A Bioactive Fullerene Peptide

Claudio Toniolo,* Alberto Bianco,† Michele Maggini,† Gianfranco Scorrano,† Maurizio Prato,‡ Mauro Marastoni,⊥ Roberto Tomatis,⊥ Susanna Spisani,∥ Giorgio Palú,§ and Edward D. Blair#

Centro di Studio sui Biopolimeri and Centro Meccanismi di Reazioni Organiche, CNR, Dipartimento di Chimica Organica, Universitá di Padova, 35131 Padova, Italy, Dipartimento di Scienze Farmaceutiche, Università di Trieste, 34127 Trieste, Italy, Dipartmento di Scienze Farmaceutiche and Dipartimento di Biochimica e Biologia Molecolare, Università di Ferrara, 44100 Ferrara, Italy, Istituto di Microbiologia, Università di Padova, 35100 Padova, Italy, and Department of Molecular Sciences, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.

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The highly hydrophobic C_{60} (buckminsterfullerene) was water solubilized by covalently linking the synthon 1,2-dihydro-1,2-methanofullerene [60]-61-carboxylic acid to the α-amino group of the hydrophilic 4–8 sequence of peptide T, known to display potent human monocyte chemotaxis. The resulting compound, characterized by a variety of analytical techniques, including a UV spectrum in *aqueous* solution, exhibits remarkable chemotactic potency, comparable to that of the parent pentapeptide. Furthermore, this fullerene-peptide conjugate inhibits, albeit weakly, HIV-1 protease.

Introduction[1]

It is not surprising that the new allotropic form of carbon C_{60} (buckminsterfullerene)[2–4] would have become a topic of considerable current interest in medicinal chemistry as a potentially biologically active compound.[5–7] Actually, the unique spherical shape of C_{60} may be envisaged as fitting the hydrophobic cleft often characterizing the structures of proteins and enzymes. When the cleft corresponds to the active site of an enzyme and the intermolecular interactions with the all carbon sphere are sufficiently strong, a significant, although nonselective, inhibitory effect may be expected.

The landmark paper in this area was published in 1993 by Friedman *et al.*[5] On the basis of computer graphics simulation, these authors found that the hydrophobic cleft of HIV-1 protease can perfectly host a C_{60} molecule. However, the problem of solubilizing C_{60} in a medium suitable for biological tests is not trivial, as the C_{60} sphere is insoluble in polar solvents including water. The only possibility lies in the preparation of specifically functionalized C_{60} derivatives,[8–12] which should contain enough polar groups to overcome its intrinsic water repulsion. This task was accomplished in Wudl's laboratory,[13] where a water-soluble fullerene derivative, bis(phenethylamino-succinate)C_{60}, was synthesized. The experimental screening of such compound confirmed the hypothesis of HIV-1 protease inhibition (EC_{50} = 6 μM).[5,6]

In an independent study Nakamura and co-workers[7] recently explored the cytotoxicity of other fullerene derivatives only sparingly soluble in water. They found that these compounds, inert in the dark, become cytotoxic when irradiated with visible light. An interesting implication of these findings is the potential use of fullerene derivatives for photodynamic therapy.

These exciting examples of C_{60} derivatives in medicinal chemistry prompted us and other groups to synthesize fullerene-based α-amino acids[14–18] and peptides.[19] All these methodologies appear promising and may lead to new interesting products. In this work we expanded such an approach to the preparation, characterization, and biological evaluation of the first water-soluble fullerene peptide, obtained by covalently linking 1,2-dihydro-1,2-methanofullerene[60]-61-carboxylic acid[15] to the α-amino group of the C-terminal (4–8) sequence of peptide T.[20–23] This pentapeptide sequence (H-Thr-Thr-Asn-Tyr-Thr-OH) is extremely hydrophilic and exhibits potent activity in a human monocyte chemotaxis assay.

Results and Discussion

Chemistry and Spectroscopy. The goal of covalently linking peptide-T [4–8] (**5b**) to fullerene [60] was achieved by preparing the versatile synthon fullerenecarboxylic acid (**4**)[15] that was subsequently coupled by the standard coupling method DCC/HOBt[24] to the α-amino group of the peptide (Scheme 1). The phenolic hydroxyl group in the Tyr side chain is relatively inert and has often been left without protection even in major syntheses. Yet, the phenolate anion, generated in the presence of base, is a good nucleophile that might compete with the α-amino group for the intermediate acylating agent.[25] Therefore, before coupling compound **4** with peptide-T [4–8] (**5b**), the reaction of **4** with the model compound H-L-Tyr-OEt (**5a**) was examined. According to the mass spectrum, product **6a** is a monoacylated derivative of **5a**. The presence of an IR absorption band at 1660 cm^{-1} (carbonyl stretching mode of a CONH moiety) and of ^1H NMR resonances at 9.17 ppm (Tyr phenolic OH proton) and 9.54 ppm (Tyr amide NH proton) is strongly in favor of the assumption that the more reactive nucleophile is the α-amino group of the amino acid derivative. Assignments of the two Tyr proton resonances were made by H ⇌ D exchange and decoupling experiments. Similarly, the mass spectrum, the IR absorption properties (in particular, the absence of the ester band in 1750–1710 cm^{-1} region), and the ^1H NMR resonances of **6b**, the product of the reaction

* Author to whom correspondence should be addressed at the Centro di Studio sui Biopolimeri, CNR, Dipartimento di Chimica Organica, Università di Padova, Via Marzolo 1, 35131 Padova, Italy.
† Dipartimento di Chimica Organica, Università di Padova.
‡ Dipartimento di Scienze Farmaceutiche, Università di Trieste.
⊥ Dipartimento di Scienze Farmaceutiche, Università di Ferrara.
∥ Dipartimento di Biochimica e Biologia Molecolare, Università di Ferrara.
§ Istituto di Microbiologia, Università di Padova.
# Department of Molecular Sciences, Wellcome Research Laboratories.
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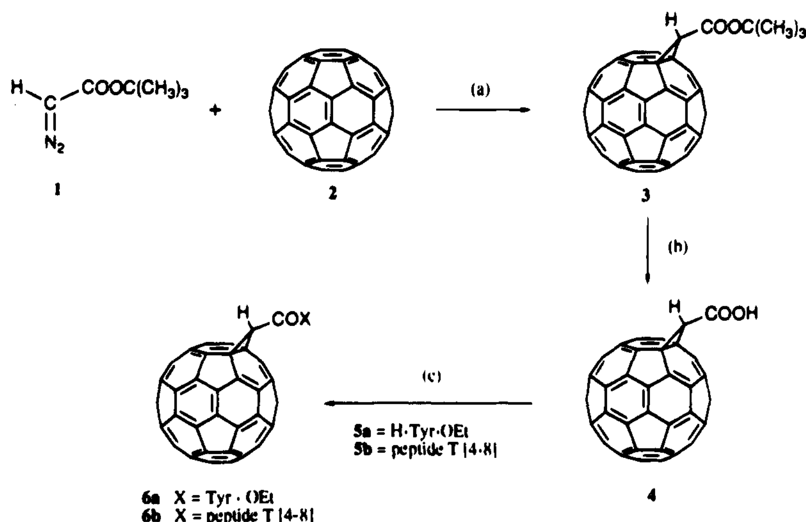
Scheme 1[a]



[a] (a) $CH_3C_6H_5$; (b) Tos-OH, $CH_3C_6H_5$; (c) DCC, HOBt, BrC_6H_5/DMSO.

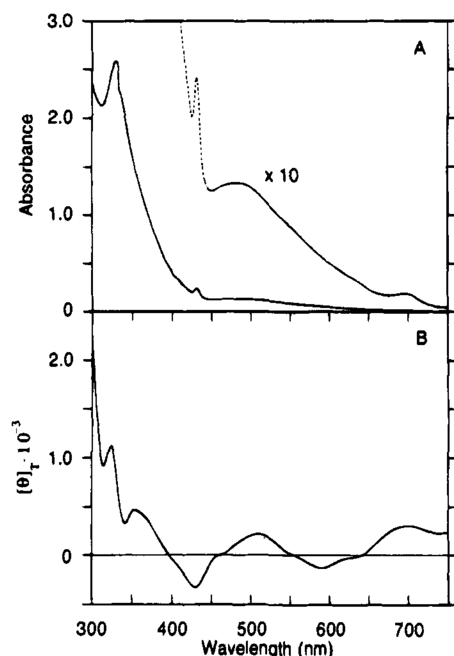


Figure 1. (A) UV–vis absorption (concentration 7.6 × 10^{-5} M) and (B) circular dichroism (concentration 4.0 × 10^{-4} M) spectra of fullerene–peptide T [4–8] (**6b**) in DMSO.

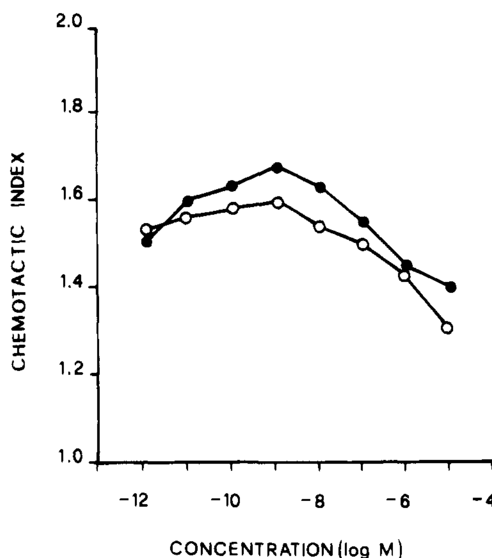


Figure 2. Chemotactic activity of human monocytes toward peptide T [4–8] (**5b**) (●) and fullerene–peptide T [4–8] (**6b**) (○) at varying concentrations. The points are means ± SE of five to seven separate experiments. SE, in the 0.02–0.08 chemotactic index range, are omitted.

between the fullerenecarboxylic acid (**4**) and peptide-T [4–8] (**5b**), allow us to conclude unambiguously that compound **6b** is the N^{α}-monoacylated peptide derivative.

The fullerene–peptide T [4–8] conjugate (**6b**) is water soluble. The UV absorption spectrum at ≃1.7 μM concentration, the limit of water solubility, shows two very strong bands at 320 and 255 nm. Interestingly, the only published spectra in water of C_{60} (solubilized in a polyvinylpyrrolidone micellar system[26] or as a γ-cyclodextrin inclusion complex[27,28]) exhibit absorption bands near 335 and 260 nm. A contribution, albeit small, to the UV absorption near 270 nm is also expected from the $\pi \rightarrow \pi^*$ transition of the phenol chromophore of the Tyr residue.[29]

A more informative absorption spectrum of fullerene–peptide T [4–8] (**6b**) was obtained in DMSO solution above 300 nm (76 μM) (Figure 1A). In addition to the UV band at 327 nm, in the visible region absorptions of decreasing intensity are located at 431, 483, and 696 nm. This spectroscopic signature, including the loss of C_{60} absorption fine structure between 500 and 650 nm,[30] is diagnostic of methanofullerenes.[13,16,18,19,31–33] The CD spectrum in the same polar solvent (Figure 1B) is extremely complex, exhibiting many more (positive and negative) maxima than those expected from the UV–vis spectrum. Analogous results were reported for other fullerene derivatives.[15,31] Conversely, the vis bands are optically inactive in a methanofullerene–benzoylpentapeptide, owing to the significant separation of the N-terminal chiral α-amino acid from the fullerene chromophoric group.[19]

Biological Activity. Fullerene–peptide T [4–8] (**6b**) was evaluated for its ability to activate human monocyte chemotaxis through the CD_4/T_4 antigen[20] and to inhibit the peptidase activity of HIV-1 protease.

Figure 2 compares the chemotactic behaviors of compound **6b** and underivatized peptide T [4–8] (**5b**). The results indicate that **5b** is a potent agonist of

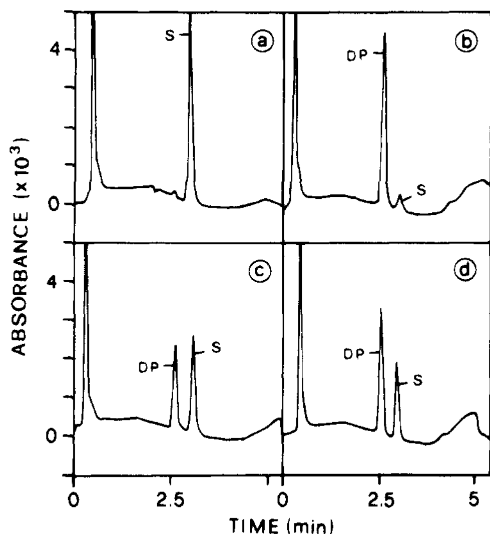


Figure 3. Hydrolysis of the peptide substrate H-Lys-Ala-Arg-Ile-Nle-Phe(NO_2)-Glu-Ala-Nle-NH_2 by HIV-1 protease. Enzyme reaction components and HPLC operating conditions are reported in the Experimental Section. Panel a shows the substrate (S) profile after incubation with the enzyme and immediate quenching with 15 mM EDTA. Substrate hydrolysis and degradation product (DP) formation after 60 min incubation are illustrated in panels b (no inhibitor), c (6 nM compound XM 323 as the inhibitor), and d (100 μM compound **6b** as the inhibitor; this DMSO/water solution was obtained as indicated in the Experimental Section).

human monocyte chemotaxis (with maximal chemotactic activity in the range $10^{-10}-10^{-8}$ M), thereby confirming the trend previously observed.[20-23] Interestingly, compound **6b** exhibits comparably high chemotactic properties. It should be recalled that the free α-amino function of peptide T [4–8] does not represent an essential requirement for its interaction with the CD_4 receptor.[21]

The anti-HIV-1 protease activities of compound **6b** and the inhibitor XM 323 (also known as DPM 323),[34] tested for comparison, are shown in Figure 3. The HPLC profiles of the peptide substrate after incubation with the enzyme for 0 min and 60 min are reported in panels *a* and *b*, respectively. Under the reaction conditions and at the incubation times that were chosen, the enzyme activity was proceeding linearly and substrate hydrolysis was not yet complete (95% yield after 60 min), thus allowing a quantitative appreciation of protease inhibition to be obtained. As shown in panels c and d, protease activity was clearly affected by addition of compound XM 323 (at the concentration 6 nM) and compound 6b (at the concentration 100 μM in a DMSO/water solution; see the Experimental Section). Inhibition values calculated from peak area integrals were about 55% for XM 323, in agreement with a recent report,[34] and 40% for **6b**. As for XM 323, inhibition produced by **6b** was dose dependent (not shown).

Conclusions

This paper describes the synthesis and detailed analytical characterization of the first water-soluble C_{60} (fullerene)–peptide conjugate. In particular, the UV spectrum in aqueous solution of C_{60} *covalently* linked to an hydrophilic "tail" has been reported for the first time. The fullerene derivative binds to a cellular receptor (the surface glycoprotein CD_4[35]) of the C-terminal pentapeptide sequence of peptide T and, consequently, induces monocyte migration. In addition, we have shown that the fullerene "ball" of this peptide conjugate interacts, albeith weakly, with the active site surface of HIV-1 protease, as already proposed from model building and experimentally demonstrated by a water-soluble, nonpeptide fullerene derivative.[5,6] In our view this "first-generation" fullerene–peptide will soon pave the way to a novel class of tailor-made, water-soluble C_{60} derivatives with more significant biological activities.

Experimental Section

Instrumentations and Materials. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AC 200, AC 250, and AM 400 spectrometers. Chemical shifts are given in parts per million (δ) relative to tetramethylsilane. Peak multiplicity is designed as s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), and m (multiplet). UV–vis absorption spectra were taken on a Perkin-Elmer Lambda 5 spectrophotometer. FT-IR spectra were recorded on a Perkin-Elmer 1720 X spectrophotometer. FAB mass spectra were obtained on a VG ZAB 2F spectrometer operating under FAB conditions (8 keV, Xe atom bombarding a 2-nitrobenzylic alcohol solution of the sample). Circular dichroism measurements were performed on a JASCO J-600 spectropolarimeter, using quartz Hellma cells of 2 or 5 mm path length. The values are given as $[\theta]_T$, total molar ellipticity (deg × cm^2 × dmol^{-1}). The amino acid composition was determined using a C. Erba 3A 30 amino acid analyzer after hydrolysis with 6 N HCl for 22 h at 110 °C in the presence of 0.2% phenol as a scavenger for Tyr. Reactions were monitored by thin layer chromatography using Merck precoated silica gel 60-F_{254} (0.25 mm thickness) plates. Flash column chromatography was performed employing 230–400 mesh silica gel (ICN Biochemicals). Solvents were distilled prior to use.

1,2-Dihydro-1,2-methanofullerene[60]-61-carboxylic acid (**4**) was prepared from the corresponding *tert*-butyl ester (**3**) according to procedure described in ref 15. Compound **3** was synthesized from *tert*-butyl diazoacetate (1) and fullerene [60] (**2**) (Hoechst) as reported in ref 32.

H-Tyr-OEt (**5a**) hydrochloride[36] was synthesized using the ethanol/HCl procedure. The trifluoroacetic acid salt of the C-terminal pentapeptide (H-Thr-Thr-Asn-Tyr-Thr-OH) of peptide T, peptide T [4–8] (**5b**), was obtained by conventional methods in solution using a procedure similar to that described and discussed previously.[21,22]

Ethyl *N*-(1,2-Dihydro-1,2-methanofullerene[60]-61-carbonyl)tyrosinate (6a). To a stirred solution of 1,2-dihydro-1,2-methanofullerene[60]-61-carboxylic acid (**4**) (7.0 mg, 0.009 mmol) and HOBt (1.5 mg, 0.0099 mmol) in a 5:1 mixture of bromobenzene–DMSO (1.2 mL) was added DCC (2.0 mg, 0.0099 mmol) at room temperature under nitrogen. After stirring for 30 min, H-Tyr-OEt hydrochloride (2.2 mg, 0.009 mmol), dissolved in DMSO (0.1 mL) in the presence of *N*-methylmorphine (1 μL, 0.009 mmol), was added. After being stirred for 24 h at room temperature, the reaction mixture was chromatographed using first toluene and then a 98:2 toluene–2-propanol mixture as eluants. The product was isolated as a brownish solid: 3.2 mg (yield 46%); UV–vis (DMSO) λ_{max} (nm) 695, 482, 431, 327; IR (KBr) ν (cm^{-1}) 3426, 1735, 1660, 1614, 1515, 1202, 1187, 576, 527; ^{1}H NMR (200 MHz, DMSO-d_6) δ 9.54 (d, 1H, Tyr H, J = 7.63 Hz), 9.17 (s, 1H, Tyr OH), 7.10 (d, 2H, Tyr phenyl CH, J = 8.54 Hz), 6.64, (d, 2H, Tyr phenyl CH, J = 8.54 Hz), 5.41 (s, 1H, C61 CH), 4.67 (m, 1H, Tyr αCH), 4.14 (q, 2H, ethoxy CH_2, J = 7.02 Hz), 3.06 (m, 2H, Tyr β-CH_2), 1.18 (t, 3H, ethoxy CH_3, J = 7.02 Hz); ^{13}C NMR (62.5 MHz, DMSO) δ 171.47, 164.01, 149.54, 144.74, 144.63, 144.60, 144.56, 144.27, 144.21, 144.01 143.87, 143.70, 143.65, 143.34, 143.32, 142.88, 142.83, 142.67, 142.58, 142.52, 142.47, 142.42, 142.24, 140.54, 140.30, 139.15, 139.02, 130.33, 127.68, 126.96, 115.25, 72.83, 60.96, 14.23; MS-FAB$^+$ (rel intensity) m/z 733 [$(C_{60} + CH)^+$, 50], 720 (C_{60}^+, 100).

(1,2-Dihydro-1,2-methanofullerene[60]-61-carbonyl)-threonylthreonylasparaginyltyrosylthreonine (6b). To a stirred solution of 1,2-dihydro-1,2-methanofullerene[60]-61-carboxylic acid (**4**) (9.25 mg, 0.0119 mmol) and HOBt (2.35 mg, 0.0154 mmol) in a 6:1 mixture of bromobenzene–DMSO (1.5 mL) was added DCC (3.17 mg, 0.0154 mmol) at room temperature under nitrogen. After stirring for 30 min the trifluoroacetic acid salt of the peptide T [4–8] (**5b**) (8.47 mg, 0.0119 mmol), dissolved in DMSO (0.3 mL) in the presence of *N*-methylmorpholine (1.3 μL, 0.0119 mmol), was added. After the mixture was stirred for 22 h at room temperature, the insoluble material was removed with the aid of a centrifuge (5000 rpm). The clear solution was diluted with chloroform (2 mL), and the crude product was precipitated by adding *n*-hexane. The solid material was washed in a centrifuge tube with toluene (2 mL), a 1:1 acetonitrile–water solution (1 mL) to remove the unreacted peptide T [4–8], and acetonitrile (2 mL) and then dried in vacuo. Pure product (**6b**) (5.0 mg, yield 31%) was isolated as a brownish solid: IR (KBr) ν (cm^{-1}) 3366, 1666, 1613, 1516, 1208, 1187, 576, 527; ^1H NMR (400 MHz, DMSO-d_6) δ 9.26 (d, 1H, Thr^1 NH, J = 8.30 Hz), 9.05 (s, 1H, Tyr OH), 8.07 (d, 1H, Asn NH, J = 8.30 Hz), 7.98 (d, 1H, Thr^2 NH, J = 8.30 Hz), 7.93 (d, 1H, Tyr NH, J = 8.30 Hz), 7.60 (d, 1H, Thr^5 NH, J = 8.30 Hz), 7.37 (s, 1H, Asp NH_2), 7.01 (d, 2H, Tyr phenyl CH, J = 8.30 Hz), 6.89 (s, 1H, Asp NH_2), 6.62 (d, 2 H Tyr phenyl CH, J = 8.30 Hz), 5.58 (s, 1H, C61 CH), 4.64 (m, 1H, Thr^1 αCH), 4.54 (m, 1H, Asn αCH), 4.41 (m, 1H, Tyr αCH), 4.35 (m, 1H, Thr^2 αCH), 4.18 (m, 1H, Thr^1 βCH), 4.08 (m, 1H, Thr^2 βCH), 4.02 (m, 2H, Thr^5 αCH and βCH), 2.95–2.91 (dd, 1H, Tyr βCH_2, J = 14.16 and 4.39 Hz), 2.75–2.69 (dd, 1H, Tyr βCH_2, J = 14.16 and 8.30 Hz), 2.58–2.53 (dd, 1H, Asn βCH_2, J = 15.63 and 5.37 Hz), 2.43–2.38 (dd, 1H, Asn βCH_2, J = 15.63 and 7.32 Hz), 1.20 (d, 3H, Thr^1 γCH_3 J = 6.35 Hz), 1.07 (d, 3H, Thr^2 γCH_3, J = 6.35 Hz), 0.98 (d, 3H, Thr^5 γCH_3, J = 5.86 Hz); ^{13}C NMR (62.5 Hz, DMSO) δ 171.94, 170.77, 169.94, 169.76, 164.42, 155.89, 149.88, 148.14, 147.87, 145.90, 145.85, 145.46, 144.75, 144.73, 144.68, 144.64, 144.59, 144.22, 144.02, 143.99, 143.89, 143.85, 143.69, 143.62, 143.55, 143.39, 143.36, 142.89, 142.84, 142.73, 142.58, 142.51, 142.44, 142.32, 142.29, 141.93, 141.89, 141.80, 141.77, 141.73, 141.69, 140.55, 140.30, 140.28, 139.69, 139.60, 135.91, 135.87, 130.30, 127.94, 115.09, 73.41, 73.17, 42.48, 19.85, 19.53; MS-FAB$^+$ (rel intensity) m/z 1397 [$(M + K)^+$, 4], 1381 [$(M + Na)^+$, 13], 1359 [$(M + H)^+$, 8], 733 [$(C_{60} + CH)^+$, 72], 720 (C_{60}^+, 100). Amino acid analysis: Thr 2.96 (3.0), Tyr 1.00 (1.0), Asp 1.03 (1.0).

Monocyte Chemotaxis. Peptides **5b** and **6b** were evaluated for their ability to stimulate monocyte directed migration (chemotaxis). Mononuclear cells were isolated from heparinized blood of normal human volunteers, and chemotaxis was performed in a modified Boyden chamber as described in ref 23. Each peptide was dissolved in DMSO at 10^{-2} M concentration, diluted before use with Krebs-Ringer phosphate buffer, and tested at a final concentration in the range 10^{-5}–10^{-12} M. To obtain an accurate comparison, the results for the individual compounds are expressed in terms of the chemotactic index, which is the ratio of migration towards the test attractant to migration towards the buffer. Migration in the presence of buffer alone was 35 μm ± SE. In order to confirm that synthetic peptides bound to the CD_4 receptor, chemotactic effects induced by compounds related to peptide T were blocked by low doses (0.1–0.2 μg/mL) of OKT4, a specific monoclonal antibody for the CD_4 molecule.

HIV-1 Protease Inhibition. The activity against HIV-1 protease was assayed by monitoring the inhibition of cleavage of a synthetic peptide on HPLC. The protease was expressed as fusion protein with IFN in HMS174(DE3)LysS bacteria under control of the T7/lac promoter of pET11D; it was purified from insoluble material by gel filtration in 5M guanidine on Sephacryl G200 and then by ion exchange chromatography on MonoQ column. Activity of the enzyme was determined as the quantity giving 50% cleavage in 60 min. The peptide substrate H-Lys-Ala-Arg-Ile-Nle-Phe(NO_2)-Glu-Ala-Nle-NH_2 was synthesized according to the Fmoc procedure on an ABI 430A peptide synthesizer. The K_m of this substrate for HIV-1 protease is 45 μM, and the K_{cat} is 35 s^{-1}.[37] The assay was conducted at 37 °C for 60 min in 100 mM sodium acetate (pH 5.6) buffer and stopped by adding 15 mM EDTA. The inhibitor XM 323[34] was used as the positive control. To establish the cleavage, the reaction mixture was analyzed by HPLC on a Porus type II reverse-phase column (Porus 20; 4.6 × 100 mm) using a gradient elution of 0–60% (v/v) acetonitrile in 0.1% trifluoroacetic acid. Products were detected by absorbance at 300 nm. A typical reaction mixture consists of enzyme (250 ng), substrate (21 μM), and different concentrations of inhibitor in a 60 μL solution of sodium acetate (pH 5.6) buffer. The inhibitor **6b** was dissolved in DMSO at 10^{-2} M concentration and diluted before use with the sodium acetate buffer.

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(1) Symbols and abbreviations for amino acids and peptides are in accord with the recommendations of the IUPAC-IUB Commission on Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977–989). The optically active α-amino acids are of L-chirality. Other abbreviations: HIV, human immunodeficiency virus; DMSO, dimethyl sulfoxide; Tos-OH, toluene-4-sulfonic acid; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; OEt, ethoxy. Numbering of the fullerene C atoms is according to Taylor, R. C_{60}, C_{70}, C_{76}, C_{78} and C_{84}: numbering, π-bond order calculations and addition pattern considerations. *J. Chem. Soc., Perkin Trans. 2* **1993**, 813–824.

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