POSSIBLE DRUGS LABELLED WITH ^{14}C. III.[x] SYNTHESIS OF D-[3-^{14}C]PHENYLALANINYL-L-PROPYL-L-ARGININE ALDEHYDE AND L-TYROSINYL-D-METHIONYL-GLYCYL-L-[3-^{14}C]PHENYLALANINYL-L--PROLINE AMIDE

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SUMMARY

D-[3-^{14}C]Phenylalaninyl-L-prolyl-L-arginine aldehyde (GYKI-14166) and L-tyrosyl-D-methionyl--glycyl-L-[3-^{14}C]phenylalaninyl-L-proline amide (GYKI-14238) were prepared starting from D-[3-^{14}C]phenylalaninyl-L-proline and L-[3-^{14}C]phenylalaninyl--L-proline, respectively. The diastereomeric pairs obtained by coupling 2,4,5-trichlorophenyl benzyloxycarbonyl-D,L-[3-^{14}C]phenylalaninate to L-proline and by subsequent deprotection were separated on silica gel.

Key Words: [3-^{14}C]phenylalanine, resolution

INTRODUCTION

D-Phenylalaninyl-prolyl-arginine aldehyde (GYKI-14166, as sulphate),[xx] a highly potent anticoagulant (1-3), and tyrosyl-D--methionyl-glycyl-phenylalaninyl-proline amide (GYKI-14238, as acetate), a superactive analogue of Met/Leu enkephalins (4) had been prepared at our Institute. Recently, both peptides were

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[x] Part II: E. Koltai, G. Zólyomi, P. Komáromy, D. Bánfi, T. Szüts and K. Takács - this Journal 18: 1107 (1981).

[xx] Unless otherwise stated the L enantiomer of amino acids should be considered.

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required to be labelled with ^{14}C for pharmacokinetic and metabolism studies. The fact that the tripeptide aldehyde contains a D-Phe-Pro sequence and a Phe-Pro fragment can be found in the pentapeptide amide, gave us the idea of labelling phenylalanine with ^{14}C, and using the diastereomeric pair of H-D,L-[3-^{14}C]Phe--Pro-OH as a common precursor in the preparation of these peptides. Moreover, Cbz-D-Phe-Pro-OH dipeptide was an intermediate in the original synthesis of GYKI-14166 (2), and several enkephalin analogues were prepared in our institute via 3+2 couplings using the Phe-Pro sequence (4,5).

Therefore, Cbz-D,L-[3-^{14}C]phenylalanine was synthesized, it was coupled to proline and the diastereomeric pairs of H-D,L--[3-^{14}C]Phe-Pro-OH were separated by chromatography. Further on the syntheses of GYKI-14166 and GYKI-14238 followed the routes described earlier.

RESULTS

A. Synthesis of Cbz-D,L-[3-^{14}C]Phe-OH

Due to the rather high price of commercially available ^{14}C-phenylalanine, the possibilities of the synthesis of labelled phenylalanine were studied and an improved method for the preparation of D,L-[3-^{14}C]phenylalanine was elaborated.

There are three basic ways published to obtain ^{14}C labelled phenylalanine:

1) Strecker synthesis affords [1-^{14}C]phenylalanine in 28% yield based on $Na^{14}CN$ (6).

2) Azlactone synthesis leads to [3-^{14}C]phenylalanine in a yield of 70% based on [7-^{14}C]benzaldehyde (7).[x]

3) Alkylation of diethyl formamido- or acetamidomalonate

[x] When the reaction was reversed using labelled hippuric acid, D,L-phenylalanine was prepared in our laboratory in a yield of 40% based on KCN.

results in D,L-[3-^{14}C]phenylalanine with a yield of 40% based on [7-^{14}C]benzoic acid (8).

Our synthesis followed the latter route. Diethyl formamidomalonate was alkylated with [7-^{14}C] benzyl bromide adapting the method described for the preparation of [1-^{14}C]n-octyl bromide (9). [7-^{14}C]Benzoic acid was esterified with ethereal diazomethane and reduced with $LiAlH_4$, and the obtained alcohol was refluxed in a mixture of 48% HBr and conc. H_2SO_4 to give [7-^{14}C]benzyl bromide. The alkylation was carried out in dried ethyl acetate using NaH as base. The molar ratio of [7-^{14}C]benzyl bromide,[x] NaH and diethyl formamidomalonate was 10:11:12. The obtained diethyl 2-[7-^{14}C]benzyl-2-formamidomalonate was purified by chromatography,[xx] then hydrolysed to D,L-[3-^{14}C]phenylalanine, which was acylated with benzyl chloroformate to give Cbz-D,L-[3-^{14}C]phenylalanine in 65% yield based on benzoic acid.[xxx]

B. Resolution

Cbz-D,L-[3-^{14}C]phenylalanine was coupled to proline by the active ester method using 2,4,5-trichlorophenyl ester, then the protecting group was removed by catalytic hydrogenolysis. Diastereomers of H-D,L-[3-^{14}C]Phe-Pro-OH were separated by chromatography on silica gel and then both compounds were reacylated with benzyl chloroformate. The optical purity checked by TLC was found to be 98% for D, and 93% for L isomers. The overall yield

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x The exact quantity of [7-^{14}C]benzyl bromide was not determined, it was supposed to be molar equivalent with [7-^{14}C]benzoic acid.

xx Radiochemical yield was 85% based on [7-^{14}C]benzoic acid. Albertson and Archer (10) reported 90% yield when benzyl chloride were used in ethanol as alkylating agent and sodium ethoxyde as base, respectively; the reproduction of the experiment, however, did not afford pure substance, and after purification the yield decreased to about 50%.

xxx In the hot run Cbz-D,L-[3-^{14}C]phenylalanine was not crystallized, but used as an oil, consequently the yield is derived from the inactive runs.

of the resolution including the removal and reconstruction of the protecting group, were equally about 40%.

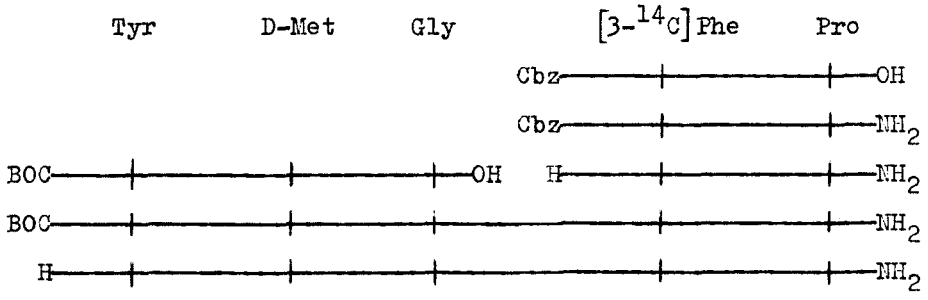
As thin-layer chromatography revealed, both D and L H-Phe-Pro--OH transformed to their diketopiperazine derivatives in mild basic or acidic solutions. The rate of the transformation was 2-3 times higher in the case of D isomer than that in the case of L isomer. Therefore, deprotection, separation and reprotection had to be carried out immediately after one another, because standing could have catastrophically decreased the yield.

C. Synthesis of GYKI-14166

GYKI-14166 was synthetised according to the reported route (1-3). Cbz-D-[3-^{14}C]Phe-Pro-OH was coupled to ω-Cbz-arginine-lactame by the mixed anhydride method and the tripeptide was then reduced with $LiAlH_4$ in tetrahydrofuran. Deprotection was performed by catalytic hydrogenolysis. The purity of the product was checked by TLC and its biological activity was also measured (3). 892 MBq (24.1 mCi) of GYKI-14166 were prepared in a yield of 4.2% based on [7-^{14}C]benzoic acid, and 20.5% based on Cbz-D--[3-^{14}C]Phe-Pro-OH.

D. Synthesis of GYKI-14238

Cbz- 3-^{14}C Phe-Pro-OH was transformed to GYKI-14238 according to the described method outlined in the following scheme:



1.83 GBq (49.4 mCi) of GYKI-14238 were obtained in a yield of 8.6% based on 7-^{14}C benzoic acid, and 39% on Cbz-3-^{14}C Phe-Pro--OH. The purity was checked by TLC.

EXPERIMENTAL

Chromatography was performed on silica gel 60 (MERCK; 70-230 mesh) and pre-coated silica gel 60 F_{254} sheets (MERCK), respectively. The spots were located by chlorine-tolidine spray reagent and quantified by a Berthold TLC scanner. Radioactivity was measured in a Packard Tri-Carb 2660 liquid scintillation spectrometer.

[7-^{14}C]Benzyl bromide

1.387 g (11.36 mmoles; 21.31 GBq=576 mCi) of [7-^{14}C]benzoic acid was esterified with ethereal diazomethane.

The ethereal solution was cooled to 0°C and $LiAlH_4$ (1.5 g; 40 mmoles) was added in small portions. To the mixture being stirred at room temperature for one hour, methanol (7 ml) was added and the solvent was evaporated. The flask was immersed in an ice bath and 48% hydrobromic acid (24 ml) and conc. H_2SO_4 (11 ml) were dropped cautiously through the reflux condenser into the mixture which was then refluxed for one hour. By the end of the reflux the mixture became clear and a brown oil separated. After cooling, water was added (25 ml) to dissolve the solids that precipitated. The solution was extracted with ether (4x25 ml), the combined extracts were washed with 5% $NaHCO_3$ (20 ml) and 25% NaCl (20 ml) successively, dried over Na_2SO_4 and evaporated to give 2.015 g (more than calculated) of a colourless oil.

Diethyl 2-[7-^{14}C]benzyl-2-formamidomalonate

To a solution of diethyl formamidomalonate (3.05 g; 15 mmoles) in dry ethyl acetate (30 ml) 560 mg of 50% sodium hydride suspension (14 mmoles) was added. The mixture was stirred till the bubbling ceased (about 15 minutes), and after addition of the above [7-^{14}C]benzyl bromide dissolved in dry ethyl acetate (20 ml) it was refluxed with stirring for 2.5 hours, then cooled and washed with water (2x20 ml), dried over Na_2SO_4 and evaporated.

The remaining white solid contained beside the substance (R_f 0.55) 9% of radioactive impurity (R_f 0.7) and some unreacted diethyl formamidomalonate (R_f 0.4) by TLC (cyclohexane-ethyl acetate 1:1). The total activity amounted to 18.13 GBq (490 mCi). This material after purification by chromatography (eluent: cyclohexane - ethyl acetate 1:1) weighed 2.472 g (74%) and its total activity was 15.32 GBq (414 mCi). Radiochemical yield: 72% (calcd. on benzoic acid).

D,L-[3-^{14}C]Phenylalanine hydrochloride

2.472 g (8.43 mmoles) of diethyl formamido [7-^{14}C]benzyl malonate was dissolved in a mixture of acetic acid, conc. HCl and water (each 15 ml) and refluxed for 18 hours. The hot solution was decolourized with charcoal and evaporated to obtain 1.559 g (92%) of a light brown oil.[x]

Benzyloxycarbonyl-D,L-[3-^{14}C]phenylalanine

D,L-[3-^{14}C]Phenylalanine hydrochloride (1.559 g; 7.73 mmoles) was dissolved in a mixture of 2N NaOH (10 ml) and 5% $NaHCO_3$ (40 ml). To the solution benzyloxycarbonyl chloride (1.6 ml; 1.91 g; 11.2 mmoles) was added under cooling with ice and the mixture was stirred at 0°C for one hour and at room temperature overnight. The pH was adjusted to 11 with 2N NaOH and the alkaline solution after washing with ether (2x15 ml) was acidified to pH 1 with conc. HCl (caution, violent bubbling!) and extracted with ethyl acetate (3x20 ml). The combined extracts were washed with 25% aqueous NaCl, dried over Na_2SO_4 and evaporated to afford 2.126 g of Cbz-D,L-[3-^{14}C]phenylalanine as a sticky white solid[xx] homogenous by TLC (ethyl acetate-pyridine--acetic acid-water 240:20:6:11; R_f 0.7). Total activity: 13.08

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[x] In acetone crystals could be prepared with some loss of weight.

[xx] When it was crystallized in ether in a cold run, the overall yield decreased.

GBq (353 mCi). Radiochemical yield: 65% based on benzoic acid.

Benzyloxycarbonyl-D,L-[3-^{14}C]phenylalaninyl-proline

To a solution of Cbz-D,L-[3-^{14}C]phenylalanine (2.126 g; 7.1 mmoles) in tetrahydrofuran (30 ml), 2,4,5-trichlorophenol (1.823 g; 9.2 mmoles) and dicyclohexyl carbodiimide (1.90 g; 9.2 mmoles) dissolved in tetrahydrofuran (10 ml) were added while the temperature was kept below 5°C. Stirring was continued at 5°C for one hour and at room temperature overnight. Next day the dicyclohexylurea was filtered off and the filtrate was evaporated. The residue was dissolved in dry pyridine (25 ml), then triethylamine (3.0 ml; 2.18 g; 21.5 mmoles) and proline (0.98 g; 28.5 mmoles) were added to the mixture which was then stirred at room temperature overnight. After evaporation, the residue was dissolved in a mixture of ether (50 ml) and water (50 ml) containing 840 mg (10 mmoles) of $NaHCO_3$. The layers were separated, the ethereal phase was extracted with water and the combined aqueous extracts were acidified with 2N HCl to pH 2 and extracted again with ethyl acetate (3x30 ml). The organic extracts were combined and washed with 25% NaCl (30 ml), dried over Na_2SO_4 and evaporated. The residue, a thick brown oil, weighed 2.39 g (85%). (Crystallization could be achieved in ether to the detriment of the yield.) Impurities not more than 10% were found by TLC (ethyl acetate-pyridine-acetic acid-water 240:20:6:11; R_f 0.3).

D- and L-[3-^{14}C]Phenylalaninyl-proline

Cbz-D,L-[3-^{14}C]Phenylalaninyl-proline (2.39 g; 6.03 mmoles) was dissolved in ethanol (40 ml) containing 2N HCl (4 ml) and hydrogenated over 10% Pd/C catalyst. After filtration and evaporation, the residue, a thick, light-brown oil (1.95 g; more than calcd.) showed two spots by TLC (chloroform-ethanol-water 60:60:10); one at R_f 0.3, corresponding to H-L-Phe-Pro-OH and

the second at R_f 0.2, identical with H-D-Phe-Pro-OH. The diastereomeric compounds were separated by chromatography (eluent: chloroform-ethanol-water 60:60:10) to obtain 1.1 g of L and 1.3 g of D isomers each as an oil. Their optical purity checked by TLC was found 98% for D and 93% for L isomers. Both diastereomers were reacted immediately with benzyl chloroformate in order to obtain protected dipeptides and, in this way, to avoid the formation of piperazine-2,5-dione derivatives.

Cbz-[3-^{14}C]Phe-Pro-OH

The L isomer was dissolved in 5% $NaHCO_3$ (35 ml) and after addition of benzyl chloroformate (1.5 ml; 1.79 g; 10.5 mmoles) the solution was stirred at room temperature overnight. 8N NaOH (5 ml) and ether (30 ml) were then added and after being stirred for 30 minutes the ether was separated, the aqueous phase was acidified with conc. HCl to pH 2, and extracted with ethyl acetate (3x20 ml). The organic extracts were combined and washed with 25% NaCl (2x15 ml), dried over Na_2SO_4 and evaporated to give 1.108 g (2.8 mmoles) of a colourless oil which contained 5% of impurities by TLC (ethyl acetate-pyridine:acetic acid-water 120:20:6:11; R_f 0.6). Total activity: 4.68 GBq (127 mCi). Radiochemical yield: 42% (calcd. on Cbz-D,L-Phe-Pro-OH).

Cbz-D-[3-^{14}C]Phe-Pro-OH

The reaction was carried out as described for the L isomer. In this case the major part of the radioactivity was found in the ethereal phase as Cbz-D-[3-^{14}C]Phe-Pro-OBz.[x] The ether was evaporated and the residue dissolved in a mixture of methanol (30 ml) and 5N NaOH (3 ml) was allowed to stand at room tempera-

[x] Although it has not been investigated exactly, it migth be due to the benzyl chloride contamination of benzyloxycarbonyl chloride. Sometimes, mostly using old benzyl chloroformate in large excess, this side-reaction was observed.

ture overnight. After evaporation of the solvent, the remaining substance was added to the aqueous phase and processed as above. 0.968 g (2.44 mmoles) of Cbz-D-[3-^{14}C]Phe-Pro-OH was obtained. Its purity checked by TLC (see L isomer) was 95%. Total activity: 4.36 GBq (118 mCi). Radiochemical yield: 39% (calcd. on Cbz-D,L--Phe-Pro-OH).

Cbz-D-[3-^{14}C]Phe-Pro-Arg(Cbz) lactam

To a solution of 968 mg of Cbz-D-[3-^{14}C]Phe-Pro-OH (2.44 mmoles) in dry dimethylformamide (4 ml) N-methylmorpholine (0.27 ml; 2.44 mmoles), and after cooling to -30°C, isobutyl chloroformate (0.33 ml; 2.44 mmoles) were added and the mixture was stirred for 15 minutes. Then arginine(Cbz) lactam hydrochloride (1.04 g; 3.2 mmoles) and triethylamine (1.4 ml; 10 mmoles) dissolved in dimethylformamide (4 ml) were added to the mixture, and stirring was continued at -20°C for 1.5 hours and at 0°C for one hour. The reaction mixture was diluted with dry benzene (20 ml), filtered and the filtrate was shaken with water. The aqueous phase was separated and extracted with benzene (20 ml) then the combined organic phases were washed with 0.1N HCl (3x15 ml), 10% Na_2CO_3 (3x15 ml) and 25% NaCl (2x15 ml) successively. The solution was dried over Na_2SO_4 and evaporated to give a thick colourless oil (1.61 g), 72% of which proved to be the required substance checked by TLC (benzene - tetrahydrofuran 3:1; R_f 0.3). It was chromatographed (eluent: benzene-tetrahydrofuran 3:1) to obtain 680 mg (42%)[x] of a white chrystalline powder which showed only one spot by TLC (ethyl acetate-pyridine-acetic acid-water 960:20:6:11; R_f 0.8). Total activity: 1.42 GBq (38.4 mCi).[xx]

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[x] In cold runs using pure Cbz-D-Phe-Pro-OH the yield was 80-90% and the product needed no purification.

[xx] The specific activity decreased from 1.70 GBq/mmoles to 1.40 GBq/mmole.

Cbz-D-[3-^{14}C]Phe-Pro-Arg(Cbz) aldehyde

To the tripeptide lactam (680 mg; 1.02 mmoles) dissolved in dry tetrahydrofuran and cooled to -30°C, 0.4 mmoles of $LiAlH_4$ dissolved in tetrahydrofuran was added and stirred for 10 minutes. After the reduction was complete (monitored by TLC), the mixture was decomposed at -30°C by adding 1N H_2SO_4 (15 ml) and water (10 ml), then extracted with dichloromethane (3x15 ml). The combined organic extracts were washed with water (2x20 ml) 5% $NaHCO_3$ (2x20 ml) and water (2x20 ml) successively, dried over Na_2SO_4 and evaporated. The oily residue gave a white powder (523 mg; 76%) in a mixture of ether and petroleum ether (1:1). Total activity: 1.08 GBq (29.2 mCi). The material showed one spot with a tail by TLC (ethyl acetate-pyridine-acetic acid-water 120:20:6:11; R_f 0.4).

H-D-[3-^{14}C]Phe-Pro-Arg-H sulphate (GYKI-14166)

To a solution of the protected aldehyde (523 mg; 0.78 mmoles; 1.08 GBq) in tetrahydrofuran (20 ml), 1N H_2SO_4 (0.7 ml) and 10% palladium charcoal catalist (0.2 g) were added and hydrogen was bubbled through the liquid for 4 hours. The catalyst was filtered off and washed with water (3x5 ml), the filtrate was adjusted to pH 5.0-5.2 with anion exchange resin (in OH^- cycle), and tetrahydrofuran was removed by evaporation. The aqueous residue was washed with butanol (3x20 ml, saturated with water) and after freeze-drying 364 mg of GYKI-14166 was obtained as a voluminous white solid. Total activity: 892 MBq (24.1 mCi).[x] The product was identical with an authentic sample by TLC (ethyl acetate-pyridine--acetic acid-water 30:20:6:11; R_f 0.5), and by biological activity (90%).

Cbz-[3-^{14}C]Phe-Pro-NH_2

To 924 mg (2.33 mmoles; 3.88 GBq) of Cbz- 3-^{14}C Phe-Pro-OH

[x] The specific activity was about 70% of the calculated one.

dissolved in ahydrous ethyl acetate (20 ml), N-methylmorpholine (0.28 ml; 2.6 mmoles) and after cooling to -20°C, isobutyl chloroformate (0.33 ml; 2.54 mmoles) were added and the mixture was stirred for 15 minutes. Then NH_4Cl (160 mg; 3.0 mmoles) and an additional portion of N-methylmorpholine (0.33 ml; 3.0 mmoles) were added to the mixture and stirring was continued at -15°C for 20 minutes, at 0°C for one hour and at room temperature overnight. After addition of water (15 ml), the layers were separated and the aqueous phase was extracted with ethyl acetate (2x15 ml). The combined extracts were washed with 1N HCl (2x15 ml), 5% $NaHCO_3$ (2x15 ml) and 25% NaCl (2x15 ml) successively, dried over Na_2SO_4 and evaporated. The purity of the residue, 776 mg of a resin (85%), was about 90% checked by TLC (ethyl acetate-pyridine--acetic acid-water 240:20:6:11; R_f 0.5).

H-[3-^{14}C]Phe-Pro-NH_2 hydrochloride

The protecting carbobenzyloxy group was removed by catalytic hydrogenation over 10% Pd/C in ethanol (20 ml) and 1N HCl (2 ml). After filtering off the catalyst, the solvent was evaporated to give 734 mg (more than calcd.) of a crystalline material. It proved to be fairly pure (about 90%) by TLC (ethyl acetate--pyridine-acetic acid-water 60:20:6:11; R_f 0.6).

BOC-Tyr-D-Met-Gly-[3-^{14}C]Phe-Pro-NH_2

To 940 mg (2.0 mmoles) of BOC-Tyr-D-Met-Gly-OH, prepared as described by Bajusz et al. (4), dissolved in tetrahydrofuran (20 ml) N-methylmorpholine (0.24 ml; 2.2 mmoles) was added. It was cooled to -20°C and, when the mixture became clear, isobutyl chloroformate (0.28 ml; 2.2 mmoles), then after being stirred for 15 minutes, a cold solution of the above H-[3-^{14}C]Phe-Pro-NH_2 in tetrahydrofuran (10 ml) and N-methylmorpholine (0.30 ml; 2.6 mmoles) were added. Stirring was continued at -20°C for 20 minutes, at 0° for one hour and at room temperature overnight.

Next day the solvent was evaporated, the residue was suspended in water (20 ml) and extracted with ethyl acetate (3x15 ml). The combined extracts were washed with cold 1N HCl (2x15 ml), 5% Na_2HCO_3 (2x15 ml) and 25% NaCl (2x15 ml) successively, dried over Na_2SO_4 and evaported. The purity of the residue was about 90% by TLC (ethyl acetate-pyridine-acetic acid-water 120:20:6:11; R_f 0.6), and after purification by chromatography (eluent: acetone), 758 mg of a white powder was obtained, which showed only one spot by TLC. Total activity: 1.94 GBq (53.5 mCi).

H-Tyr-D-Met-Gly-[3-^{14}C]Phe-Pro-NH_2 acetate (GYKI-14238)

The protected pentapeptide (758 mg; 1.06 mmoles; 1.94 GBq) was stirred in 3.6 N HCl/ethyl acetate (30 ml) at room temperature for 2 hours, then ether (60 ml) was added to the mixture and it was stirred for an additional 2 hours. The crystals were filtered off and washed with ether. The white powder (669 mg), which was pure by TLC (ethyl acetate-pyridine-acetic acid-water 30:20:6:11; R_f 0.6), was dissolved in water (5 ml) and Cl^- was exchanged for acetate by VARION AD (50 mesh) cation exchange resin (in acetate cycle). The water was removed by freeze-drying to give 670 mg of GYKI-14238 as a voluminous white solid. Total activity: 1.83 GBq (49.4 mCi). The substance was 98% pure, checked by TLC. Radiochemical yield: 47% calculated on Cbz-Phe-Pro-OH.

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