Phosphinyl- and Phosphinothioylamino Acids and Peptides. V. Preparation of Dimethylphosphinothioylamino Acids and Solid Phase Peptide Synthesis

Masaaki UEKI,* Toshiyuki INAZU, and Shigeru IKEDA

Department of Chemistry, Science University of Tokyo, 1-3 Kagurazaka, Shinjuku-ku, Tokyo 162

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The use of the dimethylphosphinothioyl(Mpt) group for protection of N^{α}-amino functions of amino acids has been studied as the most readily removable group in the phosphinothioyl series. The Mpt-amino acids have been prepared by the alkaline hydrolysis of Mpt-amino acid esters and Schotten-Baumann type reactions of free amino acids. The Mpt group has been removed using a solution of triphenylphosphine dihydrochloride in dichloromethane. L-Leu^5- and D-Ala^2, L-Leu^5-enkephalins have been synthesized by a solid phase method and synthetic L-Leu^5-enkephalin exibited identical activity with an authentic sample.

Recently it has been established that a series of phosphinothioyl groups serve to protect the N^{α}-amino functions of amino acids.[1,2] In a previous paper it was shown that the diphenylphosphinothioyl(Ppt) group was the most useful group since diphenylphosphinothioyl chloride(Ppt–Cl) is readily available from the Friedel-Crafts reaction of benzene and thiophosphoryl chloride and could be used for the preparation of Ppt-amino acids by the Schotten-Baumann type reaction.[3,4] The Ppt group was removed by hydrogen chloride reagents to reproduce the Ppt–Cl. The chloride was relatively inactive to the indole moiety of tryptophan and no other active intermediate was generated during the course of deprotection, and consequently the Ppt-amino acids were successfully utilized for the solid phase synthesis of oligopeptides containing tryptophan.[5] The conditions for removal of the Ppt-group however apper inappropriate for the synthesis of larger peptides on solid supports. In this paper the use of the dimethylphosphinothioyl(Mpt) group has been studied as the most readily removable group in the phosphinothioyl series.

Cleavage of the phosphinothioyl groups is greatly facilitated in the presence of methyl group as substituent on the phosphorus atom.[1] The dimethylphosphinothioyl group was selected in this study since this avoided the introduction of a new asymmetric center on the phosphorus atom. Dimethylphosphinothioyl chloride (**1**) was obtained by chlorinating tetramethyldiphosphine disulfide[6] with sulfuryl chloride.[7]

The Mpt-amino acids were prepared by two methods: the alkaline hydrolysis of Mpt-amino acid esters (Method A) and the direct phosphinothioylation of free amino acids (Method B).

(Method A)

$$\underset{\mathbf{1}}{(CH_3)_2P(=S)\text{–}Cl} + H_2NCHRCO_2R' \xrightarrow{(C_2H_5)_3N} Mpt\text{–}NHCHRCO_2R' \xrightarrow[2)\ H^+]{1)\ OH^-} Mpt\text{–}NHCHRCO_2H$$

(Method B)

$$\mathbf{1} + H_2NCHRCO_2H \xrightarrow[2)\ H^+]{1)\ OH^-} Mpt\text{–}NHCHRCO_2H$$

The chloride **1** reacted rapidly with the amino acid esters to give the corresponding Mpt-amino acid esters, which were subsequently hydrolysed by aqueous sodium hydroxide to yield Mpt-amino acids. In the case of Mpt-glycine the product was partially extracted by organic solvents from the acidified aqueous reaction mixture saturated with sodium chloride. A small amount of the analytically pure sample of the dicyclohexylamine salt was obtained by laborious extraction, but regeneration of the free acid before use proved impossible. Homologous diethylphosphinothioyl(Ept)-glycine was prepared in a similar manner and substituted for Mpt-glycine in the following peptide synthesis. No difficulty was encounterd in the preparation and isolation of this compound and also in the deprotection of the Ept group as described later. The tyrosine ester reacted with **1** to give a mixture of N^{α}-Mpt and N^{α}-, *O*-bis-Mpt derivatives. Mpt-tyrosine was obtained by alkaline hydrolysis in a similar manner to that for the Ppt-derivatives.[4]

TABLE 1. PREPARATION OF Mpt-L-METHIONINE DCHA SALT

Temperature	Mpt–Cl(eq)	Base	Yield(%)
RT	1.0	1 M NaOH	0
0 °C	1.0	1 M NaOH	58
0 °C	1.0	2 M NaOH	48
0 °C	1.1	1 M NaOH	59
0 °C	1.1(ether solution)	1 M NaOH	68
0 °C	1.2	1 M NaOH	78

In the direct synthesis of Mpt-amino acids by the Schotten-Baumann type reaction several problems were encountered. **1** was rapidly hydrolysed in an alkaline solution, a reaction not found with Ppt–Cl. This problem was solved by conducting the reaction at low temperature with an excess of reagent. The addition of **1** in an ether solution improved the yield as shown in Table 1. The hydrolysis product of **1**, dimethylphosphinothioic acid was not extractable by organic solvents in acidified aqueous solution, and consequently the use of excess reagent did not complicate the purification. The Mpt-amino acids were stored in their dicyclohexylamine(DCHA) or cyclohexylamine(CHA) salts. The physical properties and elemental analysis data are summarized in Table 2.

Removal of the Mpt group was accomplished by hydrogen chloride in organic solvents, in a similar manner to that for the Ppt group, but much more

Table 2. Dimethylphosphinothioyl- and diethylphosphinothioylamino acid salts

Mpt deriv. of	Yield, % (Method)[a]	Mpt–Cl (eq)	Mp (°C)	$[\alpha]_D^{25}$ (deg)[b]	Found (Calcd), % C	H	N
Gly·DCHA	(A)		131—133		55.54 (55.19)	9.62 (9.48)	8.11 (8.04)
Gly·DCHA[c]	78 (A)		181—183		57.12 (57.46)	10.13 (9.83)	7.69 (7.44)
L-Ala·DCHA	57 (B)	1.1	147—148	−10.0	53.66 (53.70)[e]	9.73 (10.22)[e]	7.57 (7.36)[e]
L-Val·DCHA	54 (B)	1.1	148—149	−12.5	58.13 (58.33)	10.49 (10.22)	7.25 (7.15)
L-Leu·DCHA	59 (B)	1.1	146—156	−30.0	59.82 (59.42)	10.66 (10.14)	6.84 (6.93)
L-Ile·CHA	63 (B)	1.1	146—158	−13.8	50.03 (49.43)[f]	9.86 (9.70)[f]	8.55 (8.23)[f]
L-Phe·DCHA	65 (B)	1.1	165—170	−7.5	62.89 (63.02)	9.05 (8.90)	6.49 (6.39)
L-Pro·DCHA	48 (B)	1.1	131—133	−75.0	57.88 (57.45)[g]	9.55 (9.57)[g]	7.14 (7.05)[g]
L-Met·DCHA	78 (B)	1.1	147—149	−10.0	53.34 (52.91)[i]	9.13 (9.27)[h]	6.35 (6.49)[h]
L-Cys(Bzl)·DCHA	67 (B)	3.0	162—164(dec)	+10.0	59.55 (59.51)	8.39 (8.46)	5.43 (5.78)
L-Asp(OBu^t)·DCHA	41 (B)	1.1	154	−20.0	57.38 (57.16)	9.55 (9.30)	5.61 (6.06)
L-Tyr·DCHA	65 (A)		160—161	−10.0	58.04 (58.49)[i]	8.81 (8.80)[i]	6.17 (5.93)[i]
L-Tyr(Bzl)·DCHA	23 (B)	1.1	169—172	−10.0	66.03 (66.19)	8.35 (8.27)	4.81 (5.14)
L-Lys(Z)·DCHA	16 (B)	1.1	123—124	+5.0	60.14 (59.80)[j]	8.67 (8.71)[j]	7.32 (7.47)[j]
L-Trp·DCHA	47 (B)	1.1	180—185(dec)	−12.5[d]	62.48 (62.91)	8.10 (8.38)	8.49 (8.80)
D-Ala·DCHA	41 (B)	2.1	172—173(dec)	+7.5	55.62 (55.63)[k]	10.16 (9.75)[k]	7.89 (7.63)[k]

a) Described in Experimental. b) *c* 1 in EtOH unless otherwise stated. c) Diethylphosphinothioyl derivative. d) *c* 1 in MeOH. e) Calcd for $C_{17}H_{35}N_2O_2PS\cdot H_2O$. f) Calcd for $C_{14}H_{31}N_2O_2PS\cdot H_2O$. g) Calcd for $C_{25}H_{43}N_2O_2PS\cdot 1/2\,H_2O$. h) Calcd for $C_{19}H_{39}N_2O_2PS_2\cdot 1/2\,H_2O$. i) Calcd for $C_{23}H_{39}N_2O_3PS\cdot H_2O$. j) Calcd for $C_{28}H_{48}N_3O_4PS\cdot 1/2\,H_2O$. k) Calcd for $C_{17}H_{35}N_2O_2PS\cdot 1/4\,H_2O$.

rapidly. A solution of triphenylphosphine dihydrochloride in dichloromethane[2,5] effectively increased the rate of removal of the Mpt by a factor of 60 compared to the *t*-butoxycarbonyl(Boc) group;[8] this reagent appears to be very convenient in solid phase synthesis. Deprotection of Mpt-L-phenylalanine resin was completed within 10 min by treatment with 0.5 M (1 M= 1 mol dm^{-3}) hydrogen chloride in dichloromethane containing 0.25 M triphenylphosphine and within 30 min with 0.25 M hydrogen chloride solution.[2]

To demonstrate the synthetic utility of Mpt-amino acids, the solid phase syntheses of L-Leu^5-enkephalin (L-Tyr–Gly–Gly–L-Phe–L-Leu) and the D-Ala^2 analog were attempted. Mpt-L-leucine was esterified with a chloromethyl resin by the caesium salt method.[9] Deprotection of the Mpt group was achieved by treatment with 0.2 M hydrogen chloride solution in dichloromethane containing 0.2 M triphenylphosphine for each 30 min (twice). Coupling of the Mpt- and Ept-amino acids were mediated with the oxidation-reduction condensation method[10] using a 3-fold excess of tris(*p*-methoxyphenyl)phosphine[11] and 2,2′-dithiodipyridine. This enabled tyrosine could be used without protection of the side chain hydroxyl group. Crude peptides were removed from the resin by treatment with hydrogen bromide in trifluoroacetic acid[12] in the presence of anisole. Separation by preparative thin layer

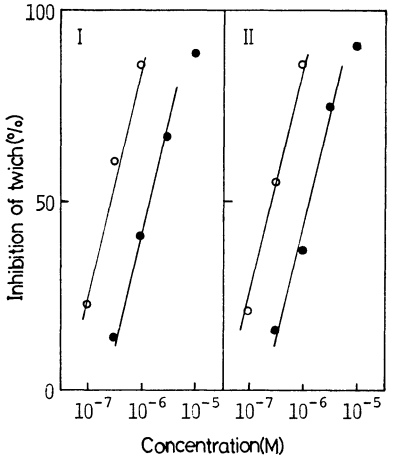

Fig. 1. Dose-response curve of synthetic L-Leu^5-enkephalin (I) and an authentic sample (II). (○: agonist alone, ●: agonist+naloxone 10^{-8} M).

chromatography on silica gel and purification by gel chromatography on Sephadex LH-20 gave the desired compounds in 52 and 40% yields, respectively. The activity of the synthetic L-Leu^5-enkephalin as an opioid agonist in logitudial muscle strips of guinea-pig ileum was identical with that of an authentic sample (Protein Research Foundation, Osaka) as shown in Fig. 1. The activity of D-Ala^2, L-Leu^5-enkephalin is now being studied and the results will be published in due course.

Experimental

Thin layer chromatography (TLC) was performed on silica gel plates (Merck $60F_{254}$) in the following solvent systems: chloroform–methanol–aqueous ammonia (60:30:5, R_f^1), 1-butanol–acetic acid–ethyl acetate–water (1:1:1:1, R_f^2) and ethanol–water (7:3, R_f^3). The peptides were detected on the TLC plates using ultraviolet light, iodine vapor and ninhydrin.

Dimethylphosphinothioyl chloride was prepared according to the literature.[6,7] Distillation of the product was achieved after decomposition of the contaminating dimethylphosphinyl chloride by shaking with water. The chloride **1** solidified on cooling and was stored for several months, without change, in a refrigerator.

N^{α}-Dimethylphosphinothioylamino acids were prepared by the alkaline hydrolysis of N^{α}-dimethylphosphinothioylamino acid esters obtained by the reaction of dimethylphosphinothioyl chloride with amino acid esters(Method A) and the reaction of an ethereal solution of dimethylphosphinothioyl chloride and α-amino acids (or α-amino acids bearing a protected side-chain functional group) in aqueous alkaline solution at controlled pH. The dimethylphosphinothioylamino acids thus prepared were isolated as the dicyclohexylamine(DCHA) or cyclohexylamine(CHA) salts.

Method A. To a suspension of the amino acid ester hydrochloride (5 mmol) in chloroform (10 ml) and triethylamine (10 mmol), Mpt–Cl (5 mmol) in chloroform (5 ml) was added at 0 °C. After stirring at room temperature for 6 h, the solution was washed with water, ice-cold 5% citric acid solution, water, 5% $NaHCO_3$ solution and saturated NaCl solution, dried and evaporated to dryness. The oily residue was dissolved in ethanol (5 ml) and to this solution was added 1 M NaOH solution (5 ml). The reaction mixture was stirred at room temperature for 4 h. After removal of ethanol *in vacuo*, the aqueous solution was extracted twice with ethyl acetate and the ethyl acetate extracts back extracted with 5% $NaHCO_3$ solution. The combined aqueous solutions were acidified at 0 °C to pH 4—5 with solid citric acid, saturated with NaCl and extracted 5 times with ethyl acetate. The ethyl acetate extracts were effectively washed with saturated NaCl solution and dried over anhydrous Na_2SO_4. After removal of the drying reagent, DCHA was added to effect separation of the corresponding salt. On certain occasions the addition of the same volume of ether was necessary to induce precipitation. The product was filtered off and washed with ethyl acetate or ether.

Method B. The amino acid (50 mmol) was dissolved in 1 M NaOH solution (50 ml), and Mpt–Cl (55 mmol) in ether (50 ml) added dropwise at 0 °C. The mixture was vigorously stirred to initiate immediate reaction. 1 M NaOH solution was added at a rate which maintained the pH of the solution at 9.5—10.0. Subsequently the reaction mixture was treated as described in Method A. The product was isolated as the DCHA or CHA salt and purified by recrystallization from ethanol, ethyl acetate or a methanol–ether mixture.

Dichloromethane Solution of Triphenylphosphine Dihydrochloride. Dry hydrogen chloride was passed through a dichloromethane solution of triphenylphosphine and saturated at room temperature. An aliquot of the solution was added to water and titrated with 1 M NaOH solution using phenolphthalein as the indicator to determine the concentration of HCl.

Solid Phase Synthesis of L-*Leu*^{5}-*enkephalin.* Mpt-L-leucine was esterified on resin support by treatment of the caesium salt with a chloromethyl resin prepared by chloromethylating the Bio-Beads S-X1 (Bio-Rad Laboratories) (Leu content; 0.63 mmol/g). The ester resin (1 g) was placed in the reaction vessel of the Beckman model 990 peptide synthesizer using the program described before.[4] Removal of the Mpt group was affected by 0.2 M HCl in dichloromethane solution containing 0.2 M triphenylphosphine (twice for 30 min). After neutralization with 10% triethylamine in dichloromethane, the couplings were mediated with the oxidation-reduction condensation method using tris(*p*-methoxyphenyl)phosphine and 2,2′-dithiodipyridine. Glycine was used as the Ept derivative. The Mpt-L-Leu^5-enkephalin-resin was finally deprotected, washed and dried *in vacuo*. A sample was hydrolysed in 12 M HCl–propionic acid at 130 °C for 2 h[13] to give the amino acid ratios: $Tyr_{0.99}$, $Gly_{1.89}$, $Phe_{1.06}$, $Leu_{0.99}$. The penta-peptide was removed from the resin by bubbling a stream of anhydrous hydrogen bromide through a suspension of the peptide resin in trifluoroacetic acid (10 ml) containing 50 equivalents of anisole for 90 min at room temperature. The filtrate and three trifluoroacetic acid (30 ml) washings were combined and evaporated *in vacuo*. The residue was separated and purified by silica gel preparative layer chromatography using the solvent system 1. The desired band (R_f=0.3—0.4) was eluted with methanol and evaporated *in vacuo* to give a white solid. The solid was dissolved in a small amount of methanol and applied to a Sephadex LH-20 (3×80 cm) column in order to remove the silica gel contaminents. The individual fractions collected (8 ml each) were examined by UV absorbancy at 280 nm. The eluates containing a single component (tube Nos. 27—33) were combined and evaporated to give white crystals; 167 mg (52% from Mpt-L-Leu-resin). An analytical sample was obtained by recrystallization from methanol, mp 158—160 °C (lit,[14] 157—167 °C, lit,[15] 206—208 °C); $[\alpha]_D^{25}$ +27.8° (*c* 0.9, MeOH) (lit,[15] +32.3° (*c* 0.9, MeOH), lit,[16] +20° (*c* 1, MeOH)); R_f^1 0.35 (lit,[17] 0.33), R_f^2 0.73 (lit,[16] 0.85), R_f^3 0.74 (lit,[17] 0.77). Amino acid ratios in hydrolysate by 6 M HCl at 110 °C for 24 h: $Tyr_{0.98}$, $Gly_{2.02}$, $Phe_{0.99}$, $Leu_{1.01}$. Found: C, 59.14; H, 6.95; N, 12.30%. Calcd for $C_{28}H_{38}N_5O_7 \cdot 1/2H_2O$: C, 59.46; H, 6.95; N, 12.38%.

D-*Ala*^{2}, L-*Leu*^{5}-*enkephalin.* This peptide was synthesized in a similar manner to above using Mpt-D-alanine at the third acylation step. The desired compound was obtained in 40% yield (from Mpt-L-Leu-resin) as white crystals. An analytical sample was obtained by recrystallization from methanol, mp 182—184 °C; $[\alpha]_D^{25}$ −25.0° (*c* 0.1, MeOH); R_f^1 0.49, R_f^2 0.83, R_f^3 0.71. Amino acid ratios in hydrolysate by 6 M HCl at 110 °C for 24 h: $Tyr_{0.99}$, $Ala_{0.99}$, $Gly_{0.97}$, $Phe_{1.02}$, $Leu_{1.00}$. Found: C, 58.75; H, 7.47; N, 10.94%. Calcd for $C_{29}H_{39}N_5O_7 \cdot 2MeOH$: C, 58.75; H, 7.48; N, 11.05%.

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