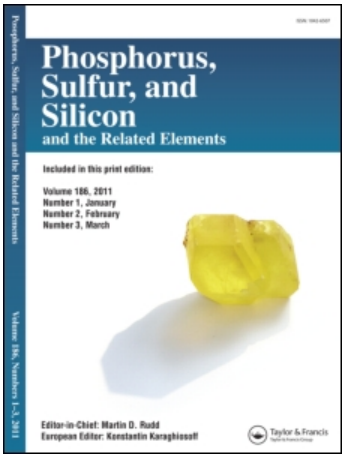

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Synthesis of Ursolic Phosphonate Derivatives as Potential Anti-HIV Agents

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Synthesis of Ursolic Phosphonate Derivatives as Potential Anti-HIV Agents

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In order to search for new anti-tumor and anti-viral agents, a series of α-aminophosphonate conjugates of 3-O-β-acetyl ursolic acid were prepared. Their biological activities as cytotoxic and anti-HIV agents were evaluated. The preliminary bioassays indicate that synthesized compounds **7a–j** *have anti-HIV activity (targeting HIV-1 gp120 and CD4) and no cytotoxicity on HT-29 cells (human colon adenocarcinoma cell line).*

Keywords α-aminophosphonate; anti-HIV activity; conjugate; cytotoxicity; Ursolic acid

INTRODUCTION

Ursolic Acid (UA) **1** (Figure 1) is a pentacyclic triterpene, widely distributed in the vegetable kingdom in aglycone, glycosides, or acylated

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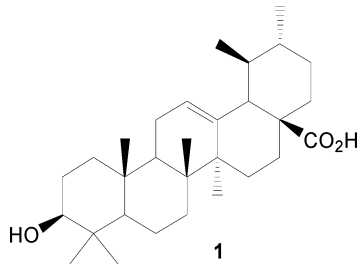


FIGURE 1 Structure of UA.

form. In past decades, UA has attracted considerable interest owing to its significant biological activities and promising clinical application as a chemotherapeutic and chemopreventive agent. Indeed, UA and its derivatives exhibit attractive pharmacological properties, including anti-inflammatory activity and,[1] anti-microbial activity[2,3] and being an inhibitor of mutagenesis in bacteria.[4] It also shows a dose-dependent cytotoxic activity in vivo through inhibiting implanted ascetic tumor cells.[5] UA is known as an anti-melanoma agent.[6] It is obviously a potent inhibitor of MCF-7 growth in the light of both cytostatic and cytotoxic activities.[7] Moreover, UA is able to inhibit dimerisation of HIV-1 protease, although slight toxicity is observed. Some dicarboxylic acid hemiesters at C-3 of ursolic acid also show anti-HIV activity. However, the action mechanism of these compounds is not clear.[8–10]

Literature review reveals that only a few synthetic analogs of UA have been reported for the purpose of cytotoxicity evaluation.[11–14] Results from a more extensive investigation using a great number of derivatives are needed, and criteria may be eventually established for the design and synthesis of more effective terpenoid-derived chemotherapeutic and chemopreventive agents. On the other hand, our recent work upon the chemical modification of UA as glucoside at C-28 and cinnamoyl esters at C-3 position showed that some of those derivatives possessed cytotoxicity on HT-29 and anti-HIV activity.[15] These exciting discoveries prompted us to do more chemical modification of the carboxyl group to meet the demand of bioactivity screening and the Quantitative Structure-Activity Relationship (QSAR) study.

It is well known that α-aminophosphonic acids and their derivatives, as analogs of natural α-amino acids, have attracted wide attention in chemistry, medicine, and agricultural science. They play an important role in living systems, as mimetic agents of the tetrahedral transition state of amide, esters, and peptides hydrolysis. α-Aminophosphonic acid derivatives have been used as antibiotics, herbicides, anti-tumor agents and enzyme inhibitors.[16,17] In the past three decades, there has been a growing interest in the chemistry of α-aminophosphonic acids

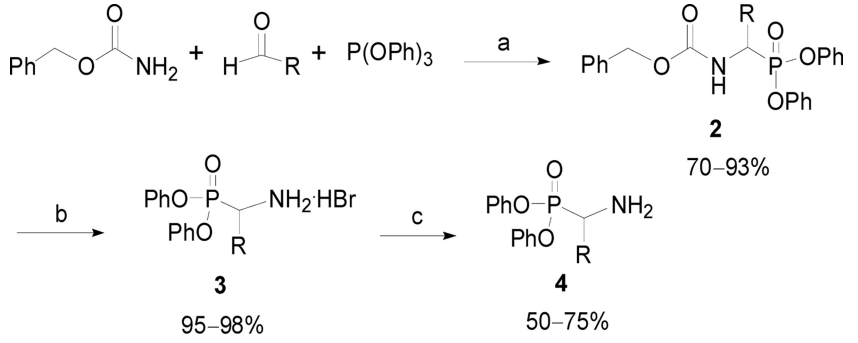
with an emphasis on their synthesis and diverse potential biological significance in metabolic processes of life.[18] Herein we are interested in the introduction of α-aminophosphonates to the parent structure of UA. Conjugation of these two entities may improve bioactivities and minimize to some extent the frequently encountered problems such as bioavailability, solubility, and pharmacokinetic asssociated with UA derivatives. In the present article, a variety of new phosphorus-containing conjugates of this triterpenoid were therefore synthesized and biologically evaluated as cytotoxic agents on HT-29 cells (human colon adenocarcinoma cell line) and anti-HIV agents.

The preliminary bioassays reveal that such compounds have some anti-HIV activity and no cytotoxicity on HT-29 cells.

RESULTS AND DISCUSSION

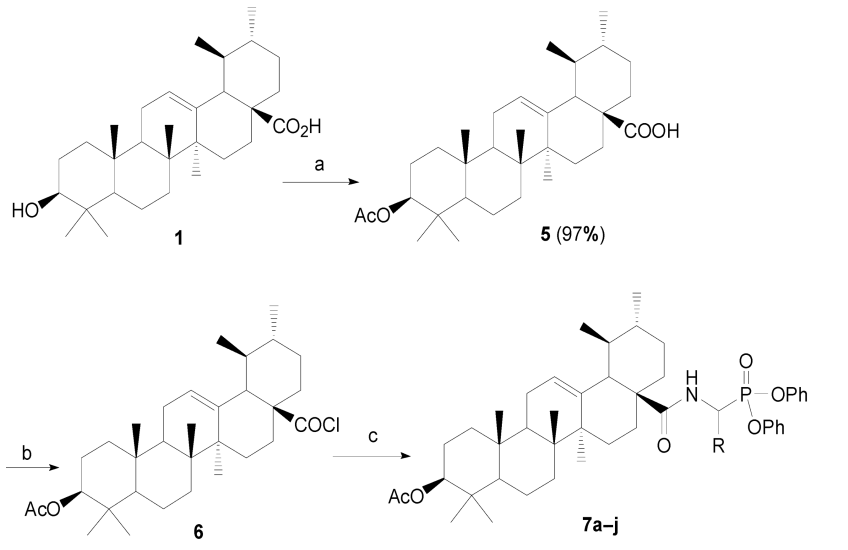
Synthesis of α-Aminophosphonates

A variety of N-blocked α-aminophosphonates were prepared in good to excellent yields by a one-pot three-component (aldehydes, benzyl carbomate, and triphenyl phosphite) condensation reaction according to the literature.[19] Ammonium hydrobromides **3** were sequentially obtained in quantitative yields after deprotection with a solution of hydrobromide in acetic acid. The subsequent neutrolization was carried out by treatment with triethylamine (>3 equivalents) in THF. The filtration and evaporation under reduced pressure resulted in free amines **4** as oils with yields of 50–75% (Scheme 1). Without further purification, the obtained α-aminophosphonates (**4**) were then dissolved in anhydrous THF and used directly in the next step.



R = H, *n*-Pr, *p*-MeOPh, *n*-Bu, Ph, *p*-MePh, *p*-ClPh, *o*-ClPh, 2, 4-Cl_2Ph, *o*-NO_2

SCHEME 1 Reagents and conditions: (a) AcOH, 80–85°C, 2 h; (b) HBr/AcOH, RT, 2 h; (c) NEt_3/CH_2Cl_2.



SCHEME 2 Reagents and conditions: (a) Ac_2O, DMAP, pyridine, RT, 2 h; (b) $SOCl_2$, 60–75°C, 5 h; (c) **4**, NEt_3/THF, 0°C, 0.5 h; RT, 5 h.

Synthesis and Structural Characterization of Ursolic Phosphonates 7a–j

To prepare the designed α-aminophosphonate conjugates of UA, a straightforward approach was first attempted, which involved a one-step condensation of UA or acetylated UA **5** with amines **4** (1 equiv.) in the presence of DiCyclohexylCarbodiimide (DCC) (1.2 equiv.) and 4-Dimethylamino-Pyridine (DMAP) in THF (or dichloromethane). Unfortunately, the reaction did not run to the desired product, presumably due to the hindrance of both reactants. To facilitate the coupling reaction, UA **1** was then transformed into acyl chloride. However, protection of hydroxyl group at 3 position was accordingly required prior to coupling reaction. Thus, acetylation of UA was conducted first according to the literature.[20] It should be indicated that improving the yield of the desired product **5** required one equivalent of DMAP when a large excess of acetic acid anhydride was used. It should be noted that the side reactions were increased by heating or strong light irradiation.

Conversion of 3β-acetoxy-urs-12-ene-28-oic acid **5** to acid chloride **6** was accomplished when treated with excess thionyl chloride at 60–75°C

TABLE I ^{31}P NMR Data of Compounds 7a–j (δ, ppm)

Compds	7a	7b	7c	7d	7e	7f	7g	7h	7i	7j
Major isomer	17.19	19.71	11.20	19.25	14.89	10.85	14.23	13.75	13.64	14.51
Minor isomer	—	19.24	12.16	19.72	15.86	11.91	15.09	14.70	14.50	13.43
d.e. (%)	0	10	13	12	24	14	3	25	22	11

for 5 h under nitrogen atmosphere, which was slightly different from the procedure reported in the literature.[21] The excess thionyl chloride was removed by distillation under reduced pressure. Complete removal of thionyl chloride required further distillation on high vacuum (oil pump) at r.t. for another 1 h. The obtained acid chloride was then used directly in the next step.

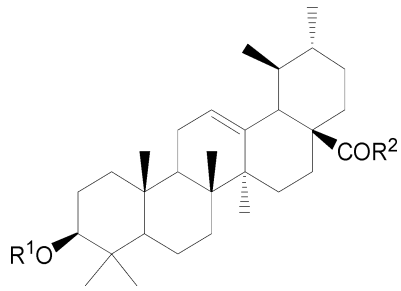
The coupling reaction of acid chloride **6** with α-aminophosphonates was routinely performed in the presence of excess triethylamine on an ice bath. It should be indicated that both reactants should be well dissolved in anhydrous THF prior to a coupling reaction. This could improve the yield of the product. Purification of the products by flash column chromatography gave the expected compounds **7a–j** as oils in 48–82% yields, which crystallized after standing for 3 days at r.t. (Scheme 2).

Products 7**a–j** were characterized by spectroscopic (^{1}H NMR, ^{31}P NMR, ^{13}C NMR, IR) and microanalytical methods. ^{31}P NMR data of compounds 7**a–j** are summarized in Table I. For each of compounds 7**b–j**, the ^{31}P NMR spectrum showed two peaks with chemical shift difference ranging from 0.47 to 1.06 ppm, indicating the existence of diastereoisomers. The corresponding ^{1}H NMR spectrum also implied the existence of isomers by appearance of two doublets of doublets at 4.70–6.80 ppm corresponding to CH moiety adjacent to a phosphorus atom and two broad single peaks corresponding to the unsaturated CH residue at C-12 position. The diastereoisomeric excess was in the range 3–25%.

Biological Activity

The preliminary bioassay of compounds **7a–j** was conducted on an HT-29 colon cancer cell line at the highest concentration (500 μM). The results showed that these new phosphonate derivatives of UA had no cytotoxicity.

Our previous study upon global anti-HIV activity revealed that some of the UA derivatives (Figure 2) had good activity.[15] For example, ursolic glucopyranosyl ester (**9**) and 3-*O*-3-(3-pyridyl)-prop-2-enoyl ursolic



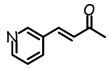
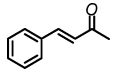
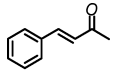
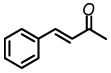
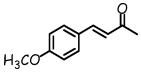
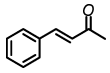
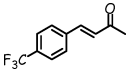
Compounds	R^1	R^2	Compounds	R^1	R^2
UA	H	-OH	13		—OH
8	H	O—β—D—Glc(OAc)$_4$	14		—OH
9	H	—O—β—D—Glc	15		—O—β—D—Glc
10		—O—β—D—Glc(OAc)$_4$	16	Ac	—OH
11		—OH	17	H	
12		—OH			

FIGURE 2 Structures of the reported compounds.

acid (**13**) were potent anti-HIV candidates with EC_{50} at 4 μM and 7.5 μMμ, respectively (Table II). Both displayed higher activity than UA (10 μM). These results prompted us to examine the anti-HIV property of compounds **7a–j**. It is noteworthy that these compounds showed specific anti-HIV activity in some degree (Table III). They proved to be entry inhibitors capable of inhibiting CD4-gp120 interaction. However, the HIV-1 entry is a multiple-step process ([a] interaction of the viral envelope gp120 glycoprotein with the host cell receptor CD4;[b] interaction of gp120-CD4 complex with CCR5 or CXCR4 chemokine receptors; [c] insertion of the envelope fusion domain into the host cell membrane; and [d] fusion of the virus' cell membrane); each of these steps may represent a potential target of our new compounds. In order to search the real target of our derivatives, assay inhibiting HIV-1 entry in

TABLE II Anti-HIV Activity of the Reported compounds

Compounds	**UA**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
[a]EC_{50} μM	10	[c]	4	[d]	30	[d]	7.5	[c]	[c]	[c]	[c]
[b]Inhibition %	24	13	16	2	9	**7**	11	13	0	16	11

[a]Global anti-HIV activity; EC_{50}: the agent concentration that inhibited viral replication in H9 cell by 50%.
[b]HIV entry inhibitor activity (10^{-4}M).
[c]Low activity.
[d]No activity.

lymphocyte cells was performed. This experiment was intended to target gp120, more specifically, the binding site between gp120 and CD4. The ability of compounds **7a–j** to inhibit CD4M33-F1 binding to gp120 was tested in florescence polarization assay. In this test, a small and fully functionalized CD4 mimic CD4M33 was engineered, which binds the recombinants gp120, gp40 proteins, and HIV-1 particles with CD4-like affinity. A florescent-labelled CD4M33 derivative was also synthesized and used as florescence tracer; additionally, a competition assay was developed to characterize different gp120 ligands.

In contrast to the reported results in Table II, compounds **7a–j** showed a comparable anti-HIV activity. Three of them (**7a**, **7b**, and **7c**) exhibited higher inhibition activity (18, 17, and 20 at 10^{-4}M) than the rest of the others except **UA**. It was found that the variation of substituents at α position of phosphoryl group did affect anti-HIV activity in some degree. Based on the results in Table III, it can be concluded that electron-donating substituents generally lead to higher activity than the electron-withdrawing counterparts. On the other hand, EC_{50} of compounds **8**∼**17** in Table II implies that free hydroxyl at 3 position and hydroxyl or carboxylic acid at 28 position are important for their activity. Therefore, keeping hydroxyl moiety at 3 position intact may improve bioactivity of UA derivatives. In this connection, removal of acetyl at 3 position and hydrolysis of phosphonate of compounds **7a–j** will be our task in the next program.

TABLE III HIV Entry Inhibitor Activity (10^{-4} M)

Compounds	**7a**	**7b**	**7c**	**7d**	**7e**	**7f**	**7g**	**7h**	**7i**	**7j**
Inhibition (%)	18	17	20	0	11	6	0	5	12	2

In conclusion, we have synthesized a series of α-aminophosphonate conjugates of 3-O-β-acetyl UA. All of the prepared compounds have been biologically evaluated. The bioassay results reveal that these compounds have no cytotoxicity on HT-29 cells. Interestingly, these new derivatives of UA exhibit specific anti-HIV activity. They prove to be an HIV-1 entry inhibitor by inhibiting gp120-CD4 interaction. These results suggest that our UA derivatives may be potential candidates to inhibit HIV infection. Nevertheless, with these results in hand, we still cannot find the target of our compounds. To understand the role and the mechanism of action of these **UA** derivatives, further work needs to be done in the future.

EXPERIMENTAL

Reagents and solvents were purchased from commercial sources and used without further purification unless indicated otherwise. Tetrahydrofuran was dried over sodium and distilled before use. Thionyl chloride and triethylamine were freshly distilled before use. Melting points were determined with a Kofler bench and uncorrected. ^{1}H, ^{13}C, ^{31}P NMR spectra were recorded on a BRUKER AC-P300 instrument. Elemental analyses were carried out on a Yanaco MT-3 instrument. All spectra were recorded in $CDCl_3$ or DMSO-d_6; TMS was used as an internal standard for ^{1}H NMR, and 85% phosphoric acid (H_3PO_4) was used as an external standard for ^{31}P NMR spectrum; chemical shifts (δ) were expressed in ppm. Analytical TLC was performed on Merck silica gel $60F_{254}$ pre-coated plates. Column chromatography was performed using commercially obtained silica gel H 40. All reactions involving air- or water-sensitive compounds were routinely conducted under nitrogen atmosphere.

General Procedure for the Preparation of Diphenyl-α-Aminophosphonates Hydrobromides (3)

To a flask containing N-benzyloxycarbonyl-diphenyl-α-aminophosphonates **2** (0.01 mol) was added a solution of HBr in AcOH (25 mL, 30%). The mixture was stirred at r.t. for 2 h. When evolution of bubbles stopped, excess HBr and solvent were subsequently removed by distillation under reduced pressure. The residue was washed with diethyl ether (30 mL), producing a white solid. Further filtration and washing with diethyl ether yielded white powder. The spectral data of all known products are in accordance with that reported in the literature.

Diphenyl[1-(1-amino)methyl]phosphonate Hydrobromide (3a)[22]

R = H, yield: 96%; m.p. 134–136°C; ^{1}H NMR (DMSO-d_6): δ-3.89 (d, 2H, $^2J_{P\text{-}H}$ = 17.96 Hz, CH_2), 6.74-7.46 (m, 10H, H_{arom}), 8.81 (brs, 3H, $^+NH_3$); ^{31}P NMR (DMSO-d_6): δ = 14.6.

Diphenyl [1-(1-amino)butyl]phosphonate Hydrobromide (3b)

R = *n*-Pr, yield: 97%; mp 195–197°C; ^{1}H NMR (DMSO-d_6): δ = 0.93 (t, 3H, CH_3), 1.50–1.65 (m, 2H, $\underline{CH_2}CH_3$), 1.85–2.03 (m, 2H, $\underline{CH_2}CH_2CH_3$), 4.20 (m, 1H, CH), 7.10–7.49 (m, 10H, H_{arom}), 8.84 (brs, 3H, $^+NH_3$); ^{31}P NMR (DMSO-d_6): δ = 15.83.

Diphenyl [1-(1-amino)methyl-p-methoxyphenyl]phosphonate Hydrobromide (3c)[23]

R = *p*-MeOPh, yield: 98%; mp 193.5°C; ^{1}H NMR (DMSO-d_6): δ = 3.77 (s, 3H, OCH_3), 5.58 (d, 1H, $^2J_{P\text{-}H}$ = 18.08 Hz, CH), 6.91–7.64 (m, 14H, H_{arom}), 9.37 (brs, 3H, $^+NH_3$); ^{31}P NMR (DMSO-d_6): δ = 12.29.

Diphenyl [1-(1-amino)pentyl]phosphonate Hydrobromide (3d)

R = *n*-Bu, yield: 98%; mp 172.5°C; IR (ν cm^{-1}): 3403, 2958, 1559, 1488, 1234, 1206, 1175, 1159, 936, 764; ^{1}H NMR (DMSO-d_6): δ 0.87 (t, 3H, CH_3, $^3J_{H\text{–}H}$ = 7.20 Hz), 1.30–1.58 (m, 4H), 1.92–2.05 (m, 2H), 4.20 (m, 1H, CH), 7.22–7.45 (m, 10H, H_{arom}), 8.89 (brs, 3H, $^+NH_3$); ^{13}C NMR (DMSO-d_6): δ = 13.57 (s), 21.67 (s), 27.02 (d, $^2J_{P\text{-}C}$ = 9.0 Hz), 27.62 (s), 46.36 (d, $^1J_{P\text{-}C}$ = 156 Hz), 120.50 (d, $^3J_{P\text{-}C}$ = 4.1 Hz), 120.57 (d, $^3J_{P\text{-}C}$ = 5.3 Hz), 125.77 (s), 130.02 (s), 149.30 (d, $^2J_{P\text{-}C}$ = 10.1 Hz); ^{31}P NMR (DMSO-d_6): δ 15.73.

Diphenyl [1-(1-amino)benzyl]phosphonate Hydrobromide (3e)[23]

R = Ph, yield: 99%; mp 195–196°C; ^{1}H NMR (DMSO-d_6): δ = 5.65 (d, 1H, $^2J_{P\text{-}H}$ = 18.42 Hz, CH), 6.86–7.68 (m, 15H, H_{arom}), 9.46 (br.s, 3H, $^+NH_3$); ^{31}P NMR (DMSO-d_6): δ = 12.22.

Diphenyl [1-(1-amino)methyl-p-tolyl]phosphonate Hydrobromide (3f)[23]

R = *p*-MePh, yield: 99%; mp 196–198°C; ^{1}H NMR (DMSO-d_6): δ = 2.34 (s, 3H, CH_3), 5.59 (d, 1H, $^2J_{P\text{-}H}$ = 18.82 Hz, CH), 6.91–7.59 (m, 14H, H_{arom}), 9.41 (br.s, 3H, $^+NH_3$); ^{31}P NMR (DMSO-d_6): δ = 12.10.

Diphenyl [1-(1-amino)methyl-p-chlorophenyl]phosphonate Hydrobromide (3g)[23]

R = *p*-ClPh, yield: 98%; mp 191.4°C; ^{1}H NMR (DMSO-d_6): $\delta = 5.70$ (d, 1H, CH, $^2J_{P\text{-}H} = 18.6$ Hz), 6.92 (d, 2H, $^3J_{H\text{-}H} = 8.10$ Hz), 7.19–7.97 (m, 12H, H_{arom}), 9.53 (br.s, 3H, $^+NH_3$); ^{31}P NMR (DMSO-d_6): $\delta = 10.48$.

Diphenyl [1-(1-amino)methyl-o-chloropheny]phosphonate Hydrobromide (3h)[23]

R = *o*-ClPh, yield: 99%; mp 203.1°C; ^{1}H NMR (DMSO-d_6): $\delta = 5.74$ (d, 1H, CH, $^2J_{H\text{-}P} = 18.3$ Hz), 6.96–7.74 (m, 14H, H_{arom}), 9.53 (br.s, 3H, $^+NH_3$); ^{31}P NMR (DMSO-d_6): $\delta = 11.51$.

Diphenyl [1-(1-amino)methyl-2, 4-dichlorophenyl]phosphonate Hydrobromide (3i)

R = 2,4-Cl_2Ph, yield: 99%; mp 196.9°C; IR (ν cm^{-1}): 3402, 3101, 2973, 2798, 2570, 1589, 1505, 1490, 1268, 1206, 1174, 959, 936, 760; ^{1}H NMR (DMSO-d_6) : $\delta = 5.72$ (d, 1H, CH, $^2J_{H-P} = 18.6$ Hz), 6.97 (d, 2H, $^3J_{H-H} = 8.40$ Hz), 7.18-7.45 (m, 8H, H_{arom}), 7.69 (d, 1H, $^3J_{H-H} = 10.50$ Hz), 7.83 (s, 1H), 7.95 (d, 1H, $^3J_{H-H} = 8.40$ Hz), 9.49 (br.s, 3H, $^+NH_3$); ^{13}C NMR: $\delta = 149.27$ (d, $^2J_{P-C} = 8.5$ Hz), 149.21 (d, $^2J_{P-C} = 11.0$ Hz), 135.25 (d, $^3J_{P-C} = 3.3$ Hz), 134.52 (d, $^2J_{P-C} = 7.5$ Hz), 131.17, 130.14 (s), 130.00 (s), 129.61, 128.19, 127.46 (d, $^3J_{P-C} = 4.4$ Hz) , 125.99 (s), 125.83 (s), 120.32 (d, $^3J_{P-C} = 4.5$ Hz), 119.88 (d, $^3J_{P-C} = 4.3$ Hz), 47.18 (d, $^1J_{P-C} = 158$ Hz); ^{31}P NMR (DMSO-d_6): $\delta = 10.15$.

Diphenyl [1-(1-amino) methyl-o-nitrophenyl]phosphonate Hydrobromide (3j)

R = *o*-NO_2Ph, yield: 96%; mp 191.4°C; IR (ν cm^{-1}): 3423, 2990, 2550, 1589, 1540, 1489, 1347, 1264, 1205, 1175, 1150, 961, 941, 774; ^{1}H NMR (DMSO-d_6): $\delta = 6.14$ (d, 1H, CH, $^2J_{H-H} = 19.8$ Hz), 6.94 (d, 2H, $^3J_{H-H} = 8.29$ Hz), 7.13–7.43 (m, 8H, H_{arom}), 7.74–8.20 (m, 4H), 9.43 (brs, 3H, $^+NH_3$); ^{13}C NMR: $\delta = 46.25$ (d, $^1J_{P-C} = 158$ Hz), 119.96 (d, $^3J_{P-C} = 4.3$ Hz), 120.27 (d, $^3J_{P-C} = 4.3$ Hz), 125.07, 125.12, 125.87, 125.99, 129.72, 129.99 (s), 130.09 (s), 131.04, 134.25, 149.17 (d, $^2J_{P-C} = 9.9$ Hz), 149.25 (d, $^2J_{P-C} = 9.9$ Hz); ^{31}P NMR (DMSO-d_6): $\delta = 11.00$.

General Procedure for the Preparation of Diphenyl-α-Aminophosphonates (4)

To a suspension of diphenyl-α-aminophosphonate hydrobromide **3** (0.25 mmol) in anhydrous dichloromethane (20 mL) was added triethylamine (0.3 mL, 2.15 mmol) dropwise at r.t.[24] The reaction mixture was

then kept stirring for 3 h. The precipitate was filtered off, and the solvent was evaporated under reduced pressure. The resulting colorless sticky oil was used directly without further purification.

Preparation of 3β-Acetoxy-urs-12-ene-28-oic Acid (5)

To a 50-mL flask was added UA (0.1 g, 0.22 mmol), pyridine (10 mL), acetic acid anhydride (2 mL, 36.67 mmol), and DMAP (0.027 g, 0.22 mmol). The mixture was kept stirring at r.t. under nitrogen for 2 h. Ground ice was then added. After the ice dissolved completely, the mixture was extracted with methylene chloride (15 mL $\times$ 3). The extracted organic layers were combined and washed with water (20 mL $\times$ 5), dried over sodium sulfate, and followed by filtration and evaporation on a rotavapor. The residue was allowed to evaporate with dry toluene (20 mL) under reduced pressure. After repetition of the addition and evaporation of toluene (4 times), the crude pale powder was obtained. Recrystallization from ethyl acetate/hexane (1/6, v/v) afforded a white solid in 95% yield. m.p. 289-290°C. (Lit. [22], m.p. 289–290°C).

Preparation of 3-β-Acetoxy-urs-12-ene- 28-oyl Chloride (6)

To a 10-mL flask was added 3β-acetoxy-urs-12-ene-28-oic acid (43 mg, 0.086 mmol) and freshly distilled thionyl chloride (2 mL). The reaction was warmed to 60–75°C and kept stirring for about 5 h. The solution was then concentrated by distillation under reduced pressure at 60°C, followed by evaporation on vacuum (oil pump) for another 1 h. The obtained acid chloride was dissolved in anhydrous THF and used immediately in the next reaction.

General Procedure for the Synthesis of 3β-Acetoxy-ursolic Phosphonate Derivatives (7a–j)

To a solution of α-aminophosphonates **4** (0.2 mmol) in anhydrous THF (10 mL) was added triethylamine (0.2 mL). The solution was then cooled with stirring on an ice bath. A solution of acid chloride **6** (0.1 mmol) in anhydrous THF (5 mL) was added dropwise over 10 min. After completion of the addition, the reaction was allowed to stir at 0°C for 0.5 h and at r.t. for another 5 h. The precipitate was then filtered off, and the solvent was removed on a rotavapor. The residue was subject to column chromatography using a mixture of ethyl acetate/hexane (1/4, by volume) as an eluent. The fractions (R_f = 0.3–0.4) were collected. The subsequent evaporation gave pure products **7a–j** as oils. After standing for 3 days, the products solidified as a yellow powder.

Diphenyl [(N-3β-acetoxyurs-12-en-28-oyl)aminomethyl] Phosphonate (7a)

Yield: 75%; mp 48–50°C; IR (ν cm^{-1}): 3395, 2922, 1737, 1715, 1528, 1244, 1027; ^{1}H NMR ($CDCl_3$): δ = 0.65–1.95 (m, 43H, parent structure of UA unless otherwise indicated), 1.80 (d, 1H, $^3J_{H-H}$ = 9.62 Hz, H-18), 1.98 (s, 3H, CH_3C=O), 3.51 (dddd, H_a, $^2J_{Ha\text{-}Hb}$ = 15.90 Hz, $^2J_{P-Ha}$ = 11.9 Hz, $^3J_{Ha\text{-}H}$ = 3.67 Hz, CH_aH_bP), 4.22–4.30 (m, 1H_b, CH_aH_bP), 4.40 (m, 1H, H-3), 5.25 (m, 1H, H-12), 6.29 (t, 1H, $^3J_{H-H}$ = 3.87 Hz, NH), 7.10–7.30 (m, 10H, H_{arom}); ^{13}C NMR ($CDCl_3$): δ = 14.8, 15.9, 17.0, 17.1, 17.4, 21.5, 21.7, 23.7, 23.9, 24.5, 28.1, 28.4, 29.4, 30.1, 32.9, 37.0, 37.1, 38.1, 38.6, 38.9, 39.4, 39.8, 42.6, 47.8, 48.4, 53.4, 55.5, 81.5 (C3), 116.1, 120.8, 126.9 (C12), 130.1, 139.1 (C13), 156.2, 171.9 (O=C-(CH_3)), 181.5 (C28); ^{31}P NMR($CDCl_3$): δ = 17.19; Analysis of compound $C_{45}H_{62}NO_6P$ calcd.: C, 72.65; H, 8.40; found: C, 72.44; H, 8.60.

Diphenyl [(N-3β-Acetoxyurs-12-en-28-oyl) (Aminomethyl-n-propyl)] Phosphonate (7b)

Yield: 80%; mp 63–65°C; IR (ν cm^{-1}): 3371, 2928, 1737, 1700, 1595, 1489, 1244, 1024; ^{1}H NMR ($CDCl_3$): δ = 0.69–1.96 (m, 50H, $CH_2CH_2CH_3$ + parent structure of UA unless otherwise indicated), 1.97 (s, 3H, CH_3C=O), 2.08 (d, 1H, $^3J_{H-H}$ = 10.74 Hz, H-18), 4.40 (m, 1H, H-3), 4.78–4.84 (m, 1H, CHP), 5.26 (m, 1H, H-12), 5.97 and 6.17 (d, 1H, $^3J_{H-H}$ = 9.42 Hz, NH), 6.73–7.25 (m, 10H, H_{arom}); ^{13}C NMR ($CDCl_3$): δ = 14.1, 14.5, 15.9, 17.1, 17.3, 17.5, 17.8, 18.1, 21.5, 21.8, 23.5, 23.6, 27.3, 28.4, 30.1, 31.2, 37.2, 38.0, 38.6, 38.9, 39.1, 39.9, 40.0, 42.8, 42.9, 47.8, 48.7, 48.9, 55.6, 81.5 (C3), 115.8, 120.8, 126.8 (C12), 129.9, 138.1, 139.0 (C13), 156.2, 171.9 (O=C-(CH_3)), 181.5 (C28); ^{31}P NMR ($CDCl_3$): δ = 19.24, 19.71; Analysis of compound $C_{48}H_{68}NO_6P$ calcd.: C, 73.34; H, 8.72, found: C, 73.69; H, 8.34.

Dipheny[(N-3β-Acetoxyurs-12-en-28-oyl) Aminomethyl-p-methoxyphenyl)]Phosphona te (7c)

Yield: 75%; mp 127–128°C; IR (ν cm^{-1}): 3392, 2926, 1737, 1700, 1592, 1513, 1247, 1030; ^{1}H NMR ($CDCl_3$): δ = 0.68–1.97 (m, 43H, parent structure of UA unless otherwise indicated), 2.07 (s, 3H, CH_3C=O), 2.15 (d, 1H, $^3J_{H-H}$ = 10.80 Hz, H-18), 3.80 and 3.81 (s, 3H, OCH_3,), 4.48 (m, 1H, H-3), 5.29 and 5.41 (m, 1H, H-12), 5.75 and 5.93 (dd, 1H, $^3J_{H-H}$ = 8.18 Hz, $^2J_{P\text{-}H}$ = 19.45 Hz, CHP), 6.60 (br s, 1H, NH), 6.64–7.42 (m, 14H, H_{arom}); ^{13}C NMR ($CDCl_3$): δ = 14.1, 15.2, 15.6, 16.7, 17.0, 17.3, 21.1, 21.3, 22.7, 23.0, 23.5, 28.1, 29.7, 36.6, 36.8, 37.1, 37.6, 38.8, 39.4, 39.5, 42.3, 42.4, 47.9, 48.1, 55.2, 80.8 (C3), 114.2, 115.4, 120.3, 126.6 (C12), 129.5, 138.1, 137.9 (C13), 155.9, 171.1 (O=C–(CH_3)), 181.5 (C28); ^{31}P NMR

($CDCl_3$): $\delta = 11.20, 12.16$. Analysis of compound $C_{52}H_{68}NO_7P$ calcd.: C, 73.47; H, 8.06, found: C, 73.38; H, 8.22.

Diphenyl [(N-3β-acetoxyurs-12-en-28-oyl) (aminomethyl-n-butyl)] Phosphonate (7d)

Yield: 82%; mp 87–89°C; IR (ν cm^{-1}): 3369, 2925, 1737, 1715, 1624, 1518, 1242, 1027; ^1H NMR ($CDCl_3$): δ = 0.70–1.97 (m, 52H, $CH_2CH_2CH_2CH_3$ + parent structure of UA unless otherwise indicated), 2.05 (s, 3H, CH_3CO), 2.15 (d, 1H, $^3J_{H-H} = 11.10$ Hz, H-18), 4.48 (m, 1H, H-3), 4.84–4.94 (m, 1H, CHP), 5.34 (m, 1H, H-12), 6.04 and 6.25 (d, 1H, $^3J_{H-H} = 9.54$ Hz, NH), 7.11–7.36 (m, 10H, H_{arom}); ^{13}C NMR ($CDCl_3$): δ = 12.7, 12.8, 13.2, 14.4, 14.5, 15.7, 16.0, 16.4, 16.8, 17.1, 18.2, 18.4, 20.0, 20.1, 20.3, 22.0, 22.3, 22.5, 23.7, 26.8, 27.0, 28.7, 29.8, 29.9, 32.0, 35.8, 36.2, 36.6, 37.3, 37.7, 38.5, 38.6, 41.4, 41.5, 41.6, 46.4, 46.5, 47.4, 47.5, 52.3, 52.8, 54.2, 59.5, 80.0 (C3), 114.4, 119.0, 119.4, 119.5, 119.7, 124.0, 124.4, 125.0 (C12), 128.5, 128.6, 128.8, 137.7(C13), 155.2, 170.4 (O=C–(CH_3)), 178.2 (C28); ^{31}P NMR ($CDCl_3$): $\delta = 19.25, 19.72$; Analysis of compound $C_{49}H_{70}NO_6P$ calcd.: C, 73.56; H, 8.82; found: C, 73.49; H, 8.91.

Diphenyl[(N-3β-Acetoxyurs-12-en-28-oyl) (aminomethylphenyl)]Phosphonate (7e)

Yield: 70%; mp 98–101°C; IR (ν cm^{-1}): 3416, 2924, 1736, 1715, 1595, 1497, 1244, 1024; ^1H NMR ($CDCl_3$): δ = 0.59–2.03 (m, 43H, parent structure of UA unless otherwise indicated), 2.11 (s, 3H, CH_3CO), 2.22 (d, 1H, $^3J_{H-H} = 10.28$ Hz, H-18), 4.55 (m, 1H, H-3), 5.35 and 5.46 (m, 1H, H-12), 5.92 and 6.06 (dd, 1H, $^3J_{H-H} = 9.42$ Hz, $^2J_{P-H} = 19.80$ Hz, CHP), 6.64–7.87 (m, 15H, H_{arom}), 7.95 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ = 15.6, 16.0, 16.7, 16.9, 17.0, 17.3, 17.4, 17.6, 18.3, 21.4, 21.5, 21.7, 23.5, 23.6, 28.1, 28.4, 29.7, 30.1, 36.7, 37.0, 37.2, 38.0, 38.1, 39.7, 39.8, 40.0, 42.6, 42.8, 47.8, 48.4, 48.6, 48.8, 55.6, 81.6 (C3), 115.8, 120.6, 120.8, 121.0, 129.4 (C12), 129.9, 130.3, 138.2 (C13), 139.2, 156.3, 172.0 (O=C-(CH_3)), 181.5 (C28); ^{31}P NMR ($CDCl_3$): $\delta = 14.89, 15.86$; Analysis of compound $C_{51}H_{66}NO_6P$ calcd.: C, 74.70; H, 8.11; found: C, 74.89; H, 8.23.

Diphenyl [(N-3β-Acetoxyurs-12-en-28-oyl) (aminomethyl-p-tolyl)] Phosphonate (7f)

Yield: 78%; mp 112–114°C; IR (ν cm^{-1}): 3412, 2925, 1737, 1715, 1596, 1500, 1243, 1026; ^1H NMR ($CDCl_3$): δ = 0.56–2.04 (m, 43H, parent structure of UA unless otherwise indicated), 2.14 (s, 3H, CH_3CO), 2.22 (d, 1H, $^3J_{H-H} = 10.90$ Hz, H-18), 2.42 (s, 3H, CH_3), 4.54 (m, 1H, H-3),

5.36 and 5.49 (m, 1H, H-12), 5.85 and 6.02 (dd, 1H, $^3J_{H-H} = 9.46$ Hz, $^2J_{P-H} = 20.29$ Hz, CHP), 6.69–7.44 (m, 14H, H_{arom}), 7.70 (d, 1H, $^3J_{H-H} = 9.34$ Hz, NH); ^{13}C NMR ($CDCl_3$): $\delta = 14.1, 14.6, 15.2, 15.6, 15.7, 16.0, 20.0, 20.1, 20.3, 22.1, 23.7, 26.7, 27.0, 28.7, 29.7, 31.8, 35.6, 35.7, 36.6, 37.7, 38.2, 38.4, 38.6, 41.2, 41.4, 46.4, 47.2, 50.3, 53.1, 54.1, 59.5, 79.9$ (C3), 114.4, 119.0, 119.3, 119.4, 119.5, 124.1, 124.3, 125.0, 127.9 (C12), 128.0, 128.4, 128.5, 128.6, 128.7, 129.3, 138.0, 137.9 (C13), 149.0, 149.2, 155.3, 170.2 (O=C-(CH_3)), 176.2, 176.4, 181.5 (C28); ^{31}P NMR ($CDCl_3$): $\delta = 10.85, 11.91$. Analysis of compound $C_{52}H_{68}NO_6P$ calcd.; C, 74.88; H, 8.22, found: C, 74.50; H, 8.51.

Diphenyl [(N-3β-Acetoxyurs-12-en-28-oyl) (aminomethyl-p-chlorophenyl)] Phosphonate (7g)

Yield: 67%; mp 116–118°C; IR (ν cm^{-1}): 3383, 2925, 1733, 1715, 1669, 1594, 1490, 1243, 1088; ^1H NMR ($CDCl_3$): $\delta = 0.66–2.01$ (m, 43H, parent structure of UA unless otherwise indicated), 2.07 (s, 3H, CH_3C=O), 2.13 (d, 1H, $^3J_{H-H} = 10.56$ Hz, H-18), 4.47 (m, 1H, H-3), 5.26 and 5.46 (m, 1H, H-12), 6.33 and 6.72 (dd, 1H, $^3J_{H-H} = 8.76$ Hz, $^2J_{P-H} = 22.23$ Hz, CHP), 6.62–7.48 (m, 14H, H_{arom}), 7.72 (d, 1H, $^3J_{H-H} = 8.46$ Hz, NH); ^{13}C NMR ($CDCl_3$): $\delta = 14.1, 15.7, 15.9, 16.0, 20.0, 20.1, 20.3, 22.1, 25.9, 27.0, 28.7, 29.7, 31.8, 35.7, 36.6, 38.3, 38.4, 41.4, 46.4, 47.1, 53.1, 54.1, 80.2$ (C3), 114.4, 119.1, 119.4, 119.5, 125.5, 127.7, 127.9 (C12), 128.1, 128.5, 128.7, 129.0, 136.7, 137.9 (C13), 154.8, 170.6 (O=C-(CH_3)), 178.9 (C28); ^{31}P NMR ($CDCl_3$): $\delta = 14.23, 15.09$. Analysis of compound $C_{51}H_{65}ClNO_6P$ calcd.: C, 71.69; H, 7.67, found: C, 71.57; H, 7.40.

Diphenyl [(N-3β-Acetoxyurs-12-en-28-oyl) (aminomethyl-o-chlorophenyl)] Phosphonate (7h)

Yield: 61%; mp 125–127°C; IR (ν cm^{-1}): 3409, 2925, 1737, 1718, 1656, 1595, 1490, 1242, 1069; ^1H NMR ($CDCl_3$): $\delta = 0.61–2.06$ (m, 43H, parent structure of UA unless otherwise indicated), 2.09 (s, 3H, CH_3C=O), 2.16 (d, 1H, $^3J_{H-H} = 10.68$ Hz, H-18), 4.51 (m, 1H, H-3), 5.31 and 5.44 (m, 1H, H-12), 5.76 and 5.98 (dd, 1H, $^3J_{H-H} = 9.40$ Hz, $^2J_{P-H} = 20.25$ Hz, CHP), 6.64–7.45 (m, 14H, H_{arom}), 7.78 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): $\delta = 14.1, 15.7, 15.9, 16.0, 20.0, 20.1, 20.3, 22.1, 25.9, 27.0, 28.7, 29.7, 31.8, 35.7, 36.6, 38.3, 38.4, 41.4, 46.4, 47.1, 53.1, 54.1, 80.2$ (C3), 114.4, 119.1, 119.4, 119.5, 125.5, 127.7, 127.9 (C12), 128.1, 128.5, 128.7, 129.0, 136.7, 137.9 (C13), 154.8, 170.6 (O=C-(CH_3)), 178.9 (C28); ^{31}P NMR ($CDCl_3$): $\delta = 13.75, 14.70$. Analysis of compound $C_{51}H_{65}ClNO_6P$ calcd.: C, 71.69; H, 7.67; found: C, 71.66; H, 7.33.

Diphenyl[(N-3β-Acetoxyurs-12-en-28-oyl) (aminomethyl-2,4-dichlorophenyl)]phosphornate (7i)

Yield: 57%; mp 142–145°C; IR (ν cm^{-1}): 3405, 2924, 1737, 1718, 1654, 1589, 1488, 1247, 1071; ^{1}H NMR ($CDCl_3$): $\delta = 0.65$–2.04 (m, 42H, parent structure of UA unless otherwise indicated), 2.07 (s, 3H, $CH_3C{=}O$), 2.22 (d, 1H, $^3J_{H-H} = 10.35$ Hz, H-18), 4.50 (m, 1H, H-3), 5.26 and 5.44 (m, 1H, H-12), 6.26 and 6.64 (dd, 1H, $^3J_{H-H} = 7.57$ Hz, $^2J_{P\text{-}H} = 14.26$ Hz, CHP), 6.60–7.59 (m, 14H, H_{arom}), 7.82 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ = 14.1, 14.9, 15.2, 16.2, 16.7, 17.0, 17.1, 21.0, 21.3, 22.7, 23.1, 23.3, 28.0, 29.7, 36.7, 37.6, 38.2, 38.4, 38.8, 39.3, 39.6, 42.3, 42.4, 42.7, 48.2, 53.9, 55.0, 55.2, 80.8 (C3), 119.7, 119.9, 120.0, 120.4, 120.5, 125.2, 125.4, 126.5 (C12), 129.5, 129.6, 129.7, 129.9, 138.1 (C13), 148.0, 150.0, 150.2, 156.3, 171.1 (O=C-(CH_3)), 179.8, 179.9 (C28); ^{31}P NMR ($CDCl_3$): δ = 13.64, 14.50. Analysis of compound $C_{51}H_{64}Cl_2NO_6P$ calcd.: C, 68.91; H, 7.26; found: C, 69.21; H, 7.17.

Diphenyl[(N-3β-Acetoxyurs-12-en-28-oyl) (aminomethyl-o-nitrophenyl)]phosphonate (7j)

Yield: 48%; mp 112–114°C; IR (ν cm^{-1}): 3419, 2924, 1733, 1716, 1654, 1540, 1488, 1245, 1070; ^{1}H NMR ($CDCl_3$): $\delta = 0.65$–2.02 (m, 43H, parent structure of UA unless otherwise indicated), 2.03 (s, 3H, $CH_3C{=}O$), 2.15 (d, 1H, $^3J_{H-H} = 9.12$ Hz, H-18), 4.45 (m, 1H, H-3), 5.23 and 5.46 (m, 1H, H-12), 6.64 and 6.90 (dd, 1H, $^3J_{H-H} = 6.60$ Hz, $^2J_{P\text{-}H} = 18.84$ Hz, CHP), 6.54–8.07 (m, 14H, H_{arom}), 8.04 (brs, 1H, NH); ^{13}C NMR ($CDCl_3$): δ = 14.1, 15.2, 15.3, 16.4, 16.7, 21.3, 22.7, 24.4, 28.0, 29.3, 29.7, 30.7, 30.8, 31.9, 36.7, 37.6, 39.0, 39.3, 39.6, 47.3, 48.3, 53.9, 55.2, 59.5, 73.8, 79.7 (C3), 98.5, 111.9, 113.9, 114.4, 119.8, 120.0, 120.4, 120.7, 125.1, 129.5 (C12), 129.8, 138.0 (C13), 170.9 (O=C-(CH_3)), 172.7, 176.2, 176.4, 181.5 (C28); ^{31}P NMR ($CDCl_3$): $\delta = 13.43$, 14.51. Analysis of compound $C_{51}H_{65}N_2O_8P$ calcd.: C, 70.81; H, 7.57, found: C, 70.87; H, 7.28.

Cell and Drug Cytotoxicity Assay

The HT-29 colon cancer cell line originated from a colic adenocarcinoma in an untreated patient and was obtained from American Type Culture Collection (ATCC) (Rockville, MD). The culture medium was a mixture of Ham F10 medium (Bio Whittaker, Ververs, Belgium) and 10% fetal calf serum (Boerhinger Ingelheim, Gagny, France). Cells were grown as monolayers in a controlled atmosphere (37°C, 5% CO_2) in Ham's F-10 medium supplemented with 10% fetal calf serum.[25] All test compounds were solubilized in DMSO and were tested twice at different concentrations.

HT29 cells (2×10^4 per well) were seeded in 96-well culture plates and cultured for two days before treatment.[25] The prepared compounds were dissolved immediately before use in a mixture of DMSO and absolute EtOH (1:1, v/v), then diluted in serum-free Ham's F-10 medium. Final concentration of DMSO and EtOH, which did not exceed 1%, did not affect cell survival. Cells were treated for 3 h with drugs alone. After treatment, cells were washed twice with Ham's F-10 and cultured again for 7 days in a drug-free culture medium. Cell survival was measured by the crystal violet colorimetric assay. In brief, cells were rinsed with phosphate buffered saline, and then surviving adherent cells were fixed for 5 min by pure ethanol. After drying, cells were stained by crystal violet (5g/L in distilled water). Dye in excess was flushed off using tap water. Cell-fixed dye was eluted by 33% acetic acid.

Fluorescence Polarization Assay Specific to the CD4 Binding Site of HIV-1 Glycoproteins[26]

Anisotropy measurements in the Beacon 2000 analyzer are quite reproducible, but require 200 μL total volume and 20 min equilibration time after each determination. Therefore, it is rather consuming in terms of material and time. The assay was thus performed in 384-well microplates a method that uses a 10-fold smaller total volume and allows multiple determinations in a short time. The fluorescence anisotropy of CD4M33-Fl was evaluated in quadruplicate experiments, with 16 two-fold dilutions of 200 nM of gp120. CD4M33-Fl was tested at different concentrations (0.1, 0.5, 1.0, and 10 nM) to minimize the use of gp120. At 0.1 nM, the intensity of fluorescence was very weak (the signal-to-noise ratio was less than 10), while 1 nM was found to be the best compromise between an acceptable signal-to-noise ratio and the use of gp120.

Measurements were made in an Lev J. Leytes analyst (LJL Biosystems, Sunnyvale, CA) microplate reader, using the fluorescence polarization detection mode, 485 nm excitation, and 530 nm emission filters, and an additional 505 nm dichroic filter to limit blank response. A 1.2-G parameter was used. Samples were in 10 mM sodium phosphate buffer, pH 7.0, containing 135 mM NaCl and 0.05% Tween 20. The binding assay was performed in a final volume of 20 mL (titration experiments) or 21 mL (competitive experiments) in a 384-well small-volume black microtitration plate by using 1 nM fluorescein–CD4M33. Titration assays were performed in quadruplicate by the addition of 10 mL of fluorescein ligand (1 nM final concn.) to 10 mL of gp120 (two-fold dilutions in 16 wells and starting at 200 nM). Competition assays were performed in triplicate by mixing 7.0 mL of competing unlabelled ligand, 7.0 mL of fluorescein-labelled ligand (1 nM final concn.), and 7.0 mL of gp120

(12.5 nM final concn.). Fluorescence anisotropy was determined after 40 min equilibration at 25°C.

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