A New Efficient Method for S–CH_2–S Bond Formation and Its Application to a Djenkolic Acid-Containing Cyclic Enkephalin Analog[1)]

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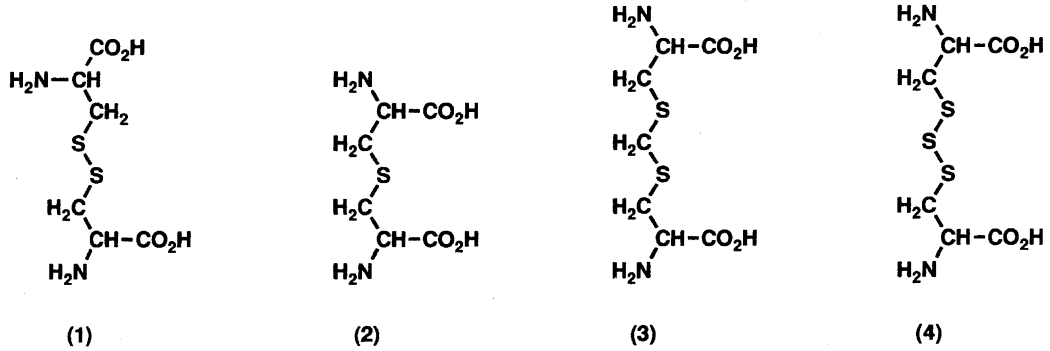
An efficient methylene insertion reaction to construct an S–CH_2–S bridge between two cysteine residues occurred when the thiol-protecting dimethylphosphinothioyl (Mpt) group of Z–Cys(Mpt)–OMe was removed with tetrabutylammonium fluoride hydrate in CH_2Cl_2. The thiol-free form gave similar results, albeit the yields were somewhat lower. In both cases, the best yields were obtained using 2 molar amounts of the reagent. Higher amounts of the reagent reduced the yield because of dehydroalanine formation. In the case of penicillamine, the thiol-free form was better in reactivity than the *S*-Mpt form, which required double the amount of the reagent to give the same yield. The reaction was successfully used in a synthesis of a cyclic enkephalin analog with the S–CH_2–S bridge.

Cyclic structures are included in many biologically active peptides. Conformational restriction of linear peptides by cyclization has also been a general method for improvement of biological activity by increasing receptor selectivity.

Cystine (**1**) has an important role in the construction of the intra- and interchain disulfide bridge structure in many biologically active peptides. Lanthionine (3,3′-thiodialanine, **2**) is also found in nisin and related lantibiotics.[2)] Djenkolic acid (3,3′-(methylenedithio)dialanine, **3**), isolated from the djenkol bean,[3)] has a unique methylenedithioether structure corresponding to a monocarba analog of the bis-cysteine trisulfide (**4**) (Scheme 1). Recently, a trisulfide derivative of the biosynthetic human growth hormone produced in *Escherichia coli* was found.[4)] This finding aroused strong interest in the activities of trisulfide analogs of biologically active peptides.[5,6)] However, the trisulfide bond was not stable at pH 7 and rapidly converted to disulfide at pH 9.[5)] On the other hand, no natural peptide containing **3** has so far been reported. Only synthetically S–CH_2–S bond-forming reactions were used earlier for the modification of wool[7)] and, more recently, for the study of structure-activity relationships of opioid peptides.[8,9)] In the latter work, methylenedithioether-containing analogs were obtained in uncertain yields by treating the free thiol-containing peptides with dibromomethane under strongly basic conditions. Reactions of 1,2-dibromoethane and 1,3-dibromopropane, respectively, suffered from low conversion to give the corresponding dithioether-containing peptides only in 20% yields.[10)] Recently, we found a very mild and efficient method for S–CH_2–S bond formation, which occurs simultaneously when the thiol-protecting dimethylphosphinothioyl (Mpt) group[11)] is removed with tetrabutylammonium fluoride hydrate (TBAF·xH_2O).[12)] This paper describes a detailed study of the new reaction and its application to the synthesis of a cyclic methylenedithioether analog of enkephalin.

Results and Discussion

The Mpt group is a thiol-protecting group suited for the Boc[13)] strategy synthesis of peptides.[11)] Removal of the *S*-Mpt group can be done by heavy metal ion-catalyzed hydrolysis[11)]



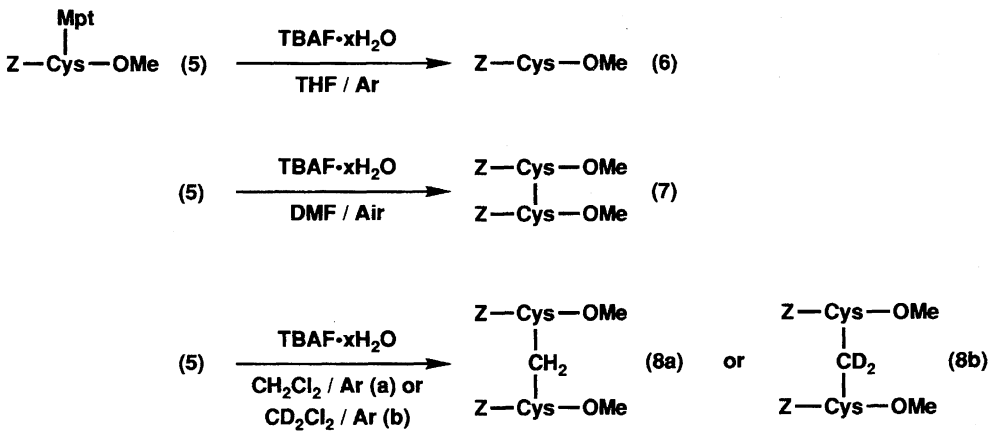
Scheme 1.

and more efficiently by fluorolysis with TBAF·$x$$H_2O$ in anhydrous organic solvents.[12] The fluorolytic removal had already been used for removal of the *S*-diphenylphosphinothioyl (Ppt) group[14,15] by Horner et al.[15] They reported the P–S bond cleavage of Ppt–Cys(Ppt)–OMe with TBAF in abs. chloroform, dichloromethane (DCM) and tetrahydrofuran (THF); however, experimental details on regeneration of Ppt–Cys–OMe were given only for the reaction in THF. We reported that the cleavage of the *S*-Mpt group with TBAF·$x$$H_2O$ in DCM, $CHCl_3$, THF, and acetonitrile was faster than that of the *S*-Ppt group;[12] however, we did not note the variable states of the Cys residue after the fluorolysis. Recently, we precisely investigated the reaction products of Z–Cys(Mpt)–OMe (**5**) with TBAF·$x$$H_2O$ in various solvents and discovered a novel S–CH_2–S bond-forming reaction.[1a]

A reaction of **5** with TBAF·$x$$H_2O$ in THF, especially under an argon atmosphere, regenerated the thiol form, Z–Cys–OMe (**6**) (Scheme 2). In *N*,*N*-dimethylformamide (DMF), oxidation was prone to occur to give the corresponding disulfide **7** as the sole product under the atmosphere of air. Most interestingly, when DCM was used as the solvent, a third product was obtained. The structure of the new product was identified by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry (FAB-MS, m/z = 551.2 $[M+H]^+$) to be a djenkolic acid derivative **8a**. Incorporation of the DCM used as the solvent into the reaction was unambiguously shown by isolation of the two mass number higher product **8b** (FAB-MS, m/z = 553.1 $[M+H]^+$) using CD_2Cl_2 as the solvent. Thus, three modes of deprotection of the *S*-Mpt group to give the thiol, disulfide, and methylenedithioether forms were established.[1a]

The low reactivity of dichloromethane in bimolecular nucleophilic substitution reactions is generally known. Contrary to this, the above methylene insertion reaction was very fast and selective to give the methylenedithioether as the major product. The simultaneous formation of $(CH_3)_2P(S)$–F was ascertained by ^{31}P NMR spectroscopy (δ = 121.3 ppm relative to the external H_3PO_4, d, J_{PF} = 990 Hz). This fact suggested that the initial step of the reaction was an attack of the fluoride ion on the phosphorus atom. This attack generates an unsolvated thiolate ion, which would be reactive enough to attack, in turn, the carbon atom of CH_2Cl_2. Such mechanistic considerations prompted us to investigate the possibility for other polyhaloalkanes to incorporate into the reactions. Table 1 shows the results of reactions of **5** with TBAF·$x$$H_2O$ using various polyhaloalkanes as the solvent. However, new alkylidene and alkylene insertion reactions were not observed. Only dibromomethane (Entry 8) gave a mixture of products containing **8a**; however, its isolation in the pure state was apparently not easy. In $CHCl_3$, CH_3CHCl_2, and $Cl_2CHCHCl_2$ (Entries 3, 10, and 11), thiol **6** was obtained as the major product together with a small amount of the disulfide **7**. When CCl_4 (Entry 4) was used, quantitative oxidation occurred to give **7**. Fluoromethanes (Entries 5, 6, and 7) also gave the disulfide **7** as the major product. On the other hand, primary vicinal dihalides (Entries 9 and 12) gave only monosubstituted products. Therefore, the new type of insertion reaction is limited so far to the methylene insertion reaction using CH_2Cl_2. The concentration of **5** had no effect on the yields (Entries 1 and 2). This fact suggested the possibility of the new methylene insertion reaction for use in intramolecular cyclization of peptides.

The new S–CH_2–S bond-forming reaction was discovered using the cysteine derivative **5** with the Mpt group as the thiol-protecting group. However, the real formation of the S–CH_2–S bond would occur after the removal of the *S*-Mpt group with TBAF·$x$$H_2O$. Generation of the same situation would be possible using other compounds with various thiol-protecting groups removable with TBAF·$x$$H_2O$. Based on this consideration, effects of *S*-substituents on the structure and yield of products were then checked for three acyl-type protecting groups, Ppt,[14,15] trichloroacetyl (Tca),[16] and benzyloxycarbonyl (Z),[17] and an alkyl-type acetamidomethyl (Acm)[18] group. The thiol-free form was also added for comparison. As shown in Table 2, among the three acyl groups, Tca (Entry 3) showed the highest reactivity; however, the product was the disulfide **7**. Ppt and thiol-free forms (Entries 2 and 6) were comparable to the *S*-Mpt form in product selectivity; however, yields were somewhat lower and a quarter of the starting material remained unchanged in the case of the thiol-free form. Since an RS^- ion would be generated by the attack of the fluoride ion on the *S*-acyl group, the difference



Scheme 2.

Table 1. Reactions of **5** with TBAF·xH$_2$O in Various Polyhaloalkane Solvents under Argon[a)]

Entry	Polyhaloalkane	Concentration of **5** / mol dm^{-3}	Product (Yield/%)
1	CH_2Cl_2	0.05	**8a** (76) + **6** (18)
2	CH_2Cl_2	0.25	**8a** (77) + **6** (15)
3	$CHCl_3$	0.05	**6** (79) + **7** (20)
4	CCl_4	0.25	**7** (quantitative)
5	CF_2Cl_2[b)]	0.05	**7** (71) + **6** (10)
6	CF_2Br_2[b)]	0.05	**7** (70) + **6** (13)
7	CF_4[b)]	0.05	**7** (43) + **6** (38) + **5** (10)
8	CH_2Br_2	0.25	**8a** (89)[c)]
9	$ClCH_2CH_2Cl$	0.05	Z–Cys(CH_2CH_2Cl)–OMe (92)
10	CH_3CHCl_2	0.25	**6** (68) + **7** (30)
11	$Cl_2CHCHCl_2$	0.25	**6** (83) + **7** (15)
12	$BrCH_2CH_2Br$	0.25	Z–Cys(CH_2CH_2Br)–OMe (62)
13	$Br_2CHCHBr_2$/THF[d)]	0.25	**7** (85)

a) Conditions: Compound **5** : TBAF·xH$_2$O = 1 : 2 (mol/mol) at R.T. for 4 h. b) Ten per cent solution in THF was used. c) Unseparable impurities are included. HPLC content of **8a** was 51%. d) Compound **5** : $Br_2CHCHBr_2$ = 1 : 0.25 (mol/mol).

Table 2. Effects of *S*-Substituent X on Structure and Yield of Products in Reactions of Z–Cys(X)–OMe with TBAF·xH$_2$O in CH_2Cl_2[a)]

Entry	X	Product (Yield/%)	Recovery / %
1	Mpt	**8a** (76) + **6** (18)	0
2	Ppt	**8a** (56) + **6** (10)	6
3	Tca	**7** (64)	0
4	Z	**8a** (35)	64
5	Acm	**9** (6)	65
6	H	**8a** (61)	25

a) Conditions: Concentration of the substrate = 0.05 mol dm^{-3}; Z–Cys(X)–OMe : TBAF·xH$_2$O = 1 : 2 (mol/mol) at R.T. for 4 h.

between the *S*-Mpt and the thiol-free forms coincides with the general order of nucleophilicity $RS^- > RSH$. The Z derivative (Entry 4) gave **8a** in low yield. In the case of the *S*-Acm derivative (Entry 5), slow decomposition by β-elimination occurred to give Z–ΔAla–OMe (**9**) (ΔAla = dehydroalanine). The highest reactivity and nonoxidative nature would be attributed to the best result for the *S*-Mpt derivative. However, considering the easy availability of the thiol-free form, the optimization of the reaction conditions was tried for both the *S*-Mpt and the thiol-free forms.

As shown in Table 3, 2 molar amounts of TBAF·xH$_2$O for 4—8 h (Entries 2, 3, 7, and 8) gave the best results. Increase of the amounts of TBAF·xH$_2$O (Entries 4—6 for *S*-Mpt and 9—11 for free SH) reduced the yield of **8a** by increasing the tendency to β-elimination. Thus, treatment of the *S*-Mpt derivative with 2 molar amounts of TBAF·xH$_2$O for 4 h was adopted as the standard conditions for the S–CH_2–S bond formation between two cysteine residues.

The effect of the thiol structure was also examined. Penicillamine (Pen) has been used as a substitute for cysteine to increase the conformational constraints of cyclic cystine peptides since the discovery of [Pen^1, Leu^2]oxytocin as a potent hormone inhibitor.[19)] According to these results, Boc–D-Pen–NHBzl (**10**) and its *S*-Mpt derivative (**11**) were

Table 3. Comparison of the *S*-Mpt and Thiol-Free Forms of Z–Cys–OMe in the S–CH_2–S Bond Formation

Entry	Substrate[a)]	*S*-Sub.	TBAF·xH$_2$O / molar amount	Time / h	Yield (%) **8a**	**9**	**6**	**5**
1	**5**	Mpt	1	4	45	0	40	8
2	**5**	Mpt	2	4	76	0	18	0
3	**5**	Mpt	2	8	78	0	15	0
4	**5**	Mpt	3	4	65	8	20	0
5	**5**	Mpt	4	4	46	22	23	0
6	**5**	Mpt	10	4	0	40	25	0
7	**6**	H	2	4	61	0	25	
8	**6**	H	2	8	64	0	23	
9	**6**	H	3	4	53	7	17	
10	**6**	H	4	4	39	20	28	
11	**6**	H	10	4	0	35	22	

a) Concentration of the substrate : 0.05 mol dm^{-3}.

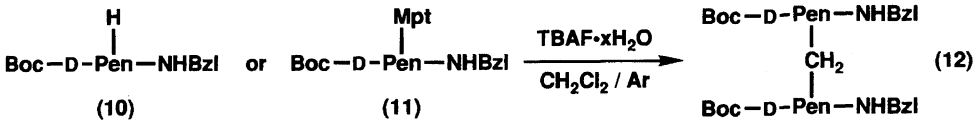
prepared and treated with TBAF·$x$$H_2O$ in DCM (Scheme 3).

In the Cys derivatives the *S*-Mpt-form was more reactive than the thiol-free form, giving higher yields under the same conditions (Table 3, Entries 2 and 7). On the contrary, the preference order between the *S*-Mpt- and thiol-free forms was reversed in the Pen derivatives as shown in Table 4. When **11** was treated with 2 molar amounts of TBAF·$x$$H_2O$ for 4 h, cleavage of the *S*-Mpt group was fast as in the case of the cysteine derivative **5**; however, the methylene insertion product (**12**) was obtained in lower yield along with the thiol-free form **10** (Entry 2). When the amount of TBAF·$x$$H_2O$ was doubled (Entry 3), the yield of **12** increased to the same level as in the reaction of **10** with 2 molar amounts of TBAF·$x$$H_2O$ (Entry 1). The fact that the increase of the yield of **12** was caused by the increase of the amount of TBAF·$x$$H_2O$ suggested the presence of two routes to **12** from **11**. The thiolate ion, generated from **11** by consumption of a part of TBAF·$x$$H_2O$, is bulky and less reactive than that from **5**, and protonated before its attack on CH_2Cl_2 to the less nucleophilic thiol form. The thiol form, in turn, reacted rather slowly with CH_2Cl_2 with the assistance of the remaining TBAF·$x$$H_2O$.[20]

The S–CH_2–S bond-forming reaction is a two-step reaction. At the initial step, a thiolate ion, RS^-, reacts with CH_2Cl_2 to produce a chloromethyl sulfide, $RSCH_2Cl$. To provide a methylenedithioether, $RSCH_2SR$, this intermediate should react with the second RS^-, thus overcoming the large excess of CH_2Cl_2 present as the solvent. In our experiments, however, the presence of the chloromethyl sulfide has not been observed. It is interesting to know what factor governs this high selectivity for the S–CH_2–S bond formation (Scheme 4).

Two possibilities for the selective reaction of $RSCH_2Cl$ with the second RS^- to form the *S*–CH_2–S bond were considered. The first one is the special role of a small amount of water existing in the form of TBAF·$x$$H_2O$ (x = nearly 3[21]). If the cleavage reaction of RS–Mpt with TBAF·$x$$H_2O$ would occur at the boundary between CH_2Cl_2 and the water phase, locally high concentration of RS^- would accomplish selective trapping of the $RSCH_2Cl$ just generated. To ascertain this possibility, dilution of CH_2Cl_2 with water-miscible THF was tried. The reaction of **5** with TBAF·$x$$H_2O$ in such a low concentration as 10% DCM in THF gave the *S*–CH_2–S compound **8a** in 74% yield, which was almost the same as that obtained in pure DCM. Therefore, the two-phase mechanism was clearly ruled out.

The other explanation would depend on the exceptionally high reactivity of $RSCH_2Cl$ in the nucleophilic substitution reaction. This chloride dissociates partly to $RSCH_2^+$, the cation of which is strongly stabilized by resonance with the S atom. This cationic nature of the intermediate would electrostatically attract the second RS^- to produce selectively

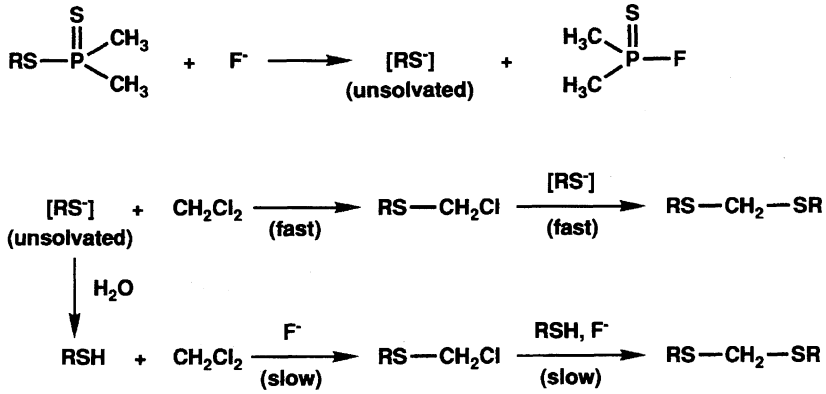


Scheme 3.

Table 4. Comparison of the *S*-Mpt and Thiol-Free Forms of Boc–D-Pen–NHBzl in the S–CH_2–S Bond Formation

Entry	Substrate[a]	*S*-Sub.	TBAF·$x$$H_2O$	Time	Yield (%)		Recovery
			molar amount	h	**12**	**10**	%
1	**10**	H	2	4	76	8 (recovery)	
2	**11**	Mpt	2	4	63	23	0
3	**11**	Mpt	4	4	79	8	0

a) Concentration of the substrate : 0.05 mol dm^{-3}.



Scheme 4.

methylene-inserted products.

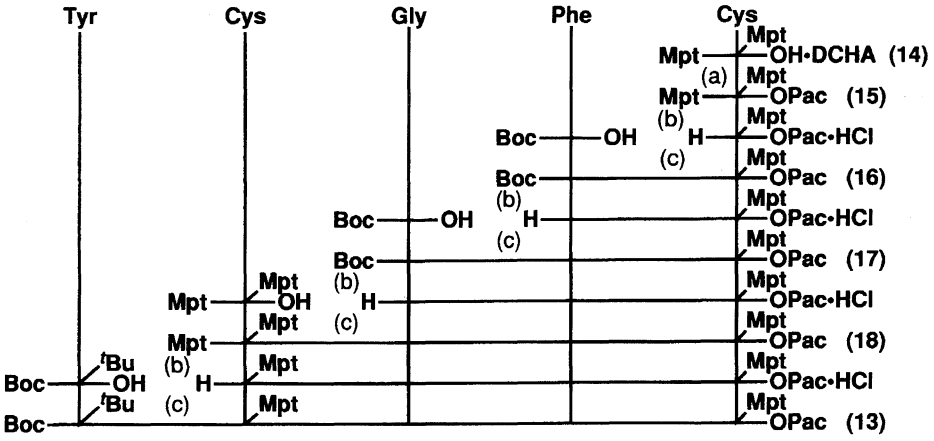
As the most promising application of the new methylene insertion reaction, intramolecular cyclization of a peptide containing two Cys residues was investigated. Cyclization in solution of a peptide with a [Cys^2, Cys^5]-enkephalin sequence was selected as a model reaction. As summarized in Scheme 5, a linear precursor, Boc–Tyr(^{t}Bu)–Cys(Mpt)–Gly–Phe–Cys(Mpt)–OPac (**13**) was prepared by the Boc strategy except for Cys^2 and Cys^5, which were incorporated using *N*,*S*-bis-Mpt-cysteine (**14**).[11]

Compound **13** was dissolved in DCM in a concentration of 1 mM and treated at R.T. with 2 molar amounts of TBAF·xH$_2$O for each *S*-Mpt-Cys residue (molar ratio of **13** : TBAF·xH$_2$O = 1 : 4). Rapid reaction occurred and a simple work-up after a total reaction time of 2 h gave the pure monomeric cyclic product **19** in a high yield of 94%. Since applicability of a higher concentration would be more convenient for preparative purposes, the reaction in the concentration of 2 mM was also tried to give **19** in the same yield. As shown in Fig. 1, analysis of these two crude products by FAB-MS assured the monomeric cyclization.

The 15-membered cyclic structure of **19** corresponds to that of a cyclic pentapeptide. Cyclization of penta- and hexapeptides, especially those consisting of all-*l*-configured amino acids, is often accompanied with dimeric cyclization.[22] In this point, this rapid and selective monomer-giving cyclization reaction would provide a new tool for the study of structure-activity relationships of biologically active peptides. It should also be noted that the C-terminal phenacyl (Pac) ester group, which is highly susceptible to TBAF·xH$_2$O,[23] remained untouched during the cyclization. This implies that the fluoride ion attacks the *S*-Mpt group preferably to the Pac group with consumption of the fluoride ion. The reduced amounts of TBAF·xH$_2$O would not have sufficient potency for the cleavage of the Pac group.

Two-step deprotection of **19** was done with Zn reduction in 85% acetic acid followed by treatment with 46% TFA in DCM containing 4% each of *m*-cresol and thioanisole to give a cyclic enkephalin analog **20** in 84% yield (Scheme 6). After purification by preparative HPLC, the purity was measured by an analytical HPLC as shown in Fig. 2.

In conclusion, we were able to establish a new side-chain



Reagents: (a) Pac-Br in DMF at R.T. overnight; (b) 4M HCl in EtOAc at 0°C 1h; (c) EDC·HCl + HOBt + DIEA at R.T. overnight.

Scheme 5. Synthetic route to the protected linear precursor **13**.

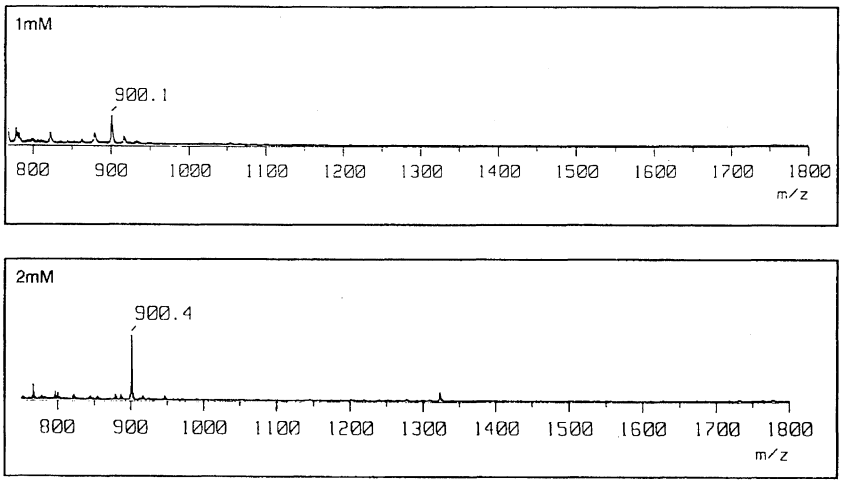
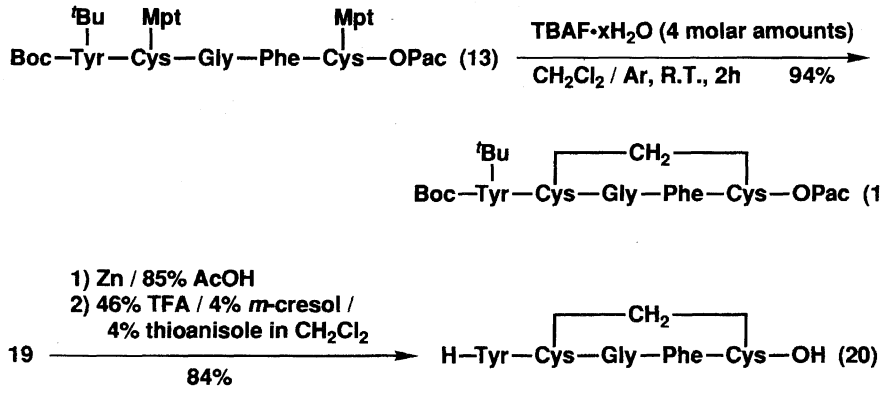


Fig. 1. FAB mass spectra of crude cyclization products obtained by reactions in concentrations of 1 mM and 2 mM, respectively.



Scheme 6. S–CH_2–S bond-forming cyclization and final deprotection.

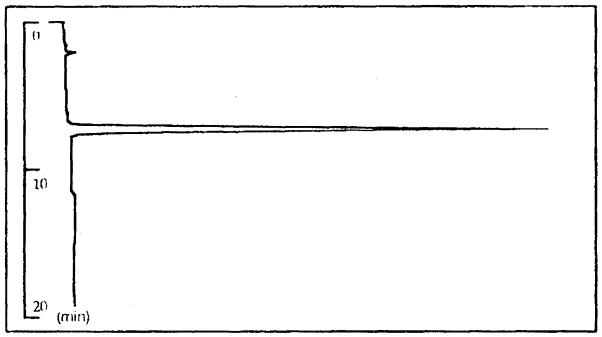


Fig. 2. Analytical HPLC profile of purified [Cys^2, Cys^5]-enkephalin methylenedithioether **20**.

cyclization method for cysteine-containing peptides. This would provide a new tool in the study of structure-activity relationships of biologically active peptides. Application of the method to other enkephalin analogs and solid-phase cyclization considering combinatorial use is now under investigation in our laboratory. Biological activities of enkephalin analogs will be discussed elsewhere in due time.

The new S–CH_2–S bond-forming reaction exemplified in peptides containing amide and fluoride ion-susceptible ester functions would be versatile in organic synthesis.

Experimental

TLC was done using Merck silica gel plates $60F_{254}$ in the following systems: (a) hexane–ethyl acetate (1 : 1) and (b) chloroform–methanol (10 : 1). Column chromatography was done on a Wakogel C-300 (Wako Pure Chemical Industries, Ltd., Osaka). Analytical HPLC was done on a Waters 625 LC system containing 5 μm μBondasphere C18 (3.9 mm×150 mm) with a Waters 484 tunable absorbance detector. Preparative HPLC was done using a Waters 600E LC system containing 5 μm μBondasphere C18 (19 mm×150 mm). ^{1}H NMR spectra were recorded on a Bruker Avance DPX300 (300 MHz) or a JEOL JNM-Rambda500 (500 MHz) using tetramethylsilane (TMS) as the internal standard. ^{13}C NMR spectra were recorded on the Bruker spectrometer operating at 75.5 MHz or the JEOL spectrometer operating at 125.65 MHz. Samples were dissolved in the solvent indicated and chemical shifts were measured relative to $CDCl_3$ assigned at 77.00 ppm. ^{31}P NMR spectra were recorded on the Bruker spectrometer operating at 121.5 MHz. Chemical shifts were measured relative to external phosphoric acid assigned to zero. Mass spectra were obtained using a JEOL JMS-AX505HA spectrometer. Optical rotations were measured in a JASCO DIP-360 apparatus. Melting points were measured on an Ishii-shoten melting point apparatus without correction. Elemental analyses were done on a Yanaco MT-5 apparatus. Amino acid analyses were done on a Hitachi L-8500 Amino Acid Analyzer.

***N*-Benzyloxycarbonyl-*S*-(dimethylphosphinothioyl)cysteine Methyl Ester: Z–Cys(Mpt)–OMe (5).** To an ice-cooled solution of HCl·H–Cys(Mpt)–OMe[11] (1.37 g, 5.18 mmol) and triethylamine (TEA) (1.59 ml, 11.4 mmol) in DCM (10 ml) was added benzyl chloroformate (912 μl, 5.70 mmol) in DCM (3 ml), and the mixture was stirred at 0 °C for 1 h and then at R.T. overnight. The solvent was converted to ethyl acetate and the solution washed with water, 5% aq $NaHCO_3$, and saturated NaCl solution, and dried over anhydrous Na_2SO_4. After removal of the solvent, the product was obtained as a colorless oil by column chromatography on silica gel using chloroform for elution.

Yield 1.87 g (quantitative), $[\alpha]_D^{25}$ −24.7° (*c* 1.0, CH_3OH), R_f^a 0.53. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.95—1.97 (3H, m, *P*-CH_3), 1.99—2.02 (3H, m, *P*-CH_3), 3.30–3.58 (2H, m, β-CH_2), 3.76 (3H, s, *O*-CH_3), 4.59—4.67 (1H, m, α-CH), 5.07 (2H, s, $C\underline{H}_2Ph$), 5.81—5.94 (1H, m, NH), 7.29—7.36 (5H, m, $CH_2\underline{Ph}$).

^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 26.53, 27.49 (*P*-CH_3), 33.46 (β-CH_2), 52.62 (*O*-CH_3), 54.05 (α-CH), 66.89 ($\underline{C}H_2Ph$), 127.91, 127.96, 128.26, 135.87 ($CH_2\underline{Ph}$), 155.55 (CO/urethane), 170.29 (CO/Cys). ^{31}P NMR ($CDCl_3$) δ = 58.31—58.98 (m).

FAB-MS: Found: *m/z* 362.1. Calcd for $C_{14}H_{21}NO_4PS_2$: $(M+H)^+$, 362.1. Found: C, 46.63; H, 5.63; N, 3.78; S, 17.69%. Calcd for $C_{14}H_{20}O_4NS_2P$: C, 46.53; H, 5.58; N, 3.88; S, 17.74%.

Reactions of 5 in Various Polyhaloalkane Solvents: To a solution of **5** (181 mg, 0.500 mmol) in one of the solvents shown in Table 1 (10 ml) under argon was added TBAF·xH_2O (316 mg, 1.00 mmol) and the solution stirred at R.T. for 4 h. The reaction was quenched by addition of 5% aq citric acid solution (10 ml). The organic layer was separated and washed with water and saturated NaCl solution, and dried. After removal of the solvent, products were obtained as amorphous solid by preparative silica-gel TLC using hexane–ethyl acetate (2 : 1) for development.

[Z–Cys–OMe]$_2$$CH_2$ (8a): $[\alpha]_D^{22}$ −44.4° (*c* 1.0, CH_3OH), R_f^a 0.51. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 3.05—3.24 (4H, m, β-CH_2), 3.72 (2H, s, SCH_2S), 3.82 (6H, s, *O*-CH_3), 4.68—4.71 (2H, m, α-CH), 5.20 (4H, s, $C\underline{H}_2Ph$), 5.87—5.89 (2H, m, NH), 7.36 (10H, m, $CH_2\underline{Ph}$). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 32.89 (β-CH_2), 36.19 (SCH_2S), 52.57 (*O*-CH_3), 53.21 (α-CH), 66.96 ($\underline{C}H_2Ph$), 127.95, 128.05, 128.36, 135.94 ($CH_2\underline{Ph}$), 155.59 (CO/urethane), 170.96 (CO/Cys).

FAB-MS: Found: *m/z* 551.2. Calcd for $C_{25}H_{31}N_2O_8S_2$: $(M+H)^+$,

551.2. Found: C, 53.85; H, 5.89; N, 5.02; S, 11.50%. Calcd for $C_{25}H_{30}N_2O_8S_2$ (+ 1/2H_2O): C, 53.65; H, 5.58; N, 5.01; S, 11.46%.

[Z–Cys–OMe]$_2$CD$_2$ (8b): $[\alpha]_D^{22}$ −44.4° (*c* 1.0, CH_3OH), R_f^a 0.51. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 3.04—3.25 (4H, m, β-CH_2), 3.80 (6H, s, *O*-CH_3), 4.65—4.69 (2H, m, α-CH), 5.24 (4H, s, C$\underline{H_2}$Ph), 5.88—5.90 (2H, m, NH), 7.42 (10H, m, $CH_2\underline{Ph}$). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 32.56 (β-CH_2), 36.43 (SCD_2S), 52.23 (*O*-CH_3), 53.12 (α-CH), 67.05 (C$\underline{H_2}$Ph), 127.75, 127.97, 128.32 135.86 ($CH_2\underline{Ph}$), 155.54 (CO/urethane), 171.10 (CO/Cys).

FAB-MS: Found: *m/z* 553.1. Calcd for $C_{25}H_{29}D_2N_2O_8S_2$: $(M+H)^+$, 553.2.

Found: C, 54.02; H, 5.50; N, 4.84; S, 11.74%. Calcd for $C_{25}H_{28}D_2N_2O_8S_2$ (+1/4CH_3OH): C, 54.09; H, 5.21; N, 5.00; S, 11.44%.

Z–Cys(CH_2CH_2Cl)–OMe: Mp 56—57 °C, $[\alpha]_D^{26}$ −13.2° (*c* 1.0, CH_3OH), R_f^a 0.71. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.84 (2H, t, *J* = 7.9 Hz, S$\underline{CH_2}CH_2Cl$), 2.93—3.11 (2H, m, β-CH_2), 3.58 (2H, t, *J* = 7.9 Hz, $SCH_2\underline{CH_2}Cl$), 3.77 (3H, s, *O*-CH_3), 4.58—4.63 (1H, m, α-CH), 5.12 (2H, s, C$\underline{H_2}$Ph), 5.69—5.78 (1H, m, NH), 7.31—7.36 (5H, m, $CH_2\underline{Ph}$). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 34.53 (β-CH_2), 34.58 (S$\underline{CH_2}CH_2Cl$), 42.70 ($SCH_2\underline{CH_2}Cl$), 52.71 (*O*-CH_3), 53.69 (α-CH), 67.10 (C$\underline{H_2}$Ph), 128.05, 128.19, 128.47, 135.94 ($CH_2\underline{Ph}$), 155.59 (CO/urethane), 170.80 (CO/Cys).

FAB-MS: Found: *m/z* 332.1. Calcd for $C_{14}H_{19}ClNO_4S$: $(M+H)^+$, 332.1. Found: C, 50.96; H, 5.55; N, 4.11; S, 9.70; Cl, 10.67%. Calcd for $C_{14}H_{18}ClNO_4S$: C, 50.68; H, 5.47; N, 4.22; S, 9.66; Cl, 10.68%.

Z–Cys(CH_2CH_2Br)–OMe: Mp 64—65 °C, $[\alpha]_D^{25}$ −12.9° (*c* 1.0, CH_3OH), R_f^a 0.70. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.93 (2H, t, *J* = 7.9 Hz, S$\underline{CH_2}CH_2Br$), 2.98—3.11 (2H, m, β-CH_2), 3.42 (2H, t, *J* = 7.9 Hz, $SCH_2\underline{CH_2}Br$), 3.77 (3H, s, *O*-CH_3), 4.58—4.65 (1H, m, α-CH), 5.12 (2H, s, C$\underline{H_2}$Ph), 5.68—5.70 (1H, m, NH), 7.31—7.37 (5H, m, $CH_2\underline{Ph}$). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 29.93 (S$\underline{CH_2}CH_2Br$), 34.42 (β-CH_2), 34.46 ($SCH_2\underline{CH_2}Br$), 52.76 (*O*-CH_3), 53.70 (α-CH), 67.13 (C$\underline{H_2}$Ph), 128.08, 128.19, 128.47, 135.94 ($CH_2\underline{Ph}$), 155.58 (CO/urethane), 170.75 (CO/Cys).

FAB-MS: Found: *m/z* 376.0. Calcd for $C_{14}H_{19}BrNO_4S$: $(M+H)^+$, 376.0. Found: C, 44.55; H, 4.79; N, 3.46; S, 8.05; Br, 20.51%. Calcd for $C_{14}H_{18}BrNO_4S$(+1/5CH_3OH): C, 44.56; H, 4.93; N, 3.65; S, 8.35; Br, 20.80%.

Reactions of Z–Cys–OMe and Its Variously *S*-Protected Derivatives with TBAF·*x*H_2O: To a solution of Z–Cys(X)–OMe (X = H, Ppt, Tca, Z, and Acm) (0.50 mmol) in DCM (10 ml) under argon was added TBAF·*x*H_2O (316 mg, 1.00 mmol) and the solution stirred at R.T. for 4 h. Work-up was done as above. Results are shown in Table 2.

***N*-(*t*-Butoxycarbonyl)-D-penicillamine Benzylamide: Boc–D-Pen–NHBzl (10).** To an ice-cooled solution of Boc–D-Pen–OH (511 mg, 2.05 mmol) and DIEA (393 µl, 2.25 mmol) in chloroform (5 ml) was added dimethylphosphinothioyl chloride[24,25] (290 mg, 2.25 mmol) in chloroform (2 ml) and the solution was stirred for 30 min at 0 °C. To this was added benzylamine (247 µl, 2.25 mmol) drop by drop and the mixture was stirred at 0 °C for 1 h and then at R.T. overnight. After evaporation, the residue was dissolved in ethyl acetate, washed in the usual manner, and dried. After removal of the solvent, compound **10** was obtained by silica-gel column chromatography using hexane–ethyl acetate (4 : 1 to 3 : 1) for elution.

Yield 660 mg (95%), mp 123—125 °C, $[\alpha]_D^{25}$ +4.3° (*c* 1.0, CH_3OH), R_f^a 0.74. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.32 (3H, s, γ-CH_3), 1.39 (9H, s, $C(CH_3)_3$), 1.50 (3H, s, γ-CH_3), 2.54 (1H, s, SH), 4.14—4.17 (1H, m, α-CH), 4.34—4.49 (2H, m, NHC$\underline{H_2}$Ph), 5.70—5.73 (1H, m, α-NH), 6.97 (1H, brs, N$\underline{H}CH_2$Ph), 7.21—7.32 (5H, m, $NHCH_2\underline{Ph}$). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 28.42 (C($\underline{C}H_3)_3$), 30.92 (γ-CH_3), 43.36 (α-CH), 45.98 (β-C), 62.15 (NH$\underline{C}H_2$Ph), 80.09 ($\underline{C}(CH_3)_3$), 127.38, 127.59, 128.58, 137.77 ($NHCH_2\underline{Ph}$), 155.81 (CO/urethane), 170.05 (CO/Pen).

FAB-MS: Found: *m/z* 339.2. Calcd for $C_{17}H_{27}N_2O_3S$: $(M+H)^+$, 339.2. Found: C, 60.22; H, 7.61; N, 8.21; S, 9.56%. Calcd for $C_{17}H_{26}N_2O_3S$: C, 60.33; H, 7.74; N, 8.28; S, 9.47%.

***N*-(*t*-Butoxycarbonyl)-*S*-dimethylphosphinothioyl-D-penicillamine Benzylamide: Boc–D-Pen(Mpt)–NHBzl (11).** To a ice-cooled solution of **10** (68 mg, 0.20 mmol) in DMF (2 ml) was added NaH (8.8 mg, 0.22 mmol) and the mixture stirred at 0 °C for 30 min. After the absence of gas evolution was confirmed, the mixture was warmed up to R.T. To this was added dimethylphosphinothioyl chloride (28 mg, 0.22 mmol) in DMF (1 ml) and the solution stirred at R.T. overnight. After evaporation, the residue was dissolved in ethyl acetate, washed in the usual manner, and dried. After removal of the solvent, compound **11** was obtained by silica-gel column chromatography using hexane–ethyl acetate (4 : 1) for elution.

Yield 75 mg (87%), mp 97—99 °C, $[\alpha]_D^{25}$ +24.9 ° (*c* 1.0, CH_3OH), R_f^a 0.56. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.43—1.47 (9H, m, $C(CH_3)_3$), 1.58—1.62 (3H, m, *P*-CH_3), 1.77 (6H, s, γ-CH_3), 2.08—2.16 (3H, m, *P*-CH_3), 4.25 (1H, dd, *J* = 14.5 and 4.6 Hz, NHC$\underline{H_2}$Ph), 4.72 (1H, dd, *J* = 14.5 and 6.9 Hz, NHC$\underline{H_2}$Ph), 5.49—5.52 (2H, m, α-CH and α-NH), 7.22—7.39 (5H, m, $NHCH_2\underline{Ph}$), 7.68 (1H, brs, N$\underline{H}CH_2$Ph). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 26.59, 26.79 (*P*-CH_3), 28.89 (C($\underline{C}H_3)_3$), 29.31, 30.00 (γ-CH_3), 43.33 (α-CH), 55.85 (β-C), 58.06 (NH$\underline{C}H_2$Ph), 79.81 ($\underline{C}(CH_3)_3$) 127.31, 128.34, 128.61, 137.86 ($NHCH_2\underline{Ph}$), 155.14 (CO/urethane), 169.59 (CO/Pen). ^{31}P NMR (121.5 MHz, $CDCl_3$) δ = 49.66—49.86 (m).

EI-MS: Found: *m/z* 431.2. Calcd for $C_{19}H_{31}N_2O_3PS_2$: M^+, 431.2. Found: C, 53.30; H, 7.41; N, 6.48; S, 14.75%. Calcd for $C_{19}H_{31}N_2O_3PS_2$: C, 53.00; H, 7.26; N, 6.51; S, 14.89%.

Reactions of 10 and 11 with TBAF·*x*H_2O: To a solution of Boc–D-Pen(X)–NHBzl (**10**: X = H, **11**: X = Mpt) (0.50 mmol) in DCM (10 ml) under argon was added TBAF·*x*H_2O (1.0 mmol) and the solution stirred at R.T. for 4 h. The reaction was quenched by addition of 5% aq citric acid solution (10 ml). The organic layer was separated, washed with water, and saturated NaCl solution, and dried. After removal of the solvent, products were obtained by preparative silica-gel TLC using hexane–ethyl acetate (4 : 1) for development. Results were summarized in Table 4.

[Boc–D-Pen–NHBzl]$_2$$CH_2$: Mp 161—162 °C, $[\alpha]_D^{26}$ −10.7° (*c* 1.0, CH_3OH), R_f^a 0.60. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.33—1.38 (30H, m, $C(CH_3)_3$ and γ-CH_3), 3.73 (2H, s, SCH_2S), 4.25—4.39 (4H, m, NHC$\underline{H_2}$Ph), 4.45—4.57 (2H, m, α-CH), 5.89—5.92 (2H, brs, α-NH), 7.20—7.26 (10H, m, $NHCH_2\underline{Ph}$), 7.57 (2H, brs, N$\underline{H}CH_2$Ph).

^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 24.82 (γ-CH_3), 27.30 (SCH_2S), 28.26 (C($\underline{C}H_3)_3$), 43.42 (α-CH), 49.16 (β-C), 60.01 (NH$\underline{C}H_2$Ph), 79.76 ($\underline{C}(CH_3)_3$) 127.23, 127.59, 128.47, 137.92 ($NHCH_2\underline{Ph}$), 156.01 (CO/urethane), 169.70 (CO/Pen).

FAB-MS: Found: *m/z* 689.3. Calcd for $C_{35}H_{53}N_4O_6S_2$: $(M+H)^+$, 689.3. Found: C, 61.05; H, 7.66; N, 8.06; S, 9.20%. Calcd for $C_{35}H_{52}N_4O_6S_2$: C, 61.02; H, 7.61; N, 8.13; S, 9.31%.

***N*,*S*-Bis(dimethylphosphinothioyl)cysteine Phenacyl Ester: Mpt–Cys(Mpt)–OPac (15).** To an ice-cooled solution of Mpt–Cys(Mpt)–OH·DCHA[11] (2.43 g, 5.00 mmol) in DMF (5 ml) was added phenacyl bromide (975 mg, 4.90 mmol) and the mixture was stirred at 0 °C for 1 h and then at R.T. overnight. After removal of DMF in vacuo, crystalline salts were removed by filtra-

tion and washing with ethyl acetate. The filtrate and washings were washed with 5% $NaHCO_3$, water, and saturated NaCl solution, and dried. After removal of the solvent in vacuo, the title compound was obtained as an oil by silica-gel column chromatography using hexane–ethyl acetate (3 : 1) for elution.

Yield 1.55g (73%), $[\alpha]_D^{26}$ −7.3° (*c* 1.0, CH_3OH), R_f^a 0.41. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.85—1.92 (6H, m, *P*-CH_3), 2.02—2.12 (6H, m, *P*-CH_3), 3.38—3.63 (3H, m, β-CH_2 and NH), 4.63—4.65 (1H, m, α-CH), 5.34—5.55 (2H, m, C$\underline{H_2}$COPh), 7.47—7.66 (3H, m, aromatic 3,4-H/CH_2CO$\underline{Ph}$), 7.89-7.92 (2H, m, aromatic 2-H/CH_2CO$\underline{Ph}$). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 23.63, 24.08, 26.67, 27.52 (*P*-CH_3), 34.56 (β-CH_2), 54.12 (α-CH), 66.82 ($\underline{C}H_2$COPh), 127.68, 128.87, 133.72, 134.07 (CH_2CO$\underline{Ph}$), 171.59 (CO/Cys), 191.16 ($CH_2\underline{C}$OPh). ^{31}P NMR (121.5 MHz, $CDCl_3$) δ = 58.31—58.98 (m, *S*-P(S)$(CH_3)_2$), 59.41-60.28 (m, *N*-P(S)-$(CH_3)_2$).

FAB-MS: Found: *m/z* 424.0. Calcd for $C_{15}H_{24}NO_3P_2S_3$: $(M+H)^+$, 424.0. Found: C, 42.14; H, 5.54; N, 3.20; S, 22.66%. Calcd for $C_{15}H_{23}NO_3P_2S_2$ (+ 1/5H_2O): C, 42.18; H, 5.52; N, 3.28; S, 22.52%.

***N*-(*t*-Butoxycarbonyl)-*S*-(dimethylphosphinothioyl)phenylalanylcysteine Phenacyl Ester: Boc–Phe–Cys(Mpt)–OPac (16).** Compound **15** (1.55 g, 3.65 mmol) was treated with 4 M (1 M = 1 mol dm^{-3}) HCl in ethyl acetate (25 ml) at 0 °C for 1 h. After removal of the cleaving reagent in vacuo, addition of ether gave HCl·H–Cys(Mpt)–OPac. 1.34 g (quantitative).

To an ice-cooled solution of Boc–Phe–OH (1.07 g, 4.02 mmol) and HOBt·H_2O (615 mg, 4.02 mmol) in DMF (10 ml) were added EDC·HCl (770 mg, 4.02 mmol), DIEA (637 µl, 3.65 mmol), and then HCl·H–Cys(Mpt)–OPac (1.34 g, 3.65 mmol). The mixture was stirred at 0 °C for 1 h and at R.T. overnight and evaporated in vacuo. The residue was dissolved in ethyl acetate, washed as usual, and dried. After removal of the solvent, the title compound was obtained by column chromatography on silica gel using chloroform–methanol (200 : 1 to 50 : 1) for elution. 2.11 g (90%) The compound was homogeneous on TLC (R_f^a 0.65) and used without further purification.

***N*-(*t*-Butoxycarbonyl)-*S*-(dimethylphosphinothioyl)glycylphenylalanylcysteine Phenacyl Ester: Boc–Gly–Phe–Cys(Mpt)–OPac (17).** Compound **16** (2.11 g, 3.33 mmol) was treated with 4 M HCl in ethyl acetate (20 ml) at 0 °C for 1 h. After removal of the cleaving reagent in vacuo, addition of ether gave HCl·H–Phe–Cys(Mpt)–OPac. 1.68 g (98%).

To an ice-cooled solution of Boc–Gly–OH (628 mg, 3.59 mmol) and HOBt·H_2O (549 mg, 3.59 mmol) in DMF (10 ml) were added EDC·HCl (688 mg, 3.59 mmol), DIEA (568 µl, 3.26 mmol), and then HCl·H–Phe–Cys(Mpt)–OPac (1.68 g, 3.26 mmol). The mixture was stirred at 0 °C for 1 h and at R.T. overnight and evaporated in vacuo. The residue was dissolved in ethyl acetate, washed as usual, and dried. After removal of the solvent, the title compound was obtained by column chromatography on silica gel using chloroform–methanol (200 : 1 to 50 : 1) for elution. 2.05 g (quantitative). The compound was homogeneous on TLC (R_f^a 0.54) and used without further purification.

***N*,*S*,*S*′-Tris(dimethylphosphinothioyl)cysteinylglycylphenylalanylcysteine Phenacyl Ester: Mpt–Cys(Mpt)–Gly–Phe–Cys(Mpt)–OPac (18).** Compound **17** (2.05 g, 3.26 mmol) was treated with 4 M HCl in ethyl acetate (16 ml) at 0 °C for 1 h. After removal of the cleaving reagent in vacuo, addition of ether gave HCl·H–Gly–Phe–Cys(Mpt)–OPac. 1.86 g (quantitative).

To an ice-cooled solution of Mpt–Cys(Mpt)–OH·DCHA (1.61 g, 3.58 mmol) and HOBt·H_2O (548 mg, 3.58 mmol) in DMF (10 ml) were added EDC·HCl (686 mg, 3.58 mmol), DIEA (567 µl, 3.26 mmol), and then HCl·H–Gly–Phe–Cys(Mpt)–OPac (1.86 g, 3.26 mmol). The mixture was stirred at 0 °C for 1 h and at R.T. overnight and evaporated in vacuo. The residue was dissolved in ethyl acetate, washed as usual, and dried. After removal of the solvent, the title compound was obtained by column chromatography on silica gel using chloroform–methanol (100 : 1 to 50 : 1) for elution. 1.20 g (44%). The compound was homogeneous on TLC (R_f^b 0.74) and used without further purification.

***N*-(*t*-Butoxycarbonyl)-*O*-(*t*-butyl)-*S*,*S*′-bis(dimethylphosphinothioyl)tyrosylcysteinylglycylphenylalanylcysteine Phenacyl Ester: Boc–Tyr(^{t}Bu)–Cys(Mpt)–Gly–Phe–Cys(Mpt)–OPac (13).** Compound **18** (1.20 g, 1.46 mmol) was treated with 4 M HCl in ethyl acetate (20 ml) at 0 °C for 1 h. After removal of the cleaving reagent in vacuo, addition of ether gave HCl·H–Cys(Mpt)–Gly–Phe–Cys(Mpt)–OPac. 1.12 g (quantitative).

To an ice-cooled solution of Boc–Tyr(^{t}Bu)–OH (543 mg, 1.61 mmol) and HOBt·H_2O (247 mg, 1.61 mmol) in DMF (10 ml) were added EDC·HCl (309 mg, 1.61 mmol), DIEA (281 µl, 1.61 mmol), and then HCl·H–Cys(Mpt)–Gly–Phe–Cys(Mpt)–OPac (1.12 g, 1.46 mmol). The mixture was stirred at 0 °C for 1 h and at R.T. overnight and evaporated in vacuo. The residue was dissolved in ethyl acetate, washed as usual, and dried. After removal of the solvent, the title compound was obtained by column chromatography on silica gel using chloroform–methanol (100 : 1 to 50 : 1) for elution.

Yield 1.02 g (66%), mp 118—119 °C, $[\alpha]_D^{27}$ −29.5° (*c* 1.0, CH_3OH), R_f^b 0.70. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 1.33 (9H, s, CH_3/^{t}Bu), 1.41 (9H, s, CH_3/Boc), 2.01—2.04 (6H, m, *P*-CH_3), 2.05—2.08 (6H, m, *P*-CH_3), 2.61—2.67 (2H, m, β-CH_2/Cys^2), 2.93—2.95 (2H, m, β-CH_2/Tyr), 3.09—3.11 (2H, m, β-CH_2/Cys^5), 3.14—3.16 (2H, m, β-CH_2/Phe), 3.48—3.52 (1H, m, CH_2/Gly), 3.65—3.68 (1H, m, CH_2/Gly), 4.59 (1H, br, α-CH/Cys^2), 4.83—4.85 (1H, m, α-CH/Cys^5), 4.88—4.92 (1H, m, α-CH/Tyr), 5.11—5.12 (1H, m, α-CH/Phe), 5.38—5.49 (2H, m, C$\underline{H_2}$COPh), 6.84—6.86 (2H, m, aromatic 2-H/Tyr), 6.99—7.00 (1H, m, NH/Cys^2), 7.13—7.14 (2H, m, aromatic 3-H/Tyr), 7.17—7.29 (5H, m, aromatic 2,3,4-H/Phe), 7.50—7.55 (2H, m, aromatic 3-H/CH_2CO$\underline{Ph}$), 7.65—7.69 (1H, m, aromatic 4-H/CH_2CO$\underline{Ph}$), 7.90—7.93 (2H, m, aromatic 2-H/CH_2CO$\underline{Ph}$), 8.07—8.09 (1H, m, NH/Tyr), 8.11—8.14 (1H, m, NH/Phe), 8.16—8.18 (1H, m, NH/Cys^5), 8.57 (1H, brs, NH/Gly). ^{13}C NMR (125.65 MHz, $CDCl_3$) δ = 26.35, 26.62 (*P*-CH_3), 26.80, 27.08 (*P*-CH_3), 28.37 (CH_3/Boc), 28.87 (CH_3/^{t}Bu), 31.16 (β-CH_2/Tyr), 32.75 (β-CH_2/Phe), 37.00 (β-CH_2/Cys^5), 37.56 (β-CH_2/Cys^2), 43.41 (CH_2/Gly), 53.04 (α-CH/Tyr), 54.42 (α-CH/Phe), 54.90 (α-CH/Cys^5), 57.15 (α-CH/Cys^2), 66.89 ($\underline{C}H_2$COPh), 76.83 ($\underline{C}(CH_3)_3$/Boc), 77.07 ($\underline{C}(CH_3)_3$/^{t}Bu), 124.50 (aromatic C3, C5/Tyr), 126.62 (aromatic C4/Phe), 127.84 (aromatic C2, C6/CH_2COPh), 128.43 (aromatic C2, C6/Phe), 128.91 (aromatic C3, C5/CH_2CO$\underline{Ph}$), 129.45 (aromatic C3, C5/Phe), 129.64 (aromatic C2, C6/Tyr), 130.42 (aromatic C1/Tyr), 133.98 (aromatic C4/CH_2CO$\underline{Ph}$), 134.04 (aromatic C1/Phe), 137.24 (aromatic C1/CH_2CO$\underline{Ph}$), 154.72 (aromatic C4/Tyr), 156.12 (CO/urethane), 168.71 (CO/Gly), 169.27 (CO/Cys^2), 170.55 (CO/CH_2COPh), 171.19 (CO/Phe), 173.64 (CO/Tyr), 191.44 (CO/Cys^5).

FAB-MS: Found: *m/z* 1050.3. Calcd for $C_{47}H_{66}N_5O_{10}P_2S_4$: $(M+H)^+$, 1050.3. Amino acid ratios (4% anisole/6 M HCl 120 °C, 24 h): Tyr (1) 0.99, Cys (2) 1.43, Gly (1) 1.00, Phe (1) 1.28. Found: C, 53.56; H, 6.12; N, 6.62; S, 12.09%. Calcd for $C_{47}H_{65}N_5O_{10}P_2S_4$: C, 53.75; H, 6.24; N, 6.67; S, 12.21%.

***N*-(*t*-Butoxycarbonyl)-*O*-(*t*-butyl)-tyrosylcysteinylglycylphenylalanylcysteine Phenacyl Ester *S*,*S*′-Methylene Deriva-**

tive: [Boc–Tyr(^{t}Bu)–Cys–Gly–Phe–Cys–OPac]CH_2 (19). To a solution of **13** (110 mg, 0.104 mmol) in DCM (100 ml) under an argon atmosphere was added TBAF·xH$_2$O (0.132 mg, 0.420 mmol) and the mixture stirred at R.T. for 1 h. After the disappearance of **13** was confirmed, stirring was continued for additional 1 h. The reaction was quenched by addition of 5% aq citric acid solution. The organic layer was separated, washed with water and saturated NaCl solution, and dried over anhydrous Na_2SO_4. After removal of the solvent, the product was obtained by crystallization and washing with ether. 85.8 mg (94%). The compound was homogeneous on TLC.

Mp 180—181 °C, $[\alpha]_D^{27}$ −16.0° (c 1.0, CH_3OH), R_f^b 0.68. ^{1}H NMR (500 MHz, DMSO-d_6) δ = 1.24 (9H, s, CH_3/^{t}Bu), 1.28 (9H, s, CH/Boc), 2.63—2.68 (2H, m, β-CH_2/Cys^2), 2.91—2.97 (2H, m, β-CH_2/Tyr), 3.10—3.11 (2H, m, β-CH_2/Cys^5), 3.13—3.15 (2H, m, β-CH_2/Phe), 3.50—3.54 (1H, m, CH_2/Gly), 3.72—3.76 (1H, m, CH_2/Gly), 3.79 (2H, s, SCH_2S), 4.19 (1H, br, α-CH/Cys^2), 4.42—4.47 (1H, m, α-CH/Cys^5), 4.48—4.50 (1H, m, α-CH/Tyr), 4.73—4.77 (1H, m, α-CH/Phe), 5.47—5.59 (2H, m, C$\underline{H_2}$COPh), 6.83—6.84 (2H, m, aromatic 2-H/Tyr), 6.98—6.99 (1H, m, NH/Cys^2), 7.12—7.14 (2H, m, aromatic 3-H/Tyr), 7.16—7.27 (5H, m, aromatic 2,3,4-H/Phe), 7.54—7.57 (2H, m, aromatic 3-H/CH_2CO$\underline{Ph}$), 7.67—7.70 (1H, m, aromatic 4-H/CH_2CO$\underline{Ph}$), 7.95—7.96 (2H, m, aromatic 2-H/CH_2CO$\underline{Ph}$), 8.04—8.06 (1H, m, NH/Tyr), 8.10—8.12 (1H, m, NH/Phe), 8.14—8.16 (1H, m, NH/Cys^5), 8.58 (1H, br, NH/Gly). ^{13}C NMR (125.65 MHz, DMSO-d_6) δ = 28.06 (CH_3/Boc), 28.51 (CH_3/^{t}Bu), 33.33 (β-CH_2/Tyr), 33.84 (β-CH_2/Phe), 35.27 (SCH_2S), 35.91 (β-CH_2/Cys^5), 36.46 (β-CH_2/Cys^2), 43.02 (CH_2/Gly), 52.28 (α-CH/Tyr), 53.31 (α-CH/Phe), 54.19 (α-CH/Cys^5), 55.61 (α-CH/Cys^2), 67.14 ($\underline{C}H_2$COPh), 77.53 ($\underline{C}(CH_3)_3$/Boc), 78.13 ($\underline{C}(CH_3)_3$C/^{t}Bu), 123.25 (aromatic C3, C5/Tyr), 126.26 (aromatic C4/Phe), 127.81 (aromatic C2, C6/CH_2CO$\underline{Ph}$), 128.21 (aromatic C2, C6/Phe), 128.91 (aromatic C3, C5/CH_2CO$\underline{Ph}$), 129.09 (aromatic C3, C5/Phe), 129.67 (aromatic C2, C6/Tyr), 132.66 (aromatic C1/Tyr), 133.68 (aromatic C4/CH_2CO$\underline{Ph}$), 134.05 (aromatic C1/Phe), 137.89 (aromatic C1/CH_2CO$\underline{Ph}$), 153.26 (aromatic C4/Tyr), 156.12 (CO/urethane), 168.99 (CO/Gly), 169.45 (CO/Cys^2), 170.51 (CO/CH_2COPh), 170.76 (CO/Phe), 171.51 (CO/Tyr), 192.16 (CO/Cys^5).

FAB-MS: Found: m/z 878.3. Calcd for $C_{44}H_{56}N_5O_{10}S_2$: $(M+H)^+$, 878.3. Amino acid ratios (4% anisole/6 M HCl 120 °C, 24 h): Tyr (1) 0.95, Gly (1) 1.04, Phe (1) 1.18, Djenkolic acid (1) 0.97. Found: C, 59.54; H, 6.34; N, 7.69; S, 7.48%. Calcd for $C_{44}H_{55}N_5O_{10}S_2$(+1/2H_2O): C, 59.58; H, 6.36; N, 7.89; S, 7.23%.

Tyrosylcysteinylglycylphenylalanylcysteine *S,S′*-Methylene Derivative: [H–Tyr–Cys–Gly–Phe–Cys–OH]CH_2 (20). To an ice-cooled solution of **19** (77.0 mg, 0.087 mmol) in 85% aq acetic acid solution (5 ml) was added zinc powder (548 mg, 8.77 mmol) and the mixture stirred at 0 °C for 1 h and then at R.T. for 3 h. Zinc powder was removed by filtration and washing with methanol. The filtrate and washings were evaporated in vacuo with toluene. The residue was dissolved in ethyl acetate, washed with 5% aq citric acid solution, water, and saturated NaCl solution, and dried over anhydrous Na_2SO_4. After removal of the solvent, addition of ether gave [Boc–Tyr(^{t}Bu)–Cys–Gly–Phe–Cys–OH]CH_2. 66.6 mg (quantitative).

This compound (66.6 mg, 0.088 mmol) was treated with trifluoroacetic acid (TFA) (5 ml) containing anisole (0.514 ml, 4.38 mmol) and *m*-cresol (0.458 ml, 4.38 mmol) at 0 °C for 4 h. After removal of volatile materials in vacuo, the product was obtained by crystallization and washing with ether. 44.5 mg (84%). Pure material was obtained by preparative HPLC (gradient, done over 20 min, of 0.1% aq TFA and CH_3CN from 25/75 to 35/65). Purity of the final product was checked by an analytical HPLC (conditions: linear gradient elution, CH_3CN-0.1% aq TFA 25/75 to 35/65 over 20 min; flow rate, 1.0 ml min^{-1}; detection 220 nm) and its profile was shown in Fig. 2. Retention time was 7.58 min.

Mp 166 °C (gradually decomposed), $[\alpha]_D^{25}$ +5.3° (c 1.0, DMF). ^{1}H NMR (500 MHz, DMSO-d_6) δ = 2.64—2.67 (2H, m, β-CH_2/Cys^2), 2.91—2.96 (2H, m, β-CH_2/Tyr), 3.09—3.11 (2H, m, β-CH_2/Cys^5), 3.12—3.15 (2H, m, β-CH_2/Phe), 3.49—3.53 (1H, m, CH_2/Gly), 3.70—3.74 (1H, m, CH_2/Gly), 3.78 (2H, s, SCH_2S), 4.17 (1H, br, α-CH/Cys^2), 4.45—4.47 (1H, m, α-CH/Cys^5), 4.49—4.52 (1H, m, α-CH/Tyr), 4.74—4.79 (1H, m, α-CH/Phe), 6.82—6.84 (2H, m, aromatic 2-H/Tyr), 6.97—6.98 (1H, m, NH/Cys^2), 7.13—7.14 (2H, m, aromatic 3-H/Tyr), 7.17—7.29 (5H, m, aromatic 2,3,4-H/Phe), 8.04—8.06 (2H, m, NH_2/Tyr), 8.10—8.11 (1H, m, NH/Phe), 8.13—8.14 (1H, m, NH/Cys^5), 8.59 (1H, br, NH/Gly), 14.02 (1H, br, COOH/Cys^5). ^{13}C NMR (125.65 MHz, DMSO-d_6) δ = 33.21 (β-CH_2/Tyr), 33.78 (β-CH_2/Phe), 35.35 (SCH_2S), 35.86 (β-CH_2/Cys^5), 36.50 (β-CH_2/Cys^2), 42.98 (CH_2/Gly), 52.09 (α-CH/Tyr), 53.25 (α-CH/Phe), 54.20 (α-CH/Cys^5), 55.54 (α-CH/Cys^2), 122.96 (aromatic C3, C5/Tyr), 127.01 (aromatic C4/Phe), 128.07 (aromatic C2, C6/Phe), 129.21 (aromatic C3, C5/Phe), 129.88 (aromatic C2, C6/Tyr), 132.57 (aromatic C1/Tyr), 134.09 (aromatic C1/Phe), 153.22 (aromatic C4/Tyr), 170.01 (CO/Gly), 170.12 (CO/Cys^2), 170.87 (CO/Phe), 171.81 (CO/Tyr), 191.98 (CO/Cys^5).

FAB-MS: Found: m/z 603.2. Calcd for $C_{27}H_{33}N_5O_7S_2$: M^+, 603.2. Amino acid ratios (4% anisole/6 M HCl 120 °C, 24 h): Tyr (1) 0.93, Gly (1) 1.10, Phe (1) 1.17, Djenkolic acid (1) 0.94.

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13) Abbreviations: Symbols for amino acids and peptides are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: *Biochem. J.*, **219**, 345 (1984). The following other abbreviations were used: Acm, acetamidomethyl; Bzl, benzyl; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; ΔAla, dehydroalanine; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EI-MS, electron ionization-mass spectrometry; FAB-MS, fast atom bombardment-mass spectrometry; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Mpt, dimethylphosphinothioyl; NMR, nuclear magnetic resonance; OMe, methyl ester; Pen, penicillamine; Ppt, diphenylphosphinothioyl; TBAF·xH$_2$O, tetrabutylammonium fluoride hydrate; Tca, trichloroacetyl; TEA, triethylamine; THF, tetrahydrofuran; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

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