[Chem. Pharm. Bull. 34(10)4356—4361(1986)]

Studies on Peptides. CXLIII.[1,2] Evaluation of β-Menthylaspartate for Peptide Synthesis

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(Received May 2, 1986)

The β-*l*-menthyl ester of aspartic acid, Asp(OMen), was found to be stable to trifluoroacetic acid (TFA) in an ice-bath for 3 h, but to be cleaved by HF or 1 M trifluoromethanesulfonic acid–thioanisole in TFA in an ice-bath within 60 min. Asp(OMen) was employed for the synthesis of tetragastrin, for which the use of diphenylsulfide, as an additional scavenger, is recommended to accelerate the acidolytic cleavage of this protecting group. This protecting group is superior to other available protecting groups so far examined in terms of suppression of base-catalyzed succinimide formation.

Keywords——β-menthylaspartate; base-catalyzed succinimide formation; acid-catalyzed succinimide formation; hydrogen fluoride deprotection; trifluoromethanesulfonic acid deprotection; cation scavenger; diphenylsulfide; tetragastrin

Base-catalyzed succinimide formation of the Asp (OBzl) residue[3] in peptides is known to be sequence-dependent.[4] This side reaction in peptide synthesis takes place predominantly when the carboxyl side of the Asp (OBzl) residue is adjacent to amino acids with sterically less hindered side chains, such as Gly and Ser or occasionally particular amino acids, such as Asn[5] and Phe.[6] In order to suppress this side reaction, the β-cyclopentyl (Cpe) and the β-cyclohexyl (Chx) esters of aspartic acid were introduced for use in solid-phase peptide synthesis by Blake[7] and Tam *et al.*,[8] respectively. Recently we reported that the cycloheptyl (Chp) and the cyclooctyl (Coc) esters similarly have an ability to suppress this side reaction.[9] When five model peptides, Z(OMe)–Ala–Asp(OR)–Gly–OBzl (R = Bzl, Cpe, Chx, Chp and Coc), were exposed to Et_3N, the Bzl ester derivative gave the largest amount of the side product, while the Coc ester was least susceptible to base, though the differences between these cycloalkyl esters so far tested were not large. Thus, the steric nature of the β-protecting groups seems to play an important role in suppressing this side reaction. Thus, we have evaluated the properties of β-*l*-menthylaspartate [H–Asp(OMen)–OH], the protecting group of which has two bulky side chains compared to the Chx group, and we found that this group was less susceptible to base than the cycloalkyl esters mentioned above. Boc–Asp(OMen)–OH was easily prepared according to the procedure of Tam *et al.*[8] for the preparation of the corresponding Chx ester. Boc–Asp–OBzl[10] was esterified with easily available *l*-menthol with the aid of DCC and DMAP, and the Bzl group was removed from the resulting diester by hydrogenolysis to give the desired ester as a crystalline compound (Fig. 1).

First, the stability of the Men group to TFA was examined. In an ice-bath, the Boc group was selectively cleaved by TFA within 60 min, but no Asp was detected on thin layer chromatography (TLC), even after 180 min. The result indicated that this group survives under the TFA treatment (usually 60 to 80 min) required for N^{α}-deprotection in practical

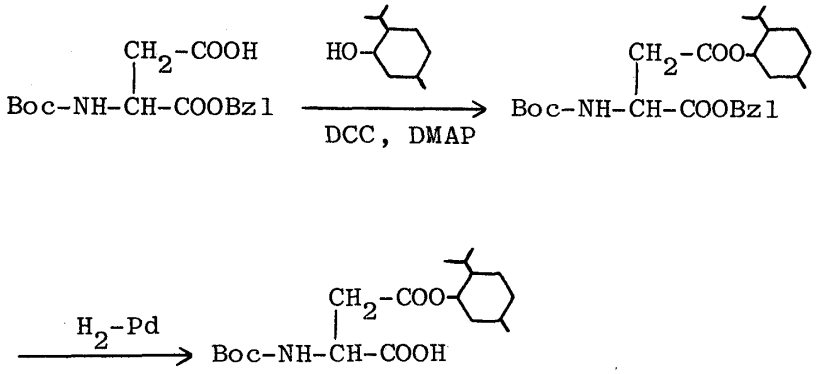

Fig. 1. Preparation of Boc–Asp(OMen)–OH

TABLE I. Succinimide Formation (%) from Z(OMe)–Ala–Asp(OR)–Gly–OBzl upon Base and Acid Treatments

R	Et_3N treatment (24 °C)		Acid treatment (0 °C)	
	20 h	40 h	HF	1 M TFMSA–thioanisole/TFA
Men	0.4	3.9	1.3	0.7
Bzl[9]	25.0	36.7	6.8	2.4
Coc[9]	1.9	5.2	5.4	3.2

peptide synthesis. Next, the susceptibility of this protecting group to HF[11] or 1 M TFMSA–thioanisole in TFA[12] was examined. This group was found to be cleaved quantitatively at ice-bath temperature within 60 min in both cases. However, as described later, we noticed that when Asp(OMen) was involved in the peptide chain, there was some sequence–dependency in the rate of acidolytic cleavage of this protecting group, as well as in the degree of succinimide formation. Next, using a model peptide, Z(OMe)–Ala–Asp(OMen)–Gly–OBzl, we examined the sensitivity of this protecting group to Et_3N. The above tripeptide ester in DMF exposed to Et_3N (1 eq) at 24 °C and the corresponding imide[9] formed after 20 and 40 h, were quantitated with a dual-wavelength TLC scanner. The amounts of the succinimide derivative detected were less than those in the case of the Bzl and the Coc derivatives[9] (Table I). Within 20 h, the amount formed can be judged to be negligible. Thus, this protecting group with two bulky side chains strongly suppressed the ring closure in the most base-sensitive Asp–Gly bond. Next, succinimide formation under acid deprotecting conditions was examined. This side reaction seems to depend mainly on the acid conditions employed rather than the nature of the protecting groups. The amount of succinimide formed with 1 M TFMSA–thioanisole in TFA was only 0.7%, and that in the case of HF treatment was 1.3%. Thus, as far as these model experiments are concerned, this protecting group exhibited attractive properties with both base and acid.

Next, the usefulness of Asp(OMen) for practical peptide synthesis was examined by using tetragastrin, Trp–Met–Asp–Phe–NH_2,[13] as an example, since Asp(OBzl)–Phe was reported to be relatively sensitive to base and acid.[14] Boc–Trp(Mts)–Met–Asp(OMen)–Phe–NH_2 was prepared in a stepwise manner by the active ester procedure, followed by reduction of Met(O) with trimethylphenylthiosilane[15] (Fig. 2). In the preparation step of Boc–Asp(OMen)–Phe–NH_2, the acyl component, Boc–Asp(OMen)–OSu, had to be used in a slight excess (1.4 eq) to complete the coupling reaction, presumably due to the bulkiness of its side chain. The rest of the reactions proceeded as usual without any side product detectable on TLC. The results

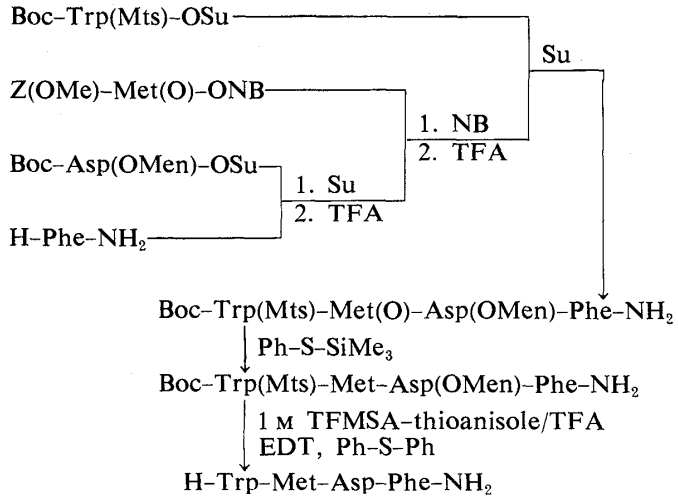

Fig. 2. Synthetic Scheme for Tetragastrin

TABLE II. Effect of Various Cation Scavengers for Deprotection of Boc–Asp(OMen)–Phe–NH_2 with 1 M TFMSA–Thioanisole/TFA (50 eq) at 0 °C

Scavengers (20 eq)	H–Asp–Phe–NH_2 (%)[a]	
	60 min	120 min
m-Cresol	36.1	41.4
Ethanedithiol	43.5	80.7
Dimethylselenide	46.1	62.1
Diisopropylsulfide	54.2	78.3
o-Thiocresol	74.8	90.8
p-Thiocresol	68.6	91.0
Dimethylsulfide	50.4	91.3
Thiophenol	71.3	92.2
Diphenylsulfide	95.3	≒100

a) Determined by using a TLC scanner.

indicated that care is necessary for the introduction of Asp(OMen) into the peptide chain, but later reactions can be performed without particular difficulty. In the deprotecting step, we found that the Men group could not be removed from Boc–Trp(Mts)–Met–Asp(OMen)–Phe–NH_2 by treatment with 1 M TFMSA–thioanisole/TFA (30 to 100 eq of TFMSA) within 120 min at 0 °C, in spite of the fact that under identical conditions, the same group was completely cleaved from Z(OMe)–Ala–Asp(OMen)–Gly–OBzl. Presumably the *l*-configuration of the menthyl moiety does not participate in resistance to this acidolytic deprotection. EDT, used to prevent indole-alkylation of Trp,[16] was unable to accelerate this acidolytic cleavage. Then Boc–Asp(OMen)–Phe–NH_2 was treated with the above acid in the presence of various additional scavengers such as dimethylselenide,[17] *p*-thiocresol,[18] dimethylsulfide,[19] and thiophenol,[20] as shown in Table II. We found that this protecting group could be cleaved completely by addition of diphenylsulfide (20 eq), a sulfur compound with higher nucleophilicity than thioanisole. With the aid of this additional scavenger, we were able to obtain tetragastrin in 65% yield.

When the crude products were examined by HPLC, a certain amount of the succinimide derivative[14] was detected. Problems concerning the sequence-dependency involved in removal of various protecting groups, as well as in acid-catalyzed succinimide formation of Asp, have never been investigated. However, the above results suggested that this tendency must be

taken into account in any deprotecting reaction. In the above deprotecting reaction, replacement of thioanisole by diphenylsulfide is impractical, since diphenylsulfide is not freely soluble in TFA. From these experimental results, we conclude that Asp(OMen) is an attractive derivative for the synthesis of peptides containing particularly base-sensitive Asp residues, such as Asp–Gly. Application of Asp(OMen) to solid-phase peptide synthesis seems worthy of examination in the future.

Experimental

The *Rf* values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems: Rf_1 $CHCl_3$, Rf_2 $CHCl_3$–MeOH (10:0.5), Rf_3 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_4 *n*-BuOH–AcOH–AcOEt–H_2O (1:1:1:1), Rf_5 *n*-BuOH–pyridine–AcOH–H_2O (4:1:1:2), Rf_6 *n*-BuOH–pyridine–AcOH–H_2O (3:1:1:1). After spraying of ninhydrin reagent, a plate was heated in an oven at 90 °C for 15 min and the color intensity was measured with a Shimadzu dual-wavelength TLC scanner (model CS-900). High performance liquid chromatography (HPLC) was conducted with a Waters 204 compact model. Leucine aminopeptidase (Lot. No. L-6007) was purchased from Sigma.

Boc–Asp(OMen)–OBzl——DCC (3.84 g, 1.2 eq) was added to a solution of Boc–Asp–OBzl [prepared from 7.54 g (14.94 mmol) of the DCHA salt], *l*-menthol (3.63 g, 1.5 eq) and DMAP (0.19 g, 0.1 eq) in AcOEt and the mixture was stirred in an ice-bath overnight. The solution was filtered. The filtrate was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and concentrated. The residue was treated with *n*-hexane to afford a powder, which was recrystallized from MeOH and *n*-hexane; yield 6.12 g (89%), mp 90—91 °C, $[\alpha]_D^{18}$ −48.4° (*c*=1.0, MeOH), Rf_1 0.52. *Anal.* Calcd for $C_{26}H_{39}NO_6$: C, 67.65; H, 8.52; N, 3.03. Found: C, 67.92; H, 8.55; N, 3.19.

Boc–Asp(OMen)–OH——Boc–Asp(OMen)–OBzl (2.62 g, 5.68 mmol) in MeOH–H_2O (30 ml–1 ml) containing a few drops of AcOH was hydrogenated over a Pd catalyst for 3h. The catalyst was removed by filtration, the filtrate was concentrated and the residue was treated with *n*-hexane to afford a solid, which was recrystallized from MeOH and *n*-hexane; yield 1.84 g (87%), mp 135—137 °C, $[\alpha]_D^{18}$ −17.7° (*c*=1.0, MeOH), Rf_3 0.63. *Anal.* Calcd for $C_{19}H_{33}NO_6$: C, 61.43; H, 8.95; N, 3.77. Found: C, 61.59; H, 9.07; N, 3.89.

Treatment of Boc–Asp(OMen)–OH with TFA——A sample (2.5 mg) was exposed to TFA–anisole (150 μl–2 μl) for 180 min. The Boc group was cleaved within 60 min. Rf_4 of H–Asp(OMen)–OH was 0.78. No spot corresponding to Asp was observed on TLC.

Treatment of Boc–Asp(OMen)–OH with HF——A sample (5 mg) was treated with HF (approximately 1 ml) in the presence of thioanisole (10 eq) in an ice-bath for 60 min. After evaporation of HF, the residue was washed with ether and analyzed in an amino acid analyzer. Recovery of Asp was 97.0%. Besides Asp, no other peak was observed.

Treatment of Boc–Asp(OMen)–OH with 1 M TFASA–Thioanisole/TFA——A sample (5 mg) was treated with 1 M TFMSA–thioanisole in TFA (0.3 ml) in the presence of *m*-cresol (10 eq) in an ice-bath for 60 min, then dry ether was added. The residue was examined with an amino acid analyzer. Recovery of Asp was 100%. Besides Asp, no other peak was detected.

Boc–Asp(OMen)–Gly–OBzl——DCC (0.73 g, 3.55 mmol) and HOSu (0.45 g, 3.88 mmol) were added to an ice-chilled solution of Boc–Asp(OMen)–OH (1.20 g, 3.23 mmol) in THF (8 ml) and the mixture, after being stirred at room temperature for 5 h, was filtered. The filtrate was added to a solution of H–Gly–OBzl [prepared from 1.08 g (3.23 mmol) of the tosylate] in DMF (10 ml) and the mixture, after being stirred overnight, was concentrated. The residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was further purified by column chromatography on silica gel (1.2 × 16 cm) using $CHCl_3$ as an eluant to give the protected dipeptide ester as an oil; yield 1.16 g (69%), Rf_2 0.90.

Z(OMe)–Ala–Asp(OMen)–Gly–OBzl——A TFA treated sample of Boc–Asp(OMen)–Gly–OBzl (0.27 g, 0.52 mmol) was dissolved in DMF (4 ml) together with Et_3N (0.07 ml, 1 eq), Z(OMe)–Ala–OSu (0.22 g, 1.2 eq) and NMM (0.05 ml, 1 eq) and the mixture was stirred overnight, then concentrated. Treatment of the residue with 5% citric acid afforded a powder, which was washed with 5% $NaHCO_3$ and H_2O and recrystallized from MeOH and ether; yield 0.28 g (82%), mp 114—116 °C, $[\alpha]_D^{14}$ −39.4° (*c*=1.0, MeOH), Rf_2 0.74. *Anal.* Calcd for $C_{35}H_{47}N_3O_9$: C, 64.30; H, 7.25; N, 6.43. Found: C, 64.38; H, 7.31; N, 6.47.

Treatment of Z(OMe)–Ala–Asp(OMen)–Gly–OBzl with Et_3N——In the presence of Et_3N (1 eq), a solution of a sample (5 mg) in DMF (50 μl) was kept standing at 24 °C. After 20 and 40 h, an aliquot was examined by TLC in the solvent system of $CHCl_3$–MeOH (10:0.5). An authentic sample of Z(OMe)–Ala–Asc–Gly–OBzl[9] served to monitor the reaction. Each spot was measured with a dual-wavelength TLC scanner, and the results are shown in Table I.

Treatment of Z(OMe)–Ala–Asp(OMen)–Gly–OBzl with 1 M TFMSA–Thioanisole/TFA——A sample (6.5 mg) was treated with 1 M TFMSA–thioanisole/TFA (300 μl, 30 eq) in the presence of *m*-cresol (30 eq) in an ice-bath for 120 min. The product was precipitated with ether and examined with a TLC scanner using an authentic sample of H–Ala–Asc–Gly–OH[9] as a reference. The result is listed in Table I. Besides the Asc-derivative (Rf_6 0.31) and H–Ala–

Asp–Gly–OH[9] (Rf_6 0.25), no other spot was detected.

Treatment of Z(OMe)–Ala–Asp(OMen)–Gly–OBzl with HF——A sample (6.5 mg) was treated with HF (*ca.* 1 ml) in the presence of thioanisole (30 eq) in an ice-bath for 120 min. HF was removed by evaporation and the residue was examined by TLC as described above. The result is listed in Table I.

Boc–Asp(OMen)–Phe–NH_2——A mixture of H–Phe–NH_2 [prepared from 0.20 g (0.61 mmol) of the Z(OMe)-derivative], Boc–Asp(OMen)–OSu (0.32 g, 1.1 eq) and NMM (0.08 ml, 1 eq) in DMF (1 ml) was stirred overnight, then additional Boc–Asp(OMen)–OSu (0.08 g, 0.3 eq) was added. Stirring was continued for an additional 4 h and the solution was concentrated. The residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and concentrated. Trituration of the residue with *n*-hexane afforded a powder, which was recrystallized from MeOH and *n*-hexane; yield 0.24 g (76%), mp 179—181 °C, $[\alpha]_D^{18}$ −52.0° (c=1.0, MeOH), Rf_2 0.49. *Anal.* Calcd for $C_{28}H_{43}N_3O_6$: C, 64.96; H, 8.37; N, 8.12. Found: C, 65.18; H, 8.55; N, 8.15.

Z(OMe)–Met(O)–Asp(OMen)–Phe–NH_2——A mixture of DCC (0.26 g, 1.27 mmol), Z(OMe)–Met(O)–OH (0.38 g, 1.16 mmol) and HONB (0.25 g, 1.39 mmol) in THF–DMF (5 ml–1 ml) was stirred for 4 h and filtered. The filtrate and NMM (0.11 ml, 0.97 mmol) were added to a solution of a TFA-treated sample of Boc–Asp(OMen)–Phe–NH_2 (0.50 g, 0.97 mmol) in DMF (2 ml) containing Et_3N (0.13 ml, 0.97 mmol) and the solution was stirred overnight, then concentrated. Treatment of the residue with 5% citric acid afforded a powder, which was precipitated from DMF with AcOEt; yield 0.65 g (92%), mp 212—214 °C, $[\alpha]_D^{18}$ −52.3° (c=0.7, DMF), Rf_3 0.67. *Anal.* Calcd for $C_{37}H_{52}N_4O_8S$: C, 60.97; H, 7.19; N, 7.69. Found: C, 60.78; H, 7.20; N, 7.81.

Boc–Trp(Mts)–Met(O)–Asp(OMen)–Phe–NH_2——A mixture of Boc–Trp(Mts)–OH [prepared from 0.66 g (0.99 mmol) of the DCHA salt], DCC (0.22 g, 1.09 mmol) and HOSu (0.14 g, 1.19 mmol) in THF (3 ml) was stirred for 5 h and filtered. The filtrate and NMM (0.09 ml, 0.82 mmol) were added to a solution of a TFA-treated sample of the above tripeptide (0.60 g, 0.82 mmol) in DMF (3 ml) containing Et_3N (0.11 ml, 0.82 mmol) and the solution, after being stirred overnight, was concentrated. Treatment of the residue with 5% citric acid afforded a powder which was precipitated from DMF with ether; yield 0.62 g (73%), mp 176—178 °C, $[\alpha]_D^{18}$ −31.0° (c=1.0, DMF), Rf_2 0.24, Rf_3 0.84. *Anal.* Calcd for $C_{53}H_{72}N_6O_{11}S_2$: C, 61.60; H, 7.02; N, 8.13. Found: C, 61.58; H, 7.29; N, 8.42.

Boc–Trp(Mts)–Met–Asp(OMen)–Phe–NH_2——Under an Ar atmosphere, the above Met(O)-derivative (100 mg, 97 μmol) in DMF (2 ml) was incubated with trimethylphenylthiosilane (0.37 ml, 20 eq) at 40 °C for 15 min. On TLC, the starting material (Rf_2 0.24) disappeared and a new spot (Rf_2 0.72) was detected. The solvent was removed by evaporation. Treatment of the residue with H_2O afforded a powder, which was recrystallized from DMF and isopropyl ether; yield 95 mg (95%), mp 178—181 °C, $[\alpha]_D^{20}$ −44.0° (c=1.0, DMF). Amino acid ratios in a 4 N MSA hydrolysate: Trp 0.99, Met 0.89, Asp 1.02, Phe 1.00 (recovery of Phe 84%). *Anal.* Calcd for $C_{53}H_{72}N_6O_{10}S_2 \cdot H_2O$: C, 61.48; H, 7.20; N, 8.12. Found: C, 61.59; H, 7.26; N, 8.20.

Deprotection of Boc–Trp(Mts)–Met–Asp(OMen)–Phe–NH_2 with 1 M TFMSA–Thioanisole/TFA——A sample (5 mg, 5 μmol) was treated with 1 M TFMSA–thioanisole/TFA (0.50 ml, 100 eq of TFMSA) in the presence of EDT (4.7 μl, 10 eq) in an ice-bath for 120 min, then *n*-hexane was added. In order to remove the scavenger, the residue was dissolved in CH_3CN–0.1% TFA (1 : 1, 0.5 ml) and applied to a column of Sephadex LH-20 (2.2 × 40 cm), which was eluted with the same solvent. The fractions (4.5 ml each) corresponding to the front peak (monitored by measuring the UV absorption at 280 nm) were combined and the solvent was removed by evaporation. The residue was examined by HPLC on a Nucleosil $5C_{18}$ column (4 × 150 mm), which was eluted first by isocratic elution with 30% CH_3CN for 3 min, then by gradient elution with CH_3CN (30—50%, 20 min) in 0.1% TFA aq at the flow rate of 0.8 ml per min. Every peak was integrated to determine the amount of each product: (A) H–Trp–Met–Asp–Phe–NH_2

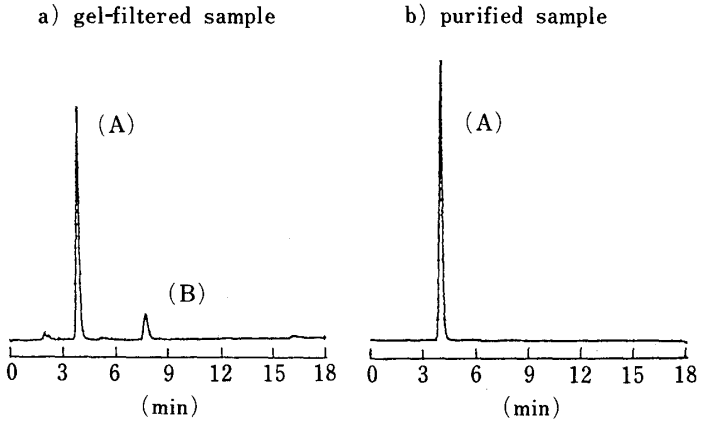

Fig. 3. HPLC of Synthetic Tetragastrin

(A) H–Trp–Met–Asp–Phe–NH_2. (B) H–Trp–Met–Asc–Phe–NH_2.
Column: Nucleosil 5C18 (4 × 150 mm). Flow rate: 0.8 ml/min. Solvent: CH_3CN (30—50%, 20 min)–0.1% TFA. OD: 280 nm.

(14.0%, retention time 4.5 min), (B) H–Trp–Met–Asc–Phe–NH_2 (6.0% retention time 7.8 min), (C) H–Trp–Met–Asp(OMen)–Phe–NH_2 (62.1%, retention time 27.3 min) and (D) unidentified products presumably due to indole-modification (17.9%). For identification, each product recovered from HPLC was subjected to aminopeptidase digestion, then to amino acid analysis: (A) Trp 0.92, Met 0.93, Asp 0.97, Phe 1.00. (B) Trp 1.00, Met 1.00, Asp 0.14, Phe 0.17. (C) Trp 0.97, Met 1.01, Phe 1.00, Asp(OMen) N. D.

Characterization of tetragastrin (A): $[\alpha]_D^{19}$ $-15.1°$ ($c=0.2$, 1 N AcOH, lit.[13] $-26.5°$ in DMF), Rf_5 0.66. *Anal.* Calcd for $C_{29}H_{36}N_6O_6S \cdot CF_3COOH \cdot 2H_2O$: C, 49.86; H, 5.53; N, 11.26. Found: C, 50.13; H, 5.21; N, 11.61.

Deprotection of Boc–Trp(Mts)–Met–Asp(OMen)–Phe–NH_2 with 1 M TFMSA–Thioanisole/TFA in the Presence of Diphenylsulfide——The reaction was performed on the same scale in the presence of diphenylsulfide (10 eq) and the products isolated as stated above were examined by HPLC (Fig. 3): (A) H–Trp–Met–Asp–Phe–NH_2 82.1%, (B) H–Trp–Met–Asc–Phe–NH_2 11.5%, (C) H–Trp–Met–Asp(OMen)–Phe–$NH_2=O$, (D) unidentified products 6.4%. In a preparative run (sample 50 mg), the isolation yield of the HPLC-purified sample was 65%.

Acknowledgement This investigation was supported in part by a Grant-in-Aid (No. 61771808) from the Ministry of Education, Science and Culture, Japan.

References and Notes

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2) Amino acids used in this study are of the L-configuration. The following abbreviations are used: Asc = aminosuccinimidyl, Boc = *tert*-butoxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, Mts = mesitylenesulfonyl, DCC = dicyclohexylcarbodiimide, HONB = *N*-hydroxy-5-norbornene-2,3-dicarboximide, Su = *N*-hydroxysuccinimidyl, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, THF = tetrahydrofuran, EDT = ethanedithiol, DMAP = dimethylaminopyridine, MSA = methanesulfonic acid, DCHA = dicyclohexylamine, HOSu = *N*-hydroxysuccinimide, NMM = *N*-methylmorpholine.
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