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Anti-AIDS Agents. Part 36:[1] 17-Carboxylated Steroids as Potential Anti-HIV Agents

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Abstract—In our search for novel anti-HIV agents, seven 17-carboxylated steroid derivatives were synthesized and evaluated as potential anti-HIV agents. Compound **13** exhibited potent anti-HIV activity in acutely infected H9 lymphocytes with EC_{50} and therapeutic index values of 0.8 μM and 300, respectively.

Introduction

HIV infection leads to the disease called acquired immunodeficiency syndrome (AIDS), which has been a life-threatening health problem since 1981.[2] The magnitude of the AIDS pandemic and the need for more effective therapy have prompted much exploration of new potent anti-HIV agents. Many strategies, including FDA approved anti-HIV reverse transcriptase (RT) and protease agents, have been investigated to prevent and/or control the spread of virus infection.[3] Current therapy can effectively control plasma viremia, but the virus is suppressed rather than eradicated in HIV-infected individuals.[4–6] To circumvent the existing therapeutic difficulties, the discovery of new structures with significant bioactivity is meaningful, not only to obtain more active agents, but also to find new molecular targets for drug intervention.

Steroids show a broad spectrum of biological activities and numerous steroidal drugs are widely used in the clinic. However, the anti-HIV activity of steroids is still a new research area. Recent studies revealed that some steroids (Fig. 1) are significantly active in inhibiting HIV infection; for example, suberosol (**1**),[7] isolated from *Polyalthis suberosa*, was found to show anti-HIV replication activity in H9 lymphocyte cells with an EC_{50} value of 3 μg/mL. In addition, some sulfated sterols **2**–**5**[8] obtained from marine invertebrates showed EC_{50} values of 3–13 μM against HIV-1 and 2–8 μM against HIV-2. Cosalane **6** and its analogue **7**[9] displayed EC_{50} values of 5.1 and 0.55 μM, respectively, as inhibitors of HIV-1_{RF}. More interestingly, a number of structurally more simple synthetic keto/epoxy steroids (**8**) have also been reported to be effective HIV-1 Tat inhibitors, with IC_{50} values at micromolar concentrations and therapeutic index values of 1.6–6.5.[10]

During routine anti-HIV screening, we recently discovered that some 17-carboxylated steroid derivatives from our compound library show anti-HIV activity. We report herein on the synthesis and anti-HIV activity of this structurally unique class of anti-HIV agents.

Chemistry

The 17-carboxylated steroid derivatives **10**, **11** and **12** were synthesized by literature methods[11] as presented in Scheme 1, but as shown in Experimental, the reported tedious isolation of **11** was improved. After completing the Oppennauer oxidation of **10**, if the excess cyclohexanone and the cyclohexanol formed in the reaction are removed exhaustively, the product **11** can be easily isolated. In addition, we include spectral data of **10**–**12** that are not given in the literature. The 17β-carboxylic acid (**12**) was reacted either with oxalyl chloride or thionyl chloride to give the corresponding

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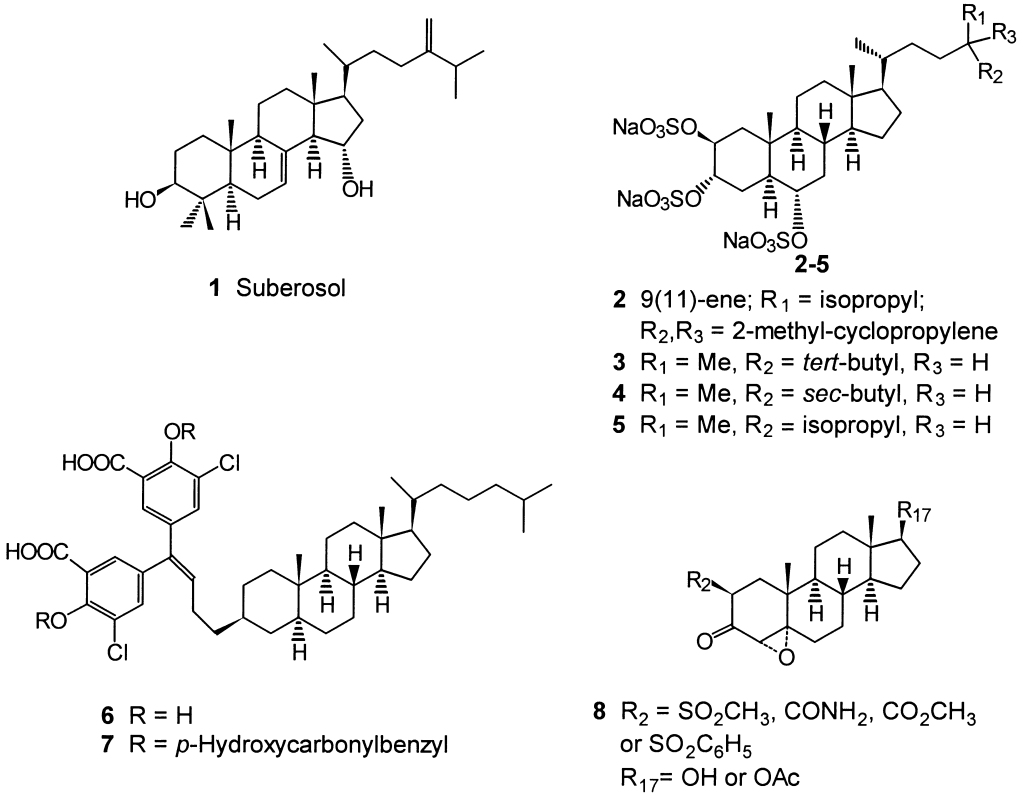
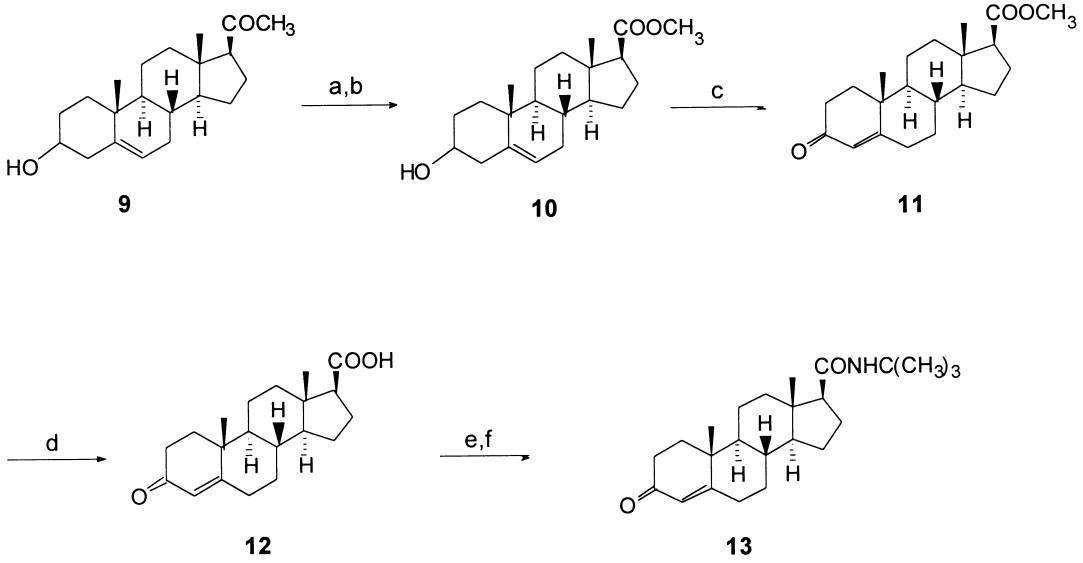


Figure 1. Anti-HIV steroids.

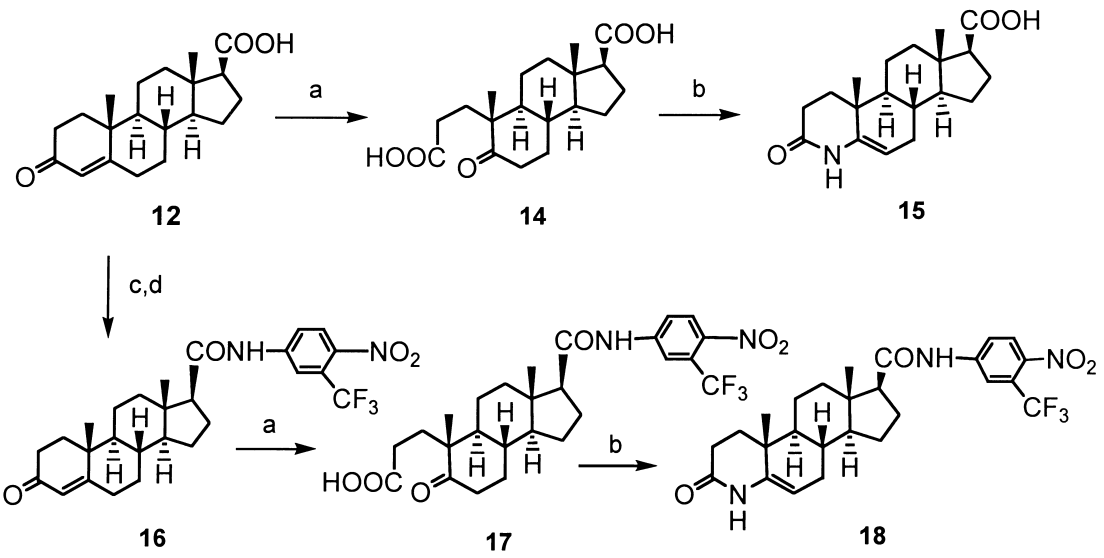
acid chloride, which was further treated in situ with *tert*-butylamine to afford the 17β-caboxyamide (**13**). The successful chlorination with thionyl chloride depends on appropriate reaction conditions including reaction medium, concentration of thionyl chloride and reaction temperature (see Experimental). A reaction temperature over 65°C and a higher concentration of thionyl chloride will lead to destruction of the product.

Scheme 2 shows the synthetic route to **14**–**18**. The A-nor-3,5-seco acid **14** was easily prepared by oxidative cleavage of 3-keto-Δ^4-17β-carboxylic acid **12**.[12] Although the synthesis of enelactam **15** is known in the literature,[12] prior methods needed high temperature and resulted in low yields. We found that the ring closure of various A-nor-3,5-seco acids, including **14**, could be smoothly completed in high yield by treating of substrate with ammonium acetate in acetic acid at reflux.

Compound **18** was synthesized previously by us.[13] As shown in Scheme 2, compound **12** was converted into the ene-lactam (**18**) via chlorination of the 17β-carboxyl



Scheme 1. (a) I_2/Py; (b) CH_3ONa/CH_3OH; (c) Al(*i*-PrO)$_3$, cyclohexanone/toluene; (d) KOH/CH_3OH; (e) $SOCl_2$/toluene; (f) $(CH_3)_3CNH_2$/toluene.



Scheme 2. (a) $NaIO_4$, $KMnO_4$, Na_2CO_3/*t*-BuOH; (b) CH_3COONH_4/CH_3COOH; (c) $SOCl_2$/toluene; (d) 3-trifluoromethyl-4-nitroaniline/toluene.

Table 1. Anti-HIV activity of **10**–**18** in acutely infected H9 lymphocytes

Compound	IC_{50} (μM)[a]	EC_{50} (μM)[b]	TI[c]
10	39.4	18.0	2.2
11	15.3	8.47	1.8
12	67.3	No suppression	
13	240	0.8	300
14		No suppression	
15	14.1	3.85	3.67
18	4.8	3.09	1.55
AZT	1875	0.045	41,667

[a] Concentration that inhibits uninfected H9 cell growth by 50%.
[b] Concentration that inhibits viral replication by 50%.
[c] TI = therapeutic indec IC_{50}/EC_{50}.

with thionyl chloride in toluene, amidation in situ with 3-trifluoromethyl-4-nitroaniline, oxidative cleavage of ring A with $NaIO_4$-$KMnO_4$ and Na_2CO_3 in refluxing *tert*-BuOH, and ring closure of the A-nor-3,5-seco acid with ammonium acetate in acetic acid at high temperature. These improved procedures are easy to carry out and produce high yields.

Anti-HIV activity

Table 1 shows the anti-HIV activities of **10**–**15** and **18** with AZT included in the same experiment for comparison. The results of the HIV growth inhibition assay indicated that 3-ketone-4-ene 17-(*N*-*tert*-butyl) amide (**13**) displayed potent anti-HIV activity in acutely infected H9 lymphocytes with an EC_{50} value of 0.8 μM and a good therapeutic index (TI) of 300. However, replacing the 17-amide with a 17-carboxylic acid methyl ester gave compound **11**, which was 10-fold less active (EC_{50} = 8.47 μM) than **13**. Furthermore, compound **11** displayed a greater than 160-fold lower TI value. In particular, a hydrophilic carboxylic group at 17-position (**12**) abolished the anti-HIV activity. Thus, in the 3-ketone 4-ene system, the substituents at the 17-position have great effects on the anti-HIV activity and 17-amide derivatives seem to be beneficial for increased anti-HIV activity and selectivity. Compound **10** was also examined, it has the same 17-acid methyl ester group as compound **11**, but different structural features in the A-B rings (a 3β-hydroxy-5-ene moiety). Both compounds had similar therapeutic index values, although compound **11** showed slightly greater anti-HIV activity with an EC_{50} value about twofold lower than that of **10**. Opening the A ring to give compound **14** abolished the anti-HIV activity. Inserting a N atom at 4-position formed 3-oxo-17β-carboxylated 4-azasteroids **15** and **18**; these two 4-aza compounds had similar therapeutic index values, but were not comparable with compound **13**. Thus, modification of the 17-position seemed to be more meaningful than that of the A-B rings.

In summary, the discovery of the considerable anti-HIV activity of steroid 3-ketone-4-ene 17β-amide (**13**) has significance in opening a new research area into a novel structural class of anti-HIV agent. Therefore, we have selected **13** as a promising, new lead compound for further development of anti-HIV agents. The mechanism and pharmacological action of this compound are currently under investigation.

Experimental

Melting points were obtained in capillary tubes and are uncorrected. The IR spectra were obtained in potassium bromide discs on a Perkin–Elmer 783 spectrophotometer. The ^1H NMR spectra were determined on a JNM-FX-400 spectrometer with TMS as internal reference. The MS spectra were recorded on an HP5989A instrument. Elemental analyses (C, H, and N) were performed by Shanghai Institute of Organic Chemistry. Specific rotations were measured on a WZZ-2 instrument. All reactions were monitored by TLC on silica gel plate.

Methyl 3β-Hydroxy-5-androstene-17β-carboxylate (10). Iodine (43.33 g, 0.17 mol) was added portionwise over 30 min to a solution of pregnenolone **9** (50.00 g, 0.16 mol) in 134 mL of pyridine at 90°C under stirring. After refluxing 1.5 h, the reaction mixture was cooled to room temperature. Filtration, washing with a pyridine and petroleum ether, and drying in air afforded 82.40 g of crude pregnonolone 21-pyridinium iodide as a yellow-tan powder, mp 224–226°C. (lit.[11] mp 228–230°C). The crude pyridinium iodide (66.96 g, 0.13 mol) was added portionwise over 30 min to a refluxing solution of sodium methoxide (9.18 g, 0.17 mol) in 200 mL of methanol. The dark solution was refluxed for another 1 h. After cooling to 45°C, 200 mL of water was added slowly under stirring followed by neutralization with 13 mL of 6 N HCl solution. Filtration, washing with 1:1 methanol:water, and drying gave 41.67 g of crude methyl 3β-hydroxy-5-androstene-17β-carboxylate (**10**), mp 170–174°C (97.25% from pregnenolone **9**). Crystallization from EtOAc offered the analytical sample, mp 174–176°C. MS *m/z*: 332 (M^+), 313; ^{1}H NMR ($CDCl_3$): δ0.67 (s, 3H, C_{13}-CH_3), 1.01 (s, 3H, C_{10}-CH_3), 3.67 (s, 3H, -OCH_3), 5.34–5.35 (m, 1H, 6-H)

Methyl 3-Oxo-4-androstene-17β-carboxylate (11). A solution of crude **10** from above reaction (5.00 g, 15.04 mmol) and 6.25 mL of cyclohexanone in 50 mL of toluene was heated until 20 mL of distillate was removed. After cooling to 70°C, Al(i-PrO)$_3$ (1.44 g, 7.05 mmol) was added and the mixture was refluxed for 4 h followed by reduced distillation in vacuum (20 mm Hg, 170°C) to exhaustively remove the solvent, the excess cyclohexanone, and the cyclohexanol formed from the reaction. After cooling to room temperature, the residue solidified when a little methanol was added. Filtration and washing with acetone gave 3.51 g of methyl 3-oxo-4-androstene-17β-carboxylate (**11**) as white crystals (70.62%), mp 126–128°C. MS *m/z*: 330 (M^+); ^{1}H NMR ($CDCl_3$): δ 0.69 (s, 3H, C_{13}-CH_3), 1.17 (s, 3H, C_{10}-CH_3), 3.66 (s, 3H, -OCH_3), 5.72 (s, 1H, 4-H).

3-Oxoandrost-4-ene-17β-carboxylic acid (12). 18 mL of 10% aq KOH was added to a solution of methyl 3-oxo-4-androstene-17β-carboxylate (**11**) (6.91 g, 0.02 mol) in 56 mL of MeOH. The reaction mixture was refluxed for 5 h and poured into 300 mL of ice-water. Filtration and washing with water gave, on drying, 6.63 g of 3-oxoandrost-4-ene-17β-carboxylic acid (**12**) (100%), mp 244–250°C. MS (*e/z*): 316 (M^+); ^{1}H NMR ($CDCl_3$): δ 0.77 (s, 3H, C_{13}-CH_3), 1.28 (s, 3H, C_{10}-CH_3), 5.73 (s, 1H, 4-H).

***N*-*tert*-Butyl-3-oxoandrost-4-ene-17β-carboxamide (13).** 3-Oxoandrost-4-ene-17β-carboxylic acid (**12**) (1.43 g, 4.52 mmol) was dissolved in 100 mL of dry toluene; 25 mL of toluene was distilled and pyridine (2.43 mL) was added under cooling with an ice-water bath. After stirring a few min, thionyl chloride (0.794 mL, 10.88 mmol) was added dropwise at 5°C. The mixture was stirred at the same temperature for 3 h. After removing excess thionyl chloride by distillation under reduced pressure at 60°C; *tert*-butylamine (4.15 mL, 39.49 mmol) was added at 5°C, then stirred at the same temperature for 0.5 h. The reaction mixture was diluted with EtOAc and washed with water. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated to give *N*-*tert*-Butyl-3-oxoandrost-4-ene-17β-carboxamide (**13**), 1.67 g; mp 198–202°C. Recrystallization from acetone gave an analytical sample 1.50 g (89.34%); mp 218–219°C. Anal. calcd for $C_{24}H_{37}NO_2$: C, 77.58; H, 10.04; N, 3.77. Found: C, 77.37; H, 10.26; N, 3.61; MS: *m/z* 371 (M^+), 271; IR (KBr): 3420, 2920, 1725, 1500 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.72 (s, 3H, C_{13}-CH_3), 1.20 (s, 3H, C_{10}-CH_3), 5.10 (s, 1H, N-H), 5.70 (s, 1H, 4-H).

17β-Carboxy-5-oxo-A-nor-3,5-secoandrostan-3-oic acid (14). 12 mL of sat aq sodium carbonate was added to a solution of 3-oxoandrost-4-ene-17β-carboxylic acid (**12**) (2.00 g, 6.36 mmol) in 40 mL of *tert*-butyl alcohol. Under refluxing, a solution of $NaIO_4$ (11.52 g, 53.86 mmol) and $KMnO_4$ (64.00 mg, 0.40 mmol) in 30 mL of water was added dropwise. The reaction mixture was refluxed for 3 h then cooled to room temperature. After filtration and washing with water, the combined filtrates were concentrated under reduced pressure to remove most of *tert*-butyl alcohol. The aq residue was cooled and acidified with 6 N HCl to pH 2. The product was extracted with CH_2Cl_2, washed with water and dried over anhydrous Na_2SO_4. Filtration and removal of solvent gave the crude product. Crystallization from EtOAc gave 17β-carboxy-5-oxo-A-nor-3,5-secoandrostan-3-oic acid (**14**) 1.50 g (70.42%), mp 190–193°C (lit.[12] mp 189–192°C).

3-Oxo-4-aza-5-androstene-17β-carboxylic acid (15). To a solution of 17β-carboxy-5-oxo-A-nor-3,5-secoandrostan-3-oic acid (**14**) (0.50 g, 1.50 mmol) in glacial acetic acid (20 mL) was added ammonium acetate (1.15 g, 14.95 mmol). The reaction mixture was refluxed for 10 h and cooled to room temperature. Distillation under reduced pressure removed most of acetic acid. The residue as poured into 100 mL of ice-water. Filtration, washing with water and drying gave 3-oxo-4-aza-5-androstene-17β-carboxylic acid (**15**) 0.385 g (81.6%), mp >310°C; Elemental analysis for $C_{19}H_{27}NO_3$ was verified; MS: *m/z* 317 (M^+), 318 ($M^+ + 1$), 302, 274, 256; IR (KBr): 3210, 2918, 1730, 1680, 1650, 1445 cm^{-1}; ^{1}H NMR (pyridine-d_5): δ 1.0 (s, 3H, C_{13}-CH_3), 1.12 (s, 3H, C_{10}-CH_3), 5.21 (m, 1H, 6-H).

***N*-(3′-Trifluoromethyl-4′-nitrophenyl)-3-oxoandrost-4-ene-17β-carboxamide (16).** Prepared as for **13** with the substitution of 3-trifluoromethyl-4-nitroaniline for *tert*-butyl and stirred overnight at room temperature. After dilution with EtOAc and washing with water, the organic layer was dried over anhydrous Na_2SO_4. Removal of the solvent and crystallization from acetone gave *N*-(3′-trifluoromethyl-4′-nitrophenyl)-3-oxoandrost-4-ene-17β-carboxamide (**16**) 0.57 g (71.50%), mp 226–228°C; Elemental analysis for $C_{27}H_{31}N_2O_4F_3$ was verified; MS: *m/z* 504 (M^+); IR (KBr): 3300, 2940, 1700, 1660, 1520 cm^{-1}; ^{1}H NMR ($CDC1_3$): δ 0.80 (s, 3H, C_{13}-CH_3), 1.25 (s, 3H, C_{10}-CH_3), 5.75 (s, 1H, 4-H), 7.97 (d, $J = 8.76$ Hz, 1H, Ar-H), 8.07 (s, 1H, Ar-H), 8.10 (s, 1H, N-H), 8.14 (d, $J = 8.76$ Hz, 1H, Ar-H).

17β-[(*N*-3′-Trifluoromethyl-4′-nitrophenyl)aminocarbonyl]-5-oxo-A-nor-3,5-secoandrostan-3-oic acid (17). Prepared from **16** in same manner as **14** from **12**. Removal of the solvent gave crude product (**17**) 0.52 g (94.70%); mp 198–220°C. Recrystallization from EtOAc afforded an analytical sample, 0.33 g, mp 240–242°C; Elemental analysis for $C_{26}H_{31}N_2O_6F_3$ was verified; MS: *m*/*z* 524(M^+), 506, 319, 273 (base peak); IR (KBr): 1725, 1700, 1685, 1545, 1525, 1320 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.82 (s, 3H, C_{13}-CH_3), 1.14 (s, 3H, C_{10}-CH_3), 7.62 (s, 1H, 17-NH), 7.94–8.00 (m, 3H, Ar-H).

***N*-(3′-Trifluoromethyl-4′-nitrophenyl)-3′-oxo-4-azaandrost-5-ene-17β-carboxamide (18).** Prepared from **17** in same procedure as **15** from **14**. *N*-(3′-Trifluoromethyl-4′-nitrophenyl)-3′-oxo-4-azaandrost-5-ene-17β-carboxamide (**18**) 0.95 g (65.5%); mp 267–269°C; Elemental analysis for $C_{26}H_{30}N_3O_4F_3$ was verified; MS: *m*/*z* 506 ($M^+ + 1$), 505 (M^+); 490, 475, 300, 247; IR (KBr): 1670, 1650, 1540, 1520, 1340 cm^{-1}; ^1H NMR (pyridine-d_5): δ 1.08 (s, 3H, C_{13}-CH_3), 1.15 (s, 3H, C_{10}-CH_3), 2.75 (m, lH, 17-H), 5.20 (m, 1H, 6-H), 8.20–8.65 (m, 3H, Ar-H), 10.36 (s, lH, 4-NH), 11.20 (s, 1H, 17-CONH-).

Anti-HIV assay. The T cell line, H9, was maintained in continuous culture with complete medium (RPMI 1640 with 10% fetal calf serum (FCS) 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 of growth. Test samples were first dissolved in dimethyl sulfoxide (DMSO). The following were the final drug concentrations routinely used for screening: 100, 20, 4 and 0.8 μ/mL, but for active agents, additional dilutions were prepared for subsequent testing so that an accurate EC_{50} value could be achieved. As the test samples were being prepared, an aliquot of the T cell line, H9, was infected with HIV-1 (IIIB isolate) while another aliquot was mock-infected with complete medium. The mock-infected was used for toxicity determinations (IC_{50}). 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 (m.o.i.) between 0.1 and 0.01 Infectious Units/cell was added to the first aliquot of H9 cells. The other aliquot of H9 cells only received culture medium and then was incubated under identical conditions as the HIV-infected H9 cells. 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) or test sample or AZT.

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