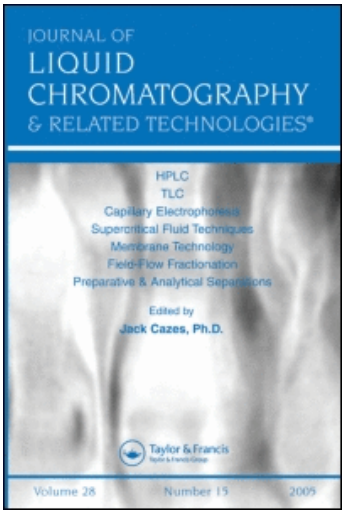

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Linear and Cyclic Enkephalin Pseudopeptides. Use of RP-HPLC to Separate and Identify Diastereomers Formed during Synthesis

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LINEAR AND CYCLIC ENKEPHALIN PSEUDOPEPTIDES. USE OF RP-HPLC TO SEPARATE AND IDENTIFY DIASTEREOMERS FORMED DURING SYNTHESIS

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ABSTRACT

During the course of synthesis of both a linear and a cyclic enkephalin pseudopeptide containing a ψ[CH_2S] amide bond replacement, two isomeric products were produced in each case in virtually equimolar quantities. RP-HPLC was used to: 1) isolate and characterize both pairs of products; 2) confirm their sulfide content by partial oxidation to their ψ[CH_2SO] equivalents, with two new pairs of diastereomeric sulfoxides formed in each case, and 3) confirm that the isomers were formed by epimerization of the C-terminal alpha carbon of the pseudopeptide, H-Tyr-D-Ala-Gly-Pheψ[CH_2S]Leu-OH and its cyclic counterpart in an early synthetic step. Both the presence and the absolute configurations of the new epimeric center were further established by RP-HPLC. This involved acid catalyzed hydrolysis and comparison of the resulting HPLC-isolated pseudodipeptides with authentic species of Pheψ[CH_2S]Leu and Pheψ[CH_2S]D-Leu prepared by controlled stereochemical routes.

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INTRODUCTION

The chromatographic separation of peptide diastereomers in which one or more chiral centers exist as both the S and R configuration is of current interest in obtaining the following: 1) more optically pure peptides (1), 2) separation of peptides modified with racemic amino acids for purposes of isotope labeling, 3) structure function studies involving diastereomeric analogs (5), and 4) the relation of differences in configuration to changes in conformation (3). The application of reverse phase high performance liquid chromatography to the separation of diastereomeric peptides has been reported in a number of instances involving a variety of both linear and cyclic peptides (4-7). Neurohypophyseal hormone analogs in which D-amino acids are introduced in an otherwise all L sequence have in most cases been shown to be more lipophilic and thus more retentive on reversed phase columns (4,5,7). However, some reports of earlier eluting D-amino acid-containing analogs have appeared further suggesting the unpredictability of conformational changes as a result of a particular substitution (3). The application of RP-HPLC to the separation of a diastereomeric mixture of synthetic cyclic peptides has also been reported such as in the case of the enkephalin retro inverso analog, H-Tyr-cyclo[D-A_2bu-Gly-g Phe-mLeu] (or the Pheψ[NHCO]Leu] cyclic enkephalin analog) (13).

Backbone modified peptides have proven useful in structure activity and conformation studies (10). Enkephalin structure function studies have been reported for such amide bond

substitutions as ψ[CH_2NH], ψ[CH=CH], [$COCH_2$], ψ[NHCO], and ψ[CH_2S] (10). Our efforts have concentrated on incorporation of ψ[CH_2S] as an amide bond surrogate. Peptide analogs in which an amide bond is replaced by another group or functionality are referred to as pseudopeptides (8a). The structure of one of the linear enkephalin pseudopeptide analogs included in this study is shown in Fig. 1. An interesting consequence of incorporating a thiomethylene ether linkage, -CH_2S-, as an amide bond substitution is the potential conversion of this group to its sulfoxide diastereomers to yield the new amide bond substitution, ψ[CH_2SO]. Due to the generation of a new achiral center at sulfur and the non-stereoselectivity of the oxidation, equivalent formation of the R and S isomers of the sulfoxides occurs. These diastereomeric sulfoxides are usually separable on RP-HPLC (Fig. 2).

The study reported here demonstrates the application of reverse phase HPLC for first establishing the synthetic origin and subsequently for the determination of the linear and cyclic diastereomers of two enkephalin pseudopeptide analogs Tyr-D-Ala-Gly-Pheψ[CH_2S]Leu and Tyr-cyclo[D-Lys-Gly-Pheψ[CH_2S]Leu] the final reverse phase purification yielded two isomers (Fig. 2). In each case identical molecular weights were established by FAB mass spectrometry. This anomalous isomeric phenomenon was viewed as probably arising from one of the following reactions: 1) epimerization or rearrangement during synthesis of the precursor pseudodipeptide Boc-Pheψ[CH_2S]Leu; 2) epimerization

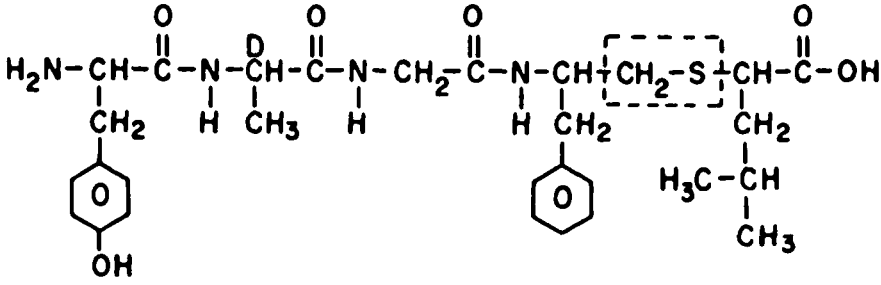

Fig. 1. Structure of [D-Ala^2,PheΨ[CH_2S]Leu]-enkephalin.

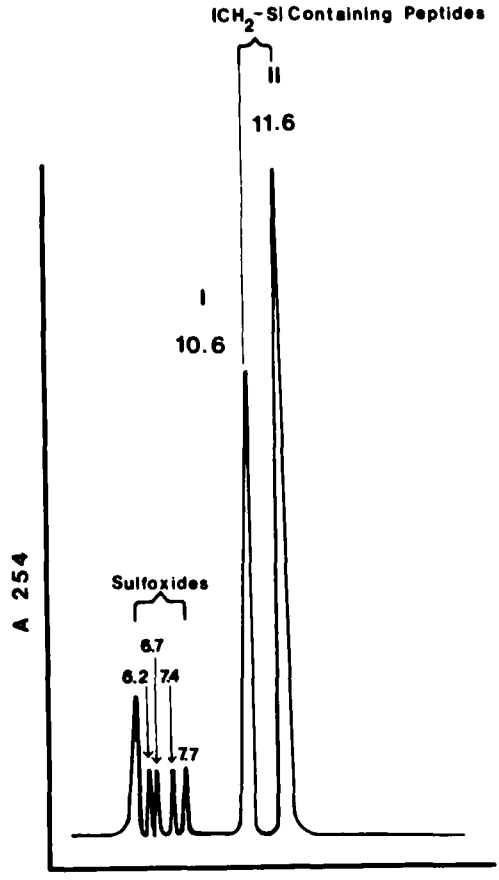

Fig. 2. Gradient elution of cyclic enkephalin pseudopeptide isomers and the oxidized sulfoxide products. Solvent: methanol and 0.41 M ammonium acetate, pH 4.1; MeOH gradient 60-80%, 20 min (2 ml/min); column used: DuPont Zorbax C-18 analytical.

during solid phase synthesis coupling reactions; 3) rearrangement during solid phase synthesis due to employment of acidic and basic deprotection reagents; or 4) epimerization or rearrangement during hydrogen fluoride cleavage of the peptide from Merrifield resin.

MATERIALS AND METHODS

Apparatus and Reagents

Chromatographic separations were carried out on a Dupont Model 850 Liquid Chromatographic System with a continuous wavelength detector (200-600 nm) and a Glenco Model CR-10-2 chart recorder. A Zorbax C-18 column (6.2 mm x 25 cm) was employed for both analytical and semipreparative separations. Fisher brand HPLC grade methanol was used. Water was deionized and distilled. Fischer HPLC grade ammonium acetate was employed.

Experimental Preparation of Pseudopeptides and Pseudodipeptides

The linear and cyclic enkephalin pseudopeptides were prepared at the University of Louisville by both solid and solution phase peptide synthetic methods developed in our laboratory and described elsewhere (10,12). The pseudodipeptide diastereomers Boc-Pheψ[CH_2S]Leu and Boc-Pheψ[CH_2S]D-Leu were prepared by reaction in DMSO of (S)-[2-tert-butyloxycarbonyl-1-amino-3-phenyl-propanyl-p-toluenesulfonate with the disodium salt of (R) or (S)-2-mercapto-4-methyl pentanoic acid and subjected to conventional work-up and purification techniques as described elsewhere (8a).

Oxidation of Thiomethylene Ether Containing Pseudopeptides to Diastereomeric Sulfoxides

An approximate 2 ml solution of the pseudodipeptide (1-5 mg) or pseudopeptide enkephalin was prepared in the appropriate HPLC eluant solvants. To this sample solution was added 0.1 ml of a 5% solution of hydrogen peroxide in 50% aqueous 0.4 M ammonium acetate/methanol solution. The sample was allowed to stand at 0°C for 10 minutes whereupon typically a 1 ml injection onto a reversed phase column (Zorbax C-18) was made. Oxidation to the diastereomeric sulfoxides, as monitored on HPLC, is usually signaled by the appearance of two closely eluting peaks having retention times considerably earlier than the -CH_2S-containing pseudopeptide (Fig. 2 and Fig 3, parts b and c). The peaks associated with the diastereomeric sulfoxides increased with time in succeeding injections, and have been previously characterized in other backbone substituted -CH_2S-containing peptides (12).

Attachment of Pseudodipeptides to Chloromethylated Polystyrene Resin Followed by HF Cleavage

The pseudodipeptide diastereomers of Boc-Pheψ[CH_2S]Leu were coupled to chloromethylated polystyrene resin using the Gisin procedure (9). This employed an overnight reaction of one millimole of the protected pseudodipeptide cesium salt per one milliequivalent of chloromethyl resin and 9 ml DMF/gm resin in a round bottom flask placed on a shaker and immersed in a thermostated water bath at 50°C. The protected pseudodipeptide

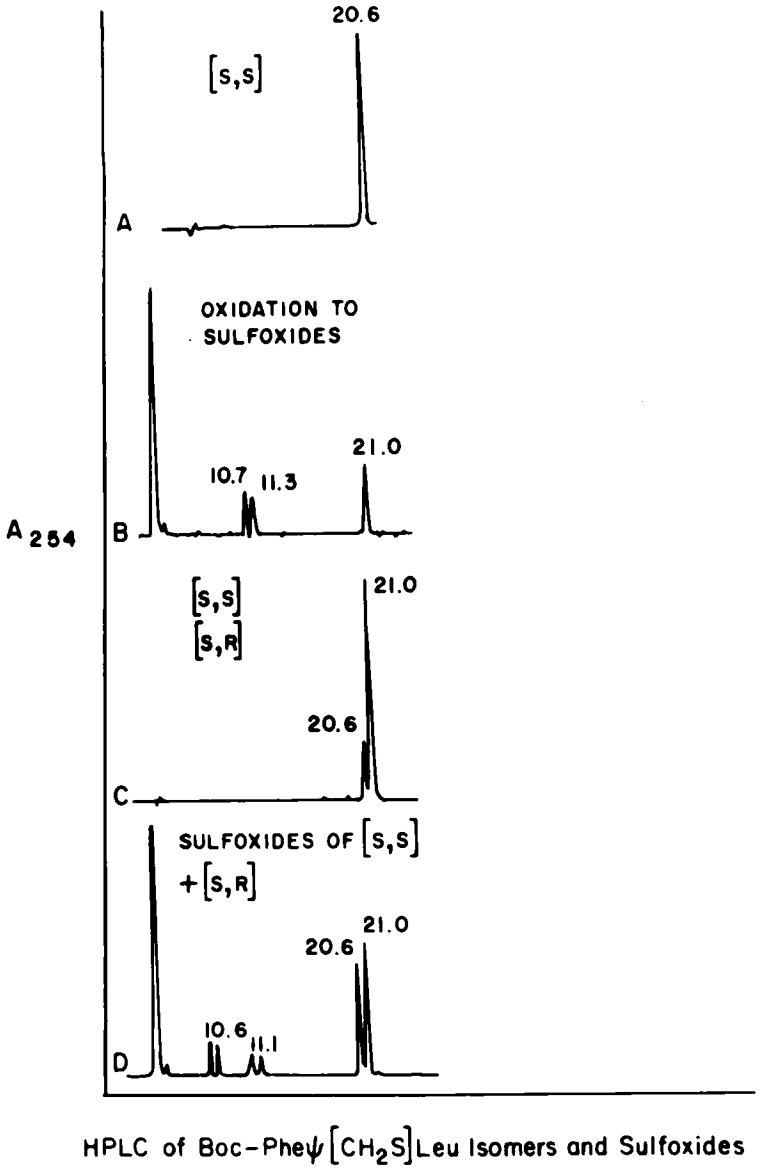

Fig. 3. Gradient elution of protected pseudodipeptide isomers and the sulfoxide diastereomers. Solvents: methanol and 0.41 M ammonium acetate, pH 4.1; MeOH gradient 60-80%, 25 min (2 ml/min) A. Elution of [S,S] isomer; B. Oxidation of [S,R] isomer to sulfoxides; C. Coelution of [S,S] and [S,R] isomers; D. Oxidation and coelution of [S,S] and [S,R] isomers; Zorbax C-18 column.

chloromethyl resin ester (1.5 gm) was dried and then treated with cobalt trifluoride-dried hydrogen fluoride in the presence of 1.5 ml anisole and 0.5 ml of methyl ethyl sulfide for 1 hour at 0°C. Following removal of hydrogen fluoride under reduced pressure the resin was dried, stirred with ethyl ether (30 ml x 3), and filtered. The pseudodipeptide was then extracted with an aqueous acetic acid solution (30%, 40 ml x 3). The extracts were pooled and lyophilized. A sample of the pseudodipeptide was dissolved in methanol and ammonium acetate and analyzed by HPLC (Fig. 4).

Hydrolysis Experiments for Assignment of Absolute Configuration of the Pseudopeptide Enkephalins

One milligram each of the enkephalin pseudopeptide epimers of Tyr-D-Ala-Gly-Pheψ[CH_2S]Leu and Tyr-cyclo[D-Lys-Gly-Pheψ[CH_2S]Leu] were treated with a 1 ml of 6N HCl in the presence of two drops of mercaptoethanol in a vacuum sealed hydrolysis vial. The samples were hydrolyzed at 110°C for 24 hours and then filtered through a sintered glass funnel. In addition, Boc-Pheψ[CH_2S]Leu and Boc-Pheψ[CH_2S]D-Leu were subjected to identical conditions for use as standards. The hydrolysates were then lyophilized from a 30% aqueous acetic acid solution, and subsequently analyzed by HPLC (Fig. 5).

DISCUSSION

RP-HPLC was initially employed to determine at which of two stages in the syntheses of the pseudopeptides the isomeric

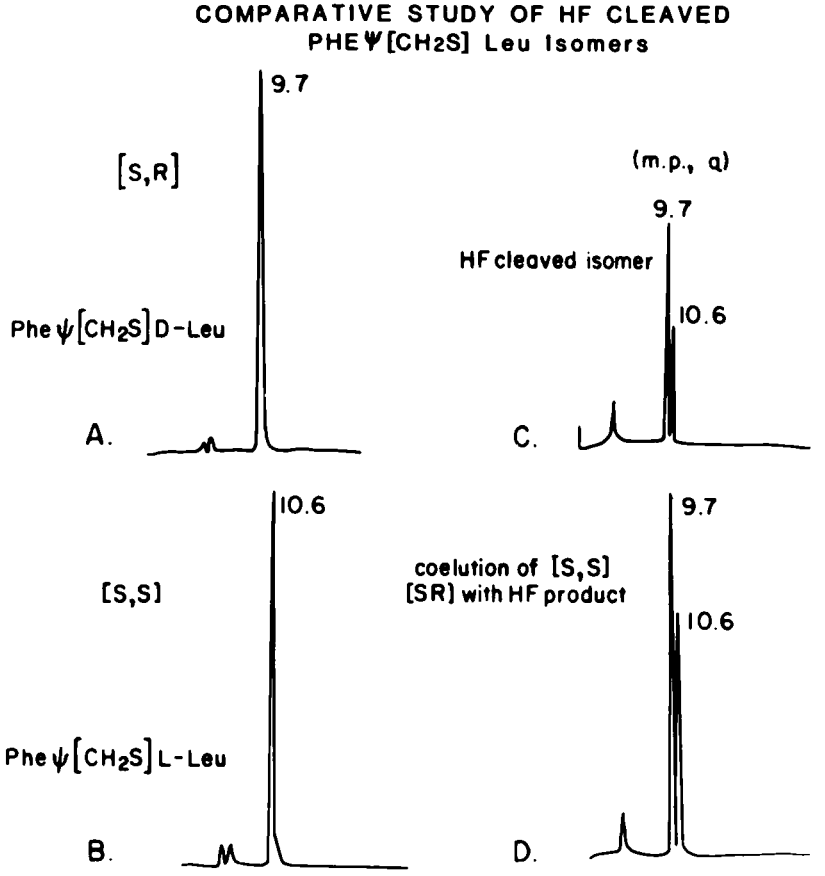

Fig. 4. Gradient elution of pseudodipeptide isomers and products following Merrifield resin HF cleavage. Solvent: methanol and 0.41 M ammonium acetate, pH 4.1; MeOH gradient 70-80%; 20 min (1 ml/min) A. Elution of [S,R] isomer; B. Elution of [S,S] isomer, C. Products of HF resin cleavage; D. Coelution of [S,S] and [S,R] isomer with HF product. Zorbax C-18 column.

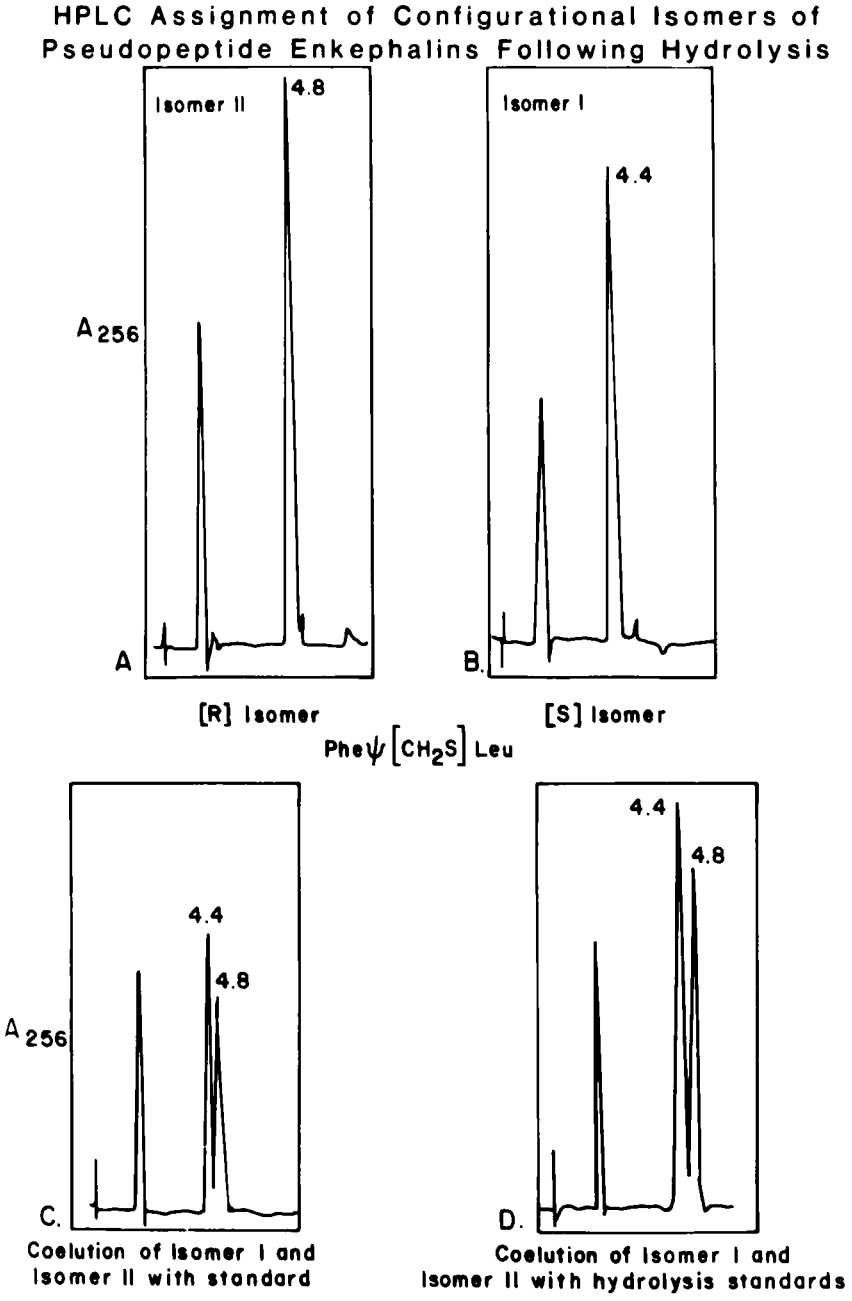

Fig. 5. Gradient elution of pseudopeptide hydrolysis products and pseudodipeptide standards: 0.41 M ammonium acetate, pH 4.1; MeOH gradient, 70-80%; 20 min (1 ml/min). A. Elution of [S,R] isomer following hydrolysis; B. Elution of [S,S] isomer; C. Coelution of [S,S] and [S,R] isomers with standards; D. Coelution of [S,S] and [S,R] isomers with hydrolysis standards; Zorbax C-18 column.

species arose. Investigation of the origin of the assumed epimerization or rearrangement was carried out by first examining the precursor reaction products from the synthesis of Boc-Pheψ[CH_2S]Leu. The solid phase reactions involving coupling of Boc-Pheψ[CH_2S]Leu to Merrifield resin were then studied using both analytical and semipreparative RP-HPLC.

To determine whether epimerization of Boc-Pheψ[CH_2S]Leu had occurred during the synthesis of the protected pseudodipeptide, the [S,R] isomer was synthesized and compared in oxidation and coelution experiments with the original [S,S] isomer employed in the preparation of Tyr-D-Ala-Gly-Pheψ[CH_2S]Leu and Tyr-cyclo[D-Lys-Gly-Pheψ[CH_2S]Leu]. The stereochemical integrity was investigated by comparing the [S,R] diastereomer and its sulfoxide diastereomers with the [S,S] isomer.

The results shown in Fig. 3 demonstrate that no epimerization occurred during the synthesis of β-mercapto-leucine from L-leucine and its subsequent conversion to the pseudodipeptide. Part A shows the chromatogram of Boc-Pheψ[CH_2S]Leu (the [S,S] isomer) with a characteristic retention time of 20.6 min. This compound was then partially oxidized with H_2O_2 to give the pair of diastereomeric sulfoxides with retention times of 10.7 and 11.3 min as seen in part B. In part C is shown the chromatogram of the Boc-Pheψ[CH_2S]L-Leu after it was spiked with Boc-Pheψ[CH_2S]D-Leu (the [S,R] isomers). These were both then oxidized and co-chromatographed giving two pairs of diastereomeric sulfoxides as shown in Part D.

The baseline separation of the S,S and S,R pseudodipeptides shown here confirms the stereochemistry of the starting materials. This experiment also excludes possible epimerization of the [S,S] isomer during synthesis to the [R,S] diastereomer, since the latter would have an enantiomeric relationship with the [S,R] compound, and thus must also elute at 20.6 min.

Having confirmed that no epimerization had occurred during the synthesis of the precursor Boc-Pheψ[CH_2S]Leu, we decided to examine in a similar manner the coupling reaction of the pseudodipeptide to the Merrifield resin that precedes solid phase synthesis. The anchoring reaction of Boc-Pheψ[CH_2S]Leu with the resin was studied for possible epimerization by comparing the products of a pseudodipeptide-resin cleavage resin versus deprotected samples of Pheψ[CH_2S]Leu and Pheψ[CH_2S]D-Leu. Thus, a sample of Boc-Pheψ[CH_2S]Leu was coupled to Merrifield resin using the Gisin procedure which employs formation of the cesium salt of the pseudopeptide followed by resin esterification at 50°C overnight.

The resin bound pseudodipeptide was then subjected to HF cleavage conditions followed by analysis on reverse phase HPLC. As seen in Fig. 4, the Pheψ[CH_2S]D-Leu and Pheψ[CH_2S]D-Leu controls (parts A and B) showed identical retention times with the products of the HF cleaved pseudodipeptide (part C). Co-elution of the [S,S] and [S,R] isomers with the HF cleaved isomer was observed (part D). Finally semi-preparative HPLC of the [S,S] and [S,R] isomers from the HF cleaved products yielded products with

TABLE I. Physical Constants of HF-Cleaved and Standard Pheψ[CH_2S]Leu Isomers

Isomers of Pheψ[CH_2S]Leu (acetate salts)	m.p.		$[\alpha]_D^{25}$	
	HF cleaved	standards	standards	HF cleaved
[S,S]	85-86.5°C	87-89°C	-4.26 (c=2,MeOH)	-4.8 (c=2,MeOH)
[S,R]	195-197°C	196-198°C	+46.7 (c=4,MeOH)	+44.5 (c=4,MeOH)

nearly identical melting points and optical rotations with the known [S,S] and [S,R] isomers (Table 1). The [S,R] isomer demonstrated the property of crystallizing in the eluant solution following separation.

The above experiments provided convincing evidence that epimerization had in fact occurred during the resin-attachment reaction, but did not indicate which enkephalin isomer corresponded to which leucine side chain orientation. A relatively unambiguous assignment of configuration at the α-carbon of the leucine moiety in Pheψ[CH_2S]Leu pseudodipeptide isomers was possible by virtue of the previously established resistance of the thiomethylene ether linkage to normal peptide hydrolysis conditions (6N HCl; 110°C for 24 hours). As seen in Fig. 5, examination of hydrolysis samples of each peptide isomer under reverse phase conditions revealed two identifiable peaks which

co-eluted with Pheψ[CH_2S]Leu and Pheψ[CH_2S]D-Leu in the case of each peptide isomer. The Pheψ[CH_2S]Leu [S,S] isomer elutes at 4.4 min (part B) whereas the [S,R] isomers elute at 4.8 min (part A). As seen in part C Fig. 5, the [S,S] and [S,R] isomers of Pheψ[CH_2S]Leu coelute with hydrolyzed samples of the linear and cyclic pseudopeptides. The retention times of these samples were identical with samples of Pheψ[CH_2S]Leu and Pheψ[CH_2S]D-Leu (part D) subjected to the same hydrolysis condition.

The above results outline an application of RP-HPLC for the isolation and investigative identification of cyclic and linear backbone modified synthetic peptide epimers. Both pairs of linear and cyclic peptide isomers were found to arise from a common synthetic origin. The RP-HPLC coelution isolation, and identification of the [S,R] and [S,S] isomers of Pheψ[CH_2S]Leu following the anchoring of the protected pseudodipeptide Boc-Pheψ[CH_2S]Leu to the Merrifield resin, and the absence of evidence for epimerization during resin HF cleavage suggest that epimerization occurs during the anchoring of the protected pseudodipeptide to the resin. Since no evidence of epimerization has been observed with other synthetic pseudodipeptides, it appears that this reaction is a consequence of having a chiral carbon adjacent to both a sulfur atom and a benzyl ester linkage. Previous syntheses of thiomethylene containing pseudopeptides incorporated the pseudodipeptide in other portions of the sequence or had an achiral glycine at the C-terminus. The solid phase synthesis of 4-5 amide bond modified enkephalin analogs described

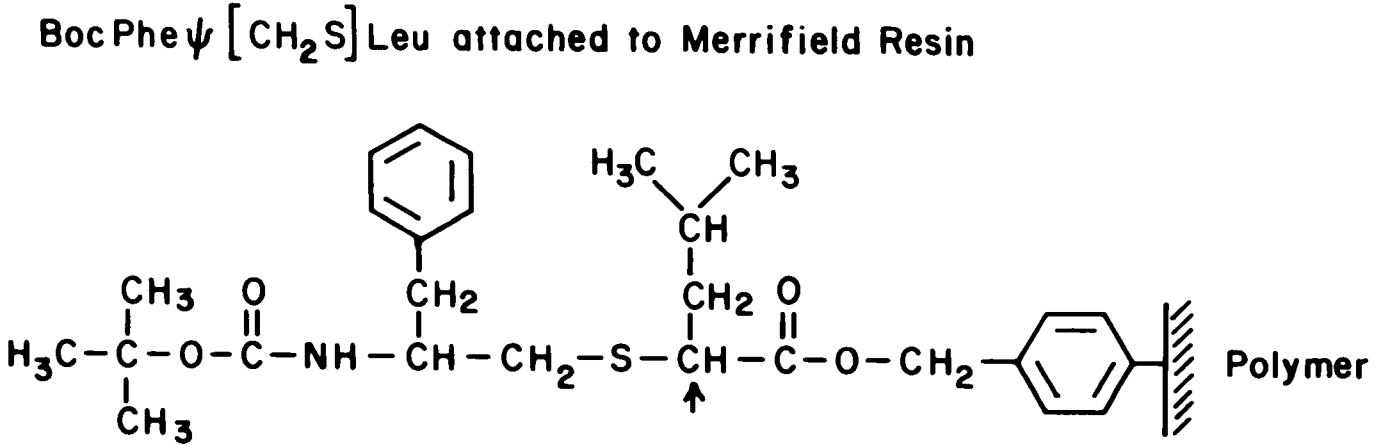

Fig. 6. Structure of Merrifield resin bound Boc-PheΨ[CH_2S]Leu. Arrow denotes site of proposed epimerization.

in this study necessitated anchoring of the pseudodipeptide directly to the resin. Since cesium coupling of a protected amino acid has not been observed to induce racemization, it is assumed that epimerization of the pseudodipeptide occurs as a result of the lability of the α-carbon hydrogen of the pseudo-leucine moiety. The structure of the protected pseudodipeptide resin (Fig. 6) indicates the proposed site of epimerization.

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