Solution-Phase Synthesis of an Anti-human Immunodeficiency Virus Peptide, T22 ([Tyr^{5,12}, Lys^{7}]-Polyphemusin II), and the Modification of Trp by the *p*-Methoxybenzyl Group of Cys during Trimethylsilyl Trifluoromethanesulfonate Deprotection[1)]

Hirokazu TAMAMURA,[a] Akira OTAKA,[a] Wataru TAKADA,[a] Yoshihiro TERAKAWA,[a] Hidenori YOSHIZAWA,[a] Masao MASUDA,[a] Toshiro IBUKA,[a] Tsutomu MURAKAMI,[b] Hideki NAKASHIMA,[c] Michinori WAKI,[d] Akiyoshi MATSUMOTO,[d] Naoki YAMAMOTO,[b] and Nobutaka FUJII*,[a]

Faculty of Pharmaceutical Sciences, Kyoto University,[a] Sakyo-ku, Kyoto 606, Japan, Department of Microbiology, Tokyo Medical and Dental University, School of Medicine,[b] Bunkyo-ku, Tokyo 113, Japan, Department of Microbiology, Yamanashi Medical University,[c] Tamaho-cho, Yamanashi 409–38, Japan, and SEIKAGAKU Corporation,[d] Chuo-ku, Tokyo 103, Japan. Received July 15, 1994; accepted August 27, 1994

T22 ([Tyr^{5,12}, Lys^{7}]-polyphemusin II) was previously synthesized by a solid-phase method and was found to have a strong anti-human immunodeficiency virus (HIV) activity, comparable to that of 3′-azido-2′,3′-dideoxythymidine (AZT). In the present study, the solution-phase synthesis of T22 was attempted in order to produce this peptide on a large scale. An 18-residue peptide amide corresponding to the entire amino acid sequence of T22 was synthesized by assembling four peptide fragments and two amino acid derivatives, followed by thioanisole-mediated deprotection with 1 M trimethylsilyl trifluoromethanesulfonate (TMSOTf) in trifluoroacetic acid followed by air-oxidation. During this deprotection, a significant by-product derived from the transfer of the *p*-methoxybenzyl (MBzl) group from the sulfhydryl group of the cysteine residue to the side chain of the tryptophan residue was formed. This side reaction was found to be efficiently suppressed by adopting a two-step deprotection procedure using silver trifluoromethanesulfonate (AgOTf)–TMSOTf or trimethylsilyl bromide (TMSBr)–TMSOTf.

Keywords T22; anti-human immunodeficiency virus peptide; tachyplesin; polyphemusin; solution-phase synthesis; Trp

Tachyplesins I, II and III and polyphemusins I and II, which are abundant in hemocyte debris of the Japanese horseshoe crab (*Tachypleus tridentatus*) and the American horseshoe crab (*Limulus polyphemus*), respectively, inhibit the growth of gram-positive and gram-negative bacteria and some fungi.[2)] Furthermore, these peptides were shown to have antiviral activity against vesicular stomatitis virus, influenza A virus and HIV-1.[3,4)] We have synthesized more than 100 peptide analogs of tachyplesin and polyphemusin by the solid-phase method. Among these peptides, we found a novel compound, T22 ([Tyr^{5,12}, Lys^{7}]-polyphemusin II), which showed strong anti-HIV activity and relatively low cytotoxicity *in vitro*.[5)] The 50% inhibitory concentration of T22 was 2.6 nM, a value comparable to that of AZT (5.2 nM). T22 is an 18-residue peptide amide possessing two disulfide bonds, and takes an antiparallel β-sheet structure with a type-II β-turn as determined by ^{1}H-NMR.[6)] Our recent studies showed that T22 exerted its effect on a process, most probably the virus-cell fusion, immediately after virus adsorption,[7)] and that the anti-HIV activity of T22 was mediated through interaction with a chiral component(s) of the cell or virus.[8)] In order to examine in detail the mechanism of the strong anti-HIV activity of T22, and its stability and cytotoxicity *in vivo*, *etc.*, a large quantity of T22 is required. In the present study, T22 was synthesized by a solution-phase method, which is suitable for synthesis on a large scale. We also report the details of the side reaction observed during the deprotection, and the optimal conditions to suppress this side reaction.

Results and Discussion

Synthesis of T22 Four peptide fragments and two amino acid derivatives were selected as building blocks to construct the entire peptide backbone, as shown in Fig. 1, and amino acid derivatives bearing protecting groups removable by treatment with 1 M TMSOTf–thioanisole/TFA[9)] were employed, *i.e.*, Cys(MBzl),[10)] Lys(Z), Arg(Mts),[11)] Tyr(Cl_2Bzl),[12)] and Trp(Mts).[13)] Each fragment ([1], [2], [4], or [6]) was prepared in a stepwise manner by the WSCD[14)] plus HOBt procedure, the Su active ester procedure[15)] or the MA procedure,[16)] followed by the usual hydrazine treatment (except for [1]) (see Experimental). Four peptide fragments thus obtained and two amino acid derivatives were then successively assembled according to the route illustrated in Fig. 1. The fragment condensations using the azide procedure[17)] proceeded from the C-terminal in DMF or DMF–NMP (1 : 1), each with the acyl component being employed in slight excess (1.2 to 1.7 eq). Every reaction was carried out without significant problems and each product was purified by precipitation from DMF with MeOH. Consequently, 19 g of the protected T22 was obtained in the good yield of 67% as calculated from the C-terminal fragment [1].

In the final step, the protected T22 was treated with 1 M TMSOTf–thioanisole/TFA to remove all protecting groups. The deprotected peptide was air-oxidized to form two disulfide bridges (Fig. 2). HPLC examination of the crude air-oxidized product showed the presence of two major components (Fig. 3). Each product was isolated by HPLC. The product obtained from the front eluate was

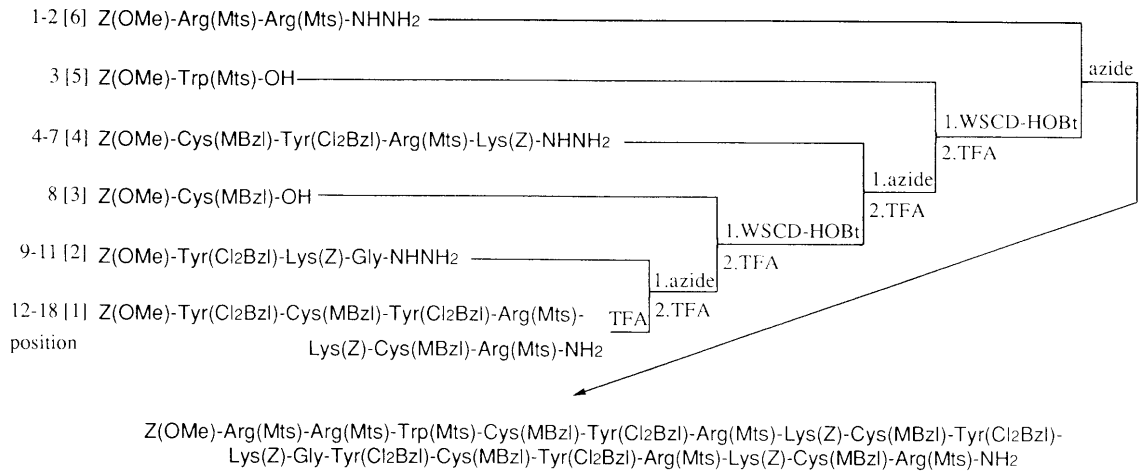

Fig. 1. Synthetic Scheme for the Protected T22

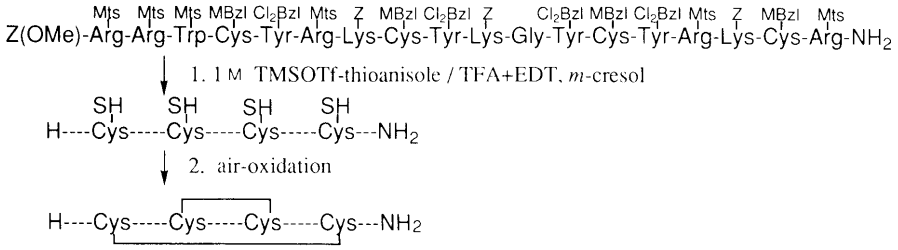

Fig. 2. Deprotection by 1 M TMSOTf–Thioanisole/TFA for the Synthesis of T22

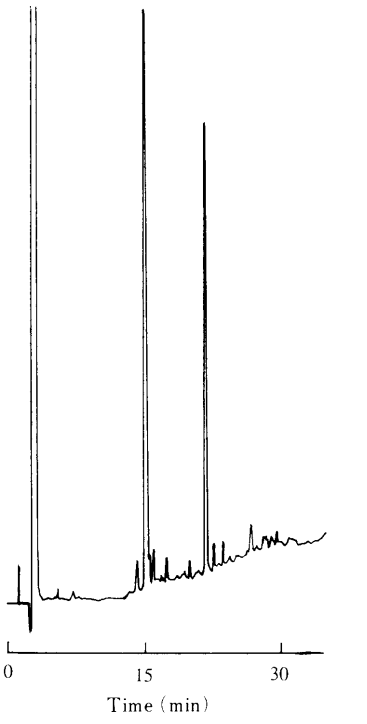

Fig. 3. Analytical HPLC of Crude T22 Deprotected by 1 M TMSOTf–Thioanisole/TFA

found to be T22, based on the retention time on HPLC, ion spray mass spectrometric examination and amino acid analysis. Its disulfide-pairings proved to be identical with those of T22 based on ion spray mass analysis of peptide fragments derived from the tryptic digest of the synthetic

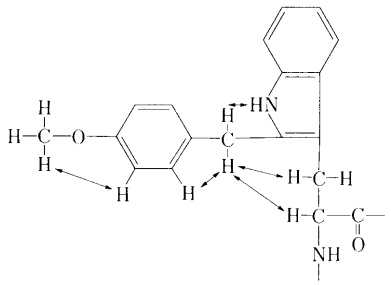

Fig. 4. NOE Connectivities between the Protons of the MBzl Group and the Trp[3] Residue in NMR Analysis of the By-product

peptide. The yield of T22 was low (12%, calculated from the protected T22), due to the generation of the by-product, corresponding to the later eluate (T22: by-product = 1 : 0.63, w/w).

Characterization of the By-product The ion spray mass value of the by-product was 120 atomic mass units higher than that of T22. This mass difference corresponds to an MBzl moiety. Amino acid analysis after LAP digestion of this by-product detected no Trp. These results suggested that the Trp residue had been modified by the MBzl group. With reference to the NMR assignment of all protons of T22,[6] NMR analysis of this by-product was performed. In its spectrum, the signal at position 2 of the indole ring (2′-H) of Trp[3] had disappeared and additional signals corresponding to an MBzl moiety appeared. Based on the presence of NOEs between the protons of the MBzl group and the Trp[3] residue shown in Fig. 4, the structure of this by-product was assigned as [Trp(2′-MBzl)[3]]-T22. Its disulfide-pairings proved to be identical with those of T22.

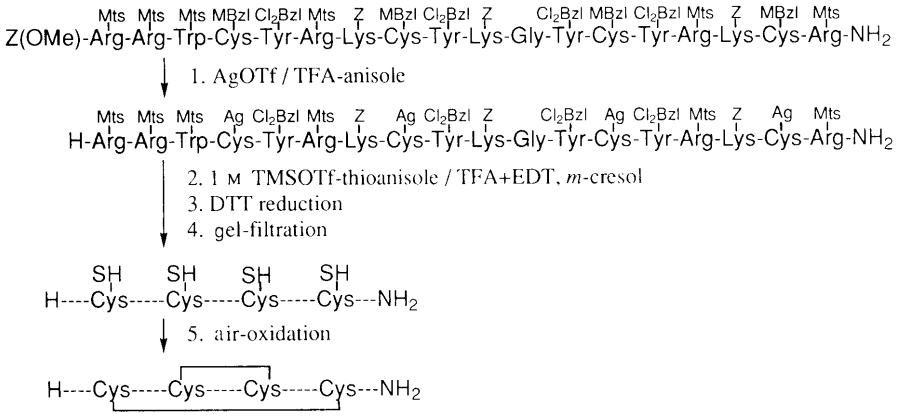

Fig. 5. Deprotection by Sequential AgOTf/TFA and 1 M TMSOTf–Thioanisole /TFA Treatments for the Synthesis of T22

This side reaction was similarly detectable, when an N^{α}-deprotected sample of the protected T22 (obtained by treatment of the protected T22 with TFA–anisole) was treated with 1 M TMSOTf–thioanisole/TFA. In addition, when an equimolar mixture of Boc–Trp(Mts)–OH and Z(OMe)–Cys(MBzl)–OH was treated with 1 M TMSOTf–thioanisole/TFA at a high concentration (15 mM), the parent amino acid, Trp, was quantitatively recovered and no modification of Trp occurred (data not shown). These findings suggested that the source of the MBzl group concerned in the modification of the Trp residue was the protecting group of the side chain of the Cys residue(s), not the N^{α}-protecting group (Z(OMe)), and that this transfer was due to an intramolecular mechanism, not an intermolecular one. The stability of the reactive cation, MBzl, liberated by acid, its electrophilic and steric properties, the cation-trapping ability of the indole moiety as a scavenger, and the distance between the side chain of the Cys residue(s) and the indole ring of Trp^3 were related to the modification of the Trp residue. In terms of the spatial distance, the MBzl group might transfer from the side chain of Cys^4 to the 2′ position of Trp^3. In 1978, Yajima *et al.* reported that by-products, such as H–Trp(2′-MBzl)–OH, were formed during the treatment of Z(OMe)–Trp–OH with TFA, and that this type of side reaction was efficiently suppressed by the use of a scavenger system, such as thioanisole containing 2% EDT–skatole or dimethylsulfide containing 2% EDT–skatole.[18] However, the present side reaction could not be suppressed either by increasing amounts of scavengers (2 M thioanisole, EDT 300—600 eq) or by the addition of indole (5—200 eq) to the deprotection system (data not shown). The result indicated that the present modification of the Trp residue might be attributed to the short spatial distance between the side chain of Cys^4 and the indole ring of Trp^3. Several other papers regarding the modification of the 2′ position of Trp have been published, *i.e.*, generation of 2′-phenylthiotryptophan by treatment of Boc–Trp(Mts)–OH with 1 M TFMSA–thioanisole/TFA,[19] modification by the Pmc protecting group of Arg during TFA deprotection,[20] modification during treatment with mercury(II) acetate/2-mercaptoethanol in aqueous acetic acid,[21] *etc.* Formation of the 1′-substituted by-product by *tert*-butyl cation during N^{α}-deprotection of Boc with TFA was also reported.[22]

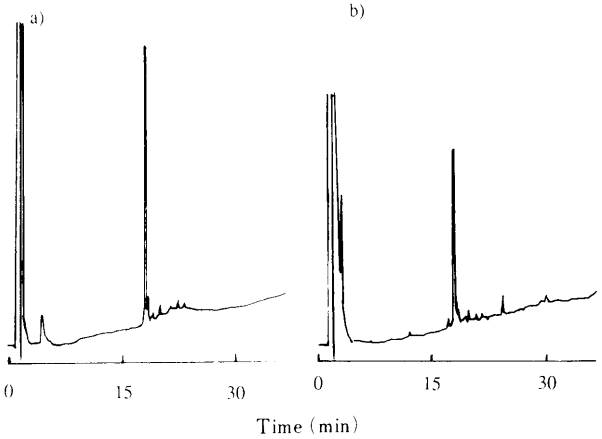

Fig. 6. Analytical HPLC of Crude T22 Deprotected by Sequential AgOTf/TFA and 1 M TMSOTf–Thioanisole/TFA Treatments (a) or by Sequential 1 M TMSBr–Thioanisole/TFA and 1 M TMSOTf–Thioanisole/TFA Treatments (b)

Studies on the Optimal Deprotection Conditions We next looked for efficient deprotection conditions for suppressing the modification of the Trp residue. First, the protected T22 was treated with AgOTf/TFA–anisole,[23] then with 1 M TMSOTf–thioanisole/TFA. The former treatment was performed to remove MBzl groups from Cys residues while leaving the other side chain protecting groups intact (Fig. 5). All of the other protecting groups were removed by the latter reagent. The deprotected peptide was treated with DTT to reduce Cys(Ag) to Cys(SH), followed by gel-filtration and air-oxidation. The HPLC elution pattern of the crude peptide is shown in Fig. 6a. This deprotection procedure almost completely suppressed the generation of the by-product, and consequently the yield of T22 was increased to 19%. However, this procedure has some problems: use of an expensive reagent and heavy metal (AgOTf), and many steps (reduction and gel-filtration).

Next, a two-step deprotection procedure involving two consecutive hard acid treatments was attempted. The protected T22 was treated with the weak hard acid, 1 M TMSBr–thioanisole/TFA,[24] then with the stronger hard acid, 1 M TMSOTf–thioanisole/TFA (Fig. 7). The usefulness of this procedure for practical solid-phase peptide synthesis was demonstrated by comparison with other

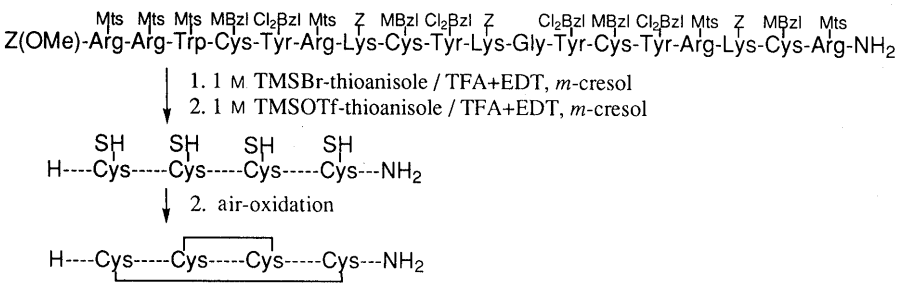

Fig. 7. Deprotection by Sequential 1 M TMSBr–Thioanisole/TFA and 1 M TMSOTf–Thioanisole/TFA Treatments for the Synthesis of T22

deprotection methods.[25] The first treatment cleaves the potential carbocation forming benzylic protecting groups sensitive to TMSBr under mild conditions. The resulting crude product is precipitated from the reaction mixture by addition of Et_2O with removal of the benzyl cation by an Et_2O-wash. In the second step, the other protecting groups, which are left intact in the first step, are completely removed from the peptide. In this peptide, the MBzl group is completely cleaved from Cys residues by 1 M TMSBr–thioanisole/TFA; however, the Mts group of the Trp residue is less cleavable, and thus is completely removed by 1 M TMSOTf–thioanisole/TFA. The HPLC elution pattern of the air-oxidized product is shown in Fig. 6b. This procedure also suppressed this side reaction. As a result, 152 mg of the purified T22 was obtained from 1 g of the protected T22 in the yield of 26%.

These two deprotection procedures efficiently suppressed the modification of the Trp residue. In both procedures, the MBzl groups on the Cys residues were cleaved prior to cleavage of the Mts group on the Trp residue. In the one-step deprotection with 1 M TMSOTf–thioanisole/TFA, the MBzl group on Cys and the Mts group on Trp were cleaved at almost the same time. The presence of the Mts group in the Trp residue during cleavage of the MBzl group on the Cys residue may be indispensable to avoid the modification of the Trp residue by the MBzl group. Possibly, an electron-withdrawing substituent, Mts, acts to protect the indole moiety of Trp against attack of the MBzl cation.[13] As a general deprotection procedure in the synthesis of Trp and the adjacent Cys(MBzl)-containing peptides, we recommend the use of the two-step deprotection procedure consisting of consecutive TMSBr and TMSOTf treatments.

Conclusion

An anti-HIV peptide, T22, was synthesized by a solution-phase method. During its deprotection, a by-product derived from the modification of Trp by the MBzl group was generated. The presence of MBzl in the 2′ position of Trp^3 in the modified peptide was identified by ion spray mass and NMR spectrometric analyses. Consideration of the spatial distance led to the speculation that the MBzl group of the side chain of Cys^4 might transfer to the 2′ position of Trp^3. This side reaction will also be investigated by examination of the effect of the relative position of Trp and Cys(MBzl) residues in the peptide chain on the extent of this modification using model compounds. The data presented here provide useful information for the synthesis of peptides containing Trp and adjacent Cys(MBzl). The two-step deprotection procedure involving consecutive TMSBr and TMSOTf treatments was efficient in suppressing this type of side reaction. We have demonstrated the advantageous features of a two-step hard acid deprotection procedure. By applying this condition, methodology suitable for supplying T22 on a large scale was established. The EC_{50} of the sample synthesized by this solution-phase method was 2.5 nM, and it was judged to be equivalent to that of the sample synthesized by a solid-phase method (2.6 nM).[5] With a large quantity of T22 in hand, the mechanism of its anti-HIV activity can be examined in detail.

Experimental

N^{α}-Deprotection The N^{α}-protecting group, Z(OMe)[26] or Boc, was cleaved by TFA (*ca.* 10 ml per 1 g of a peptide) in the presence of anisole (2 eq or more) at ice-bath temperature for 1 h. If the N^{α}-deprotected sample was condensed with the next fragment using the WSCD plus HOBt procedure, 4 M HCl solution in dioxane (1.5 eq) was added to the reaction mixture to convert the TFA salt to the HCl salt.[27] After evaporation of the TFA (HCl/dioxane) *in vacuo* at 30 °C or below, the product was precipitated with Et_2O; the resulting powder was collected by filtration, dried over NaOH *in vacuo* for 2 h and then used for the condensation reaction.

Condensation Reactions Every reaction was continued until the solution became negative to the ninhydrin test. The active ester[15] and WSCD[14]–HOBt condensation reactions were performed at room temperature (17—25 °C). The azide was prepared with isoamyl nitrite, and the reaction was usually conducted at 4 °C.[17] A mixed anhydride was prepared with isobutyl chloroformate and the reaction was performed in an ice-bath.[16]

Purification Unless otherwise stated, protected peptides were purified by the following procedure A or B.

Procedure A: For purification of protected peptides soluble in AcOEt, the extract was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and concentrated. The residue was recrystallized from appropriate solvents.

Procedure B: For purification of protected peptides less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$, and H_2O and recrystallized or precipitated from appropriate solvents.

TLC was performed on silica (Kieselgel G, Merck). *Rf* values refer to the following solvent systems (v/v): Rf_1 $CHCl_3$–MeOH (10:0.5), Rf_2 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_3 $CHCl_3$–MeOH–AcOH (9:1:0.5). Amino acid analysis was conducted using a Hitachi 835 instrument. HPLC was performed on a Waters 600E or a Waters LC Module I equipped with a Waters 741 Data Module. The solvents for HPLC were H_2O and CH_3CN, both containing 0.1% (v/v) TFA. For analytical HPLC, μBondasphere 5μ C18-100 Å (3.9 × 150 mm) was eluted with a linear gradient of CH_3CN (10—40%, 30 min) at a flow rate of 1 ml/min. Preparative HPLC was performed on a YMC Packed Column (ODS, 20 × 250 mm) at a flow rate of 7 ml/min. The elute was monitored by UV absorption measurement at 220 nm. Ion spray mass spectra were obtained using a Sciex API IIIE Biomolecular Mass Analyzer. LAP (microsomal type VI-S, lot no. 61H7045) and trypsin (TPCK treated type XIII from bovine pancreas, lot no. 100H8130) were purchased from

Sigma.

Z(OMe)–Cys(MBzl)–Arg(Mts)–NH_2 An N^{α}-deprotected sample of Boc–Arg(Mts)–NH_2 (20.65 g, 45.3 mmol) was dissolved in DMF (200 ml), then Z(OMe)–Cys(MBzl)–OH (18.38 g, 45.3 mmol), HOBt·H_2O (6.94 g, 45.3 mmol), WSCD (7.89 ml, 45.3 mmol) and TEA (3.0 ml, 21.6 mmol) were added and the mixture was stirred overnight. An N^{α}-deprotected sample of Boc–Arg(Mts)–NH_2 (13.2 g, 28.9 mmol), HOBt·H_2O (4.42 g, 28.9 mmol), WSCD (5.03 ml, 28.9 mmol) and TEA (2.0 ml, 14.4 mmol) were added and the reaction was continued for an additional 18 h. The product was purified by procedure A, followed by recrystallization from AcOEt and Et_2O; yield 24.67 g (73%), mp 84—86 °C, $[\alpha]_D^{20}$ −9.35° (c=0.2, DMF), Rf_2 0.77. *Anal.* Calcd for $C_{35}H_{46}N_6O_8S_2$: C, 56.58; H, 6.25; N, 11.32. Found: C, 56.36; H, 6.54; N, 11.30.

Z(OMe)–Lys(Z)–Cys(MBzl)–Arg(Mts)–NH_2 An N^{α}-deprotected sample of the above dipeptide amide (28.38 g, 38.2 mmol) was dissolved in DMF (200 ml), then Z(OMe)–Lys(Z)–OH (18.68 g, 42.0 mmol), HOBt·H_2O (6.43 g, 42.0 mmol) and WSCD (7.32 ml, 42.0 mmol) were added and the mixture was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF and AcOEt; yield 27.70 g (72%), mp 176—178 °C, $[\alpha]_D^{20}$ −12.2° (c=0.2, DMF), Rf_2 0.75. *Anal.* Calcd for $C_{49}H_{64}N_8O_{11}S_2$: C, 58.54; H, 6.42; N, 11.15. Found: C, 58.32; H, 6.37; N, 11.06.

Z(OMe)–Arg(Mts)–Lys(Z)–Cys(MBzl)–Arg(Mts)–NH_2 An N^{α}-deprotected sample of the above tripeptide amide (27.70 g, 27.6 mmol) was dissolved in DMF (200 ml), then Z(OMe)–Arg(Mts)–OH [prepared from 20.5 g (33.1 mmol) of the CHA salt] in DMF (100 ml), HOBt·H_2O (5.06 g, 33.1 mmol), WSCD (5.76 ml, 33.1 mmol) and TEA (1.91 ml, 13.8 mmol) were added and the mixture was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF and EtOH; yield 26.56 g (72%), mp 114—115 °C, $[\alpha]_D^{20}$ −6.19° (c=0.2, DMF), Rf_2 0.74. *Anal.* Calcd for $C_{64}H_{86}N_{12}O_{14}S_3 \cdot H_2O$: C, 56.37; H, 6.65; N, 12.33. Found: C, 56.67; H, 6.41; N, 12.31.

Z(OMe)–Tyr(Cl_2Bzl)–Arg(Mts)–Lys(Z)–Cys(MBzl)–Arg(Mts)–NH_2 An N^{α}-deprotected sample of the above tetrapeptide amide (26.56 g, 19.8 mmol) was dissolved in DMF (200 ml), then Z(OMe)–Tyr(Cl_2Bzl)–OH (11.0 g, 21.8 mmol), HOBt·H_2O (3.33 g, 21.8 mmol), WSCD (3.79 ml, 21.8 mmol) and TEA (1.38 ml, 9.9 mmol) were added and the mixture was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF and EtOH; yield 29.76 g (90%), mp 154—156 °C, $[\alpha]_D^{20}$ −11.23° (c=0.1, DMF), Rf_2 0.92. *Anal.* Calcd for $C_{80}H_{99}Cl_2N_{13}O_{16}S_3$: C, 57.68; H, 5.99; N, 10.93. Found: C, 57.42; H, 6.21; N, 10.80.

Z(OMe)–Cys(MBzl)–Tyr(Cl_2Bzl)–Arg(Mts)–Lys(Z)–Cys(MBzl)–Arg(Mts)–NH_2 An N^{α}-deprotected sample of the above pentapeptide amide (29.76 g, 17.9 mmol) was dissolved in DMF (200 ml), then Z(OMe)–Cys(MBzl)–OH (8.69 g, 21.1 mmol), HOBt·H_2O (3.24 g, 21.1 mmol), WSCD (3.68 ml, 21.1 mmol) and TEA (1.24 ml, 8.9 mmol) were added and the mixture was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF and MeOH; yield 32.34 g (96%), mp 159—160 °C, $[\alpha]_D^{20}$ −15.3° (c=0.4, DMF), Rf_2 0.93. *Anal.* Calcd for $C_{91}H_{112}Cl_2N_{14}O_{18}S_4$: C, 57.86; H, 5.98; N, 10.38. Found: C, 57.59; H, 5.98; N, 10.42.

Z(OMe)–Tyr(Cl_2Bzl)–Cys(MBzl)–Tyr(Cl_2Bzl)–Arg(Mts)–Lys(Z)–Cys(MBzl)–Arg(Mts)–NH_2 [1] An N^{α}-deprotected sample of the above hexapeptide amide (32.34 g, 17.1 mmol) was dissolved in DMF (200 ml), then Z(OMe)–Tyr(Cl_2Bzl)–OH (10.36 g, 20.5 mmol), HOBt·H_2O (3.15 g, 20.5 mmol), WSCD (3.58 ml, 20.5 mmol) and TEA (1.19 ml, 8.6 mmol) were added and the mixture was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF and MeOH; yield 31.28 g (83%), mp 200—203 °C, $[\alpha]_D^{20}$ −16.28° (c=0.3, DMF), Rf_2 0.91. *Anal.* Calcd for $C_{107}H_{125}Cl_4N_{15}O_{20}S_4 \cdot H_2O$: C, 57.65; H, 5.74; N, 9.42. Found: C, 57.67; H, 5.63; N, 9.57.

Z(OMe)–Lys(Z)–Gly–OMe HCl·H–Gly–OMe (7.00 g, 55.8 mmol) was dissolved in DMF (100 ml), then Z(OMe)–Lys(Z)–OH (24.8 g, 55.8 mmol), HOBt·H_2O (8.54 g, 55.8 mmol), WSCD (9.71 ml, 55.8 mmol) and TEA (5.0 ml, 36.0 mmol) were added and the mixture was stirred overnight. The product was purified by procedure A, followed by recrystallization from MeOH and Et_2O; yield 21.0 g (73%), mp 112—114 °C, $[\alpha]_D^{20}$ −12.22° (c=0.3, MeOH), Rf_2 0.90. *Anal.* Calcd for $C_{26}H_{33}N_3O_8$: C, 60.57; H, 6.45; N, 8.15. Found: C, 60.58; H, 6.49; N, 8.18.

Boc–Tyr(Cl_2Bzl)–Lys(Z)–Gly–OMe An N^{α}-deprotected sample of the above dipeptide (24.80 g, 48.1 mmol) was dissolved in DMF (200 ml) containing TEA (6.69 ml, 1 eq), then Boc–Tyr(Cl_2Bzl)–OSu (31.01 g, 57.7 mmol) and TEA (6.69 ml, 48.1 mmol) were added and the mixture was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF and isopropyl alcohol; yield 35.33 g (95%), mp 165—167 °C, $[\alpha]_D^{20}$ −9.65° (c=0.3, DMF), Rf_1 0.19. *Anal.* Calcd for $C_{38}H_{46}Cl_2N_4O_9 \cdot 1/2H_2O$: C, 58.31; H, 6.05; N, 7.16. Found: C, 58.37; H, 6.03; N, 11.32.

Z(OMe)–Tyr(Cl_2Bzl)–Lys(Z)–Gly–OMe An N^{α}-deprotected sample of the above tripeptide (31.22 g, 40.4 mmol) was dissolved in DMF (200 ml) containing TEA (5.56 ml, 1 eq), then MZ-SDP (18.40 g, 60.5 mmol) and TEA (8.41 ml, 60.5 mmol) were added and the mixture was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF and EtOH; yield 29.00 g (86%), mp 175—179 °C, $[\alpha]_D^{20}$ −10.46° (c=0.5, DMF), Rf_1 0.39. *Anal.* Calcd for $C_{42}H_{46}Cl_2N_4O_{10} \cdot 1/2H_2O$: C, 59.58; H, 5.59; N, 6.62. Found: C, 59.62; H, 5.50; N, 6.79.

Z(OMe)–Tyr(Cl_2Bzl)–Lys(Z)–Gly–$NHNH_2$ [2] The above protected tripeptide ester (29.00 g, 34.6 mmol) in DMF (150 ml) was treated with hydrazine hydrate (16.78 ml, 10 eq) overnight. The product was precipitated from DMF with H_2O (400 ml), and then washed with H_2O, followed by recrystallization from DMF and EtOH; yield 26.99 g (93%), mp 188—192 °C, $[\alpha]_D^{20}$ −15.82° (c=0.3, DMF), Rf_2 0.67. *Anal.* Calcd for $C_{41}H_{46}Cl_2N_6O_9 \cdot H_2O$: C, 57.54; H, 5.65; N, 9.82. Found: C, 57.84; H, 5.65; N, 10.09.

Boc–Arg(Mts)–Lys(Z)–OMe HCl·H–Lys(Z)–OMe (21.20 g, 64.1 mmol) was dissolved in DMF (200 ml), then Boc–Arg(Mts)–OH (29.3 g, 64.1 mmol), HOBt·H_2O (9.00 g, 64.1 mmol), WSCD (11.16 ml, 64.1 mmol) and TEA (4.4 ml, 32.0 mmol) were added and the mixture was stirred overnight. The product was purified by procedure A, followed by recrystallization from AcOEt and isopropyl ether; yield 46.0 g (98%), mp 54—60 °C, $[\alpha]_D^{20}$ −6.11° (c=0.4, MeOH), Rf_3 0.60. *Anal.* Calcd for $C_{35}H_{52}N_6O_9S \cdot H_2O \cdot 1/4$AcOEt: C, 56.94; H, 7.23; N, 11.07. Found: C, 57.03; H, 7.19; N, 11.10.

Boc–Tyr(Cl_2Bzl)–Arg(Mts)–Lys(Z)–OMe An N^{α}-deprotected sample of the above dipeptide (30.00 g, 40.9 mmol) was dissolved in DMF (200 ml), then Boc–Tyr(Cl_2Bzl)–OH (18.05 g, 40.9 mmol), HOBt·H_2O (6.25 g, 40.9 mmol), WSCD (7.12 ml, 40.9 mmol) and TEA (5.70 ml, 40.9 mmol) were added and the mixture was stirred overnight. The product was purified by procedure A, followed by column chromatography on silica (Kieselgel 60-H, Merck) using AcOEt–$CHCl_3$ (4 : 1) as an eluant; yield 32.0 g (74%), mp 90—91 °C, $[\alpha]_D^{20}$ −0.80° (c=0.5, MeOH), Rf_1 0.41. *Anal.* Calcd for $C_{51}H_{65}Cl_2N_7O_{11}S$: C, 58.05; H, 6.21; N, 9.29. Found: C, 58.08; H, 6.46; N, 9.03.

Z(OMe)–Cys(MBzl)–Tyr(Cl_2Bzl)–Arg(Mts)–Lys(Z)–OMe An N^{α}-deprotected sample of the above tripeptide (30.00 g, 28.43 mmol) was dissolved in DMF (200 ml), then Z(OMe)–Cys(MBzl)–OH (12.68 g, 31.3 mmol), HOBt·H_2O (4.78 g, 31.3 mmol), WSCD (5.44 ml, 31.3 mmol) and TEA (1.98 ml, 14.3 mmol) were added and the mixture was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF and AcOEt; yield 24.0 g (63%), mp 138—139 °C, $[\alpha]_D^{20}$ −13.75° (c=0.5, DMF), Rf_1 0.32. *Anal.* Calcd for $C_{66}H_{78}Cl_2N_8O_{14}S_2 \cdot H_2O$: C, 58.27; H, 5.93; N, 8.24. Found: C, 58.30; H, 5.83; N, 8.50.

Z(OMe)–Cys(MBzl)–Tyr(Cl_2Bzl)–Arg(Mts)–Lys(Z)–$NHNH_2$ [4] The above protected tetrapeptide ester (24.00 g, 17.9 mmol) in DMF (50 ml) was treated with hydrazine hydrate (8.69 ml, 10 eq) overnight. The product was precipitated from DMF with H_2O (400 ml), and then washed with H_2O, followed by recrystallization from DMF and Et_2O; yield 23.6 g (98%), mp 190—191 °C, $[\alpha]_D^{20}$ −12.89° (c=0.5, DMF), Rf_2 0.80. *Anal.* Calcd for $C_{65}H_{78}Cl_2N_{10}O_{13}S_2$: C, 58.16; H, 5.86; N, 10.43. Found: C, 57.97; H, 5.99; N, 10.44.

Z(OMe)–Arg(Mts)–Arg(Mts)–$NHNH_2$ [6] A mixed anhydride [prepared from 11.94 g (23.0 mmol) of Z(OMe)–Arg(Mts)–OH] in AcOEt (100 ml) was added to an ice-chilled solution of an N^{α}-deprotected sample of Z(OMe)–Arg(Mts)–OMe (12.27 g, 23.0 mmol) in DMF (100 ml) containing TEA (3.19 ml, 1 eq), then the solution was stirred overnight. The product was purified by procedure A. The concentrated protected dipeptide ester in MeOH (150 ml) was treated with hydrazine hydrate (5.97 ml, 5 eq) overnight. The product was purified by procedure A, followed by recrystallization from AcOEt and Et_2O; yield 16.77 g (84%), mp 121—127 °C, $[\alpha]_D^{20}$ −1.56° (c=0.4, MeOH), Rf_3 0.66. *Anal.* Calcd for $C_{39}H_{56}N_{10}O_9S_2 \cdot H_2O$: C, 52.67; H, 6.56; N, 15.72. Found: C, 52.65; H, 6.61; N, 15.98.

Synthesis of Protected T22 Successive azide or WSCD–HOBt condensations of the four fragments and two amino acid derivatives were

TABLE I. Characterization of the Protected T22 and Its Intermediates

	Yield (%)	Rf_3	mp (°C)	$[\alpha]_D^{20}$ (°) (c=0.3, DMF)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N
Z(OMe)–(9—18)–NH_2	99	0.59	230—233	−15.20	$C_{139}H_{159}Cl_6N_{19}O_{26}S_4$	58.52	5.62	9.33	58.49	5.59	9.23
Z(OMe)–(8—18)–NH_2	98	0.64	252—255	−16.44	$C_{150}H_{172}Cl_6N_{20}O_{28}S_5$	58.57	5.64	9.11	58.37	5.80	9.39
Z(OMe)–(4—18)–NH_2	92	0.92	260—270	−10.97	$C_{206}H_{238}Cl_8N_{28}O_{38}S_7 \cdot 3H_2O$	57.86	5.75	9.17	57.74	5.84	9.17
Z(OMe)–(3—18)–NH_2	85	0.55	273—277	−14.32	$C_{226}H_{258}Cl_8N_{30}O_{41}S_8 \cdot H_2O$	58.90	5.69	9.12	58.75	5.73	9.12
Z(OMe)–(1—18)–NH_2	88	0.35	269—272	−7.16	$C_{256}H_{302}Cl_8N_{38}O_{47}S_{10}$	58.37	5.78	10.10	58.10	5.75	10.08

carried out according to the indicated route (Fig. 1). Prior to condensation, the Z(OMe) group was removed from the respective amino component. An N^{α}-deprotected sample was dissolved in DMF or DMF–NMP (1 : 1 for condensations of [2], [4], and [6]) containing TEA (1 eq). The corresponding azide of [2], [4], and [6] (the amount was from 1.2 to 1.7 eq as chain elongation progressed) in DMF or NMP (for [4]) and TEA (1 eq to peptide azide) were added to the above ice-chilled solution and the mixture was stirred at 4 °C until the solution became negative to the ninhydrin test. H_2O was added and the resulting powder was purified by procedure B, followed by precipitation from DMF with MeOH. The amino acid derivative of [3] (or [5]) (1.5 eq), HOBt (1.5 eq), WSCD (1.5 eq) and TEA (0.5 eq) were added to the above solution of the corresponding N^{α}-deprotected peptide and the mixture was stirred at 4 °C. The product was purified by procedure B, followed by recrystallization from DMF and MeOH. Yields, physical constants and analytical data of the protected T22 and its protected intermediates are listed in Table I. The yield of the protected T22 was 19.30 g.

Deprotection of the Protected T22 by 1 M TMSOTf–Thioanisole/TFA for the Preparation of T22 and Isolation of the By-product Protected T22 (309 mg, 58.7 μmol) was treated with 1 M TMSOTf–thioanisole in TFA (20.7 ml)–*m*-cresol (1.5 ml, 250 eq)–EDT (600 μl, 125 eq) in an ice-bath for 2 h, then dry Et_2O was added. The resulting powder was collected by centrifugation. After being washed three times with dry Et_2O, the product was dissolved in 50% AcOH (5 ml). The total volume of solution was brought to 500 ml with H_2O and then its pH was adjusted to 8 with concentrated NH_4OH. The HPLC elution pattern of the solution after a 2-day treatment is shown in Fig. 3. Two major peaks (retention time 16 min (T22) and 24 min (by-product)) were observed. The pH of the solution was adjusted to 5 with AcOH followed by the addition of Diaion HP-20 resin (*ca.* 10 g). The mixture was stirred for 1 h, and the resin was collected by filtration. Peptides were eluted from the the resin with 80% CH_3CN in 1 M AcOH (100 ml). The solvent was removed by evaporation and lyophilization. Each major product was purified by HPLC; [T22] yield 22.0 mg (12% based on the protected T22). Amino acid ratios after 6 M HCl hydrolysis and LAP digestion (numbers in parentheses): Gly × 1, 1.13 (0.89), cystine × 2, N.D. (not determined) (1.52), Tyr × 4, 3.98 (3.74), Lys × 3, 3.06 (2.84), Trp × 1, N.D. (1.00), Arg × 5, 5.00 (5.62). Ion spray mass (reconstructed) *m/z*: 2485.6 (Calcd for $C_{109}H_{164}N_{38}O_{22}S_4$: 2485.2), ion spray mass of peptide fragments derived from tryptic digest (reconstructed) *m/z*: 1070.5 (Calcd for $C_{47}H_{66}N_{12}O_{13}S_2$: 1070.4, H–Cys(S-)–Tyr–Lys–OH H–Gly–Tyr–Cys(S-)–Tyr–Arg–OH), 1198.5 (Calcd for $C_{53}H_{78}N_{14}O_{14}S_2$: 1198.5, H–Cys(S-)–Tyr–Lys–OH H–Gly–Tyr–Cys(S-)–Tyr–Arg–Lys–OH), 901.5 (Calcd for $C_{38}H_{55}N_{13}O_9S_2$: 901.4, H–Trp–Cys(S-)–Tyr–Arg–OH H–Cys(S-)–Arg–OH), 1029.5 (Calcd for $C_{44}H_{67}N_{15}O_{10}S_2$: 1029.5, H–Trp–Cys(S-)–Tyr–Arg–Lys–OH H–Cys(S-)–Arg–OH). $[\alpha]_D^{20}$ 13.02° (c=0.1, H_2O). [By-product, [Trp(2′-MBzl)^3]-T22], yield 13.9 mg. Amino acid ratios after 6 M HCl hydrolysis and LAP digestion (numbers in parentheses): Gly × 1, 1.00 (1.00), cystine × 2, N. D. (1.40), Tyr × 4, 3.75 (3.71), Lys × 3, 2.84 (2.81), Trp(2′-MBzl) × 1, N.D. (N.D.), Arg × 5, 4.90 (5.22). Ion spray mass (reconstructed) *m/z*: 2605.7 (Calcd 2605.2 for $C_{117}H_{172}N_{38}O_{23}S_4$), ion spray mass of peptide fragments derived from tryptic digest (reconstructed) *m/z*: 1070.5 (Calcd for $C_{47}H_{66}N_{12}O_{13}S_2$: 1070.4, H–Cys(S-)–Tyr–Lys–OH H–Gly–Tyr–Cys(S-)–Tyr–Arg–OH),1199.0 (Calcd for $C_{53}H_{78}N_{14}O_{14}S_2$: 1198.5, H–Cys(S-)–Tyr–Lys–OH H–Gly–Tyr–Cys(S-)–Tyr–Arg–Lys–OH), 1121.5 (Calcd for $C_{46}H_{63}N_{13}O_{10}S_2$: 1121.4, H–Trp(2′-MBzl)–Cys(S-)–Tyr–Arg–OH H–Cys(S-)–Arg–OH), 1149.0 (Calcd for $C_{52}H_{75}N_{15}O_{11}S_2$: 1149.5, H–Trp(2′-MBzl)–Cys(S-)–Tyr–Arg–Lys–OH H–Cys(S-)–Arg–OH). $[\alpha]_D^{20}$ 10.98° (c=0.1, H_2O).

NMR Spectroscopy of By-product {[Trp(2′-MBzl)^3]-T22} The by-product was dissolved at a 10 mM concentration in either 99.9% D_2O or a mixture of H_2O and D_2O (9 : 1). The pH was adjusted to 3.0 in the NMR tube with μl increments of 0.1 M DCl in D_2O. The spectra of T22 were recorded on a Bruker AM 600 spectrometer at 600 MHz ^1H frequency and at 28 °C. Chemical shifts are given relative to the internal standard 4,4-dimethyl-4-silapentane-1-sulfonate. DQF-COSY,[28] HOHAHA,[29] and NOESY[30] were all recorded in the phase-sensitive mode according to States *et al.*[31] For the H_2O solution sample, the huge water resonance was suppressed by selective saturation using irradiation. The mixing time for the NOESY experiments was set at 250 ms. δ (2′-MBzl in Trp^3, D_2O): 3.77 (3H, s, OMe), 4.08 (2H, s, benzyl H), 7.18 (2H, d, benzyl C2, 6H), 6.87 (2H, d, benzyl C3, 5H). NOEs observed between the protons of the MBzl group and the Trp^3 residue are shown in Fig. 4.

Deprotection of the Protected T22 by AgOTf/TFA + 1 M TMSOTf–Thioanisole/TFA and the Preparation of T22 Protected T22 (200 mg, 38.0 μmol) was treated with AgOTf (1.17 g, 4.56 mmol)/TFA (11 ml)–anisole (200 μl) in an ice-bath for 2 h, then dry Et_2O was added. The resulting powder was collected by centrifugation. After being washed three times with Et_2O, the product was treated with 1 M TMSOTf–thioanisole/TFA (13.8 ml)–*m*-cresol (1.0 ml, 250 eq)–EDT (400 μl, 125 eq) in an ice-bath for 2 h, then dry ether was added. The resulting powder was dissolved in 0.2 M Tris–HCl buffer pH 7.5 containing 6 M guanidine hydrochloride (3.0 ml) followed by incubation with DTT (200 mg, 1.30 mmol) at room temperature overnight. The solution was then applied to a column of Sephadex G-15 (2.1 × 43 cm), which was eluted with 4 M AcOH. The fractions corresponding to the front main peak (monitored by UV adsorption measurement at 280 nm) were collected, then the total volume of solution was brought to 300 ml with H_2O and its pH was adjusted to 8 with concentrated NH_4OH. The HPLC elution pattern of the solution after a 2-day treatment is shown in Fig. 6a. The product was isolated by adsorption on Diaion HP-20 and purified by HPLC as described in the previous section: yield 18.0 mg (19%).

Two-Step Hard Acid Deprotection of the Protected T22 and the Preparation of T22 Protected T22 (1 g, 190 μmol) was treated with 1 M TMSBr–thioanisole/TFA (75 ml)–*m*-cresol (5.0 ml, 250 eq)–EDT (2.0 ml, 125 eq) in an ice-bath for 1 h, and then the solution was concentrated *in vacuo*. Dry Et_2O was added to precipitate the product. The product was treated with 1 M TMSOTf–thioanisole/TFA (69 ml)–*m*-cresol (5.0 ml, 250 eq)–EDT (2.0 ml, 125 eq) in an ice-bath for 1 h, then dry ether was added. The product was dissolved in 50% AcOH (15 ml). The total volume of solution was brought to 1 l with H_2O and its pH was adjusted to 8 with concentrated NH_4OH. The HPLC elution pattern of the solution after a 2-day treatment is shown in Fig. 6b. The product was isolated by adsorption on Diaion HP-20 and purified by HPLC: yield 152 mg (26%).

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References and Notes

1) The amino acids used here are of the L-configuration. The following abbreviations are used: T22 = [Tyr^{5,12}, Lys^7]-polyphemusin II, HIV = human immunodeficiency virus, AZT = 3′-azido-2′,3′-dideoxythymidine, TMSOTf = trimethylsilyl trifluoromethanesulfonate, MBzl = *p*-methoxybenzyl, AgOTf = silver trifluoromethanesulfonate, TMSBr = trimethylsilyl bromide, NMR = nuclear magnetic resonance, Z = benzyloxycarbonyl, Mts = mesitylene-2-sulfo-

nyl, EC_{50} = 50% effective concentration, Cl_2Bzl = 2,6-dichlorobenzyl, WSCD = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, HOBt = 1-hydroxybenzotriazole, Su = *N*-succinimidyl, MA = mixed anhydride, DMF = *N*,*N*-dimethylformamide, NMP = *N*-methylpyrrolidone, TFA = trifluoroacetic acid, LAP = leucine aminopeptidase, NOE = nuclear Overhauser effect, DTT = dithiothreitol, EDT = 1,2-ethanedithiol, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, TEA = triethylamine, CHA = cyclohexylamine, MZ-SDP = S-*p*-methoxybenzyloxycarbonyl-4,6-dimethyl-2-mercaptopyrimidine, DQF-COSY = double quantum filtered correlation spectroscopy, HOHAHA = homonuclear Hartmann-Hahn spectroscopy, NOESY = nuclear Overhauser effect spectroscopy, Tris = tris(hydroxymethyl)aminomethane.

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