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Stability of Methionyl Residues towards Oxidation during Solid Phase Peptide Synthesis

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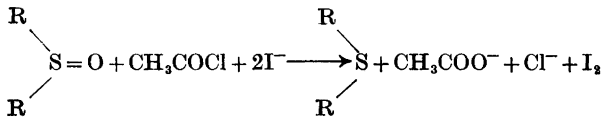
The transformation Met→Met(O)* as a possible side reaction was studied during the solid phase synthesis of the protected hexapeptide amide Boc-Ala-Phe-Ile-Gly-Leu-Met-NH_2. No sulfoxide formation could be detected, using an iodometric titration method directly on the peptide resin. The same result was obtained with an *N*-Ac-Met-resin after 32 simulated coupling cycles in an automatic peptide synthesizer. However, sulfoxide formation by air-oxidation of the protected hexapeptide amide dissolved in HOAc was demonstrated. A new synthesis of Boc-Met-resin by reduction of Boc-Met(O)-resin with NaI and CH_3COCl in DMF is described.

During synthesis and purification of peptides containing methionine, the thioether function of this amino acid may undergo various reactions. Thus it is known that exposure to atmospheric oxygen of dilute solutions of biologically active peptides containing methionine can lead to a considerable loss of activity due to oxidation of methionine to methionine sulfoxide.[1] The ease with which methionyl residues are oxidized to methionyl sulfoxide residues has also been discussed in connection with biological oxidative phosphorylation.[2]

Recently, a protected C-terminal Lys[5]-heptapeptide of eledoisin[3] was synthesized. Oxidation of methionyl residues in this synthesis seemed to be responsible for the formation of by-products. In order to examine whether any oxidation of thioether groups to sulfoxide groups takes place during the solid phase synthesis of peptides containing methionyl residues, it was decided to investigate the solid phase synthesis of the protected C-terminal hexapeptide of eledoisin: *t*-butyloxycarbonyl-L-alanyl-L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methionine amide. For elucidation of the problem, a method for the determination of the sulfoxide content in peptides after cleavage from the resin as well as in resin bound peptides was needed.

*Methionyl sulfoxide. Abbreviations follow the rules of the IUPAC-IUB Commission on Biochemical Nomenclature: *J. Biol. Chem.* **241** (1966) 2491.

A reliable method for quantitative determination of sulfoxides consists of treatment of the sulfoxide dissolved in glacial acetic acid with acetyl chloride and potassium iodide followed by addition of dilute hydrochloric acid and titration of the liberated iodine with a standard solution of sodium thiosulfate:[4]



The procedure was found suitable for the determination of methionine sulfoxide and Boc-methionine sulfoxide (Table 1), but not for the determination of the sulfoxide content of Boc-L-methionyl sulfoxide resins. This, because

Table 1.

Sulfoxide	Equivalent weight	
	Found	Calc.
Methionine sulfoxide	82.3	82.6
Boc-methionine sulfoxide	132.9	132.7

part of the liberated iodine was so strongly adsorbed to the resin, that it could not be titrated with thiosulfate. If, however, dimethylformamide (DMF) was used as reaction medium, sodium iodide substituted for potassium iodide, and a small volume of dilute hydrochloric acid was added just before the titration, the adsorption was avoided. This modified procedure allows a determination of the sulfoxide content of resinbound peptides with a reasonable degree of accuracy (Table 2).

Preparation of Boc-L-methionyl sulfoxide resin. Three different Boc-L-methionyl sulfoxide resins were prepared according to the general method of Merrifield,[5] by reaction between the triethylammonium salt of Boc-L-methionine sulfoxide* and chloromethylated copolystyrene-2 % divinylbenzene in refluxing ethanol. The content of sulfoxide of the esterified resins was determined by iodometric titration as follows:

a. Determination of unreacted Boc-L-methionine sulfoxide in the filtrate from the esterification.
b. Direct titration of Boc-L-methionyl sulfoxide resin.
c. Determination of Boc-L-methionine sulfoxide amide formed by ammonolysis of Boc-L-methionyl sulfoxide resin.

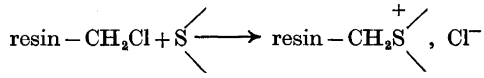
The results are presented in Table 2.

*A mixture of the two diastereoisomeric compounds Boc-2(*S*), *S*(*S*)- and Boc-2(*S*), *S*(*R*)-methionine sulfoxide was used.

Table 2.

Procedure of analysis	Sulfoxide content in mmol/g		
	Resin 1	Resin 2	Resin 3
a	0.383	0.677	0.641
b	0.377	0.668	0.640
c	0.378	–	0.626

Preparation of Boc-L-methionyl resin. In the case of Boc-methionine, the normal esterification procedure [5] for the attachment of Boc-amino acids to a chloromethylated resin proceeds unsatisfactorily, probably because part of the Boc-methionine is alkylated at the thioether function:[6]



This difficulty can be avoided by first preparing a Boc-L-methionyl sulfoxide resin in the normal way, and then reducing the sulfoxide to thioether. By this procedure it is furthermore possible to calculate the content of Boc-L-methionyl residues of the resin by determining iodometrically the amount of Boc-L-methionyl sulfoxide residues attached to the resin before the reduction step. The preparative reduction of Boc-L-methionyl sulfoxide resin to Boc-L-methionyl resin in 98 % yield was performed with sodium iodide and acetyl chloride in dimethylformamide.

A small sample of Boc-L-methionyl resin was reoxidized to Boc-L-methionyl sulfoxide resin with a solution of hydrogen peroxide in acetic acid. The resulting resin was titrated iodometrically in DMF, whereby the same content of sulfoxide as that of the original Boc-L-methionyl sulfoxide resin was found.

Ammonolysis of a sample of the Boc-L-methionyl resin with a methanolic solution of ammonia gave a nearly quantitative yield of Boc-L-methionine amide, the optical purity of which was demonstrated by conversion into L-methionine amide acetate and comparison of the specific optical rotation with the value reported in the literature.[7]

RESULTS

Boc-L-alanyl-L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methionyl resin was synthesized in a stepwise manner, according to the procedure of Merrifield,[5] and the sulfoxide content of the di-, tetra-, and hexapeptide resins was determined by iodometric titration. Ammonolysis of these resins with a methanolic solution of ammonia gave the corresponding protected peptide amides, which also were titrated iodometrically. As will be seen from Table 3, no oxidation of methionyl residues to methionyl sulfoxide residues could be detected, either during the synthesis on the resin or during the cleavage from the resin.

Table 3.

Resin	Determination of sulfoxide content of resins in mmol/g		Theoretical methionine content of resins (based on resin 3) in mmol/g
	Procedure b	Procedure c	
Boc-Met-resin	0.015	0.007	0.65
Boc-Leu-Met-resin	0.004	0.000	0.61
Boc-Ile-Gly-Leu-Met-resin	0.014	0.004	0.55
Boc-Ala-Phe-Ile-Gly-Leu-Met-resin	0.010	0.003	0.49

That oxidation of methionyl residues as a side reaction during automated solid phase peptide synthesis apparently does not occur, was demonstrated by the following experiment. A Boc-methionyl resin was converted into an *N*-acetyl-methionyl resin, and this resin subjected to thirty-two identical cycles in an automatic peptide synthesizer.[8] Each cycle was similar to a cycle of deblocking, neutralization, and coupling in the synthesis of the Boc-hexapeptide resin, except that no Boc-amino acids were added and all operations were performed under a nitrogen atmosphere. Iodometric titration of the resin after the thirty-two cycles showed that no sulfoxide had been formed.

Both this resin and the original *N*-acetyl-methionyl resin were oxidized with hydrogen peroxide in acetic acid, followed by iodometric titration. The difference between the two titration values showed that 14 % of the methionine present before the thirty-two cycles in the peptide synthesizer had been lost, probably by cleavage from the resin during treatments with N hydrochloric acid in acetic acid. This corresponds to an average loss of 0.4 % per cycle. An average loss per cycle of 1.4 % of peptide was reported in the synthesis of a polypeptide with ribonuclease A activity.[9]

While no sulfoxide formation was observed as long as the above-mentioned methionine containing hexapeptide was attached to the resin or during ammonolysis from the resin, some oxidation took place when a solution of Boc-L-alanyl-L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methionine amide in glacial acetic acid was left standing in the air and without special protection against light. After standing for 96 h, iodometrical titration showed that 10 % of the original peptide had been oxidized to the corresponding sulfoxide. The formation of the sulfoxide could easily be followed by thin layer chromatography. The R_F-value of the slower moving sulfoxide was identical with that of the sulfoxide resulting from the oxidation of Boc-hexapeptide with hydrogen peroxide in acetic acid.

EXPERIMENTAL

Melting points were determined with a Büchi capillary melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 141 automatic digital readout polarimeter, tube length 1 dm. Ascending thin-layer chromatography was performed on commercial plates (DC-Fertigplatten, Kieselgel F 254, E. Merck AG, Darmstadt). The chromatograms were visualized by spraying with tertiary butyl hypochlorite, followed by *p*-tolidine/potassium iodide. The simulated coupling cycles were performed by an automatic peptide synthesizer from Schwarz BioResearch, Orangeburg, New York. The resin (Bio-beads S-X2, 200–400 mesh) was obtained from

Bio-Rad Laboratories, Richmond, California, and was chloromethylated according to Merrifield[10]. Methylene chloride (May and Baker Ltd., Dagenham, Essex) was stored over potassium carbonate and distilled before use. Acetic acid ("Pronalys" glacial acetic acid, May and Baker Ltd.) was used as such. Ethanol was commercial absolute ethanol. Eledoisin trifluoroacetate was obtained from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England. The Boc-amino acids were prepared according to Schnabel.[11] L-Methionine sulfoxide was prepared by oxidation of L-methionine suspended in glacial acetic acid with hydrogen peroxide, according to the method of Iselin;[12] $[\alpha]_D^{22} = 19.8°$ ($c = 0.94$ in acetic acid). Iodometric titration [4] gave an equivalent weight of 82.3 (calc. 82.6).

Boc-L-methionine sulfoxide. L-Methionine sulfoxide (12.0 g, 73 mmol) was dissolved in a mixture of water (90 ml) and dioxane (70 ml). *t*-Butylazidoformate (11.5 g, 80 mmol) was added, and the mixture was stirred at room temperature, while the pH was kept automatically at 9.2 by addition of 4 N sodium hydroxide solution (36 ml, 0.14 mol) over a period of 20 h. The resulting, nearly clear, colorless solution was acidified to pH 2.5 by dropwise addition of concentrated hydrochloric acid (12 ml, 0.15 mol). Sodium chloride (about 50 g) was added, and the mixture extracted with six 50 ml portions of ethyl acetate. The combined ethyl acetate extracts were dried over magnesium sulfate, filtered, and evaporated to dryness on a water bath (40°) under reduced pressure. The remaining clear yellowish, viscous oil (21.0 g) crystallized on standing at room temperature overnight. Recrystallization from ethyl acetate (70 ml) and drying (20°, 0.1 mm) overnight gave pure Boc-L-methionine sulfoxide (14.3 g; 74 % yield) as white crystals, m.p. 118–120° (decomp.); $[\alpha]_D^{22} = 12.0°$ ($c = 2.0$ in acetic acid). [Found: C 45.1; H 7.1; N 5.3; S 12.2. Calc. for $C_{10}H_{19}NO_5S$ (265.3): C 45.3; H 7.2; N 5.3; S 12.1.] Iodometric titration [4] gave an equivalent weight of 132.9 (calc. 132.7).

By addition of petroleum ether (boiling range 40–65°) to the mother liquor, a further crop of Boc-L-methionine sulfoxide could be obtained as white crystals (1.6 g; 8 % yield), m.p. 117–119° (decomp.).

Boc-L-methionyl sulfoxide resin (resin 3, Table 2). Boc-L-methionine sulfoxide (7.96 g, 30.0 mmol) and freshly distilled triethylamine (4.20 ml, 30.0 mmol) in absolute ethanol (120 ml) were added to a chloromethylated copolystyrene-2 % divinylbenzene (16.4 g, 30.0 mequiv. Cl). The reaction mixture was stirred under reflux in the dark under nitrogen for 24 h. The resin was isolated by filtration and washed with seventeen 50 ml portions of a solution, which changed gradually from ethanol to water, and then progressively to methanol. After drying overnight (20°, 0.1 mm), Boc-L-methionyl sulfoxide resin (19.5 g) was obtained as a white powder. The combined washings were diluted with methanol to 1000 ml in a volumetric flask. 25 ml of this solution was pipetted off and evaporated to dryness under reduced pressure. The white, crystalline residue was dissolved in glacial acetic acid (10 ml) and titrated iodometrically by the method of Allenmark.[4] The total sulfoxide content of the filtrate was thus found to be 17.5 mmol, which corresponds to a substitution of 0.641 mmol Boc-L-methionyl sulfoxide per g of Boc-aminoacyl resin.

Boc-L-methionyl sulfoxide resin was analyzed for quaternary ammonium chloride groups by suspending in dilute nitric acid, followed by Volhard titration. A content of 0.12 mequiv. $-CH_2\overset{+}{N}Et_3$, Cl^- per g of Boc-aminoacyl resin was found.

Boc-L-methionyl sulfoxide resin (0.450 g) was suspended in pyridine (5 ml) and the mixture heated under reflux for 1 h. After evaporation to dryness on a water bath (40°) under reduced pressure, the residue was suspended in dilute nitric acid and titrated as above. A content of 0.59 mequiv. Cl per g of Boc-aminoacyl resin was found, which corresponds to a content of 0.47 mequiv. $-CH_2Cl$ per g of Boc-aminoacyl resin.

Similarily were prepared two other Boc-L-methionyl sulfoxide resins, the analytical data of which are given in Table 2.

Boc-L-methionine sulfoxide amide. Boc-L-methionyl sulfoxide resin (resin 3, 1.456 g) was stirred for 70 h in a sealed flask at room temperature with a methanolic solution of ammonia (30 ml), prepared by saturating absolute methanol with dry ammonia (distilled from sodium) at 0°. After filtration, the resin was washed with four 10 ml portions of methanol. The combined methanol washings were diluted to 100 ml in a volumetric flask. 25 ml of this solution was pipetted off and evaporated to dryness on a water bath (35°) under reduced pressure. The white, crystalline residue was titrated iodometrically.[4] From the result, a content of sulfoxide of the resin of 0.626 mmol/g was calculated.

The remaining methanolic filtrate was evaporated to dryness to give a white product (207 mg, 98 % yield*). The crude cleavage product was stirred with glacial acetic acid (2.5 ml). The mixture was filtered, and the ammonium chloride on the filter was washed with three 0.3 ml portions of glacial acetic acid. The combined filtrates were evaporated to dryness on the water bath (35°) under reduced pressure, and the residue dried overnight (20°, 0.1 mm), whereby crude Boc-L-methionine sulfoxide amide (180 mg, 98 % yield) was obtained as a yellowish, viscous oil. By stirring with dry ether (5 ml), the oil slowly crystallized. The ether was decanted off, and the remaining material was recrystallized from ethyl acetate-ether (3 : 1), giving white crystals (155 mg, 84 % yield), m.p. 95 – 99° (the melting range was not very narrow, possibly because the compound is a mixture of two diastereoisomeric sulfoxides). [Found: C 45.4; H 7.8; N 10.5; S 11.9. Calc. for $C_{10}H_{20}N_2O_4S$ (264.3): C 45.4; H 7.6; N 10.6; S 12.1.]

Iodometric titration of Boc-L-methionyl sulfoxide resin. Boc-L-methionyl sulfoxide resin (resin 3, 290 mg, 0.186 mmol sulfoxide) was allowed to settle in DMF (45 ml). A few pieces of dry ice were added, whereafter the walls of the flask were rinsed with DMF (5 ml). Sodium iodide (1 g) and acetyl chloride (0.30 ml) were added, and the mixture was stirred for 10 min. Then N hydrochloric acid (5 ml) was added, and the iodine liberated titrated dropwise with a 0.1000 N sodium thiosulfate solution under vigorous stirring. Near the end of the titration, the titrant was added very slowly, finishing at a rate of 1 drop per min. A blank determination on a chloromethylated resin was carried out in order to correct for any air oxidation of iodide to iodine. 3.71 ml of 0.1000 N sodium thiosulfate was used, corresponding to a sulfoxide content of the resin of 0.640 mmol/g. The two other Boc-L-methionyl sulfoxide resins were similarily titrated (Table 2).

Boc-L-methionyl-resin. Boc-L-methionyl sulfoxide resin (resin 3, 15.0 g, 9.6 mmol sulfoxide) was suspended in DMF (1 l). With efficient mechanical stirring, sodium iodide (20 g) and acetyl chloride (7.5 ml) were added, causing the formation of iodine. After stirring for 10 min, 50 % aqueous acetic acid (200 ml) was added, whereby all the sodium iodide dissolved. Then a 0.200 N sodium thiosulfate solution (100 ml, 20.0 mmol) was added dropwise over a period of 20 min. The white suspension was filtered, and the resin washed with twenty-one 50 ml portions of a solution, which changed gradually from DMF to water, and then progressively to methanol. After drying overnight (20°, 0.2 mm), Boc-L-methionyl resin (14.8 g) was obtained as a white powder.

Iodometric titration of the resin as described above showed that 98 % of the sulfoxide groups of the Boc-L-methionyl sulfoxide resin had been reduced, corresponding to a sulfoxide content of 0.015 mmol/g.

A small portion of the Boc-L-methionyl resin (57.3 mg, theor. 37 μmol**) was reoxidized by stirring with a solution of 10.5 M aqueous hydrogen peroxide (10 μl, 105 μmol) in glacial acetic acid (5 ml) for 2 h at room temperature. After evaporation under reduced pressure (6 – 7 mm, bath temperature 25°) on a rotatory evaporator, three 5 ml portions of glacial acetic acid were added, and after each addition the mixture was evaporated to dryness. Finally, the resin was dried (95°, 0.5 mm) for 20 min. The resin was titrated iodometrically in DMF (20 ml) by the method described for Boc-L-methionyl sulfoxide resin. The result corresponded to a methionine content of the resin of 0.64 mmol/g.

Boc-L-methionine amide. Boc-L-methionyl resin (1.515 g, theor. 0.984 mmol) was ammonolyzed as described for Boc-L-methionyl sulfoxide resin. After filtration, the resin was washed with six 5 ml portions of methanol. The combined methanol washings were diluted to 100 ml in a volumetric flask. 25 ml of this solution was pipetted off and evaporated to dryness on a water bath (35°) under reduced pressure. The white crystalline residue was titrated iodometrically [4] in acetic acid (7 ml). From the result, a sulfoxide content of the resin of 0.007 mmol/g could be calculated.

The remaining methanolic filtrate was evaporated to dryness on a water bath (35°) under reduced pressure to give a white product (211.5 mg) which was dissolved in a mixture of methylene chloride (5 ml) and water (5 ml). The phases were separated and the aqueous phase extracted with two 5 ml portions of methylene chloride. The combined

*Theoretically, based on a sulfoxide and non-ionic chloride content of the Boc-L-methionyl sulfoxide resin of 0.64 mmol/g and 0.47 mequiv/g, respectively, a mixture of 184 mg of Boc-L-methionine sulfoxide amide and 27 mg of ammonium chloride should be formed.

** Based on Boc-L-methionyl sulfoxide resin.

organic phases were dried over magnesium sulfate, filtered and evaporated to dryness on a water bath (35°) under reduced pressure, whereby Boc-L-methionine amide was obtained as white crystals (179.6 mg, 98 % yield), m.p. 116 – 118°. Recrystallization from ethyl acetate-petroleum ether (boiling range 40 – 65°) (2 : 6, 8 ml) gave white crystals (156.9 mg, 89 % yield), m.p. 115 – 116°.

L-Methionine amide acetate. Boc-L-methionine amide (157 mg, 0.63 mmol) was stirred at room temperature with N HCl in acetic acid (8 ml). Dry ether (10 ml) was added to the thick, white suspension and the precipitate isolated by filtration, washed with four 1 ml portions of dry ether and dried (20°, 0.1 mm). L-Methionine amide hydrochloride (107 mg, 92 % yield) was isolated as a white powder; m.p. 224 – 225° (decomp.) (reported [13] m.p. 192°). The hydrochloride (107 mg, 0.58 mmol) was converted into the acetate as described in the literature.[7] After one recrystallization from ethyl acetate, L-methionine amide acetate (85 mg, 70 % yield) was obtained as white crystals, m.p. 100 – 101° (reported [7] m.p. 100 – 102°); $[\alpha]_D^{19} = 16.0°$ ($c = 0.53$ in water) (reported [7] $[\alpha]_D^{19} = 15.2°$ ($c = 0.88$ in water).

Boc-L-leucyl-L-methionyl resin. Boc-L-methionyl resin (9.00 g, theor. 5.85 mmol) was placed in a weighed cylindrical reaction vessel provided with a sintered glass filter,[14] and the Boc group was cleaved by shaking the resin with two 80 ml portions of N HCl in acetic acid for 5 and 30 min, respectively.* After washing with three 70 ml portions of acetic acid, three 70 ml portions of ethanol, and three 80 ml portions of methylene chloride, the resin was shaken with a solution of triethylamine (10.0 ml, 72 mmol) in methylene chloride (70 ml) for 20 min. After washing with five 80 ml portions of methylene chloride, the resin was shaken for 30 min with a solution of Boc-L-leucine in methylene chloride prepared by adding Boc-L-leucine hydrate (7.29 g, 29.3 mmol) to methylene chloride (65 ml), followed by filtration through magnesium sulfate. Now a solution of *N,N'*-dicyclohexylcarbodiimide (6.03 g, 29.3 mmol) in methylene chloride (10 ml) was added and the coupling allowed to proceed for 17 h. After washing with four 80 ml portions of methylene chloride and six 80 ml portions of ethanol, the filtrates from the last ethanol washing did not leave any residue on evaporation. The resin was dried in the reaction vessel on a rotatory evaporator under reduced pressure and slow rotation. After drying (20°, 0.1 mm) overnight, the resin was obtained as a faintly yellow powder (9.55 g). A small sample of the resin (246 mg) was titrated iodometrically as described for Boc-L-methionyl sulfoxide resin. A sulfoxide content of the resin of 0.004 mmol/g was found.

Boc-L-leucyl-L-methionyl resin (1.50 g) was ammonolyzed as above. Iodometric titration of the filtrate showed that the sulfoxide content was below the limit of detection.

Boc-L-isoleucyl-glycyl-L-leucyl-L-methionyl resin. Boc-L-leucyl-L-methionyl resin (7.01 g, theor. 4.29 mmol) was subjected to a cycle of deprotection, neutralization, and coupling with Boc-glycine (3.76 g, 21.5 mmol). After washing with methylene chloride and ethanol, and drying (20°, 0.1 mm) overnight, the resin was obtained as a beige, quite voluminous powder (7.20 g). In an analogous manner the resulting resin was coupled with Boc-L-isoleucine (4.96 g, 21.5 mmol) whereby a beige, voluminous resin (7.62 g) was obtained. A sample of the resin (253 mg) was titrated iodometrically as described above. A sulfoxide content of the resin of 0.014 mmol/g was found.

Boc-L-isoleucyl-glycyl-L-leucyl-L-methionyl resin (1.02 g) was ammonolyzed as above. Iodometric titration of the filtrate showed that 0.004 mmol of sulfoxide was present.

Boc-L-alanyl-L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methionyl resin. Boc-L-isoleucyl-glycyl-L-leucyl-L-methionyl resin (5.61 g, theor. 3.09 mmol) was subjected to a cycle of deprotection, neutralization, and coupling with Boc-L-phenylalanine (4.19 g, 15.8 mmol). After washing and drying as above, the resin was obtained as a nearly white powder (5.96 g). In an analogous manner the resulting resin was coupled with Boc-L-alanine (2.99 g, 15.8 mmol) whereby the resin was obtained as a nearly white, free-flowing powder (6.12 g).

By iodometric titration of the resin (249 mg) in DMF, as described above, a sulfoxide content of 0.010 mmol/g was found.

*The combined filtrates from these treatments developed a brownish color on standing in the air, probably due to formation of iodine by air oxidation of iodide incorporated into the quaternary ammonium groups of the resin during the reduction of Boc-L-methionyl sulfoxide resin. The color disappeared on the addition of 0.1 N sodium thiosulfate solution.

A sample of the resin (70.7 mg, theor. 35 μmol) was oxidized by stirring with a solution of aqueous 10.5 M hydrogen peroxide (10 μl, 105 μmol) in glacial acetic acid (5 ml) for 2 h at room temperature. Work up and titration as described for Boc-L-methionyl resin gave a value for the methionine content of the resin of 0.45 mmol/g. Theoretically, a value of 0.49 mmol/g should be expected, based on Boc-L-methionyl sulfoxide resin. Hydrolysis of another sample (17.31 mg) in 6 N hydrochloric acid-acetic acid (1 : 1) at 110° for 96 h in an evacuated and sealed vial, followed by amino acid analysis gave the following values in μmol/mg: Ala 0.44; Phe 0.42; Ile 0.44; Gly 0.44; Leu 0.47; Met 0.42.

Boc-L-alanyl-L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methionine amide. Boc-L-alanyl-L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methionyl resin (1.521 g, theor. 0.745 mmol) was ammonolyzed under the same conditions as described for Boc-L-methionyl sulfoxide resin. The thick, white suspension was filtered, and the peptide was dissolved on the filter by washing with four 25 ml portions of warm (50°) DMF. The remaining resin was then washed with three 10 ml portions of methanol, and the combined faintly yellow filtrates were diluted to 250 ml in a volumetric flask. 50 ml of this solution was pipetted off and evaporated to dryness on a water bath (55°) under reduced pressure (10 mm). The crystalline residue was dried (20°, 0.1 mm), and glacial acetic acid (7 ml) was added. Iodometric titration [4] showed that the hexapeptide resin had a sulfoxide content of 0.003 mmol/g. The remaining filtrate (200 ml) was evaporated to dryness as above. After drying (20°, 0.05 mm) overnight, crude Boc-hexapeptide amide (415 mg) was obtained as a slightly yellow, crystalline material. Recrystallization from acetic acid-water (1 : 4, 18 ml) gave pure Boc-L-alanyl-L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methionine amide (301 mg, 67 % yield); m.p. in an evacuated tube 252 – 254° (decomp.) (reported m.p. 248 – 250°,[15] 250 – 254°,[15] 254 – 256°[16]). [Found: C 58.0; H 8.0; N 12.9; S 3.8. Calc. for $C_{36}H_{59}N_5O_8S$ (750.0): C 57.7; H 7.9; N 13.1; S 4.3.] Amino acid analysis, given in mol per 750 g of substance: Ala 0.98; Phe 0.98; Ile 0.98; Gly 0.98; Leu 1.01; Met 0.98. $[\alpha]_D^{22} = -28.4°$ ($c = 0.5$ in DMF) (reported $[\alpha]_D^{22} = -28°$ ($c = 0.5$ in DMF),[16] $[\alpha]_D^{22} = -29.7°$ ($c = 1$ in DMF),[15] $[\alpha]_D^{22} = -30.6°$ ($c = 1$ in DMF)[15]).

A small sample of the Boc-hexapeptide amide (50 mg, 67 μmol) was dissolved in glacial acetic acid (2 ml). The solution was left standing in an open flask away from direct sunlight. At 24 h intervals, thin layer chromatography of the mixture was performed in chloroform-methanol (9 : 1), in which the starting material had $R_F = 0.5$. The progressive formation of the corresponding sulfoxide was followed by observing the appearance of a new spot ($R_F = 0.2$). After 96 h, the mixture was titrated iodometrically,[4] whereby it was found that 10 % of the original peptide had been oxidized to the corresponding sulfoxide. In a similar experiment, but with the initial addition of 1 drop of 10.5 M aqueous hydrogen peroxide, the sulfoxide formation was followed thin layer chromatographically by the distinct appearance of the same spot ($R_F = 0.2$) as above within 30 min.

Bioassay. The estimated hypotensive effect of the Boc-hexapeptide amide in the rabbit blood pressure test was 61 % compared on a weight base with that of eledoisin. The blood pressure was measured in the femoral artery of a rabbit under urethane anæsthesia. 50 – 100 μl of solutions containing 0.5 – 1 μg peptide per ml was given in a femoral vein. These solutions were prepared from a solution of 1 mg of peptide in 1 ml of glacial acetic acid (Boc-hexapeptide amide) or dimethylformamide (eledoisin) by dilution with 0.9 % aqueous sodium chloride. In the dog, the hypotensive effect of the Boc-hexapeptide amide has been reported [16] to be 25 % relative to eledoisin.

N-Acetyl-L-methionyl resin. Boc-L-methionyl resin (2.89 g, 1.88 mmol) was deprotected with N HCl in acetic acid and neutralized with triethylamine as described above for the synthesis of Boc-L-leucyl-L-methionyl resin. After washing with three 15 ml portions of methylene chloride and three 15 ml portions of DMF, the resin was shaken for 1 h with a solution of acetic anhydride (2.0 ml, 21 mmol) and triethylamine (1.2 ml, 9 mmol) in DMF (15 ml). After washing with three 15 ml portions of DMF and six 20 ml portions of ethanol, the resin was dried in the reaction vessel on a rotatory evaporator under reduced pressure. After drying (20°, 0.1 mm) overnight, *N*-acetyl-L-methionyl resin was obtained as a slightly yellow powder (2.70 g).

A small sample of the resin (213 mg) was analyzed for sulfoxide as above. The sulfoxide content of the resin was found to be 0.014 mmol.

Another sample (54.7 mg) was oxidized to the corresponding *N*-acetylmethionyl sulfoxide resin as described for Boc-L-methionyl resin, followed by iodometric titration. A methionine content of the resin of 0.64 mmol/g was found.

Table 4.

Code	Reagent	Code	Function
A	HOAc	0	Filling *via* measuring cylinder I
B	CH_2Cl_2	1	Filling *via* measuring cylinder II
C	EtOH	2	Shaking for 2 min
E	N HCl in HOAc	3	Shaking for 5 min
F	1.8 M Et_3N in CH_2Cl_2	4	Shaking for 10 min
G	0.15 M DCC in CH_2Cl_2	5	Shaking for 30 min
		6	Shaking for 60 min
		9	Draining of reaction vessel

Repeated simulated coupling cycles with N-acetyl-L-methionyl resin. *N*-Acetyl-L-methionyl resin (2.0 g, 1.28 mmol) was placed in the reaction vessel of an automatic peptide synthesizer and subjected to 32 simulated coupling cycles without addition of Boc-amino acids. Each cycle was similar to that described for the synthesis of Boc-L-leucyl-L-methionyl resin. According to Table 4, one cycle can be described by the following coding:

A029A029A029E03ŞE059A029A029A029C129C129C129B129B129B129F1449
B129B129B129B129B129B14G1669B129B129B129B129C129C129C129C129.

The volumes employed were: 80 ml of A, B, C, E, and F; 45 ml of G. The duration of each cycle was 4 h 55 min. All operations were performed under a nitrogen atmosphere (99.8 – 99.9 % N_2).

After the last cycle had been completed, the slightly yellow resin was removed from the reaction vessel and dried (20°, 0.1 mm) overnight. A small sample of the resin (201 mg) was titrated iodometrically. A sulfoxide content of the resin of 0.005 mmol/g was found.

Another sample (65.4 mg) was oxidized with hydrogen peroxide in acetic acid as described above, followed by iodometric titration. The methionine content of the resin was found to be 0.56 mmol/g. As the methionine content of the original *N*-acetyl-L-methionyl resin was 0.64 mmol/g, the decrease can be explained by assuming that some *N*-acetyl-L-methionine has been cleaved from the resin, probably by the treatments with N HCl in acetic acid. This corresponds to a loss of 14 % of the *N*-acetyl-L-methionine present on the original resin, giving an average of 0.4 % per cycle. In the calculation, correction has been made as to the difference in the ratio *N*-acetyl-L-methionine/polymeric support of the substituted resins.

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