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Synthesis of Fluorescent Peptides and Their Phosphorylation by the Catalytic Subunit of cyclicAMP-dependent Protein Kinase†

Hiroki KONDO,* Katsunori TAKAKI, Ryoichi KUROKI, Akihiro TADA, Kunihiro FUKUMOTO, and Junzo SUNAMOTO

Department of Industrial Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 852

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The following two fluorescent peptides were prepared as substrates of the cyclicAMP-dependent protein kinase reaction: Leu-Arg-Arg-Ala-Ser-Leu-X; **1** (X= 2-(dansylamino)ethylamino or DAE) and **2** (X=4-methyl-2-oxo-7-chromenylamino or MCA). Both peptides were phosphorylated by ATP and the catalytic subunit of cyclicAMP-dependent protein kinase from bovine heart in a stoichiometric manner. Phosphorylation of peptide **1** is accompanied by a 10% increase in the fluorescence intensity at 550 nm. This enables one to assay peptide phosphorylation by fluorescence spectroscopy. The kinetic parameters obtained by this and the conventional phosphocellulose paper method for this phosphorylation were the following: K_m 7.8 μM and V_{max} 35 μmol·min^{-1}·mg^{-1} for **1**, and K_m 7.4 μM and V_{max} 16 μmol·min^{-1}·mg^{-1} for **2**.

Phosphorylation and dephosphorylation of proteins are one of the major mechanisms by which functions of proteins are regulated in biological systems.[1,2] A number of protein kinases, which mediate protein phosphorylation by ATP, are now known, but despite their importance in cellular regulation the detailed mechanism of enzymatic phosphoryl transfer reactions remains largely obscure. cAMP-dependent protein kinase is one of the most ubiquitous protein kinases[3] and is the only protein kinase whose primary structure has been reported.[4-6] This enzyme recognizes the two successive basic amino acid residues placed N-terminal to the phosphorylatable Ser or Thr of a protein.[7-9] Model peptides such as **3** that bears this feature of the amino acid sequence serve as good a substrate as do the natural protein substrates.[8] This fact as well as

Leu-Arg-Arg-Ala-Ser-Leu-Gly

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the known primary sequence of the protein makes this enzyme system suitable for mechanistic studies. We have undertaken a study of enzyme-substrate interaction by use of fluorescent peptides and fluorescence spectroscopy. Besides mechanistic studies, such peptides are useful for assaying peptide phosphorylation spectroscopically and continuously. To date a few peptides bearing a fluorophore or chromophore at the N-terminus or in the middle of the sequence of **3** have been reported.[10-12] Unfortunately, however, a large spectral change was not observed before and after phosphorylation of the peptide and in the presence or absence of enzyme. In this aritcle we have attempted a C-terminal modification of peptide **3** with a fluorophore to examine if the resulting peptide is a sensitive probe of phosphorylation and/or the polarity of the enzyme active site.

† Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; TFA, trifluoroacetic acid; Boc, *t*-butoxycarbonyl; Bzl, benzyl (ester); DCUrea, dicyclohexylurea; NEM, *N*-ethylmorpholine; Mops, 4-morpholinepropanesulfonic acid; EGTA, ethyene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The abbreviations used for amino acids follow the nomenclature recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.*, **138**, 9 (1984)).

Results and Discussion

Synthesis. The synthesis of peptides **1** and **2** has been accomplished by the route illustrated in Fig. 1. The peptides were bisected between Ala and Ser. The resulting two fragments were prepared separately and then combined at the late stage of the synthesis. The C-terminal fragment which contains the fluorophore was assembled by a stepwise elongation of the sequence from the C terminus. The N-terminal fragment was further divided into two dipeptides mainly for synthetic reasons. It was first attempted to synthesize the tetrapeptide also by the stepwise elongation method, but a difficulty was encountered at the synthesis of tripeptide Boc-Arg(NO_2)-Arg(NO_2)-Ala-OMe(**4**). A coupling of Boc-Arg(NO_2)-OH with H-Arg(NO_2)-Ala-OMe by the DCC method or the mixed anhydride method with ethyl chloroformate gave impure **4** in a low yield (<30%, depending on the coupling method). The poor solubility of **4** in most common solvents except MeOH hampered purification by recrystallization or chromatograpy. In one case, tetrapeptide

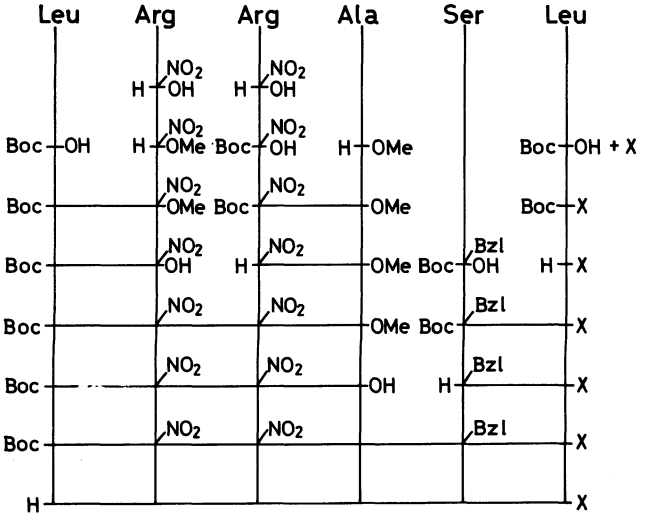


Fig. 1. Synthetic route to peptides **1** and **2** (X=DAE or MCA).

TABLE 1. ANALYTICAL DATA FOR PEPTIDES **1** AND **2**

Peptide	TLC R_f		Paper electrophoresis[b]
	Solvent[a] A	B	F(pH 3.6)
1	0.49	0.23	0.85
2	0.47	0.40	0.88

a) For solvents see text. b) Mobility relative to Arg.

Boc-Leu-Arg(NO_2)-Arg(NO_2)-Ala-OMe was prepared by coupling of Boc-Leu-OH with H-Arg(NO_2)-Arg-(NO_2)-Ala-OMe, derived from partially purified **4**, by the DCC-HOBt method. The product purified by recrystallization from CH_3CN-ether was analytically pure and identical within experimental error in optical rotations with the peptide obtained by the fragment condensation of Boc-Leu-Arg(NO_2)-OH and H-Arg-(NO_2)-Ala-OMe; the former product, $[\alpha]_D^{26}$ −26.8° (*c* 1.0, MeOH); the latter peptide, $[\alpha]_D^{27}$ −27.7° (*c* 1.0, MeOH). This suggests that racemization of Arg has been suppressed in the DCC fragment condensation of dipeptides in the presence of HOBt.

A final condensation of the N-terminal tetrapeptide with the C-terminal fragment by either EEDQ or DCC-HOBt method afforded fully protected hexapeptides with the fluorophore at their C-termini in moderate yields (50—70%). The peptides were subjected to HF cleavage followed by chromatography on Sephadex G-10 and CM-cellulose. The purified peptides were characterized by various analytical techniques such as TLC and ^1H NMR. The data obtained were consistent with the proposed structure for the peptides (Table 1). Peptides **1** and **2** exhibit absorption maxima at 333 and 327 nm, respectively, and when excited at their respective absorption maximum wavelength, they emit strong fluorescence with maxima at 550 and 393 nm. This observation and the ready phosphorylation by the enzyme provide final proof of the identity of both peptides.

Fluorescence Assay of Phosphorylation. Both peptides **1** and **2** are phosphorylated by ATP and the catalytic subunit of cAMP-dependent protein kinase from bovine heart. Phosphorylation of peptide **1** gives rise to a slight blue shift (4 nm) of the emission maximum and a 10% increase in the fluorescence intensity (Fig. 2), while no discernible change was observed in the fluorescence spectrum of **2** before and after phosphorylation (data not shown). The magnitude of intensity change in **1** is half of that observed for the peptide bearing a Trp residue just N-terminal to the phosphorylatable Ser.[12] Peptide phosphorylation can be assayed by taking advantage of the spectral change in **1** as well as by the conventional phosphocellulose paper method.[13] As the time course experiment given in Fig. 3 demonstrates, phosphorylation of **1** in the presence of a saturating concentration of ATP, *i.e.*, about fifty times the $K_{m,ATP}$,[14] is rapid and stoichiometric; 0.96±0.03 mol of phosphate is incorporated to **1** and **2** in ≈10 min. For identification of the phosphorylated peptides, the reaction mixture was subjected to high voltage paper electrophoresis at pH 3.6. As the reaction progresses, a spot migrating toward the cathode more slowly than

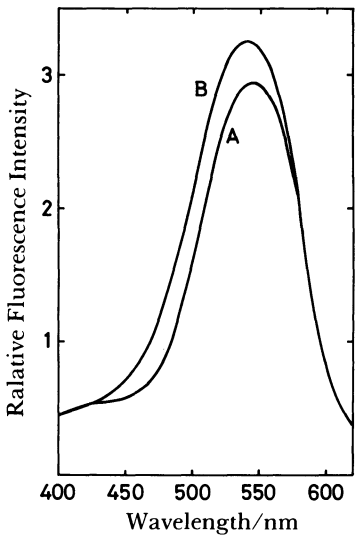


Fig. 2. Fluorescence spectra of peptide **1** in 50 mM Mops, pH 7.0, before (A) and after phosphorylation (B).

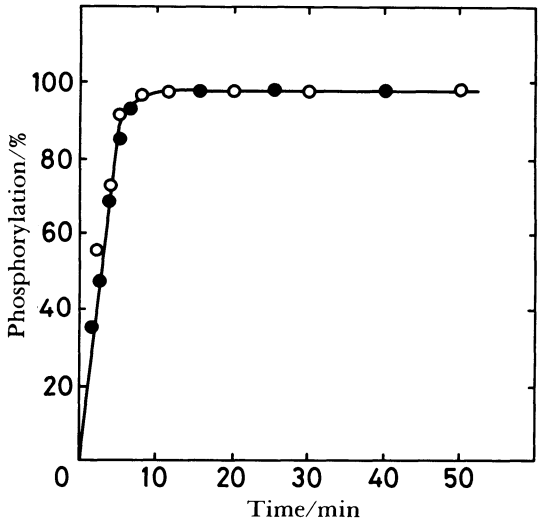


Fig. 3. Time course of peptide **1** phosphorylation at 30°C.
The extent of reaction was determined by the fluorescence (○) or phosphocellulose paper method (●). The assay mixture consisted of 250 μM [γ-^{32}P]ATP, 12.5 mM magnesium acetate, 0.25 mM EGTA, 140 μM **1**, 0.1 mg/ml BSA, and 1.8 μg/ml enzyme in 50 mM Mops, pH 7.0.

the unphosphorylated peptide becomes visible (data not shown). This is as expected, since phosphorylation introduces 1—2 negative charges into substrate.

Initial rate data of peptide phosphorylation at a low enzyme concentration were analyzed in terms of Henri-Michaelis-Menten formulation.[15] The results are summarized in Table 2. The kinetic parameters for **1** obtained by the fluorescence assay are identical within experimental error with those determined by the phosphocellulose paper method; *e.g.*, K_m was 7.5 μM for the former and 7.8 μM for the latter. The K_m values for **1** and **2** are smaller than that of peptide **3**. This result is consistent with the previous observation that a hydrophobic substituent placed C-terminal to the

Table 2. Kinetic parameters for phosphorylation of peptides **1** and **2** by ATP and the catalytic subunit of cAMP-dependent protein kinase at pH 7.0 and 30 °C

Peptide	K_m/μM	V_{max}/μmol·min^{-1}·mg^{-1}	Reference
1	7.8	35	This work
2	7.4	16	This work
3	16	20	8

phosphorylatable Ser increases the binding ability of peptide substrate to the enzyme.[16] In addition to K_m, the catalytic rates (V_{max}) are high and comparable with those of peptide **3** and protein substrates. This excellent substrate activity of peptides **1** and **2** makes them useful in mechanistic studies of enzyme-substrate interaction by, for example, fluorescence spectroscopy.

Experimental

Materials. L-Amino acids were obtained from Protein Research Foundation or Wako Pure Chemical Ind. Co., both Osaka. DCC, HOBt, and EEDQ were purchased from the former source, while TFA and dansyl chloride were from the latter. Sephadex G-10 and CM-cellulose were obtained from Pharmacia and Brown, respectively. Carrier-free [^{32}P] inorganic phosphate was the product of Japan Atomic Energy Research Institute. Scintillation reagents 2,5-diphenyloxazole (PPO) and 2,2′-(*p*-phenylene)bis[5-phenyloxazole] (POPOP) were obtained from Nakarai Chemical Ltd., Kyoto. 4-Methyl-7-aminocoumarin was prepared according to the literature,[17] whereas 2-(dansylamino)ethylamine was synthesized by the route illustrated in the Scheme. [γ-^{32}P]ATP of specific radioactivity 20—200 cpm/pmol was prepared by the method of Glynn and Chappell.[18]

Apparatus. Electronic absorption and fluorescence spectra were taken on a Shimadzu UV-150 and Hitachi 650-10S spectrophotometer, respectively. ^{1}H NMR spectra were recorded on a Jeol JNM-MH-100 spectrometer. Optical rotations were read on a Jasco DIP-4. Paper electrophoresis was run with a Toyo HPE-406 apparatus. Radioactivity was determined on an Aloka liquid scintillation counter LSC-703.

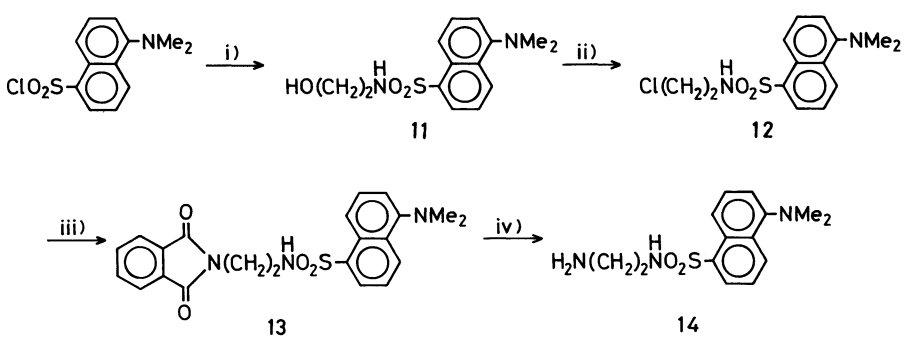
Miscellaneous. TLC was run on a silica-gel plate in the following solvent systems: (A) AcOH: Pyridine: *n*-BuOH: H_2O=1:1:4:2 (B) AcOH:*n*-BuOH:H_2O=4:2:1 (C) MeOH (D) $CHCl_3$:MeOH=5:1 (E) $CHCl_3$:MeOH:benzene=5:1:1. Paper chromatography was carried out on a Toyo Roshi filter paper No.51 in one of the solvents described above. High-voltage paper electrophoresis was run on the same filter paper in AcOH:Pyridine:H_2O=10:1:99 (solvent F) at pH 3.6 and 2,500 V for ≈2 h. Peptide concentrations were determined spectroscopically by using ε_{329} 4780 of *N*-ethyl-5-dimethylamino-1-naphthalenesulfonamide[19] for **1** and ε_{324} 26800 of Leu-MCA·HCl[20] for **2**.

Enzyme Preparation and Assay. The catalytic subunit of bovine heart cAMP-dependent protein kinase was prepared by the method of Demaille *et al.*[16] The preparation gave a single band on SDS-PAGE that corresponds to a molecular weight of ≈41000.[4] The enzyme concentration was determined on the basis of $A_{280}^{1\%}$14.9.[16] The enzyme activity was assayed by the phosphocellulose paper method of Witt and Roskoski[13] with a slight modification.[21,22] The components of an assay solution and the assay conditions are given in the caption of Fig. 3. The radioactivity associated with the peptide was counted with 5 ml of a toluene solution of 4 g/l PPO and 0.1 g/l POPOP.

Boc-Arg(NO$_2$)-Ala-OMe (5). A mixture of Boc-Arg(NO_2)-OH (9.6 g, 30 mmol), HOBt (4.1 g, 30 mmol), and DCC (6.2 g, 30 mmol) in CH_3CN (50 ml)–DMF (20 ml) was stirred at −5 °C for 20 min. A pre-cooled solution of H-Ala-OMe·HCl (4.2 g, 30 mmol) and NEM (3.8 ml, 30 mmol) in CH_2Cl_2 (50 ml) was added and the whole mixture was stirred at −5 °C for 1 h and then at room temperature overnight. The precipitate formed (DCUrea) was removed by filtration. The filtrate was kept in a refrigerator for 4 h and DCUrea was filtered off again. Evaporation of the solvent *in vacuo* gave a semi-solid. It was taken up in AcOEt (300 ml) and the insoluble material was removed by filtration. The filtrate was washed with 70 ml each of the following ice-cold solutions three times; 4% $NaHCO_3$, aq saturated NaCl, 10% citric acid, aq saturated NaCl. After drying over Na_2SO_4, the solvent was evaporated *in vacuo* to yield a solid. It was recrystallized from CH_3CN–Et_2O (7.0 g, 58%), mp. 149.5—151 °C. Found: C, 44.66; H, 7.11; N, 20.63%. Calcd for $C_{15}H_{28}N_6O_7$: C, 44.55; H, 6.98; N, 20.78%. Use of 1.5-equiv DCC and HOBt increased the yield to 62%.

Boc-Leu-Arg (NO$_2$)-OMe (6). To a stirred solution of Boc-Leu-OH·H_2O (7.5 g, 30 mmol) and NEM (3.8 ml, 30 mmol) in THF (40 ml) chilled at −15 °C was added isobutyl chloroformate (4.0 ml, 30 mmol). Ten min later, a pre-cooled solution of H-Arg(NO_2)-OMe·HCl (8.1 g, 30 mmol) and NEM (3.8 ml, 30 mmol) in CH_2Cl_2 (40 ml)–DMF (50 ml) was added. The mixture was stirred at −15 °C for 2 h and then at room temperature overnight. The precipitate formed was filtered off and washed with CH_2Cl_2. The solvents were evaporated *in vacuo*. The resulting syrup was taken up in 500 ml of AcOEt. The solution was washed and worked up essentially as described above. The crude product was recrystallized from aqueous ethanol (12.1 g, 90%), mp 146—148 °C. Found: C, 48.36; H, 7.82; N, 18.95%. Calcd for $C_{18}H_{34}N_6O_7$: C, 48.42; H, 7.67; N, 18.82%.

Boc-Leu-Arg(NO$_2$)-Arg(NO$_2$)-Ala-OMe(7). A sample of **6** (4.2 g, 10 mmol) was treated with 20 g of TFA (175 mmol) in 35 ml of CH_2Cl_2 at room temperature for 40 min. The solvent and excess TFA were evaporated *in vacuo*. The residual syrup was taken up in CH_2Cl_2 and the solvent was



Scheme. Reagents: i, $HO(CH_2)_2NH_2$; ii, $SOCl_2$; iii, potassium phthalimide; iv, H_2NNH_2.

evaporated *in vacuo*. This procedure was repeated until constant weight was reached for H-Arg(NO_2)-Ala-OMe (**8**)·2TFA. Boc-Leu-Arg(NO_2)-OH (**9**) was preapred by saponification of **6** in the way identical with that described for **10** below. A mixture of **8**·2TFA (1.5 g, 2.8 mmol), **9** (1.2 g, 2.8 mmol), NEM (0.65 g, 5.6 mmol), HOBt (0.38 g, 2.8 mmol), and DCC (0.58 g, 2.8 mmol) in 30 ml of THF was allowed to react at 0 °C for 2 h and then at room temperature overnight. The precipitate formed was filtered off. The filtrate was concentrated to dryness. The residue was taken up as much as possible in 1 l each of AcOEt. The insoluble material was extracted three more times with 1 l each of AcOEt. The extract was washed and worked up as described above for **5**. The product was recrystallized from CH_3CN-Et_2O (1.9 g, 93%), mp 140—142 °C. TLC R_f 0.69 (C).

Boc-Leu-Arg(NO_2)-Arg(NO_2)-Ala-OH (10). A sample of **7** (0.72 g, 1.0 mmol) in 20 ml of MeOH was allowed to react with 1.7 ml of 1 M NaOH at room temperature for 7 h. The solution was diluted with 20 ml of water and MeOH was evaporated *in vacuo*. After washing with Et_2O, the aqueous solution was chilled in ice and the pH was brought to 3—4 with ice-cold 10% citric acid. The precipitate formed was collected by filtration and recrystallized from MeOH-Et_2O (0.69 g, 98%), mp 205—208 °C. TLC R_f 0.53 (C). Found: C, 43.31; H, 6.90; N, 23.01%. Calcd for $C_{26}H_{48}N_{21}O_{11}\cdot H_2O$: C, 43.21; H, 6.97; N, 23.26%.

2-(5-Dimethylamino-1-naphthalenesulfonamido)ethanol (11) Dansyl chloride (2.0 g, 7.4 mmol) was added dropwise to a 33% aqueous 2-aminoethanol solution (2 ml). The mixture was heated at 80 °C until the dansyl chloride disappeared as inspected by TLC. The solution was made weakly acidic (pH 3—4) and the product was extracted with AcOEt. The extract was dried over Na_2SO_4 and then concentrated to dryness. The crude product obtained was recrystallized from ether-petroleum ether (2.10 g, 96%), mp 102.5—104 °C. TLC R_f 0.71 (D), 0.55 (E). Found: C, 56.66; H, 6.21; N, 9.23%. Calcd for $C_{14}H_{18}N_2O_3S$: C, 57.12; H, 6.16; N, 9.52%.

1-Chloro-2-(5-dimethylamino-1-naphthalenesulfonamido)ethane (12). To a solution of **11** (0.42 g, 1.4 mmol) in chloroform (30 ml) was added dropwise 1.7 g (14 mmol) of thionyl chloride in chloroform (25 ml). The mixture was refluxed for 4 h. The excess reagent and solvent were evaporated *in vacuo* to leave a solid, which was taken up in water. The solution was made weakly alkaline and the precipitate formed was extracted with AcOEt and treated as above for **11** (yield 95%), mp 87—90 °C. TLC R_f 0.75 (E).

N-[(5-Dimethylamino-1-naphthalenesulfonamido)ethyl]phthalimide (13). A mixture of **12** (0.42 g, 1.3 mmol) and potassium phthalimide (0.29 g, 1.5 mmol) in 30 ml of DMF was stirred at 100 °C for 3 h. The solution was mixed with 200 ml of 0.1 M NaOH (1 M=1 mol dm^{-3}) and extracted with 50 ml each of chloroform four times. The extract was washed with 0.2 M NaOH and water, and dried over Na_2SO_4. The following work-up procedures were the same as that for **11**. The product was obtained in 76% yield, mp 160—165 °C. TLC R_f 0.81 (D).

2-(5-Dimethylamino-1-naphthalenesulfonamido)ethylamine (14). A methanolic solution (25 ml) of **13** (0.43 g, 1.0 mmol) and hydrazine hydrate (0.10 g, 2.0 mmol) was refluxed for 2 h. The mixture was diluted with water (25 ml) and methanol was removed *in vacuo*. The turbid solution left was heated with conc. HCl for 2 h. The solution was made alkaline and the precipitate formed was extracted with AcOEt. The product was obtained in 72% yield by the usual work-up described above, mp 155—157 °C. TLC R_f 0.10 (E). Found: C, 57.24; H, 6.53; N, 13.71%. Calcd for $C_{14}H_{19}N_3O_2S$: C, 57.32; H, 6.53; N, 14.32%.

Boc-Leu-DAE (15). A mixture of **14** (0.58 g, 2.0 mmol), Boc-Leu-OH·H_2O (0.55 g, 2.2 mmol), HOBt (0.30 g, 2.2 mmol), NEM (2.2 mmol), and DCC (0.45 g, 2.2 mmol) in 25 ml of CH_2Cl_2 was stirred at 0 °C for 2 h and then at room temperature overnight. The precipitated DCUrea was filtered off. The filtrate was concentrated, diluted with 200 ml of AcOEt and washed as described earlier for **5**. After drying over Na_2SO_4, the solution was concentrated to dryness, 0.94 g (94%), mp 63—64.5 °C. TLC R_f 0.53 (E). Found: C, 59.11; H, 7.63; N, 10.47%. Calcd for $C_{25}H_{38}N_4O_5S$: C, 59.26; H, 7.56; N, 11.06%.

Boc-Ser(Bzl)-Leu-DAE (17). H-Leu-DAE (**16**)·3TFA was prepared from **15** as described for **8**. The reaction of **16**·3TFA (1.25 g, 1.7 mmol) with Boc-Ser(Bzl)-OH (0.54 g, 1.8 mmol) in the presence of NEM (0.58 g, 5.0 mmol), HOBt (0.25 g, 1.8 mmol), and DCC (0.38 g, 1.8 mmol) in CH_2Cl_2 (5 ml) and AcOEt (25 ml) and the usual work-up of the reaction mixture gave 1.1 g (94%) of **17** (AcOEt-petroleum ether), mp 68—70 °C. TLC R_f 0.63 (E). Found: C, 60.94; H, 7.25; N, 10.22%. Calcd for $C_{35}H_{49}N_5O_7S$: C, 61.47; H, 7.22; N, 10.24%.

Boc-Leu-Arg(NO_2)-Arg(NO_2)-Ala-Ser(Bzl)-Leu-DAE (19). H-Ser(Bzl)-Leu-DAE (**18**)·3TFA was prepared from **17** as described for **8**. A mixture of **18**·3TFA (1.3 g, 1.4 mmol), **10** (1.1 g, 1.5 mmol), NEM (0.49 g, 4.2 mmol), DCC (0.31 g, 1.5 mmol), and HOBt (0.21 g, 1.5 mmol) was allowed to react at 2 °C for 2 h and then at room temperature overnight. The precipitate formed was filtered off. The filtrate was kept in a refrigerator for 4 h to ensure precipitation of DCUrea, which was again removed by filtration. The filtrate was concentrated to a small volume and the product was precipitated with 100 ml of AcOEt in 52% yield (0.93 g), mp 211—212 °C. TLC R_f 0.31 (E).

Boc-Ser(Bzl)-Leu-MCA (21). H-Leu-MCA (**20**) was prepared by the deprotection of Boc-Leu-MCA[20] with TFA in CH_2Cl_2. A mixture of **20**·TFA (1.37 g, 3.4 mmol), Boc-Ser(Bzl)-OH (1.0 g, 3.4 mmol), NEM (0.43 ml, 3.4 mmol), and EEDQ (0.84 g, 3.4 mmol) in DMF (5.5 ml) was stirred at room temperature for 12 h. Following evaporation of the solvent, the residue was taken up in AcOEt and washed with acid and alkaline solutions as above to give 1.5 g of the product in 78% yield. Recrystallized from MeOH-ether, mp 152—154 °C. Found: C, 65.25; H, 7.25; N, 7.30%. Calcd for $C_{31}H_{39}N_3O_7\cdot 0.5\ H_2O$: C, 64.79; H, 7.02; N, 7.31%.

Boc-Leu-Arg(NO_2)-Arg(NO_2)-Ala-Ser(Bzl)-Leu-MCA (23). H-Ser(Bzl)-Leu-MCA (**22**)·TFA was prepared from **21** as above. An equimolar mixture (1.9 mmol) of **10**, **22**·TFA, and NEM was allowed to react with EEDQ in DMF at room temperature for 12 h. The solvent was evaporated *in vacuo* and the product was precipitated with EtOAc in 70% yield. Recrystallized from MeOH-ether, mp 176—178 °C. TLC R_f 0.36 (E).

Peptide 2. A sample of **23** (550 mg) was treated with 20 ml of HF and 8 ml of anisole at 0 °C for 50 min. The HF was removed *in vacuo* and the residue was taken up in 10% AcOH. Anisole was removed by extraction with 10 ml each of AcOEt three times. The aqueous phase was concentrated to *ca.* 5 ml and then applied to a column of Sephadex G-10 (3.2×56 cm). The column was eluted with 1% AcOH and the peak fractions were pooled and lyophilized. The peptide was further purified by chromatography on a column of CM-cellulose (1.8×24 cm) with a linear gradient elution with 25 mM ammonium acetate, pH 5.0, and 700 mM ammonium acetate, pH 6.0. The desired fractions were combined, concentrated to *ca.* 10 ml, and then applied to the same Sephadex G-10 column. The column was first washed with water for desalting and the peptide was eluted with 1% AcOH.

Peptide **1** was obtained also by an analogous treatment of **19**.

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