Convenient one-pot synthesis of cystine-containing peptides using the trimethylsilyl chloride–dimethyl sulfoxide/trifluoroacetic acid system and its application to the synthesis of bifunctional anti-HIV compounds[1]

Hirokazu Tamamura,*[,a] Tsunehito Ishihara,[a] Hiromi Oyake,[a] Makoto Imai,[a] Akira Otaka,[a] Toshiro Ibuka,[a] Rieko Arakaki,[b] Hideki Nakashima,[b] Tsutomu Murakami,[c] Michinori Waki,[d] Akiyoshi Matsumoto,[d] Naoki Yamamoto[c] and Nobutaka Fujii*[,a]

[a] *Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan*
[b] *Department of Microbiology and Immunology, Kagoshima University Dental School, Sakuragaoka, Kagoshima 890, Japan*
[c] *Tokyo Medical and Dental University, School of Medicine, Bunkyo-ku, Tokyo 113, Japan*
[d] *Tokyo Research Institute, SEIKAGAKU Corporation, Higashiyamato, Tokyo 207, Japan*

A one-pot synthesis of cystine-containing peptides is achieved by treatment of protected peptidyl resins with trimethylsilyl chloride–dimethyl sulfoxide/trifluoroacetic acid in the presence of anisole. This methodology has been successfully applied to the synthesis of highly active anti-HIV peptides conjugated with 3′-azido-3′-deoxythymidine.

Introduction

Many biologically active peptides containing disulfide bonds have been isolated from natural sources to date. Analogues have also been prepared in association with structure–activity relationship (SAR) studies.[2] In order to facilitate SAR studies on peptide-lead drugs, development of a new, efficient synthetic methodology for the rapid and convenient preparation of cystine-containing peptides is needed. Recently, we and others have reported several disulfide-bond-forming reactions using sulfoxide-mediated oxidations.[3–5] As an example, the trimethylsilyl chloride (TMSCl)–dimethyl sulfoxide (DMSO)/trifluoroacetic acid (TFA) system can oxidize *S*-Acm†-cysteine[6] into cystine with a 1 h treatment.[5b] However, cleavage from the corresponding peptidyl resin and removal of all the protecting groups except for the Acm group on the Cys(Acm)-residues are required to give *S*-Acm-cysteine peptides prior to disulfide-bond formation. It would be extremely advantageous if cleavage/deprotection can be performed simultaneously with disulfide-bond formation in one pot. A favourable feature of the TMSCl–DMSO/TFA system is that it also has the ability to remove several protecting groups. Herein we report a convenient one-pot synthesis of cystine-containing peptides by treatment of protected peptidyl resins with the TMSCl–DMSO/TFA system. Furthermore, we report its application to the synthesis of highly active, bifunctional anti-HIV compounds, which involve tachyplesin analogues linked with AZT.

† *Abbreviations*: Acm = acetamidomethyl, HIV = human immunodeficiency virus, AZT = 3′-azido-3′-deoxythymidine, Fmoc = fluoren-9-ylmethoxycarbonyl, Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl, Boc = *tert*-butoxycarbonyl, Bu^t = *tert*-butyl, TMSBr = trimethylsilyl bromide, DMAP = 4-(dimethylamino)pyridine, DIPCDI = 1,3-diisopropylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, EC_{50} = 50% effective concentration, CC_{50} = 50% cytotoxic concentration, SI = selectivity index (CC_{50}/EC_{50}), MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MAP = multiple antigen peptide, ISM-S = ion spray mass spectrometry, THF = tetrahydrofuran, FAB = fast-atom bombardment.

Results and discussion

We initially examined the behaviour of the side-chain-protecting groups on several Fmoc-amino acid derivatives [Fmoc-Arg(Pmc)-OH, Fmoc-Asp(OBu^t)-OH, Fmoc-Glu(OBu^t)-OH, Fmoc-His(Boc)-OH, Fmoc-Ser(Bu^t)-OH, Fmoc-Thr(Bu^t)-OH, Fmoc-Tyr(Bu^t)-OH and Fmoc-Lys(Boc)-OH] during treatment with TMSCl–DMSO/TFA by high-performance liquid chromatography (HPLC) analysis. The results demonstrated complete removal of each protecting group of the side-chain within 30 min without significant side-reactions. However, one potential limitation to the use of this system is the modification of Trp and Met due to undesirable oxidative modifications, as seen in the sulfoxide-mediated oxidations.[3,5]

Next, the behaviour of amino acids on two peptide linkers, *p*-alkoxybenzyl alcohol linker (Alko linker)[7] and 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy linker (Rink amide linker),[8] was examined by amino acid analyses. Treatment with TMSCl–DMSO/TFA at 4 °C for 1 h did not bring about the complete cleavage of Leu and Arg from the H-Leu-Alko resin or the H-Arg(Pmc)-Rink amide resin, respectively (data not shown). However, this incomplete reaction presents no significant problems associated with its practical use since preliminary treatment of the above amino acid resins with TMSCl/TFA at room temp. resulted in efficient cleavage of the amino acids from the resins (see Experimental section). Based on the above preliminary experiments, we established optimized synthetic conditions: addition of DMSO at 4 °C after 1 h treatment with TMSCl/TFA at room temp.

In order to examine the feasibility of the TMSCl–DMSO/TFA system in a one-pot synthesis of cystine-containing peptides, two model peptides [T131(OH) and T131(NH_2)] were synthesized. These peptides are tachyplesin analogues,[2] which were found by us to have anti-HIV activity. The protected peptide resins were constructed using standard Fmoc-based solid-phase techniques (Scheme 1).[9] An Alko linker and a Rink amide linker were utilized for T131(OH) and T131(NH_2),

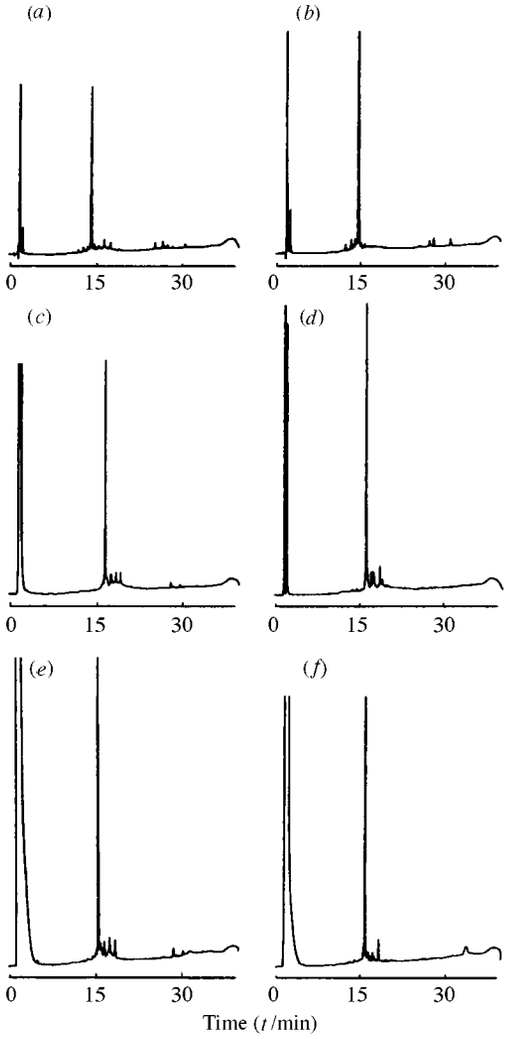
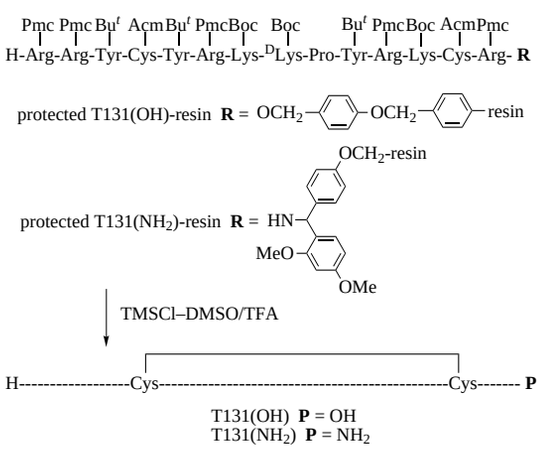


Fig. 1 Analytical HPLC of the crude T131(OH) (*a*) and T131(NH_2) (*b*) synthesized by the one-pot procedure, the crude T131(OH) (*c*) and T131(NH_2) (*d*) synthesized by a stepwise procedure (TMSBr deprotection and TMSCl–DMSO oxidation) and the crude T131(OH) (*e*) and T131(NH_2) (*f*) synthesized by a stepwise procedure (TMSBr deprotection and iodine oxidation)



Scheme 1

respectively, in combination with the following Fmoc-protected amino acids: Arg(Pmc), Cys(Acm), Lys(Boc) and Tyr(Bu^t). The protected peptide resins were treated in a one-pot manner with TMSCl/TFA followed by addition of DMSO. The resulting crude deprotected cystine-peptides exhibited sharp main peaks on analytical HPLC [Fig. 1(*a*) and (*b*)] without significant side-products involving dimers or polymers. Subsequent HPLC purification gave T131(OH) and T131(NH_2) in satisfactory yields: T131(OH), 33% and T131(NH_2), 56% (calculated from the corresponding protected peptide resins).‡

As a comparative study, two stepwise strategies of deprotection/cleavage and disulfide-bond formation were used for the parallel syntheses of both peptides. First, the crude T131(OH) and T131(NH_2) were prepared by a strategy comprising the use of 1 mol dm^{-3} TMSBr–thioanisole/TFA[10] and TMSCl–DMSO/TFA.[5b] Their analytical HPLC profiles were almost identical with those given by the products of the corresponding one-pot synthesis [Fig. 1(*c*) and (*d*)]. The yields of the purified peptides were 36% for T131(OH) and 55% for T131(NH_2). Next, a strategy comprising TMSBr deprotection and conventional iodine oxidation[11] also gave similar results [Fig. 1(*e*) and (*f*)]: yield, 44% for T131(OH) and 59% for T131(NH_2). In view of its convenience and rapidity, a one-pot protocol is much preferred.

Subsequently the present procedure was applied to the synthesis of more complicated peptidyl derivatives. Our recent studies on the development of anti-HIV compounds derived from tachyplesin analogues suggest that a highly active analogue, T22 ([Tyr^{5,12}, Lys^7]-polyphemusin II), blocks an HIV-cell fusion,[12] and that it binds to gp120, CD4 and CXCR4/fusin, which are closely associated with the virus–cell fusion.[13] In this study, we attempted to synthesize bifunctional anti-HIV compounds, which are composed of a tachyplesin analogue and AZT, since we previously demonstrated that a combined use of T22 and AZT exhibited a synergistic effect for anti-HIV activity *in vitro* (unpublished data). AZT is a reverse transcriptase inhibitor, which has been used most frequently for clinical therapy.[14] Herein, we describe the design of an AZT-T131(OH)-conjugate: the N-terminal α-amino group of T131(OH) is linked to the 5′-hydroxy group of AZT through a succinyl or glutaryl linker.

At first, treatment of AZT **1** with succinic anhydride **2** and glutaric anhydride **3** in the presence of DMAP afforded AZT 5′-hemisuccinate **4** and AZT 5′-hemiglutarate **5**, respectively (Scheme 2). Subsequent condensation of compounds **4** and **5** on the protected T131(OH)-resin using DIPCDI and HOBt provided the protected AZT-Suc-T131(OH)-resin **6** and the protected AZT-Glu-T131(OH)-resin **7**, respectively. Synthesis of AZT-Suc-T131(OH) **8** proved considerably more troublesome than first envisaged. Deprotection of protecting groups with cleavage from the resin by treatment of compound **6** with 1 M TMSBr–thioanisole/TFA, followed by air oxidation, did not yield AZT-Suc-T131(OH) **8** in satisfactory yield. This failure was due to a release of AZT from the peptide during air oxidation in basic media (data not shown). Therefore, we investigated the behaviour of AZT-Suc-T131(OH) **8** in aqueous media at pH 8.0. As depicted in Scheme 3, release of AZT **1** by the concomitant formation of a succinimide derivative of T131(OH), compound **10**, was confirmed by HPLC analysis and ion-spray mass spectrometry (ISMS). Therefore, for deprotection/cleavage and disulfide-bond formation of AZT-Suc-T131(OH) **8**, we used the present one-pot procedure, which is performed in acidic media unaccompanied by the above side-reactions. Treatment of compounds **6** and **7** with TMSCl–DMSO/TFA, followed by HPLC purification, afforded the corresponding conjugates **8** and **9**, respectively. The crude AZT-Suc-T131(OH) **8** exhibited a sharp main peak on analytical HPLC [Fig. 2(*a*)].

The anti-HIV activity of these compounds is shown in Table 1. In the MTT assay,[15] EC_{50}-values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells [HIV strain: a T cell-line tropic (T-tropic) strain, HIV-1(III_B)]. In the p24 antigen assay, two types of HIV-1 strains were used: a T-tropic

‡ The one-pot procedure is compatible with *S*-trityl protection in Cys residues.

Table 1 MTT assay

	EC_{50} (nM)	CC_{50} (μM)	SI
T22	8.4	27	3200
T131(OH)	17	>80	>4600
AZT-Suc-T131(OH)	1.1	6.8	5700
AZT-Glu-T131(OH)	11	29	2600
$(AZT\text{-}Suc)_8$-T131(OH)	0.18	1.7	9700
AZT	2.5	5.6	2300

p24 antigen assay

	EC_{50} (nM)	
	NL4-3 (T-tropic)	JR-CSF (M-tropic)
T22	36	>3300
T131(OH)	480	>4000
AZT-Suc-T131(OH)	31	31
AZT-Glu-T131(OH)	340	67
$(AZT\text{-}Suc)_8$-T131(OH)	12	<1.6
AZT	49	1.7

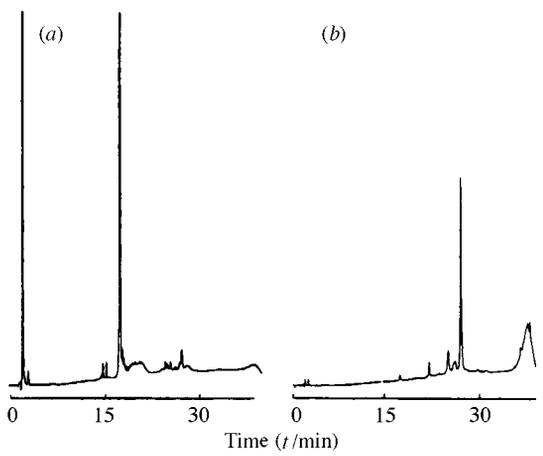
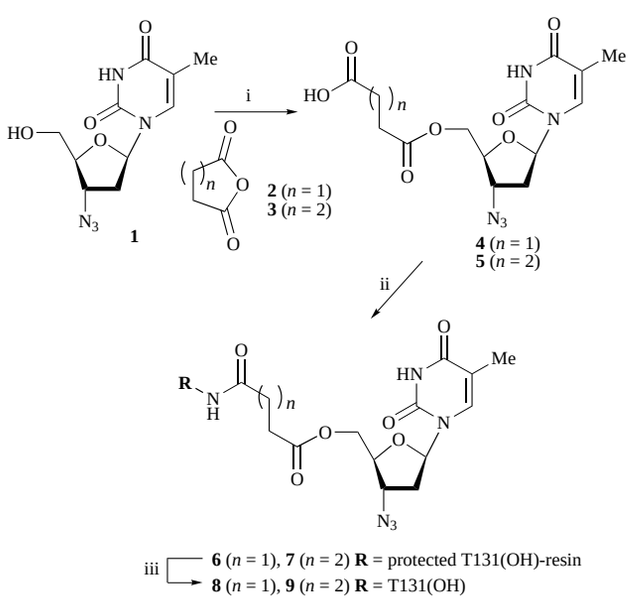
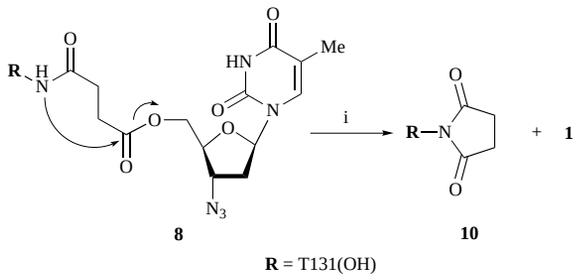


Fig. 2 Analytical HPLC of the crude AZT-Suc-T131(OH) (*a*) and $(AZT\text{-}Suc)_8$-T131(OH) (*b*) synthesized by the one-pot procedure



Scheme 2 *Reagents*: i, DMAP; ii, protected T131(OH)-resin, DIPCDI, HOBt; iii, TMSCl–DMSO/TFA

strain, NL4-3, and a macrophage tropic (M-tropic) strain, JR-CSF, and EC_{50}-values are based on the inhibition of expression of HIV-1 p24 antigen.[16] Our previous study showed that T22 specifically inhibits the entry of T-tropic HIV-1 into target

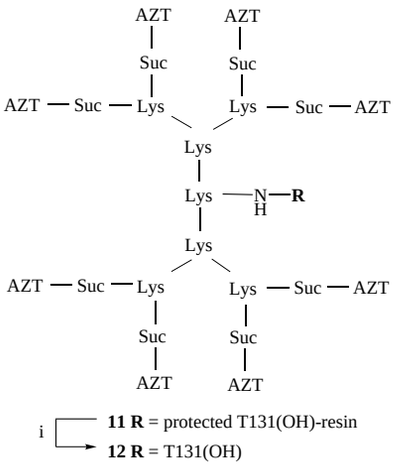


Scheme 3 *Reagents and conditions*: i, 0.4 mol dm^{-3} aq. $AcONH_4$, pH 8.0, room temp.

cells.[12] In the MTT assay, AZT-Suc-T131(OH) showed higher activity than T22, T131(OH) and AZT. AZT-Suc-T131(OH) seems to be preferred to AZT-Glu-T131(OH) as a conjugate compound. This is probably because a desirable controlled release of AZT by formation of a succinimide ring from AZT-Suc-T131(OH) may occur at a much faster rate than a release of AZT from AZT-Glu-T131(OH) in aqueous assay media at pH 7.2. AZT-Suc-T131(OH) and AZT-Glu-T131(OH) acquired the ability to inhibit the infection of both T-tropic and M-tropic HIV-1 strains, whereas T22 and T131(OH) exhibited anti-HIV activity against only a T-tropic HIV-1, but not against an M-tropic HIV-1 (p24 antigen assay). These results suggest that AZT, which was released during the assay, and the peptide exhibit a synergistic effect for anti-HIV activity, and that bifunctional anti-HIV compounds composed of a tachyplesin analogue and AZT are useful as anti-HIV agents.

Next, for the purpose of further enhancing the activity, an AZT-dendrimeric conjugate, $(AZT\text{-}Suc)_8$-T131(OH) **12**, was synthesized as follows. Repeated condensations (3 times) of Fmoc-Lys(Fmoc)-OH on the protected T131(OH)-resin using DIPCDI and HOBt, followed by condensation of AZT 5′-hemisuccinate **4**, provided the protected $(AZT\text{-}Suc)_8$-T131(OH)-resin **11**. Subsequent one-pot treatment of compound **11** with TMSCl–DMSO/TFA and HPLC purification gave $(AZT\text{-}Suc)_8$-T131(OH) **12** in relatively good yield [Scheme 4 and Fig. 2(*b*)]. The AZT-dendrimer **12** contains octameric AZT anchored on a MAP-type branched lysine[17] in the N-terminal end of T131(OH) *via* a succinyl linker. The present one-pot procedure was useful for the synthesis of such a complicated peptidyl derivative as an AZT-dendrimeric peptide. The anti-HIV activity of $(AZT\text{-}Suc)_8$-T131(OH) was much higher than that of AZT-Suc-T131(OH) in the MTT and p24 antigen assays (Table 1). This result indicates that an AZT-dendrimeric conjugate is a valuable lead compound for anti-HIV agents. Taken together, AZT–tachyplesin analogue conjugates have several potential advantages. (1) These compounds have binding sites to surface proteins of HIV and T cell (gp120, CD4 and fusin/CXCR4), and tachyplesin analogues can probably work as a carrier targetting T cells and HIV-infected T cells. (2) There are practicable possibilities of development of effective prodrugs of AZT based on AZT–tachyplesin analogue-conjugates possessing the potency of an efficient release of AZT. An ester bond between AZT and a succinyl linker may be susceptible not only to formation of a succinimide ring, but also to esterase metabolism. (3) These conjugates have two different antiviral mechanisms. (4) They exhibit anti-HIV activity against T-tropic and M-tropic strains. (5) A synergistic effect by bifunctional agents enables drug dosage to be reduced, and thus it may effectively suppress toxic side-effects and the emergence of drug-insensitive HIV strains, which are serious problems in the use of AZT for clinical therapy.[14]

In conclusion, the present procedure provides a convenient methodology for the rapid preparation of cystine-containing peptides. This procedure features a one-pot protocol for cleavage/deprotection of protected peptide-resins with con-



Scheme 4 *Reagents*: i, TMSCl–DMSO/TFA

comitant formation of disulfide bonds. Furthermore, this procedure was successfully applied to the synthesis of complicated bifunctional anti-HIV compounds, which were composed of a tachyplesin analogue linked with AZT by a succinyl linker (unstable in aqueous media at pH 8).

Experimental

General

Amino acid analysis was conducted using a Hitachi 835 instrument (Tokyo, Japan). The solvents for HPLC were water and MeCN, both containing 0.1% (v/v) TFA. For analytical HPLC, a Waters μBondasphere 5μC18-100 Å column (3.9 × 150 mm, Nihon Millipore Ltd., Tokyo, Japan) was eluted with a linear gradient of MeCN (gradient I, 25–55%, 30 min; gradient II, 10–40%, 30 min; gradient III, 10–50%, 30 min) at a flow rate of 1 cm^3 min^{-1} on a Waters LC Module I equipped with a Waters 741 Data Module. Preparative HPLC was performed on a Waters Delta Prep 4000 equipped with a Cosmosil 5μC18-AR column (20 × 250 mm, Nacalai Tesque Inc., Kyoto, Japan) at a flow rate of 7 cm^3 min^{-1}. The eluate was monitored by UV absorption at 220 nm. ^{1}H NMR spectra were recorded using a JEOL EX-270 (270 MHz) spectrometer (Tokyo, Japan) for samples in deuteriochloroform. Chemical shifts are reported in parts per million downfield from internal tetramethylsilane. *J*-Values are given in Hz. ISMS were performed with a Sciex API*III*E triple-quadrupole mass spectrometer (Toronto, Canada). Nominal (LRMS) and exact mass (HRMS) spectra were recorded on a JEOL JMS-01SG-2 or JMS-HX 110A mass spectrometer (Tokyo, Japan). Optical rotations of the peptides in water were measured with a JASCO DIP-360 digital polarimeter (Tokyo, Japan); $[a]_D$-values are given in 10^{-1} deg cm^2 g^{-1}. Fmoc-protected amino acids and Alko resins were purchased from Watanabe Chemical Industries, Ltd (Hiroshima, Japan). Rink amide resins were obtained from Calbiochem-Novabiochem Japan, Ltd. (Tokyo, Japan). All the other chemicals were purchased from either Nacalai Tesque Inc. or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Chemical behaviour for Fmoc-amino acid side-chain-protecting groups

Treatment of the following side-chain-protected Fmoc-amino acids (15 μmol each), Fmoc-Arg(Pmc)-OH, Fmoc-Asp(OBu^t)-OH, Fmoc-Glu(OBu^t)-OH, Fmoc-His(Boc)-OH, Fmoc-Ser(Bu^t)-OH, Fmoc-Thr(Bu^t)-OH, Fmoc-Tyr(Bu^t)-OH and Fmoc-Lys(Boc)-OH, with TMSCl (25 mol equiv.)–DMSO (750 mol equiv.) in TFA (total 6 cm^3) was performed at 4 °C. At intervals (5, 10, 30, 60 and 90 min), an aliquot (0.1 cm^3 each) was diluted with water–MeCN (1:1) (0.9 cm^3), followed by HPLC analysis (0.02 cm^3). Loss of starting material and generation of the corresponding side-chain-deprotected Fmoc-amino acid derivatives were quantitated from the HPLC peak area (gradient I).

Recovery of amino acids from corresponding Alko and Rink amide resins

H-Leu-Alko resin (0.60 mmol/g; 2 μmol) or H-Arg(Pmc)-Rink amide resin (0.32 mmol/g; 2 μmol) and Boc-Gly-OH (internal standard, 3 μmol) were treated with TMSCl (25 mol equiv.) in TFA in the presence of anisole (0.01 cm^3) at room temp. for 1 h. DMSO (750 mol equiv.) was then added to the reaction mixture at 4 °C (total solution volume 1 cm^3) and the reaction was allowed to continue. After 1 h, an aliquot (0.1 cm^3 each) was sampled and diluted with 0.02 mol dm^{-3} HCl aq. (0.9 cm^3). Regeneration of H-Leu-OH or H-Arg-OH was quantitated by an amino acid analyzer (H-Leu-OH, 100%; H-Arg-OH, 87%). The cleavage of Val from H-Val-Rink amide resin required a 2 h treatment with TMSCl in TFA–anisole (recovery of H-Val-OH, 87%).

Synthesis of T131(OH) using the one-pot procedure

The protected T131(OH)-resin was manually constructed using the Fmoc-based solid-phase method on Fmoc-Arg(Pmc)-Alko-resin (0.52 mmol/g; 0.1 mmol scale). The Fmoc-protected amino acid derivatives (2.5 mol equiv.) were then condensed using DIPCDI (2.5 mol equiv.) in the presence of HOBt (2.5 mol equiv.) according to the reported procedure.[18] The following side-chain-protected Fmoc-amino acids were used: Cys(Acm), Lys(Boc), Arg(Pmc), Tyr(Bu^t) and D-Lys(Boc). The given protected T131(OH)-resin (50 mg, 9.6 μmol) was treated with TMSCl (0.105 cm^3, 10 mol equiv.) in TFA (13 cm^3) in the presence of anisole (0.15 cm^3) at room temp. After 1 h, DMSO (1.9 cm^3, 300 mol equiv.) was added to the reaction mixture at 4 °C and the reaction was allowed to continue for 1 h. After removal of the resin by filtration, ice-cold dry diethyl ether (30 cm^3) was added to the filtrate. The resulting powder was collected by centrifugation and was then washed three times with ice-cold, dry diethyl ether (20 cm^3 × 3). The analytical HPLC pattern of the crude T131(OH) is shown in Fig. 1(*a*). The crude peptide was purified by preparative HPLC using a linear gradient of MeCN (14–16%, 30 min). The solvent was removed by lyophilization to give a fluffy powder [6.2 mg, 33%, calculated from the protected T131(OH)-resin] [ISMS (reconstructed) Found: M_r, 1974.0. Calc. for $C_{86}H_{140}N_{32}O_{18}S_2$: *M*, 1973.2]. Retention time t_R on analytical HPLC (gradient II) 14.2 min. Amino acid ratios after hydrolysis with 6 mol dm^{-3} HCl (values in parentheses are theoretical): cystine not determined (1), Tyr 3.00 (3), Lys and D-Lys 2.86 (3), Arg 5.11 (5), Pro 0.95 (1); $[a]_D^{26}$ −24.5 (*c* 0.2, water).

Synthesis of T131(NH_2) using the one-pot procedure

The protected T131(NH_2)-resin was similarly constructed on Rink amide resin (0.34 mmol/g; 0.1 mmol scale). The given protected T131(NH_2)-resin (50 mg, 7.6 μmol) was treated in the same way as in the synthesis of T131(OH), to obtain purified T131(NH_2) [8.4 mg, 56%, calculated from the protected T131(NH_2)-resin] [ISMS (reconstructed) Found: M_r, 1972.5. Calc. for $C_{86}H_{141}H_{33}O_{17}S_2$: *M*, 1972.1]. The analytical HPLC pattern of the crude T131(NH_2) is shown in Fig. 1(*b*). t_R on analytical HPLC (gradient II) 14.8 min. Amino acid ratios after hydrolysis with 6 mol dm^{-3} HCl (values in parentheses are theoretical): cystine not determined (1), Tyr 3.00 (3), Lys and D-Lys 2.80 (3), Arg 5.13 (5), Pro 0.88 (1); $[a]_D^{28}$ −18.8 (*c* 0.2, water).

Synthesis of T131(OH) and T131(NH_2) using a stepwise procedure consisting of TMSBr deprotection and TMSCl–DMSO oxidation

T131(OH). The protected T131(OH)-resin (48 mg, 9.2 μmol) was treated with 1 mol dm^{-3} TMSBr–thioanisole/TFA (5 cm^3) in the presence of *m*-cresol (0.25 cm^3, 300 mol equiv.) and

ethane-1,2-dithiol (0.10 cm^3, 180 mol equiv.) at 4 °C for 2 h. After removal of the resin by filtration, the filtrate was concentrated *in vacuo*. Ice-cold dry diethyl ether (30 cm^3) was then added, and the resulting powder was collected by centrifugation. After being washed three times with ice-cold dry diethyl ether (20 cm^3 × 3), the crude [Cys(Acm)[4,13]]-T131(OH) was subsequently treated with TMSCl (0.105 cm^3, 10 mol equiv.) and DMSO (1.9 cm^3, 300 mol equiv.) in TFA (13 cm^3) in the presence of anisole (0.15 cm^3) at 4 °C. After 1 h, ice-cold dry diethyl ether (30 cm^3) was added to the reaction mixture. The resulting powder was collected by centrifugation and then was washed three times with ice-cold dry diethyl ether (20 cm^3 × 3). The analytical HPLC pattern of the crude T131(OH) is shown in Fig. 1(*c*). HPLC purification afforded the purified T131(OH) (6.6 mg, 36%).

T131(NH_2). T131(NH_2) was similarly prepared from the protected T131(NH_2)-resin (34 mg, 5.2 μmol). The analytical HPLC pattern of the crude T131(NH_2) is shown in Fig. 1(*d*). Yield of the purified T131(NH_2) was 5.6 mg (55%).

Synthesis of T131(OH) and T131(NH_2) using a stepwise procedure consisting of TMSBr deprotection and iodine oxidation

T131(OH). The crude [Cys(Acm)[4,13]]-T131(OH) [obtained from TMSBr treatment of the protected T131(OH)-resin (48 mg, 9.2 μmol)] was dissolved in MeOH–water (8 : 2) (4.7 cm^3) in the presence of 15 mol equiv. of HCl. A MeOH solution (1.5 cm^3) of I_2 (20 mol equiv.) was added at room temp. After 2.5 h, the reaction was stopped by addition of saturated aq. ascorbic acid (6 cm^3). The analytical HPLC pattern of the crude T131(OH) is shown in Fig. 1(*e*). HPLC purification gave purified T131(OH) (8.1 mg, 44%).

T131(NH_2). T131(NH_2) was similarly prepared from the protected T131(NH_2)-resin (25 mg, 3.7 μmol). The analytical HPLC pattern of the crude T131(NH_2) is shown in Fig. 1(*f*). Yield of the purified T131(NH_2) was 4.3 mg (59%).

3′-Azido-3′-deoxythymidine 5′-hemisuccinate 4[19]

Succinic anhydride **2** (200 mg, 2.0 mmol) and DMAP (49 mg, 0.40 mmol) were added to a solution of 3′-azido-3′-deoxythymidine **1** (530 mg, 2 mmol) in THF (10 cm^3). The mixture was stirred at 4 °C for 48 h. Subsequently, further succinic anhydride **2** (400 mg, 4.0 mmol) was added to the above mixture, and stirring of the mixture was continued for 24 h. The mixture was extracted with AcOEt, and the extract was washed with brine. The crude product was extracted from the AcOEt layer with saturated aq. $NaHCO_3$. The aqueous layer was acidified with citric acid until pH 4, and the crude product was extracted with AcOEt. The extract was washed with brine, and dried over $MgSO_4$. The solvent was removed under reduced pressure. The residue **4** was used for the next reaction without any further purification and was obtained as an oil (570 mg 77%), $[\alpha]_D^{24}$ 70.6 (*c* 1.5, $CHCl_3$); δ_H(270 MHz; $CDCl_3$) 1.87 (d, *J* 0.99, 3 H), 2.34–2.93 (m, 6 H), 4.11–4.13 (m, 1 H), 4.26 (dd, *J* 12.7 and 3.8, 1 H), 4.34–4.39 (m, 1 H), 4.85 (dd, *J* 12.7 and 1.5, 1 H), 5.90 (dd, *J* 7.26 and 5.28, 1 H), 7.45 (d, *J* 0.99, 1 H) and 10.68 (s, 1 H); LRMS (FAB) *m*/*z* 368 (MH^+), 242 (base peak), 127, 101 and 81; HRMS (FAB) [Found: MH, 368.1215. Calc. for $C_{14}H_{18}N_5O_7$: *m*/*z* 368.1206].

3′-Azido-3′-deoxythymidine 5′-hemiglutarate 5

3′-Azido-3′-deoxythymidine **1** (530 mg, 2 mmol) was similarly treated with glutaric anhydride **3** (230 mg, 2.0 mmol) and DMAP (49 mg, 0.40 mmol) in THF (10 cm^3) to give title compound **5** as an oil (350 mg, 46%); $[\alpha]_D^{24}$ 19.0 (*c* 0.9, $CHCl_3$); δ_H(270 MHz; $CDCl_3$) 1.91 (s, 3 H), 1.96–2.05 (m, 2 H), 2.36–2.57 (m, 6 H), 4.05 (dd, *J* 5.9 and 3.3, 1 H), 4.24–4.33 (m, 2 H), 4.53 (dt, *J* 12.5 and 4.5, 1 H), 6.07 (t, *J* 6.1, 1 H), 7.39 (s, 1 H) and 10.27 (s, 1 H); LRMS (FAB) *m*/*z* 382 (MH^+), 279, 256, 221, 207 (base peak), 167, 149, 147, 136, 127, 115 and 113; HRMS (FAB) [Found: MH, 382.1358. Calc. for $C_{15}H_{20}N_5O_7$: *m*/*z* 382.1363].

AZT-Suc-T131(OH) 8

Hemisuccinate **4** (78 mg, 210 μmol) was condensed with the protected T131(OH)-resin (500 mg, 84 μmol), using DIPCDI (210 μmol, 2.5 mol equiv.) and HOBt (210 μmol, 2.5 mol equiv.). The thus-formed protected AZT-Suc-T131(OH)-resin **6** (50 mg, 8.0 μmol) was treated in the same one-pot manner using the TMSCl–DMSO/TFA system as in the synthesis of T131(OH). Yield of title product **8** was 5.5 mg (30%, calculated from **6**) [ISMS (reconstructed) Found: M, 2323.2. Calc. for $C_{100}H_{155}N_{37}O_{24}S_2$: *M*, 2322.1]. The analytical HPLC pattern of the crude product **8** is shown in Fig. 2(*a*); t_R on analytical HPLC (gradient III) 17.5 min. Amino acid ratios after hydrolysis (6 mol dm^{-3} HCl) (values in parentheses are theoretical): cystine not determined (1), Tyr 3.00 (3), Lys and D-Lys 3.00 (3), Arg 5.37 (5), Pro 1.24 (1); $[\alpha]_D^{18}$ −16.7 (*c* 0.1, 1 mol dm^{-3} AcOH).

AZT-Glu-T131(OH) 9

Hemiglutarate **5** (31 mg, 83 μmol) was condensed with the protected T131(OH)-resin (200 mg, 33 μmol), using DIPCDI (83 μmol, 2.5 mol equiv.) and HOBt (83 μmol, 2.5 mol equiv.). The thus-prepared protected AZT-Glu-T131(OH)-resin **7** (100 mg, 16 μmol) was treated in the same one-pot manner using the TMSCl–DMSO/TFA system as in the synthesis of T131(OH). Yield of product **9** was 2.3 mg (6.3%, calculated from **7**) [ISMS (reconstructed) Found: M, 2337.7. Calc. for $C_{101}H_{157}N_{37}O_{24}S_2$: *M*, 2336.2]; t_R on analytical HPLC (gradient III) 17.4 min. Amino acid ratios after hydrolysis (6 mol dm^{-3} HCl) (values in parentheses are theoretical): cystine not determined (1), Tyr 2.92 (3), Lys and D-Lys 3.00 (3), Arg 5.08 (5), Pro 1.22 (1); $[\alpha]_D^{20}$ −38.2 (*c* 0.3, 1 mol dm^{-3} AcOH).

$(AZT\text{-}Suc)_8$-T131(OH) 12

Fmoc-Lys(Fmoc)-OH (2.5 mol equiv. for free amino group) was condensed repeatedly three times with the protected T131(OH)-resin (90 mg, 11 μmol) using DIPCDI (2.5 mol equiv. for free amino group) and HOBt (2.5 mol equiv. for free amino group). After each condensation step, the resin was treated with 20% piperidine–DMF (v/v, 10 cm^3, 1 min and 15 min) for the purpose of removing the Fmoc groups. Successively, the hemisuccinate **4** (83 mg, 230 μmol) was similarly condensed. The thus-formed protected $(AZT\text{-}Suc)_8$-T131(OH)-resin **11** (110 mg, 9.8 μmol) was treated in the same one-pot manner using the TMSCl–DMSO/TFA system as in the synthesis of T131(OH) to give title product **12** (7.3 mg, 13%, calculated from **11**) [ISMS (reconstructed) Found: M, 5665.5. Calc. for $C_{240}H_{346}N_{86}O_{73}S_2$: *M*, 5664.5]. The analytical HPLC pattern of the crude product **12** is shown in Fig. 2(*b*); t_R on analytical HPLC (gradient III) 26.5 min. Amino acid ratios after hydrolysis (6 mol dm^{-3} HCl) (values in parentheses are theoretical): cystine not determined (1), Tyr 2.87 (3), Lys and D-Lys 10.00 (10), Arg 5.42 (5), Pro 1.26 (1); $[\alpha]_D^{18}$ 22.6 (*c* 0.1, 1 mol dm^{-3} AcOH).

Behaviour of AZT-Suc-T131(OH) 8 in aqueous media at pH 8.0

AZT-Suc-T131(OH) **8** (1 mg, 2.3 μmol) was dissolved in 0.4 mol dm^{-3} aq. $AcONH_4$ (pH 8.0; 10 cm^3) at room temp. At intervals (1, 3, 7 and 18 h), an aliquot (0.5 cm^3) was sampled and examined by analytical HPLC (gradient III). HPLC peaks of the starting material **8** and generated products were identified by ISMS, and their amounts were quantitated from the peak areas.

Evaluations of anti-HIV activity and cytotoxicity

A strain of HIV-1, HIV-1(III_B), was used for the anti-HIV assay (MTT assay). This virus was obtained from the culture supernatant of HIV-1 persistently infected MOLT-4/HIV-1(III_B) cells. Antiviral activity against HIV-1 was determined

based on the protection against virus-induced cytopathogenicity in MT-4 cells. Various concentrations of the test compounds were added to HIV-1-infected MT-4 cells at a multiplicity of infection (MOI) of 0.01, and placed in wells of a flat-bottomed microtiter tray (2.5×10^4/well). After 5 days' incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined using the MTT method.[15] Cytotoxicity of compounds was determined based on the viability of mock-infected cells using the MTT method. Anti-HIV-1 activity was also determined as the inhibitory effect on HIV-1 p24 antigen production in the culture supernatant. In the p24 antigen assay, two types of HIV-1 strains were used: a T-tropic strain, NL4-3, and an M-tropic strain, JR-CSF. Phytohaemagglutinin-stimulated peripheral blood mononuclear cells (PBMC) were infected with the above HIV-1 strains at MOI of 0.01. The HIV-1-infected PBMC were cultured with various concentrations of the test compounds. On day 7 after infection, the amount of HIV-1 p24 antigen in the culture supernatant was determined by the commercial enzyme-linked immunosorbent assay (Cellular Products).

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Paper 7/06545K
Received 8*th September* 1997
Accepted 13*th October* 1997