potential antileishmanial compounds rhodacyanine derivatives **68** and **69**.

The common flavonoids luteolin **70** and quercetin **71** (Figure 21) were assayed *in vivo* for visceral leishmaniasis against *L. donovani*-infected golden hamsters.[118] Luteolin **70** showed good activity by an over 80% reduction in splenic parasites. Quercetin **71** reduced the splenic parasite load by 90% at four times higher concentration. *In vitro* studies suggested that leishmanicidal activity was related to inhibition of promastigotes DNA synthesis and promotion of topoisomerase II-mediated linearization of kDNA minicircles. Quercetin **71** was almost equally potent to the standard antileishmanial drug sodium stibogluconate **35** in lowering the parasite load in the spleen of *L. donovani*-infected hamsters (reductions 77% and 82%, respectively).[119] Combination therapy with quercetin **71** and stibogluconate **35** showed improved synergistic activity with a 93% reduction of parasites in hamster spleen.

Hydroxynaphthoquinone-based buparvaquone **72** showed moderate antileishmanial activity in *L. donovani* infected mice at very high concentration.[120] Potency of buparvaquone **72** was increased several-fold with the formulation containing buparvaquone **72** (BPQ) and phosphatidylserine (PS) entrapped in liposomes (BPQ–PS-LP).[121] BPQ–PS-LP was evaluated *in vivo* against *L. infantum (syn.*

L. chagasi) infected hamsters. BPQ–PS-LP reduced the number of amastigotes by 89% in the spleen and by 67% in the liver, compared to 84% and 99.7%, respectively, with meglumine antimonate **36**. Buparvaquone **72** alone failed to treat the hamsters when compared to the untreated group.

Natural compound 2',6'-dihydroxy-4'-methoxychalcone (DMC) **73** induced only 23% reduction in parasites in *L. amazonensis*-infected mice. When chalcone **73** was formulated with polylactide to form DMC-PLA nanoparticles, activity was improved to 53% parasite reduction. Moreover, lesion size was reduced by about 60%. Thirty days after the initiation of treatment, the parasite load in the lesions was reduced by 90% and the effect was comparable to equivalent doses of the antileishmanial drug meglumine antimonate **36**.[122] In the follow up study, it was demonstrated that DMC **73** alters the sterol composition of *L. amazonensis*, suggesting that the target is different from other known sterol inhibitors.[123]

A treatment of hamsters infected with *L. donovani* with intraperitoneal administration of natural chalcone derivative, licochalcone A **74**, resulted in a 96% reduction of parasite load in the liver and the spleen.[124] Oral administration resulted in 65% and 85% reductions of *L. donovani* parasite loads in the liver and the spleen, respectively. In a subsequent study by the same authors, activity of the oxygenated chalcones was studied.[125] The best derivative, '35m4ac' **75**, showed very good *in vivo* activity. *L. donovani* parasite load in hamster liver was reduced by 97% after intraperitoneal administration. The chalcone derivative **75** inhibits parasite respiration and activity of mitochondrial dehydrogenases.

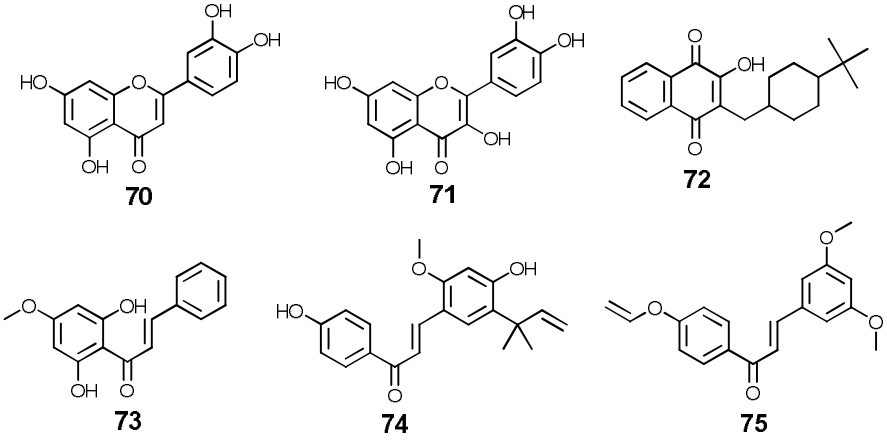


Figure 21. Flavonoids **70** and **71**, naphthoquinone **72** and chalcones **73**–**75** displaying antileishmanial activity *in vivo*.

The *seco*-iridoid glycoside amarogentin **76** (Figure 22) was investigated in an *in vivo* hamster model against visceral leishmaniasis caused by *L. donovani*.[126] The free form of amarogentin **76** displayed a 34% reduction in parasite load in the spleen. When amarogentin **76** was applied with liposomal or niosomal non-ionic

surfactant vesicles, its activity was improved considerably with a parasite load reduction in the spleen of 90% and 69%, respectively.

Argentilactone **77** showed very good *in vivo* antileishmanial activity when administered to *L. amazonensis*-infected mice by oral or subcutaneous routes.[127] Argentilactone showed the same efficacy as the reference drug meglumine antimonate **36**, reducing parasite load in lesions by 96% and in the spleen by 50%.

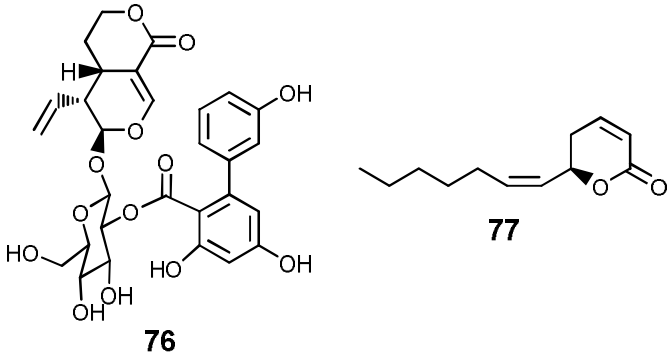


Figure 22. Lactone derivatives having antileishmanial activity *in vivo*.

Four potential anticancer alkyl-lysophopholipids were assayed by oral administration against two strains of *L. donovani* in a mouse model.[128] The currently approved antileishmanial drug miltefosine **41** displayed the best parasitic reduction in liver (97% and 99% inhibition against strain LV9 and Patna I) at a 30 mg/kg dose. Ilmofosine **78** (Figure 23) displayed good activity with 67% and 92% inhibition. Edelfosine **79** was moderately active. In a study by Cabrera-Serra et al., miltefosine-related derivatives edelfosine **79** and perifosine **80** were orally administered to *L. amazonensis*-infected mice.[129] The edelfosine-treated mouse group showed 49% and perifosine **80** 38% inhibition in footpad lesions. Biopsies obtained from mice treated with edelfosine **79** showed a 19% parasitic burden when compared to the non-treated control. In perifosine-treated mice, the parasitic burden was only 7% when compared to the control. The researchers concluded that perifosine **80** should be studied further in preclinical studies.

In vivo intraperitoneal administration of the osteoporosis bisphosphonate drug pamidronate **81** to mice infected with cutaneous leishmaniasis caused by *L. mexicana amazonensis* resulted in long-term disappearance of lesions.[130] In another study, pamidronate **81** and related bisphosphonate risedronate **82** were studied against *L. donovani*.[131] Intravenous administration of pamidronate **81** and risedronate **82** was found to inhibit *L. donovani* amastigotes parasite burden in mouse liver by 92% and 99%, respectively. However, at high doses of risedronate **82** toxicity was also observed.

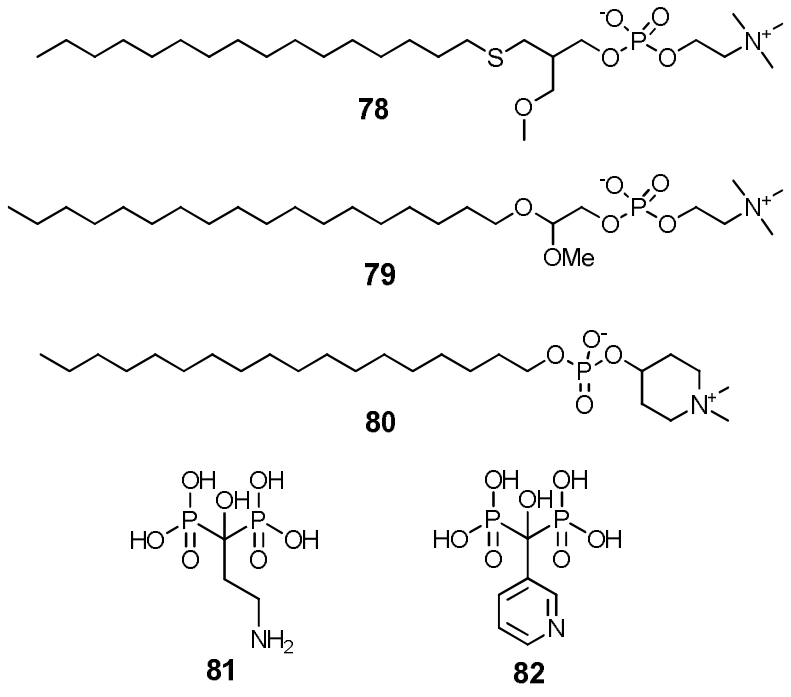
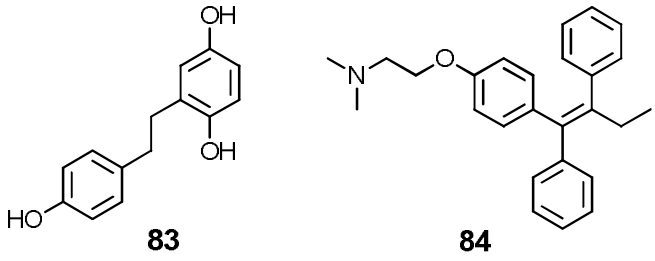


Figure 23. Phosphorus-containing antileishmanial compounds.

Hydroxybibenzyl 14-hydroxylunularin **83** (Figure 24) was highly active in an *in vitro* assay against culture and intracellular forms of *Leishmania* spp., without cytotoxicity to mammalian cells.[132] In addition, 14-hydroxylunularin **83** showed high antiprotozoal activity in *Leishmania spp*.-infected mice by subcutaneous and oral administration routes. Treatment caused the lesion weight to decrease by 96% and the parasite load by 93% with the subcutaneous route, and 90% and 69% with oral administration. The authors of that study suggest that **83** could be chosen as a new candidate in the development of leishmanicidal therapy.

Antiestrogen drug tamoxifen **84**, which is used as a chemotherapeutic and chemopreventive agent against breast cancer, showed excellent antileishmanial activity in mice infected with *L. amazonensis* promastigotes.[133] Parasite burden in tamoxifen-treated animal lesions was reduced by 99.7% in treated groups, as compared to untreated animals. In addition, the reduction in parasite burden was sustained for 6 weeks after the end of treatment. Thus, the results support the proposal for further testing of this drug in other models of leishmaniasis.



83 **84**

Figure 24. Antileishmanial compounds 14-hyroxylunularin **83** and antiestrogen agent tamoxifen **84**.

Natural oleane triterpene saponin maesabalide III **85** (MB-III, PX-6518, Figure 25) demonstrated *in vivo* activity against *L. donovani* in golden hamsters.[134] It was concluded that administration of a single dose of MB-III has efficacy comparable to that of a single dose of liposomal amphotericin B **38**. However, severe toxicity was observed as several animals died during the experiment series and further development is required. Activity of MB-III **85** was further evaluated *in vivo* with mice infected with *L. mexicana*, *L. panamensis* or *L. major*.[135] MP-III **85** completely healed *L. mexicana* and *L. panamensis* lesions, whereas *L. major* lesions were reduced by 50%, thus demonstrating broad-spectrum curative efficacy. In the follow up study, the same authors synthesized several semisynthetic MB-III derivatives to study the structure-activity relationships, but in *in vitro* studies none of the derivatives showed increased activity compared to **85**.[136]

Monoterpenoid cantharidin **86** is a natural poisonous terpenoid secreted by male blister beetles (*Lytta vesicatoria*). The effect of different doses of cantharidin **86** on *L. major* were investigated both *in vitro* (promastigote and amastigote viability) and in infected mice (skin lesions) using ointment or soluble cantharidin. Two weeks of topical treatment with 0.1% cantharidin ointment was an effective method for treating cutaneous leishmaniasis in infected mice and skin lesions were totally healed. However, in the follow up study, after two months a relapse of lesions was observed for two mice out of eight.

The widely used antimalarial drug sesquiterpene lactone, which contains an unusual peroxide bridge, artemisinin **87**, demonstrated only moderate antileishmanial activity against promastigotes of a wide range of *Leishmania* species, including strains responsible for visceral leishmaniasis (*L. donovani, L. infantum*), cutaneous leishmaniasis (*L. tropica*, *L. mexicana*) and mucocutaneous leishmaniasis (*L. amazonensis*, *L. braziliensis*) promastigotes, the IC_{50} value being approx. 100 µM.[137] However, in a mouse model of visceral leishmaniasis, orally administered artemisinin **87** (10 mg/kg and 25 mg/kg body weight) effectively reduced both splenic weight and parasite burden by 83% and 86%, respectively. The reference drug sodium stibogluconate **35** demonstrated a similar significant 85% decrease in parasite burden.

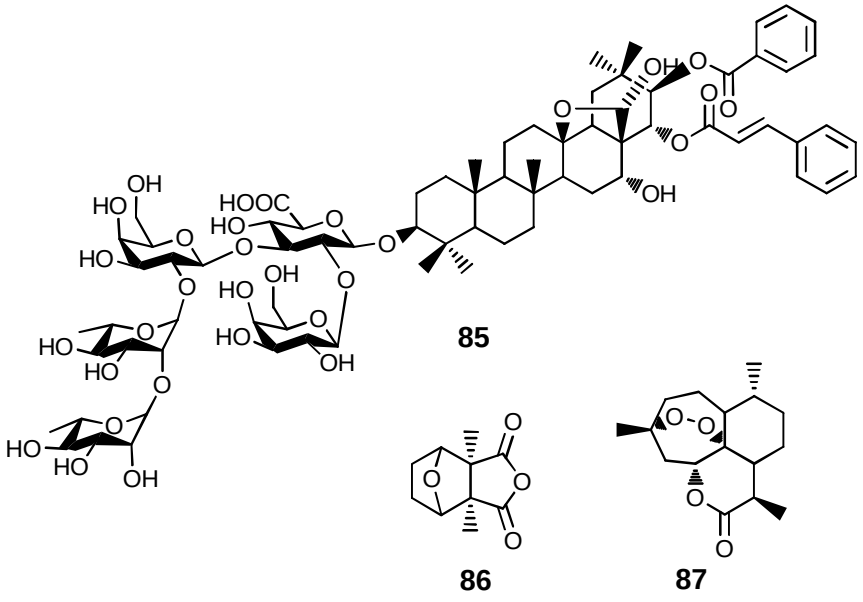


Figure 25. Terpenes and terpenoids possessing antileishmanial activity *in vivo*.

Betulin-derived compounds

In a study by Sauvain et al., betulinic aldehyde **88** (Figure 26), obtained from *Doliocarpus dentatus* (Aubl.) Stand. showed weak *in vitro* activity against *L. amazonensis* amastigotes with a survival index of 12% at 136 µM and 42% at 68 µM.[138] However, at these doses **88** also showed toxicity against peritoneal macrophages with survival indices of 70% and 80%, respectively. At a concentration of 34 µM, aldehyde **88** was ineffective against *L. amazonensis* as well as nontoxic to macrophages.

In another study, in which a library of natural compounds was screened for antileishmanial activity, betulinic acid **2** was found to be weakly active against *L. major* with an IC_{50} value of 88 µM.[139] It was also noted that in triterpenes having a ursane, oleanane or lupane skeleton, a carboxy substituent was required for antileishmanial activity.

In a study by Chowdhury et al., dihydrobetulinic acid **89** was found to inhibit growth of *L. donovani* promastigotes and amastigotes with an IC_{50} value of 2.6 and 4.1 µM, respectively.[140] It acts by targeting DNA topoisomerase I and II, preventing DNA cleavage and formation of the enzyme-DNA complex, ultimately inducing apoptosis. Leishmanial parasitic burden in golden hamsters was reduced by 92% after 6-week treatment with dihydrobetulinic acid **89** (10 mg/kg body weight). In a subsequent study by the same authors, six betulin and six dihydrobetulin esters were assayed against sodium stibogluconate sensitive and resistant

L. donovani strains *in vitro*.[141] Derivatives having small ester groups were active against both *L. donovani* strains. 3,28-Disuccinoylbetulin **90**, 3,28-disuccinoyldihydrobetulin **91** and 3,28-diglutaroylbetulin **92** showed the best inhibition activity against amastigote growth inside macrophages, with EC_{50} values between 6.1 and 10.5 µM. The suggested biochemical mechanism of these betulin esters was similar to dihydrobetulinic acid **89**, as they inhibited in a reversible manner the relaxation activity of enzyme type IB topoisomerase of the parasite as well as topoisomerase I–DNA complex formation.

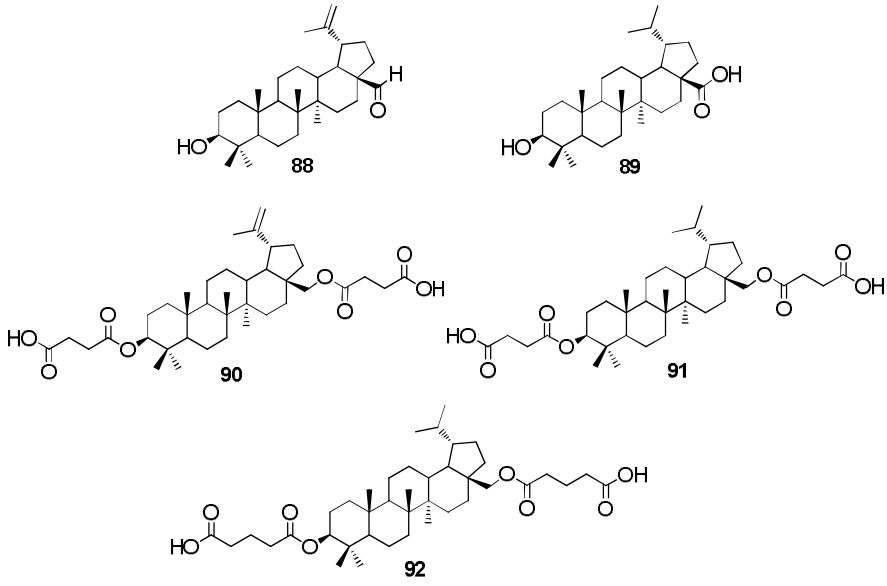


Figure 26. Betulin derivatives with antileishmanial activity.

2.4 Chlamydia pneumoniae

2.4.1 Species, taxonomy and life cycle

The order Chlamydiales comprises four families and 13 species, with the *C. pneumoniae* species belonging to Family I: Chlamydiaceae and Genus I: *Chlamydia* (Table 6).[142] In addition to *C. pneumoniae*, *C. trachomatis* is the only other bacterial species in the order Chlamydiales that infects humans. *C. trachomatis* causes urogenital infections and also trachoma, which causes roughening of the inner eyelids and can lead to blindness. The remaining species are infectious to animals only, including insects, birds and other mammals.

Table 6. Taxonomy of *Chlamydia pneumoniae*.

Order	**Chlamydiales**
Family	Chlamydiaceae
Genus I	*Chlamydia*
Species	*Chlamydia pneumoniae*

The chlamydial life cycle consists of two distinct morphological forms: the elementary body (EB) and reticulate body (RB). EB is the metabolically inactive, infective extracellular stage, and RB the metabolically active, replicating, intracellular form.

In the *C. pneumoniae* life cycle (Figure 27):[143]

1) EB attaches to the host cell (yet unknown receptors).
2) Entry of EB to host cell via phagocytosis[144] or receptor-mediated endocytosis.[145]
3) Inside the host cell EB is surrounded by double-membrane inclusion. EB is converted to RB.
4) Inclusion is enlarged and filled with new replicates of RB.
5) RBs are converted back to infectious EBs and exit of the EBs can occur via lysis of the host cell or via extrusion of the EB packed inclusion.

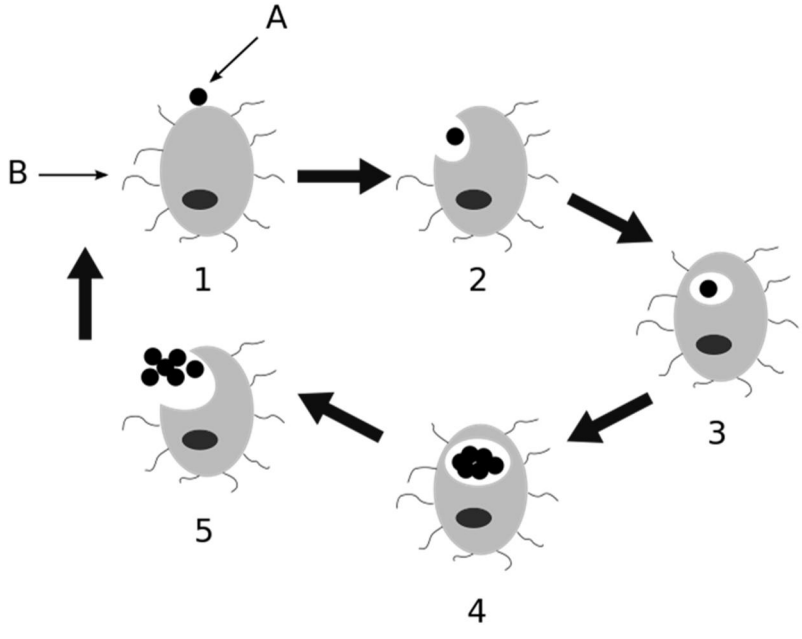


Figure 27. Life cycle of *C. pneumoniae.* A = *C. pneumoniae* elementary body (EB). B = Host cell, usually lung cell.[146]

2.4.2 Chlamydial persistence

Chlamydial persistence can last for years and it is defined as a viable but non-cultivable growth stage inside the infected host cell.[142] In the persistence stage, *C. pneumoniae* is not metabolically active and not replicating. Persistence is caused by several stress factors that are unfavourable for bacterial growth, such as insufficient nutrients (e.g. amino acids), iron depletion, non-lethal antibiotics, heat and growth in monocytes.[147] In all cases, the developmental cycle is halted and the RBs are not converted back to the infective extracellular EB stage. Instead of the normal round shape of EBs, enlarged aberrant bodies (AB) are formed. This is clinically highly relevant, since a persistent culture cannot be subcultured for diagnostic purposes. However, chlamydial persistence can be detected from chlamydial DNA or marker proteins in the absence of viable *Chlamydiae*.[148] It has been suggested that *C. pneumoniae* can persist for many years after initial respiratory infection.[149]

2.4.3 Chemotherapy against infections caused by *C. pneumoniae*

C. pneumoniae is susceptible to antibiotics that interfere with DNA and protein synthesis, including tetracyclines, macrolides, quinolones and rifamycins.[16] These classes of antibiotics are most widely studied and used for treatment of human infections. However, *C. pneumoniae* is resistant to some common antibiotic classes, such as trimethoprim, sulphonamides, aminoglycosides and glycopeptides.[150]

Clinical manifestation and treatment

Asymptomatic infection or unrecognized, mildly symptomatic illnesses, such as prolonged cough and mild fever, are very common symptoms of *C. pneumoniae* infection.[151] Severe systemic infections with *C. pneumoniae* alone are not common. Even in mild cases, complete recovery is slow, despite appropriate antibiotic therapy, and cough and malaise may persist for many weeks after the acute illness. It has been estimated that 10% of cases of pneumonia and approximately 5% of bronchitis and sinusitis cases in adults have been attributed to this organism.[152] In addition, there is strong evidence that *C. pneumoniae* is associated with other severe diseases, such as atherosclerotic cardiovascular diseases as well as some neurodegenerative diseases, such as Alzheimer's disease and multiple sclerosis.[17,18]

The intracellular location of *C. pneumoniae* limits and hampers the efficacy of some antibiotics. The antichlamydial agent must penetrate several biological membranes in therapeutic concentrations: the host cell membrane, the inclusion membrane, and, finally, the inner and outer membrane of the Gram-negative bacterium itself. Thus, antimicrobial agents need good intracellular penetration properties to be effective. The following regimen guidelines (Table 7) for adults can be followed in clinical treatment of *C. pneumoniae* infection.[16] However, some patients may need re-treatment.

Table 7. Current treatment guidelines for the treatment of respiratory infection due to *C. pneumoniae.*[16]

Antibiotic class	Drug	Dosing	Regimen
Fluoroquinolone	Levofloxacin **101**	500 mg once a day	Orally or intravenously 7–14 days
	Moxifloxacin **103**	400 mg once a day	Orally 10 days
Tetracycline	Doxycycline **112**	100 mg twice a day	Orally 14–21 days
	Tetracycline **114**	250 mg four times a day	Orally 14–21 days
Macrolide	Azithromycin **115**	1.5 g once a day	Orally 5 days
	Clarithromycin **116**	500 mg twice a day	Orally 10 days

Fluoroquinolones

Many of the commonly used fluoroquinolone antibiotics show very good *in vitro* antichlamydial activity with low micromolar MIC values. The fluoroquinolone class of antibiotics share a quinoline ring system with a fluorine atom at C-6. Piperazine or another ring system is attached to C-7. Fluoroquinolones block DNA replication and repair by inhibiting topoisomerase II function.[153]

The chemical structures of fluoroquinolone antibiotics and some experimental fluoroquinolones (and a few quinolones) screened *in vitro* against *C. pneumoniae* are presented in Figure 28 and their *in vitro* MIC values in Table 8. In most of the cases, susceptibility of *C. pneumoniae* to antibiotics is tested using a HEp-2 cell line or, in some cases, a HeLa 229 cell line, as a host. Several antibiotics, such as garenoxacin **97** (MIC 0.015–0.03 µg/mL), sitafloxacin **107** (MIC 0.031–0.0125 µg/mL) and experimental BMS-284756 **94** (MIC 0.015–0.03 µg/mL) show very good *in vitro* activities (Table 8). The MIC values from different studies are very consistent, and usually only small variations can be seen between HEp-2 or HeLa 229 host cell lines. The current recommendation for human treatment is to use levofloxacin **101** (MIC 0.25–1 µg/mL) or moxifloxacin **103** (MIC 0.06–1 µg/mL) as the first-choice antibiotic of this class.[16]

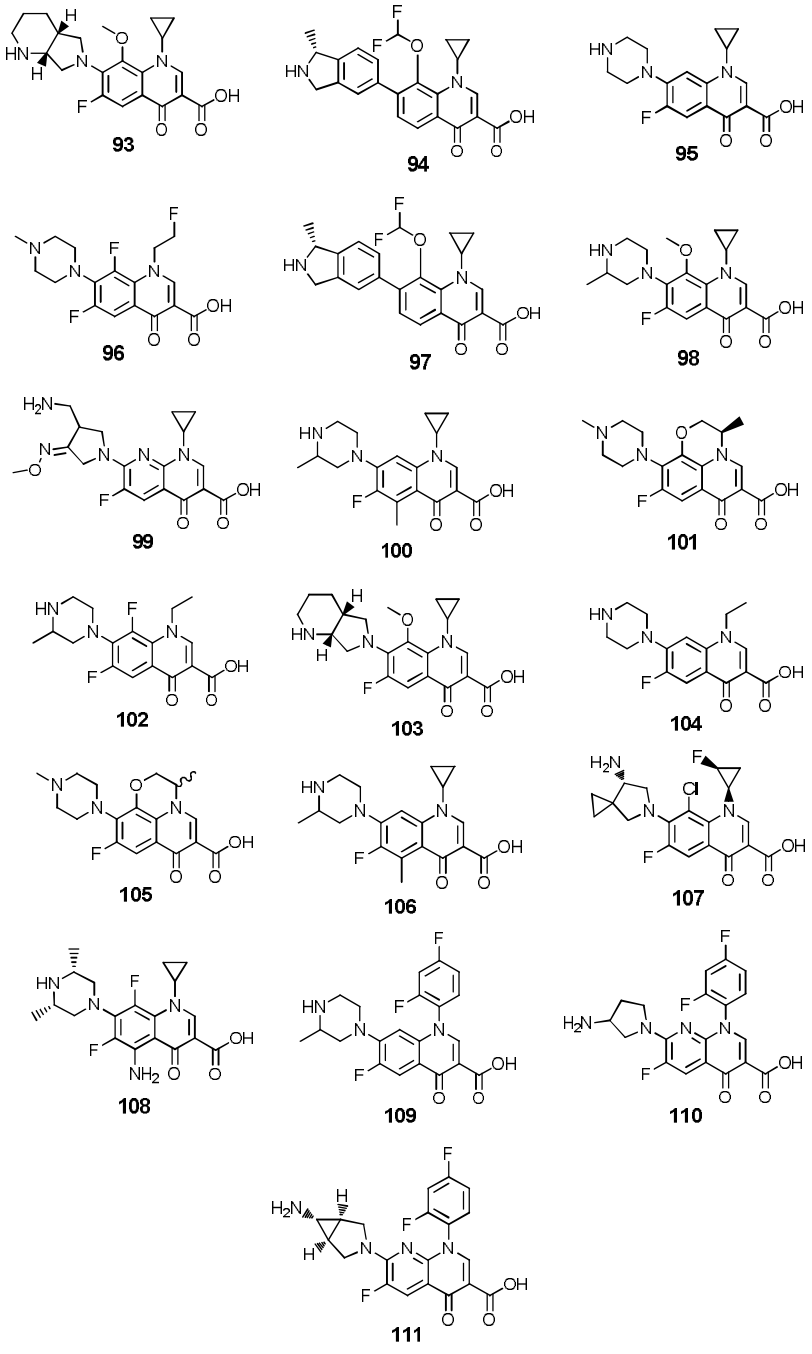


Figure 28. Chemical structures of the fluoroquinolone and quinolone classes of antibiotics with antichlamydial activity.

Table 8. *In vitro* efficacy of fluoroquinolones and some quinolones against *C. pneumoniae*.

Quinolone or fluoroquinolone	MIC range µg/mL	Cell line	Ref.
BAY 12-8039 **93**	0.5–1	HEp-2	154
BMS-284756 **94**	0.015–0.03	HEp-2	155
	0.004–0.008	HEp-2	156
Ciprofloxacin **95**	1.0–4.0	HEp-2	157
	0.25–4	HeLa 229, HEp-2	158
Fleroxacin **96**	2–8	HeLa 229, HEp-2	158
Garenoxacin **97**	0.008	HEp-2	159
	0.015–0.03	HEp-2	160
Gatifloxacin (AM-1155) **98**	0.125	HEp-2	159
	0.125–0.25	HEp-2	161
	0.063–0.125	HeLa 229	162
Gemifloxacin **99**	0.125–0.25	HEp-2	163
Grepafloxacin **100**	0.063–0.125	HeLa 229	162
Levofloxacin **101**	0.5–1.0	HEp-2	155
	0.25	HEp-2	159
	0.5	HEp-2	156
Lomefloxacin **102**	2	*	164
Moxifloxacin **103**	0.125–1.0	HEp-2	155
	0.06–0.12	HEp-2	156
	0.063	HEp-2	159
Norfloxacin **104**	16	*	164
Ofloxacin **105**	0.5–2.0	HEp-2	157
	0.5–2.0	HEp-2	165
	0.5–1	HEp-2	156
L-Ofloxacin	0.125–0.5	HeLa 229, HEp-2	166
OPC-17116 **106**	0.25–0.5	HEp-2	165
Sitafloxacin (DU-6859a) **107**	0.031–0.125	HEp-2	157
	0.031–0.063	HeLa 229	162
Sparfloxacin **108**	0.031–0.125	HEp-2	157
	0.016	HEp-2	159
	0.06–0.25	HEp-2	165
	0.06–0.25	HeLa 229, HEp-2	158
Temafloxacin **109**	0.125–1	HeLa 229, HEp-2	158
Tosulfloxacin **110**	0.063–0.25	HEp-2	157
	0.125	*	164
Trovafloxacin **111**	0.5–1	HEp-2	167

* Cell line not mentioned in publication.

Tetracyclines

The first tetracycline antibiotics, chlortetracycline and oxytetracycline, were discovered in the late 1940s and they are produced by the bacteria *Streptomyces aureofaciens* and *S. rimosus*, respectively.[168] Tetracycline antibiotic molecules consist of four linearly fused rings, to which a variety of functional groups are attached. Tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome.[169]

The chemical structures of tetracycline antibiotics, natural tetracycline **114**, and semisynthetic derivatives doxycycline **112** and minocycline **113**, screened *in vitro* against *C. pneumoniae*, are presented in Figure 29, and their *in vitro* MIC values using HEp-2 or HeLa 229 host cell lines are given in Table 9. All three compounds display good efficacy (MIC 0.05–0.25 µg/mL) against *C. pneumoniae*. Currently, doxycycline **112** and tetracycline **114** are recommended antibiotics for treatment of chlamydial infection in humans.[16]

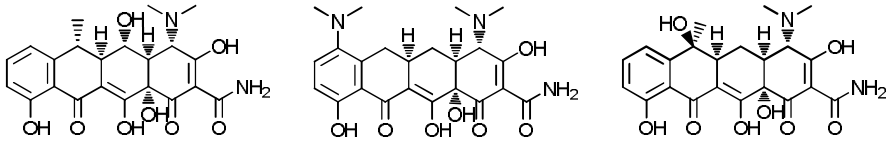


Figure 29. Chemical structures of tetracycline-class antibiotics with antichlamydial activity.

Table 9. *In vitro* efficacy of tetracyclines against *C. pneumoniae*.

Tetracycline	MIC range	Cell line	Ref.
Doxycycline **112**	0.06–0.25	HEp-2	166
Minocycline **113**	0.0625	HEp-2	170
Tetracycline **114**	0.05–0.1	HeLa 229	171

Macrolides

Macrolide antibiotics are usually used to treat infections caused by Gram-positive bacteria.[172] The macrolide antibiotics have a large, usually 14-, 15-, or 16-membered macrocyclic lactone ring with one or more deoxy sugars attached. Macrolides belong to the polyketide class of natural products, and they act by inhibiting the bacterial protein synthesis machinery, the ribosome.

The chemical structures of macrolide antibiotics and some experimental macrolides screened *in vitro* against *C. pneumoniae* are presented in Figure 30, and their *in vitro* MIC values using a HEp-2 host cell line are given in Table 10.

Azithromycin **115**, clarithromycin **116** and erythromycin **118** display very good antichlamydial *in vitro* inhibition activity (MIC 0.004–0.5 µg/mL), whereas dirithromycin and roxithromycin show slightly diminished activities. MIC results from different studies are very consistent. The current recommendation for human treatment is to use azithromycin **115** (MIC 0.06–0.5 µg/mL) or clarithromycin **116** (MIC 0.004–0.063 µg/mL) as the first-choice antibiotic of this class.[16]

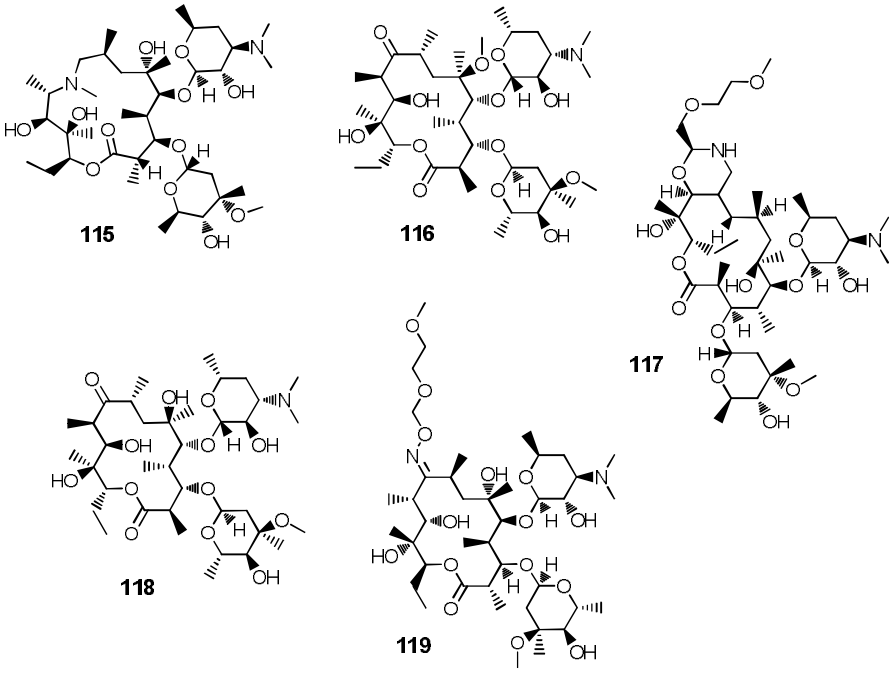


Figure 30. Chemical structures of macrolide-class antibiotics with antichlamydial activity.

Table 10. *In vitro* efficacy of macrolides against *C. pneumoniae*.

Macrolide	MIC range	Cell line	Ref.
Azithromycin **115**	0.125–0.5	HEp-2	173
	0.06–0.25	HEp-2	166
Clarithromycin **116**	0.016–0.063	HEp-2	173
	0.015–0.06	HEp-2	155
	0.004–0.03	HEp-2	166
Dirithromycin **117**	0.5–4	HEp-2	174
Erythromycin **118**	0.063–0.25	HEp-2	173
	0.016–0.5	HEp-2	174
Roxithromycin **119**	0.0625–2	HEp-2	175

Ketolides

Ketolide antibiotics are broad spectrum 14-membered semi-synthetic derivatives of the macrolide erythromycin A.[176] Main modifications are replacement of the neutral L-cladinose sugar at position C-3 in erythromycin by a 3-ketone carbonyl, and attachment of a cyclic carbamate group in the lactone ring. These structural modifications give ketolides improved pharmacodynamic and pharmacokinetic properties, such as improved acid stability, reduced risk of resistance induction and higher lipophilicity when compared with 14-membered macrolides.[177] Similarly to macrolides, ketolides act by inhibiting the bacterial protein synthesis machinery, the ribosome.

Figure 31 presents the chemical structures of ketolide antibiotics and some experimental ketolides screened *in vitro* against *C. pneumoniae,* and Table 11 shows their *in vitro* MIC values using HEp-2 host cells. Cethromycin **120** (MIC 0.008–0.031 µg/mL) and telithromycin **121** (MIC 0.031–0.25 µg/mL) show very good antichlamydial efficacy. However, none of the ketolide antibiotics are included in current treatment recommendations for humans.[16]

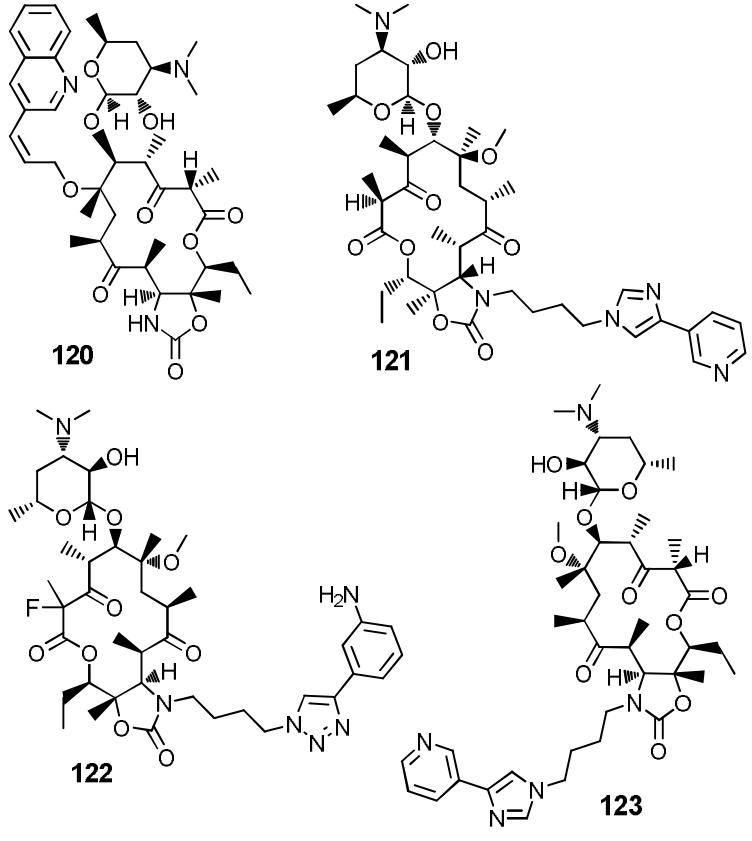


Figure 31. Chemical structures of ketolide-class antibiotics with antichlamydial activity.

Table 11. *In vitro* efficacy of ketolides against *C. pneumoniae*.

Ketolide	MIC range	Cell line	Ref.
Cethromycin (ABT-773) **120**	0.016–0.031	HEp-2	[173]
	0.008–0.015	HEp-2	[178]
HMR 3647 **121**	0.031–2	HEp-2	[175]
Solithromycin (CEM-101, OP-1068) **122**	0.25–1.0	HEp-2	[179]
Telithromycin **123**	0.031–0.25	HEp-2	[173]

Rifamycins

The molecular structure of rifamycins consists of an aromatic moiety bridged at nonadjacent positions by an aliphatic chain.[180] The aromatic moiety can be a naphthalene or naphthoquinone ring system, as in rifamycin, or a benzene or benzoquinone ring. The rifamycins were first isolated by Sensi and co-workers in 1959.[181]

The rifamycins display a broad spectrum of antibiotic activity, especially against Gram-positive bacteria.[180] After evaluation of a large number of semisynthetic analogues, rifampicin **126** with excellent oral bioavailability was developed. Rifampicin **126** shows more pronounced activity against Gram-positive bacteria, in particularly mycobacteria. In addition, activity against Gram-negative bacteria is improved. The antibacterial action of rifampicin **126** results from its inhibition of DNA-dependent RNA synthesis.[182]

The chemical structures of semisynthetic rifamycin antibiotic derivatives screened *in vitro* against *C. pneumoniae* are presented in Figure 32, and their *in vitro* MIC values using a HEp-2 host cell line are given in Table 12. All compounds display excellent efficacy (MIC 0.00125–0.02 µg/mL) against *C. pneumoniae*, however, rifamycins are currently not recommended for antichlamydial antibiotics in humans.[16]

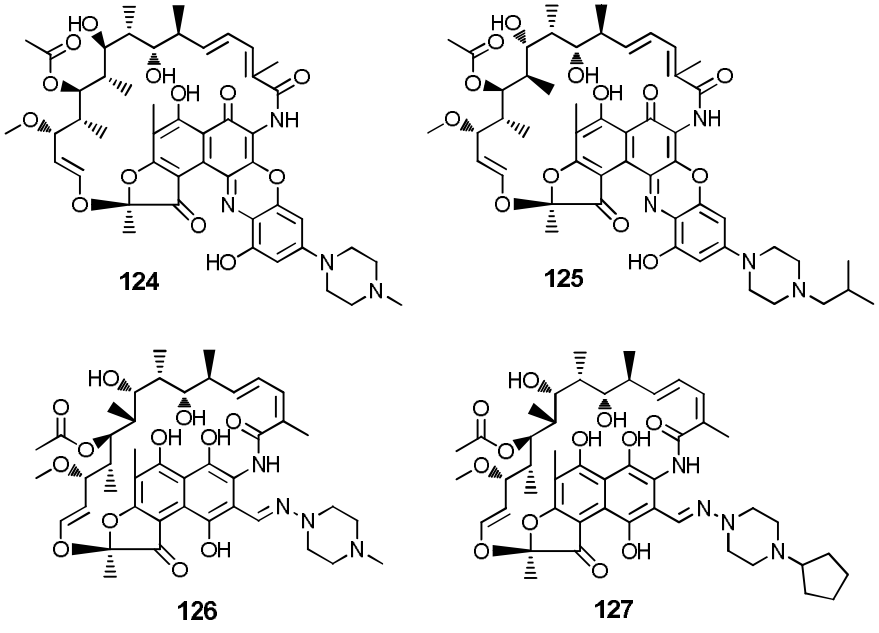


Figure 32. Chemical structures of rifamycin-class antibiotics with antichlamydial activity.

Table 12. *In vitro* efficacy of rifamycins against *C. pneumoniae*.

Rifamycins	MIC range	Cell line	Ref.
ABI-1131 **124**	0.00125–0.0025	HEp-2	[183]
Rifalazil (ABI-1648) **125**	0.00125–0.0025	HEp-2	[183]
Rifampicin **126**	0.005	HEp-2	[184]
Rifapentin **127**	0.04–0.02	HEp-2	[185]

Natural non-antibiotic-derived compounds

Little research has been conducted on the antichlamydial properties of non-antibiotic natural compounds.

In a study by Alvesalo et al., a library of 57 natural phenolic compounds or their derivatives were tested against *C. pneumoniae in vitro* using HL cells.[186] The compounds included flavones, flavonols, flavonones, isoflavonones, coumarins, catechins, phenolic acids, gallates, a stilbene or their semisynthetic derivatives. Active compounds were found in several compound groups, especially in gallates (dodecyl gallate **128**, MIC = 18 µM), but also flavones (luteolin **70** MIC = 8.8 µM) and flavonols (myricetin **131**, MIC = 29 µM) with no cytotoxicity to host cells (Figure 33).

In a related study, two flavonoids luteolin **70**, quercetin **71** and octyl gallate **129** were tested in an *in vivo* mouse model.[187] Results were contradictory: octyl gallate **129** had no significant effect; quercetin **71** increased both the inflammatory responses and the chlamydial load in the lungs. On the other hand, luteolin **70** suppressed the presence of *Chlamydia* and inflammation in lung tissue as well as development of *C. pneumoniae*-specific antibodies.

A tea polyphenol product "Polyphenon 70S", consisting of a mixture of flavan-3-ols, gallocatechins and catechins, was found to inhibit *C. pneumoniae* growth in HL cells completely at 0.8–1.6 mg/mL concentration, depending on the bacterium strain.[188]

Corn mint (*Mentha arvensis* L.) extract, and its main flavone components, acacetin **132**, linarin (diglycoside acacetin); and rosmarinic acid were evaluated against *C. pneumoniae* infection.[189] *In vitro* both flavones showed roughly 100% inhibition at 100 µM concentration with no toxicity to the HL host cell line, whereas the activity of corn mint extract and rosmarinic acid were lower. Corn mint extract was tested in a mouse model in which the extract was able to reduce the inflammatory parameters related to *C. pneumoniae* infection and the number of *C. pneumoniae* genome equivalents at biologically relevant amounts.

The antichlamydial properties of retinoic acid (Vitamin A) **130** have been studied *in vitro* and *in vivo*. The *in vitro* results showed two separate effects of retinoic acid **130**: firstly, retinoic acid prevents attachment of the organism to the host endothelial cell surface and, secondly, inhibits growth in both epithelial and endothelial cells.[190] The *in vivo* results showed that retinoic acid **130** prevents *C. pneumoniae*-induced foam cell development in a mouse model of atherosclerosis.[191]

Betulinic acid **2** has been found to be inactive against *Staphylococcus aureus*, *Escherichia coli*,[192] *Bacillus subtilis*, *Micrococcus luteus*,[193] as well as *Enterococcus faecalis* and *Pseudomonas aeruginosa*.[194] Generally, betulin derivatives seem to have rather poor antibacterial activity.

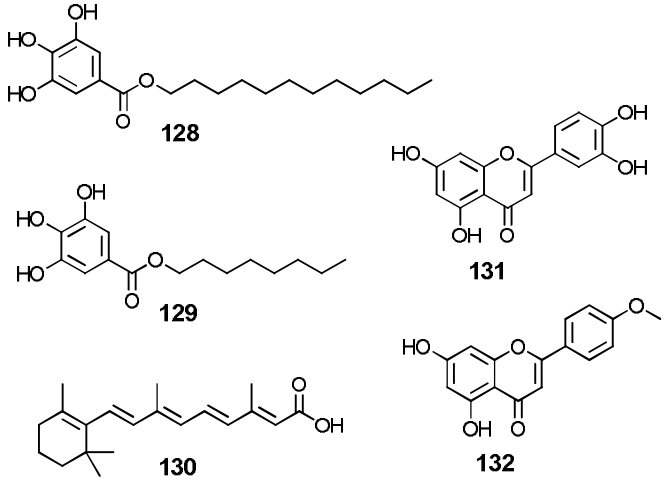


Figure 33. Natural compounds with antichlamydial activity.

3. Aims of the study

The aim of this study was to use the triterpene skeleton of an abundant birch bark extractive betulin **1** to synthesize a library of derivatives with various functional groups for bioactivity screening.

More specifically, the aims were:

- to prepare a library of betulin derivatives with diverse functional groups by modifying reactive moieties of the betulin skeleton at 3-OH, 28-OH and isopropenyl group at C22–C29
- to investigate the structure-activity relationships of betulin derivatives against intracellular pathogenic bacteria, protozoan parasites and viruses
- to synthetize a library of novel heterocyclic betulin derivatives with more hydrophilic drug-like properties and optimize their bioactivities against *Leishmania donovani*.

4. Experimental

4.1 Materials and methods

Commercially available reagents were used without further purification and all solvents were HPLC grade. Anhydrous solvents were purchased from Sigma-Aldrich. All reactions in anhydrous solvents were performed in oven-dried glassware under an inert atmosphere of anhydrous argon or nitrogen. Thin layer chromatography (TLC) was performed on E. Merck Silica Gel 60 aluminium packed plates, with visualization accomplished by UV illumination and staining with 5% H_2SO_4 in MeOH. The ^{1}H NMR spectra were measured on a Varian Mercury-VX 300 MHz or a Chemagnetics CMX 400 MHz spectrometer with chemical shifts reported as parts per million (in $CDCl_3$ at 23 °C, solvent peak at 7.26 ppm as an internal standard or in DMSO-d_6 at 23 °C, solvent peak at 2.50 ppm as an internal standard). The ^{13}C NMR spectra were obtained on a Varian Mercury-VX 75 MHz or a Chemagnetics CMX 100 MHz spectrometer with chemical shifts reported as parts per million (in $CDCl_3$ at 23 °C, solvent peak at 77.0 ppm as an internal standard or in DMSO-d_6 at 23 °C, solvent peak at 39.50 ppm as an internal standard). HPLC-MS and elemental analyses were performed to determine the purity of all tested compounds. The purity of all tested compounds was >95%. Mass spectra were measured on a Bruker Daltonik Esquire-HPLC spectrometer, with an XTerra MS RP18 column (4.6 × 30 mm, 2.5 µm) or on a JEOL JMS-AX505 spectrometer with direct input and electron ionization (EI). Elemental analyses were performed by Robertson Microlit Laboratories, Madison, NJ, USA. Melting points were obtained with a Sanyo Gallenkamp apparatus without correction. Fourier transform infrared (FTIR) spectra were recorded on a Bruker Vertex 70 spectrometer with Pike MIRacle diamond crystal or with a Bruker Equinox 55 spectrometer including IRScope II and diamond anvil.

Biological assays, cell lines and methodology are described in details in the original publications I–IV.

4.2 Results and discussion

The overall objective in the synthesis work was to produce desired test compounds for bioactivity screening. Neither synthesis method development nor optimization

of the reaction conditions was performed and in many cases yields are only moderate, but still sufficient to obtain enough pure material for bioactivity assays.

The first objective was to prepare a library of betulin derivatives with diverse functional groups by modifying the reactive moieties of the betulin skeleton at 3-OH, 28-OH and isopropenyl group between C22–C29. Details of the chemical synthesis procedures and characterization data are presented in Publications I, II and IV. The chemical structures of all synthesized simple betulin derivatives are presented in Table 13.

The second objective of the study was to synthesize a library of novel heterocyclic betulin derivatives for bioactivity screening. Details of the chemical synthesis procedures and characterization data are presented in Publication II. The chemical structures of all synthesized heterocyclic betulin derivatives are presented in Table 14.

4.2.1 Synthesis of simple betulin derivatives (I, II, IV)

Synthesis of betulin esters and related derivatives (I, II, IV)

Many of the natural terpenes or aromatic acids possess numerous bioactivities in nature, such as pheromones and semiochemicals in insects.[195] At least 30 hydroxy- and polyhydroxybenzoic acids have been reported in the last 10 years to have biological activities with potential use in pharmaceutical and agricultural products to improve human health and nutrition.[196] In addition, hydroxycinnamic acids are a large group of phenolic compounds (e.g. caffeic, *p*-coumaric and ferulic acids), which have shown antioxidant and anticancer activities.[197] Because both betulin **1** and its naturally occurring carboxylic acid derivatives have been shown to possess numerous bioactivities, it was hypothesized that it would be very interesting to try combining these kinds of moieties in a single compound. Thus, several such carboxylic acids were chosen and esterified with betulin **1** to yield a set of betulin esters and related derivatives for bioactivity screening [(Figure 34), see Table 13 for the chemical structures of the compounds **133–139**)]. Some of the aromatic betulin derivatives were initially designed for cosmetic applications as possible bioactive agents having UV-activity in a single molecule. These substances were also included in the bioactivity assays.

Ethyl chrysanthemate was hydrolyzed to produce chrysanthemic acid, which was subsequently converted with oxalyl chloride to chrysanthemoyl chloride. Finally, chrysanthemoyl chloride was reacted with **1** to afford a 1:3 mixture of *cis* and *trans* 28-*O*-chrysanthemoylbetulin **133**. The treatment of carvacrol with chloroacetic acid in an aqueous solution of sodium hydroxide gave carvacryloxyacetic acid,[198] which was esterified with **1** using titanium(IV) isopropoxide as a catalyst to produce betulinyl 28-carboxymethoxycarvacrolate **134**. Treatment of **1** with nicotinic acid using DCC as a coupling reagent gave 28-*O*-nicotinoylbetulin **135**. Cinnamic acid was converted to cinnamoyl chloride with thionyl chloride, which was subsequently esterified with **1** to give 28-*O*-cinnamoylbetulin **136**. *N*-Acetylanthranilic acid was treated with oxalyl chloride to produce *N*-acetylanthraniloyl chloride, which was

treated immediately with **1** to give 28-*O*-(*N*-acetylanthraniloyl)betulin **137**. Betulin **1** was treated with *t*-BuOK followed by addition of methyl bromoacetate to give 28-*O*-bromoacetylbetulin **138**. 3,28-Di-*O*-levulinoylbetulin **139** was obtained by esterifying of **1** with levulinic acid using PPTS as a catalyst.

Treatment of **1** with DHP produced a diastereomeric mixture of the corresponding tetrahydropyranyl ether **140**. The THP-protected betulin was subsequently acetylated to give **141**. Removal of the THP group with PPTS produced 3-*O*-acetylbetulin **142**,[22] which was treated with mesyl chloride to give 3-*O*-acetyl-28-*O*-mesylbetulin **143**.

Betulin **1** was diacetylated to **144** with excess acetic anhydride, and the resulting diacetylbetulin was converted by acidic (HBr) treatment to **145** with double-bond migration to position C18–C19. The alkene **145** was subsequently epoxidized with 3-chloroperoxybenzoic acid (*m*CPBA) to yield the intermediate **146**.

Finally, 28-*O*-acetylbetulin **147** was obtained by treating betulin **1** with 1.05 equiv of acetic anhydride. The subsequent oxidation of **147** with PCC afforded 28-*O*-acetyl-3-oxobetulin **148**.[199]

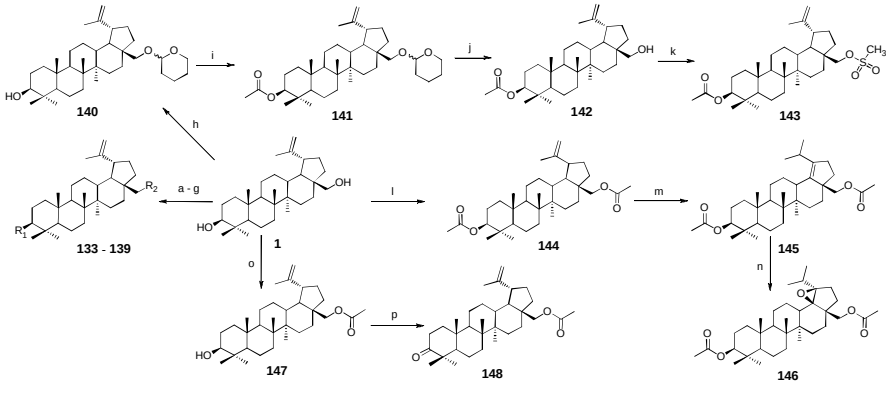


Figure 34. Synthesis of betulin esters and related betulin derivatives. Conditions: (a) (i) ethyl chrysanthemate, NaOH, MeOH-THF (2:1), 80 °C, 4 h, 91%, (ii) chrysanthemic acid, (COCl)2, CH2Cl2, rt, 6 h, 81%, (iii) chrysanthemoyl chloride, DMAP, py, 40 °C, 48 h, 63%; (b) (i) carvacrol, chloroacetic acid, NaOH, Δ, 3 h, 45%, (ii) carvacryloxyacetic acid, Ti(OPr-*i*)*4*, PhMe, Δ, 6 h, 55%; (c) nicotinic acid, DCC, DMAP, CH2Cl2, rt, 23 h, 31%; (d) (i) cinnamic acid, SOCl2, 40 °C, 2 h, 92%, (ii) cinnamoyl chloride, DMAP, pyridine, 40 °C, 22 h, 21%; (e) (i) *N*-acetylanthranilic acid, $(COCl)_2$, rt, 3 d, 99%, (ii) *N*-acetylanthraniloyl chloride, DMAP, py, 40 °C, 40 h, 25%; (f) *t*-BuOK, methyl bromoacetate, THF, 75 °C, 10 min, 15%; (g) levulinic acid, PPTS, PhMe, 175 °C, 23 h, 23%; (h) DHP, PPTS, CH_2Cl_2, rt, 2 d, 30%; (i) Ac_2O, DMAP, py, CH_2Cl_2, rt, 20 h, 95%; (j) PPTS, EtOH, rt, 14 d, 94%; (k) CH_3SO_2Cl, TEA, CH_2Cl_2, 0 °C, 2 h, 99%; (l) Ac_2O (6 equiv), DMAP, py, CH_2Cl_2, rt, 17 h, 97%; (m) HBr, Ac_2O, AcOH, PhMe, rt, 21 d, 42%; (n) *m*CPBA, Na_2CO_3, $CHCl_3$, rt, 2 h, 65%; (o) Ac_2O (1.05 equiv), DMAP, py, CH_2Cl_2, rt, 22 h, 45%; (p) PCC, CH_2Cl_2, rt, 24 h, 57%. THF = tetrahydrofuran; DCC = *N,N'*-dicyclohexylcarbodiimide; DHP = 3,4-dihydro-2*H*-pyran; PPTS = pyridinium *p*-toluenesulfonate; DMAP = 4-(dimethylamino)pyridine; py = pyridine; PCC = pyridinium chlorochromate; *m*CPBA = 3-chloroperoxybenzoic acid.

Synthesis of betulin oxidation products (I, IV)

Based on a thorough literature survey,[6] it was hypothesized that oxidized betulin derivatives may have improved bioactivity when compared to the starting material betulin. Thus, a set of betulin oxidation products was synthesized for bioactivity screening [(Figure 35), see Table 13 for chemical structures of compounds **2**, **88** and **149–155**)].

Oxidation of betulin **1** with Jones reagent ($CrO_3/H_2SO_4/H_2O$) gave betulonic acid **149**, which was subsequently methylated with $TMSCHN_2$ to give methyl betulonate **150**. The subsequent treatment of **149** with oxalyl chloride gave betulonoyl chloride,[200] which was immediately reacted with vanillin or L-aspartic acid dimethyl ester to produce vanillyl betulonate **151** and L-aspartyl amide of betulonic acid **152**, respectively.

Reduction of betulonic acid **149** with $NaBH_4$ gave betulinic acid **2**,[55] which was subsequently methylated with $TMSCHN_2$ to give methyl betulinate **153**.[201] 28-Oxyallobetulone **154** was synthesized by refluxing betulonic acid **149** with *p*-TSA.

When betulin **1** was oxidized with excess PCC, betulonic aldehyde **155**[202] was formed. When a smaller molar amount of PCC was used, betulin aldehyde **88** was isolated from a 3:1 mixture of betulonic aldehyde **155** and betulin aldehyde **88**.

The synthesis of two additional oxidized betulin derivatives 28-*O*-acetyl-3-oxobetulin **148** and dihydrobetulonic acid **158** is presented in Figure 34 and Figure 36.

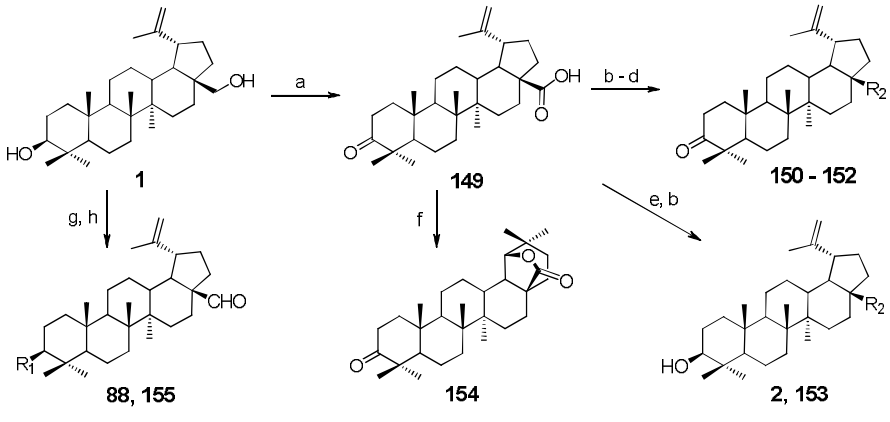


Figure 35. Synthesis of oxidized betulin derivatives. Conditions: (a) H_2CrO_4, acetone, 0 °C → rt, 21 h, 44%; (b) $TMSCHN_2$, PhMe-MeOH (3:2), rt, 40 min, 66% **152**, 89% **153**; (c) (i) $(COCl)_2$, CH_2Cl_2, rt, 22 h, 85%, (ii) vanillin, DMAP, py, 40 °C, 21 h, 20%; (d) L-aspartic acid dimethyl ester hydrochloride, TEA, CH_2Cl_2, rt, 19 h, 42%; (e) $NaBH_4$, *i*-PrOH, rt, 2.5 h, 82%; (f) *p*-TSA, H_2O, $CHCl_3$, Δ, 3 h, 19%; (g) PCC (1.8 equiv), CH_2Cl_2, rt, 40 min, 18%; (h) PCC (6 equiv), CH_2Cl_2, rt, 1 h, 82%. TMS = trimethylsilyl; TEA = triethylamine. *p*-TSA = *p*-toluenesulfonic acid monohydrate.

Synthesis of miscellaneous betulin derivatives (I, IV)

To gain a more comprehensive understanding of the structure-activity relationships (SAR) of different betulin derivatives, a set of miscellaneous betulin derivatives with diverse functional groups were synthesized (Figure 36, Table 13).

Allobetulin **156** was synthesized by refluxing a mixture of betulin **1** and *p*-TSA in chloroform.[203]

Dihydrobetulin **157** was obtained by catalytic hydrogenation of **1** in the presence of palladium on carbon as a catalyst. The subsequent oxidation of dihydrobetulin **157** with Jones reagent produced dihydrobetulonic acid **158**.

3-Deoxy-2,3-didehydrobetulin **159** was prepared by treating **1** with a mixture of DEAD, PPh_3 and 3,3-dimethylglutarimide.[204] Subsequent acetylation of **159** gave 3-deoxy-2,3-didehydro-28-*O*-acetylbetulin **160**.

Betulin **1** was oxidized with PCC to a 3:1 mixture of betulonic aldehyde **155** and betulin aldehyde **88**,[202] which was subsequently treated with excess hydroxylamine hydrochloride to produce oximes **161** and **162**.[205] Separate treatment of **161** and **162** with neat acetic anhydride gave the nitriles **163** and **164**, respectively.

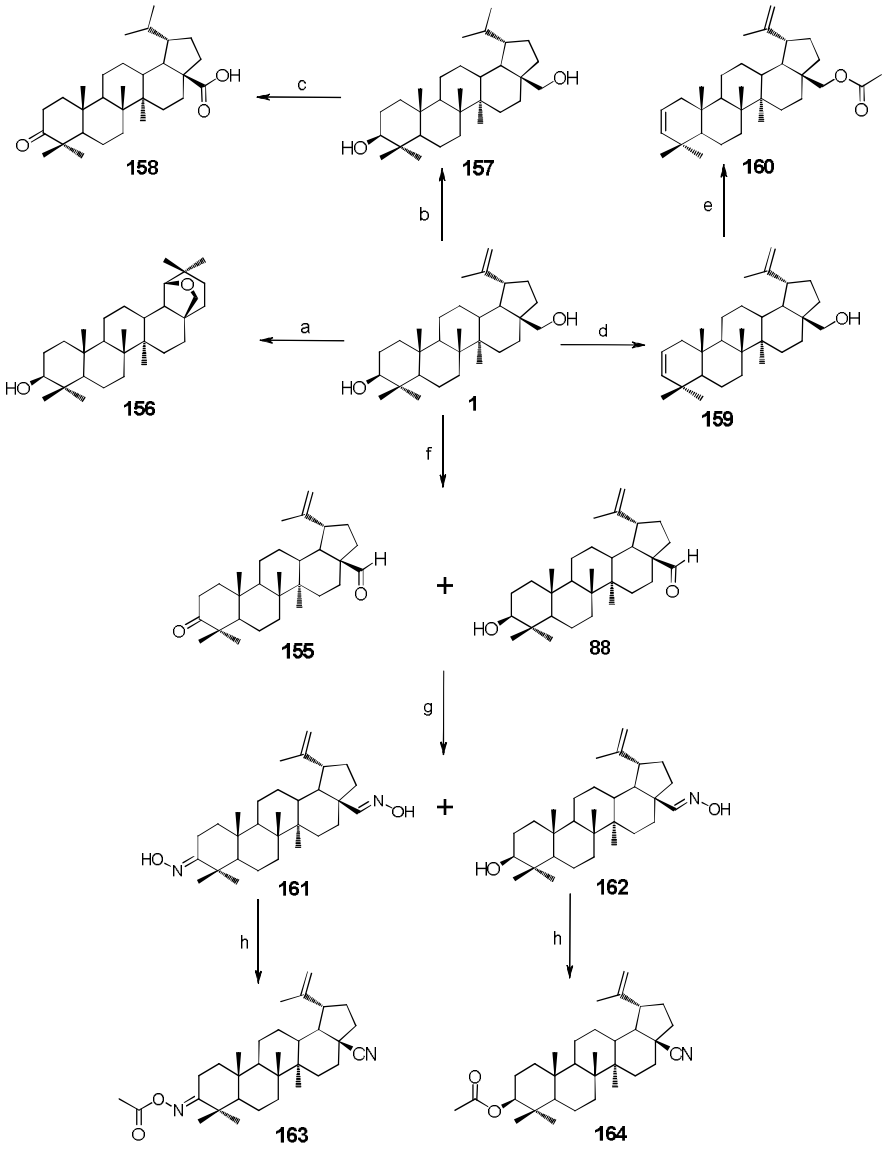
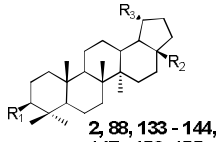
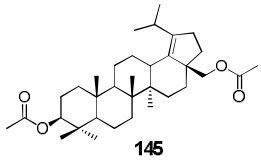


Figure 36. Synthesis of miscellaneous betulin derivatives. Conditions: (a) *p*-TSA, H_2O, $CHCl_3$, Δ, 3 h, 93%; (b) H_2, 5% Pd/C, THF-MeOH (1:2), rt, 22 h, 99%; (c) H_2CrO_4, acetone, rt, 20 h, 31%; (d) DEAD, PPh_3, 3,3-dimethylglutarimide, THF, 0 °C → rt, 24 h, 31%; (e) Ac_2O, DMAP, py, CH_2Cl_2, rt, 22 h, 81%; (f) PCC (1.8 equiv), CH_2Cl_2, rt, 40 min, **88**:**155** (1:3); (g) $NH_2OH{\cdot}HCl$, py-EtOH (1:3), 100 °C, 18 h, 10% **161**, 33% **162**; (h) Ac_2O, 120 °C, 2 h, 34% **163**, 46% **164**. DEAD = diethyl azodicarboxylate.

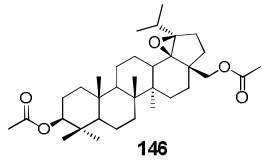
Table 13. Summary and chemical structures of the simple betulin derivatives.



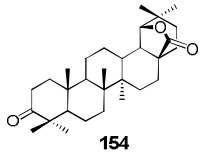
2, 88, 133 - 144, 147 - 153, 155, 157 - 164



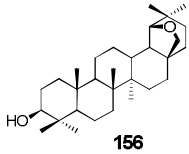
145



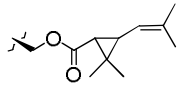
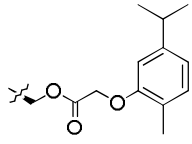
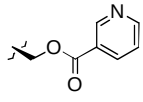
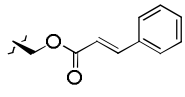
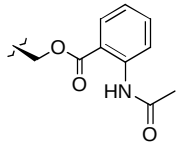
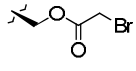
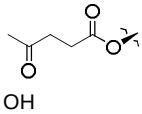
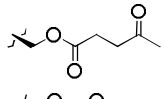
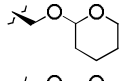
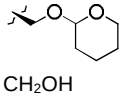
146

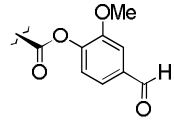
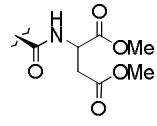


154



156

Compound	R_1	R_2	R_3
1	OH	CH_2OH	CH_3-C=CH_2
133	OH		CH_3-C=CH_2
134	OH		CH_3-C=CH_2
135	OH		CH_3-C=CH_2
136	OH		CH_3-C=CH_2
137	OH		CH_3-C=CH_2
138	OH		CH_3-C=CH_2
139			CH_3-C=CH_2
140	OH		CH_3-C=CH_2
141	OAc		CH_3-C=CH_2
142	OAc	CH_2OH	CH_3-C=CH_2
143	OAc	CH_2OMs	CH_3-C=CH_2

144	OAc	CH_2OAc	CH_3-C=CH_2
145 (*vide supra*)	OAc	CH_2OAc	CH_3-CH-CH_3
146 (*vide supra*)	OAc	CH_2OAc	CH_3-CH-CH_3
147	OH	CH_2OAc	CH_3-C=CH_2
148	O=	CH_2OAc	CH_3-C=CH_2
149	O=	CO_2H	CH_3-C=CH_2
150	O=	CO_2Me	CH_3-C=CH_2
151	O=		CH_3-C=CH_2
152	O=		CH_3-C=CH_2
2	OH	CO_2H	CH_3-C=CH_2
153	OH	CO_2Me	CH_3-C=CH_2
154 (*vide supra*)	O=	*	*
155	O=	CHO	CH_3-C=CH_2
88	OH	CHO	CH_3-C=CH_2
156 (*vide supra*)	OH	*	*
157	OH	CH_2OH	CH_3-CH-CH_3
158	O=	CO_2H	CH_3-CH-CH_3
159	-	CH_2OH	CH_3-C=CH_2
160	-	CH_2OAc	CH_3-C=CH_2
161	=NOH	CH=NOH	CH_3-C=CH_2
162	OH	CH=NOH	CH_3-C=CH_2
163	=NOAc	CN	CH_3-C=CH_2
164	OAc	CN	CH_3-C=CH_2

4.2.2 Synthesis of heterocyclic betulin derivatives (II)

It is clear from the chemical structure of betulin **1** that most of the betulin derivatives presented in Table 13 are lipophilic and thus poorly soluble in water. This may have important implications for the interpretation of the results of the bioactivity assays. Observed differences in bioactivity between different modified betulin derivatives may be explained at least partly by different partition coefficients (log *P*) of these compounds. This prompted us to convert these hydrophobic triterpenoids to more soluble and drug-like compounds by introducing a fused heterocyclic system to the lupane skeleton.

A treatment of **146** (see Figure 34 for synthesis of **146**) with pyridine *p*-toluenesulfonate (PPTS) gave a mixture (4:1) of conjugated dienes, 3,28-di-*O*-acetyllupa-12,18-diene **165a** and 3,28-di-*O*-acetyllupa-18,21-diene **165b** (Figure 37).[206] Heterocycloadducts **166–188** were synthesized by reaction of a mixture of betulin-derived dienes **165a/165b** and urazines **195**, which were generated *in situ* by

oxidation of urazoles **194** with the hypervalent iodine reagent, (diacetoxyiodo)benzene (Figure 38).[207] In addition, reactions of commercially available 4-phenyl- or 4-methyl-1,2,4-triazoline-3,5-dione with a mixture of dienes **165a/165b** gave the corresponding heterocycles **178** and **179**. The subsequent hydrolysis of heterocycles **178** and **179** with NaOH provided the diols **180** and **181** as the hydrolysis products.

In order to synthesize heterocyclic derivatives with different acyl groups at C3 and C28, the dienes **165a**/**165b** were hydrolyzed with NaOH treatment and the obtained diols **189a/189b** were subsequently acylated with various acyl chlorides. The resulting acylated dienes **190a/190b** (see Table 14 for acyl R_2 groups, betulin derivatives **182–188**) were treated with 4-methyl- or 4-phenyl-1,2,4-triazoline-3,5-dione to give the corresponding heterocycles **182–187**. In addition, heterocycloadduct **188**, with bulky R_1 and R_2 groups, was synthesized by treating **190a/190b** with *tert*-butyl-substituted *in situ*-generated urazine **195**. In all cases, the only isolable heterocyclic betulin derivatives **166–188** were those obtained from the Diels-Alder reaction between the sterically least hindered diene **165a** and urazines **195**. The [4+2] cycloaddition reactions between the mixture of dienes **165a**/**165b** and other less reactive dienophiles, such as maleic anhydride, *N*-phenylmaleimide, diethyl azodicarboxylate and dimethyl acetylenedicarboxylate were unsuccessful.

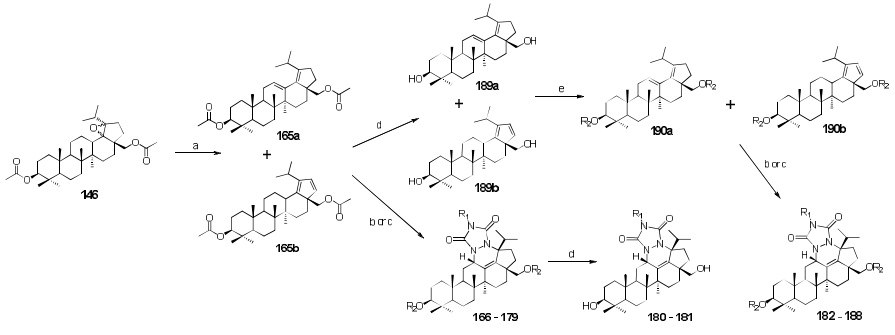
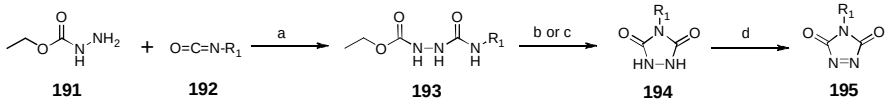


Figure 37. Synthesis of conjugated betulin dienes **165a**/**165b** and heterocyclic betulin derivatives **166**–**188**. Conditions: (a) PPTS, Ac_2O, PhMe, reflux, 3 h, 68%; (b) 4-substituted urazole **194**, $PhI(OAc)_2$, CH_2Cl_2, THF, rt, 20 h; (c) 4-methyl- or 4-phenyl-1,2,4-triazoline-3,5-dione, CH_2Cl_2, THF, rt, 20 h; (d) NaOH, MeOH, THF, rt, 18 h, **189a**/**189b** 85%; (e) R_2Cl, DMAP, py, CH_2Cl_2, 40 °C, 20 h.

The dienophilic urazines **195** and their corresponding urazole precursors **194** were synthesized as follows (Figure 38).[208] Reaction of a stoichiometric amount of ethyl hydrazinecarboxylate **191** and isocyanates **192** yielded carbethoxysemicarbazides **193**. When the substituent R_1 of the carbethoxysemicarbazides **193** was aromatic (see Table 14 for R_1 groups, betulin derivatives **166–174**), cyclization to the corresponding urazoles **194** was carried out by heating in aqueous KOH solution. Acidification (HCl) of the basic solution afforded urazoles **194**. When the substituent R_1 was aliphatic (see Table 14 for R_1 groups, betulin derivatives **175**, **176** and **188**),

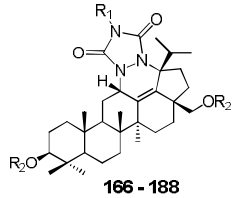
the cyclization reaction was carried out by sodium ethoxide in refluxing ethanol. Acidification (HCl) of the basic solution afforded urazoles **194**.



191 **192** **193** **194** **195**

Figure 38. Synthesis of urazines **195**. Conditions: (a) PhMe, rt, 2 h to 80 °C, 2 h, 90–99%; (b) (i) 4 M KOH in H_2O, 70 °C, 90 min, (ii) 37% HCl (see Table 14 for R_1 groups, betulin derivatives **166–174**) 37–88%; (c) (i) Na, EtOH, 85 °C, 24 h, (ii) 1.25 M HCl in EtOH (see Table 14 for R_1 groups, betulin derivatives **175**, **176** and **188**), 94–96%; (d) in situ $PhI(OAc)_2$, CH_2Cl_2, THF.

Table 14. Summary and chemical structures of heterocyclic betulin derivatives.



166 - 188

Compound	R_1	R_2
166	Bn	Ac
167	3-MeO-Ph	Ac
168	4-F-Ph	Ac
169	3-NO_2-Ph	Ac
170	4-Cl-Ph	Ac
171	4-Ac-Ph	Ac
172	1-naphthyl	Ac
173	indan-5-yl	Ac
174	1,3-dioxol-5-yl	Ac
175	*n*-Bu	Ac
176	Et	Ac
177	H	Ac
178	Ph	Ac
179	Me	Ac
180	Ph	H
181	Me	H
182	Me	COEt
183	Me	COPr
184	Me	COi-Pr
185	Me	COcHex
186	Me	COPh
187	Ph	COPh
188	*t*-Bu	COcHex

4.2.3 SAR studies: *Semliki Forest virus* (I)

In the primary screen, the inhibition activity of 33 simple betulin derivatives and 18 heterocyclic betulin derivatives was assayed against SFV at 50 µM concentration. In addition, the cytotoxicity of the compounds to a Huh-7 cell line was determined (Figure 39, Figure 40). Based on the results from the primary screen, a total of 18 (marked by * in Figure 39 and Figure 40) promising compounds having good antiviral activity combined with low cytotoxicity to the Huh-7 cell line were selected for determination of IC_{50} values (Figure 41).

In the set of simple betulin derivatives, only three derivatives having an unmodified hydroxy group at C3 showed good activity combined with low cytotoxicity. The starting material betulin **1** inhibited SFV replication with an IC_{50} value of 46 µM, and betulinic acid **2** and 28-*O*-tetrahydropyranylbetulin **140** displayed improved activity with IC_{50} values 15 µM and 17 µM, respectively. Five compounds out of seven having an acetyl group at C3 performed well, 3,28-di-*O*-acetylbetulin **144** having the best SFV inhibition with an IC_{50} value of 9 µM. Compounds having a carbonyl group at C3 also performed well, with four out of seven compounds showing good performance, of which betulonic acid **149** displayed best activity with an IC_{50} value of 13 µM. For comparison, a standard SFV inhibitor, ribavirin, has an IC_{50} value of 95 µM.

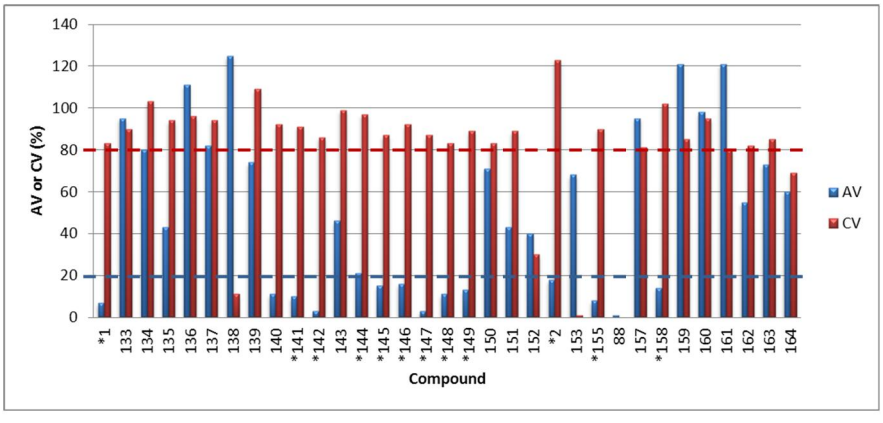


Figure 39. Antiviral effect of simple betulin derivatives against SFV in the primary screen at 50 µM. For chemical structures, see Table 13. Compounds marked (*), yielding <20% (dashed blue line) remaining viral replication (AV) and >80% (dashed red line) cell viability (CV), were selected for determination of IC_{50} values.

In the subgroup of heterocyclic betulin derivatives, all compounds that displayed good inhibition combined with low cytotoxicity had acetyl R_2 groups at C3 and C28 and a bulky R_1 substituent at N-4, except for compound **183** (IC_{50} = 30 µM), which had propanoyl groups at C3 and C28 and methyl at N-4 (Figure 40). From the group of betulin derivatives having bulky aromatic R_1 moiety at N-4, benzyl **166**

(IC_{50} = 36 µM), phenyl **178** (IC_{50} = 20 µM) as well derivatives having an electron-withdrawing group on the aromatic ring, performed well: 3-nitrophenyl **169** (IC_{50} = 23 µM) and 3-chlorophenyl **170** (IC_{50} = 22 µM) showed good activity.

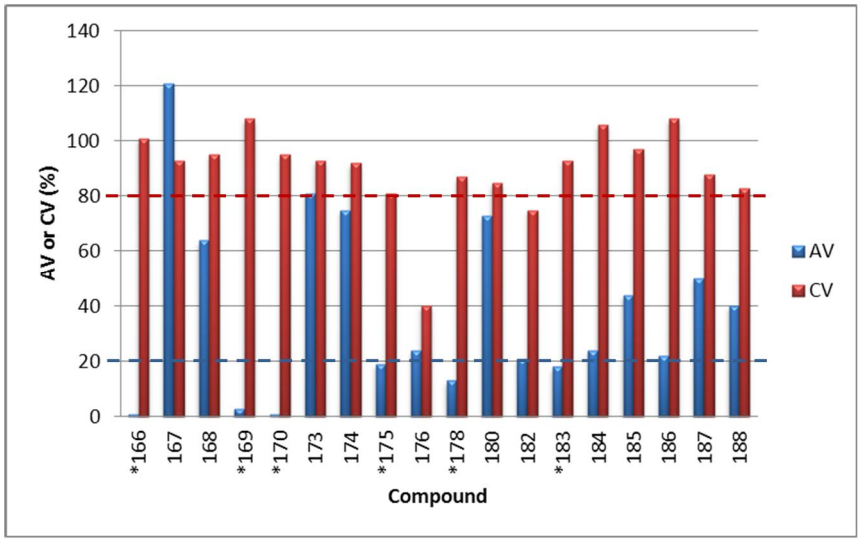


Figure 40. Antiviral effect of heterocyclic betulin derivatives against SFV in the primary screen at 50 µM. For chemical structures of compounds see Table 14. Compounds marked (*), yielding <20% (dashed blue line) remaining viral replication (AV) and >80% (dashed red line) cell viability (CV), were selected for IC_{50} determination.

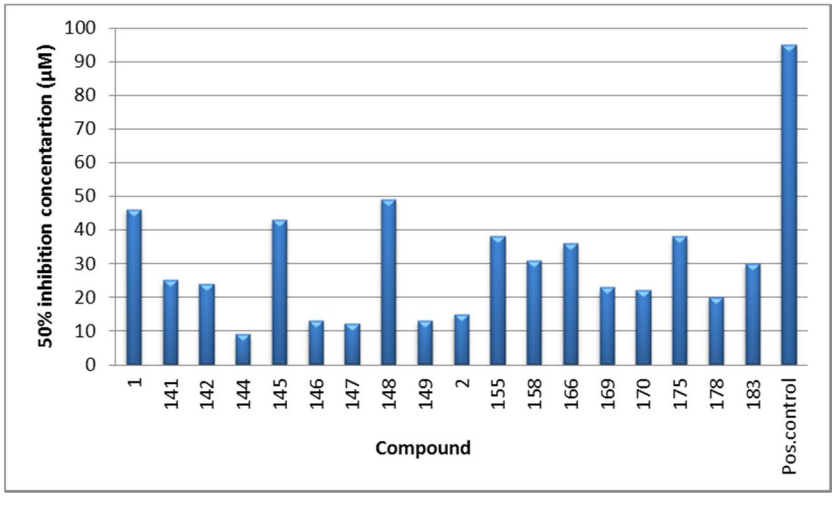


Figure 41. IC_{50} values of betulin-derived compounds against *Semliki Forest virus* (SFV). Positive control = ribavirin.

It is reported that antiviral inhibition of betulin-derived compounds takes place during the early or late stages of the virus life cycle.[24,204] On the other hand, it can be speculated that the inhibitory effect of the SFV inhibitor nucleoside analogue 3′-amino-3′-deoxyadenosine (3′-NH_2-3′-dAdo) occurs during the replication phase.[209] Thus, synergism of the three representative and potent betulin derivatives 28-*O*-tetrahydropyranylbetulin **140**, betulinic acid **2** and heterocycloadduct **178** was assayed in combination with (3′-NH_2-3′-dAdo). The best synergism was achieved by combination of 5 µM 3′-NH_2-3′-dAdo and 2 µM of the heterocycle **178**, showing an interaction index (I) value of 0.16.

Finally, the three compounds 28-*O*-tetrahydropyranylbetulin **140**, betulinic acid **2** and heterocycloadduct **178** were assayed against another alphavirus Sindbis (SIN). All three compounds were also effective against SIN, with lower IC50 values when compared to SFV, betulinic acid **2** having the best activity at a IC_{50} value of 0.5 µM.

4.2.4 SAR studies: *Leishmania donovani* (II)

Initial antileishmanial activity of heterocyclic compounds **166–181** was screened against *Leishmania donovani* axenic amastigotes at 50 µM on a microplate assay with alamarBlue as previously described.[210] The screening results of the first batch with derivatives having acetyl R_2 groups at C3 and C28 showed that small R_1 substituents at the nitrogen atom gave the best antileishmanial activity (Figure 42). Derivatives **176** (R_1 = Et; inhibition = 88%), **177** (R_1 = H; inhibition = 88%) and **179** (R_1 = Me; inhibition = 98%) displayed the best activity at 50 µM.

Therefore, a second batch of heterocyclic betulin derivatives **182–186** was synthesized such that the acyl groups at R_2 were altered and the R_1 group was kept as methyl. In addition, two cycloadducts **187** and **188** with bulky R_1 and R_2 groups were synthesized. Also in this case, the screening results showed that small acyl R_2 groups at C3 and C28 (**182** R_2 = Et; inhibition = 93%) were more active than derivatives with bulky acyl groups.

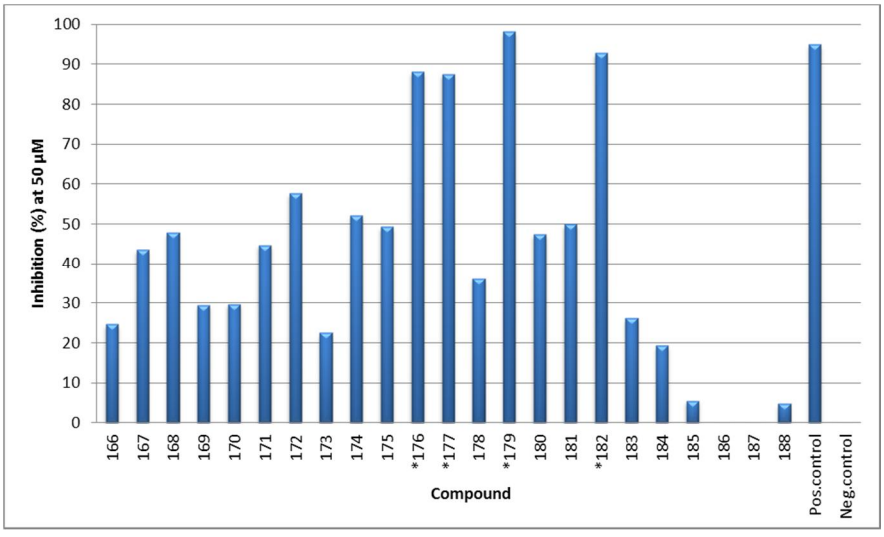


Figure 42. Antileishmanial activity of heterocyclic betulin derivatives against *L. donovani* axenic amastigotes. Determined at 50 µM concentration of betulin derivative with the exception of compounds **183** and **184**, which were used at 15 µM due to their poor solubility. The most potent compounds are marked (*). Pos. control = amphotericin B (1 µM). Neg. control = culture medium and DMSO. See Table 14 for chemical structures.

The most potent derivatives (**176**, **177**, **179** and **182**, Figure 42) with a small acyl group at R_2 and small substituent R_1 (Me or Et) at the nitrogen on the initial micro-plate assay were selected for further investigation: determination of GI_{50} on axenic amastigotes, cytotoxicity for THP-1 macrophages, and antileishmanial activity against *L. donovani* amastigotes growing inside macrophages (Figure 43). The methyl derivative **179** had the best GI_{50} value at 8.9 µM. However, it showed cyto-toxicity against macrophage cell line THP-1. Other compounds showed slightly higher but very similar GI_{50} values and displayed cytotoxicity only at highest 50 µM concentration.

Finally, the compounds were tested for antileishmanial activity against L. donovani amastigotes growing inside macrophages at concentrations that showed less than 40% cytotoxicity to the macrophages. When compared to initial screening with axenic amastigotes at 50 µM, compound **182** showed similar strong activity against L. donovani amastigotes growing inside macrophages. At 25 µM, **176**, **177** and **182** still showed good activity and the relatively cytotoxic derivative 179 showed good inhibition even at 12.5 µM concentration.

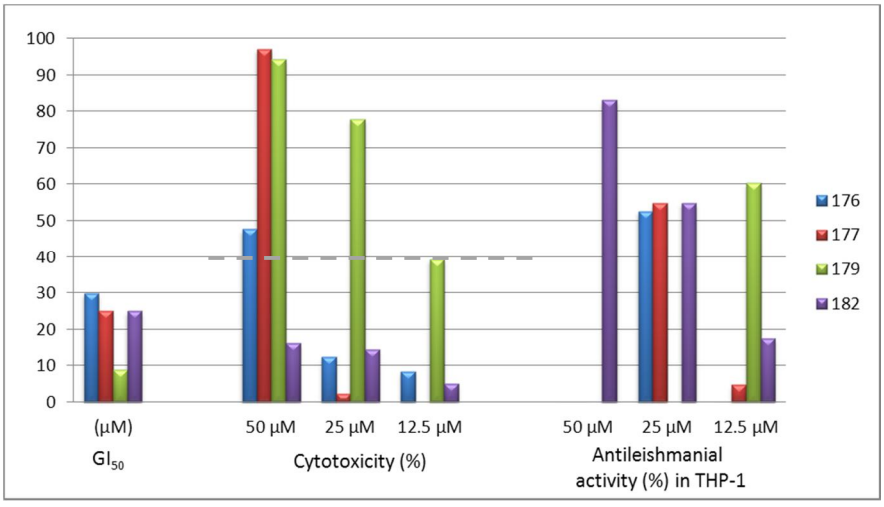


Figure 43. GI_{50}, cytotoxicity to THP-1 cells as well as antileishmanial activity of **176**, **177**, **179** and **182** against *L. donovani* amastigotes growing in THP-1 cells at concentrations showing <40% (dashed line) cytotoxicity to THP-1 cells.

4.2.5 SAR studies: *Leishmania donovani* (III)

Similarly to Publication II, the antileishmanial activities of simple betulin derivatives at 50 µM were screened using a fluorescent viability microplate assay with *L. donovani* axenic amastigotes (Figure 44).

Betulin **1** showed moderate antileishmanial activity against *L. donovani* axenic amastigotes, showing 35% inhibition at 50 µM in a microplate assay. Acetylation, esterification or etherification of the hydroxy groups at C3 or C28 in most cases retained antileishmanial activity. Only 28-*O*-(*N*-acetylanthraniloyl)betulin **137** and 28-*O*-bromoacetylbetulin **138** showed improved leishmanicidal activity, compared with **1**.

Oxidized betulin derivatives seem to have similar or increased antileishmanial activity when compared to **1**. Furthermore, 28-*O*-Acetyl-3-oxobetulin **148**, methyl betulonate **150**, betulonic aldehyde **155** and betulinic acid **2** all showed moderate 40% inhibition at 50 µM. L-Aspartyl amide of betulonic acid **152**, betulin aldehyde **88** and dihydrobetulonic acid **158** displayed improved antileishmanial growth inhibition between 64–72%, and betulonic acid **149** had remarkable antileishmanial activity with 98% inhibition at 50 µM.

In addition, oxime derivatives **161** and **162** and the betulin derivative **164** SAL-II-141 with a nitrile group at C28 showed increased inhibition (63–73%) at 50 µM.

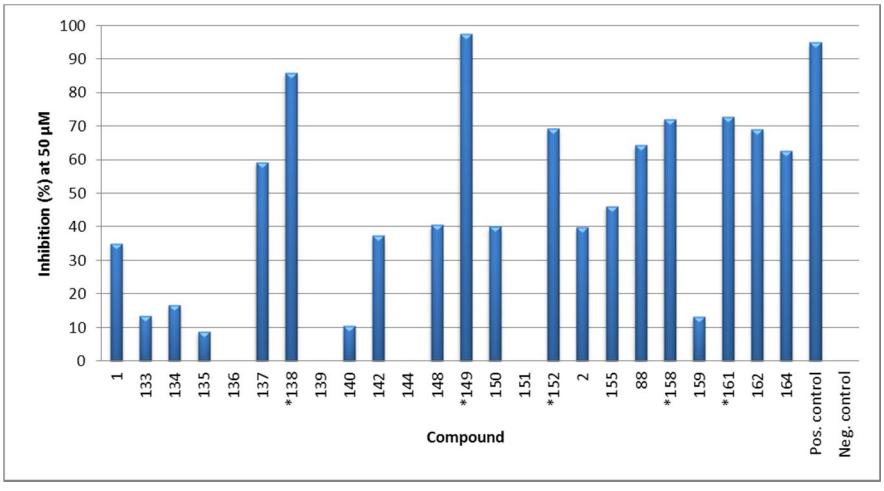


Figure 44. Antileishmanial activity of simple betulin derivatives at 50 µM against *L. donovani* axenic amastigotes. The most potent compounds are marked (*). Pos. control = amphotericin B (1 µM). Neg. control = culture medium and DMSO. See Table 13 for chemical structures.

The most potent betulin derivatives (**138**, **149**, **152**, **158** and **161**) from initial screening were selected for further investigation: GI_{50} values, cytotoxicity to macrophage THP-1 cell line, as well as activity against *L. donovani* growing inside macrophages were determined (Figure 45).

Betulonic acid **149** showed the best GI_{50} value of 14.6 µM. However, it also showed cytotoxicity against the THP-1 cell line at all test concentrations. The L-aspartyl amide derivative **152** displayed good GI_{50} values of 21.2 µM with no cytotoxicity to the THP-1 cell line. The oxime derivative **161** also displayed good GI_{50} values of 22.8 µM, but also increased general cytotoxicity. 28-*O*-bromoacetylbetulin **138** had a moderate GI_{50} value of 34.9 µM with no toxicity to the THP-1 cells. Dihydrobetulonic acid **158** had a moderate GI_{50} value (56.0 µM), but also increased general toxicity. Compounds **138** and **152** were moderately active (inhibition >50%) against *L. donovani* amastigotes growing in THP-1 cells at 50 µM. At 12.5 µM concentration, only oxime derivative **161** showed moderate inhibition, whereas the rest of the compounds were relatively inactive.

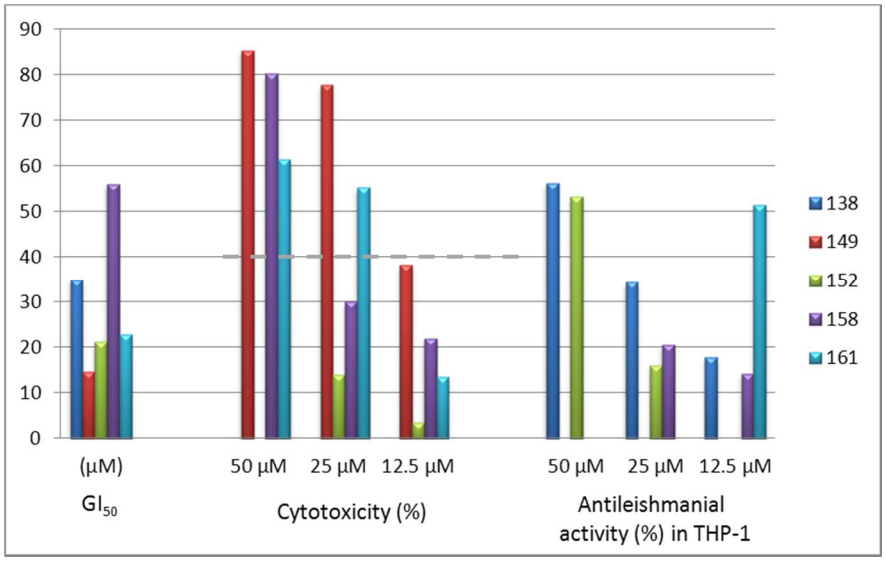


Figure 45. GI_{50}, cytotoxicity to THP-1 cells, and antileishmanial activity of **138**, **149**, **152**, **158** and **161** against *L. donovani* amastigotes growing in THP-1 cells at concentrations showing <40% (dashed line) cytotoxicity to THP-1 cells.

4.2.6 SAR studies: *Chlamydia pneumoniae* (IV)

A total of 32 betulin derivatives, including 5 heterocyclic adducts (compounds **176**, **178**, **182**, **185** and **187**), were screened for activity against *Chlamydia pneumoniae* at 1 µM concentration (Figure 46). In addition, the cytotoxicity of the compounds was screened at 1 and 8 µM concentration against HL cells (only the results of the 8 µM assay are presented in Figure 46). Five derivatives, methyl betulonate **150**, dihydrobetulonic acid **158**, betulin-3,28-dioxime **161**, betulin-28-oxime **162** and 3-acetoxymebetulinyl-28-nitrile **164** showed high (>70% growth inhibition) antichlamydial activity.

These five compounds were selected for dose–response experiments to determine MIC concentrations. Only compound **161** was able to completely eradicate *C. pneumoniae* from the HL cells, having a MIC value of 1 µM. Furthermore, a 50% inhibition activity of betulin-3,28-dioxime **161** was achieved with a nanomolar concentration of 290 nM or 125 nM depending on the assay method. For comparison, one of the most potent antichlamydial compounds known, rifampicin **126**, displayed a MIC value of 12 nM.

Chlamydia stimulates the production of host cytosolic phospholipase A_2 ($cPLA_2$). Preventing this enzyme from functioning is known to cause inhibition of chlamydial growth.[211] Thus, the effect of ten betulin derivatives was assayed against the PLA_2 enzyme of HL cells. Betulin **1** showed PLA_2 inhibition of 62.5% at 0.5% (w/v) concentration. The potent antichlamydial derivatives betulin-3,28-dioxime **161** and betulin-28-oxime **162** displayed the highest PLA_2 inhibition of 75% at

0.5% (w/v) concentration. Despite good PLA_2 inhibition activity of compounds **161** and **162**, no clear correlation was observed between antichlamydial activity and PLA_2 inhibition of the betulin derivatives.

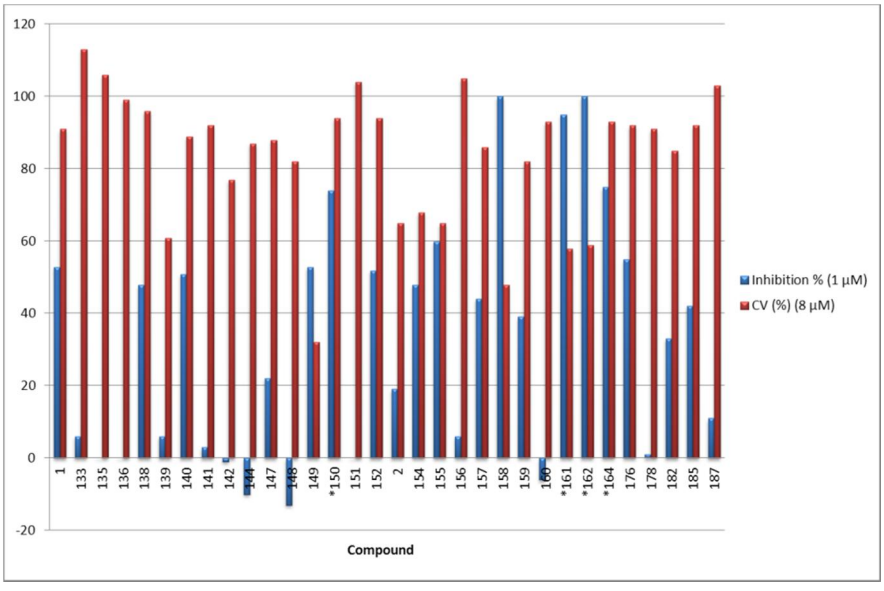


Figure 46. Antichlamydial activity of betulin derivatives at 1 µM against *Chlamydia pneumoniae* and host cell viability at 8 µM. The most potent compounds are marked (*). See Table 13 and Table 14 for chemical structures.

4.2.7 Cytotoxicity of betulin compounds (I, II, III, IV)

The cytotoxic effects of the test compounds to cell viability were evaluated with two methods using three cell lines (Figure 47, Figure 48). The methods included an ATP assay using HL and Huh-7 cell lines, and an alamarBlue assay using an THP-1 cell line. In general, betulin compounds were well tolerated by the test cell lines at a concentration of 8 µM or 50 µM. However, in some cases there were clear differences between the cell line tolerances to certain betulin derivatives. For example, **138** was found to be toxic to the Huh-7 cell line, but nontoxic to the other two cell lines tested. Conversely, betulonic acid **149** and dihydrobetulonic acid **158** were nontoxic to the Huh-7 cell line, but relatively toxic to HL and THP-1 cells. These kinds of toxicity differences should be taken into an account when testing cytotoxicity, and several cell lines should be used to exclude possible false non-toxic compounds.

The cytotoxicity of the heterocyclic betulin derivatives was also relatively low (Figure 48). However, in this case, not all derivatives were tested extensively with all three cell lines. Unfortunately, the most active heterocyclic betulin derivatives against *Leishmania donovani*, i.e. **48** and **50**, were also significantly toxic to the THP-1 cell line.

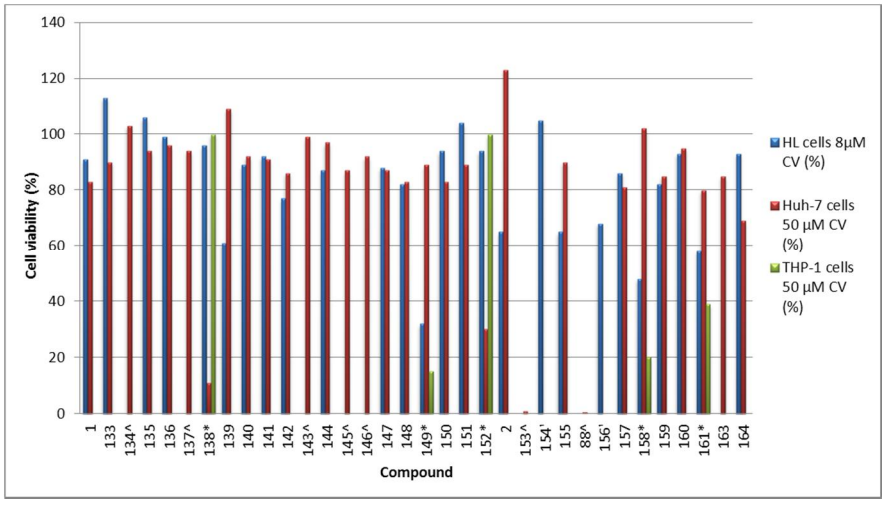


Figure 47. Cytotoxicity of simple betulin derivatives to HL cells (8 µM), Huh-7 cells (50 µM) and THP-1 cells (50 µM). Bars represents cell viability after treatment with the test compound. Compounds marked (^) were not assayed with HL cells and compounds marked (') were not assayed with Huh-7 cells. Toxicity to THP-1 cells was assayed only with compounds marked (*).

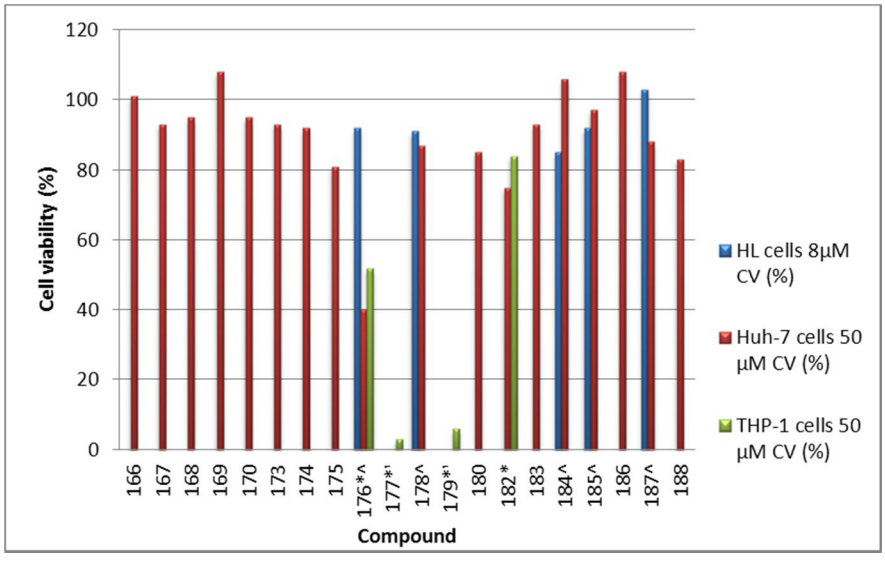


Figure 48. Cytotoxicity of heterocyclic betulin derivatives to HL cells (8 µM), Huh-7 cells (50 µM) and THP-1 cells (50 µM). Bars represent cell viability after treatment with the test compound. Toxicity to HL cells was assayed only with compounds marked (^) and toxicity to THP-1 cells tested only with compounds marked (*). Compounds marked (') were not tested with Huh-7 cells.

4.3 Conclusion

New inexpensive therapeutic agents are urgently needed due to increasing drug resistance of disease agents and to ensure the availability of needed drugs in the developing countries. In this study, two sets of betulin derivatives were synthesized and screened for their antiviral, antileishmanial and antibacterial activities.

The first set of 34 compounds included relatively easily synthesizable betulin derivatives, such as esters, various oxidation products (e.g. carboxylic acids, ketones and aldehydes); and miscellaneous betulin derivatives such as oximes and nitriles or compounds where hydroxyl group of ring A was cleaved or double bond in betulin isopropenyl group was hydrogenated.

The second set of 23 compounds consisted of novel heterocyclic betulin derivatives in which the triazole ring is fused by the Diels-Alder reaction to the lupane skeleton of betulin. First, mixture of conjugated dienes 3,28-di-*O*-acetyllupa-12,18-diene **165a** and 3,28-di-*O*-acetyllupa-18,21-diene **165b** was prepared in four steps. Formed mixture was reacted with urazines, which were generated *in situ* by oxidation of urazoles with the hypervalent iodine reagent, (diacetoxyiodo)benzene. Diversity of heterocyclic betulin derivatives was increased by using different urazole intermediates (which were synthesized in two steps starting from ethyl hydrazinecarboxylate and various isocyanates) and different ester groups at 3-OH and 28-OH in the betulin skeleton.

In the antiviral assays against SFV, betulin acetyl esters performed well; for example 28-*O*-tetrahydropyranylbetulin **140** and 3,28-di-*O*-acetylbetulin **144** displayed IC_{50} values of 17 µM and 9 µM, respectively. Additionally, oxidation products betulinic acid **2** and betulonic acid **149** displayed good activity, with IC_{50} values of 13 µM and 15 µM, respectively. In the subgroup of heterocyclic betulin derivatives, compounds having acetyl R_2 groups at C3 and C28 and an aromatic group R_1 at N-4 displayed generally good activity. Derivative **178** (IC_{50} = 20 µM) with a phenyl at R_1, as well as derivatives having an electron-withdrawing group on the aromatic ring performed well.

In the antileishmanial studies, L-aspartyl amide derivative **152** displayed a good GI_{50} value of 21.2 µM with no cytotoxicity to the THP-1 cell line. Furthermore, the dioxime derivative **161** and 28-*O*-bromoacetylbetulin **138** displayed relatively good GI_{50} values. Unfortunately, betulonic acid **149**, while having good antileishmanial activity, also showed cytotoxicity against the THP-1 cell line. The heterocyclic betulin derivatives with small R_1 (Me or Et) substituents at the nitrogen atom of the triazole moiety combined with the sterically least hindered acyl groups at the R_2 positions in the betulin skeleton promoted antileishmanial activity. The most potent derivatives against *Leishmania donovani* amastigotes were compounds **176**, **177**, **179** and **182**, compound **179** having the best GI_{50} value of 8.9 µM.

Five betulin derivatives, methyl betulonate **150**, dihydrobetulonic acid **158**, betulin-3,28-dioxime **161**, betulin-28-oxime **162** and 3-acetoxymebetulinyl-28-nitrile **163**, showed high (>70% growth inhibition) antichlamydial activity against *Chlamydia pneumoniae*. The most potent derivative, betulin-3,28-dioxime **161**, displayed 50%

inhibition activity at a nanomolar concentration of 290 nM or 125 nM depending on the assay method.

We have shown that, by simple chemical modifications, the antibacterial, antiprotozoal and antiviral activities of the ubiquitous naturally occurring triterpene, betulin, can be improved considerably. It is possible to derive potent antimicrobial compounds with low micromolar or even nanomolar inhibition values. In most cases, a single derivative showed very different activities at different bioassays. For example, several *O*-betulinyl acetates were active against SFV, but totally inactive against *L. donovani* and *C. pneumoniae*. Thus, it is also quite impossible to predict the general activity of a certain derivative against other microbial or viral strains. For example, several potent antichlamydial compounds were totally inactive against other bacterial and fungal species such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes, Enterococcus faecalis* and *Candida albicans* (unpublished data, manuscript in preparation).

Further studies are needed to develop more potent betulin derivatives with antimicrobial properties, improved water solubility, and zero cytotoxicity to host cell lines. Moreover, thorough early ADME (absorption, distribution, metabolism and excretion), biological mechanism and animal studies are needed to evaluate the potency of betulin-derived compounds *in vivo*.

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PUBLICATION I

Betulin-derived compounds as inhibitors of alphavirus replication

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Betulin-Derived Compounds as Inhibitors of Alphavirus Replication

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This paper describes inhibition of Semliki Forest virus (SFV) replication by synthetic derivatives of naturally occurring triterpenoid betulin (**1**). Chemical modifications were made to OH groups at C-3 and C-28 and to the C-20−C-29 double bond. A set of heterocyclic betulin derivatives was also assayed. A free or acetylated OH group at C-3 was identified as an important structural contributor for anti-SFV activity, 3,28-di-*O*-acetylbetulin (**4**) being the most potent derivative (IC_{50} value 9.1 μM). Betulinic acid (**13**), 28-*O*-tetrahydropyranylbetulin (**17**), and a triazolidine derivative (**41**) were also shown to inhibit Sindbis virus, with IC_{50} values of 0.5, 1.9, and 6.1 μM, respectively. The latter three compounds also had significant synergistic effects against SFV when combined with 3′-amino-3′-deoxyadenosine. In contrast to previous work on other viruses, the antiviral activity of **13** was mapped to take place in virus replication phase. The efficacy was also shown to be independent of external guanosine supplementation.

The genus *Alphavirus* consists of enveloped viruses with a single-stranded positive-sense RNA genome of approximately 11.5 kilobases. These widely distributed viruses infect avian and mammalian hosts, spreading in nature by using *Aedes* sp. mosquitoes as vectors. In vertebrate cells, the infection is acute and cytopathic; most of the amplification occurs in small rodents, whereas humans and other larger mammals are usually dead-end hosts.[1] One of the most prominent human epidemics caused by alphaviruses was the recent Chikungunya outbreak, which occurred at different sites surrounding the Indian Ocean in 2006 and involved more than 1.5 million cases.[2] In 2007, an outbreak of 205 confirmed cases in northern Italy was also reported, raising awareness of the potential for rapid transmission of tropical arthropod-borne diseases to temperate areas.[3,4] Chikungunya and other alphaviruses found on the Eurasian and African continents primarily cause polyarthritis, accompanied by rash-like symptoms and myalgia.[5] In contrast, viruses of the same genus found on the American continents, such as Western, Eastern, and Venezuelan equine encephalitis viruses, are primarily associated with small epidemics of encephalitis in both humans and domestic animals.[6] Even though alphaviruses are considered a potential cause of both economic loss and human suffering and mortality, currently available pharmacotherapy for alphavirus-borne diseases is limited to relatively inefficient ribavirin and interferon combinations and to symptomatic relief.

Betulin **1** (lup-20(29)-ene-3β,28-diol), a pentacyclic, lupane-type triterpene, is a major constituent of the bark of white birches (*Betula* sp.) that are found in abundance in northern temperate zones. A more water-soluble compound, betulinic acid, is also present in birch bark in minor quantities (0.3% of dry weight in *B. pendula*[7]). However, the distribution of these compounds in nature is not limited to this genus but covers a variety of plant species, including well-known medicinal plants on most continents.[8−10] The spectrum of naturally occurring betulin-related compounds also includes betulonic acid, 3-*O*-sulfates,[11] 28-*O*-glycosides, and esters such as nicotinates and caffeates.[12,13]

Due to the ease of isolation in large quantities and accessibility for chemical modification of the hydroxy groups at positions C-3 and C-28, betulin derivatives have been investigated for a variety of applications. Betulin by itself is quite inactive in pharmaceutical applications; however, it can be oxidized with the Jones' reagent (CrO_3/aq. H_2SO_4) to betulonic acid. Betulonic acid, in turn, can be reduced with $NaBH_4$ selectively to betulinic acid,[14] which is an important and pharmaceutically more active precursor for further modifications. The chemistry and therapeutic potential of betulin-derived compounds have been most widely studied for use against certain cancers and human immunodeficiency virus type 1 (HIV-1), and different betulin derivatives are currently undergoing clinical trials for both indications.[15] In anti-HIV therapy, two separate mechanisms of action have been proposed, involving both early and late stages in the virus infection cycle. A C-3-substituted betulinic acid derivative, bevirimat [3-*O*-(3′,3′-dimethylsuccinyl)-betulinic acid], has been shown to inhibit HIV-1 maturation by a previously undescribed mechanism, i.e., by blocking the processing of Gag polyprotein between the capsid and p2 spacer sequences and leading to aberrant maturation and decreased infectivity of the virions.[16] Phase II clinical trials with bevirimat were positively reported in 2007, indicating favorable pharmacokinetics and preliminary data on efficacy in patients with HIV.[17,18] On the other hand, the C-28-substituted aminoalkyl betulin derivatives ICH9564 and A43-D inhibit HIV-1 entry by targeting the V3 loop of HIV gp120.[19]

Beyond antiretroviral therapy, reports on the antiviral properties of betulin derivatives mainly involve the effects of naturally occurring derivatives on DNA viruses. As such, betulin alone and in combination with aciclovir has been reported to inhibit *Herpes simplex* virus types I and II (HSV I and II), showing approximately 10-fold increased sensitivity to HSV-I when compared to HSV-II.[20] Betulinic acid and betulonic acid are also active against HSV, as well as against influenza A and ECHO-6 picornavirus. Betulinic acid was reported to be more potent in the two former cases and betulonic acid in the latter case.[21] Furthermore, the naturally occurring 3-*epi*-betulinic acid 3-*O*-sulfate was recently demonstrated to inhibit HSV, influenza A, and respiratory syncytial virus (RSV).[22] A small set of synthetic C-3- and C-28-substituted betulin derivatives has also been assayed against HSV and influenza A, emphasizing the potential role of C-28-substitution in antiviral activity.[23]

In the present study, anti-alphaviral properties of 51 betulin derivatives were assayed against Semliki Forest virus (SFV), which is an extensively studied member of the *Alphavirus* genus. Another

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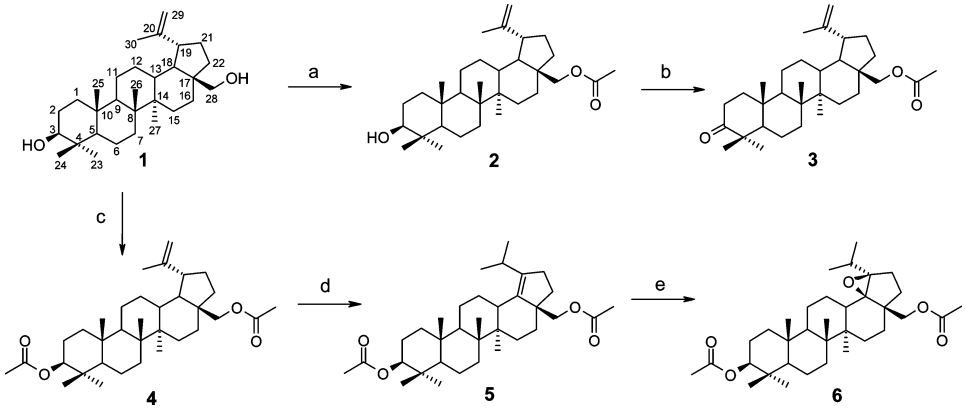
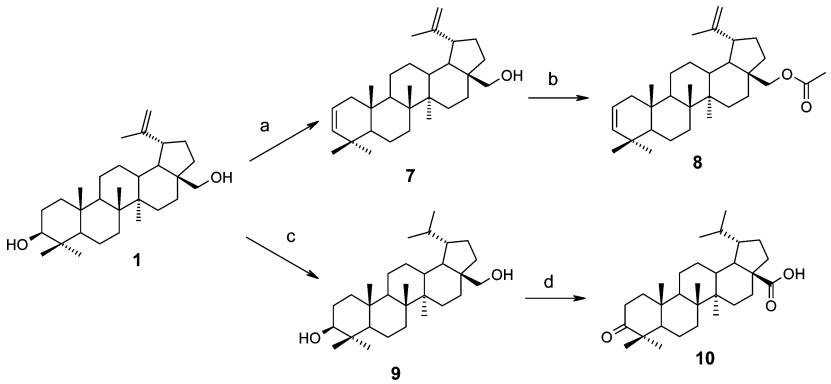
Scheme 1. Synthesis of Compounds **2**−**6**[a]



[a] Conditions: (a) Ac_2O (1.05 equiv), DMAP, py, CH_2Cl_2, rt, 22 h, 45%; (b) PCC, CH_2Cl_2, rt, 24 h, 57%; (c) Ac_2O (6 equiv), DMAP, py, CH_2Cl_2, rt, 17 h, 97%; (d) HBr, Ac_2O, AcOH, PhMe, rt, 21 days, 42%; (e) *m*CPBA, Na_2CO_3, $CHCl_3$, rt, 2 h, 65%. DMAP = 4-(dimethylamino)pyridine; py = pyridine; PCC = pyridinium chlorochromate; *m*CPBA = 3-chloroperoxybenzoic acid.

Scheme 2. Synthesis of Compounds **7**−**10**[a]



[a] Conditions: (a) DEAD, PPh_3, 3,3-dimethylglutarimide, THF, 0 °C → rt, 24 h, 31%; (b) Ac_2O, DMAP, py, CH_2Cl_2, rt, 22 h, 81%; (c) H_2, 5% Pd/C, THF−MeOH (1:2), rt, 22 h, 99%; (d) H_2CrO_4, acetone, rt, 20 h, 31%. DEAD = diethyl azodicarboxylate; THF = tetrahydrofuran.

member, Sindbis virus (SIN), was also shown to be sensitive to selected betulin-derived compounds. The anti-SFV activity of betulinic acid was mapped into the replication phase of the virus, and the derivatives of different structural subgroups were shown to exhibit strong synergism in SFV inhibition when combined with 3′-amino-3′-deoxyadenosine.

Results and Discussion

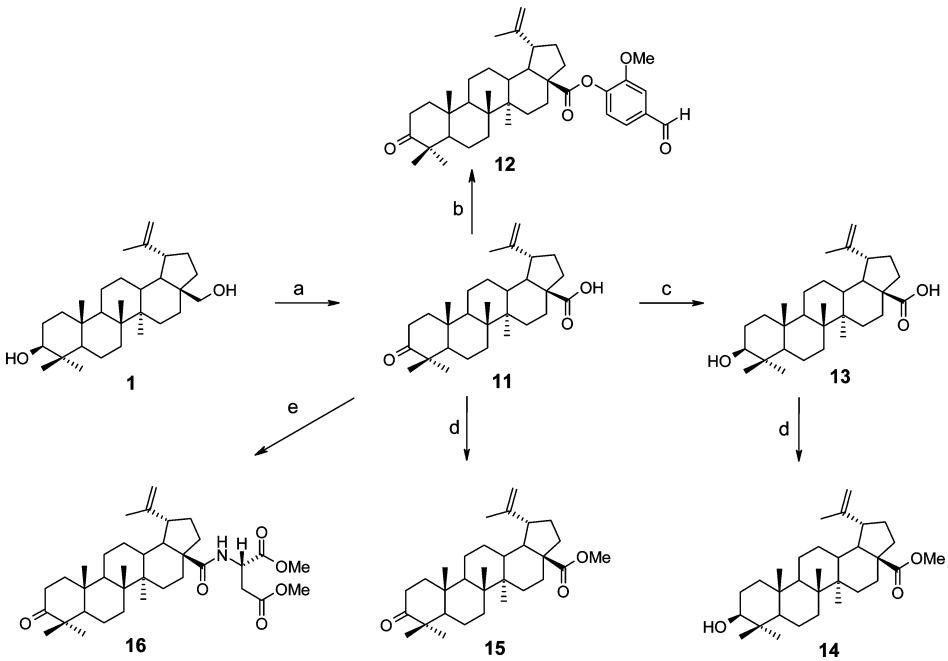
To date, only a limited number of organic small molecules have been found to inhibit alphavirus replication, and most of the existing reports concern nucleoside analogues with often nonoptimal selectivity indices (see ref 24 for review). However, the need for wider structural diversity among the inhibitors of these viruses has been emphasized by the recent epidemic outbreaks. Natural products have often been proven invaluable in the search for novel antimicrobial agents. In the context of alphavirus inhibitors, a *seco*-pregnane steroid and steroidal glycosides were recently investigated as inhibitors of SFV subgenomic RNA production.[25] The current study elucidates the effects of lupane-type triterpenoids on SFV. Even though betulin-derived compounds are known for their various antimicrobial and antineoplastic properties, their effectiveness on RNA viruses remained uncharacterized.

Synthesis of Betulin Derivatives. Compound **2**, 28-*O*-acetylbetulin, was obtained in moderate yield (45%) by treating **1** with acetic anhydride in the presence of DMAP and pyridine in CH_2Cl_2 (Scheme 1). Subsequent oxidation of **2** with PCC in CH_2Cl_2 afforded 28-*O*-acetyl-3-oxobetulin (**3**) in 57% yield.[26] 3,28-Di-*O*-acetylbetulin (**4**), in turn, was obtained in excellent (97%) yield by treating **1** with excess acetic anhydride. Treatment of **4** with HBr in toluene caused the migration of the C-20−C-29 double bond of **4** to the C-18−C-19 position, giving 3,28-di-*O*-acetyllup-18-ene (**5**) in 42% yield.[27−29] The C-18−C-19 double bond of **5** was epoxidized with *m*CPBA in $CHCl_3$ to provide the intermediate **6** in 65% yield.

3-Deoxy-2,3-didehydrobetulin (**7**) was prepared in 31% yield by treating **1** with a mixture of DEAD, PPh_3, and 3,3-dimethylglutarimide in THF[30] (Scheme 2). Subsequent acetylation of **7** gave 3-deoxy-2,3-didehydro-28-*O*-acetylbetulin (**8**) in 81% yield. Dihydrobetulin (**9**) was obtained in 99% yield after the catalytic hydrogenation of **1** using Pd/C as a catalyst. Subsequent oxidation with the Jones reagent in acetone produced the target compound, dihydrobetulonic acid (**10**), in 31% yield.

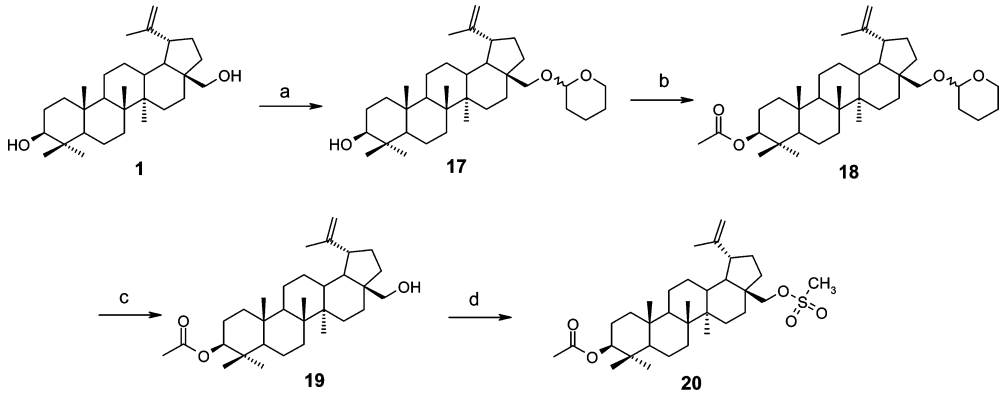
Oxidation of **1** with the Jones reagent in acetone afforded betulonic acid (**11**)[14] in 44% yield (Scheme 3). Subsequent

Scheme 3. Synthesis of Compounds **12**−**16**[a]



[a] Conditions: (a) H_2CrO_4, acetone, 0 °C → rt, 21 h, 44%; (b) (i) $(COCl)_2$, CH_2Cl_2, rt, 22 h, 85%, (ii) vanillin, DMAP, py, 40 °C, 21 h, 20%; (c) $NaBH_4$, *i*-PrOH, rt, 2.5 h, 82%; (d) $TMSCHN_2$, PhMe−MeOH (3:2), rt, 40 min, 89% **14**, 66% **15**; (e) L-aspartic acid dimethyl ester hydrochloride, TEA, CH_2Cl_2, rt, 19 h, 42%. TMS = trimethylsilyl; TEA = triethylamine.

Scheme 4. Synthesis of Compounds **17**−**20**[a]



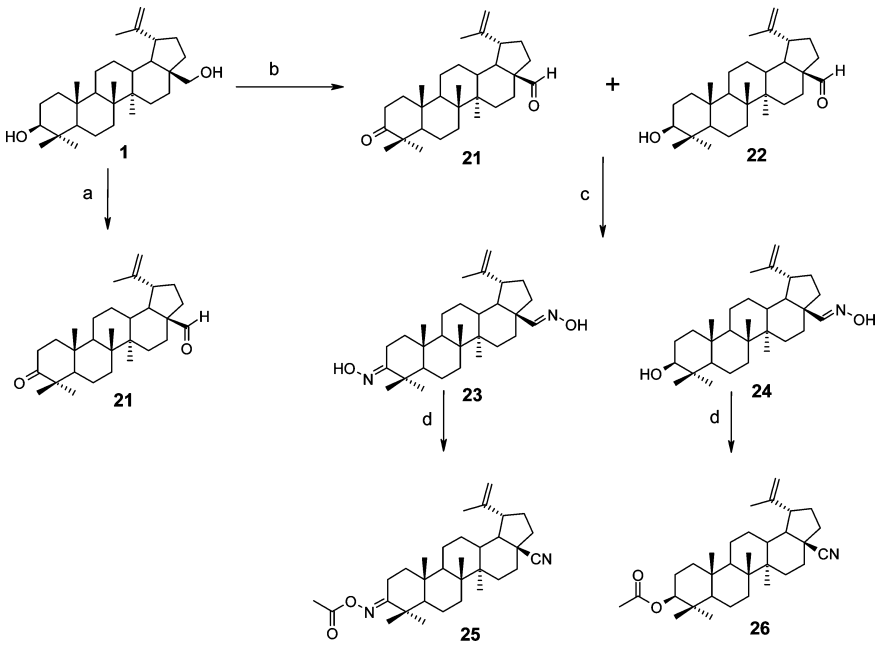
[a] Conditions: (a) DHP, PPTS, CH_2Cl_2, rt, 2 days, 30%; (b) Ac_2O, DMAP, py, CH_2Cl_2, rt, 20 h, 95%; (c) PPTS, EtOH, rt, 14 days, 94%; (d) CH_3SO_2Cl, TEA, CH_2Cl_2, 0 °C, 2 h, 99%. DHP = 3,4-dihydro-2*H*-pyran; PPTS = pyridinium *p*-toluenesulfonate.

treatment of **11** with oxalyl chloride in CH_2Cl_2 gave betulonoyl chloride,[31] which was immediately allowed to react with vanillin in the presence of DMAP in pyridine to produce vanillyl betulonate (**12**) in 20% yield. Reduction of **11** with $NaBH_4$ in 2-propanol gave betulinic acid (**13**) in 82% yield,[32] which was subsequently methylated with $TMSCHN_2$ in PhMe−MeOH to give **14** in 89% yield.[33] Similarly, treatment of **11** with $TMSCHN_2$ in PhMe−MeOH produced methyl betulonate (**15**) in 66% yield. Treatment of **11**, in turn, with oxalyl chloride in CH_2Cl_2 followed by L-aspartic acid dimethyl ester in the presence of TEA in CH_2Cl_2 gave the corresponding L-aspartyl amide of betulonic acid (**16**) in 42% yield.[31]

Treatment of **1** with PPTS and DHP in CH_2Cl_2 produced a diastereomeric mixture of the corresponding tetrahydropyranyl ether (**17**) in 30% yield (Scheme 4). The THP-protected betulin was subsequently acetylated to give **18** in excellent 95% yield. Removal of the THP group with PPTS in EtOH produced 3-*O*-acetylbetulin (**19**) in 94% yield.[29] Subsequent treatment of **19** with CH_3SO_2Cl in the presence of TEA in CH_2Cl_2 gave 3-*O*-acetyl-28-*O*-mesylbetulin (**20**) in 99% yield.

Betulin (**1**) was oxidized with PCC (6 equiv) in CH_2Cl_2 to give betulonic aldehyde (**21**) in 82% yield (Scheme 5).[34] When a smaller molar amount of PCC (1.8 equiv) was used, a 3:1 mixture of **21** and betulin aldehyde (**22**) was produced. Part of the mixture was

Scheme 5. Synthesis of Compounds **21**−**26**[a]



[a] Conditions: (a) PCC (6 equiv), CH_2Cl_2, rt, 1 h, 82%; (b) PCC (1.8 equiv), CH_2Cl_2, rt, 40 min, **22**:**21** (1:3); (c) $NH_2OH \cdot HCl$, py−EtOH (1:3), 100 °C, 18 h, 10% **23**, 33% **24**; (d) Ac_2O, 120 °C, 2 h, 34% **25**, 46% **26**.

separated by SiO_2 column chromatography, and **22** was isolated in 18% yield. The rest of the mixture was treated with an excess of hydroxylamine hydrochloride in pyridine−EtOH to produce oximes **23** and **24** in 10% and 33% yields, respectively.[35] Separate treatment of **23** and **24** with neat acetic anhydride at 120 °C gave nitriles **25** and **26** in 34% and 46% yields, respectively.

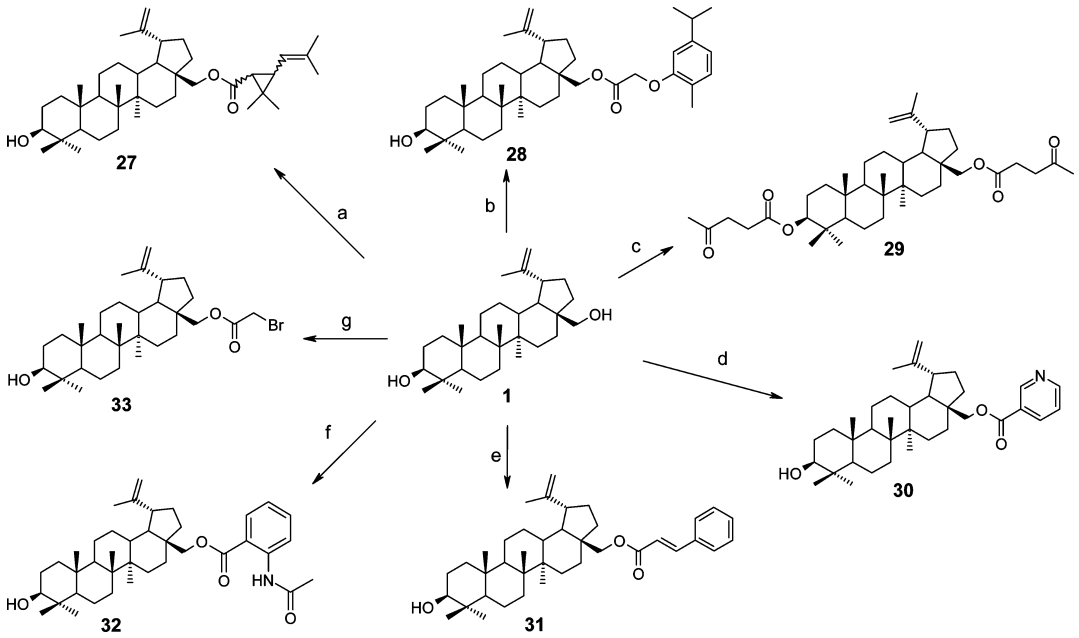
The last series of the synthetic triterpenoids commenced by treating ethyl chrysanthemate with NaOH in THF−MeOH to produce chrysanthemic acid (91% yield), which was subsequently allowed to react with oxalyl chloride in CH_2Cl_2 to give chrysanthemoyl chloride in 81% yield (Scheme 6). Chrysanthemoyl chloride was reacted with **1** to produce a 1:3 mixture of *cis*- and *trans*-28-*O*-chrysanthemoylbetulin (**27**) in 63% yield. Treatment of carvacrol in the presence of chloroacetic acid and NaOH in water gave carvacryloxyacetic acid (45% yield),[36] which was reacted with **1** in PhMe using titanium(IV) isopropoxide as an esterification catalyst to produce betulinyl 28-carboxymethoxycarvacrolate (**28**) in 55% yield. A mixture of **1**, levulinic acid, and PPTS was reacted in PhMe to produce 3,28-di-*O*-levulinoylbetulin (**29**) in 23% yield. Treatment of **1** with nicotinic acid in the presence of DCC and DMAP in CH_2Cl_2 gave 28-*O*-nicotinoylbetulin (**30**) in 31% yield. Cinnamic acid was treated with thionyl chloride to produce cinnamoyl chloride, which was treated immediately with **1** to give 28-*O*-cinnamoylbetulin (**31**) in 21% yield. *N*-Acetylanthranilic acid was treated with oxalyl chloride to produce *N*-acetylanthraniloyl chloride, which was treated immediately with **1** to give 28-*O*-(*N*-acetylanthraniloyl)betulin (**32**) in 25% yield. Finally, betulin **1** was treated with *t*-BuOK in THF followed by addition of methyl bromoacetate to give 28-*O*-bromoacetylbetulin (**33**) in 15% yield.

For the synthesis of heterocyclic betulin derivatives **34**−**51** (Scheme 7, Table 1), 3,28-di-*O*-acetyl-18,19-epoxylupane (**6**) was treated with PPTS in PhMe to give a mixture (4:1) of conjugated dienes, 3,28-di-*O*-acetyllupa-12,18-diene and 3,28-di-*O*-acetyllupa-18,21-diene, in 68% yield.[37] Reactions of 4-phenyl- or 4-methyl-1,2,4-triazoline-3,5-dione or reactions of various 4-substituted urazoles[38] with a mixture of dienes gave the corresponding heterocycles (**34**−**43**) with acetyl R_2 groups in moderate (16% to 62%) yields after the urazoles were oxidized to the corresponding urazines with iodobenzene diacetate in situ.[39] For the synthesis of heterocycles (**44**−**51**) with different R_2 ester groups, a mixture of 3,28-di-*O*-acetyllupa-12,18-diene and 3,28-di-*O*-acetyllupa-18,21-diene was treated with NaOH in THF−MeOH to remove the acetyl groups. Subsequent acylation with various acyl chlorides yielded a mixture of dienes (**44**−**51**) with new R_2 ester groups. Synthesis of betulin heterocycloadducts will be described in detail elsewhere.

Inhibition of SFV by Betulin Derivatives. The primary screen of 51 betulin-derived compounds against SFV, combined with a counterscreen for Huh-7 cell viability, was run in order to determine the tentative inhibitory potential of each derivative. A relatively high test concentration (50 μM) was selected for the primary screen with the added intention of tracking weakly active derivatives for structure−activity comparisons. The results of the primary screen were expressed as surviving fractions (remaining percentages of viral replication or cell viability) after exposure to each compound. These data were used to divide the derivatives according to their properties into the following four clusters: *cluster 1,* selective and efficient antiviral activity (compounds yielding <20% remaining viral replication and >80% cell viability); *cluster 2a,* moderate but selective antiviral activity (remaining viral replication <50% and cell viability >80%); *cluster 2b*, efficient but moderately selective antiviral activity (remaining viral replication <20% and cell viability between 50% and 80%); *cluster 3,* antivirally inactive derivatives and compounds with unacceptable cytotoxicity (virus replication >50%/cell viability <50%).

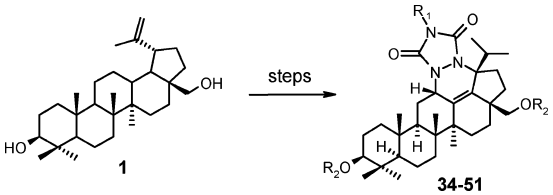
Table 2 presents the results of the primary screen, listing the antiviral and cell viability surviving fractions and the corresponding cluster number for each compound. Compounds in cluster 1 represent the best lead candidates and were thus selected for further evaluation by dose−response experiments. The anti-SFV IC_{50} (50% inhibitory concentration) values for this set of compounds, derived from the fitting of data into a sigmoidal dose−response curve model,

Scheme 6. Synthesis of Compounds **27**−**33**[a]



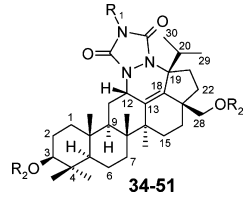
[a] Conditions: (a) (i) ethyl chrysanthemate, NaOH, MeOH−THF (2:1), 80 °C, 4 h, 91%, (ii) chrysanthemic acid, $(COCl)_2$, CH_2Cl_2, rt, 6 h, 81%, (iii) chrysanthemoyl chloride, DMAP, py, 40 °C, 48 h, 63%; (b) (i) carvacrol, chloroacetic acid, NaOH, Δ, 3 h, 45%, (ii) carvacryloxyacetic acid, Ti(OPr-*i*)$_4$, PhMe, Δ, 6 h, 55%; (c) levulinic acid, PPTS, PhMe, 175 °C, 23 h, 23%; (d) nicotinic acid, DCC, DMAP, CH_2Cl_2, rt, 23 h, 31%; (e) (i) cinnamic acid, $SOCl_2$, 40 °C, 2 h, 92%, (ii) cinnamoyl chloride, DMAP, pyridine, 40 °C, 22 h, 21%; (f) (i) *N*-acetylanthranilic acid, $(COCl)_2$, rt, 3 days, 99%, (ii) *N*-acetylanthraniloyl chloride, DMAP, py, 40 °C, 40 h, 25%; (g) *t*-BuOK, methyl bromoacetate, THF, 75 °C, 10 min, 15%. DCC = *N*,*N*′-dicyclohexylcarbodiimide.

Scheme 7. Synthesis of Compounds **34**−**51**[a]



[a] Synthesis of heterocyclic betulin derivatives **34**−**51** will be described in detail elsewhere.

Table 1. Heterocyclic Betulin Derivatives **34**−**51**



compound	R_1	R_2
34	3-MeO-Ph	Ac
35	1,3-dioxol-5-yl	Ac
36	indan-5-yl	Ac
37	4-F-Ph	Ac
38	3-NO_2−Ph	Ac
39	3-Cl-Ph	Ac
40	$PhCH_2$	Ac
41	Ph	Ac
42	*n*-Bu	Ac
43	Et	Ac
44	Ph	H
45	Me	COEt
46	Me	COPr
47	Me	CO*i*-Pr
48	Me	COcHex
49	Me	COPh
50	Ph	COPh
51	*t*-Bu	COcHex

are shown in Table 3. As discussed in more detail in the structure−activity relationship section, the values range from 9.1 μM (compound **4**) to 48.5 μM (compound **3**). For comparison, a standard SFV inhibitor, ribavirin, has an IC_{50} value of 95 μM in the assay. In an extended cytotoxicity analysis at 500 μM concentration performed on all cluster 1 compounds, only betulonic aldehyde (**21**) affected the surviving fraction of Huh-7 cells (cell viability 52% after 24 h exposure). For other cluster 1 compounds, this high concentration, which is close to the solubility limits, was well tolerated (cell viability values >80%).

Structure−Activity Relationships. The primary screening data and the results of the potency analysis were used to examine the structural determinants for the anti-alphaviral activity of betulin derivatives and to study the chemical space of antivirally active betulin-derived compounds. Betulin (**1**) inhibited SFV replication with an IC_{50} value of 45.5 μM. Removal of the C-20−C-29 double bond yielded compound **9**, which failed to show antiviral activity. Both betulinic acid (**13**) and betulonic acid (**11**) yielded improved antiviral potency compared to **1** (IC_{50} values 14.6 and 13.3 μM and *p* values in Student's *t* test <0.05 in both cases). Oxidation of the OH moieties also compensated for the loss of double-bond exclusion activity, even though the potency of the 20−29-saturated dihydrobetulonic acid (**10**) remained inferior to its unsaturated counterpart **11**. Removal or oxidation of the secondary OH group at C-3 disturbed the anti-SFV activity, as demonstrated by the

Table 2. Antiviral (AV) and Cytotoxic Effects (CV) of Betulin-Derived Compounds

compound	virus replication (%)	cell viability (%)	cluster
1	7	83	1
2	3	87	1
3	11	83	1
4	21	97	2a
5	15	87	1
6	16	92	1
7	121	85	3
8	98	95	3
9	95	81	3
10	14	102	1
11	13	89	1
12	43	89	2a
13	18	123	1
14	68	1	3
15	71	83	3
16	40	30	3
17	11	92	1
18	10	91	1
19	3	86	1
20	46	99	2a
21	8	90	1
22	1	0	3
23	121	80	3
24	55	82	3
25	73	85	3
26	60	69	3
27	95	90	3
28	80	103	3
29	74	109	3
30	43	94	2a
31	111	96	3
32	82	94	3
33	125	11	3
34	121	93	3
35	97	92	3
36	81	93	3
37	64	95	3
38	3	108	1
39	1	95	1
40	1	101	1
41	13	87	1
42	19	81	1
43	24	40	3
44	73	85	3
45	21	75	2b
46	18	93	1
47	24	106	2a
48	44	97	2a
49	22	108	2a
50	50	88	2a
51	40	83	2a

The data present results from the primary screen of betulin-derived compounds in anti-SFV and ATP cell viability assays (see the Supporting Information for experimental details). The numbers represent surviving fractions (the remaining percentages of viral replication or cell viability) in each assay. All experiments were made in triplicate using a concentration of 50 μM of each compound.

inactive 3-deoxy-2,3-didehydro derivatives **7** and **8**, as well as the inactivity of oxime derivative **23**. Interestingly, betulonic aldehyde (**21**) demonstrated selective antiviral activity in the primary screen, whereas betulin aldehyde (**22**) had an inverse activity profile. However, in the extended cytotoxicity assay at higher concentration, **22** also had indications of host cell toxicity.

Conversely to the inactive methyl esters of both betulinic acid and betulonic acid (compounds **14** and **15**, respectively), 28-*O*-acetylbetulin (**2**) was a potent SFV inhibitor (IC_{50} value 12.1 μM). The inhibitory capacity was retained, yet with loss in potency, in the presence of the individually inactivating 28-*O*-acetyl-3-oxo-betulin (**3**). Acetylation of both the C-3 and C-28 hydroxy groups in **1** to yield **4** improved the antiviral activity, yielding an IC_{50} value of 9.1 μM. Migration of the terminal double bond from C-20−C-29 to C-18−C-19 [3,28-di-*O*-acetyllup-18-ene (**5**)] reduced the antiviral activity to 43 μM. Further introduction of 18−19-epoxide resulted in equally effective and potent activity (compound **6**; IC_{50} value 13 μM), when compared to **4**. However, acetylation of **1** at C-3 as such (derivative **19**) or when combined with 28-*O*-tetrahydropyranyl derivatization in **18**, as well as addition of the 28-*O*-tetrahydropyranyl (THP) moiety alone (**17**), yielded cluster 1 compounds **17**, **18**, and **19** with IC_{50} values of 17.2, 24.7, and 24.2 μM, respectively. On the other hand, mesylation of the 3-*O*-acylation product at C-28 resulted in only moderately active compound **20**, scoring into cluster 2a with surviving viral fraction of 46% (Table 2). Introducing a nitrile moiety at C-28 led to even more diminished antiviral activity (**26** in cluster 3 and **25** in cluster 2a).

Table 3. IC_{50} Values of Betulin-Derived Compounds against Semliki Forest Virus (SFV)[a]

compound	IC_{50} μM (pIC_{50})
1	45.5 (−4.34 ± 0.18)
2	12.1 (−4.92 ± 0.16)
3	48.5 (−4.32 ± 0.15)
4	9.1 (−5.04 ± 0.26)
5	43.2 (−4.37 ± 0.20)
6	13.3 (−4.94 ± 0.45)
10	30.6 (−4.52 ± 0.23)
11	13.3 (−4.88 ± 0.15)
13	14.6 (−4.84 ± 0.28)
17	17.2 (−4.76 ± 0.24)
18	24.7 (−4.61 ± 0.15)
19	24.2 (−4.62 ± 0.25)
21	38.3 (−4.42 ± 0.28)
38	22.9 (−4.64 ± 0.16)
39	22.1 (−4.65 ± 0.15)
40	35.9 (−4.44 ± 0.12)
41	19.7 (−4.71 ± 0.13)
42	37.9 (−4.42 ± 0.20)
46	30.1 (−4.52 ± 0.18)
ribavirin	95.1 (−4.02 ± 0.27)

[a] Dose−response experiments were performed using a luminometric anti-SFV assay (see Supporting Information) using serial dilutions of each derivative and fitting the data into sigmoidal dose−response curves; values are means ± SD (n = 6).

As indicated by the lack of antiviral activity of 3-deoxy and 3-oxime derivatives, maintaining the secondary OH group at C-3 contributed to the anti-SFV activity of the betulin-derived compounds. However, the IC_{50} values for derivatives with free versus acetylated C-3 OH groups did differ from each other, 3,28-di-*O*-acetylbetulin (**9**) being the most potent derivative. Thus, the influence of C-28 substitution on anti-SFV activity was further examined with C-3-unmodified derivatives. Unfortunately, the activity of betulin C-28 esters of naturally occurring and biologically active terpenoid or aromatic carboxylic acids was poor. Esters of betulin and chrysanthemic acid **27**, carvacryloxyacetic acid **28**, *N*-acetylanthranilic acid **32**, or cinnamic acid **31** as well as the 3,28-dilevulinate of betulin (**29**) had no detectable antiviral activity, whereas the 28-nicotinate of betulin (**30**) was moderately active, scoring into cluster 2a (Table 2). The antiviral activity of cytotoxic 28-*O*-bromoacetylbetulin (**33**) was poor. 28-Vanillinyl betulonate (**12**) scored into cluster 2a, whereas the L-aspartyl amide of betulonic acid (**16**) was cytotoxic.

In addition to the C-3- and C-28-modified derivatives, a set of heterocyclic compounds was synthesized by the [4+2] cycloaddition reaction between N-substituted 1,2,4-triazolidine-3,5-diones (urazines) and 3β,28-diacyloxylupa-12,18-dienes (**34**−**51**; see Table 1). The heterocycloadducts with 4-*n*-butyl and 4-ethyl substituents, **42** and **43**, were active against SFV, even though the 4-ethyl derivative **43** also affected Huh-7 cell viability (Table 2). The heterocycloadduct with phenyl at N-4 (compound **41**) inhibited SFV, with an IC_{50} value of approximately 20 μM, and a switch to the N-4 benzyl group was accompanied by a slight loss in potency (derivative **40**

IC_{50} value 36 μM). Derivatives having an electron-withdrawing group on the aromatic ring, 3-chlorophenyl (**39**) and 3-nitrophenyl (**38**), were selective and potent SFV inhibitors, having IC_{50} values of 22 and 23 μM, respectively (Table 3). However, 4-fluorophenyl-substituted **37** had poor activity. Derivatives having an electron-donating group in the aromatic ring, 3-methoxyphenyl (**34**), 1,3-dioxol-5-yl (**35**), and indan-5-yl (**36**), had no activity against SFV.

However, further removal of the acetyl groups from the antivirally active heterocycle **41** resulted in loss of activity (compound **44**), but substitution of the acetyl groups by benzoyl at both positions (**50**) yielded a moderately active compound (cluster 2a; Table 2). In addition, the 4-methyl-1,2,4-triazoline-3,5-dione adducts **47**, **48**, and **49**, combined with either isopropanoyl, cyclohexanoyl, or phenyl 3,28-diesters of betulin, scored into cluster 2a. Indication of cytotoxicity was observed in the case of the corresponding propanoyl 3,28-diester (**45**). Derivate **51**, with bulky substituents (*tert*-butyl group in N-4, cyclohexanoyl groups in C-3, and hydroxy groups in C-28), scored into cluster 2a. However, the most efficient inhibition of SFV among this subset of heterocycles was achieved with a cycloadduct between 4-methylurazine and 3,28-di-*O*-butyrylbetulin-12,18-diene (**46**). This compound scored into cluster 1 in the primary screen and yielded an IC_{50} value of 30 μM.

Inhibition of SIN by Betulin-Derived Compounds. Sindbis virus (SIN), the causative agent of Pogosta disease (also known as Carelian fever), is another alphavirus that is widely distributed over the European, Asian, and African continents. SIN and SFV represent separate clusters in the alphavirus phylogenetic tree, generated by comparing E1 glycoprotein sequences. SIN and SFV also fall into different serocomplexes according to antibody cross-reactivity. However, the replicase proteins are relatively highly conserved within the genus.[1]

Three betulin derivatives from different structural subclasses showing antiviral potency and selectivity, i.e., **13**, **17**, and **41**, were assayed for inhibitory potency against SIN using a radiometric RNA labeling assay. The dose−response curves presented in Figure 1 demonstrate the sensitivity of SIN toward these compounds; the IC_{50} values extracted from the data were 0.5 μM (pIC_{50} -6.34 ± 0.09), 1.9 μM (pIC_{50} -5.72 ± 0.10), and 6.1 μM (pIC_{50} -5.21 ± 0.16) for **13**, **17**, and **41**, respectively. In all three cases, the studied SIN strain exhibited greater sensitivity toward the derivatives compared to SFV.

Synergism Studies. Antiviral therapy is often conducted as a combination of multiple drugs targeting different sites in virus replication.[40] Parallel administration of inhibitors with different molecular targets is considered beneficial in terms of improved efficacy and/or prevention of resistance. Previously we reported the anti-alphaviral efficacy of several modified nucleosides, including 3′-amino-3′-deoxyadenosine (3′-NH-3′-dAdo) with an IC_{50} value of 18 μM in the reporter gene assay.[23] The inhibition of the virus life cycle by nucleoside analogues is likely to take place during the replication phase, targeting viral polymerases or components of cellular nucleoside metabolism, whereas betulin-derived compounds have been related to a variety of antiviral mechanisms mainly in the early and late stages of the virus life cycle (see below). Thus, we investigated the possibility of synergistic inhibition of SFV by 3′-NH-3′-dAdo together with derivatives **13**, **17**, and **41**.

The IC_{50} values for each interaction partner were determined by using varying concentrations of 3′-NH-3′-dAdo and test compound in combination (see Experimental Section and Supporting Information for experimental details). The strong Loewe synergism that was demonstrated in these experiments is visualized by the bending of the isobolograms below the additivity-indicating diagonal line in Figure 2. Calculation of Berembel interaction indices for individual combinations indicated that the most intense Loewe synergism was achieved when 5 μM 3′-NH-3′-dAdo was combined with low or moderate concentrations of each betulin derivative. At this nucleoside concentration, 0.08 μM and 0.4 μM **13** yielded

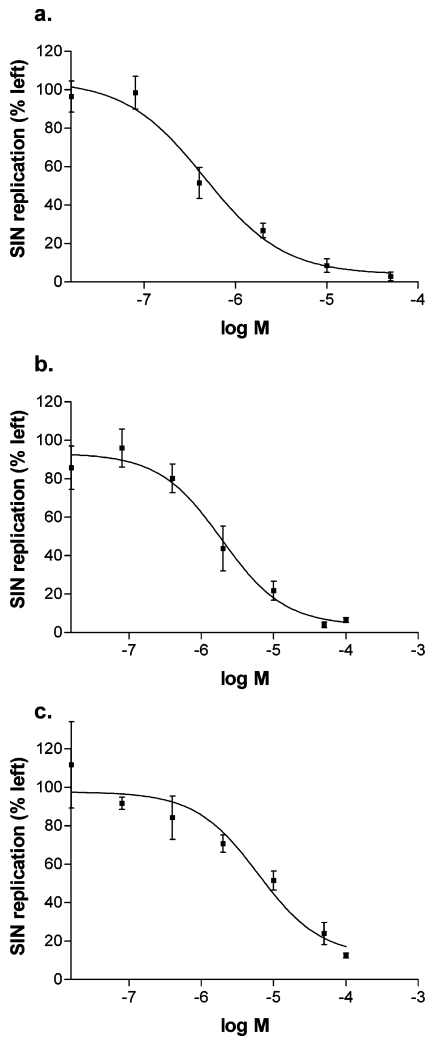


Figure 1. Dose-dependent activity of (a) betulinic acid (**13**), (b) 28-*O*-tetrahydropyranylbetulin (**17**), and (c) 4-phenyl-substituted betulin heterocycle **41** against Sindbis virus (SIN). The dose−response experiments were performed using the radiometric RNA labeling assay (see Supporting Information) using serial dilutions ranging from 16 pM to 50 μM. Nonlinear regression was used to fit the data into sigmoidal dose−response curves; values are means ± SD ($n = 4$).

interaction index values of $I = 0.28$ and 0.25, respectively. For 0.4 μM 28-*O*-tetrahydropyranylbetulin (**17**), an I value of 0.24 was obtained, whereas combining 5 μM nucleoside with the heterocycle **41** resulted in the most intense synergism at 2 μM ($I = 0.16$). Increasing compound **17** and **41** concentrations closer to their IC_{50} values gave moderate or strong synergism (I values at 10 μM concentration of betulin derivatives with different 3′-NH-3′-dAdo concentration ranged from 0.25 to 0.47), whereas similar conditions with **13** yielded additive rather than synergistic inhibition (I values from 0.52 to 1.26). Complete tables of interaction indices for all three compounds with 3′-NH-3′-dAdo, as well as the equations used to calculate the indices, are provided as part of the Supporting Information.

Mapping of the Target Site for Anti-SFV Activity. Since the antiviral effects of betulin derivatives are associated with a range

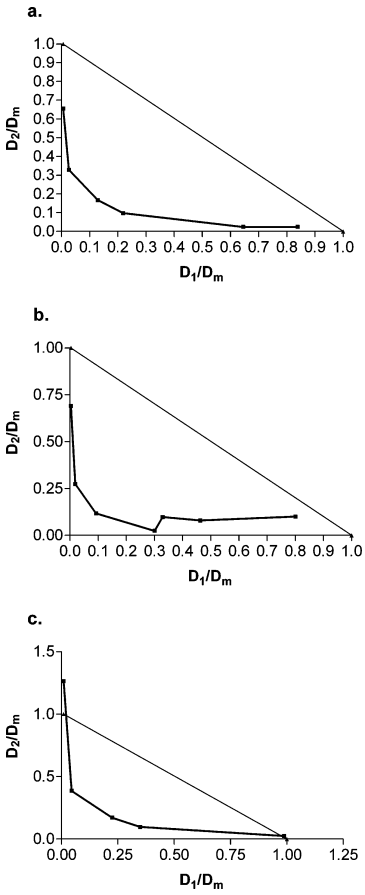


Figure 2. Synergistic activities of (a) betulinic acid (**13**), (b) 28-*O*-tetrahydropyranylbetulin (**17**), and (c) 4-phenyl-substituted betulin heterocycle **41** in combination with 3′-amino-3′-deoxyadenosine against SFV presented as isobolograms. The synergism studies were performed by titrating different concentrations (from 0.5 to 50 μM) of 3′-amino-3′-deoxyadenosine against the serial dilutions of each betulin derivative (from 80 pM to 50 μM). D_1/D_{m1} and D_2/D_{m2} values were derived from the data according to ref 48. Ratios were calculated for IC_{50} values of each compound alone and in the presence of different concentrations of 3′-amino-3′-deoxyadenosine in the combination. The diagonal line in each figure presents a visualization of Loewe additive effects.

of target sites, an administration time experiment using a high-multiplicity infection of SFV-Rluc was applied in order to gain preliminary information on the target site of the reported anti-SFV activity. Compounds **13**, **17**, and **41** were administered into cell cultures at different time points in conditions where the majority of cells were infected at once. The time scale of this experiment represents a single virus replication cycle, as the luciferase reporter gene was expressed by the translation of viral nonstructural proteins, and the readout was thus taken at 4.5 h (see the Supporting Information for experimental details). As illustrated in Figure 3, none of the three betulin derivatives showed antiviral efficacy when present in the cultures only at the time of viral adsorption (0−1 h). The same was also observed for the 3′-amino-3′-deoxyadenosine that was used for comparison. On the other hand, delivery of the compounds just after the removal of the viral inocula (at 1 h) yielded inhibition comparable to the effect obtained when the agent was present throughout the experiment. Furthermore, postponing the

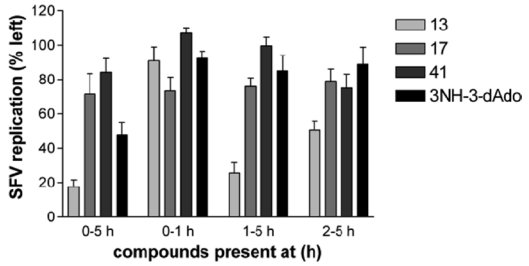


Figure 3. Effect of administration time on antiviral effect of betulin-derived compounds **13**, **17**, and **41** against SFV. Each of the compounds was present in high-multiplicity infected cultures (5 PFU/cell) either throughout the experiment (0−5 h), during virus adsorption (0−1 h), at 1−5 h, or at 2−5 h. The results represent the surviving virus fraction, determined as remaining luciferase reporter gene activity in each sample at 5 h; values are means ± SD (n = 4).

administration to 2 h led to attenuated efficacy, yet the response was still detectable in the case of **13**.

Even though physicochemical and kinetic features, such as hydrophobicity of the triterpenoids and nucleoside phosphorylation, may affect the interpretation, the entry phase does not appear to be the target of the reported anti-alphaviral activity. In the case of betulinic acid, the activity is associated with the early replication phase, whereas the two other betulin-derived compounds give less obvious results. The end point in the experimental setup is in the translation and processing of viral polyprotein (into which the *Rluc* gene is inserted; see ref 24). Thus, inhibitors of viral maturation would give seemingly negative results in this particular setup. On the other hand, our previous work indicated that the sensitivity of an in vitro antiviral assay is highly dependent on the infection multiplicity used (L. Pohjala, unpublished results). However, repeating the experiment with a higher concentration (200 μM) yielded similar results, as shown in Figure 3, at 50 μM. Yet the effect of inferior potency cannot be completely ruled out by this means since the target site for the anti-alphaviral activity of **17** and **41** may lie in steps of the virus life cycle occurring after the production of nonstructural polyproteins. Each of the replication phases consists of several substages, which are typically inhibited by different sets of chemical agents.[40]

As betulin-derived compounds are known to have a wide spectrum of antimicrobial, anti-inflammatory, and antineoplastic effects,[15] these findings could be reconciled by a general underlying mechanism, such as interference with cellular nucleoside metabolism. Indeed, certain classical inhibitors of RNA virus replication that also share anti-inflammatory properties (e.g., ribavirin and mycophenolic acid) exert their action via depletion of cellular GTP pools.[41,42] This mode of action is characterized by the loss of inhibitory effect when the cultures are supplemented with external guanosine to compensate for depletion in cellular guanosine biosynthesis. However, the betulin-derived compounds **13**, **17**, and **41** maintained their antiviral activity in the presence of 50 μg/mL (177 μM) guanosine supplementation also (data not shown), implying that any contribution of this mechanism to the observed anti-SFV activity is minor at most. The existence of more specific virus-related targets is also supported by the distinct structure−activity relationships reported for different pharmacological uses.[15]

The study of antiviral mechanisms of betulin-derived compounds has involved several molecular targets. For relatively simple, naturally occurring compounds such as betulin and betulinic acid, the proposed targets include HIV-1 reverse transcriptase,[43] HIV gp41,[44] and severe acute respiratory syndrome coronavirus (SARS-CoV) 3_{CL} protease.[45] Recent work on the SARS protease implies that modulation of a single molecular target may not correlate with

in vivo antiviral efficacy: Wen and co-workers[45] conclude that both betulinic acid and betulonic acid inhibit SARS-CoV replication, but only betulinic acid inhibits 3_{CL}-purified 3_C protease. Previous work on the effects of triterpenoid drugs on HSV also supported the hypothesis that the antiviral efficacy of such agents may be a combination of different activities in cell culture, rather than directly associated with any specific phase in the virus life cycle.[46] Such findings may put into question the usefulness of the compounds from a rational drug design viewpoint, but may reflect nature's strategy for sustained bioactivity. On the other hand, chemically modified betulin derivatives, such as those in anti-HIV drug discovery, are thought to exert their activity via more limited sets of targets, which is also supported by the generation of resistant HIV strains via point mutations.[47,48] The results from the current screen elucidate a pattern in which most of the relatively simple derivatives inhibit SFV replication, whereas, among the more complex structures, the antiviral activity is not ubiquitous but shared only by certain structural subclasses. On the other hand, betulinic acid (**13**) is distinguished from the two derivatives (**17** and **41**) on the basis of its behavior in the administration time experiments.

Betulin-derived compounds form a family of natural compounds that, along with their synthetic derivatives, have a broad spectrum of antineoplastic and antimicrobial activities. The present results, together with prior reports on SARS-CoV and ECHO-6 picornavirus, provide evidence for the sensitivity of positive-stranded RNA viruses toward betulin-derived compounds. The inhibitory activity against Semliki Forest virus and Sindbis virus, together with the lack of early signs of toxicity, raise hopes about the therapeutic potential of betulin-derived compounds used against these pathogens either alone or in combination with other antiviral therapy.

Experimental Section

General Experimental Procedures. Details of the chemical synthesis procedures and characterization as well as the biological experiments are presented in the Supporting Information.

Bioassays. Full-length infectious cDNA clones of SFV and SIN[49,50] were used to generate virus stocks for the BHK-21 cell culture infections. A validated and automated luciferase-based reporter gene assay with marker virus SFV-Rluc[23] was used to screen for anti-SFV activity in conditions where a low-multiplicity infection [0.001 plaque-forming units (PFU)/cell] was allowed to proceed for more than 2 infectious cycles during a 14 h incubation. 3′-Amino-3′-deoxyadenosine was used as a positive control in the screen; 20 μM of this nucleoside yielded surviving fractions of 12% to 25% in the assay. The counter-screen for mammalian cell viability was performed by determining the intracellular ATP contents of Huh-7 cells (a continuous cell line derived from human hepatocellular carcinoma) after 24 h exposure to each derivative, as previously described.[51] Labeling by [^{3}H]-uridine was used to determine the viral RNA synthesis rate in SIN virus studies. In the synergism studies, the Loewe additivity model[52] served to predict the expected effects of combinations of selected betulin derivatives and a nucleoside analogue, 3′-amino-3′-deoxyadenosine. The observed and expected effects were then compared by analysis of isobolograms and interaction indices (*I*).[52] In order to gain preliminary information on the target site of anti-SFV activity of the betulin-derived compounds, high-multiplicity infections [5 PFU/cell] were accompanied with administration of betulin derivatives at 0, 1, or 2 h or withdrawal of the compounds at 1 h. Here, the luciferase activity resulting from translation of *Renilla* luciferase together with SFV nonstructural proteins was analyzed at 5 h. The presence of unspecific replication inhibition by depletion of cellular guanosine pools was also studied by guanosine supplementation.

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Supporting Information Available: Experimental procedures (detailed chemical and bioactivity screening methods) and characterization data are available free of charge via the Internet at http://pubs.acs.org.

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PUBLICATION III

Anti-leishmanial activity of betulin derivatives

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ORIGINAL ARTICLE

Anti-leishmanial activity of betulin derivatives

Sami Alakurtti[1,2], Pia Bergström[2], Nina Sacerdoti-Sierra[3], Charles L Jaffe[3] and Jari Yli-Kauhaluoma[1]

Leishmanicidal activity of 24 derivatives of naturally occurring and abundant triterpenes belonging to the lupane series, betulin, betulinic acid and betulonic acid, is described in this study. The easily modified positions of the lupane skeleton, the hydroxy groups of C-3 and C-28, as well as the carbon–carbon double bond C-20–C-29 were used as a starting point to prepare a library of triterpenoid derivatives for bioactivity studies. The compounds were evaluated against *Leishmania donovani* axenic amastigotes on a microplate assay at 50 μM. GI_{50} values of the most effective compounds were evaluated, as well as their cytotoxicity on the human macrophage cell line THP-1, and anti-leishmanial activity against *L. donovani*-infected THP-1 macrophages was determined. Betulonic acid was the most potent derivative, yielding a GI_{50} value of 14.6 μM. Promising and distinct structure–activity relationships were observed, and these compounds can be regarded as significant lead molecules for further improvement and optimization.

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Keywords: antiprotozoal agents; betulin; betulinic acid; *Leishmania* sp.; Terpenoids

INTRODUCTION

Leishmaniases are diseases caused by protozoan parasites that affect millions of people in more than 88 countries worldwide. These parasites are transmitted by female sand flies belonging to the genus *Phlebotomus* and *Lutzomyia* in the Old and New World, respectively. Leishmaniasis causes three main forms of clinical disease: (1) visceral leishmaniasis, the most severe form, is usually fatal if not treated and affects internal organs such as the liver, spleen and bone marrow; (2) mucocutaneous leishmaniasis, a chronic form, causes extensive destruction and disfiguration of the nasopharynx region; and (3) cutaneous leishmaniasis, the mildest form, is usually self-healing within a few months to years, causing scarring at the site of the lesion(s). First-line drugs include pentavalent antimony (Sb^v) compounds, pentamidine or amphotericin B. All these drugs are administrated by injection and require clinical supervision or hospitalization because of the possibility of severe side effects. However, parasite resistance to Sb^v drugs has resulted in the discontinued use of these compounds in some endemic regions for visceral leishmaniasis.[1] Liposomal amphotericin B shows reduced toxicity, but is prohibitively expensive for use in less-developed countries. Recently, miltefosine, an alkylphospholipid derivative and the first orally administered drug, has been approved for use in India. However, the teratogenic effects of this drug prevent its use in pregnant women,[2,3] and parasite resistance is easily generated in the laboratory.[4] As such, there is an urgent need for the development and testing of new compounds for the treatment of all clinical forms of leishmaniasis.

Betulin **1** (lup-20(29)-ene-3β,28-diol) is an abundant naturally occurring triterpene found in the plant kingdom (Figure 1). It is the principal extractive (up to 30% of dry weight) of the bark of white-barked birch trees (*Betula* sp.).[5] This pentacyclic triterpene can be converted into betulinic acid **2**,[6] which has shown anti-inflammatory,[7] antimalarial[8] and especially cytotoxic activity against several tumor cell lines by inducing apoptosis in cells.[9,10] Some betulin derivatives have also shown remarkable anti-human immunodeficiency virus activity with new mechanisms of action.[11,12] Structure–activity relationship studies and pharmacological properties of betulin and its derivatives have been reviewed recently.[13]

Previously, dihydrobetulinic acid **3** was examined as a new lead compound for anti-leishmanial therapy.[14] It was shown that it targeted DNA topoisomerases I and II by preventing DNA cleavage and formation of an enzyme–DNA complex, which ultimately induced apoptosis in *Leishmania donovani* promastigotes and amastigotes in infected macrophages with an IC_{50} value of 2.6 and 4.1 μM, respectively. Parasitic burden in golden hamsters was reduced by 92% after a 6-week treatment with dihydrobetulinic acid **3** ($10\,mg\,kg^{-1}$ body weight). In another study, in which leishmanicidal inhibition activity of a plethora of natural products was screened, betulinic acid **2** isolated in small quantities from *Betula platyphylla* var. *japonica* was found to be weakly active against *Leishmania major* promastigotes, the extracellular form of the parasite, with an IC_{50} value of 88 μM.[15] It was also noted that in triterpenes with ursane, oleanane or lupane skeletons, a carboxyl substituent was required for anti-leishmanial activity. In a related study, it was shown that a rare natural product, betulin aldehyde **4**, obtained from *Doliocarpus dentatus* (Aubl.) showed *in vitro* activity against *Leishmania amazonensis* amastigotes in infected macrophages, reducing infection by 88% at 136 μM and by

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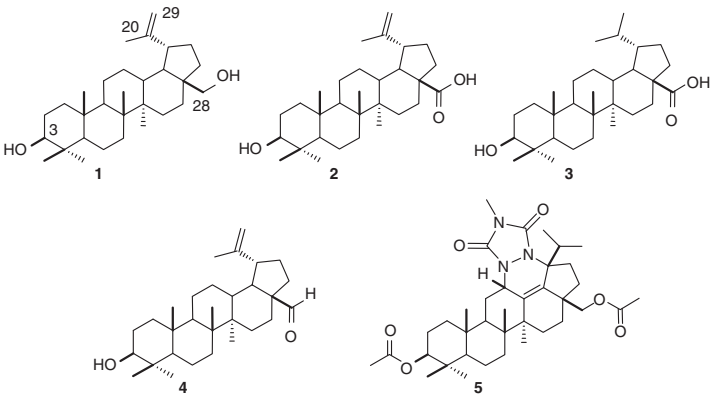


Figure 1 Chemical structures of betulin **1**, betulinic acid **2**, dihydrobetulinic acid **3**, betulin aldehyde **4** and betulin heterocycloadduct between 3,28-di-*O*-acetyllupa-12,18-diene and 4-methylurazine **5**.

58% at 68 μM.[16] At these doses, **4** also showed some toxicity against peritoneal macrophages, with survival indices of 70 and 80%, respectively. Previously, we studied anti-leishmanial activity of heterocyclic betulin derivatives, in which the heterocycloadduct between 3,28-di-*O*-acetyllupa-12,18-diene and 4-methylurazine **5** was the most effective derivative with a GI_{50} value of 8.9 μM against *L. donovani* amastigotes.[17] These results prompted us to investigate more closely the anti-leishmanial activity of 24 betulin derivatives that have been chemically modified in positions C-3, C-28 and C-20–C-29 of the lupane skeleton.

RESULTS AND DISCUSSION

We found that betulin **1** (isolated from *Betula* sp.) has moderate anti-leishmanial activity against *L. donovani* axenic amastigotes, showing 35% inhibition at 50 μM in a microplate assay (Table 1). Acetylation, esterification or etherification of the hydroxy groups at C-3 or C-28 in most cases retained anti-leishmanial activity. We observed that 28-*O*-Cinnamoylbetulin **6** was totally inactive and 28-*O*-nicotinoylbetulin **7**, 28-*O*-tetrahydropyranylbetulin **8**, 28-*O*-chrysanthemoylbetulin **9** and betulinyl-28-*O*-carboxymethoxycarvacrolate **10** were only slightly active. Only 28-*O*-(*N*-acetylanthraniloyl)betulin **11** and 28-*O*-bromoacetylbetulin **12** showed improved anti-leishmanicidal activity (59 and 86% inhibition at 50 μM, respectively), compared with **1**. In addition, 3-*O*-acetylbetulin **13** had similar anti-leishmanial inhibition activity compared with the starting material betulin **1**, whereas 3,28-di-*O*-acetylbetulin **14** and 3,28-di-*O*-levulinoylbetulin **15** were totally inactive.

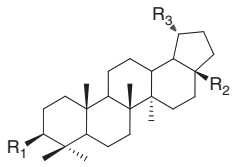
Oxidation of **1** seems to have a beneficial effect on anti-leishmanial activity. Betulin aldehyde **4** displayed improved anti-leishmanial activity with a 64% inhibition at 50 μM. Betulinic acid **2** possessed moderate anti-leishmanial activity with a 40% inhibition at 50 μM. 28-*O*-Acetyl-3-oxobetulin **16** and betulonic aldehyde **17** showed moderate anti-leishmanial activity similar to the starting material **1**, but betulonic acid **18** had remarkable anti-leishmanial activity with a 98% inhibition at 50 μM. Reduction of the carbon–carbon double bond of betulonic acid **18** to the corresponding dihydrobetulonic acid **19** decreased anti-leishmanial activity to 72% at 50 μM. Furthermore, methylation of betulonic acid **18** to methyl betulonate **20** decreased the inhibition activity at 50 μM to 40%. L-aspartyl amide of betulonic acid **21** showed reduced leishmanicidal activity compared with betulonic acid **18,** with a 69% inhibition at 50 μM. Vanillyl betulonate **22** was totally inactive.

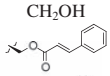
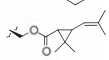
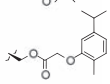
Removal of the C-3 hydroxy group of **1** resulted in 3-deoxy-2,3-didehydrobetulin **23**, the anti-leishmanial activity of which diminished to 13% at 50 μM. Oxime derivatives **24** and **25** showed good leishmanicidal activities at 50 μM, with 69 and 73% inhibition, respectively. Moreover, betulin derivative **26** with a nitrile group at C-28 showed good anti-leishmanial activity with a 63% inhibition at 50 μM.

Derivatives (**12**, **18**, **19**, **21** and **25**) that showed the best anti-leishmanial activity on microplate assay at 50 μM against *L. donovani* axenic amastigotes were selected for further investigations: GI_{50} values, cytotoxicity to the macrophage cell line THP-1 and anti-leishmanial activity against the *L. donovani*-infected macrophage cell line THP-1 were evaluated. Betulonic acid **18** showed the best GI_{50} value of 14.6 μM on microplate assay against *L. donovani* axenic amastigotes, followed by L-aspartyl amide derivative **21** and oxime derivative **25**, with GI_{50} values of 21.2 and 22.8 μM, respectively (Table 1). 28-*O*-Bromoacetylbetulin **12** and dihydrobetulonic acid **19** had moderate GI_{50} values of 34.9 and 56.0 μM, respectively. Cytotoxicity of derivatives **12**, **18**, **19**, **21** and **25** was tested against the macrophage cell line THP-1 at concentrations of 50, 25 and 12.5 μM (Table 2). Betulonic acid **18** showed cytotoxicity against the THP-1 cell line at all test concentrations. Dihydrobetulonic acid **19** and oxime derivative **25** showed cytotoxicity against the THP-1 cell line at 50 and 25 μM, but at 12.5 μM concentration, cytotoxicity of **19** and **25** was reduced to 22.0 and 13.6%, respectively. L-aspartyl amide derivative **21** and 28-*O*-bromoacetylbetulin **12** were nontoxic to macrophage cell line THP-1 at all test concentrations.

Finally, anti-leishmanial activity of compounds **12, 19**, **21** and **25** was tested against *L. donovani*-infected macrophage cell line THP-1, with concentrations that showed $<30\%$ cytotoxicity to the THP-1 cell line (Table 3). In all cases, anti-leishmanial activity was reduced when compared with that in the corresponding microplate assay with *L. donovani* axenic amastigotes. L-aspartyl amide derivative **21** and 28-*O*-bromoacetylbetulin **12** showed good anti-leishmanial activity at 50 μM, inhibiting 53 and 56% of the intracellular parasites, respectively (compared with 69 and 86% inhibition using axenic amastigotes in the microplate assay, respectively). At 25 μM, 28-*O*-bromoacetylbetulin **12** still had the best activity of the compounds examined showing 34% inhibition, dihydrobetulonic acid **19** and L-aspartyl amide derivative

Table 1 Anti-leishmanial activities at 50 μM on microplate assay and GI_{50} values for the most potent synthetic betulin derivatives against *Leishmania donovani* axenic amastigotes



Compound	R_1	R_2	R_3	*Inhibition (%) at 50 μM*	GI_{50} *(μM)*
1	OH	CH_2OH	CH_3-C=CH_2	35.0	
6	OH		CH_3-C=CH_2	0.0	
7	OH		CH_3-C=CH_2	8.8	
8	OH		CH_3-C=CH_2	10.5	
9	OH		CH_3-C=CH_2	13.4	
10	OH		CH_3-C=CH_2	16.6	
11	OH		CH_3-C=CH_2	59.2	
12	OH		CH_3-C=CH_2	86.0	34.9
13	OAc	OH	CH_3-C=CH_2	37.4	
14	OAc	CH_2OAc	CH_3-C=CH_2	0.0	
15			CH_3-C=CH_2	0.0	
4	OH	CHO	CH_3-C=CH_2	64.3	
2	OH	CO_2H	CH_3-C=CH_2	39.8	
16	O=	CH_2OAc	CH_3-C=CH_2	40.6	
17	O=	CHO	CH_3-C=CH_2	46.2	
18	O=	CO_2H	CH_3-C=CH_2	97.6	14.6
19	O=	CO_2H	CH_3CHCH_3	72.1	56.0
20	O=	CO_2Me	CH_3-C=CH_2	40.1	
21	O=		CH_3-C=CH_2	69.3	21.2
22	O=		CH_3-C=CH_2	0.0	
23	-	CH_2OH	CH_3-C=CH_2	13.2	
24	OH	CH=NOH	CH_3-C=CH_2	69.1	
25	=NOH	CH=NOH	CH_3-C=CH_2	72.9	22.8
26	OAc	CN	CH_3-C=CH_2	62.7	
Positive control[a]				95	
Negative control[b]				0.0	

Abbreviation: DMSO, dimethyl sulfoxide.
[a]Amphotericin B (1 μM).
[b]Culture medium+DMSO.

21 were only weakly active at this concentration. Finally, at 12.5 μM concentration, oxime derivative **25** showed the best anti-leishmanial activity with a 52% inhibition, whereas L-aspartyl amide derivative **21** was totally inactive and the rest showed only weak activity.

We have shown that by simple chemical modification, anti-leishmanial activity of ubiquitous naturally occurring triterpene, betulin, can be improved considerably. It is possible to derive relatively potent anti-leishmanial compounds with low micromolar GI_{50} values. In

Table 2 Cytotoxicity of the most potent synthetic betulin derivatives on macrophage cell line THP-1

	Inhibition of growth (%)		
Compound	*50 μM*	*25 μM*	*12.5 μM*
12	0.0	0.0	0.0
18	85.3	77.7	38.2
19	80.2	30.0	22.0
21	0.0	14.0	3.6
25	61.4	55.2	13.6

Table 3 Anti-leishmanial activities of the most potent synthetic betulin derivatives against macrophage cell line THP-1 infected with *Leishmania donovani*

	Inhibition of growth (%)		
Compound	*50 μM*	*25 μM*	*12.5 μM*
12	56.3	34.4	17.8
19	nt	20.6	14.3
21	53.3	16.0	0.0
25	nt	nt	51.5

Abbreviation: nt, not tested because the toxicity to the THP-1 cell line was >30% at that concentration.

general, carbonyl or carboxyl groups at C-3 or C-28 have a beneficial effect in anti-leishmanial inhibition activity, and these compounds can be regarded as significant lead molecules for further improvement and optimization. Further studies are required to develop more potent betulin derivatives with leishmanicidal properties, and with no toxicity in macrophage cell lines or in human host cells. Moreover, thorough early ADME, biological mechanism and animal studies are required to evaluate anti-leishmanial activity *in vivo*.

EXPERIMENTAL SECTION

Chemical syntheses of betulin derivatives screened in this study for anti-leishmanial activity are described in detail elsewhere.[18] Anti-leishmanial activities of betulin derivatives were screened using a fluorescent viability microplate assay with *L. donovani* (MHOM/SD/1962/1S-Cl2d) axenic amastigotes and alamarBlue (resazurin, AbD Serotec, Oxford, UK) as described previously.[19–21] Initial screening was carried out by assessing the inhibition of amastigote growth at 50 μM of betulin derivative. All compounds were tested at least twice in triplicate. Complete medium, both with and without dimethyl sulfoxide, was used as negative controls (0% inhibition of amastigote growth). The most potent betulin derivatives from initial screening were selected for further investigation. For these compounds, the GI_{50} value (concentration for 50% growth inhibition) was also determined, as well as screening for activity on infected macrophages. The latter assay was carried out as previously described using the retinoic acid-treated human macrophage cell line THP-1 infected with *L. donovani* expressing the luciferase gene (*Ld*:pSSU-int/LUC) at a 3:1 parasite:macrophage ratio.[17,22] Compounds (at 50, 25 and 12.5 μM) to be tested were added for 48 h, and luminescence was determined after adding a luciferase substrate and measuring in a microplate reader. Amphotericin B was included as a positive control on each plate and resulted in >90% inhibition at 1 μM. The effect of compounds on THP-1 cells alone was assessed using the alamarBlue viability assay.

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Series title and number
VTT Science 39

Title	**Synthesis of betulin derivatives against intracellular pathogens**
Author(s)	Sami Alakurtti
Abstract	Birch (*Betula* spp.) is utilized in huge quantities in the forest industry throughout the Northern Hemisphere, and low-value side-stream birch bark is burnt for energy. Outer birch bark is rich in (up to 30% dry weight) triterpene betulin, which is readily isolable by solvent extraction. Betulin can be used both in its raw form and as a starting material for more valuable products and fine chemicals. The increasing drug resistance of numerous microbes and viruses is an issue of global concern, and new inexpensive therapeutic agents are urgently needed. In this study two sets of betulin derivatives were synthesized and screened as antiviral, antileishmanial and antibacterial agents. The first set includes relatively easily synthesizable betulin derivatives, such as esters and various oxidation products. The second set includes novel heterocyclic betulin derivatives, where the triazole ring is fused by the Diels-Alder reaction to the lupane skeleton of betulin. Alphavirus *Semliki Forest virus* (SFV) is distributed by mosquitoes and infects avian and mammalian hosts. Some alphaviruses may cause fatal encephalitis in humans, although the number of cases is small. On the other hand, some alphaviruses have caused millions of cases of serious illnesses characterized by fever, rash and painful arthralgia. There is currently no efficient medical treatment against alphaviruses. In the antiviral assay, 18 betulin-derived compounds displayed good activity against SFV with low-micromolar 50% inhibitory concentration values combined with low cytotoxicity. In addition, three assayed potent and representative compounds displayed synergistic effect with modified nucleoside analogue against SFV, and similar good antiviral efficacy against another alphavirus, *Sindbis virus*. The neglected tropical disease leishmaniasis is caused by protozoan parasites belonging to the genus *Leishmania*, and is transmitted to mammalian hosts by sandflies. It is estimated that around 12 million people are currently infected, mostly in developing countries. The most severe form, visceral leishmaniasis, is fatal if not treated. There are currently several drugs marketed for the treatment of leishmaniasis. However, none of these are fully effective against *Leishmania*, and severe side effects, often requiring hospitalization, are common. In addition, parasite resistance to drugs is a serious growing problem. In the present study, the most potent betulin derivatives displayed low-micromolar 50% growth inhibition values against *L. donovani* amastigotes. Good inhibition activity was well retained against *L. donovani* amastigotes growing inside macrophages. However, in some cases betulin derivatives also showed cytotoxicity to host macrophage cell line. *Chlamydia pneumoniae* is a common Gram-negative human pathogen mainly causing mild respiratory infections, which can lead to pneumonia or bronchitis. There is also strong evidence that associates *C. pneumoniae* with other severe diseases, such as atherosclerotic cardiovascular diseases as well as some neurodegenerative diseases, such as Alzheimer's disease and multiple sclerosis. *C. pneumoniae* is susceptible to antibiotics that interfere with DNA and protein synthesis. However, its complex life cycle and its chlamydial persistence, which can last for years, as well as, importantly, the lack of specific diagnostic tests for detection of the organism in clinical samples, make the current treatment regimens unsatisfactory. Out of 32 betulin derivatives, five betulin derivatives showed high (>70% growth inhibition) antichlamydial activity against *C. pneumoniae* at 1 µM concentration. The most potent derivative displayed a remarkable 50% inhibition at nanomolar concentration.
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Julkaisun sarja ja numero
VTT Science 39

Nimeke	**Betuliinijohdannaisten syntetisointi solunsisäisiä patogeenejä vastaan**
Tekijä(t)	Sami Alakurtti
Tiivistelmä	Metsäteollisuus käyttää koivua raaka-aineenaan suunnattomia määriä. Sivuotteena syntyvä koivunkuori poltetaan energian tuotantoon. Koivun ulkokuori sisältää runsaasti betuliini-nimistä triterpeeniä jopa 30 % kuivapainostaan. Betuliini voidaan helposti eristää kuoresta liuotinuutolla. Betuliinia voidaan käyttää sellaisenaan tai lähtöaineena muille tuotteille ja hienokemikaaleille. Useiden pieneliöiden ja virusten kasvava lääkeresistenssi on maailmanlaajuinen ongelma, minkä takia on ilmennyt suuri tarve kehittää uusia lääkeaineita niitä vastaan. Tässä väitöskirjatyössä syntetisoitiin kaksi betuliinijohdannaisryhmää ja yhdisteiden tehokkuutta testattiin alfavirusten, Leishmania-suvun alkueläinten ja keuhkoklamydiabakteerin vastaisina yhdisteinä. Ensimmäinen ryhmä sisältää melko helposti valmistettavissa olevia johdannaisia, kuten betuliinin estereitä ja erilaisia hapetustuotteita. Toinen ryhmä sisältää uusia heterosyklisiä betuliinijohdannaisia, joissa triatsoli-rengas on fuusioitu betuliinin lupaanirakenteiseen hiilivetyrankaan. Alfaviruksiin kuuluva *Semliki Forest virus* (SFV) leviää moskiittojen välityksellä, ja se infektoi lintuja ja nisäkkäitä. Jotkin alfavirukset voivat aiheuttaa tappavaa aivotulehdusta, mutta nämä tapaukset ovat hyvin harvinaisia. Useimmiten alfavirukset aiheuttavat sairauskohtauksia, joiden oireet ilmenevät usein kuumeena, allergisena ihottumana ja kivuliaana niveltulehduksena. Tällä hetkellä alfaviruksia vastaan ei ole tehokasta ja turvallista lääkitystä. Kaikkiaan 18 betuliinijohdannaista osoitti alfavirusten vastaista aktiivisuutta mikromolaarisella konsentraatiolla. Lisäksi kolmella potentiaalisella ja kemialliselta rakenteeltaan erilaisella betuliinijohdannaisella oli synergistisiä alfaviruksen vastaisia vaikutuksia muokatun nukleosidijohdannaisen kanssa. Lisäksi valitut kolme johdannaista olivat aktiivisia myös toista alfavirusta, *Sinbis virusta*, vastaan. Leishmaniaasi-tautia aiheuttavat *Leishmania*-sukuun kuuluvat alkueläimet. Tauti esiintyy nisäkkäissä etenkin tropiikissa, ja sitä levittävät perhossääsket. Arvioiden mukaan 12 miljoonaa ihmistä sairastaa tällä hetkellä leishmaniaasia. Taudin vakavin muoto on sisäelinleishmaniaasi, joka on hoitamattomana tappava. Leishmaniaasia vastaan on käytössä useita lääkeaineita, mutta niiden tehokkuudessa ja turvallisuudessa on toivomisen varaa. Ne aiheuttavat usein vakavia sivuvaikutuksia, ja niiden käyttö edellyttää sairaalahoitoa. Lisäksi lääkeresistenssi on vakava ja kasvava ongelma. Tässä tutkimuksessa lupaavimmat betuliinijohdannaiset osoittivat *Leishmania donovanin* vastaisia vaikutuksia mikromolaarisilla konsentraatioilla. Johdannaisten estovaikutus pysyi hyvänä myös selvitettäessä niiden vaikutuksia syöjäsolujen sisällä kasvavia *L. donovani* -alkueläimiä vastaan. Valitettavasti osa betuliinijohdannaisista osoitti sytotoksisuutta myös itse syöjäsoluja vastaan. Keuhkoklamydia (*Chlamydia pneumoniae*) on yleinen Gram-negatiivinen bakteeri, joka aiheuttaa lieviä hengitystieinfektioita. Ne voivat pahimmillaan johtaa keuhkokuumeeseen tai keuhkoputkentulehdukseen. *C. pneumoniaen* aiheuttamilla infektiolla on myös osoitettu olevan yhteys useisiin muihin vakaviin sairauksiin, kuten sydän- ja verisuonisairauksiin, ja hermostoperäisiin sairauksiin, kuten Alzheimerin tautiin ja MS-tautiin. *C. pneumoniaen* aiheuttamia infektioita voidaan hoitaa antibiooteilla, jotka vaikuttavat bakteerin DNA- tai proteiinisynteesiin. Valitettavasti bakteerin monimutkainen elämänkierto, taudin vaikea diagnosointi ja bakteerille tyypillinen oireeton, jopa vuosia kestävä piilevänä sairautena pysyminen tekevät taudin hoidosta ongelmallista. Testisarjasta viisi betuliinijohdannaista esti huomattavasti *C. pneumoniae* -bakteerin kasvua 1 µM konsentraatiolla. Parhaalla johdannaisella 50 %:n inhibitioarvo saavutettiin nanomolaarisella konsentraatiolla.
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Synthesis of betulin derivatives against intracellular pathogens

White birches (*Betula* spp.) are utilized in huge quantities in the forest industry in northern latitudes of the world and low-value side-stream birch bark is burnt for energy. Outer birch bark is rich in (up to 30% dry weight) triterpene betulin and it can be easily isolated by solvent extraction. Betulin could be used as such or as starting material for more valuable products, fine chemicals and pharmaceuticals.

In this work two sets of betulin derivatives were synthesized and screened as antiviral, antileishmanial and antibacterial agents. First set includes relatively easily synthesizable betulin derivatives, such as esters and various oxidation products. Second set includes novel heterocyclic betulin derivatives, where triazolo ring is fused by Diels-Alder reaction to the lupane skeleton of betulin.

In the biological assays against human pathogens *Semliki Forest virus*; *L. donovani* parasites, which cause tropical disease leishmaniasis; and Gram-negative bacteria *Chlamydia pneumoniae*, several betulin derivatives displayed low-micromolar 50% growth inhibition values *in vitro*. In addition, most of the derivatives showed low cytotoxicity against the host cell lines.

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