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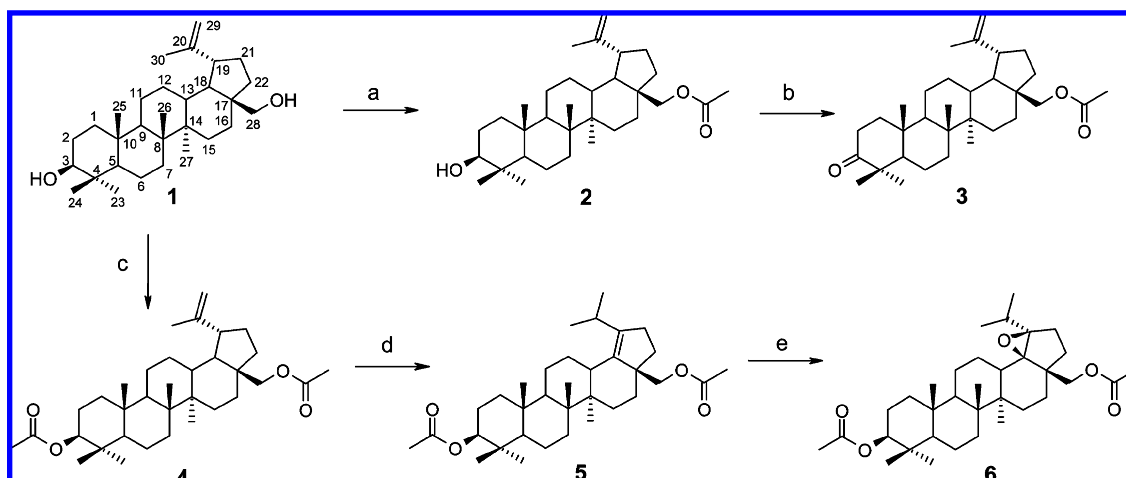
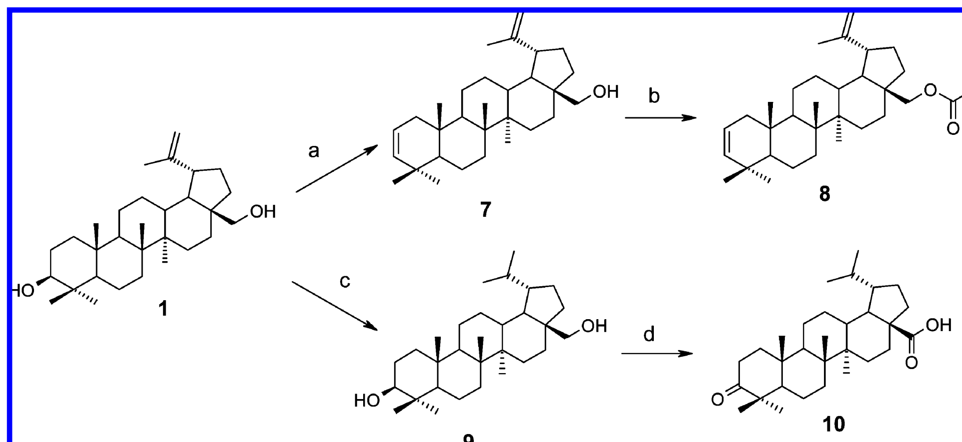
Scheme 1. Synthesis of Compounds **2**−**6**[a]



[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

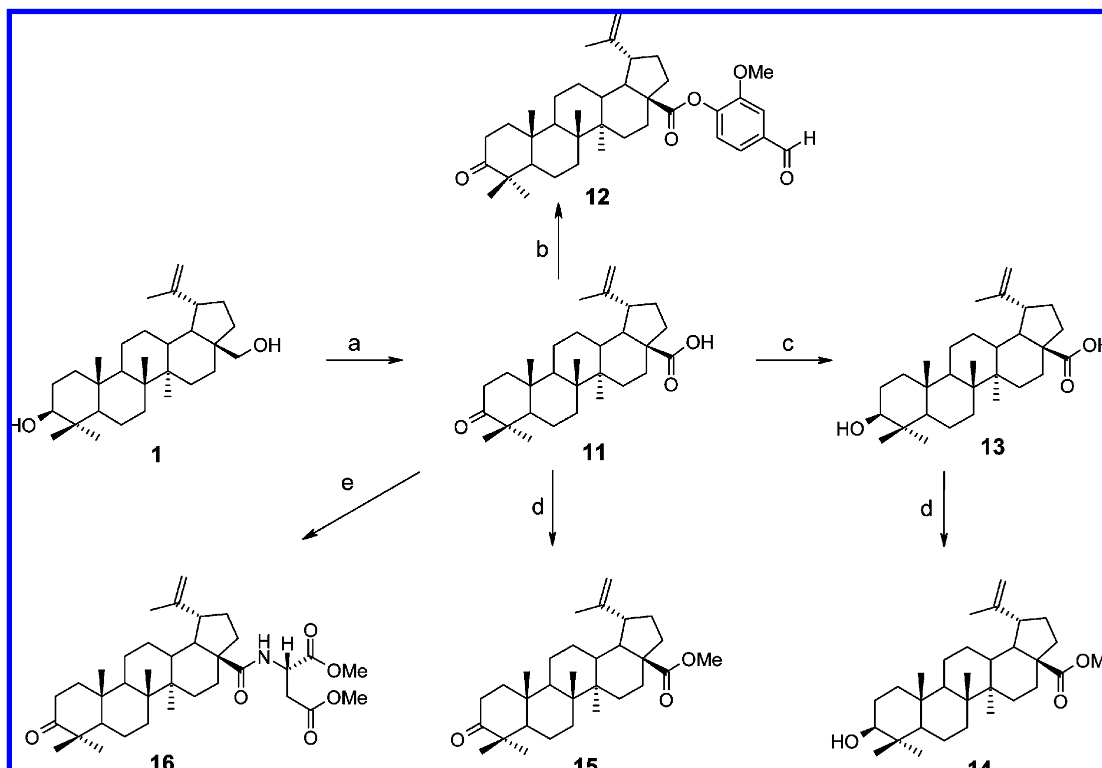
To date, only a limited number of organic small molecules have been found to inhibit alphavirus replication, and most of the existing reports concern nucleoside analogues with often nonoptimal selectivity indices (see ref 24 for review). However, the need for wider structural diversity among the inhibitors 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.

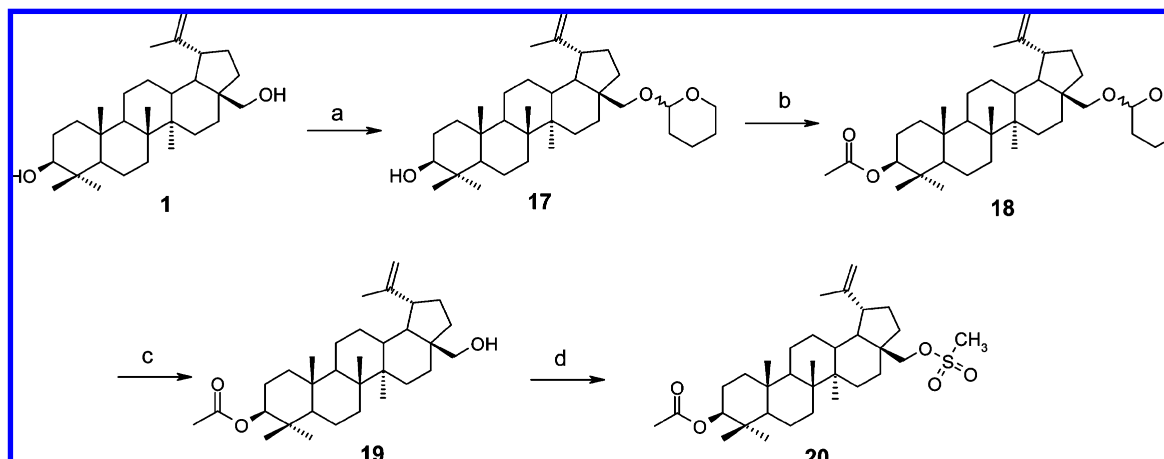
Oxidation of **1** with the Jones reagent in acetone afforded betulonic acid (11)[14] in 44% yield (Scheme 3). Subsequent

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]



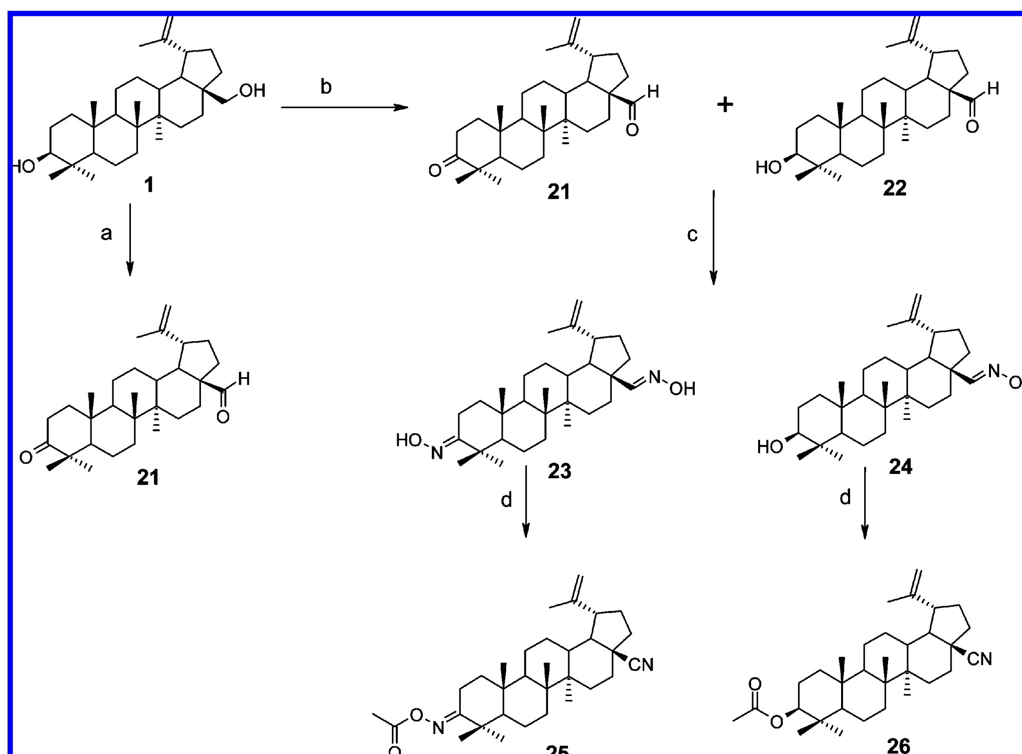
[a] Conditions: (a) DHP, PPTS, CH_2Cl_2, rt, 2 days, 30%; (b) Ac_2O, DMAP, py, CH_2Cl_2, rt, 20 h, 95%; (c) PPTS, EtOH, rt, 14 days, 94%; (d) CH_3SO_2Cl, TEA, CH_2Cl_2, 0 °C, 2 h, 99%. DHP = 3,4-dihydro-2*H*-pyran; PPTS = pyridinium *p*-toluenesulfonate.

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.

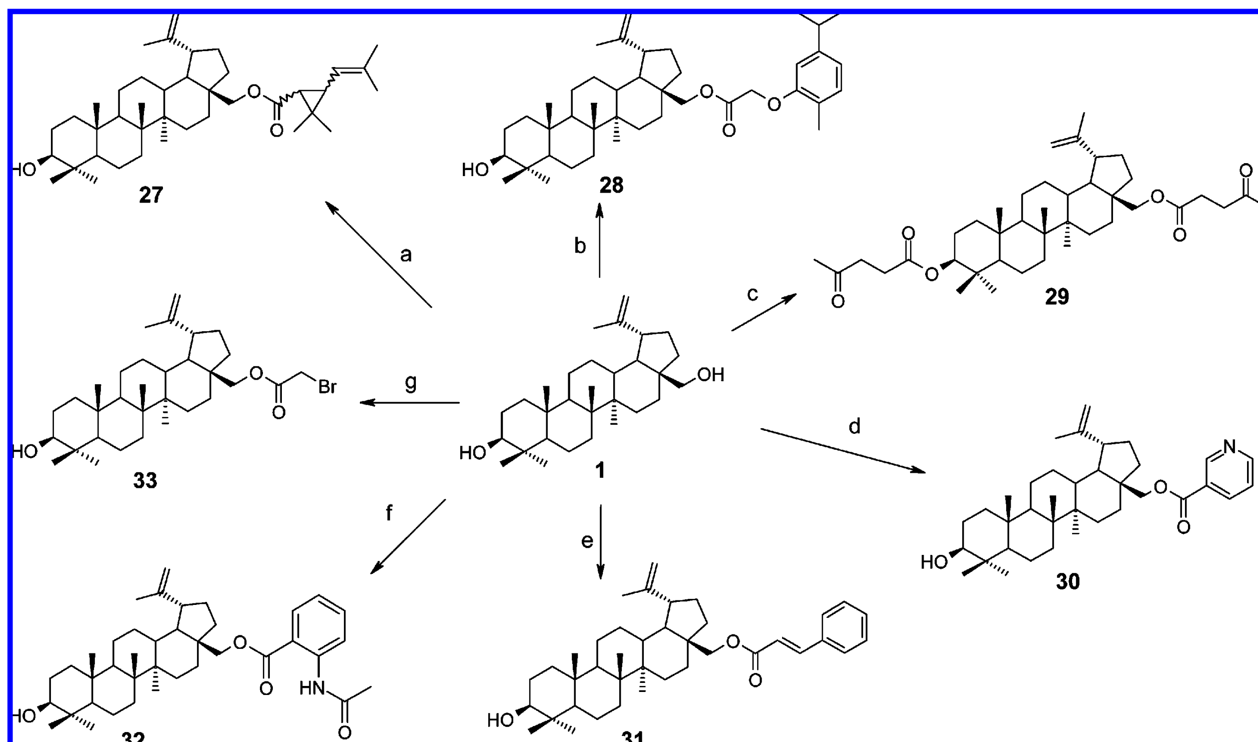
The last series of the synthetic triterpenoids commenced by treating ethyl chrysanthemate with NaOH in THF−MeOH to produce chrysanthemic acid (91% yield), which was subsequently allowed to react with oxalyl chloride in CH_2Cl_2 to give chrysanthemoyl chloride in 81% yield (Scheme 6). Chrysanthemoyl chloride 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%).

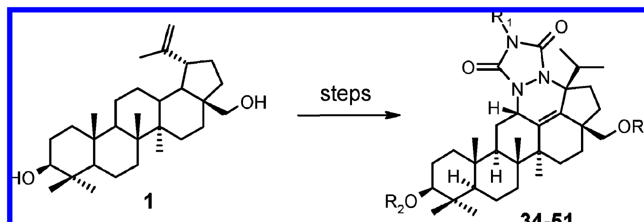
Table 2 presents the results of the primary screen, listing the antiviral and cell viability surviving fractions and the corresponding cluster number for each compound. Compounds in cluster 1 represent the best lead candidates and were thus selected for further evaluation by dose−response experiments. 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, $Ti(OPr\text{-}i)_4$, 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.

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

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	$3\text{-}NO_2$−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

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 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.

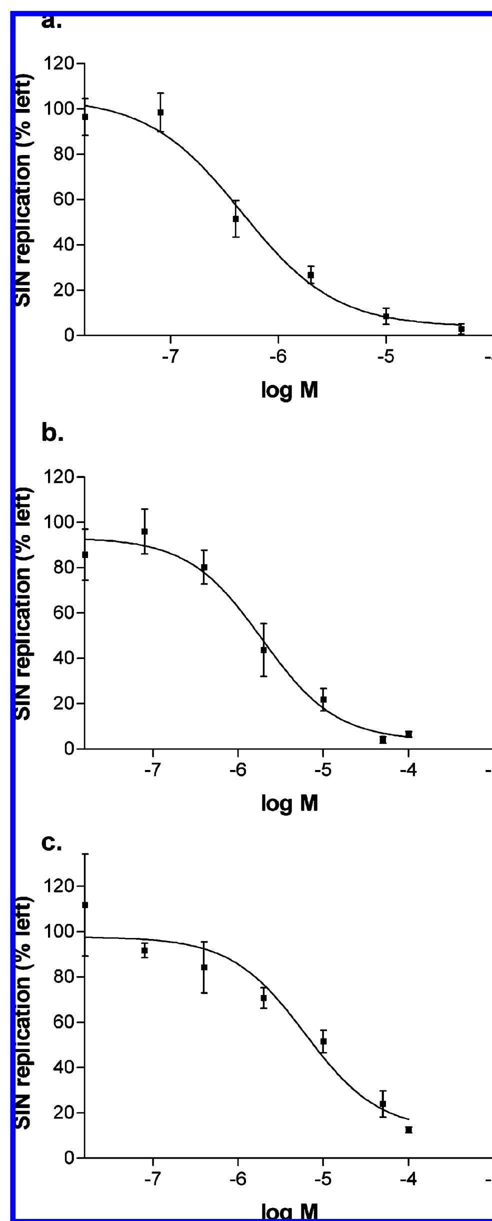


Figure 1. Dose-dependent activity of (a) betulinic acid (**13**), (b) 28-*O*-tetrahydropyranylbetulin (**17**), and (c) 4-phenyl-substituted betulin heterocycle **41** against Sindbis virus (SIN). The dose−response experiments were performed using the radiometric RNA labeling assay (see Supporting Information) 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 ($n = 4$).

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

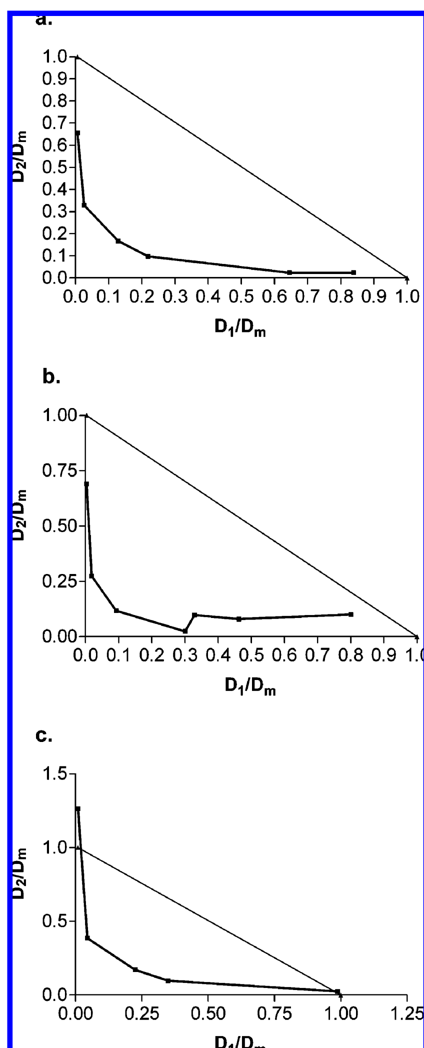


Figure 2. Synergistic activities of (a) betulinic acid (**13**), (b) 28-*O*-tetrahydropyranylbetulin (**17**), and (c) 4-phenyl-substituted betulin heterocycle **41** in combination with 3′-amino-3′-deoxyadenosine against SFV presented as isobolograms. The synergism studies were performed by titrating 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 administration to 2 h led to attenuated efficacy, yet the response was still detectable in the case of **13**.

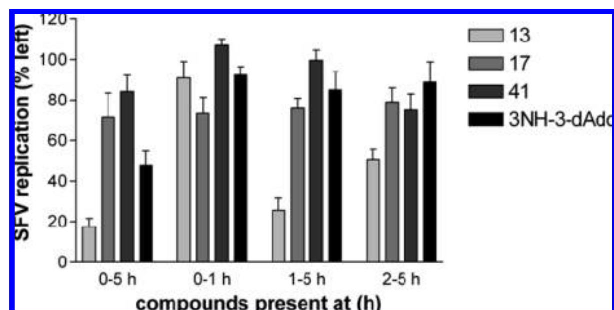


Figure 3. Effect of administration time on antiviral effect of betulin-derived compounds **13**, **17**, and **41** against SFV. Each of the compounds was present in high-multiplicity infected cultures (5 PFU/cell) either throughout the experiment (0−5 h), during virus adsorption (0−1 h), at 1−5 h, or 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 ($n = 4$).

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.

References and Notes

(1) Griffin, D. E. Alphaviruses. In *Fields Virology*, 4th ed.; Knipe, D. M., Howley, P. M., Eds.; Lippincott, Williams & Wilkins: Philadelphia, 2001; pp 917−962.
(2) Schuffenecker, I.; Iteman, I.; Michault, A.; Murri, S.; Frangeul, L.; Vaney, M. C.; Lavenir, R.; Pardigon, N.; Reynes, J. M.; Pettinelli, F.; Biscornet, L.; Diancourt, L.; Michel, S.; Duquerroy, S.; Guigon, G.; Frenkiel, M. P.; Bréhin, A. C.; Cubito, N.; Desprès, P.; Kunst, F.; Rey, F. A; Zeller, H.; Brisse, S. *PLoS Med.* **2006**, *3*, e263.
(3) Rezza, G.; Nicoletti, L.; Angelini, R.; Romi, R.; Finarelli, A. C.; Panning, M.; Cordioli, P.; Fortuna, C.; Boros, S.; Magurano, F.; Silvi, G.; Angelini, P.; Dottori, M.; Ciufolini, M. G.; Majori, G. C.; Cassone, A. *Lancet* **2008**, *370*, 1840–1846.
(4) Queyriaux, B.; Armengaud, A.; Jeannin, C.; Couturier, E.; Peloux-Petiot, F. *Lancet* **2008**, *371*, 723–734.
(5) Pialoux, G.; Gaüzère, B. A.; Jauréguiberry, S.; Strobel, M. *Lancet Infect. Dis.* **2007**, *7*, 319–327.
(6) Weaver, S. C.; Ferro, C.; Barrera, R.; Boshell, J.; Navarro, J. C. *Annu. Rev. Entomol.* **2004**, *49*, 141–174.
(7) Galgon, T.; Hoke, D.; Dräker, B. *Phytochem. Anal.* **1999**, *10*, 187–190.
(8) Nick, A.; Wright, A. D.; Rali, T.; Sticher, O. *Phytochemistry* **1995**, *40*, 1691–1695.
(9) Aponte, J. C.; Vaisberg, A. J.; Rojas, R.; Caviedes, L.; Lewis, W. H.; Lamas, G.; Sarasara, C.; Gilman, R. H.; Hammond, G. B. *J. Nat. Prod.* **2008**, *71*, 102–105.
(10) Zuo, G. Y.; Wang, G. C.; Zhao, Y. B.; Xu, G. L.; Hao, X. Y.; Han, J.; Zhao, Q. *J. Ethnopharmacol.* **2008**, *120*, 287–290.
(11) Sung, T. V.; Adam, G. *Phytochemistry* **1991**, *30*, 2717–2720.
(12) Patra, A.; Chaudhuri, S. K.; Panda, S. K. *J. Nat. Prod.* **1988**, *51*, 216–220.
(13) Asres, K.; Seyoum, A.; Veeresham, C.; Bucar, F.; Gibbons, S. *Phytother. Res.* **2004**, *19*, 557–581.
(14) Kim, D. S. H. L.; Chen, Z.; Nguyen, T.; Pezzuto, J. M.; Qiu, S.; Lu, Z.-Z. *Synth. Commun.* **1997**, *27*, 1607–1612.
(15) Alakurtti, S.; Mäkelä, T.; Koskimies, S.; Yli-Kauhaluoma, J. *Eur. J. Pharm. Sci.* **2006**, *29*, 1–13.
(16) Zhou, J.; Yuan, X.; Dismuke, D.; Forshey, B. M.; Lundquist, C.; Lee, K. H.; Aiken, C.; Chen, C. H. *J. Virol.* **2004**, *78*, 922–929.
(17) Martin, D. E.; Blum, R.; Doto, J.; Galbraith, H.; Ballow, C. *Clin. Pharmacokinet.* **2007**, *46*, 589–598.
(18) Smith, P. F.; Ogundele, A.; Forrest, A.; Wilton, J.; Salzwedel, K.; Doto, J.; Allaway, G. P.; Martin, D. E. *Antimicrob. Agents Chemother.* **2007**, *51*, 3574–3581.
(19) Lai, W.; Huang, L.; Ho, P.; Li, Z.; Montefiori, D.; Chen, C. H. *Antimicrob. Agents Chemother.* **2008**, *52*, 128–136.
(20) Gong, Y.; Raj, K. M.; Luscombe, C. A.; Gadawski, I.; Tam, T.; Chu, J.; Gibson, D.; Carlson, R.; Sacks, S. L. *Antivir. Res.* **2004**, *64*, 127–130.
(21) Pavlova, N. I.; Savinova, O. V.; Nikolaeva, S. N.; Boreko, E. I.; Flekhter, O. B. *Fitoterapia* **2003**, *74*, 489–492.
(22) Li, Y.; Jiang, R.; Ooi, L. S.; But, P. P.; Ooi, V. E. *Phytother. Res.* **2007**, *21*, 466–470.
(23) Baltina, L. A.; Flekhter, O. B.; Nigmatullina, L. R.; Boreko, E. I.; Pavlova, N. I.; Nikolaeva, S. N.; Savinova, O. V.; Tolstikov, G. A. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3549–3552.
(24) Pohjala, L.; Barai, V. N.; Azhayev, A.; Lapinjoki, S.; Ahola, T. *Antivir. Res.* **2008**, *78*, 215–222.
(25) Li, Y.; Wang, L.; Li., S.; Chen, X.; Shen, Y.; Zhang, Z.; He, H.; Xu, W.; Shu, Y.; Liang, G.; Fang, R.; Hao, X. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 8083–8088.
(26) Corey, E. J.; Suggs, J. W. *Tetrahedron Lett.* **1975**, *16*, 2647–2650.
(27) Suokas, E.; Hase, T. *Acta Chem. Scand. B* **1975**, *29*, 139–140.
(28) Suokas, E.; Hase, T. *Acta Chem. Scand. B* **1977**, *31*, 182–184.
(29) Suokas, E.; Hase, T. *Acta Chem. Scand. B* **1977**, *31*, 231–234.
(30) Sun, I.-C.; Wang, H.-K.; Kashiwada, Y.; Shen, J.-K.; Cosentino, L. M.; Chen, C.-H.; Yang, L.-M.; Lee, K.-H. *J. Med. Chem.* **1998**, *41*, 4648–4657.
(31) Petrenko, N. I.; Elantseva, N. V.; Petukhova, V. Z.; Shakirov, M. M.; Shults, E. E.; Tolstikov, G. A. *Chem. Nat. Compd.* **2002**, *38*, 331–339.
(32) Flekhter, O. B.; Nigmatullina, L. R.; Baltina, L. A.; Karachurina, L. T.; Galin, F. Z.; Zarudii, F. S.; Tolstikov, G. A.; Boreko, E. I.; Pavlova, N. I.; Nikolaeva, S. N.; Savinova, O. V. *Pharm. Chem. J.* **2002**, *36*, 484–487.

(33) Presser, A.; Hufner, A. *Monatsh. Chem.* **2004**, *135*, 1015–1022.
(34) Komissarova, N. G.; Belenkova, N. G.; Spirikhin, L. V.; Shitikova, O. V.; Yunusov, M. S. *Chem. Nat. Compd.* **2002**, *38*, 58–61.
(35) Flekhter, O. B.; Ashavina, O. Y.; Boreko, E. I.; Karachurina, L. T.; Pavlova, N. I.; Kabalnova, N. N.; Savinova, O. V.; Galin, F. Z.; Nikolaeva, S. N.; Zarudii, F. S.; Baltina, L. A.; Tolstikov, G. A. *Pharm. Chem. J.* **2002**, *36*, 303–306.
(36) Leffler, M. T.; Calkins, A. E. *Org. Synth.* **1943**, *23*, 52.
(37) Suokas, E.; Hase, T. *Acta Chem. Scand. B* **1975**, *29*, 139–140. (b) Suokas, E.; Hase, T. *Acta Chem. Scand. B* **1977**, *31*, 182–184.
(38) Cookson, R. C.; Gupte, S. S.; Stevens, I. D. R.; Watts, C. T. *Organic Syntheses*; Noland W. E., Ed.; Wiley: New York, 1988; Collect. Vol. 6, Vol. 51, pp 121−124.
(39) Moriarty, R. M.; Prakash, I.; Penmasta, R. *Synth. Commun.* **1984**, *17*, 409–413.
(40) Magden, J.; Kääriäinen, L.; Ahola, T. *Appl. Microbiol. Biotechnol.* **2005**, *66*, 612–621.
(41) Leyssen, P.; Balzarini, J.; De Clercq, E.; Neyts, J. *J. Virol.* **2005**, *79*, 1943–1947.
(42) Diamond, M. S.; Zachariah, M.; Harris, E. *Virology* **2002**, *304*, 211–221.
(43) Akihisa, T.; Ogihara, J.; Kato, J.; Yasukawa, K.; Ukiya, M.; Yamanouchi, S.; Oishi, K. *Lipids* **2001**, *36*, 507–512.
(44) Labrosse, B.; Pleskoff, O.; Sol, N.; Jones, C.; Hénin, Y.; Alizon, M. *J. Virol.* **1997**, *71*, 8230–8236.
(45) Wen, C. C.; Kuo, Y. H.; Jan, J. T.; Liang, P. H.; Wang, S. Y.; Liu, H. G.; Lee, C. K.; Chang, S. T.; Kuo, C. J.; Lee, S. S.; Hou, C. C.; Hsiao, P. W.; Chien, S. C.; Shyur, L. F. *J. Med. Chem.* **2007**, *50*, 4087–4095.
(46) Dargan, D. J.; Subak-Sharpe, J. H. *J. Gen. Virol.* **1985**, *66*, 1771–1784.
(47) Zhou, J.; Chen, C. H.; Aiken, C. *J. Virol.* **2006**, *80*, 12095–12101.
(48) Adamson, C. S.; Ablan, S. D.; Boeras, I.; Goila-Gaur, R.; Soheilian, F.; Nagashima, K.; Li, F.; Salzwedel, K.; Sakalian, M.; Wild, C. T.; Freed, E. O. *J. Virol.* **2006**, *80*, 10957–10971.
(49) Tamberg, N.; Lulla, V.; Fragkoudis, R.; Lulla, A.; Fazakerley, J. K.; Merits, A. *J. Gen. Virol.* **2007**, *88*, 1225–1230.
(50) Rice, C. M.; Levis, R.; Strauss, J. H.; Huang, H. W. *J. Virol.* **1987**, *61*, 3809–3819.
(51) Pohjala, L.; Tammela, P.; Samanta, S. K.; Yli-Kauhaluoma, J.; Vuorela, P. *Anal. Biochem.* **2007**, *362*, 221–228.
(52) Greco, W. R.; Bravo, G.; Parsons, J. C. *Pharmacol. Rev.* **1995**, *47*, 331–385.

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