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ANTI-AIDS AGENTS. 32.[1] SYNTHESIS AND ANTI-HIV ACTIVITY OF BETULIN DERIVATIVES

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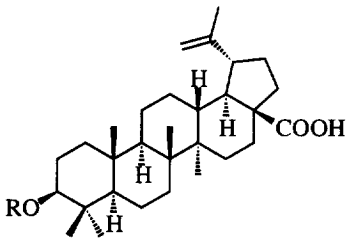
Abstract. Eleven betulin derivatives were prepared and evaluated for anti-HIV activity in H9 lymphocytes. Compound **4** was found to be the most active with EC_{50} and TI values of 0.00066 μM and 21,515, respectively.

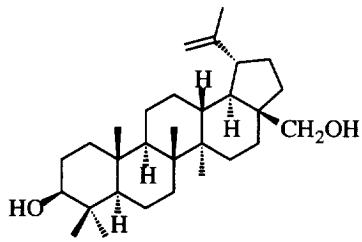
Introduction

Acquired immunodeficiency syndrome (AIDS) is one of the most frightening diseases worldwide. Extensive effort has been spent to discover therapeutic agents targeting the etiological agent of AIDS, human immunodeficiency virus type 1 (HIV-1).[2,3] Currently, the FDA has approved eleven anti-AIDS drugs including nucleoside reverse transcriptase inhibitors,[4] non-nucleoside reverse transcriptase inhibitors (NNRTIs),[5] as well as protease inhibitors.[6] Their benefits are also associated with side effects and drug resistance, which prompted a need for new agents with novel mechanisms of action.[7,8]

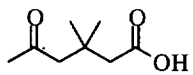
Our continuing research to find potent anti-HIV agents has discovered betulinic acid (**1**) from *Syzigium claviflorum* (Myrtaceae).[9] Previous studies in our laboratory showed that the esterification of betulinic acid (**1**) with 3',3'-dimethylglutaryl (in compound **2**) and 3',3'-dimethylsuccinyl groups produced remarkable activity.[10,11] To further explore the role of the acyl substituent, we introduced 3',3'-dimethylglutaryl and other 3'-



1 R = H (Betulinic acid)



3 (Betulin)

2 R = 

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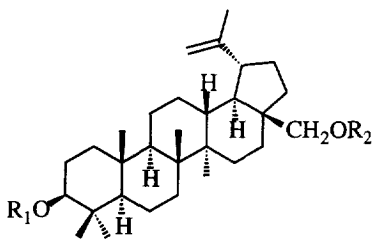
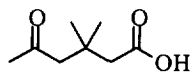
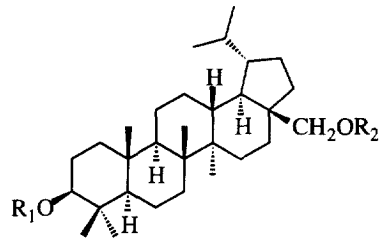
substituted glutaryl side chains at both the C-3 and C-28 positions of betulin (**3**), a natural product structurally related to betulinic acid (**1**). In addition, A ring modifications were carried out and will be discussed.

Results and Discussion

Compounds **3–6**, **8–14**, and AZT were examined for anti-HIV activity in H9 lymphocytes as shown in Table 1. Betulin (**3**) with a C-28 hydroxy group was less potent than betulinic acid (**1**) with a C-28 carboxylic acid. However, adding two 3',3'-dimethylglutaryl esters to betulin (**3**) gave **4**, which showed significantly enhanced activity with an EC_{50} value of 0.00066 μM and a remarkably high therapeutic index (TI) value of



4 $R_1 = R_2 =$ 



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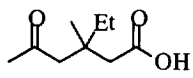
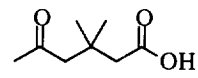
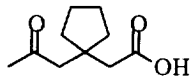
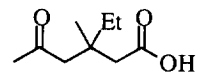
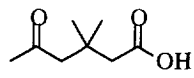
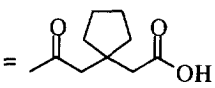
5 $R_1 = R_2 =$ 

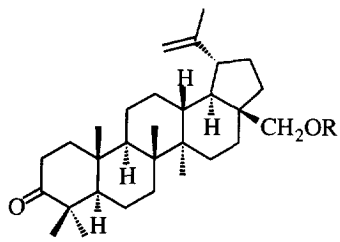
8 $R_1 = R_2 =$ 

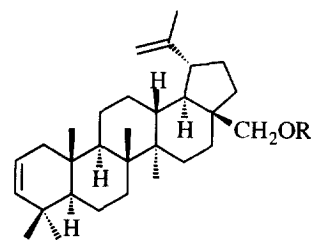
6 $R_1 = R_2 =$ 

9 $R_1 = R_2 =$ 

11 $R_1 = H, R_2 =$ 

10 $R_1 = R_2 =$ 





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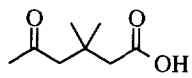
12 R = 

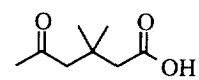
14 R = 

Table 1. Anti-HIV Activities of Betulin and Related Derivatives

Compound	Anti-HIV Activity* EC_{50} (μM)	Cytotoxicity* IC_{50} (μM)	Therapeutic Index (TI = IC_{50}/EC_{50})
1	1.4	13.0	9.3
2	0.0023	4.5	1,974
3	23	43.7	1.9
4	0.00066	14.2	21,515
5	0.0053	18.4	3,476
6	0.077	20.5	267
7	NT	NT	NT
8	0.0047	10.6	2,253
9	0.075	18.7	248
10	0.58	21.6	37
11	3.6	28.2	7.8
12	10.0	29.2	2.9
13	11.9	31.9	2.7
14	5.4	28.3	5.2
AZT	0.015	500	33,333

* all the data represented as an average of at least two experiments.

NT: not tested

21,515. Because **4** was about threefold more potent and had a tenfold higher TI than **2**, the C-28 acyl side chain led to improved activity. When the 3' substitution was changed to 3'-ethyl-3'-methyl (**5**) or 3',3'-tetramethylene (**6**), the EC_{50} values were still in the nanomolar range, but the compounds were less active compared with compound **4**. Saturation of the C20-C29 double bond in **4** gave **8** and led to a 7- and 9-fold drop in activity and in TI, respectively. Similarly, the dihydro compounds **9** and **10** showed less inhibition than the unsaturated **5** and **6**. Compounds **6** and **10**, both containing 3',3'-tetramethylene glutaryl groups, exhibited the least activity and lowest TI values among the two series of compounds (**4–6** and **8–10**, respectively). From these data, additional bulk at the 3' position does not appear favored for anti-HIV activity .

Compound **11** is esterified only at the C-28 position and is sixfold more potent compared with compound **3**. However, **11** is much less active than **4**, confirming the importance of the 3-acyl side chain for increased activity. Replacing the 3-hydroxy group of **11** with a ketone decreased activity further (compare **11** and **12**).

Dehydration of betulin's A ring gave the unsaturated **13**, which had a slightly improved EC_{50} compared with **3**. The acylated product, **14**, displayed increased anti-HIV activity, but perhaps due to the lack of a 3-acyl moiety, the EC_{50} of **14** was only 5.4 μM.

In conclusion, the diacylated betulin derivative **4** showed remarkable anti-HIV activity even greater than that of the betulinic acid derivative **2**. The C-28 acyl side chain could further increase anti-HIV activity as well as TI, whereas a C-3 acyl side chain was essential for optimal activity. The 3',3'-dimethyl glutaryl group gave the best activity among three different 3',3'-disubstituted esters. In addition, betulin derivatives (**4–6**) were more potent than their corresponding dihydrobetulin compounds (**8–10**). Further mechanism of action studies for compound **4** are ongoing and will be published at a future date.

Experimental procedure

A general synthesis for compounds **4–6** and **8–10**: Betulin or dihydrobetulin was heated overnight at 95 °C with 6 equiv of the appropriate anhydride in anhydrous pyridine in the presence of 4-(dimethylamino)pyridine. When TLC indicated complete consumption of starting material, the reaction solution was diluted with EtOAc and washed with 10% HCl solution. The EtOAc layer was then dried over $MgSO_4$ and subjected to column chromatography.

Compounds **11** and **14** were prepared by heating betulin and compound **13** overnight at 40 °C with 2 equiv of 3,3-dimethylglutaryl anhydride in anhydrous pyridine in the presence of 4-(dimethylamino)pyridine, followed by a similar workup as for compounds **4–6** and **8–10**. The residues were purified by column chromatography.

Compound **12** was synthesized by stirring compound **11** with 1.5 equiv of pyridium chlorochromate in CH_2Cl_2 at room temperature. After 2 h, the black reaction mixture was diluted with Et_2O and filtered through a short pack of Florisil. The filtrate was concentrated and chromatographed [*n*-hexane:acetone (4:1)] to yield compound **12** in a 72% yield.

A solution of betulin and triphenyl phosphine (4 equiv) in dry THF was added dropwise to diethyl azodicarboxylate (4 equiv) in an ice bath and N_2 atmonphere. The reaction solution was stirred for 12 h. After removing THF in vacuum, the residue was chromatographed with *n*-hexane:EtOAc (15:1) as eluent to afford compound **13**.

Anti-HIV Assay: The biological evaluation of HIV-1 inhibition was carried out according to established protocols.[9-11] The T cell line, H9, was maintained in continuous culture with complete medium (RPMI 1640 with 10% fetal calf serum supplemented with L-glutamine at 5% CO_2 and 37 °C). Aliquots of this cell line were only used in experiments when in log-phase growth. Test samples were first dissolved in dimethyl sulfoxide. The

following final drug concentrations were routinely used for screening: 100, 20, 4, and 0.8 μg/mL. For active agents, additional dilutions were prepared for subsequent testing so that an accurate EC_{50} value (defined below) could be achieved. As the test samples were being prepared, an aliquot of the H9 cell line was infected with HIV-1 (IIIB isolate) while another aliquot was mock-infected with complete medium. The stock virus used for these studies typically had a $TCID_{50}$ value of 10^4 Infectious Units/mL. The appropriate amount of virus for a multiplicity of infection (moi) between 0.1 and 0.01 Infectious Units/cell was added to the first aliquot of H9 cells. The other aliquot only received culture medium, and these mock-infected cells were used for toxicity determinations (IC_{50}, defined below). After a 4 h incubation at 37 °C and 5% CO_2, both cell populations were washed three times with fresh medium and then added to the appropriate wells of a 24 well-plate containing the various concentrations of the test drug or culture medium (positive infected control/negative drug control). In addition, AZT was also assayed during each experiment as a positive drug control. The plates were incubated at 37 °C and 5% CO_2 for 4 days. Cell-free supernatants were collected on Day 4 for use in our in-house p24 antigen ELISA assay. P24 antigen is a core protein of HIV and therefore is an indirect measure of virus present in the supernatants. Toxicity was determined by performing cell counts by a Coulter Counter on the mock-infected H9 cells which had either received culture medium (no toxicity), test sample, or AZT. If a test sample had suppressive capability and was not toxic, its effects were reported in the following terms: IC_{50}, the concentration of test sample which was toxic to 50% of the mock-infected H9 cells; EC_{50}, the concentration of the test sample which was able to suppress HIV replication by 50%; and Therapeutic Index (TI), the ratio of IC_{50} to EC_{50}.

Acknowledgements

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References and Note

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12 Spectroscopic data for representative compound **2**: yield 75% (after chromatography with $CHCl_3$: acetone [19:1]); an off-white amorphous powder; $[\alpha]^{25}_D$ +21.9 (c= 0.2, $CHCl_3$); ^1H NMR ($CDCl_3$): 0.84, 0.85, 0.86, 0.97, 1.03 (3H each, all s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.14 (12H, s; 3'-$(CH_3)_2$ and 3"-$(CH_3)_2$), 1.68 (3H, s; 20-CH_3), 2.42-2.50 (9H, m, H_2-2', 2", 4', 4" and H-19), 3.86, 4.30 (1H each, both d, J = 11.1 Hz; H_2-28), 4.49 (1H, dd, J = 5.2, 11.4 Hz; H-3), 4.59, 4.69 (1H each, both br s; H_2-29). Anal. calcd for $C_{44}H_{70}O_8 \cdot 1/2H_2O$: C 71.80, H 9.72; found C 71.73, H 9.66.