Chemoselective one-step purification method for peptides synthesized by the solid-phase technique

(affinity-type purification/SH precursor reagent/base-labile sulfonylthiocarbonyl linkage/iodoacetamide-resin/β-elimination cleavage)

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Communicated by Christian B. Anfinsen, April 10, 1991

ABSTRACT The specific reaction between SH and iodoacetamide groups has been explored as the basis of an affinity-type purification procedure for peptides synthesized by the solid-phase technique. For this affinity-type purification procedure, we synthesized an SH precursor reagent bearing an acid-labile S-protecting group, p-MB-SCH2CONHCH2CH2
SO2CH2CH2OCO2pNP (compound 1), in which p-MB is p-methoxybenzyl and pNP is p-nitrophenyl. Using this reagent, the procedure involves the following sequence of four steps: (i) attachment of the SH function of compound 1 to the α-amino group of a peptide-resin through a base-labile sulfonylthiocarbonyl linkage in the final step of solid-phase peptide synthesis, (ii) acid treatment to remove the S-pMB and side-chain-protecting groups employed and cleave the modified peptide from the resin, (iii) immobilization of the derived peptide-SH on an iodoacetamide-resin column, and (iv) base (5% NH4OH) treatment to release the desired peptide from the resin in nearly pure form. To facilitate this purification procedure, unreacted amino groups were acetylated in each step during solid-phase synthesis. The usefulness of this method was demonstrated by the purification of several peptides (18 to ≈44 amino acids in length) synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase technique. The principle of this affinity-type purification procedure may also be applied to the tert-butoxycarbonyl (Boc)-based solid-phase technique.

In the solid-phase synthesis of peptides (1), the purification step is a major barrier to obtaining the desired peptide in a satisfactory yield due to the accumulation of terminated or truncated peptides on the resin.

Recently, several reports relating to new separation methods have been published. However, none of these methods has been able to achieve effective one-step separation (2–7). Merrifield and coworkers (8) and Wilchek and coworkers (9, 10) have developed methods in which the target peptide alone is adsorbed to an organomercurial-agarose column by attaching Cys-Met and using the SH group of Cys, or the peptide is adsorbed to an aminod column by attaching biotinyl-Met and using the biotin group, respectively. These groups are attached to the N terminus of the synthetic peptide in both cases. Subsequent to the separation, the Met-peptide bond is cleaved by CNBr to yield the target peptide. However, these methods have limitations for peptides containing Met and Cys.

We have explored another affinity-type purification procedure, based upon the specific reaction between SH and iodoacetamide groups, which can be applied even to peptides containing Met, Cys, or both. The SH-introducing reagent S-(p-methoxybenzyl)thioglycolylamine-sulfonylthiocarbonyl p-nitrophenyl carbonate (I) was prepared. After solid-phase peptide synthesis, this SH functional group bearing an acid-labile S-protecting group was introduced onto the α-amino group of a peptide-resin through a base-labile sulfonylthiocarbonyl linkage. Then this peptide-resin was treated with acid to cleave the peptide from the resin and to remove side-chain-protecting groups together with the SH-protecting group. The resulting SH peptide was passed through an iodoacetamide resin column, allowing only the desired peptide possessing the SH functional group to be immobilized by covalent bonding to the resin. After contaminating peptides had been washed out from the resin, the target peptide was released by treatment with 5% (wt/wt) NH4OH.

MATERIALS AND METHODS

Analytical Methods. Thin-layer silica gel (Kiesel-gel G, Merck) chromatography was conducted with solvent system 1 [CHCl3/MeOH/H2O, 8:3:1 (vol/vol)] and system 2 [CHCl3/MeOH, 10:0.5 (vol/vol)]. 3H NMR spectroscopy: Bruker AC-300, using tetramethylsilane as the internal standard. Fast atom bombardment (FAB)-MS: ZAB SE spectrometer (UG Analytical, England). HPLC: Waters model 600E system. Two solvent systems, 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B), were used. Amino acid analysis was performed with a Hitachi L-8500 amino acid analyzer and peptide sequence analysis was performed with a MilliGen/Biosearch peptide sequencer (model 6600, Millipore).

Synthesis of the S-Protected Base-Labile SH-Introducing Reagent. The synthesis is outlined in Fig. 1.

Synthesis of compound A. N-(2-Bromoethyl)phthalimide (50.8 g, 0.2 mol) was dissolved in dimethylformamide (DMF) (400 ml) together with dicyclohexylamine (39.9 ml, 0.2 mol) and 2-mercaptoethanol (14.3 ml, 0.2 mol). The mixture was stirred at room temperature for 4 hr. After filtration, the filtrate was concentrated, and the resulting residue was extracted with ethyl acetate. The extract was washed with saline, dried over Na2SO4, and concentrated to produce a pale yellow oil (yield 45.9 g, 91%).

Synthesis of compound B. Compound A (43.5 g, 0.17 mol) was dissolved in MeOH (250 ml) and then treated with 50% hydrazine hydrate (9.3 ml, 0.18 mol). After being stirred for 16 hr, the solution was filtered and then evaporated under reduced pressure. The residue was triturated with CH3CN and the resulting powder was recrystallized from MeOH/CH3CN (yield 45.9 g, 86%). To an ice-chilled solution of resulting product (17 g, 0.14 mol) in water (100 ml), p-methoxybenzyl azide formate (34.7 g, 0.16 mol) and Et3N (23.6 ml, 0.17 mol) were added. After being stirred overnight, the solution was concentrated and the residue was extracted with...
FIG. 1. Synthesis of the base-libae SH-introducing reagent. Details of the synthesis of the S-protected base-libae SH-introducing reagent are given in Materials and Methods.

ethyl acetate. The extract was washed with 5% citric acid and saline, dried over Na2SO4, and evaporated under reduced pressure. The residue was treated with n-hexane and the resulting powder was recrystallized from ethyl acetate/n-hexane (yield 37.12 g, 93%). Rp in system 1 = 0.74, mp 41–41.5°C. Analysis. Calculated for C14H15NO5S: C, 54.79; H, 6.09; N, 5.47. Found: C, 54.78; H, 6.12; N, 5.46. FAB-MS: 332.1 (M + H+).

Synthesis of compound C. Compound B (18.2 g, 65 mmol) was dissolved in a mixture of water (100 ml) and MeOH (100 ml), and sodium tungstate dihydrate (65 mg) was added. Then aqueous hydrogen peroxide (16.3 ml, 114 ml/mol) was added dropwise to this well-stirred clear solution at such a rate that the ensuing exothermic reaction was maintained at about 60°C. The reaction mixture was left for 2 hr at room temperature, then treated with 5% palladium on carbon (0.5 g) to decompose the remaining hydrogen peroxide. After the evolution of oxygen had ceased, the solution was filtered and then the solvent was removed by evaporation under reduced pressure. The residue was extracted with ethyl acetate. The extract was washed with 5% citric acid, 5% NaHCO3, and saline, dried over Na2SO4, and evaporated under reduced pressure. Then the residue was treated with diethyl ether and the resulting powder was recrystallized from ethyl acetate/ether (yield 16.7 g, 81%), mp 65–66°C. Analysis. Calculated for C14H15NO5S: C, 54.79; H, 6.09; N, 5.47. Rp in system 1 = 0.52. FAB-MS: 340.1 (M + Na+). Analysis. Calculated for C14H15NO5S: C, 54.36; H, 4.88; N, 4.53. Found: C, 54.07; H, 4.85; N, 4.48. FAB-MS: 332.1 (M + Na+). (Calculated for C14H12N5O5S: 309.1.)

Synthesis of compound D. Compound C (5.0 g, 15.8 mmol) was treated with TFA/anisole (20 ml/5 ml) at 0°C for 1 hr, after which TFA was removed by evaporation and dry ether was added. The resulting residue was dissolved in DMF (50 ml) together with Et3N (4.5 ml, 31.6 mmol) and S-(p-methoxybenzyl)thioglycolic acid N-hydroxysuccinimide ester (4.87 g, 15.6 mmol). After being stirred overnight, the solution was concentrated and the residue was extracted with ethyl acetate. The extract was washed with 5% citric acid, 5% NaHCO3, and saline, dried over Na2SO4, and evaporated under reduced pressure. The residue was treated with ether and the resulting powder was recrystallized from ethyl acetate/ether (yield 4.31 g, 78%). Rp in system 1 = 0.61, mp 73–75°C. Analysis. Calculated for C14H12N5O5S: C, 48.40; H, 6.09; N, 4.03. Found: C, 48.60; H, 6.29; N, 4.11.

Synthesis of compound D (S-protected base-libae SH-introducing reagent). Compound D (4.00 g, 11.5 mmol) was dissolved in absolute pyridine (30 ml). The solution was cooled at 0°C and 2.32 g (11.5 mmol) of p-nitrophenyl chloroformate was added with stirring. This mixture was then let stand for 5 hr at 0°C and subsequently condensed under reduced pressure. The residue was treated with 1 M HCl and ether. The product, which crystallized immediately, was collected by filtration and washed with water. The crude product was recrystallized from ethyl acetate/ether (yield 4.07 g, 69%). Rp in system 2 = 0.37, mp 81–82°C. Analysis. Calculated for C14H12N5O5S: C, 49.21; H, 4.72; N, 5.47. Found: C, 49.30; H, 4.72; N, 5.28. 1H NMR (300 MHz, CDCl3): δ 8.13 (s, 2H), 3.28 (t, J = 6.0 Hz, 2H), 3.47 (t, J = 5.7 Hz, 2H), 3.75 (q, J = 6.0 Hz, 2H), 3.79 (s, 3H), 4.74 (t, J = 5.7 Hz, 2H), 6.84, 7.20 (AA′ BB′ pattern, Jortho = 8.7 Hz), 7.25 (m, 1H), 7.40, 8.28 (AA′ BB′ pattern, Jortho = 9.3 Hz). FAB-MS: 513.2 (M + H+). (Calculated for C14H12N5O5S: 512.1.)

Preparation of Iodoacetamide-Resin. 1,3-Dicyclohexylcarbodiimide (5.0 g, 24 mmol) was added to a solution of iodoacetic acid (7.4 g, 40 mmol) in CH2Cl2 (50 ml), and the mixture was stirred at room temperature for 30 min. The reaction mixture was filtered, the filtrate was mixed with pentafluorophenyl K resin (20 g, amino content, 0.2 mmol, Millipore) functionalized with ethylenediamine, and the suspension was gently shaken for 2 hr until the resin became negative to the Kaiser test (12). Then the resin was washed with DMF and CH2Cl2 and dried under reduced pressure.

Synthesis of Model Peptides (General Method). Synthesis of model peptides. The model peptides used in these experiments were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase synthesis according to the principle of Sheppard and coworkers (ref. 13 and references therein), using a MilliGen/Biosearch model 9050 automatic peptide synthesizer. The following side-chain-protected Fmoc-amino acids were used: Arg(Mtr), Lys(Boc), His(Boc), Glu(OBu)4, Asp(OBu)4, Ser(Bu)4, Thr(Bu)4, and Tyr(Bu)4, in which Mtr indicates 4-methoxy-2,3,6-trimethylbenzenesulfonyl, Boc indicates tert-butoxycarbonyl, and Bu4 indicates tert-buty1. To make the final purification step easier, the peptide-resin was treated with acetic anhydride after each condensation reaction to ensure the complete termination of the uncleaved a-amino group. Thus, only the mature peptide could be modified at the end of synthesis.

Introduction of the S-protected base-libae SH-introducing reagent. The peptide resin (200 mg) was treated with 200 μl of 20% piperidine in DMF (5 min) and 2 ml of 5% thiol in 0.1 M phosphate buffer pH 7.4 (15 min) to remove the Fmoc group. Then compound I (5 eq) was condensed to the N-terminal residue of the protected peptide-resin in the presence of 1-hydroxybenzotriazole (5 eq) in DMF. The container was shaken until the resin showed a negative
Kaiser test (usually 2 hr). The resin was then washed with DMF and CH₂Cl₂ and dried under reduced pressure.

**Deprotection and Purification (General Method).** Deprotection and cleavage of peptides from the solid-phase support. The peptide-resin (100 mg) was treated with 1 M trimethylsilyl bromide (Me₃SiBr)/thioanisole in TFA (10 ml) or 1 M trimethylsilyl triflate (Me₃SiOTf)/thioanisole in TFA (10 ml) in the presence of m-cresol (200 μl) and ethanedithiol (200 μl) at 0°C for 2 hr, after which Me₃SiBr and TFA were removed by evaporation and dry ether was added. The resulting powder was collected by centrifugation.

**Binding to iodoacetamide-resin.** The crude deprotected peptide was dissolved in 6 M guanidine-hydrochloride containing 0.4 M Tris-HCl buffer, pH 7.5, and then the solution was filtered. The filtrate was mixed with 1 eq of the iodoacetamide-resin in a column and the suspension was gently shaken for 2 hr. To this suspension, 1 eq of 2-mercaptoethanol was added and then gentle shaking was continued for additional 2 hr. Following elution of the solution from the column, the resin was washed with 50% (wt/wt) HOAc and methanol. During the binding of the modified peptide to the iodoacetamide-resin, an aliquot of the solution was collected (at 30-min intervals), and the progress of the reaction was monitored by the Ellman method and HPLC.

**Release of peptides from the iodoacetamide-resin column.** The iodoacetamide-resin column with bound peptide was treated with 5% NH₄OH for 30 min and then filtered. Then the resin was washed with 50% HOAc, and the filtrate and the washings were combined and subjected to freeze-drying to afford a fluffy powder. To determine the cleavage yield, the resins (before and after treatment with 5% NH₄OH) were hydrolyzed with 6 M HCl at 110°C for 24 hr and then the hydrolysates were processed with an amino acid analyzer.

**RESULTS AND DISCUSSION**

We have developed an effective affinity-type method for isolating a target peptide from a mixture of many kinds of immature (terminated) peptides. This objective was achieved by the development of a reagent satisfying the following two conditions. First, it was stable in the final deprotection step (where acidic reagents have conventionally been used for deprotection in peptide synthesis), and second, it was specifically cleaved under certain predetermined conditions. Therefore, we synthesized an acid-stable but base-labile cross-linking reagent that produces a linkage between peptides and iodoacetamide-resin, based on the ethoxycarbonyl group (14), since this group is easier to synthesize and has a handle with a more suitable length.

Synthesis of I was carried out according to the scheme shown in Fig. 1. The heterobifunctional reagent Z[O Me]-NHCH₂CH₂SO₂CH₂CH₂OH [Z(OMe) indicates p-methoxybenzyl group] (compound C) was obtained as an intermediate. The OH group of this intermediate can be easily converted to an active alkyloxycarbonyl. Following the removal of Z(OMe) by TFA treatment, the amino group of the other terminus can be attached to a compound containing a functional group such as SH.

In this study, we selected S-(p-methoxybenzyl)thioglycolic acid (11) as the SH precursor reagent. The SH group is known to react with iodoacetamide derivatives to form a stable thioether under neutral conditions. Under such conditions, iodoacetamide reacts specifically with the SH group, and not with Met, His, or Tyr (15, 16).

We consequently succeeded in the synthesis of a reversible cross-linking reagent—i.e., pMB-SCH₂CONHCH₂CH₂SO₂CH₂CH₂OCO₂pNP (1), in which pMB is p-methoxybenzyl and pNP is p-nitrophenyl. This compound is easily introduced to the α-amino groups of a peptide-resin within 2 hr in the presence of 1-hydroxybenzotriazole at room temperature, and it acts as a urethane-type amino protecting reagent. It is stable to acids, such as TFA, Me₂SiBr, HF, trifluoromethanesulfonic acid, and Me₃SiOTf, but is easily removed by the β-elimination reaction with a basic reagent (with 0.2 M NaOH in 50% MeOH for 5 sec or 5% NH₄OH in 50% MeOH for 5 min).

The reagent I was allowed to react with the N terminus of the completely mature peptide at the end of synthesis as shown in Fig. 2. Deprotection was subsequently performed, whereby the p-methoxybenzyl group (SH-protecting group of the linker) was removed, together with all other side-chain-protecting groups of peptides liberated from the resin. The SH group of the modified peptide thus obtained reacted with the iodoacetamide group on the resin under neutral conditions. Thus, only the mature peptide could be immobilized on the resin with a stable covalent bond, while terminated peptides and scavengers could be easily washed out from the resin. Finally, the resin was treated with 5% NH₄OH or 0.2 M NaOH in H₂O to cleave the desired peptide from the resin. The eluate was neutralized with acetic acid, then lyophilized (Fig. 3). In the present studies, only the mature peptide can be derivatized with reagent I and thus isolated selectively from impurities.

To demonstrate the usefulness of this method, S-Acethylphenemusin II (Acm indicates acetylglutamyl) (17) was synthesized as a model peptide. Synthesis was performed by the Fmoc-based solid-phase method shown in Fig. 2. Phenemusin II is an 18-residue peptide amide with two disulfide bonds. For this synthesis, the SH group of the Cys residue was protected with an acid-stable Acm group. It is essential to this method that Cys remains protected during purification, because the thiol group of each internal Cys is not protected, peptides containing Cys but lacking the amino-terminal SH group will also bind to the iodoacetamide resin. At the final step of synthesis, the synthetic reagent I was attached to the N terminus of the presence of 1-hydroxybenzotriazole (5 eq each). This introduction reaction was monitored by the Kaiser test. After 2 hr, the reaction was completed, and subsequent deprotection by the 1 M Me₂SiBr/thioanisole/TFA system (18) was carried out. The resulting crude peptide was dissolved in 0.4 M Tris-HCl buffer, pH 7.5, containing 6 M guanidine-
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**FIG. 3.** Deprotection and purification of peptides synthesized by the solid-phase technique. Step a, attachment of S-protected base-labile SH-introducing reagent (A, B, C, ... represent amino acid residues); step b, deprotection and cleavage from solid support; step c, binding to iodoacetamide-resin; step d, cleavage from iodoacetamide resin.

Hydrochloride, and the solution was introduced into a column with 1 eq of iodoacetamide-resin, after which gentle shaking was performed. The progress of this coupling reaction was monitored by Ellman's method and HPLC. After 30 min, 80% of the SH groups were incorporated into the iodoacetamide resin. Then the reaction was set for an additional 1.5 hr, after which the Ellman reaction reached a plateau (Fig. 4) and the main peak disappeared from the HPLC chromatogram. This indicated that the target peptide was completely incorporated into the iodoacetamide-resin within 2 hr. Subsequently, the resin was treated with 2-mercaptoethanol for 2 hr to cap the iodoacetamide group on the resin. After elution of the solution from the column, the resin was washed sufficiently with 50% acetic acid and methanol. As stated above, our linker reagent is easily removed by treatment with 5% NH₄OH for 5 min. To produce complete cleavage, the peptide-bound resin was treated with 5% NH₄OH for 30 min. The resulting peptide showed a single peak on HPLC (purity > 99%) and a quantitative yield (Fig. 5a). When the treated resin was hydrolyzed with 6 M HCl, amino acids of polyphemusin II were not detected by an amino acid analyzer, indicating that the peptide was completely cleaved from the resin by the 5% NH₄OH treatment.

**FIG. 4.** Binding of the linker-modified peptide to an iodoacetamide-resin column. Free SH groups were monitored by the Ellman method. hCCK-33, human 33-residue cholecystokinin; hGRF, human growth hormone-releasing factor.

As shown in Fig. 5a, the HPLC chromatogram of the crude peptide (purity ≈ 69%) had a shoulder before the main peak, but that of the purified peptide showed a sharp single peak. The results of FAB-MS analysis of the obtained peptide were identical to the theoretical value [Acm-polyphemusin II: 2714.1 (M + H⁺)], and the amino acid sequence was correct.

**FIG. 5.** HPLC elution profile of synthetic peptides. HPLC was performed on a Waters µBondasphere 5C₁₈ (100 Å) column (3.9 × 150 mm), using a solvent system B (0.1% TFA in acetonitrile) gradient at a flow rate of 1.0 ml/min. Absorbance was monitored at 220 nm. a) Acm-polyphemusin II. The column was eluted with a linear gradient of 10–40% solvent B over 30 min. (b) Unsulfated hCCK-33. The column was eluted with a linear gradient of 10–60% solvent B over 30 min. (c) hGRF. The column was eluted with a linear gradient of 25–40% solvent B over 30 min (crude) or 25–40% solvent B over 30 min (purified).
To examine the applicability of this method to the Boc-based solid-phase technique, resin-bound protected polyphenusin II was treated with 1 M Me$_2$SiOTf/thioanisole/TFA (19), which is used as the final deprotecting reagent in the Boc-based procedure. Then the target peptide was purified by the procedure described above. The resulting peptide was found to be identical to that obtained by the 1 M Me$_2$SiBr procedure and the conventional purification procedure on the basis of several criteria. This showed that our synthetic linker might be useful not only for the Fmoc-based solid-phase technique but also for Boc-based synthesis.

This method was then applied to middle-length peptide. hCCK-33 (20) is a 33-residue peptide amide containing one His, one Tyr, and two Met residues. It was synthesized by the Fmoc-based solid-phase method in the same manner as polyphenusin II, followed by introduction of the linker and deprotection. The crude product was purified essentially as described above. Assay of the obtained peptide by HPLC showed a sharp main peak (purity: crude ≈ 35%; purified > 90%) with small side peaks (Fig. 5b). The HPLC chromatogram of this peptide showed a somewhat large peak in front of the main peak. When this sample was exposed to H$_2$O$_2$, the size of this early peak increased, with a corresponding decrease in the main peak, suggesting that this peak was due to a reduction of Cys(O). The retention time of the peak of the unsulfated hCCK-33 purified by this method was identical to that of the peptide obtained by the conventional purification procedure. Additionally, amino acid sequencing and FAB-MS analysis [3865.2 (M + H$^+$)] gave results identical to the theoretical ones.

We further studied the application of this method to a longer peptide by synthesizing hGRF, a 44-residue peptide amide (21, 22). The procedure, from synthesis to cleavage from the iodoacetamide-resin, was the same as that given above. In the case of hGRF, the target peptide showed a sharp single peak on HPLC with several small peaks derived from capped terminated peptides (content of the desired peptide ≈ 28%; Fig. 5c). After purification (purity > 85%), the hGRF produced in this manner showed a retention time identical to that of the peptide obtained by a conventional purification procedure. When the treated resin was hydrolyzed with 6 M HCl, an amino acid analyzer set to identify the residues of hGRF detected 15%, indicating that 85% of the peptide was released from the resin by 5% NH$_4$OH treatment. The amino acid sequence and FAB-MS data [5037.8 (M + H$^+$)] agreed with the theoretical results.

hCCK-33 and hGRF contain Tyr, His, and Met residues, but we detected no evidence of a side reaction between the iodoacetamide groups and these amino acid side chains during purification. In all cases, the recoveries of peptides from the resin were more than 85%. However, with the increase of peptide length, the purity of the peptide recovered decreased due to a loss of capping efficiency (Fig. 5b and c).

One of the shortfalls of conventional chromatographic purification is that it is troublesome to remove terminated peptides or peptides with deletions produced due to low condensation reaction yields. Accumulation of immature peptides causes great difficulty in the assay of the target peptide by HPLC. In such cases, the purification of each assumed peak of the target peptide is required along with the identification of each peak by amino acid sequencing and FAB-MS analysis. In contrast, our method can easily identify the target peptide by comparison of HPLC assays of the purified peptide, the linker-modified peptide, and the crude peptide. But even when the crude peptide had several peaks on HPLC, the linker-modified peptide was characterized by its longer retention time due to the hydrophobicity of the linker. Therefore, the peak with its retention time extended by introduction of the linker can be identified as that of the target peptide.

The advantages of our method consist of its easiness and the purity of the product. Even peptides that have shoulder peaks can be purified with this method. In contrast to conventional HPLC purification, this method can make it possible for any researcher, without special techniques, to obtain pure peptides easily at one step in a short time by the solid-phase technique. The easily prepared cross-linking reagent introduced here will be of wide applicability for the one-step purification of peptides synthesized by the solid-phase technique. Additionally, a more readily obtainable supply of high-purity or constant-purity peptides should facilitate research in the fields of biochemistry, physiology, and medicine with respect to the physiological activities and mechanisms of action of peptides and proteins.

We are grateful to Dr. H. Tanaka (Fujisawa Pharmaceutical, Tsukuba, Japan) for FAB-MS, and Mrs. S. Funakoshi and Mr. M. Ono (Kyoto University) for $^1$H NMR. We express our sincere appreciation to Prof. H. Yajima (Niigata Pharmaceutical College) and to Profs. M. Fridkin, A. Patchornik, and M. Wilchek (The Weizmann Institute of Science, Rehovot, Israel) for valuable discussions during the preparation of the manuscript.