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DETERMINATION OF THE CHIRAL PURITY OF DIPEPTIDE ISOSTERES CONTAINING A REDUCED PEPTIDE BOND BY GAS CHROMATOGRAPHIC ANALYSIS

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SUMMARY

The stereochemical purity of $\Psi(CH_2–NH)$ dipeptides has been determined using gas chromatography–mass spectrometry. Different structures were found due to the derivatization procedures. A selective preparation of the linear bistrifluoroacetylated derivative and the monotrifluoroacetylated lactam makes it possible to monitor the chiral purity of the pseudodipeptides synthesized. Racemization occurring during peptide hydrolysis can be differentiated from racemization during the synthesis by using deuterium labelling. The method allows the optimization of the synthesis protocols and will be useful for further monitoring of the chiral purity of the pseudopeptides synthesized.

INTRODUCTION

A major drawback in the use of peptides as therapeutic agents is their rapid degradation *in vivo* by peptidases. The replacement of the scissile peptide amide bond by a non-hydrolyzable isosteric function can lead to analogues showing greater stability towards enzymatic degradation, greater selectivity in their biological action or antagonist activity[1,2]. Of the various types of isosteric replacements reported, the "reduced" peptide bond isostere $\Psi(CH_2–NH)$[3] was used successfully for the synthesis of a renin inhibitor[4], for tetragastrin[5] and somatostatin analogues[6]. The $\Psi(CH_2–NH)$ dipeptide isosteres are synthesized by reductive amination of a *tert.*-butyloxycarbonyl(Boc)-protected aminoaldehyde (1) using sodium cyanoborohydride[7] (Scheme 1).

The amine component can be an amino acid ester (2) or an oligopeptide ester (3). The resulting pseudopeptides (4 + 6) then require a carboxyl-deprotection step before further peptide couplings can occur. Recently, a reductive amination proce-

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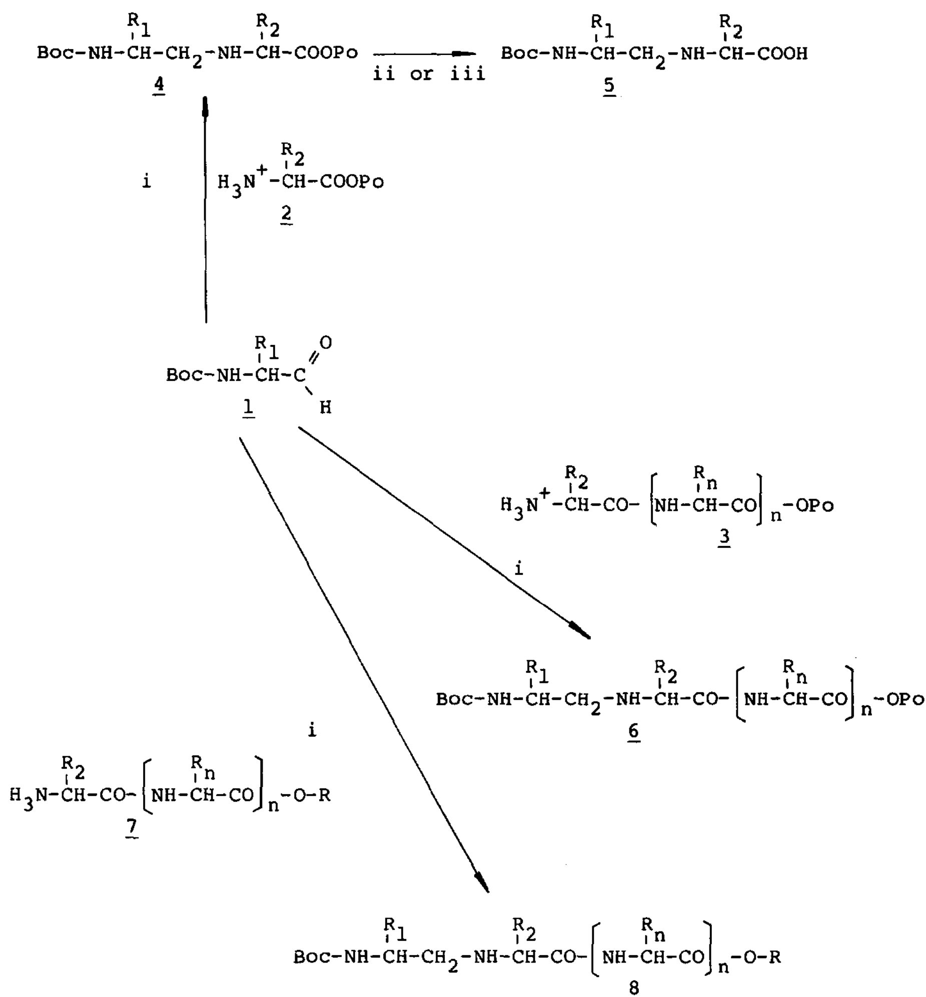
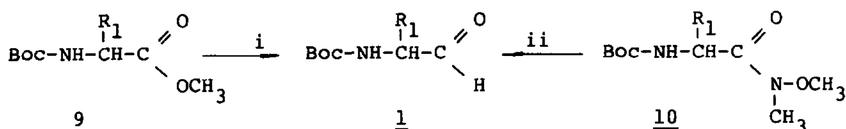


Scheme 1. Po = CH_3 or $CH_2C_6H_5$; i: $NaBH_3CN$, methanol–1% acetic acid; ii (Po = CH_3): 1 *M* sodium hydroxide–methanol (1:1), 3 h; iii: methanol, 10% Pd/C, hydrogen, 1 h; iv (R = Merrifield resin): $NaBH_3CN$, DMF–1% acetic acid.

dure on a resin-bound peptide (7) was reported[8] which allows the use of solid phase methodology in the synthesis of $\Psi(CH_2–NH)$ pseudopeptides. The chiral purity of the resulting pseudopeptides (4, 6 and 8) is dependent on the optical purity of the starting aldehyde (1), which is known to be very susceptible to racemization[9,10], and on the occurrence of racemization during the condensation and during the carboxyl-deprotection step. We have therefore examined the stereochemical purity of $\Psi(CH_2–NH)$ dipeptides, using two reductive methods for the preparation of the aldehydes (Scheme 2), using two different ester-deprotection methods, saponification and hydrogenolysis, and using the solid-phase synthesis method. It has been shown for $\Psi(CH_2–S)$ analogues that racemization in pseudopeptides may occur more easily than in the parent peptides[11].



Scheme 2. DIBAL-H, −78°C, 6 min; ii: $LiAlH_4$, −10°C, 10–30 min.

EXPERIMENTAL

Gas chromatography–mass spectrometry

GC–MS was performed on a Finnigan 3200 system connected to an on-line Finnigan Incos data system, which controlled repetitive data scanning of the mass spectrometer and acquired and processed all the data obtained. The samples were injected onto an ULTRA 2 (HP) capillary column, 50 m × 0.25 mm I.D., or a Chirasil-Val (Alltech) capillary column, 50 m × 0.25 mm I.D. The operating conditions were as follows: injection port temperature, 250°C; splitting ratio, 1/30; carrier gas, helium.

Preparation of bis(trifluoroacetyl) methyl esters (25)

A 1-mg amount of the Boc-protected methyl ester (4) of the pseudodipeptide was dissolved in 200 μl trifluoroacetic acid–acetonitrile (1:1) solution. The reaction mixture was kept at room temperature for 1 h. After evaporation of the solvents, the residue was taken up in 100 μl acetonitrile and 70 μl trifluoroacetic anhydride and allowed to react for 0.5 h at room temperature. The resulting solution was evaporated under a gentle stream of nitrogen.

Preparation of monotrifluoroacetyl lactam (28)

After the elimination of the Boc group with trifluoroacetic acid as described above, the cyclization was carried out in a solution of triethylamine–acetonitrile (1:1) for 1 h at 120°C. The resulting mixture was evaporated and a trifluoroacetyl group was added as described above.

Peptide hydrolysis

The hydrolysis was performed on a 4-mg amount of the peptide in a 1-ml Pierce Reactivial with 0.5 ml of 6 *M* deuterium chloride. The head space was purged with nitrogen gas and the vial tightly capped. The product was hydrolysed at 110°C for 24 h. Hydrochloric acid was removed to dryness under a flow of nitrogen at 65°C. The residue was taken up in 2 × 0.5 ml dichloromethane and again evaporated to dryness under a stream of nitrogen.

Preparation of methyl ester

The hydrolysate was dissolved in 400 μl of 2 *M* hydrochloric acid in methanol. The reaction mixture was purged by a stream of nitrogen, tightly capped and kept at 60°C for 5 h. Methanol was removed under a stream of nitrogen.

General procedure for preparation of pseudodipeptides (4)

Boc-aminoaldehyde (1) was prepared either by reduction of the methyl ester

according to ref. 12 using 2.5 equiv. diisobutylaluminium hydride at −78°C for 6 min, or by reduction of the dimethylhydroxamate according to ref. 13, using 1.25 equiv. lithium aluminium hydride in diethyl ether or tetrahydrofuran (THF) at −10°C for 10 to 30 min. To 1 equiv. of the Boc-aminoaldehyde (1) in dry methanol containing 1% acetic acid, were added 1.1 equiv. of the methyl ester hydrochloride salt or of the benzyl ester tosyl salt, and molecular sieves (0.8 g/mmol). Sodium cyanoborohydride (3 equiv.) was added in portions over 10 min at 0°C, and the mixture was stirred for another 30 min. After filtration and evaporation, the residue was taken up in chloroform, washed successively with saturated sodium bicarbonate and water, dried over magnesium sulphate and evaporated. The crude compound was purified by flash chromatography on silica gel using a chloroform–ethyl acetate gradient. Average yield: *ca.* 46–50%.

The pseudodipeptides investigated are collected in Table I.

RESULTS AND DISCUSSION

Gas chromatography was chosen for its high resolution, precision and reproducibility. The comparison of peak areas provides a precise measurement of the relative composition, not affected by impurities. GC–MS allows the identification of minor amounts of stereoisomers without having to prepare reference compounds and allows the use of the isotope labelling technique during the peptide hydrolysis (see below). This is not the case for high-performance liquid chromatographic (HPLC) separations of diastereomeric pseudodipeptides[6,11,14]. Gas chromatographic separations of dipeptides, including diastereomers, derivatized as N-acyl methyl esters[15] or as trimethylsilyl derivatives[16] have been described.

Moreover the enantiomeric composition of amino acid mixtures can easily be obtained using a Chirasil-Val column[17]. We chose the N-trifluoroacetyl methyl ester derivatives for the pseudodipeptides because of their good retention times[18].

TABLE I

RETENTION TIMES OF THE DIFFERENT STRUCTURES OBTAINED DURING DERIVATIZATION

(CH_2–NH) dipeptide	*Retention times*				
	24	*25*	*26*	*28*	*29*
L–Ala–L-AlaOCH_3 11	11′59″	13′51″	18′20″	15′19″	11′23″
L–Ala–D-AlaOCH_3 12	11′30″	12′50″	18′09″	14′23″	10′41″
D–Ala–D-AlaOCH_3 13	11′59″	13′51″	18′20″	15′19″	11′23″
D–Ala–L-AlaOCH_3 14	11′30″	12′50″	18′09″	14′23″	10′41″
D–Ala–D-AlaOBzl 15	–	15′46″	18′20″	15′19″	11′23″
L–Pro–L-PheOCH_3 16	–	31′50″	–	32′51″	–
L–Pro–D-PheOCH_3 17	–	31′50″	–	32′20″	–
L–Phe–L-ProOCH_3 18	–	30′53″	–	–	–
D–Phe–L-ProOCH_3 19	–	30′34″	–	–	–
L–Leu–L-ValOBzl 20	–	32′31″	–	34′15″	–
L–Pro–L-LeuOCH_3 21	–	–	–	25′50″	–
L–Pro–D-LeuOCH_3 22	–	–	–	26′12″	–

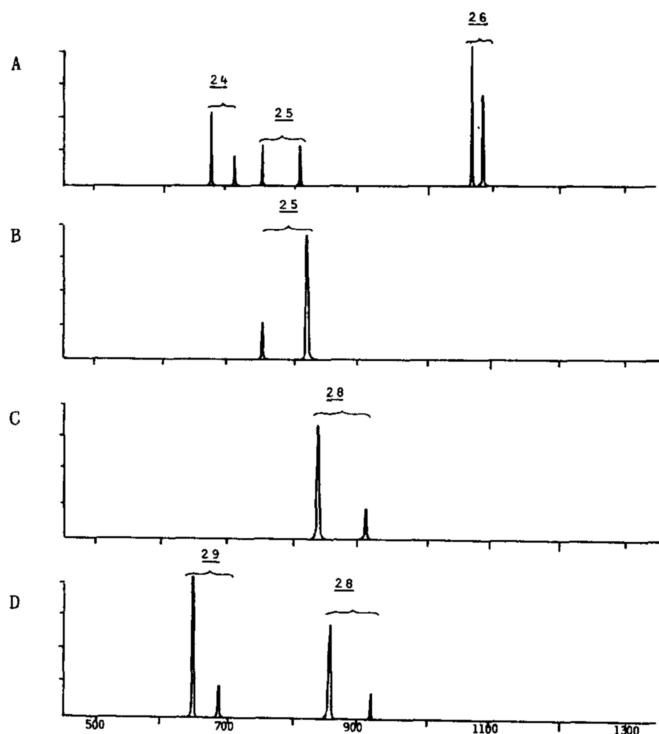
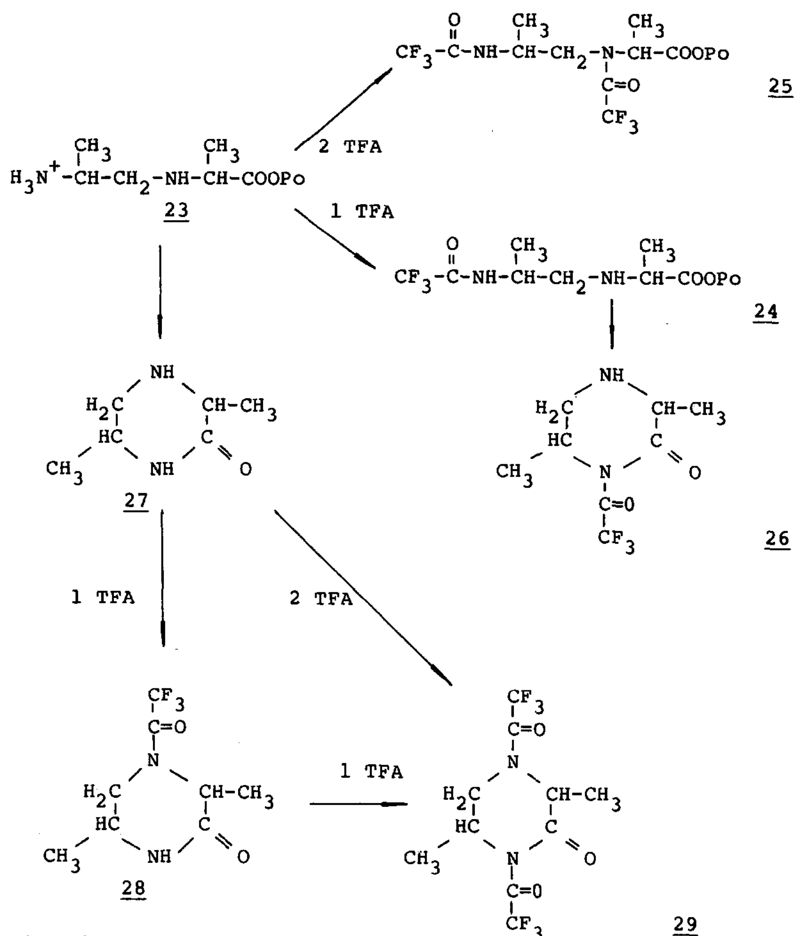


Fig. 1. GC–MS of D-Ala–$\Psi(CH_2$-NH)D-Ala-OCH_3 and D-Ala–$\Psi(CH_2$-NH)L-Ala-OCH_3. (A) Derivatization with TFAA at 100°C for 10 min which results in the linear mono- and bistrifluoroacetyl methyl esters 24 and 25, and the monotrifluoroacetyl lactam 26. (B) Derivatization to the linear bistrifluoroacetyl methyl ester 25. (C) Derivatization to the monotrifluoroacetyl lactam 28. (D) Derivatization to the mono- and bistrifluoroacetyl lactams 28 and 29. Time-scale in seconds.

In the first experiments using the Boc-protected pseudodipeptide methyl esters 4, the derivatization was performed immediately with trifluoroacetic anhydride (TFAA) at 100°C for 10 min. It was assumed that the trifluoroacetic acid (TFA) which is generated during the reaction would cleave the Boc-protecting group, so that the amino group is trifluoroacetylated. Using an artificial mixture of D-Ala–$\Psi(CH_2$–NH)D-Ala–OCH_3 (13) and D-Ala–$\Psi(CH_2$–NH)L-Ala-OCH_3 (14), this derivatization procedure resulted in three groups of peaks in the gas chromatogram, with retention times of 11.5, 13.5 and 18 min (Fig. 1A). Injection of a sample of D-Ala–$\Psi(CH_2$–NH)D-Ala-OBzl (15) also gave three peaks, the last one having the same retention time and mass spectrum as for 13. Based on this observation and on the mass spectra, it was evident that the third peak is formed by a cyclization reaction to lactam 26 (Scheme 3), while the more rapidly eluted compounds correspond to the linear mono- and bistrifluoroacetylated derivatives 24 and 25. Since the multiplicity of peaks represents a complication for the analysis of the stereochemical purity, more selective derivatization procedures were worked out. The Boc group is first cleaved by using a 50% TFA–acetonitrile solution for 1 h at room temperature, followed by treatment with a TFAA–acetonitrile (7:10) solution during 0.5 h at room



Scheme 3.

temperature. This cleanly gives only the linear bistrifluoroacetylated compound 25 (Fig. 1B). After evaporation of the reactants and dissolution in acetonitrile, the sample is stable at room temperature for over a week.

The monotrifluoroacetylated lactam 28 can be selectively obtained by cleaving the Boc group first, neutralizing the TFA salt with triethylamine and heating the solution for 1 h at 120°C. The resulting lactam 27 is derivatized with TFAA at room temperature to 28 (Fig. 1C). Raising the derivatization temperature to 110°C results also in the formation of the bistrifluoroacetylated lactam 29 (Fig. 1D).

The diastereomeric composition of the pseudodipeptides can be determined either on the linear (25) or the cyclic (28) structure. Mostly, a better separation of the diastereomers is observed for the latter (Table I). This is especially the case for L-Pro–$\Psi(CH_2$–NH)L-Phe-OCH_3 and L-Pro–$\Psi(CH_2$–NH)D-Phe-OCH_3, for which the linear derivatives could not be separated.

It is interesting that in samples containing diastereomeric pseudodipeptides a kinetic resolution is observed: the heterochiral isomers cyclize more rapidly than do the homochiral. This can be seen in a sample containing compounds 24 and 25. By

storing this sample for 2 weeks or more, only some of the heterochiral isomer cyclizes spontaneously to the corresponding lactam 26.

The samples of the pseudodipeptides were then analyzed for possible racemization during synthesis of the aldehyde, during the derivatization step or during the deprotection of the ester function. For the Ala–Ala pseudodipeptides 11–14 no racemization could be detected whether the aldehyde 1 was prepared by reduction of the methyl ester[12] or by reduction of the dimethylhydroxamate[13] (Scheme 2).

A sample of Boc-D-Ala–aldehyde was stored for 2 days at −18°C, and then coupled to D-Ala-OBzl to give compound 15. This gave rise to 5% racemization in the pseudodipeptide 15, indicating that the aldehyde should be freshly prepared before the amination step.

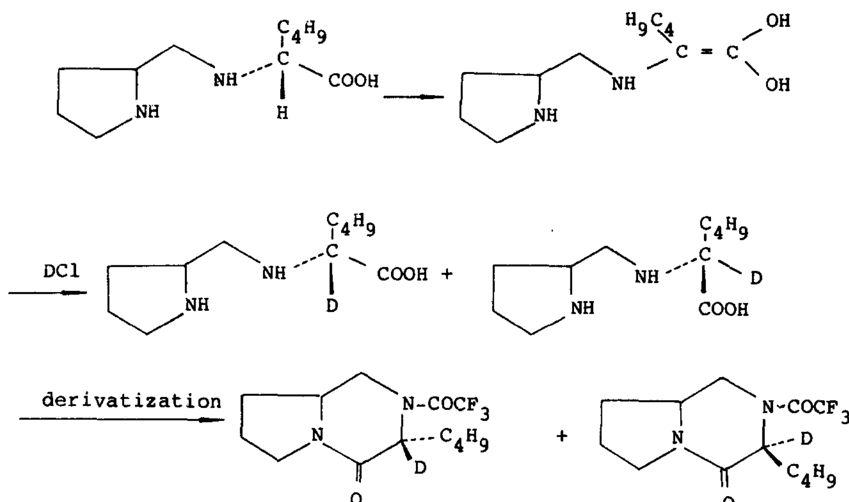
These analyses also demonstrate that the reductive amination itself proceeds without racemization. Saponification of Boc-D-Ala–Ψ(CH_2–NH)D-Ala-OCH_3 with 1 *M* sodium hydroxide during 3 h also occurs without detectable formation of the Boc-D-Ala–Ψ(CH_2–NH)L-Ala isomer. In the synthesis of Boc-L-Pro–Ψ(CH_2–NH)L-Phe-OCH_3 or of its D-Phe isomer, no racemization could be detected. However, saponification of their methyl esters with 1 *M* sodium hydroxide during 3 h occurred with 8% racemization. The analysis of L-Leu–Ψ(CH_2–NH)L-Val-OBzl (20) revealed no isomeric compound, and the free acid obtained by hydrogenolysis of the benzyl ester contained no trace of racemized product. Therefore we recommend in general the use of benzyl ester protection instead of methyl ester protection. In this case, it is imperative to stop the reductive amination which is performed in methanol immediately after its completion (1 h). An overnight reaction caused partial transesterification of the benzyl ester 15 to the methyl ester 13, and a mixture containing 20% methyl ester was obtained. No racemization was observed however.

We previously prepared L-Pro–Ψ(CH_2–NH)L-Leu–Gly-NH_2[19] by condensing Boc-L-Pro–aldehyde with the dipeptide amide. To determine the racemization of the pseudodipeptide, an acid hydrolysis in 6 *M* deuterium chloride during 20 h was carried out[20]. A molecule undergoing racemization during hydrolysis automatically incorporates a deuterium atom. Isomers formed during the hydrolysis can be distinguished by mass spectrometry from those which were present in the sample before hydrolysis (Scheme 4).

Using this technique, it was possible to demonstrate that, although 2% of the L-Pro–Ψ(CH_2–NH)D-Leu isomer was observed (Fig. 2) after derivatization to the lactams, this epimer was formed during the hydrolysis since this peak contained only deuterium-labelled compound.

As a further test, the dipeptide Phe–Pro (18) was chosen, because of the ease of racemization of the Phe residue[10], and because during the reductive amination the condensation of Boc-Phe-aldehyde with the secondary amine of Pro-OCH_3 generates an immonium function, which is expected to racemize easily. The preparation of the pseudodipeptide 18 according to the general procedure occurred with 3% racemization, probably at the Phe residue. Boc-Phe–aldehyde was also condensed with TFA-L-Pro–Gly–resin, which after further peptide synthesis resulted in the β-casomorphin analogue Tyr–Pro–Phe–Ψ(CH_2–N)Pro–Gly–O–resin[21].

Hydrolysis of a sample of the resin-bound peptide, followed by derivatization, also showed 3% racemization of the pseudodipeptide. There was no observable racemization during hydrolysis.



Scheme 4.

At this point it became of interest to determine whether the observed racemization occurred at the first or at the second residue of the pseudodipeptide. This requires the separation of the L-D enantiomer from the D-L enantiomer on a chiral stationary phase. However injection of mixtures of L-Ala–Ψ(CH_2–NH)L-Ala (11) and D-Ala–Ψ(CH_2–NH)D-Ala (13) or L-Ala–Ψ(CH_2–NH)D-Ala (12) and D-Ala–Ψ(CH_2–NH)L-Ala (14), derivatized as the linear bis-TFA compounds 25 or as the lactams 28, onto a Chirasil-Val column allowed no separation of the enantiomers.

The GC–MS method reported allows an easy and accurate determination of the diastereomeric composition of Ψ(CH_2–NH) dipeptide analogues. Racemization

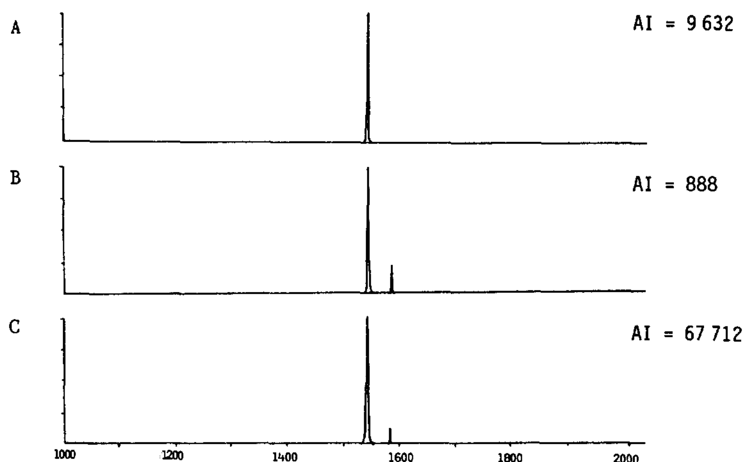


Fig. 2. GC–MS of Pro–Ψ(CH_2-NH)-Leu after acid hydrolysis in 6 *M* deuterium chloride. (A) Ion chromatogram of mass 236, $(M_1 - C_4H_9)^+$. M_1 = Molecular ion of the monotrifluoroacetyl lactam without deutrium incorporation. (B) Ion chromatogram of mass 237 $(M_2 - C_4H_9)^+$. M_2 = Molecular ion of the monotrifluoroacetyl lactam with deuterium incorporation or with a 13C-atom in its chain. (C) Total ion chromatogram: 98% L-Pro–Ψ(CH_2–NH)L-Leu; 2% L-Pro–Ψ(CH_2–NH)D-Leu. Time-scale in seconds.

occurring during peptide hydrolysis can be differentiated from racemization during the synthesis by using deuterium labelling. The present method allowed the optimization of the synthesis protocols and will be useful for further monitoring of the chiral purity of the pseudopeptides synthesized.

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