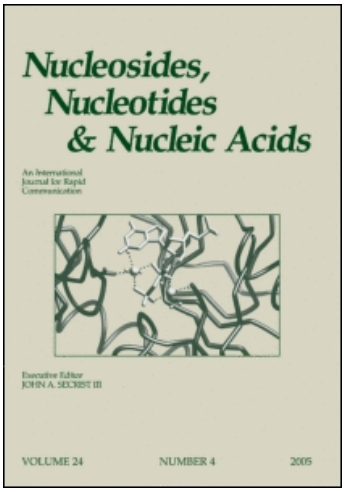

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SYNTHESIS AND ANTIVIRAL ACTIVITY OF AMINO ACID CARBAMATE DERIVATIVES OF AZT

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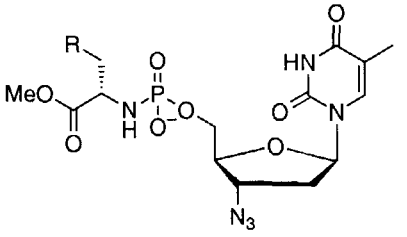
Dedicated to the memory of Dr. Gertrude B. Elion

ABSTRACT: Lipophilic amino acid methyl ester and methyl amide carbamates of 3'-azido-3'-deoxythymidine (AZT) were synthesized and their anti-HIV-1 activity in PBMCs was determined. The methyl amides were more potent (EC_{50}s = 1.8 - 4.0 μM) than the methyl esters (EC_{50}s = 2.0 – 20 μM). Carbamate hydrolysis by cell lysates and liberation of AZT was not observed for representative methyl ester or methyl amide AZT carbamates. No evidence of direct inhibition of HIV reverse transcriptase or integrase was observed.

Introduction

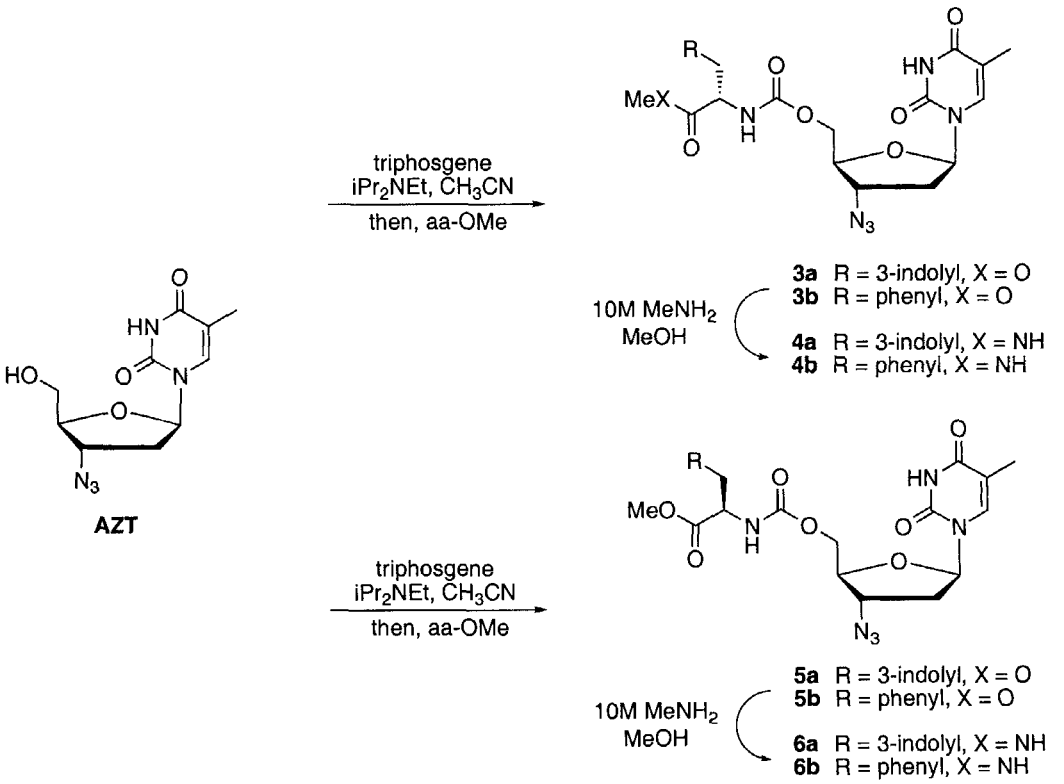
3'-Azido-3'-deoxythymidine (AZT), a nucleoside based reverse transcriptase inhibitor, has been widely used in treating HIV-1 infections. The antiviral activity of AZT requires its intracellular conversion to the 5'-triphosphate (AZT-TP) which acts as an inhibitor of reverse transcriptase and as a chain terminator of growing viral DNA. The enzymes thymidine kinase, thymidylate kinase and nucleoside diphosphate kinase are responsible for the sequential phosphorylation of AZT to AZT-TP. This dependence on cellular kinases has limited the efficacy of AZT in treating HIV-1 infection. For instance, long term AZT exposure may lead to decreased levels of thymidine kinase in patients undergoing HIV therapy.[1] This might be a factor contributing to emerging resistance to AZT treatment.[2] Furthermore, AZT itself has a short half-life and long-term treatment can lead to several undesirable side-effects, such as anemia and neutropenia.[3] To overcome some of the drawbacks associated with AZT treatment, several prodrug approaches have been explored in an attempt to deliver AZT-MP directly into cells.[4] The prodrug approach that we employ involves amino acid phosphoramidate derivatives such as **1** and **2**. We

have previously demonstrated that **1** and **2** exhibit anti-HIV-1 activity in PBMCs and CEM cells.[5,6] It was proposed that these phosphoramidates are hydrolyzed intracellularly, either chemically or enzymatically, to give AZT 5'-monophosphate which would subsequently be



1 R = 3-indolyl
2 R = phenyl

SCHEME 1



phosphorylated to the triphosphate. However, preliminary mechanistic studies revealed that **1** and **2** are only moderately metabolized by PBMCs suggesting that the intact phosphoramidates may have intrinsic antiviral activity.[7] If this were the case, then it may be possible to prepare 5'-substituted nucleoside derivatives that also have intrinsic antiviral activity, thereby eliminating the need for phosphorylation and thus avoiding the kinase

pathway. To probe this possibility, we prepared a series of compounds in which the labile phosphoramidate linkage was replaced with a more chemically stable carbamate moiety. The synthesis, biological activities and decomposition in cell lysates of these compounds is described below.

Results and Discussion

Chemistry.

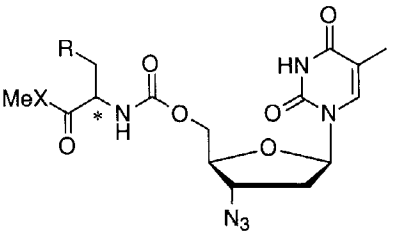
The carbamate derivatives were prepared by condensing AZT with triphosgene to give an intermediate chloroformate[8] that was immediately treated with an excess of tryptophan methyl ester or phenylalanine methyl ester to give **3a** (89% yield) and **3b** (83% yield). Because the terminal methyl ester moiety may be susceptible to esterase hydrolysis, we also prepared the methyl amide derivatives. This was achieved by treating **3a** and **3b** with 10 M methyl amine in methanol to give **4a** and **4b** in 84% and 77% yield, respectively. To investigate the role of the amino acid stereochemistry, we also prepared the D-tryptophan and D-phenyl alanine methyl esters (**5a**, **5b**) and methyl amides (**6a**, **6b**) following the same procedure.

Biological Analysis.

The ability of the AZT amino acid carbamates to inhibit production of LAI/HIV-1 in PBMCs and their ability to directly inhibit the catalytic activity of HIV-1 reverse transcriptase (HIV-1 RT) were investigated. Similar to the phosphoramidate derivatives **1** and **2**, none of the carbamates tested inhibited HIV-1 RT activity at concentrations >100 μM (Table 1). The carbamates also showed no cytotoxicity to PBMCs at concentrations ≥100 μM. The carbamate analogs inhibited viral replication with EC_{50} values of 10 μM and 20 μM, respectively. The phosphoramidates **1** and **2** were found to be 33- and 110-fold more potent than **3a** and **3b**, consistent with the dependence of the phosphoramidates on conversion to AZT-MP for antiviral activity. When the methyl ester moiety of **3a** and **3b** was converted to a methyl amide (**4a** and **4b**), a 5-fold increase in antiviral activity was observed for both cases. The increased potencey of the methyl amides **4a** and **4b** may be related to their increased stability relative to the methyl esters (vide infra).

We next investigated the effects of the stereochemistry on the amino acid side chain. Compounds **5a** and **5b** were prepared from the methyl esters of D-tryptophan and D-phenylalanine. When we compared the antiviral potencies of the L-tryptophanyl derivative **3a** with the D-tryptophanyl derivative **5a**, we found that the L-derivative was about 2-fold more potent. In contrast, the D-phenylalanine derivative **5b** was 10-fold more potent than the L-phenylalanine derivative **3b**. Both **5a** and **5b** were converted to the corresponding methyl amides **6a** and **6b**. Again, we observed similar trends in anti-HIV-1 activity. The tryptophan methyl amide **6a** was almost 5-fold more potent than the methyl ester **5a** and the phenylalanine methyl amide **6b** was slightly more potent than the methyl ester **5b**. In

TABLE 1. The Effect of AZT Carbamate on HIV-1 Replication, Reverse Transcriptase Activity and Their Cytotoxicity in PBMCs[a]



Compound	R	X	*[b]	EC_{50}[c]	CC_{50}[d]	IC_{50}[e]	Log *P*
1[f]	3-indolyl	O	L	0.3	>100	>100	–0.69
2[f]	phenyl	O	L	0.18	>100	>100	–1.84
3a	3-indolyl	O	L	10	>100	>100	1.31
3b	phenyl	O	L	20	>100	>100	1.76
4a	3-indolyl	NH	L	2.2	>100	>100	1.10
4b	phenyl	NH	L	3	>100	>100	1.16
5a	3-indolyl	O	D	18	>100	>100	1.13
5b	phenyl	O	D	2	>100	>100	1.74
6a	3-indolyl	NH	D	4	>100	>100	1.14
6b	phenyl	NH	D	1.8	>100	>100	1.13
AZT				0.010	>100	>100	–0.16

[a]Values are in μM. [b]Stereochemistry of the amino acid. [c]Effective concentration required to inhibit the replication of HIV-1 by 50%. PBMCs used were all from the same donor. [d]Concentration required to kill 50% of the cells as compared to the untreated cultures. [e]Effective concentration necessary to inhibit 50% of recombinant HIV-1 reverse transcriptase activity. [f]Phosphoramidates.

addition, the L-tryptophanyl derivative **4a** was about 2-fold more potent than the D-trypyophanyl **6a** and the D-phenylalaninyl compound **6b** was about 2-fold more potent than the L-phenylalaninyl derivative **4b**. The increased potency of the L-tryptophan deriviatives relative to the D-tryptophan derivatives, but decreased potency of the L-phenylalanine derivatives relative to the L-phenylalanine derivatives suggests that there is

no predictable stereochemical preference for the amino acid side chain. However, replacing the methyl ester with a methyl amide appears to be desirable since all of the methyl amide derivatives are more potent than their corresponding methyl ester derivatives.

Partition coefficients (log P values) were measured to determine a possible correlation between *in vitro* antiviral potency and lipophilicity. As observed in Tables 1, all of the AZT carbamates (log P = 1.10 - 1.76) were one to three orders of magnitude more lipophilic than AZT (log P = -0.16) and the AZT phosphoramidates (log P = -0.69 - 1.84). Linear regression analysis did not reveal a correlation between log P values and antiviral potency (data not shown).

Stability Studies.

The stability of compounds **3a**, **3b**, **4a** and **4b** in CEM cell lysates at 37 °C was examined (Table 1). This study revealed that the methyl esters **3a** and **3b** are both readily degraded to the carboxylic acids, with only about 10% of the compounds remaining intact at the end of a 24 hour incubation period. The phenylalanine derivative **3b** appears to be more susceptible to degradation compared to the tryptophan derivative **3a**. At 45 min, approximately 40% of compound **3b** was converted to the carboxylic acid while only 10% of compound **3a** was hydrolyzed over the same period. Thus, the indolyl side chain appears to moderately stabilize the carbomethoxy ester to enzymatic hydrolysis. In contrast, over the 24 hour incubation period, hydrolysis of the methyl amides, **4a** and **4b** was not observed.

Regardless of the carbamate, no detectable AZT was generated over the course of the 24 hour incubation period, verifying the stability of the carbamate linkage. If the antiviral activity of the carbamates was due to slow conversion to AZT, one would expect that for **4a**, for example, conversion of approximately 0.5% of the carbamate to AZT. Since the conditions of our assay would have detected as little as 0.002% conversion to AZT, the antiviral potency of the carbamates is likely due to the intact carbamate and not AZT release.

Summary

A series of tryptophan and phenylalanine carbamates of AZT was prepared and evaluated for anti-HIV-1 activity in PBMCs. These compounds showed antiviral (HIV-1) activity in the low μM range and were not toxic at concentrations >100 μM. The carbamates compounds did not exhibit inhibitory activity toward HIV-1 reverse transcriptase. In addition, neither **4a** nor **4b** were found to inhibit 3'-processing or strand transfer by HIV-1 integrase (C. Marchard and Y. Pommier, personnel communication). The stereochemical requirement for the amino acid side chain does not appear to be specific, since the L-tryptophan derivatives were found to be more potent than the D-tryptophan derivatives, while the D-phenylalanine derivatives were found to be more potent

than the L-phenylalanine derivatives. The methyl amides exhibited superior potency and stability in cell lysates compared to the corresponding methyl ester derivatives. Although the antiviral activity of this series is in the low μM range, their inability to either inhibit HIV-1 reverse transcriptase or integrase or serve as AZT prodrugs suggests that they may act through a mechanism not previously utilized by nucleoside based antivirals.

Experimental Section

Chemistry

Anhydrous acetonitrile was purchased from Aldrich Chemical Co. and was used without further purification. L-amino acid methyl esters (hydrochloride salts), and triphosgene were also purchased from Aldrich. All other solvent were reagent grade and used as received. Anhydrous methylamine was bubbled through methanol to give approximately a 10 M solution. NMR (^1H and ^{13}C) spectra were recorded on Varian VAC-200 and VAC-300 spectrometers. FAB mass spectra were obtained on a VG 7070E-HF mass spectrometer. Elemental analysis was performed by M-H-W Laboratories, Pheonix, AZ.

3'-Azido-2',3'-didehydro-5'-O-methoxy-L-tryptophanyl thymidine (3a). A strirred solution of AZT (300 mg, 1.12 mmol) in 5 mL of dry acetonitrile was cooled to 0 °C under an Ar atmosphere. Diisopropylethylamine (195 μL, 1.12 mmol) and triphosgene (465 mg, 1.57 mmol) were then added and after 5 min the reaction mixture was allowed to warm to rt. After 3 h, the reaction mixture was concentrated to a yellow oil under a stream of Ar. The residue was redissolved in 5 mL of acetonitrile and treated with diisopropylethylamine (390 μL, 2.24 mmol) and L-tryptophan methyl ester (HCl salt, 428 mg, 1.68 mmol). After stirring for 16 h, the reaction was diluted with CH_2Cl_2 (50 mL) and washed with aqueous KH_2PO_4 solution (2×) and brine. The organic portion was dried over Na_2SO_4 and concentrated under reduced pressure. Column chromatography (SiO_2, $CHCl_3$ to 3% MeOH/$CHCl_3$) gave the desired product (513 mg, 89%) as a white foam. MS (FAB) *m/z* 512.2 $(M + H)^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.791 (s, 1 H), 8.292 (s, 1 H), 7.496 (d, J = 7.9 Hz, 1 H), 7.325 (d, J = 8.1 Hz, 1 H), 7.179–7.040 (m, 3 H), 6.949 (d, J = 1.3 Hz, 1 H), 5.999 (t, J = 6.4 Hz, 1 H), 5.377 (d, J = 8.2 Hz, 1 H), 4.718 (ddd, J = 5.3, 5.5, 8.1 Hz, 1 H), 4.379 (dd, J = 3.7, 12.3 Hz, 1 H), 4.273 (dd, J = 3.1, 12.3 Hz, 1 H), 4.073–3.975 (m, 2 H), 3.728 (s, 3 H), 3.361 (dd, J = 5.3, 14.8 Hz, 1 H), 3.275 (dd, J = 5.3, 14.8 Hz, 1 H), 2.347 (m, 1 H), 2.154 (m, 1 H), 1.739 (s, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz, DEPT) δ C 173.05, 164.73, 155.79, 150.90, 136.83, 128.20, 111.55, 110.01; CH 136.12, 123.71, 122.84, 120.22, 118.91, 86.28, 82.98, 77.93,

61.04, 55.29; CH_2, 64.53, 38.06, 28.34; CH_3 53.22, 12.98; Anal. Calcd for $C_{23}H_{25}N_7O_7$: C, 54.01, H, 4.93, N, 19.17. Found C, 53.78, H, 5.19, N, 18.95.

3'-Azido-2',3'-didehydro-5'-O-methoxy-L-phenylalaninyl thymidine (3b). A stirred solution of AZT (300 mg, 1.12 mmol) in 5 mL of dry acetonitrile was cooled to 0 °C under an Ar atmosphere. Diisopropylethylamine (195 μL, 1.12 mmol) and triphosgene (465 mg, 1.57 mmol) were then added and after 5 min the reaction mixture was allowed to warm to rt. After 3 h, the reaction mixture was concentrated to a yellow oil under a stream of Ar. The residue was redissolved in 5 mL of acetonitrile and treated with diisopropylethylamine (390 μL, 2.24 mmol) and L-phenylalanine methyl ester (HCl salt, 362 mg, 1.68 mmol). After stirring for 16 h, the reaction was diluted with CH_2Cl_2 (50 mL) and washed with aqueous KH_2PO_4 solution (2×) and brine. The organic portion was dried over Na_2SO_4 and concentrated under reduced pressure. Purification by column chromatography (SiO_2, $CHCl_3$ to 3% MeOH/$CHCl_3$) gave the desired product (439 mg, 83%) as a white foam. MS (FAB) *m/z* 473.1 $(M + H)^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.502 (s, 1 H), 7.280–7.081 (m, 6 H), 6.039 (t, *J* = 6.4 Hz, 1 H), 5.292 (d, *J* = 8.4 Hz, 1 H), 4.658 (m, 1 H), 4.331 (m, 2 H), 4.116 (m, 1 H), 4.001 (dd, *J* = 4.0, 9.0 Hz, 1 H), 3.740 (s, 3 H), 3.163 (dd, *J* = 5.3, 13.9 Hz, 1 H), 3.053 (dd, *J* = 6.6, 13.9 Hz, 1 H), 2.418 (m, 1 H), 2.267 (m, 1 H), 1.868 (s, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz, DEPT) δ C 172.82, 164.85, 155.85, 151.08, 136.28, 111.77; CH 136.33, 129.87, 129.25, 127.80, 86.08, 82.77, 61.19, 55.56; CH_2, 64.67, 38.79, 38.02; CH_3 53.23, 13.18; Anal. Calcd for $C_{21}H_{24}N_6O_7$: C, 53.39, H, 5.12, N, 17.79. Found C, 53.29, H, 5.00, N, 17.74.

3'-Azido-2',3'-didehydro-5'-O-methylamino-L-tryptophanyl thymidine (4a). Compound **3a** (72 mg, 0.141 mmol) was dissolved in 3 mL of 10M methyl amine in MeOH. The flask was sealed and the reaction mixture was stirred. After 1 h, the reaction was concentrated under reduced pressure. Purification by column chromatography (SiO_2, 5% MeOH/$CHCl_3$) gave the desired product (60 mg, 84%) as a white foam. MS (FAB) *m/z* 511.2 $(M + H)^+$; ^1H NMR (CD_3CN, 300 MHz) δ 9.680 (s, 1 H), 9.226 (s, 1 H), 7.563 (d, *J* = 7.7 Hz, 1 H), 7.357 (dd, *J* = 0.9, 7.0 Hz, 1 H), 7.239 (s, 1 H), 7.121–6.958 (m, 3 H), 6.671 (d, *J* = 4.6 Hz, 1 H), 6.152 (d, *J* = 8.2 Hz, 1 H), 6.074 (t, *J* = 6.6 Hz, 1 H), 4.359 (dd, *J* = 7.7, 13.7 Hz, 1 H), 4.183 (m, 2 H), 4.138 (dd, *J* = 5.7, 10.6 Hz, 1 H), 3.962 (dd, *J* = 3.8, 7.7 Hz, 1 H), 3.222 (dd, *J* = 5.7, 14.6 Hz, 1 H), 3.063 (dd, *J* = 7.7, 14.6 Hz, 1 H), 2.624 (d, *J* = 4.8 Hz, 3 H), 2.267 (m, 2 H), 1.752 (s, 3 H); ^{13}C NMR (CD_3CN, 75 MHz, DEPT) δ C 171.98, 163.99, 155.59, 150.55, 136.43, 127.56, 110.59, 110.14; CH 135.76, 123.71, 121.53, 118.92, 118.45, 111.41, 84.49, 81.87, 60.73, 55.78; CH_2, 64.10, 36.52, 28.07; CH_3 25.37, 11.66; Anal. Calcd for $C_{23}H_{26}N_8O_6$: C, 54.11, H, 5.13, N, 21.95. Found C, 53.84, H, 5.34, N, 21.70.

3'-Azido-2',3'-didehydro-5'-O-methylamino-L-phenylalaninyl thymidine (4b). Compound **4a** (73 mg, 0.155 mmol) was dissolved in 3 mL of 10M methyl amine in MeOH. The flask was sealed and the reaction mixture was stirred. After 1 h, the reaction was concentrated under reduced pressure. Purification by column chromatography (SiO_2, 4% MeOH/$CHCl_3$) gave the desired product (56 mg, 77%) as a white foam. MS (FAB) *m/z* 472.2 $(M + H)^+$; ^1H NMR (C_5D_5N, 300 MHz) δ 8.963 (d, *J* = 8.6 Hz, 1 H), 8.835 (d, *J* = 6.6 Hz, 1 H), 7.418 (s, 1 H), 7.282–7.099 (m, 6 H), 6.500 (t, *J* = 6.6 Hz, 1 H), 4.934 (dd, *J* = 8.1, 15.2 Hz, 1 H), 4.487 (dd, *J* = 5.1, 11.9 Hz, 1 H), 4.398 (dd, *J* = 3.7, 11.9 Hz, 1 H), 4.274 (m, 1 H), 4.095 (m, 1 H), 3.425 (dd, *J* = 6.6, 13.5 Hz, 1 H), 3.165 (dd, *J* = 8.1, 13.5 Hz, 1 H), 2.783 (d, *J* = 4.8 Hz, 3 H), 2.391 (m, 2 H), 1.885 (s, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz, DEPT) δ C 172.11, 164.54, 156.46, 151.39, 138.20, 110.99; CH 135.48, 129.53, 128.46, 126.64, 84.87, 82.14, 61.08, 57.22; CH_2, 64.12, 39.21, 36.74; CH_3 25.90, 12.55; Anal. Calcd for $C_{21}H_{25}N_7O_6$: C, 53.50, H, 5.34, N, 20.80. Found C, 53.74, H, 5.17, N, 20.96.

3'-Azido-2',3'-didehydro-5'-O-methoxy-D-tryptophanyl thymidine (5a). A strirred solution of AZT (267 mg, 1.00 mmol) in 5 mL of dry acetonitrile was cooled to 0 °C under an Ar atmosphere. Diisopropylethylamine (180 μL, 1.04 mmol) and triphosgene (460 mg, 1.55 mmol) were then added and after 5 min the reaction mixture was allowed to warm to rt. After 3 h, the reaction mixture was concentrated to a yellow oil under a stream of Ar. The residue was redissolved in 5 mL of acetonitrile and treated with diisopropylethylamine (360 μL, 2.08 mmol) and D-tryptophan methyl ester (HCl salt, 390 mg, 1.54 mmol). After stirring for 16 h, the reaction was diluted with CH_2Cl_2 (50 mL) and washed with aqueous KH_2PO_4 solution (2×) and brine. The organic portion was dried over Na_2SO_4 and concentrated under reduced pressure. Column chromatography (SiO_2, 4% MeOH/CH_2Cl_2) gave partially purified material. A second column was run (SiO_2, 33% acetone/hexanes to 50% acetone/hexanes) to give the desired product (337 mg, 66%) as a white foam. MS (FAB) *m/z* 512.2 $(M + H)^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ 9.746 (s, 1 H), 8.635 (s, 1 H), 7.478 (d, *J* = 7.7 Hz, 1 H), 7.280 (d, *J* = 7.9 Hz, 1 H), 7.148–7.034 (m, 3 H), 6.945 (d, *J* = 2.0 Hz, 1 H), 6.030 (t, *J* = 6.2 Hz, 1 H), 5.588 (d, *J* = 8.2 Hz, 1 H), 4.670 (ddd, *J* = 5.5, 5.7, 8.1 Hz, 1 H), 4.352 (dd, *J* = 3.8, 12.3 Hz, 1 H), 4.242 (dd, *J* = 3.3, 12.3 Hz, 1 H), 4.095 (dd, *J* = 5.5, 12.6 Hz, 1 H), 3.925 (dd, *J* = 3.8, 8.8 Hz, 1 H), 3.697 (s, 3 H), 3.293 (m, 2 H), 2.368–2.168 (m, 2 H), 1.623 (s, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz, DEPT) δ C 172.67, 164.22, 155.54, 150.36, 136.26, 127.40, 111.16, 109.27; CH 135.60, 123.08, 122.27, 119.68, 118.26, 111.57, 85.22, 82.21, 60.27, 54.08; CH_2, 63.81, 37.40, 27.52; CH_3 52.64, 12.30; Anal. Calcd for $C_{23}H_{25}N_7O_7$: C, 54.01, H, 4.93, N, 19.17. Found C, 53.82, H, 4.90, N, 19.11.

3'-Azido-2',3'-didehydro-5'-O-methoxy-D-phenylalaninyl thymidine (5b). A strirred solution of AZT (267 mg, 1.00 mmol) in 5 mL of dry acetonitrile was cooled to 0 °C under an Ar atmosphere. Diisopropylethylamine (180 μL, 1.04 mmol) and triphosgene (470 mg, 1.58 mmol) were then added and after 5 min the reaction mixture was allowed to warm to rt. After 3 h, the reaction mixture was concentrated to a yellow oil under a stream of Ar. The residue was redissolved in 5 mL of acetonitrile and treated with diisopropylethylamine (360 μL, 2.08 mmol) and D-phenylalanine methyl ester (HCl salt, 322 mg, 1.57 mmol). After stirring for 16 h, the reaction was diluted with CH_2Cl_2 (50 mL) and washed with aqueous KH_2PO_4 solution (2×) and brine. The organic portion was dried over Na_2SO_4 and concentrated under reduced pressure. Purification by column chromatography (SiO2, 33% acetone/hexanes to 50% acetone/hexanes) gave the desired product (296 mg, 63%) as a white foam. MS (FAB) *m/z* 473.1 $(M + H)^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ 9.790 (s, 1 H), 7.277–7.073 (m, 6 H), 6.087 (t, *J* = 6.4 Hz, 1 H), 5.645 (d, *J* = 8.4 Hz, 1 H), 4.612 (dd, *J* = 6.2, 14.5 Hz, 1 H), 4.363 (dd, *J* = 4.4, 12.1 Hz, 1 H), 4.225 (dd, *J* = 3.5, 12.1 Hz, 1 H), 4.145 (dd, *J* = 5.3, 12.6 Hz, 1 H), 3.956 (dd, *J* = 4.4, 9.2 Hz, 1 H), 3.698 (s, 3 H), 3.107 (dd, *J* = 5.7, 13.9 Hz, 1 H), 3.044 (dd, *J* = 6.4, 13.9 Hz, 1 H), 2.419–2.223 (m, 2 H), 1.782 (s, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz, DEPT) δ C 172.29, 164.04, 155.40, 150.43, 135.57, 111.36; CH 135.48, 129.15, 128.73, 127.33, 85.12, 82.03, 60.42, 55.05; CH_2, 63.99, 37.92, 37.36; CH_3 52.58, 12.57; Anal. Calcd for $C_{21}H_{24}N_6O_7$: C, 53.39, H, 5.12, N, 17.79. Found C, 53.48, H, 5.26, N, 18.00.

3'-Azido-2',3'-didehydro-5'-O-methylamino-D-tryptophanyl thymidine (6a). Compound **5a** (176 mg, 0.344 mmol) was dissolved in 4 mL of 10M methyl amine in MeOH. The flask was sealed and the reaction mixture was stirred. After 1.5 h, the reaction was concentrated under reduced pressure. Purification by column chromatography (SiO_2, 6% MeOH/$CHCl_3$) gave the desired product (157 mg, 89%) as a white foam. MS (FAB) *m/z* 511.2 $(M + H)^+$; ^1H NMR (CD_3CN, 300 MHz) δ 9.969 (s, 1 H), 9.300 (d, *J* = 1.6 Hz, 1 H), 7.553 (d, *J* = 7.7 Hz, 1 H), 7.358 (d, *J* = 8.1 Hz, 1 H), 7.267 (s, 1 H), 7.118–6.979 (m, 3 H), 6.736 (d, *J* = 4.6 Hz, 1 H), 6.348 (d, *J* = 8.1 Hz, 1 H), 6.077 (t, *J* = 6.6 Hz, 1 H), 4.355 (dd, *J* = 7.5, 14.1 Hz, 1 H), 4.352–4.170 (m, 3 H), 3.925 (dd, *J* = 5.0, 9.1 Hz, 1 H), 3.213 (dd, *J* = 5.9, 14.6 Hz, 1 H), 3.069 (dd, *J* = 7.5, 14.6 Hz, 1 H), 2.606 (d, *J* = 4.6 Hz, 3 H), 2.293 (m, 2 H), 1.723 (s, 3 H); ^{13}C NMR (CD_3CN, 75 MHz, DEPT) δ C 172.38, 164.18, 155.77, 150.72, 136.02, 127.49, 110.72, 110.16; CH 136.46, 123.68, 121.55, 118.92, 118.46, 111.43, 84.54, 81.75, 60.63, 55.92; CH_2, 64.17, 36.41, 28.13; CH_3 25.47, 11.71; Anal. Calcd for $C_{23}H_{26}N_8O_6$: C, 54.11, H, 5.13, N, 21.95. Found C, 54.23, H, 5.15, N, 22.04.

3'-Azido-2',3'-didehydro-5'-O-methylamino-D-phenylalaninyl thymidine (6b). Compound **5b** (203 mg, 0.430 mmol) was dissolved in 4 mL of 10M methyl amine in MeOH. The flask was sealed and the reaction mixture was stirred. After 1 h, the reaction was concentrated under reduced pressure. Purification by column chromatography (SiO_2, 5% MeOH/$CHCl_3$) gave the desired product (203 mg, 100%) as a white foam. MS (FAB) *m/z* 472.2 $(M + H)^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ 10.196 (s, 1 H), 7.290–7.139 (m, 6 H), 6.444 (d, J = 8.2 Hz, 1 H), 6.210 (d, J = 4.8 Hz, 1 H), 6.094 (t, J = 6.5 Hz, 1 H), 4.364 (dd, J = 7.5, 15.4 Hz, 1 H), 4.261 (m, 2 H), 4.178 (dd, J = 5.8, 10.8 Hz, 1 H), 3.974 (m, 1 H), 3.000 (m, 2 H), 2.668 (d, J = 4.8 Hz, 3 H), 2.335 (m, 2 H), 1.834 (s, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz, DEPT) δ C 171.84, 164.24, 155.66, 150.58, 135.90, 111.32; CH 136.46, 129.18, 128.67, 127.10, 85.27, 81.94, 60.80, 56.75; CH_2, 64.35, 38.94, 37.13; CH_3 26.29, 12.60; Anal. Calcd for $C_{21}H_{25}N_7O_6 \cdot 1/2H_2O$: C, 52.50, H, 5.45, N, 20.41. Found C, 52.62, H, 5.10, N, 20.43.

Culture of Human PBMCs

Human PBMCs were isolated using Ficoll-Paque (Pharmacia Biotech, NJ) density sedimentation[9] and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/mL), streptomycin (100 μg/mL). Cultures were supplemented with phytohemagglutinin (PHA, Sigma, MI) (10 μg/mL) and IL-2 (10 U/mL) (Boehringer Mannheim, IN) where noted.

Antiviral Activity Assay

The procedures for the antiviral activity assays in human PBMCs were adapted from a previously reported method with minor modification.[10,11] Briefly, uninfected PHA-stimulated human PBMCs were counted using the trypan blue dye exclusion method and spun down at 1,500 rpm, at room temperature for 10 min. Infection was carried out at 37 °C for 3 h with 5,000 disintegrations of reverse transcriptase (RT) activity per min per 10^6 cells (DPM/10^6 cells) of LAI/HIV-1. Virus inoculum was prepared from infected PBMC cultures and cell free virions were quantitated by measurement of RT in the supernatant 6 days post-infection. At the end of a 3 h adsorption period, unbound virus was removed and the cells were washed three times with 15 mL of Hank's balanced salt solution (Gibco/BRL, NY) using centrifugation (1,500 rpm, 10 min, room temperature). The cells were resuspended to 2.5 x 10^5 cells/mL in RPMI 1640 medium (10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 U/mL IL-2) and cultures were set up in 24-well tissue culture plates with 1 mL/well. Compounds, prepared in DMSO, were then added to duplicate or triplicate cultures. Uninfected and untreated cultures as well as infected untreated cultures were grown at equivalent cell density as controls. The cultures were maintained in a humidified 6% CO_2-94% air incubator at 37 °C for 6 days. Samples were then collected for the supernatant RT activity.

The RT assays were performed by the method of Spira *et. al.*[12,13] with minor modification. The supernatant from each culture was clarified by centrifugation at 1,500 rpm for 10 min at room temperature. 750 μL of the supernatant was then transferred to a new tube. An equal volume of 20% PEG (polyethyleneglycol) solution was then added to each tube. The samples were mixed and the tubes were placed on ice for 1 h. Virus particles were pelleted by centrifugation at 12,000 rpm for 10 min at room temperature and then disrupted with 30 μL of virus solubilization buffer (0.5% Triton X-100, 0.8 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, MI), 20% glycerol, and 50 mM Tris-HCl, pH 7.8). 10 μL solubilized virus and 90 μL of the reaction mixture (50 mM Tris-HCl, pH 7.8, 9 mM $MgCl_2$, 5 mM DTT, 5 μg/mL poly (rA)d$(T)_{12-18}$, 140 μM dATP and 0.22 μM ^{3}H-TTP (78 Ci/mmol)) were then added to each well of 96-well flat-bottomed microtiter plates (Costar, MA). The plates were covered and incubated at 37 °C for 2 h. Then, 50 μL of trichloroacetic acid (TCA) solution (10% TCA, 0.45 mM sodium pyrophosphate) was added to each well to precipitate the DNA. After precipitation, the DNA was harvested with a cell harvester. A glass fiber filter (Grade 25, Schleicher & Schuell) was prewet with 70% ethanol for 2 sec followed by washing with 400 μL of 5% TCA, 0.45 mM sodium pyrophosphate and 400 μL of 70% ethanol five times. The glass fiber sheet was air dried and disks containing the precipitated DNA were removed and placed into scintillation vials. 4 mL of scintillation fluid (Ecolite$^+$) was added to each vial. The vials were then counted in a beta scintillation counter (Beckman Instruments, Inc., CA), and the results were expressed as disintegrations per min per 0.25 mL of clarified tissue culture supernatant. The extent of HIV-1 replication inhibition was then determined from a set of six drug concentrations and expressed as 50% effective molar concentration (EC_{50}) at which 50% HIV-1 production was inhibited.[14]

Cytotoxicity Assay

After cell density and viability determination(trypan blue dye exclusion method) determination, cells were distributed at a density of 2.5 x 10^5 cells/well into 96-well tissue culture plates to which diluted drug solutions and medium had been added. The plates were then incubated for 6 days at 37 °C in H_2O-saturated air with 10% CO_2. After incubation, 50 μL of XTT/PMS solution (XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2-H-tetrazolium-5-carboxanilide sodium salt, Sigma, MI; PMS, phenazine methosulfate, Sigma, MI) was added to each well. The XTT/PMS solution was made by first dissolving 0.15 g XTT in 144 m L of RPMI 1640 medium (without phenol red). Then, 1% (w/v) of PMS solution was prepared in PBS (phosphate buffer saline). 6 mL of PMS solution was then added to the XTT solution and mixed well before being applied to each well. The plates were incubated for 4 h at 37 °C to allow the PMS-coupled reduction of XTT. Cell viability was quantified by measuring the absorbance at 450 nm using 630 nm as a reference wavelength.

Radioactive Reverse Transcriptase (RT) Assay

In vitro RT assays were performed as described previously[15] with purified recombinant HIV-1 RT (#NEI-490, NEN Life Science Products, Inc., MA) and poly (rA)-oligo $(dT)_{12-18}$ as the primer templates. The reactions were carried out in a total volume of 80 μL in 96- well microtiter plates. To each reaction well, 20 μL of reconstituted AZT-TP standard (Moravek, CA) or compounds in 4- fold final concentrations, and 40 μL of the master mix was added. All reactions were done in triplicate. AZT was prepared in ddH_2O, carbamate derivatives were prepare in 100% DMSO. The master mix consisted of 0.2 mL of lysis buffer, 0.2 mL of 0.5 M Tris, pH 8.0/ 5 mM EDTA, 20 μL of 0.5 M $MgCl_2$, 24 μL of ^{3}H-TTP (specific activity 80 Ci/mmol), 0.2 mL of primer template (250 μg/mL stock), and 0.32 mL of ddH_2O. The reactions were initiated by adding 20 μL of diluted HIV-1 RT (1 unit/mL in 10 mM CHAPS (Sigma, MI) solution in ddH_2O) into each well and incubating at 37 °C for 2 h. To stop the reaction, 100 μL of 10 % TCA/0.45 mM $Na_4P_2O_7$ was added to each well. The plates were then chilled at -20 °C for 10 min before the DNA was harvested (see above). The extent of HIV-1 RT inhibition was determined from a set of six drug concentrations and expressed as 50% effective molar concentration at which 50% of HIV-1 RT was inhibited (IC_{50}).[16]

Stability Studies

1.5 x 10^8 CEM cells were lysed in 1 mL lysis buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) using sonication (4x, 4 sec, on ice) then 80 μL of the resulting lysate was used for each reaction. Each reaction consisted of 80 μL of lysate, 10 μL of compound from 10 mM stock (prepared in DMSO) and 10 μL lysis buffer for a final concentration of 1 mM. The total volume of the reaction was 100 μL. A lysate only control was also included. The reactions were incubated in a heat block at 37°C and were stopped at the end of 0, 45 min and 24 h, respectively, by adding 150 μL of 100% methanol and quick-freezed on dry ice. On the next day, samples were centrifuged at 12,000 rpm for 10 min at 4 °C to remove any particulate matter. The supernatant was transferred to a new tube and vacuum dried. The dried residue was then resuspended into 200 μL ddH_2O and the metabolites analyzed by reverse phase HPLC with a Phenomenex® 250 X 4.6 mm, 5-μm Luna phenyl-hexyl column. The Waters HPLC system was used for the analysis and consisted of a Waters 600E multi-solvent delivery system, a Waters 700 Satellite WISP auto-sampler, a SPD-10A vis-uv dector (Shimadzu) and a digital venturis 466 computer for data analysis. To analyze the metabolites, 20 mM ammonium acetate, pH 3.75 and 100% acetonitrile was used as the solvent. Isocratic condition was used to elute metabolites (52% ammonium acetate, 48% acetonitrile). The flow rate was 1 mL/min and the metabolites were monitored at 270 nm. The retention times for compounds **3a**, **4a**, **3b**, **4b**, **3a**-free acid, and **4a**-free acid was 7.617, 7.967, 7.200, 7.033, 2.117 and 2.117 min,

respectively.[17] The percent degradation of carbamates was derived by dividing the areas under the curve of the HPLC chromatogram by a control that received no incubation. The limit of detection for AZT was 5 ng/mL (20 nM) and 50 ng/mL (100 nM) for the carbamates (data not shown). Under these conditions, conversion of 0.002% of a 1 mM solution of the compounds to AZT would be observable.

Spectrophotometric Determination of Partition Coefficients.

Concentration curves for each compound in octanol and water were constructed with a minimum of six data points, in duplicate, on a Beckman DU7400 spectrophotometer fitted with a 12-well micro cell adapter. The concentrations for the curves, which depended on the ultraviolet absorbance of the compounds, ranged from 2 μM to 300 μM, but only concentrations in the linear range of the instrument (0 to 2.7 absorbance units) were used for the standard curves. Extinction coefficients (ε) were determined for each compound in octanol and water. 4 mL each of octanol and water were mixed and equilibrated in a mechanical shaker at 20 cycles/minute for 6 h and allowed to stand overnight. Approximately 0.5 mg of each compound was weighed and added to the equilibrated solvent mixtures in duplicate, and then resealed with a plastic cap containing a Teflon insert. The tubes were shaken vigorously and placed on a mechanical shaker (20 cycles/minute) for 1 h at room temperature. This was followed by centrifugation for 1 h at 1000 rpm and then the system was left to stand at room temperature for 30 h to achieve equilibrium. After this, 250 μL samples were withdrawn from each phase and the ultraviolet absorbance determined with either octanol or water as blank. The concentration of compound in each phase was determined using the previously determined extinction coefficients and the Beer-Lambert equation. The Log P values were determined in duplicate or triplicate and are reported as the average of the two values that were within 15% variance.

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