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Studies on Peptides. LXIX.[1,2] Selective Removal of Acid Labile α-Amino Protecting Groups with Dilute Sulfonic Acids

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Among various dilute sulfonic acids tested, 2 to 4N ethanesulfonic acid in acetic acid or methylenechloride was found suitable as a deprotecting reagent for the acid labile α-amino protecting groups, such as Boc and Z(OMe), which are currently removed by trifluoroacetic acid or dilute hydrochloric acid, since the deprotection occured selectively within 60 minutes at room temperature leaving intact other side chain protecting groups, such as Z, benzyl ester, S-*p*-methoxybenzyl and N^G-*p*-methoxybenzenesulfonyl groups. This reagent was applied to the synthesis of three model peptides; Met and Leu-enkephalin and endorphin.

Keywords——methanesulfonic acid in AcOH; 2—4N ethanesulfonic acid in AcOH; bromocamphoresulfonic acid in AcOH; *p*-toluenesulfonic acid in AcOH; Met-enkephalin; Leu-enkephalin; endorphin; Z(OMe)–Lys(Z)–OH; Boc–Lys(Z)–OH; Z(OMe)–Asp-(OBzl)–OH

Currently we have achieved the synthesis of several biologically active peptides, such as gastric inhibitory polypeptide (GIP)[4] and [27-Tyr]-cholecystokinin-pancreozymin (CCK-PZ)[5] by utilizing the TFA labile Z(OMe) group[6] as a sole α-amino protecting group. Along strategies synthesizing such complex peptides containing Lys, Glu and Asp, as well as Met, the Z(OMe) group has to be selectively removed prior to each chain elongation reaction from intermediates by TFA under limited conditions without affecting side chain protecting groups employed; *i. e.*, Lys(Z), Asp(OBzl) and Glu(OBzl). This situation is quite similar to that of the Merrifield solid phase peptide synthesis,[7] in which the Boc group[8] is selectively cleaved in most cases by 50% TFA–methylenechloride from resin bounded peptides bearing similar side chain protecting groups. It was pointed out however that the routine TFA treatment is apt to partially cleave the Z group from Lys(Z) and consequently N^{ε}-branched peptides may accumulate as a contaminant.[9] This side reaction can be prevented or suppressed by

1) Part LXVIII: H. Yajima, Y. Mori, K. Koyama, T. Tobe, M. Setoyama, H. Adachi, T. Kanno, and A. Saito, *Chem. Pharm. Bull.* (Tokyo), **24**, 2794 (1976).
2) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: *Biochem.*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, Z(OMe) or pMZ=*p*-methoxybenzyloxycarbonyl, Boc=*tert*-butoxycarbonyl, OBzl=benzyl ester, MBzl=*p*-methoxybenzyl, TFA=trifluoroacetic acid.
3) Location: *606, Sakyo-ku, Kyoto.*
4) H. Yajima, H. Ogawa, M. Kubota, T. Tobe, M. Fujimura, K. Henmi, K. Torizuka, H. Adachi, H. Imura, and T. Taminato, *J. Am. Chem. Soc.*, **97**, 5593 (1975).
5) H. Yajima, Y. Mori, Y. Kiso, K. Koyama, T. Tobe, M. Setoyama, H. Adachi, T. Kanno, and A. Saito, *Chem. Pharm. Bull.* (Tokyo), **24**, 1110 (1976).
6) *a*) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962); *b*) H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **17**, 1962 (1969); *c*) H. Yajima, F. Tamura, and Y. Kiso, *ibid.*, **18**, 2574 (1970); *d*) H. Yajima, F. Tamura, Y. Kiso, and M. Kurobe, *ibid.*, **21**, 1380 (1973).
7) R.B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
8) F.C. McKay and N.F. Albertson, *J. Am. Chem. Soc.*, **79**, 4686 (1957).
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either selecting more TFA resistant protecting groups at Lys in one hand or by applying alternate suitable deblocking reagents for the Z(OMe) or Boc groups in the other. Along the former category, the diisopropylmethyloxycarbonyl group[10] or the chloro-substituted Z groups[11] were recommended.

Along the latter category, we have examined the usefulness of various sulfonic acids in dilute solutions in order to find alternative deblocking conditions, under which the partial cleavage of the side chain protecting groups can be effectively suppressed. First, three amino acid derivatives, Z(OMe)-Lys(Z)-OH,[6a,b] Boc-Lys(Z)-OH[12] and Z(OMe)-Asp(OBzl)–OH[13] were selected as the model compound and behaviours of these protecting groups toward the action of commercially available sulfonic acids were examined in various concentrations diluted with AcOH or AcOH–CH_2Cl_2. Sulfonic acids examined here are not freely soluble in CH_2Cl_2 and therefore the latter solvent system was employed for comparison with the AcOH system. Among these reagents, concentrated methanesulfonic acid (MSA) was examined previously as a deprotecting reagent in the final step of peptide synthesis.[14]

Each sample was exposed to various dilute reagent in the presence of anisole as a cation scavenger at room temperature (18°) and solutions were examined quantitatively by the Shimadzu thin layer chromatography scanner at three intervals: one, three and 24 hours. From these experiments, following tendencies were observed (see Table I, II and III). (1) Results obtained in the MSA or ethanesulfonic acid (ESA)–AcOH system were comparable to those of the AcOH–CH_2Cl_2 system respectively. (2) Acidolytic cleavage of the Z group

Table I. Removal of the Z(OMe) Group from Z(OMe)-Lys(Z)-OH

in AcOH		Product								
		60 min			3 hr			24 hr		
		Z(OMe)-Lys	Z-Lys	Lys	Z(OMe)-Lys	Z-Lys	Lys	Z(OMe)-Lys	L-Lys	Lys
MSA	4 N	0	100	0	0	100	0	0	47.4	52.6
	2 N	0	100	0	0	100	0	0	72.9	27.1
	1 N	0	100	0	0	100	0	0	92.2	7.8
	0.5 N	0	100	0	0	100	0	0	0	0
	0.1 N	23.6	76.4	0	6.6	93.4	0	0	100	0
	0.05 N	58.5	41.5	0	11.5	88.5	0	0	100	0
	0.01 N	66.7	33.3	0	44.6	55.4	0	24.5	75.5	0
ESA	4 N	0	100	0	0	100	0	0	90.0	10.0
	2 N	0	100	0	0	100	0	0	98.0	2.0
	1 N	0	100	0	0	100	0	0	100	0
	0.5 N	0	100	0	0	100	0	0	100	0
	0.1 N	33.3	66.7	0	26.8	73.2	0	2.1	97.9	0
	0.05 N	70.9	29.1	0	51.4	48.6	0	23.1	76.9	0
	0.01 N	77.1	22.9	0	62.8	37.2	0	49.2	50.8	0
BSA or Tos-OH or Mes-SA	2 N	0	100	0	0	100	0	0	100	0
	1 N	0	100	0	0	100	0	0	100	0
Bc-8-SA or Bc-10-SA	1 N	0	100	0	0	100	0	0	100	0

MSA= methanesulfonic acid, ESA= ethanesulfonic acid, BSA= benzenesulfonic acid, Tos-OH= toluenesulfonic acid, Mes-SA=mesitylenesulfonic acid, Bc-8 or 10-SA= 3 bromocamphore-8 or 10-sulfonic acid

10) S. Sakakibara, T. Fukuda, Y. Kishida, and I. Honda, *Bull. Chem., Soc. Japan*, **43**, 3322 (1970).
11) K. Noda, S. Terada, and N. Izumiya, *Bull. Chem. Soc., Japan*, **43**, 1883 (1970); D. Yamashiro and C.H. Li, *J. Org. Chem.*, **38**, 591 (1973); B.W. Erickson and R.B. Merrifield, *J. Am. Chem. Soc.*, **95**, 3757 (1973).
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13) H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **19**, 420 (1971).
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occured within 24 hours' on-standing with 2 to 4 N MSA and ESA, but in both solvent systems, the partial cleavage was not observed within one to three hours' treatments. (3) As for the selectivity in deprotection between the Z and the Z(OMe) or Boc group, dilute aromatic sulfonic acids and bromocamphoresulfonic acids (BC–SA) seem to be better reagents than MSA or ESA, since no destruction of the Z group was observed in 1 to 2N solutions after 24 hours. (4) Conditions for selective removal between the Z(OMe) and the Boc groups seem difficult to establish, even if varying the concentration of sulfonic acids with different acidities.[15] For example both groups were partially cleaved in very dilute solutions, such as 0.01 N MSA or ESA. (5) The benzyl ester survived under various conditions tested. Thus removal of the Boc or Z(OMe) group from peptides containing Asp(OBzl) and Glu(OBzl) can be achieved more selectively than Lys(Z).

Table II. Removal of the Boc Group from Boc-Lys(Z)-OH

in AcOH		Product								
		60 min			3 hr			24 hr		
		Z Boc-Lys	Z Lys	Lys	Z Boc-Lys	Z Lys	Lys	Z Boc-Lys	Z Lys	Lys
MSA	1 N	0	100	0	0	100	0	0	87.5	12.5
	0.5 N	0	100	0	0	100	0	0	100	0
	0.1 N	34.4	65.6	0	16.9	83.1	0	13.2	86.8	0
	0.05 N	52.0	48.0	0	39.1	60.9	0	20.3	79.7	0
	0.01 N	78.7	21.3	0	50.3	49.7	0	32.3	67.3	0
ESA	1 N	0	100	0	0	100	0	0	100	0
	0.5 N	32.4	67.6	0	0	100	0	0	100	0
	0.1 N	43.2	56.8	0	33.7	66.3	0	6.4	93.6	0
	0.05 N	74.5	25.5	0	46.7	53.3	0	10.1	89.9	0
	0.01 N	91.7	8.3	0	60.5	39.5	0	20.8	79.2	0
BSA or	2 N	0	100	0	0	100	0	0	100	0
Tos-OH or Mes-SA	1 N	0	100	0	0	100	0	0	100	0
Bc-8-SA or Bc-10-SA	1 N	0	100	0	0	100	0	0	100	0

Table III. Removal of the Z(OMe) Group from Z(OMe)-Asp(OBzl)-OH

in AcOH		Product								
		60 min			3 hr			24 hr		
		OBzl pMZ-Asp	OBzl Asp	Asp	OBzl pMZ-Asp	OBzl Asp	Asp	OBzl pMZ-Asp	Bzl Asp	Asp
MSA or ESA	4 N	0	100	0	0	100	0	0	100	0
	2 N	0	100	0	0	100	0	0	100	0
	1 N	0	100	0	0	100	0	0	100	0
BSA or	4 N	0	100	0	0	100	0	0	100	0
Tos-OH or	2 N	0	100	0	0	100	0	0	100	0
Mes-SA	1 N	0	100	0	0	100	0	0	100	0

Next, we consider to select a suitable one of these deprotecting conditions for practical peptide synthesis. Usually the TFA deprotection of the Boc or Z(OMe) group is performed in the presence of anisole in an ice-bath within 60 minutes and a deprotected peptide, after

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precipitation with ether or petroleum ether, is submitted to the condensation reaction. Aromatic sulfonic acids examined here are not freely soluble in ether or petroleum ether. Therefore possibility exists that after deprotection, these reagents are precipitated together with a deprotected peptide by these solvents. In a practical standpoint, a dilute ESA solution, a much milder acid than MSA,[15] was thought to be more attractive for this deprotecting purpose, since our test indicated that MSA itself cleaved the Z group completely within 30 minutes, while ESA required more than 3 hours. If a high dilute solution were employed, difficulty may arise to precipitate a deblocked peptide quantitatively with ether or petroleum ether. We therefore judged that a solution of ESA in AcOH in concentrations around 2 to 4 N (amount around 5 equivalents) is a preferable reagent for practical peptide synthesis.

Next, utilizing such a deprotecting reagent as a main tool, three model peptides, H-Tyr-Gly-Gly-Phe-X-OH (I, X=Met, II, X=Leu) and H-Tyr-Gly-Gly-Gly-Lys-Met-Gly-OH (III) were synthesized. Among these, I and II are the compounds termed as enkephalin by Hughes, *et al.*[16a] as natural ligands for opiate receptors and were synthesized by the conventional manner[6b-d] or by the solid phase method.[6e] The latter heptapeptide is a model opioid peptide termed as endorphin by Goldstein, *et al.*[17] in 1975.

Our synthetic scheme of I is illustrated in Fig. 1. Z(OMe)-Gly-Gly-OMe and Z(OMe)-Phe-Met-OH were prepared by the DCC[18] and the *p*-nitrophenyl ester procedures[19] respectively. These peptides were exposed to a solution of 4 N ESA–AcOH in the presence of anisole in an ice-bath. When examined by thin layer chromatography, cleavage of the Z(OMe) group was completed within 30 minutes. After standing for an additional 30 minutes at room temperature, dry *n*-hexane was added to precipitate the deblocked peptides. In both cases, oily precipitates were obtained. They were washed with ether, dried over KOH pellets *in vacuo* for 3 hr and then submitted to the next coupling reactions. The former dipeptide ester was condensed with Z(OMe)-Tyr-$NHNH_2$[20] by the Honzl and Rudinger's azide procedure[21] and the resulting protected tripeptide ester was converted in the usual manner to the corresponding hydrazide, Z(OMe)-Tyr-Gly-Gly-$NHNH_2$. This hydrazide was condensed with the latter deprotected dipeptide, H-Phe-Met-OH, by the same azide procedure. The resulting protected pentapeptide, Z(OMe)-Tyr-Gly-Gly-Phe-Met-OH, was exposed to dilute ESA under conditions mentioned above and the ESA salt precipitated as a powder with ether was then converted to the corresponding acetate by Amberlite CG-4B. In order to secure the complete removal of ESA, the product was purified by column chromatography on DEAE-cellulose. Purity of synthetic Met-enkephalin (I) thus obtained was ascertained by four criteria; thin layer chromatography, elemental analysis, 3 N Tos-OH hydrolysis and aminopeptidase (AP–M) digest.[22] Leu-enkephalin (II) was obtained in essentially the same manner (Fig. 1) as stated above, though the deprotected dipeptide, H-Phe-Leu-OH, was precipitated as powder, while the pentapeptide ESA salt as an oil.

Through these model syntheses, it was noticed that deprotected peptides could be almost quantitatively precipitated from solutions with ether or *n*-hexane, though ESA salts of relatively small peptides are apt to give oily compounds. Recovery of Met in AP–M digest of I

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17) A. Goldstein, J.S. Goldstein, and B.M. Cox, *Life Science*, **17**, 1643 (1975).
18) J.S. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
19) B. Bodanszky, *Chem. Ind.*, **1955**, 1517.
20) N. Fujii and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 2446 (1975).
21) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).
22) G. Pfleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963). AP–M was purchased from the Protein Research Foundation (Osaka, Minoo).

was satisfactory indicating that this residue sensitive to concentrated trifluoromethanesufolnic acid (TFMSA)[23] and MSA,[14] remained intact through-out treatments with dilute ESA.

Endorphin, H-Tyr-Gly-Gly-Gly-Lys-Met-Gly-OH (III) is a suitable model compound for examining the selective removal of the Z(OMe) group from Lys(Z)-peptides. According to the scheme illustrated in Fig. 2, III was synthesized using 2 N ESA–AcOH, instead of 4 N ESA, with which no partial cleavage of the Z group from Lys proceeded, when examined by the scanner. The deprotected dipeptide, H-Met-Gly-OH, was precipitated with ether as an oil, while the tetrapeptide, H-Gly-Lys(Z)-Met-Gly-OH as powder. Finally, removal of the Z and Z(OMe) groups from the protected heptapeptide, Z(OMe)-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-OH, was performed by hydrogen fluoride,[24] since an alternative deblocking reagent, such as TFMSA or MSA, is known to give some side reaction at the Met residue,[14,23] satisfactory suppression of which has remained to be solved at this moment.

Through these syntheses, we could demonstrated preliminarily that 2 N ESA–AcOH is a preferable deprotecting reagent of the Z(OMe) group, possibly the Boc group also, for the synthesis of peptides containing Lys(Z) in a preparative sense and this concentration can be increased up to around 4 N, if such special functional groups are absent in target peptides. Our further tests indicated that Cys (MBzl) and Arg(*p*-methoxybenzenesulfonyl)[25] are both stable, like Asp(OBzl), during the treatment with 2 to 4 N MSA or ESA–AcOH for 24 hours. It is known that Trp is more resistant to sulfonic acids than hydrochloric acid or TFA, since the Trp residue in proteins survives and is quantitatively determined after hydrolysis at 110° with 3 N Tos-OH.[26] These facts may support a view that dilute ESA procedure can be extended to the synthesis of more complex peptides.

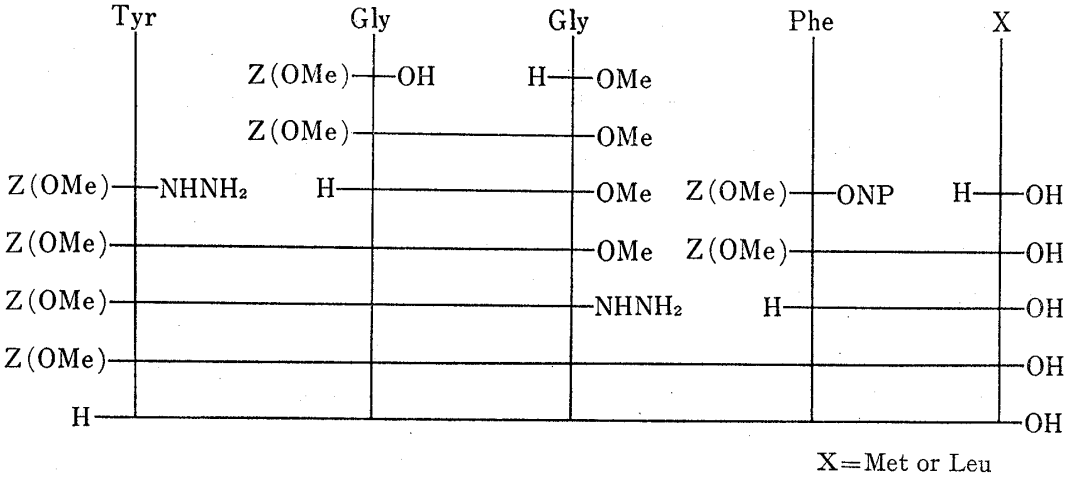

Fig. 1. Synthetic Scheme of Enkephalin

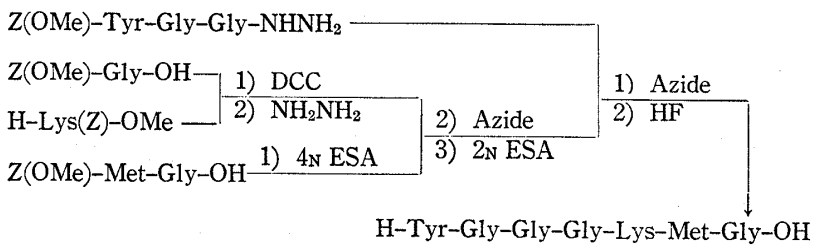

Fig. 2. Synthetic Scheme of Endorphin

23) H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J.C.S. Chem. Commun.*, **1974**, 107.
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25) O. Nishimura and M. Fujino, *Chem. Pharm. Bull.* (Tokyo), **24**, 1568 (1976).
26) T.Y. Liu and Y.H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).

Recently we have acquainted with reports of Goodacre, *et al.*[27] and Erickson and Wang[28] who applied 0.01 M Tos-OH–ethanol and 0.01 N MSA–0.01 M TFA in 1:1 (v/v) CH_2Cl_2–xylene for the deprotection of the Boc group respectively. Superior property of dilute sulfonic acids rather than hydrochloric acid or TFA seems to become apparent.

Our synthetic peptides were tested by Professor Hiroshi Takagi and his associates of our Faculty. Observable inhibitions of twitch in guinea pig ileum were similar to those expected from the literatures.[16,17]

Experimental

General experimental method employed in peptide synthesis are essentially the same as described in the Part 62[29] of this series. Ether was distilled over $LiAlH_4$ and stored over ferrous sulfate prior to its use. For the synthesis of Met-containing peptides, reactions were performed under the nitrogen atomosphere. Thin-layer chromatography (TLC) was performed on silica (Kiesel G, Merck) and *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (30:6:20:24).

Treatment of Amino Acid Derivatives with Dilute Sulfonic Acid

Treatment of three amino acid derivatives (1 mmol each), Z(OMe)–Lys(Z)–OH, Boc–Lys(Z)–OH and Z(OMe)–Asp(OBzl)–OH, with dilute sulfonic acids (5 equiv.) in AcOH or AcOH–CH_2Cl_2 (1:1, v/v) was performed in the presence of anisole (5 equiv.) at 18°. At three intervals, one, three and 24 hr, a part of the solution (1.2 μmol each) was examined by TLC. After spraying the ninhydrin reagent and heating in a oven (90°) for 15 min, the color intensities of Lys(Z), Lys, and Asp (OBzl) were measured quantitatively by Shimadzu dual wavelength TLC scanner (Model CS-900) and each amount was calculated by comparison with the standard integration of these authentic samples. The rest was deduced from these values and results are listed in Table I, II and III. Rf_1 values of reference samples: Z(OMe)–Lys(Z)–OH 0.58; H–Lys(Z)–OH 0.25; H–Lys–OH 0; Boc–Lys(Z)–OH 0.52; Z(OMe)–Asp(OBzl)–OH 0.55; H–Asp(OBzl)–OH 0.23; H–Asp–OH 0.

Z(OMe)-Gly-Gly-OMe——The title compound was prepared by condensation of Z(OMe)–Gly–OH (7.50 g) and H–Gly–OMe (prepared from 4.33 g of the hydrochloride with 4.8 ml of Et_3N) with DCC (7.12 g) in AcOEt–DMF (100 ml–100 ml). The product was isolated by the usual extraction procedure with AcOEt and recrystallized from MeOH and ether; yield 6.53 g (67%), mp 97—99°, Rf_1 0.69. *Anal.* Calcd. for $C_{14}H_{18}O_6N_2$: C, 54.19; H, 5.85; N, 9.03. Found: C, 54.38; H, 5.70; N, 9.00.

Z(OMe)-Tyr-Gly-Gly-OMe——In the presence of anisole (4.3 ml), Z(OMe)–Gly–Gly–OMe (3.10 g) was exposed to 2N ESA in AcOH (15 ml) in an ice-bath for 30 min and then at room temperature for 30 min and *n*-hexane was added. The resulting oily precipitate was washed with ether, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (30 ml) containing Et_3N (1.4 ml). Under cooling with ice-NaCl, the azide (prepared from 3.59 g of Z(OMe)–Tyr–$NHNH_2$ with 5.80 ml of 3.79N HCl–DMF, 1.48 ml of isoamylnitrite and 4.5 ml of Et_3N) in DMF (40 ml) was added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 10% citric acid and H_2O, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and then recrystallized from MeOH and ether; yield 3.94 g (83%), mp 144—146°, $[\alpha]_D^{24}$ +11.6° (c=0.3, DMF), Rf_1 0.74. *Anal.* Calcd. for $C_{23}H_{27}N_3O_8$: C, 58.34; H, 5.75; N, 8.88. Found: C, 58.60: H, 5.70; N, 8.98.

Z(OMe)-Tyr-Gly-Gly-$NHNH_2$——In the usual manner, Z(OMe)–Tyr–Gly–Gly–OMe (3.94 g) was dissolved in MeOH (70 ml) and 80% hydrazine hydrate (1.7 ml) was added. The gelatinous mass formed on standing overnight was collected by filtration and precipitated from DMF with EtOH; yield 2.62 g (66%), mp 200—204°, $[\alpha]_D^{24}$ −5.91° (c=0.3, DMSO), Rf_1 0.46. *Anal.* Calcd. for $C_{22}H_{27}O_7N_5$: C, 55.80; H, 5.75; N, 14.79. Found: C, 56.05; H, 5.77; N, 14.64.

Z(OMe)-Phe-Met-OH——Z(OMe)–Phe–ONP (10.0 g) dissolved in THF (40 ml) was added to a solution of H–Met–OH (3.30 g) in H_2O (40 ml) containing Et_3N (6.2 ml) and the mixture, after stirring at room temperature for 18 hr, was condensed. The residue was dissolved in H_2O, which after washing with ether, was acidified with 10% citric acid and the resulting precipitate was extracted with AcOEt. The organic phase was washed with 10% citric acid and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with *n*-hexane and recrystallized from MeOH and *n*-hexane; yield 9.13 g (89%), mp 148—150°, $[\alpha]_D^{24}$ −12.4° (c=0.3, MeOH), Rf_1 0.45. *Anal.* Calcd. for $C_{23}H_{28}O_6N_2S$: C, 59.98; H, 6.13; N, 6.08. Found: C, 59.92; H, 6.02; N, 6.14.

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Z(OMe)-Tyr-Gly-Gly-Phe-Met-OH——Under N_2 gas atomosphere, Z(OMe)–Phe–Met–OH (0.69 g) was treated with 4N ESA in AcOH (2.0 ml) in the presence of anisole (0.8 ml) in an ice-bath for 30 min and then at room temperature for 30 min. Dry ether was added. The resulting oily precipitate was washed twice with ether, dried over KOH pellets *in vacuo* for 2 hr and then dissolved in DMF (5 ml) containing Et_3N (0.42 ml). Under cooling with ice-NaCl, the azide (prepared from 0.66 g of Z(OMe)–Tyr–Gly–Gly–$NHNH_2$ with 0.79 ml of 3.79N HCl–DMF, 0.21 ml of isoamylnitrite and 0.63 ml of Et_3N) in DMF (10 ml) was added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was dissolved in 3% NH_4OH, which after washing with AcOEt, was acidified with 10% citric acid and the resulting oily precipitate was extracted with AcOEt. The organic phase was washed with 10% citric acid and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and the resulting powder was dissolved in a small amount of the solvent consisting of $CHCl_3$–MeOH–H_2O–DMF (8: 3: 1: 4) and the solution was applied to a column of silica (2.1×13 cm), which was eluted with the same solvent system. Fractions containing the substance of Rf_1 0.40 were combined and the solvent was evaporated. Treatment of the residue with H_2O afforded a fine powder, which was recrystallized from MeOH and AcOEt; yield 0.54 g (52%), mp 151—153°, $[\alpha]_D^{24}$ −18.7° (c=0.3, DMF). *Anal.* Calcd. for $C_{36}H_{43}O_{10}N_5S$: C, 58.60; H, 5.87; N, 9.49. Found: C, 58.48; H, 5.88; N, 9.46.

H-Tyr-Gly-Gly-Phe-Met-OH——Under N_2 gas atomosphere, the above protected pentapeptide (0.20 g) was treated with 4N ESA in AcOH (1.0 ml) in the presence of anisole (0.3 ml) in an ice-bath for 30 min and then at room temperature for 30 min and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 2 hr and then dissolved in H_2O (3 ml), which was treated with Amberlite CG-4B (type II, acetate form, approximately 1 g) for 30 min. The resin was removed by filtration and the filtrate was lyophilized. The residue was dissolved in a small amount of H_2O and the solution was applied to a column of DEAE-cellulose (2.2×7 cm) which was eluted with 0.01M NH_4HCO_3 (pH 7.8, 600 ml) through a mixing flask containing H_2O (150 ml). Individual fractions (8.5 ml each) were collected and absorbancy at 275 mμ was determined. The desired fractions (tube No. 28—50) were combined, the solvent was evaporated and the residue was lyophilized to give a fluffy powder, yield 65 mg (40%), $[\alpha]_D^{24}$ +17.7° (c=0.5, 3% AcOH), (lit.[16b] +18° in 1M AcOH; lit.[16c] +31.5° in MeOH), Rf_1 0.17. Amino acid ratios in 3N Tos–OH hydrolysate: Tyr 0.86, Gly 2.02, Phe 1.00, Met 0.81 (average recovery 85%), and in AP–M digest: Tyr 0.98, Gly 2.17, Phe 1.00, Met 0.99 (average recorvery 82%). *Anal.* Calcd. for $C_{27}H_{35}N_5O_7S$. 1.5 H_2O: C, 53.98; H, 6.38; N, 11.66. Found: C, 54.09; H, 6.05; N, 11.62.

Z(OMe)-Phe-Leu-OH——Z(OMe)–Phe–ONP (19.9 g) dissolved in THF (30 ml) was added to a solution of H–Leu–OH (4.37 g) in H_2O–pyridine (10 ml–20 ml) containing Et_3N (10 ml). The product was isolated as stated in the preparation of Z(OMe)–Phe–Met–OH and recrystallized from AcOEt and ether; yield 7.13 g (73%), mp 90—93°, $[\alpha]_D^{24}$ −6.3° (c=0.5, MeOH), Rf_1 0.58. *Anal.* Calcd. for $C_{24}H_{30}O_6N_2$: C, 65.14; H, 6.83; N, 6.33. Found: C, 64.87; H, 6.74; N, 6.35.

Z(OMe)-Tyr-Gly-Gly-Phe-Leu-OH——In the presence of anisole (0.8 ml), Z(OMe)–Phe–Leu–OH (0.66 g) was treated with 4N ESA in AcOH (3.0 ml) in an ice-bath for 30 min and then at room temperature for 30 min and *n*-hexane was added. When treated with ether, the oily precipitate turned to the solid, which was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (3 ml) containing Et_3N (0.42 ml). To this solution, the azide (prepared from 0.66 g of Z(OMe)–Tyr–Gly–Gly–$NHNH_2$ with 0.79 ml of 3.79N HCl–DMF, 0.21 ml of isoamylnitrite and 0.63 ml of Et_3N) in DMF (10 ml) was added under cooling with ice-NaCl and the mixture was stirred at 4° for 48 hr. As stated above in the purification of the Met-analogue, the product was isolated after the similar extraction followed by column chromatography on silica; yield 0.45 g (45%), mp 142—143°, $[\alpha]_D^{24}$ −27.9° (c=0.4, DMF), Rf_1 0.45. *Anal.* Calcd. for $C_{37}H_{45}O_{10}N_5$. 1/2H_2O: C, 60.97; H, 6.36; N, 9.61. Found: C, 61.06; H, 6.22; N, 9.43.

H-Tyr-Gly-Gly-Phe-Leu-OH——In the presence of anisole (0.5 ml), Z(OMe)–Tyr–Gly–Gly–Phe–Leu–OH (0.35 g) was treated with 4N ESA in AcOH (1.3 ml) in an ice-bath as stated above. Dry ether was added and the resulting oily precipitate was dried over KOH pellets *in vacuo* for 3 hr and the dissolved in H_2O (3 ml) and the solution was treated with Amberlite CG-4B (type II, acetate form, approximately 1 g) for 30 min. The resin was removed by filtration and the filtrate was lyophilized. The residue was similarly purified by column chromatography on DEAE cellulose (2.2×5.0 cm); yield 90 mg (32%), $[\alpha]_D^{24}$ +18.0° (c=0.7, 3% AcOH). (lit.[16c] +31.5° in MeOH) Rf_1 0.21. Amino acid ratios in a 3N Tos–OH hydrolysate: Tyr 0.97, Gly 2.04, Phe 1.02, Leu 1.00 (average recovery 87%). Amino acid ratios in an AP–M digest; Tyr 1.00, Gly 1.95, Phe 1.01, Leu 0.96 (average recovery 89%). *Anal.* Calcd. for $C_{28}H_{37}O_7N_5$. 1.5 H_2O: C, 57.72; H, 6.92; N, 12.02. Found: C, 57.99; H, 6.82; N, 11.87.

Z(OMe)-Gly-Lys(Z)-OMe——The title compound was prepared by condensation of Z(OMe)–Gly–OH (2.29 g) and H–Lys(Z)–OMe (prepared from 3.31 g of the hydrochloride with 1.52 ml of Et_3N) with DCC (2.26 g) in THF–DMF (10 ml–10 ml). The product was isolated by the usual extraction procedure with AcOEt and recrystallized from AcOEt and ether; yield 2.72 g (53%), mp 78—82°, $[\alpha]_D^{24}$ −6.9° (c=0.3, MeOH), Rf_1 0.75. *Anal.* Calcd. for $C_{26}H_{33}O_8N_3$: C, 60.57; H, 6.45; N, 8.15. Found: C, 60.61; H, 6.57; N, 8.33.

Z(OMe)-Gly-Lys(Z)-$NHNH_2$——Z(OMe)–Gly–Lys(Z)–OMe (2.72 g) in MeOH (20 ml) was converted to the corresponding hydrazide with 80% hydrazine hydrate (2.6 ml) in the usual manner. The product

was recrystallized from MeOH; yield 2.31 g (85%), mp 161—166°, $[\alpha]_D^{24}$ +3.3° (c=0.3, DMF), Rf_1 0.56. *Anal.* Calcd. for $C_{25}H_{33}N_5O_7$: C, 58.23; H, 6.45; N, 13.59. Found: C, 58.40; H, 6.34; N, 13.69.

Z(OMe)-Gly-Lys(Z)-Met-Gly-OH——As stated above, Z(OMe)-Met-Gly-OH (0.74 g) was treated with 4N ESA-AcOH (2.5 ml) in the presence of anisole (1.1 ml). The resulting ESA salt precipitated as an oil by ether was dissolved in DMF (5 ml), to which Et_3N (0.56 ml) and the azide (prepared from 0.93 g of Z(OMe)-Gly-Lys(Z)-$NHNH_2$ with 1.0 ml of 3.79N HCl-DMF, 0.27 ml of isoamylnitrite and 0.78 ml of Et_3N) in DMF (10 ml). The mixture was stirred at 4° for 48 hr, the solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 10% citric acid and H_2O-NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from MeOH and AcOEt; yield 1.11 g (80%), mp 138—139°, $[\alpha]_D^{24}$ −16.6° (c=0.3, DMF), Rf_1 0.38. *Anal.* Calcd. for $C_{32}H_{43}O_{10}N_5S$: C, 55.72; H, 6.28; N, 10.15. Found: C, 55.95; H, 6.32; N, 9.95.

Z(OMe)-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-OH——As stated above, Z(OMe)-Gly-Lys(Z)-Met-Gly-OH (1.11 g) was treated with 2N ESA-AcOH (8.0 ml) in the presence of anisole (1.8 ml) and the resulting ESA salt precipitated by ether as powders was then dissolved in DMF (5 ml). Et_3N (0.45 ml) and the azide (prepared from 0.71 g of Z(OMe)-Tyr-Gly-Gly-$NHNH_2$ as stated above) in DMF (10 ml) were combined and the mixture was stirred at 4° for 48 hr. The product was isolated by the usual extraction procedure and precipitated from MeOH and AcOEt; yield 1.07 g (69%), mp 165—167°, $[\alpha]_D^{24}$ −26.6° (c=0.4, DMF). Rf_1 0.22. Amino acid ratios in 3N Tos-OH hydrolysate: Tyr 0.80, Gly 4.07, Lys 1.00, Met 0.85 (average recovery 85%). *Anal.* Calcd. for $C_{45}H_{58}O_{14}N_8S$: C, 55.89; H, 6.05; N, 11.59. Found: C, 55.84; H, 6.18; N, 11.35.

H-Tyr-Gly-Gly-Gly-Lys-Met-Gly-OH——The above protected heptapeptide (0.58 g) was treated with HF (approximately 3 ml) in the presence of anisole (1.0 ml) containing 2% ethanedithiol in an ice-bath for 60 min. The excess HF was removed by evaporation at 0° and the residue, after washing with ether, was dissolved in H_2O (30 ml). The solution was treated with Amberlite CG-4B (acetate form, *ca* 3 g) for 60 min, filtered and the filtrate was lyophilized. The residue was dissolved in a small amount of H_2O and the solution was applied to a column of CM-cellulose (2.2×5.0 cm), which was eluted with 0.01M NH_4HCO_3 (pH 7.8, 800 ml) through a mixing flask containing H_2O (150 ml). Individual fractions (8 ml each) were collected and absorbancy at 275 mμ was determined. Main fractions in the gradient eluates (tube No. 59—110) were combined and the solution was lyophilized. The residue was then applied to a column of Sephadex G-10 (3×140 cm) for desalting using 0.2M AcOH as an eluent and the product was finally lyophilized as a fluffy powder; yield 132 mg (33%), $[\alpha]_D^{24}$ −3.2° (c=0.6, 3% AcOH), Rf_2 0.33, Rf_3 0.52 (lit.[17] Rf 0.33, n-BuOH: AcOH: H_2O=8: 2: 2). Amino acid ratios in 3N Tos-OH hydrolysate: Tyr 0.92, Gly 4.25, Lys 1.00, Met 0.90 (average recovery 85%). Amino acid ratios in AP-M digest: Tyr 0.94, Gly 3.91, Lys 1.02, Met 1.00 (average recovery 75%). *Anal.* Calcd. for $C_{28}H_{44}O_9N_8S \cdot CH_3COOH$: C, 49.44; H, 6.64; N, 15.38. Found: C, 49.48; H, 6.49; N, 14.94.

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