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Synthesis and Anti-HIV Activity of Some [Nucleoside Reverse Transcriptase Inhibitor] -C5′-Linker-[Integrase Inhibitor] Heterodimers as Inhibitors of HIV Replication

ELENA SUGEAC[a], CHRISTINE FOSSEY[a], DANIEL LADURÉE[a,*], SYLVIE SCHMIDT[b], GERALDINE LAUMOND[b] and ANNE-MARIE AUBERTIN[b]

[a]*Centre d'Etudes et de Recherche sur le Médicament de Normandie, U.F.R. des Sciences Pharmaceutiques, 5, Rue Vaubénard, F-14032, Caen Cedex, France;* [b]*I.N.S.E.R.M., 3, Rue Koeberlé, F-67000, Strasbourg, France*

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Selected for their expected ability to inhibit HIV replication, a series of eight heterodimers containing a Nucleoside Reverse Transcriptase Inhibitor (NRTI) and an Integrase Inhibitor (INI), bound by a linker, were designed and synthesized. For the NRTIs, d_4U, d_2U and d_4T were chosen. For the INIs, 4-[1-(4-fluorobenzyl)-1*H*-pyrrol-2-yl]-2,4-dioxobutyric acid (6) and 4-(3,5-dibenzyloxyphenyl)-2,4-dioxobutyric acid (9) (belonging to the β-diketo acids class) were chosen. The conjugation of the two different inhibitors (NRTI and INI) was performed using an amino acid (glycine or β-alanine) as a cleavable linker.

Keywords: HIV; NRTI; INI; Cleavable linker; Heterodimer; Integrase inhibitor; Reverse Transcriptase inhibitor

INTRODUCTION

The AIDS epidemic caused by the HIV virus is a serious health problem throughout the world. In the search for therapeutic drugs against this infection, the main interest has been focused on two key enzymes in the HIV machinery namely HIV reverse transcriptase (RT) and HIV protease (PR).[1]

Single-agent therapy with the currently available drugs has proved ineffective in the long-term treatment of human immunodeficiency virus (HIV) type 1 infection.[2] The introduction of combination therapy has effectively reduced the occurrence of drug-resistant strains and has transformed the prognosis for most patients receiving it. In order to prevent the emergence of a drug-resistant virus, a combination of HIV PR inhibitors and RT inhibitors has become the clinical practice.[3,4] However, these treatments do not suppress viral replication in all patients. It is therefore logical to improve the efficacy of combination therapy by the development of new compounds that target additional and different steps of the viral replication cycle. The HIV-1 integrase (IN) represents a new potential target for the development of selective antiretroviral chemotherapy.

The Reverse Transcriptase (RT) of HIV-1 is essential for its replication. Most of the antiviral drugs approved for the treatment of HIV infection inhibit this enzyme. Among the NRTIs, zidovudine,[5–7] didanosine,[8] zalcitabine[9] stavudine,[10–15] and lamivudine[16–18] are clinically used for the treatment of AIDS. These agents act competitively at the catalytic site of the enzyme, after conversion to their triphosphate form.

The integrase (IN) is one of the three enzymes encoded in the HIV-1 genome; this enzyme is essential for viral replication. Several compounds have been identified to inhibit IN activity in recent years: integric acid,[19] naphthothiazepine,[20]

*Corresponding author. Tel.: +33-(0)-2-31-93-64-58. Fax: +33-(0)-2-31-93-34-73. E-mail: laduree@pharmacie.unicaen.fr

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granulatine,[21] L-chicoric acid,[22] salicylhydrazide,[23,24] caffeic acid phenylethyl ester (CAPE),[25] 5-CITEP [1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone][26] and the β-diketo acids,[27] L-731,988 and L-708,906. Among these compounds, only β-diketo acids are authentic inhibitors of HIV-1 integration.[28]

In this paper, we describe the synthesis and biological evaluation of some heterodimers of the general formula [NRTI]-*C5′*-Linker-[INI] including both a NRTI [2′,3′-didehydro-2′,3′-dideoxyuridine (d_4U)[29] or 2′,3′-dideoxyuridine (d_2U)[29] or 2′,3′-didehydro-2′,3′-dideoxythymidine (d_4T)] and an INI (belonging to the β-diketo acids class) connected through an amino acid (glycine or β-alanine) as a cleavable linker.

MATERIALS AND METHODS

General

Dioxane and tetrahydrofuran were distilled from sodium/benzophenone immediately prior to use. Anhydrous methanol, acetone, *N,N*-dimethylformamide were purchased from E. Merk, Carlo Erba and Aldrich, respectively. Unless otherwise stated, reactions were conducted under an atmosphere of argon and monitored by thin-layer chromatography (TLC) using precoated silica gel 60 F_{254} sheets (0.2 mm layer) purchased from Macherey-Nagel, and compounds were detected by UV absorption at 254 nm. Column chromatography was effected by using Merck silica gel 60 (0.063–0.200 mm) and silica gel Si-60 for flash chromatography (40–63 μm) (Merck). All samples were kept in a drying oven over P_2O_5 for at least 24 hours prior to analysis.

Melting points were determined on a Kofler apparatus. IR spectra were recorded on a Fourier transform Mattson spectrometer Genesis DTGS using WinFIRST™ Macros and ApPro™; only noteworthy absorptions are listed. ^{1}H and ^{13}C-NMR spectra were obtained on a JEOL Lambda 400 using TMS as an internal standard. NH and OH signals appeared as broad singlets exchangeable with D_2O (s = singlet, b = broad, d = doublet, t = triplet, q = quadruplet, m = multiplet).

Chemistry

1-[1-(4-Fluorobenzyl)-1H-pyrrol-2-yl]ethanone (4)

To a solution of 2-acetylpyrrole (3.27 g; 30.0 mmol) in dimethylformamide (40 mL) was added sodium hydride (60% dispersion in oil, 1.32 g; 33.0 mmol) at 0°C and the mixture was stirred at room temperature for 15 min. To this mixture was added dropwise a solution of 4-fluorobenzyl bromide (6.24 g; 43.3 mmol) in dimethylformamide (2 mL) at 0°C and the reaction mixture was stirred at room temperature for 1h. The solution was then poured into a mixture of water and ice (100 mL) and extracted with EtOAc (3 × 100mL). The combined organic phases were washed with H_2O (200 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. The title compound **4**, obtained as a clear yellow oil (6.18 g), was taken on to the next step without further purification; Yield 95%; TLC R_f (EtOAc:Hexane = 20:80) 0.58; IR (KBr), cm^{-1}: 1651 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 2.41 (s, 3H, CH_3), 5.53 (s, 2H, $ArCH_2$), 6.18–6.20 (m, 1H, H-4:pyrrole), 6.90–6.91 (m, 1H, H-5:pyrrole), 6.94–7.01 (m, 3H, H-3:pyrrole and H-3′, H-5′), 7.07–7.11 (m, 2H, H-2′ and H-6′).

Ethyl 4-[1-(4-fluorobenzyl)-1H-pyrrol-2-yl]-2,4-dioxobutyrate (5)

To a solution of compound **4** (4.34 g; 20.0 mmol) in anhydrous dimethoxyethane (40 mL) was added, respectively: sodium hydride (60% dispersion in oil, 0.96 g; 24.0 mmol), diethyl oxalate (3.5 g; 24.0 mmol) and a drop of ethanol. The reaction mixture was heated at reflux for 2.5 h, stirred overnight at room temperature, poured into a saturated solution of $NaHCO_3/H_2O$ (200 mL) and extracted several times with EtOAc (3 × 100 mL). The combined organic phases were washed with 10% $NaHCO_3/H_2O$ (200 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was crystallized with diethyl ether to give the title compound **5** as yellow–orange crystals (2.0 g); Yield 32%; m.p. 129–130°C; TLC R_f ($CHCl_3$: MeOH : CH_3COOH = 97 : 3 : 1) 0.39; IR (KBr), cm^{-1}: 3429 (OH), 1716 (C═O : ester); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.38 (t, 3H, CH_2CH_3, 7.0), 4.36 (q, 2H, CH_2CH_3, 7.0), 5.60 (s, 2H, $ArCH_2$), 6.27–6.29 (m, 1H, H-4:pyrrole), 6.83 (s, 1H, *CH*═C–OH), 6.97–7.09 (m, 3H, H-5:pyrrole and H-3′, H-5′), 7.09–7.12 (m, 2H, H-2′ and H-6′), 7.14 (m, 1H, H-3:pyrrole).

4-[1-(4-Fluorobenzyl)-1H-pyrrol-2-yl]-2,4-dioxobutyric Acid (6)

To a solution of compound **5** (2.0 g; 6.31 mmol) in 1:1 tetrahydrofuran/methanol (30 mL) was added a solution of 1N $NaOH/H_2O$ (1.26 g; 31.6 mmol) and the mixture was stirred overnight at room temperature. Then, the reaction mixture was washed with diethyl ether (100 mL), acidified to pH = 2 with 1N HCl/H_2O and extracted several times with EtOAc (3 × 100 mL). The combined organic phases were washed with 1N HCl/H_2O (100 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was crystallized from chloroform to give the title compound **6** as yellow crystals (1.22 g);

Yield 67%; m.p. 182–184°C; TLC R_f ($CHCl_3$: MeOH : CH_3COOH = 94 : 6 : 6) 0.37; IR (KBr), cm^{-1}: 2647–2522 (OH:COOH), 1708 (C═O : COOH); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 5.59 (s, 2H, $ArCH_2$), 6.30–6.32 (m, 1H, H-4:pyrrole), 6.92 (s, 1H, C*H*═C–OH), 6.98–7.02 (m, 2H, H-3′ and H-5′), 7.05–7.12 (m, 3H, H-5:pyrrole and H-2′, H-6′), 7.18–7.21 (m, 1H, H-3:pyrrole); ^{13}C-NMR ($CDCl_3$), δ, ppm: 52.4 (Ar*C*H_2), 100.9 (*C*H═C–OH), 110.5 (C-4:pyrrole), 115.5 and 115.7 (C-3′ and C-5′), 122.0 (C-3:pyrrole), 128.6 and 128.7 (C-2′ and C-6′), 128.8 (C-1′), 133.2 (C-5:pyrrole), 133.3 (C-2:pyrrole), 160.8 (C-4′), 163.4 (CH═*C*–OH), 183.0 (C-1), 183.2 (C-4); MS *m/z*: 83.5, 109, 157, 201, 244.1, *289.1*; Anal. Calcd. for $C_{15}H_{12}NO_4F$: C, 62.28; H, 4.15; N, 4.84. Found: C, 62.30; H, 4.19; N, 4.85%.

1-(3,5-Dibenzyloxyphenyl)ethanone (7)

To a solution of 3,5-dihydroxyacetophenone (3.0 g; 19.74 mmol) in acetone (60 mL) was added, respectively: anhydrous sodium carbonate (8.2 g; 59.22 mmol) and benzyl bromide (6.75 g; 39.48 mmol). The reaction mixture was warmed to reflux for 7 h and then cooled at room temperature. The solution was then poured into water (100 mL) and extracted several times with EtOAc (3 × 100 mL). The combined organic phases were washed with H_2O (200 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. The title compound **7** was obtained as a beige solid (6.28 g) that was taken on to the next step without further purification; Yield 96%; m.p. 62°C; TLC R_f (EtOAc:Hexane = 30:70) 0.55; IR (KBr), cm^{-1}: 1678 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 2.54 (s, 3H, CH_3), 5.05 (s, 4H, 2 × C*H*$_2$Ar), 6.78–6.81 (m, 1H, H-4′), 7.16–7.19 (m, 2H, H-2′ et H-6′), 7.30–7.42 (m, 10H, Ar).

Ethyl 4-(3,5-dibenzyloxyphenyl)-2,4-dioxo butyrate (8)

To a solution of compound **7** (6.0 g; 18.0 mmol) in anhydrous dimethoxyethane (40 mL) was added, respectively: sodium hydride (60% dispersion in oil, 1.08 g; 27.0 mmol), diethyl oxalate (3.15 g; 21.6 mmol) and a drop of ethanol. The reaction mixture was warmed at 110°C for 2.5 h and then cooled at room temperature. The solution was then poured into a solution 1N HCl/H_2O (50 mL) and extracted several times with EtOAc (3 × 100 mL). The combined organic phases were dried over $MgSO_4$, filtered and concentrated in vacuo. The title compound **8** was obtained as a yellow solid (6.5 g); Yield 83%; m.p. 105°C; TLC R_f (CH_2Cl_2 100%) 0.79; IR (KBr), cm^{-1}: 3352 (OH), 1744 (C═O:ester), 1683 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.41 (t, 3H, CH_2CH_3, 7.1), 4.40 (q, 2H, CH_2CH_3, 7.1), 5.05 (s, 4H, 2 × C*H*$_2$Ar), 6.80–6.85 (m, 1H, H-4′), 7.09 (s, 1H, C*H*═C–OH), 7.18–7.23 (m, 2H, H-2′ and H-6′), 7.32–7.42 (m, 10H, Ar).

4-(3,5-Dibenzyloxyphenyl)-2,4-dioxobutyric Acid (9)

To a solution of compound **8** (6.0 g; 14.85 mmol) in 1:1 tetrahydrofuran/methanol (80 mL) was added a solution of 1M NaOH/H_2O (1.78 g; 44.5 mL; 44.5 mmol) and the mixture was stirred at room temperature for 2 h. Then, the reaction mixture was washed with diethyl ether (100 mL), acidified to pH = 2 with 1M HCl/H_2O and extracted several times with EtOAc (3 × 100 mL). The combined organic phases were washed with 1M HCl/H_2O (100 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was triturated with diethyl ether to give the title compound **9** as a yellow powder (2.66 g); Yield 47%; m.p. 171°C; TLC R_f (CH_2Cl_2 100%) 0.51; IR (KBr), cm^{-1}: 3421 (OH), 2637–2520 (OH:COOH), 1710 (C═O : COOH), 1634 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 5.06 (s, 4H, 2 × C*H*$_2$Ar), 6.80–6.84 (m, 1H, H-4′), 7.10 (s, 1H, C*H*═C–OH), 7.18–7.22 (m, 2H, H-2′ and H-6′), 7.31–7.43 (m, 10H, Ar); ^{13}C-NMR ($CDCl_3$), δ, ppm: 70.4 (2 × *C*H_2Ar), 96.4 (*C*H═C–OH), 106.8 (C-2′ and C-6′), 108.0 (C-4′), 127.5 (2 × C-II and 2 × C-VI), 128.2 (C-IV), 128.6 (2 × C-III and 2 × C-V), 135.5 (C-I), 136.1 (C-1′), 160.1 (C-3′ and C-5′), 163.4 (CH═*C*–OH), 172.0 (C═O : COOH), 187.7 (C-4); MS *m/z*: 91.1, 105.0, 181.1, 332.1, 358.1, *404.1*; Anal. Calcd for $C_{24}H_{20}O_6$: C, 71.29; H, 4.95. Found: C, 71.31; H, 4.96%.

5′-O-Glycyl(Boc)d_4U (10a)

To a solution of Boc-Gly-OH (0.416 g; 2.38 mmol) in DMF (6 mL) were added d_4U (**1**) (0.5 g; 2.38 mmol), DCC (0.490 g; 2.38 mmol) and DMAP (0.029 g; 0.238 mmol) at 0°C, and the mixture was stirred at room temperature for 17 h. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (50 mL), washed with 10% citric acid (50 mL), 5% $NaHCO_3$ (50 mL) and brine (2 × 50 mL), dried over $MgSO_4$, and concentrated in vacuo. Purification of the product by silica gel column chromatography (CH_2Cl_2:MeOH) gave the title compound **10a** as white crystals (0.48 g); Yield 55%; m.p. 70–72°C; TLC R_f (CH_2Cl_2 : MeOH = 92 : 8) 0.52; IR (KBr), cm^{-1}: 3345 (NH), 3210 (NH), 1759 (C═O), 1715 (C═O), 1698 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.38 (s, 9H, 3 × CH_3), 3.41 (d, 2H, C*H*$_2$NHBoc, 5.3), 4.23 (dd, 1H, H-5′, 8.5, 3.7), 4.38 (dd, 1H, H-5′, 8.5, 3.7), 4.99–5.03 (m, 2H, H-4′ and CH_2N*H* Boc), 5.72 (d, 1H, H-5, 8.1), 5.86 (d, 1H, H-2′, 5.9), 6.23 (d, 1H, H-3′, 5.9), 6.93 (m, 1H, H-1′), 7.38 (d, 1H, H-6, 8.1), 9.00 (bs, 1H, CON*H*CO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 28.2 (3 × CH_3), 42.2 (*C*H_2NHBoc), 65.2 (C-5′), 80.3 (*C*(CH_3)$_3$), 84.1 (C-4′),

89.9 (C-1′), 102.8 (C-5), 126.9 (C-2′), 133.4 (C-3′), 139.9 (C-6), 150.6 (C-2), 155.6 (CO:Boc), 162.9 (C-4), 170.1 ($COCH_2NHBoc$).

5′-O-Glycyl(Boc)d_2U (10b)

Compound **10b** was prepared from compound d_2U (**2**) (0.3 g; 1.42 mmol) and Boc-Gly-OH (0.247 g; 1.42 mmol) in a similar manner to that described for compound **10a**. Purification of the product by silica gel column chromatography (CH_2Cl_2:MeOH) gave the title compound **10b** as white crystals (0.41 g); Yield 79%; m.p. 84°C; TLC R_f (CH_2Cl_2 : MeOH = 92 : 8) 0.47; IR (KBr), cm^{-1}: 3399 (NH), 3177 (NH), 1765 (C═O), 1710 (C═O), 1693 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.45 (s, 9H, 3 × CH_3), 1.80–1.88 (m, 1H, H-3′), 2.06–2.12 (m, 2H, H-3′ and H-2′), 2.42–2.48 (m, 1H, H-2′), 3.95 (d, 2H, C*H*$_2$NHBoc, 5.8), 4.28–4.39 (m, 3H, H-4′ and H-5′), 5.08 (t, 1H, CH_2N*H*Boc, 5.8), 5.78 (d, 1H, H-5, 8.0), 6.04–6.06 (m, 1H, H-1′), 7.57 (d, 1H, H-6, 8.0), 8.93 (bs, 1H, CON*H*CO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 25.7 (C-3′), 28.2 (3 × CH_3), 32.3 (C-2′), 42.4 (*C*H$_2$ NHBoc), 65.4 (C-5′), 78.4 (C-4′), 80.3 (*C*(CH_3)$_3$), 86.5 (C-1′), 102.2 (C-5), 139.5 (C-6), 150.1 (C-2), 155.7 (CO:Boc), 163.0 (C-4), 170.2 ($COCH_2NHBoc$).

5′-O-Glycyl(Boc)d_4T (10c)

Compound **10c** was prepared from compound d_4T (**3**) (0.4 g; 1.79 mmol) and Boc-Gly-OH (0.312 g; 1.79 mmol) in a similar manner to that described for compound **10a**. Purification of the product by silica gel column chromatography (CH_2Cl_2:MeOH) gave the title compound **10c** as white crystals (0.53 g); Yield 78%; m.p. 120°C; TLC R_f (CH_2Cl_2 : MeOH = 92 : 8) 0.49; IR (KBr), cm^{-1}: 3396 (NH), 3172 (NH), 1759 (C═O), 1693 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.45 (s, 9H, 3 × CH_3), 1.94 (s, 3H, CH_3), 3.49 (d, 2H, C*H*$_2$NHBoc, 5.4), 4.29 (dd, 1H, H-5′, 9.7, 2.4), 4.46 (dd, 1H, H-5′, 9.7, 2.4), 5.02–5.06 (m, 2H, H-4′ and CH_2N*H*Boc), 5.90–5.93 (m, 1H, H-2′), 6.28–6.30 (m, 1H, H-3′), 6.98–7.01 (m, 1H, H-1′), 7.19 (s, 1H, H-6), 8.35 (bs, 1H, CON*H*CO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 12.6 (CH_3), 28.2 (3 × CH_3), 42.3 (*C*H$_2$NHBoc), 65.4 (C-5′), 80.3 (*C*(CH_3)$_3$), 83.9 (C-4′), 89.8 (C-1′), 111.3 (C-5), 127.3 (C-2′), 133.2 (C-3′), 135.3 (C-6), 150.5 (C-2), 155.6 (CO:Boc), 163.3 (C-4), 170.1 ($COCH_2NHBoc$).

5′-O-Glycyld_4U·HCl (11a)

To a solution of the resulting product **10a** (0.4 g; 1.09 mmol) in 4M HCl/dioxane (10 mL) at 0°C was added anisole (0.10 mL; 0.95 mmol), and the mixture was stirred at room temperature for 1 h. After removal of the solvent in vacuo, the residue was precipitated from diethyl ether to give the title compound **11a** as a green solid (0.32 g); Yield 97%; m.p. 146–148°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.09; IR (KBr), cm^{-1}: 3238 (NH), 1757 (C═O), 1683 (C═O); ^{1}H-NMR (d_6-DMSO), δ, ppm, J, Hz: 3.67–3.74 (m, 2H, C*H*$_2$NH$_2$·HCl), 4.31 (m, 2H, H-5′), 5.00 (m, 1H, H-4′), 5.63 (d, 1H, H-5, 7.9), 6.03 (d, 1H, H-2′, 5.8), 6.45 (d, 1H, H-3′, 5.8), 6.80 (m, 1H, H-1′), 7.46 (d, 1H, H-6, 7.9), 8.46 (bs, 3H, NH_2·HCl), 11.39 (s, 1H, NH); ^{13}C-NMR (d_6-DMSO), δ, ppm: 38.6 (CH_2NH_2·HCl), 65.8 (C-5′), 83.5 (C-4′), 89.3 (C-1′), 101.4 (C-5), 126.2 (C-2′), 133.7 (C-3′), 140.6 (C-6), 151.1 (C-2), 163.7 (C-4), 167.8 ($COCH_2NH_2$·HCl).

5′-O-Glycyld_2U·HCl (11b)

Compound **11b** was prepared from **10b** (0.41 g; 1.11 mmol) in a similar manner to that described for compound **11a**. Yield 97%; m.p. 112–114°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.08; IR (KBr), cm^{-1}: 3192 (NH), 1755 (C═O), 1685 (C═O); ^{1}H-NMR (d_6-DMSO), δ, ppm, J, Hz: 1.47–1.57 (m, 1H, H-3′), 1.97–2.02 (m, 2H, H-3′ and H-2′), 2.27–2.34 (m, 1H, H-2′), 3.14 (s, 2H, C*H*$_2$NH$_2$·HCl), 3.82 (d, 2H, H-5′, 3.4), 4.18–4.22 (m, 1H, H-4′), 5.62 (d, 1H, H-5, 8.0), 5.98–6.01 (m, 1H, H-1′), 7.65 (d, 1H, H-6, 8.0), 8.44 (bs, 3H, NH_2·HCl), 11.30 (s, 1H, NH); ^{13}C-NMR (d_6-DMSO), δ, ppm: 25.5 (C-3′), 30.5 (C-2′), 48.5 (CH_2NH_2·HCl), 66.2 (C-5′), 77.4 (C-4′), 85.1 (C-1′), 101.7 (C-5), 140.7 (C-6), 150.4 (C-2), 163.1 (C-4), 167.6 ($COCH_2NH_2$·HCl).

5′-O-Glycyld_4T·HCl (11c)

Compound **11c** was prepared from compound **10c** (0.22 g; 0.58 mmol) in a similar manner to that described for compound **11a**; Yield 87%; m.p. 156–158°C; TLC R_f (CH_2Cl_2 : MeOH = 85 : 15) 0.20; IR (KBr), cm^{-1}: 3211 (NH), 1749 (C═O), 1719 (C═O), 1696 (C═O); ^{1}H-NMR (d_6-DMSO), δ, ppm, J, Hz: 1.78 (s, 3H, CH_3), 3.49–3.59 (m, 2H, C*H*$_2$NH$_2$·HCl), 4.32 (d, 2H, H-5′, 3.5), 4.98 (m, 1H, H-4′), 6.02 (d, 1H, H-2′, 5.4), 6.43 (d, 1H, H-3′, 5.4), 6.80 (m, 1H, H-1′), 7.26 (s, 1H, H-6), 8.46 (bs, 3H, NH_2·HCl), 11.40 (s, 1H, CON*H*CO); ^{13}C-NMR (d_6-DMSO), δ, ppm: 12.1 (CH_3), 38.8 (CH_2 NH_2·HCl), 66.0 (C-5′), 83.2 (C-4′), 89.4 (C-1′), 109.7 (C-5), 126.7 (C-2′), 133.4 (C-3′), 136.0 (C-6), 150.8 (C-2), 163.8 (C-4), 167.6 ($COCH_2NH_2$·HCl).

5′-O-{7-[1-(4-Fluorobenzyl)-1H-pyrrol-2-yl]-5,7-dioxobutyrylglycyl}d_4U (12a)

To a solution of **6** (0.11 g; 0.38 mmol) in DMF (5 mL) was added compound **11a** (0.139 g; 0.46 mmol), HOBT (0.129 g; 0.95 mmol), TEA (0.11 mL; 0.76 mmol) and BOP (0.252 g; 0.57 mmol) at 0°C, and the solution was stirred for 24 h at room temperature. After removal of the solvent in vacuo,

the residue was dissolved in EtOAc (30 mL), washed with 10% citric acid (30 mL), 5% $NaHCO_3$ (30 mL) and brine (2 × 30 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. Purification of the product by silica gel column chromatography (CH_2Cl_2 :MeOH) gave the title compound **12a** as yellow crystals (0.080 g); Yield 40%; m.p. 103–105°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.58; IR (KBr), cm^{-1}: 3400 (NH, OH), 1756 (C═O), 1690 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 4.20 (d, 2H, NHCH_2CO, 6.0), 4.32 (dd, 1H, H-5′, 9.2, 2.9), 4.47 (dd, 1H, H-5′, 9.2, 2.9), 5.08 (m, 1H, H-4′), 5.58 (s, 2H, ArCH_2), 5.80 (d, 1H, H-5, 8.1), 5.94 (d, 1H, H-2′, 5.6), 6.27–6.32 (m, 2H, H-4:pyrrole and H-3′), 6.89 (s, 1H, C*H*═C–OH), 6.97–7.01 (m, 4H, H-1′, H-3:arom., H-5:arom. and H-5:pyrrole), 7.07–7.11 (m, 2H, H-2:arom. and H-6:arom.), 7.19–7.20 (m, 1H, H-3:pyrrole), 7.41–7.44 (m, 2H, H-6 and CH_2N*H*CO), 8.42 (bs, 1H, CON*H*CO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 41.0 (NH*C*H_2CO), 52.3 (Ar*C*H_2), 65.5 (C-5′), 84.1 (C-4′), 90.1 (C-1′), 98.5 (*C*H═C–OH), 102.8 (C-5), 110.2 (C-4:pyrrole), 115.5 and 115.7 (C-3:arom. and C-5:arom.), 121.6 (C-3:pyrrole), 127.1 (C-2′), 128.6 and 128.7 (C-2:arom. and C-6:arom.), 129.0 (C-1:arom.), 132.5 (C-5:pyrrole), 133.4 (C-3′), 133.5 (C-2:pyrrole), 139.7 (C-6), 150.4 (C-2), 160.9 (C-4), 162.2 (C-4:arom.), 162.6 (CH═*C*–OH), 163.3 (*C*ONHCH$_2$), 168.8 (NHCH$_2$ *C*O), 184.1 (*C*OCH═C–OH); Anal. Calcd for $C_{26}H_{23}N_4O_8F$: C, 57.99; H, 4.27; N, 10.41. Found: C, 58.04; H, 4.32; N, 10.48%.

5′-O-{7-[1-(4-Fluorobenzyl)-1H-pyrrol-2-yl]-5,7-dioxobutyrylglycyl}d_2U (12b)

Compound **12b** was prepared from **11b** (0.139 g; 0.46 mmol) and **6** (0.11 g; 0.38 mmol) in a similar manner to that described for compound **12a**. Purification of the product by silica gel column chromatography (CH_2Cl_2:MeOH) gave the title compound **12b** as yellow crystals (0.069 g); Yield 34%; m.p. 95–97°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.57; IR (KBr), cm^{-1}: 3406 (NH, OH), 1754 (C═O), 1687 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.78–1.88 (m, 1H, H-3′), 2.04–2.12 (m, 2H, H-3′ and H-2′), 2.41–2.50 (m, 1H, H-2′), 4.20 (d, 2H, NHCH_2CO, 5.9), 4.30–4.35 (m, 1H, H-4′), 4.41 (d, 2H, H-5′, 3.9), 5.58 (s, 2H, ArCH_2), 5.78 (d, 1H, H-5, 8.0), 6.02–6.04 (m, 1H, H-1′), 6.27–6.29 (m, 1H, H-4:pyrrole), 6.89 (s, 1H, C*H*═C–OH), 6.97–7.01 (m, 3H, H-3:arom., H-5:arom. and H-5:pyrrole), 7.07–7.11 (m, 2H, H-2:arom. and H-6:arom.), 7.17–7.20 (m, 1H, H-3:pyrrole), 7.45 (t, 1H, CH_2N*H*CO, 5.9), 7.53 (d, 1H, H-6, 8.0), 8.42 (bs, 1H, CON*H*CO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 22.7 (C-3′), 32.2 (C-2′), 41.2 (NH*C*H_2CO), 52.3 (Ar*C*H_2), 65.8 (C-5′), 78.3 (C-4′), 86.6 (C-1′), 98.5 (*C*H═C-OH), 102.2 (C-5), 110.2 (C-4:pyrrole), 115.5 and 115.7 (C-3:arom. and C-5:arom.), 121.6 (C-3:pyrrole), 128.6 and 128.7 (C-2:arom. and C-6:arom.), 129.1 (C-1:arom.), 132.5 (C-5:pyrrole), 133.5 (C-2:pyrrole), 139.4 (C-6), 150.0 (C-2), 160.9 (C-4), 162.2 (C-4:arom.), 162.7 (C*H*═C–OH), 163.4 (*C*ONHCH$_2$), 168.9 (NHCH$_2$*C*O), 184.1 (*C*OCH═C–OH); Anal. Calcd for $C_{26}H_{25}N_4O_8F$: C, 57.78; H, 4.63; N, 10.37. Found: C, 57.86; H, 4.64; N, 10.41%.

5′-O-{7-[1-(4-Fluorobenzyl)-1H-pyrrol-2-yl]-5,7-dioxobutyrylglycyl}d_4T (12c)

Compound **12c** was prepared from **11c** (0.158 g; 0.50 mmol) and **6** (0.12 g; 0.41 mmol) in a similar manner to that described for compound **12a**. Purification of the product by silica gel column chromatography (EtOAc 100%) gave the title compound **12c** as green crystals (0.079 g); Yield 35%; m.p. 90–92°C; TLC R_f (EtOAc : MeOH = 90 : 10) 0.58; IR (KBr), cm^{-1}: 3399 (NH, OH), 1757 (C═O), 1690 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.95 (s, 3H, CH_3), 2.86 (d, 2H, NHCH_2CO, 10.1), 4.32 (dd, 1H, H-5′, 8.0, 4.0), 4.49 (dd, 1H, H-5′, 8.0, 4.0), 5.06–5.08 (m, 1H, H-4′), 5.30 (bs, 1H, CH═C–*OH*), 5.59 (s, 2H, ArCH_2), 5.94 (d, 1H, H-2′, 5.1), 6.28–6.31 (m, 2H, H-4:pyrrole and H-3′), 6.89 (s, 1H, C*H*═C–OH), 6.97–7.02 (m, 4H, H-1′, H-3:arom., H-5:arom. and H-5:pyrrole), 7.07–7.12 (m, 2H, H-2:arom. and H-6:arom.), 7.17–7.19 (m, 2H, H-6 and H-3:pyrrole), 7.44 (t, 1H, CH_2N*H*CO, 10.1), 8.35 (bs, 1H, CON*H*CO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 12.6 (CH_3), 41.1 (NH*C*H_2CO), 52.3 (Ar*C*H_2), 65.7 (C-5′), 83.8 (C-4′), 89.9 (C-1′), 98.5 (*C*H═C–OH), 110.3 (C-5), 111.3 (C-4:pyrrole), 115.5 and 115.7 (C-3:arom. and C-5:arom.), 121.6 (C-3:pyrrole), 127.4 (C-2′), 128.6 and 128.7 (C-2:arom. and C-6:arom.), 129.1 (C-1:arom.), 132.5 (C-5:pyrrole), 133.1 (C-3′), 133.5 (C-2:pyrrole), 135.3 (C-6), 150.6 (C-2), 160.9 (C-4), 162.2 (C-4:arom.), 163.3 (CH═*C*–OH), 163.4 (*C*ONHCH$_2$), 168.8 (NHCH$_2$ *C*O), 184.1 (*C*OCH═C–OH); Anal. Calcd. for $C_{27}H_{25}N_4O_8F$: C, 58.70; H, 4.53; N, 10.14. Found: C, 58.76; H, 4.56; N, 10.16%.

5′-O-β-Alanyl(Boc)d_4U (13a)

To a solution of Boc-β-Ala-OH (0.360 g; 1.905 mmol) in DMF (6 mL) were added d_4U (**1**) (0.4 g; 1.905 mmol), DCC (0.392 g; 1.905 mmol) and DMAP (0.023 g; 0.19 mmol) at 0°C, and the mixture was stirred at room temperature for 18 h. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (40 mL), washed with 10% citric acid (40 mL), 5% $NaHCO_3$ (40 mL) and saturated NaCl (2 × 40 mL), dried over $MgSO_4$, and concentrated in vacuo. Purification of the product by silica gel column chromatography (CH_2Cl_2:MeOH) gave the title compound **13a** as white crystals (0.27 g); Yield 38%; m.p. 70–72°C; TLC R_f

(CH_2Cl_2 : MeOH = 90 : 10) 0.60; IR (KBr), cm^{-1}: 3396 (NH), 1694 (C=O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.35 (s, 9H, 3 × CH_3), 2.39–2.44 (m, 2H, $COCH_2CH_2$), 3.09–3.15 (m, 2H, CH_2CH_2NHBoc), 4.15 (dd, 1H, H-5′, 9.9, 2.2), 4.21 (dd, 1H, H-5′, 9.9, 2.2), 4.98 (m, 1H, H-4′), 5.68 (d, 1H, H-5, 8.0), 5.99 (d, 1H, H-2′, 5.9), 6.41 (d, 1H, H-3′, 5.9), 6.79 (m, 1H, H-1′), 6.89 (t, 1H, CH_2NHBoc, 4.9), 7.43 (d, 1H, H-6, 8.0), 11.4 (bs, 1H, CONHCO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 28.2 (3 × CH_3), 34.0 ($COCH_2CH_2$), 36.0 ($COCH_2CH_2$), 64.6 (C-5′), 77.7 ($C(CH_3)_3$), 83.8 (C-4′), 89.3 (C-1′), 102.0 (C-5), 126.4 (C-2′), 133.8 (C-3′), 140.4 (C-6), 150.7 (C-2), 155.4 (C=O:Boc), 163.1 (C-4), 171.0 ($COCH_2CH_2$).

5′-O-β-Alanyl(Boc)d_2U (13b)

Compound **13b** was prepared from compound d_2U (**2**) (0.5 g; 2.358 mmol) and Boc-β-Ala-OH (0.446 g; 2.358 mmol) in a similar manner to that described for compound **13a**. Purification of the product by silica gel column chromatography (CH_2Cl_2 : MeOH) gave the title compound **13b** as clear crystals (0.40 g); Yield 45%; m.p. 57–59°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.60; IR (KBr), cm^{-1}: 3387 (NH), 1699 (C=O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.46 (s, 9H, 3 × CH_3), 1.81–1.88 (m, 1H, H-3′), 2.04–2.12 (m, 2H, H-3′ and H-2′), 2.43–2.49 (m, 1H, H-2′), 2.58–2.61 (m, 2H, $COCH_2$), 3.41–3.51 (m, 2H, CH_2CH_2NHBoc), 4.34–4.36 (m, 3H, H-4′ and H-5′), 5.36 (t, 1H, CH_2NHBoc, 5.3), 5.79 (d, 1H, H-5, 8.0), 6.06–6.08 (m, 1H, H-1′), 7.64 (d, 1H, H-6, 8.0), 10.35 (bs, 1H, CONHCO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 25.4 (C-3′), 28.0 (3 × CH_3), 32.0 (C-2′), 34.3 ($COCH_2$), 35.8 ($COCH_2CH_2$), 64.7 (C-5′), 78.3 (C-4′), 79.1 ($C(CH_3)_3$), 86.2 (C-1′), 101.7 (C-5), 139.4 (C-6), 150.3 (C-2), 155.6 (C=O:Boc), 163.7 (C-4), 171.7 ($COCH_2CH_2$).

5′-O-β-Alanyl(Boc)d_4T (13c)

Compound **13c** was prepared from compound d_4T (**3**) (0.6 g; 2.678 mmol) and Boc-β-Ala-OH (0.506 g; 2.678 mmol) in a similar manner to that described for compound **13a**. Purification of the product by silica gel column chromatography (CH_2Cl_2:MeOH) gave the title compound **13c** as white crystals (0.43 g); Yield 41%; m.p. 80–82°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.61; IR (KBr), cm^{-1}: 3393 (NH), 1695 (C=O); ^{1}H-NMR (d_6-DMSO), δ, ppm, J, Hz: 1.35 (s, 9H, 3 × CH_3), 1.76 (s, 3H, CH_3), 2.34–2.46 (m, 2H, $COCH_2$), 3.10–3.15 (m, 2H, CH_2CH_2NHBoc), 4.15 (dd, 1H, H-5′, 9.6, 2.2), 4.24 (dd, 1H, H-5′, 9.6, 2.2), 4.96 (m, 1H, H-4′), 5.98 (d, 1H, H-2′, 5.9), 6.40 (d, 1H, H-3′, 5.9), 6.79 (m, 1H, H-1′), 6.88 (t, 1H, CH_2NHBoc, 5.4), 7.23 (s, 1H, H-6), 11.38 (bs, 1H, CONHCO); ^{13}C-NMR (d_6-DMSO), δ, ppm: 12.1 (CH_3), 28.2 (3 × CH_3), 34.0 ($COCH_2$), 35.9 (CH_2NHBoc), 64.6 (C-5′), 77.7 ($C(CH_3)_3$), 83.6 (C-4′), 89.2 (C-1′), 109.5 (C-5), 126.6 (C-2′), 133.7 (C-3′), 135.8 (C-6), 150.8 (C-2), 155.4 (C=O :Boc), 163.8 (C-4), 171.1 ($COCH_2CH_2$).

5′-O-β-Alanyld_4U·HCl (14a)

To a solution of the resulting product **13a** (0.24 g; 0.63 mmol) in 4M HCl/dioxane (6 mL) at 0°C was added anisole (0.06 mL; 0.55 mmol), and the mixture was stirred at room temperature for 1 h. After removal of the solvent in vacuo, the residue was precipitated from diethyl ether to give the title compound **14a** as a brown solid (0.18 g); Yield 90%; m.p. 161–163°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.04; IR (KBr), cm^{-1}: 3150–3030 (NH:$CH_2NH_2.HCl$), 1687 (C=O); ^{1}H-NMR (d_6-DMSO), δ, ppm, J, Hz: 2.65–2.78 (m, 2H, $COCH_2$), 2.93–3.08 (m, 2H, CH_2NH_2·HCl), 4.23 (m, 2H, H-5′), 5.01 (m, 1H, H-4′), 5.75 (d, 1H, H-5, 7.9), 6.01 (d, 1H, H-2′, 5.6), 6.43 (d, 1H, H-3′, 5.6), 6.79–6.80 (m, 1H, H-1′), 7.44 (d, 1H, H-6, 7.9), 8.00 (bs, 3H, NH_2·HCl), 11.31 (bs, 1H, CONHCO); ^{13}C-NMR (d_6-DMSO), δ, ppm: 31.2 ($COCH_2$), 34.5 (CH_2NH_2·HCl), 65.1 (C-5′), 83.7 (C-4′), 89.4 (C-1′), 102.1 (C-5), 126.5 (C-2′), 133.8 (C-3′), 142.2 (C-6), 150.8 (C-2), 163.2 (C-4), 170.2 ($COCH_2CH_2$).

5′-O-β-Alanyld_2U·HCl (14b)

Compound **14b** was prepared from **13b** (0.30 g; 0.78 mmol) in a similar manner to that described for compound **14a**. Yield 96%; m.p. 51–54°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.07; IR (KBr), cm^{-1}: 3432 (NH), 1681 (C=O); ^{1}H-NMR (d_6-DMSO), δ, ppm, J, Hz: 1.76–1.85 (m, 1H, H-3′), 1.94–2.01 (m, 2H, H-3′ and H-2′), 2.27–2.35 (m, 1H, H-2′), 2.75 (t, 2H, $COCH_2$, 6.9), 3.00 (t, 2H, $COCH_2CH_2$, 6.9), 4.18–4.27 (m, 3H, H-5′ and H-4′), 5.67 (d, 1H, H-5, 7.9), 5.96–5.98 (m, 1H, H-1′), 7.65 (d, 1H, H-6, 7.9), 8.16 (bs, 3H, NH_2·HCl), 11.29 (bs, 1H, CONHCO); ^{13}C-NMR (d_6-DMSO), δ, ppm: 25.5 (C-3′), 30.9 (C-2′), 31.3 ($COCH_2$), 34.5 ($COCH_2CH_2$), 65.4 (C-5′), 77.8 (C-4′), 85.2 (C-1′), 101.6 (C-5), 140.5 (C-6), 150.4 (C-2), 163.2 (C-4), 170.2 ($COCH_2CH_2$).

5′-O-β-Alanyld_4T·HCl (14c)

Compound **14c** was prepared from **13c** (0.43 g; 1.088 mmol) in a similar manner to that described for compound **14a**. Yield 75%; m.p. 160–162°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.10; IR (KBr), cm^{-1}: 3435 (NH), 1696 (C=O); ^{1}H-NMR (d_6-DMSO), δ, ppm, J, Hz: 1.78 (s, 3H, CH_3), 2.58–2.76 (m, 2H, $COCH_2$), 2.92–3.00 (m, 2H, CH_2NH_2·HCl), 4.23 (m, 2H, H-5′), 4.98 (m, 1H, H-4′), 6.01 (d, 1H, H-2′, 5.8), 6.43 (d, 1H, H-3′, 5.8), 6.79–6.81 (m, 1H, H-1′), 7.24 (s, 1H, H-6), 8.10 (bs, 3H, NH_2·HCl), 11.40

(bs, 1H, CONHCO); ^{13}C-NMR (d_6-DMSO), δ, ppm: 12.3 (CH_3), 31.2 ($COCH_2$), 34.4 (CH_2NH_2·HCl), 65.4 (C-5′), 83.5 (C-4′), 89.3 (C-1′), 109.7 (C-5), 126.7 (C-2′), 133.6 (C-3′), 135.8 (C-6), 150.8 (C-2), 163.8 (C-4), 170.3 ($COCH_2CH_2$).

5′-O-{8-[1-(4-Fluorobenzyl)-1H-pyrrol-2-yl]-6,8-dioxobutyryl-β-alanyl}d_4U (15a)

To a solution of **6** (0.106 g; 0.37 mmol) in DMF (5 mL) was added compound **14a** (0.140 g; 0.44 mmol), HOBT (0.124 g; 0.92 mmol), TEA (0.10 mL; 0.735 mmol) and BOP (0.244 g; 0.55 mmol) at 0°C, and the solution was stirred for 22 h at room temperature. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (30 mL), washed with 10% citric acid (30 mL), 5% $NaHCO_3$ (30 mL) and brine (2 × 30 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. Purification of the product by silica gel column chromatography (CH_2 Cl_2:MeOH) gave the title compound **15a** as yellow crystals (0.063 g); Yield 33%; m.p. 90–92°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.62; IR (KBr), cm^{-1}: 3392 (OH), 3309 (NH), 1711 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 2.58–2.65 (m, 2H, $COCH_2$), 3.62–3.66 (m, 2H, $COCH_2CH_2$), 4.32 (dd, 2H, H-5′, 9.5, 2.9), 5.05 (m, 1H, H-4′), 5.58 (s, 2H, $ArCH_2$), 5.79 (d, 1H, H-5, 7.4), 5.88 (d, 1H, H-2′, 5.4), 6.28 (m, 2H, H-4:pyrrole and H-3′), 6.88 (s, 1H, CH═C–OH), 6.97–7.01 (m, 4H, H-1′, H-3:arom., H-5:arom. and H-5:pyrrole), 7.06–7.12 (m, 2H, H-2:arom. and H-6:arom.), 7.19–7.22 (m, 1H, H-3:pyrrole), 7.39 (d, 1H, H-6, 7.4), 7.50 (bs, 1H, CH_2NHCO), 9.37 (bs, 1H, CONHCO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 33.5 ($COCH_2$), 34.8 ($COCH_2CH_2$), 52.2 ($ArCH_2$), 64.8 (C-5′), 84.1 (C-4′), 89.9 (C-1′), 98.0 (CH═C–OH), 102.7 (C-5), 110.1 (C-4:pyrrole), 115.3 and 115.5 (C-3:arom. and C-5:arom.), 121.4 (C-3:pyrrole), 127.0 (C-2′), 128.5 and 128.6 (C-2:arom. and C-6:arom.), 129.0 (C-1:arom.), 132.3 (C-5:pyrrole), 133.3 (C-3′), 133.6 (C-2:pyrrole), 139.7 (C-6), 150.6 (C-2), 160.8 (C-4), 162.0 (C-4:arom.), 163.1 (CH═C–OH), 164.2 ($CONHCH_2$), 171.5 ($COCH_2CH_2$), 184.0 (COCH═C–OH); Anal. Calcd. for $C_{27}H_{25}N_4O_8F$: C, 58.70; H, 4.53; N, 10.14; Found: C, 58.82; H, 4.57; N, 10.16%.

5′-O-{8-[1-(4-Fluorobenzyl)-1H-pyrrol-2-yl]-6,8-dioxobutyryl-β-alanyl}d_2U (15b)

Compound **15b** was prepared from **14b** (0.146 g; 0.46 mmol) and **6** (0.110 g; 0.38 mmol) in a similar manner to that described for compound **15a**. Purification of the product by silica gel column chromatography (CH_2Cl_2:MeOH) gave the title compound **15b** as yellow crystals (0.082 g); Yield 39%; m.p. 91–93°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.56; IR (KBr), cm^{-1}: 3408 (OH, NH), 1720 (C═O), 1687 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.75–1.82 (m, 1H, H-3′), 2.04–2.09 (m, 2H, H-3′ and H-2′), 2.39–2.49 (m, 1H, H-2′), 2.67 (t, 2H, $COCH_2$, 6.1), 3.68 (q, 2H, $COCH_2CH_2$, 6.1), 4.31–4.41 (m, 3H, H-5′ and H-4′), 5.58 (s, 2H, $ArCH_2$), 5.75 (d, 1H, H-5, 8.0), 6.01–6.04 (m, 1H, H-1′), 6.27–6.29 (m, 1H, H-4:pyrrole), 6.88 (s, 1H, CH═C–OH), 6.96–7.01 (m, 3H, H-3:arom., H-5:arom. and H-5:pyrrole), 7.07–7.11 (m, 2H, H-2:arom. and H-6:arom.), 7.17–7.20 (m, 1H, H-3:pyrrole), 7.43 (t, 1H, CH_2 NHCO, 5.4), 7.55 (d, 1H, H-6, 8.0), 8.40 (bs, 1H, CONHCO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 25.8 (C-3′), 32.4 (C-2′), 33.8 ($COCH_2$), 34.9 ($COCH_2CH_2$), 52.3 ($ArCH_2$), 65.2 (C-5′), 78.6 (C-4′), 86.6 (C-1′), 98.1 (CH═C–OH), 102.0 (C-5), 110.2 (C-4:pyrrole), 115.5 and 115.7 (C-3:arom. and C-5:arom.), 121.5 (C-3:pyrrole), 128.6 and 128.7 (C-2:arom. and C-6:arom.), 129.0 (C-1:arom.), 132.4 (C-5:pyrrole), 133.5 (C-2:pyrrole), 139.4 (C-6), 150.0 (C-2), 160.9 (C-4), 162.7 (C-4:arom.), 163.4 (CH═C–OH), 164.2 ($CONHCH_2$), 171.7 ($COCH_2CH_2$), 184.1 (COCH═C–OH); Anal. Calcd. for $C_{27}H_{27}N_4O_8F$: C, 58.48; H, 4.87; N, 10.11. Found: C, 58.55; H, 4.91; N, 10.16%.

5′-O-{8-[1-(4-Fluorobenzyl)-1H-pyrrol-2-yl]-6,8-dioxobutyryl-β-alanyl}d_4T (15c)

Compound **15c** was prepared from **14c** (0.151 g; 0.46 mmol) and **6** (0.110 g; 0.38 mmol) in a similar manner to that described for compound **15a**. Purification of the product by silica gel column chromatography (CH_2Cl_2:MeOH) gave the title compound **15c** as a yellow solid (0.060 g); Yield 29%; m.p. 101–103°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.54; IR (KBr), cm^{-1}: 3414 (OH, NH), 1720 (C═O), 1691 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.92 (s, 3H, CH_3), 2.59–2.67 (m, 2H, $COCH_2$), 3.65–3.67 (m, 2H, $COCH_2CH_2$), 4.25 (dd, 1H, H-5′, 9.4, 4.2), 4.43 (dd, 1H, H-5′, 9.4, 4.2), 5.05 (m, 1H, H-4′), 5.58 (s, 2H, $ArCH_2$), 5.88 (d, 1H, H-2′, 5.1), 6.26–6.29 (m, 2H, H-4:pyrrole and H-3′), 6.88 (s, 1H, CH═C–OH), 6.96–7.04 (m, 4H, H-1′, H-3:arom., H-5:arom. and H-5:pyrrole), 7.06–7.12 (m, 2H, H-2:arom. and H-6:arom.), 7.15 (s, 1H, H-6), 7.19–7.21 (m, 1H, H-3:pyrrole), 7.47 (t, 1H, CH_2 NHCO, 5.8), 8.98 (bs, 1H, CONHCO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 12.6 (CH_3), 33.6 ($COCH_2$), 34.8 ($COCH_2CH_2$), 52.3 ($ArCH_2$), 65.1 (C-5′), 83.9 (C-4′), 89.7 (C-1′), 98.1 (CH═C–OH), 110.2 (C-5), 111.2 (C-4:pyrrole), 115.4 and 115.6 (C-3:arom. and C-5:arom.), 121.4 (C-3:pyrrole), 127.4 (C-2′), 128.5 and 128.6 (C-2:arom. and C-6:arom.), 128.9 (C-1:arom.), 132.4 (C-5:pyrrole), 133.0 (C-3′), 133.5 (C-2:pyrrole), 135.2 (C-6), 150.6 (C-2), 161.8 (C-4), 163.3 (C-4:arom.), 163.5 (CH═C–OH), 164.1 ($CONHCH_2$), 171.6 ($COCH_2CH_2$), 184.1 (COCH═C–OH); Anal. Calcd. for $C_{28}H_{27}N_4O_8F$: C, 59.36; H, 4.77; N, 9.89. Found: C, 59.43; H, 4.80; N, 9.93%.

5′-O-[7-(3,5-Dibenzyloxyphenyl)-5,7-dioxobutyrylglycyl]d$_4$T (16)

To a solution of **9** (0.140 g; 0.35 mmol) in DMF (7 mL) was added compound **11c** (0.132 g; 0.42 mmol), HOBT (0.117 g; 0.87 mmol), TEA (0.10 mL; 0.70 mmol) and BOP (0.23 g; 0.52 mmol) at 0°C, and the solution was stirred for 18 h at room temperature. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (30 mL), washed with 10% citric acid (30 mL), 5% $NaHCO_3$ (30 mL) and brine (2 × 30 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. Purification of the product by silica gel column chromatography (CH_2Cl_2 : MeOH) gave the title compound **16** as beige crystals (0.060 g); Yield 26%; m.p. 92–94°C; TLC R_f (EtOAc 100%) 0.63; IR (KBr), cm^{-1}: 3419 (OH, NH), 1693 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.96 (s, 3H, CH_3), 2.56 (d, 2H, NHCH_2CO, 9.9), 4.29 (dd, 1H, H-5′, 8.0, 3.9), 4.50 (dd, 1H, H-5′, 8.0, 3.9), 4.67–4.69 (m, 1H, H-4′), 5.07 (s, 4H, 2 × CH_2Ar), 5.30 (bs, 1H, CH═C–O*H*), 5.93 (d, 1H, H-2′, 5.6), 6.30 (d, 1H, H-3′, 5.6), 6.74–6.76 (m, 1H, H-4:arom.), 6.98–6.99 (m, 1H, H-1′), 7.03 (s, 1H, C*H*═C–OH), 7.18–7.22 (m, 3H, H-6, H-2:arom. and H-6:arom.), 7.32–7.43 (m, 10H, Ar), 8.43 (bs, 1H, CON*H*CO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 12.6 (CH_3), 41.7 (NHCH_2CO), 65.6 (C-5′), 70.4 (2 × CH_2Ar), 83.8 (C-4′), 89.9 (C-1′), 105.5 (C-4:arom.), 106.2 (*C*H═C–OH), 107.3 (C-2:arom. and C-6:arom.), 111.3 (C-5), 127.4 (C-2′), 127.6 (2 × C-II:Ar and 2 × C-VI:Ar), 128.2 (2 × C-IV:Ar), 128.6 (2 × C-III:Ar and 2 × C-V:Ar), 133.2 (C-3′), 135.4 (C-6), 136.4 (2 × C-I:Ar), 139.2 (C-1:arom.), 150.6 (C-2), 156.8 (C-4), 160.1 (C-3:arom. and C-5:arom.), 163.4 (CH═*C*–OH), 167.3 (*C*ONHCH$_2$), 169.7 (NHCH$_2$*C*O), 197.7 (*C*OCH═C–OH); Anal. Calcd. for $C_{36}H_{33}N_3O_{10}$: C, 64.77; H, 4.95; N, 6.30. Found: C, 64.89; H, 5.06; N, 6.38%.

5′-O-[8-(3,5-Dibenzyloxyphenyl)-6,8-dioxobutyryl-β-alanyl]d$_4$T (17)

Compound **17** was prepared from **14c** (0.148 g; 0.45 mmol) and **9** (0.150 g; 0.37 mmol) in a similar manner to that described for compound **16**. Purification of the product by silica gel column chromatography (CH_2Cl_2:MeOH) gave the title compound **17** as beige crystals (0.040 g); Yield 16%; m.p. 97–99°C; TLC R_f (CH_2Cl_2 : MeOH = 90 : 10) 0.67; IR (KBr), cm^{-1}: 3419 (OH, NH), 1693 (C═O); ^{1}H-NMR ($CDCl_3$), δ, ppm, J, Hz: 1.91 (s, 3H, CH_3), 2.58–2.73 (m, 2H, COCH_2), 3.63–3.75 (m, 2H, COCH$_2$CH_2), 4.24 (dd, 1H, H-5′, 8.0, 4.1), 4.40 (dd, 1H, H-5′, 8.0, 4.1), 5.01–5.08 (m, 5H, H-4′ and 2 × CH_2Ar), 5.83 (d, 1H, H-2′, 5.6), 6.23 (d, 1H, H-3′, 5.6), 6.71–6.74 (m, 1H, H-4:arom.), 6.95 (s, 1H, C*H*═C–OH), 6.96–6.97 (m, 1H, H-1′), 7.13 (s, 1H, H-6), 7.19–7.22 (m, 2H, H-2:arom. and H-6:arom.), 7.31–7.43 (m, 10H, Ar), 8.54 (bs, 1H, CON*H*CO); ^{13}C-NMR ($CDCl_3$), δ, ppm: 12.6 (CH_3), 33.8 (COCH_2), 35.3 (COCH$_2$CH_2), 65.0 (C-5′), 70.3 (2 × CH_2Ar), 83.9 (C-4′), 89.8 (C-1′), 105.0 (C-4:arom.), 106.0 (*C*H═C–OH), 106.6 (C-2:arom. and C-6:arom.), 111.2 (C-5), 127.3 (C-2′), 127.6 (2 × C-II:Ar and 2 × C-VI:Ar), 128.2 (2 × C-IV:Ar), 128.6 (2 × C-III:Ar and 2 × C-V:Ar), 132.4 (C-1:arom.), 133.0 (C-3′), 135.3 (C-6), 136.3 (2 × C-I:Ar), 150.5 (C-2), 159.9 (C-3:arom. and C-5:arom.), 163.3 (C-4), 166.9 (CH═*C*–OH), 167.7 (*C*ONHCH$_2$), 171.6 (*C*OCH$_2$CH$_2$), 172.3 (*C*OCH═C–OH); Anal. Calcd. for $C_{37}H_{35}N_3O_{10}$: C, 65.20; H, 5.14; N, 6.17. Found: C, 65.26; H, 5.26; N, 6.24%.

Antiviral Test Procedures

The cultures of CEM-SS and MT4 cells were maintained at 37°C in a 5% CO_2 atmosphere in RPMI-1640 medium supplemented with 10% complement-depleted foetal bovine serum (FBS). The antiviral HIV-1 activity of a given compound in CEM-SS cells was measured by quantification of the Reverse Transcriptase activity (RT) associated with particles released from HIV-1_{LAI}-infected cells in the culture medium. CEM-SS cells were infected with 100 $TCDI_{50}$ (the virus stock was titrated under the same experimental conditions); after 30 min adsorption, free virus particles were washed out and cells were re-suspended in RPMI-1640 plus 10% foetal calf serum at a final concentration of 10^5 cells mL^{-1} in the presence of different concentrations of test compounds. After 5 days, virus production was measured by RT assay as already described.[30] The 50% inhibitory concentration (IC_{50}) was derived from the computer-generated median effect plot of the dose-effect data.[31] The cytotoxicity of the drugs was evaluated in parallel by incubating uninfected cells in the presence of different concentrations of antiviral products. The cell viability was determined by a measure of mitochondrial dehydrogenase activity, enzymes reducing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into formazan (whose quantity was measured by the absorbance at 540 nm).[32] The 50% cytotoxic concentration (CC_{50}) is the concentration of drug which reduces cell viability by 50% and was calculated with the program used in the determination of the IC_{50}. The assays using different cells and virus isolates were done according to previously published protocols,[30,33] virus production was quantified by the RT activity associated to virus particles released from the cells in the culture medium. Conditions under which the inhibitory properties were measured on HIV-1 Reverse Transcriptase *in vitro* has also been described.[30] *In vitro* RT inhibition has also been described.[30] The CEM-SS cells were obtained from P. Nara through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, Md., USA).

RESULTS AND DISCUSSION

Chemistry

The NRTIs

For the NRTIs, the compounds: d_4U (**1**), d_2U (**2**) and d_4T (**3**) were chosen. These compounds contain a 5′-hydroxyl group which is essential for conjugation with the carboxyl group of HIV integrase inhibitors through an amino acid as a cleavable linker. Furthermore, among them, d_4T is used in the clinical treatment of AIDS.

The INIs

For the INIs, we have chosen two representative compounds of the β-diketo acid class: 4-[1-(4-fluorobenzyl)-1*H*-pyrrol-2-yl]-2,4-dioxobutyric acid (L-731,988) (**6**) and 4-(3,5-dibenzyloxyphenyl)-2,4-dioxobutyric acid (L-708,906) (**9**). In the literature,[27] it has been shown that these β-diketo acids exhibit potent activity against HIV-1 integrase in vitro. Moreover, these compounds reduce viral replication in cell culture. Thus, in a single-cycle assay for acute infection, L-731,988 and L-708,906 inhibited HIV-1 replication with 50% inhibitory concentrations (IC_{50}'s) of 1 to 2 μM.[27]

Firstly, we have developed the synthesis of these INIs. The β-diketo acid **6** was obtained in three steps starting from the 2-acetylpyrrole (Scheme 1) and was subjected to an alkylation reaction using 4-fluorobenzyl bromide in dimethylformamide to provide the intermediate **4** in 95% yield. Then, reaction of **4** with diethyl oxalate, in anhydrous dimethoxyethane as solvent, in the presence of sodium hydride and a drop of ethanol, afforded the ester intermediate **5**, isolated in 32% yield. The β-diketo acid **6** was obtained after hydrolysis of the ester intermediate **5** using sodium hydroxide 1M in tetrahydrofuran: methanol (1:1); the INI **6** was isolated in 67% yield.

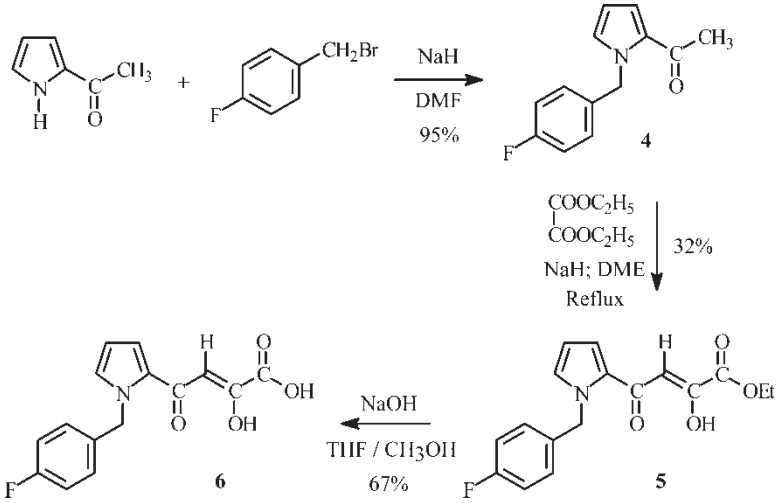
The synthetic route for the β-diketo acid **9** was carried out in a similar manner starting from 3,5-dihydroxyacetophenone (Scheme 2). In the first step, the acetophenone was subjected to a benzylation reaction using benzyl bromide in acetone as solvent, in the presence of anhydrous sodium carbonate. The resulting 1-(3,5-dibenzyloxyphenyl)ethanone (**7**) was then converted to the corresponding ester, ethyl 4-(3,5-dibenzyloxyphenyl)-2,4-dioxobutyrate (**8**), in 83% yield. In the last step, hydrolysis of the ester intermediate **8** provided the INI **9,** isolated in 47% yield.

Conjugation Strategy

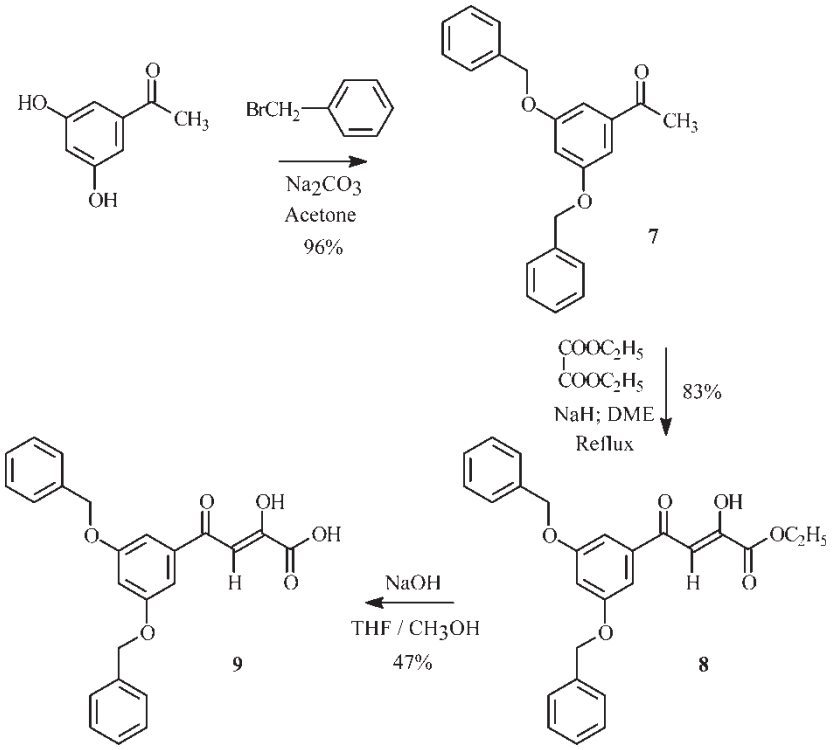
For the conjugation, an amino acid (glycine or β-alanine) was chosen as a cleavable linker.

The presence of the carboxyl group of the β-diketo acids is responsible for their insufficient cell membrane permeability. To improve cell membrane permeability and antiviral activity, we envisaged "double-drug" strategy that combined an INI and a NRTI in a single molecule bound by a linker. The essential criteria in the choice of an amino acid as a linker is based on the following premises: firstly, the heterodimer must be stable outside the target cell; secondly, after the penetration across the cell membrane, the heterodimer must regenerate the parent inhibitors (Figure 1).[34,35]

This concept of anti-HIV double-drugs containing a cleavable linker was applied to the synthesis of the heterodimers including a NRTI (d_4U, d_2U or d_4T) conjugated with a INI (**6** or **9**). After the penetration across the cell membrane,



SCHEME 1 Synthesis of the INI: 4-[1-(4-fluorobenzyl)-1*H*-pyrrol-2-yl]-2,4-dioxobutyric acid (**6**).



SCHEME 2 Synthesis of the INI: 4-(3,5-dibenzyloxyphenyl)-2,4-dioxobutyric acid (**9**).

the disintegration of the heterodimer should regenerate the NRTI and the INI.

Synthesis of the Heterodimers 12a–c, 15a–c, 16 and 17

The synthesis of a series of heterodimers of the general formula [NRTI]-*C5′*-Linker-[INI] is reported. For the NRTIs: d_4U (**1**), d_2U (**2**) and d_4T (**3**), the hydroxyl group on the C-5′ position was connected with the carboxyl group of INI (**6** or **9**) through an amino acid (glycine or β-alanine) as a cleavable linker.

Scheme 3 illustrates the synthetic procedure for conjugation of the NRTIs (d_4U, d_2U and d_4T) with the INI **6**. These NRTIs were coupled with Boc-Gly-OH or Boc-β-Ala-OH using dicyclohexylcarbodiimide (DCC) in the presence of *N,N*-dimethylaminopyridine

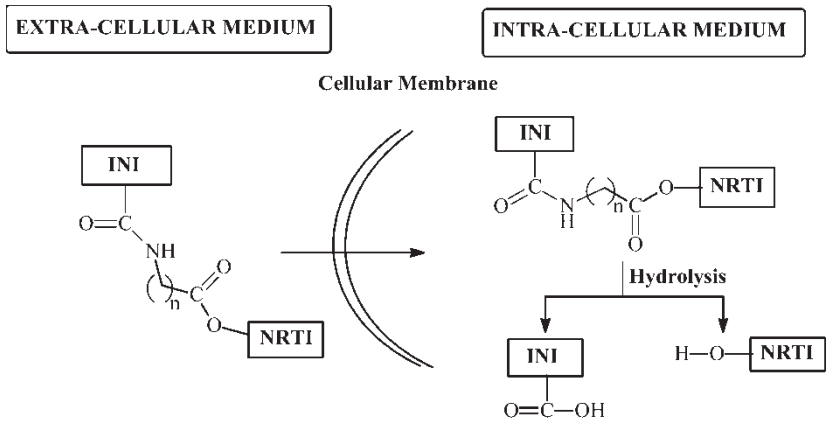
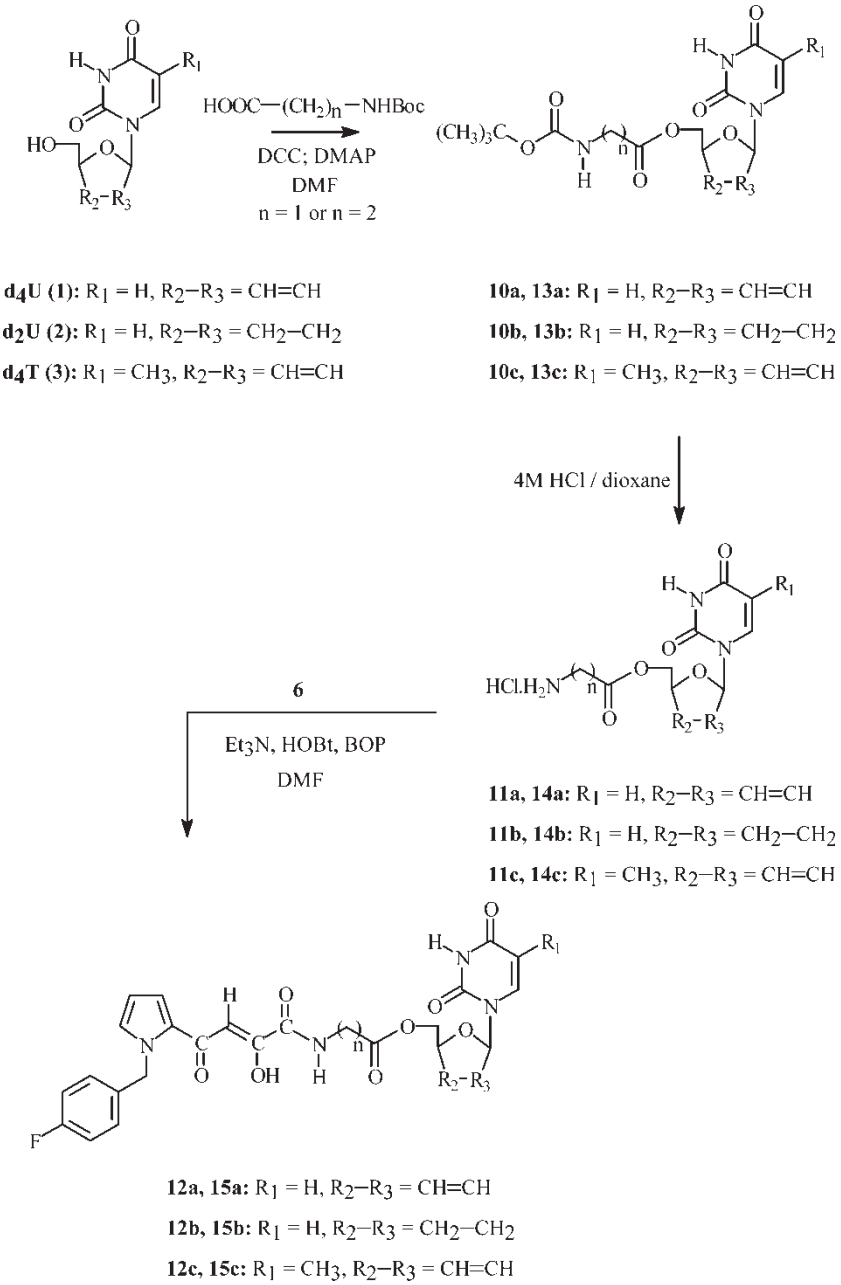


FIGURE 1 Concept of anti-HIV double-drugs containing a cleavable linker.



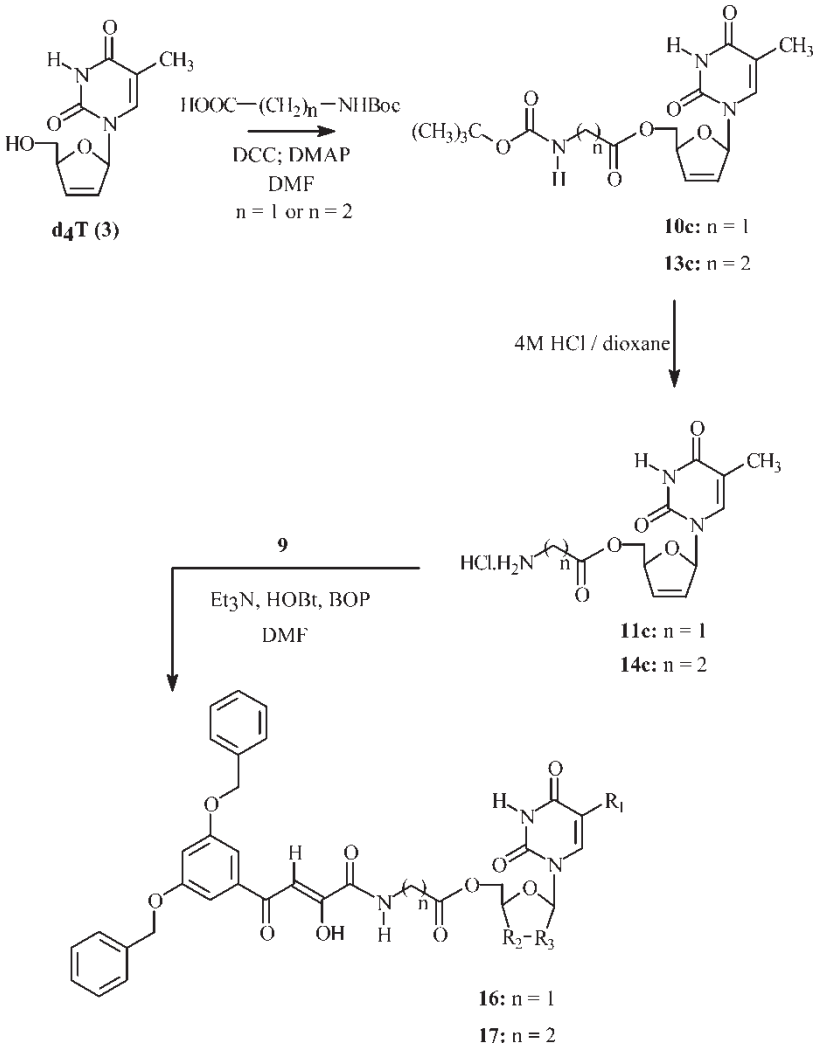
SCHEME 3 Synthesis of the heterodimers [NRTI-*C5′*-Linker-[INI] **12a–c** and **15a–c**.

(DMAP) in dimethylformamide to give the corresponding compounds **10a–c** and **13a–c**. Final deprotection of the Boc group using 4M HCl/dioxane afforded the hydrochloride compounds **11a–c** and **14a–c**. Condensation of compounds **11a–c** and **14a–c** with the β-diketo acid **6** using benzotriazol-1-yloxy-tris(dimethylamino)phosphoniumhexafluorophosphate/1-hydroxybenzotriazole (BOP/HOBT), in the presence of triethylamine in dimethylformamide, afforded the heterodimers **12a–c** and **15a–c** in 29 to 40% yields.

The conjugation of the INI **9** on the C-5′ position of NRTI (d_4T, **3**) is shown in Scheme 4. Firstly, d_4T was coupled with Boc-Gly-OH or Boc-β-Ala-OH using DCC in the presence of DMAP in DMF as solvent, and the following deprotection by 4M HCl/dioxane afforded **11c** and **14c**. Condensation of compounds **11c** and **14c** with the β-diketo acid **9** using BOP/HOBT, in the presence of TEA in DMF, afforded the heterodimers **16** and **17** in 26% and 16% yields, respectively.

Biological

The heterodimers [NRTI]-*C5′*-Linker-[INI], **12a–c**, **15a–c**, **16** and **17**, were evaluated by comparison to



SCHEME 4 Synthesis of the heterodimers [d4T]-*C5′*-Linker-[INI] **16** and **17**.

AZT for inhibition of HIV-1 multiplication in lymphocytic cell lines (CEM-SS and MT4). As shown in Table I, only the heterodimers [d_4T]-*C5′*-Linker-[INI] (**12c**, **15c**, **16** and **17**) showed significant antiviral activity with IC_{50} values of 3.0, 5.0, 3.5 and 5.6 μM against HIV-1_{LAI} in CEM-SS cells, respectively. Interestingly, heterodimers **12c** and **16** containing a glycine linker showed better antiviral activity that heterodimers **15c** and **17** containing a β-alanine linker. These results support our hypothesis that the synthesised heterodimers could penetrate across the cell membrane and then the regenerated active species could inhibit different targets (RT and IN).

Furthermore, we also evaluated the anti-HIV activities of the heterodimers [d_4T]-*C5′*-Linker-[INI] (**12c**, **15c**, **16** and **17**) against HIV-1_{Bal} and HIV-2_{D194} in PBMC cells by comparison with AZT (Table II). As mentioned above, these

TABLE I Antiviral and cytotoxicity evaluation of β-diketo acids **6** and **9** and Heterodimers [NRTI]-*C5′*-Linker-[INI] **12a–c**, **15a–c**, **16** and **17**

	HIV-1_{LAI} in CEM-SS cells		HIV-1_{IIIB} in MT-4 cells	
Compd	IC_{50} (M)[a]	CC_{50} (M)[b]	CC_{50} (M)[b]	CC_{50} (M)[b]
6	1.1×10^{-7}	$>10^{-4}$	5.7×10^{-6}	$>10^{-4}$
9	4.3×10^{-7}	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
12a	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
12b	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
12c	3×10^{-6}	$>10^{-5}$	7.3×10^{-7}	$>10^{-5}$
15a	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
15b	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
15c	5×10^{-6}	$>10^{-5}$	4.5×10^{-6}	$>10^{-5}$
16	3.5×10^{-6}	$>10^{-5}$	1.8×10^{-6}	$>10^{-5}$
17	5.6×10^{-6}	$>10^{-5}$	2.2×10^{-6}	$>10^{-5}$
AZT	1.2×10^{-8}		1.4×10^{-8}	

[a] IC_{50} is the concentration required to inhibit HIV-1 multiplication by 50%.
[b] CC_{50} is the concentration of drug which causes 50% cytotoxicity to uninfected cells.

TABLE II Antiviral and cytotoxicity evaluation of β-diketo acids **6** and **9** and heterodimers [NRTI]-*C5′*-Linker-[INI] **12c**, **15c**, **16** and **17**

	HIV-1_{Bal} in PBMC cells		HIV-2_{D194} in PBMC cells	
Compd	IC_{50} (M)[a]	CC_{50} (M) [b]	CC_{50} (M) [b]	CC_{50} (M) [b]
6	5.6×10^{-6}	$>10^{-4}$	4.2×10^{-6}	$>10^{-4}$
9	1.1×10^{-6}	$>10^{-5}$	8.6×10^{-6}	$>10^{-5}$
12c	3.2×10^{-7}	$>10^{-5}$	2.9×10^{-6}	$>10^{-5}$
15c	1.5×10^{-6}	$>10^{-5}$	4.4×10^{-6}	$>10^{-5}$
16	8.3×10^{-7}	$>10^{-5}$	4.2×10^{-6}	$>10^{-5}$
17	9.3×10^{-7}	$>10^{-5}$	5.9×10^{-6}	$>10^{-5}$
AZT	8×10^{-9}		1.1×10^{-8}	

[a] IC_{50} is the concentration required to inhibit HIV-1 multiplication by 50%. [b] CC_{50} is the concentration of drug which causes 50% cytotoxicity to uninfected cells.

prodrugs exhibited significant antiviral activity and among them, prodrug **12c** showed the best antiviral activity ($IC_{50} = 3.2 \times 10^{-7}$ M, HIV-1_{Bal}/PBMC cells).

Acknowledgements

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