SYNTHESIS OF LABELED L-CYSTINYL-BIS-L-VALINE AND BIS-6-(L-2-AMINOADIPYL)-L-CYSTINYL-BIS-L-VALINE

H. Vanderhaeghe and P. Adriaens
Rega Institute, University of Leuven, Minderbroedersstraat 10,
B-3000 Leuven, Belgium.

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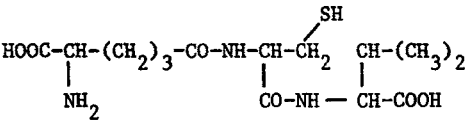
SUMMARY

The synthesis of two peptides, L-cystinyl-bis-L-valine and bis-6-(L-2-aminoadipyl)-L-cystinyl-bis-L-valine, labeled with L-valine-^{14}C(U) or L-cystine-3,3'-T, is described.

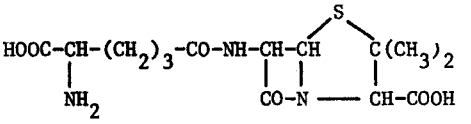
Key Words : Peptides, Carbon 14, Tritium, Penicillium

INTRODUCTION

Since the discovery of the tripeptide 6-(2-aminoadipyl)-cysteinylvaline in the mycelia of Penicillium (1,2) and Cephalosporium (3,4,5), this compound, because of its structural relationship with isopenicillin N, has been considered as a possible intermediate in the biosynthesis of the β-lactam antibiotics (6).



tripeptide

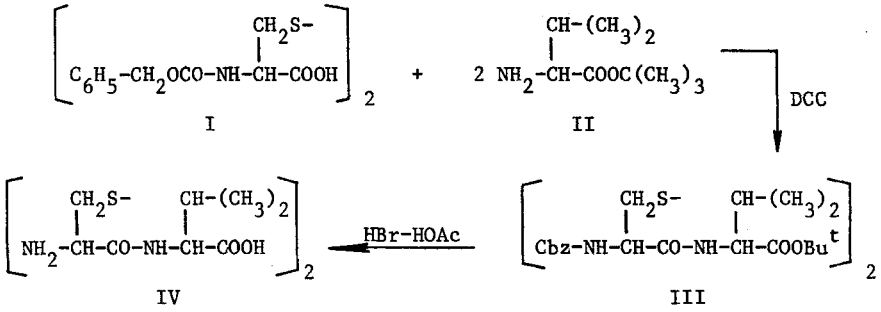


isopenicillin N

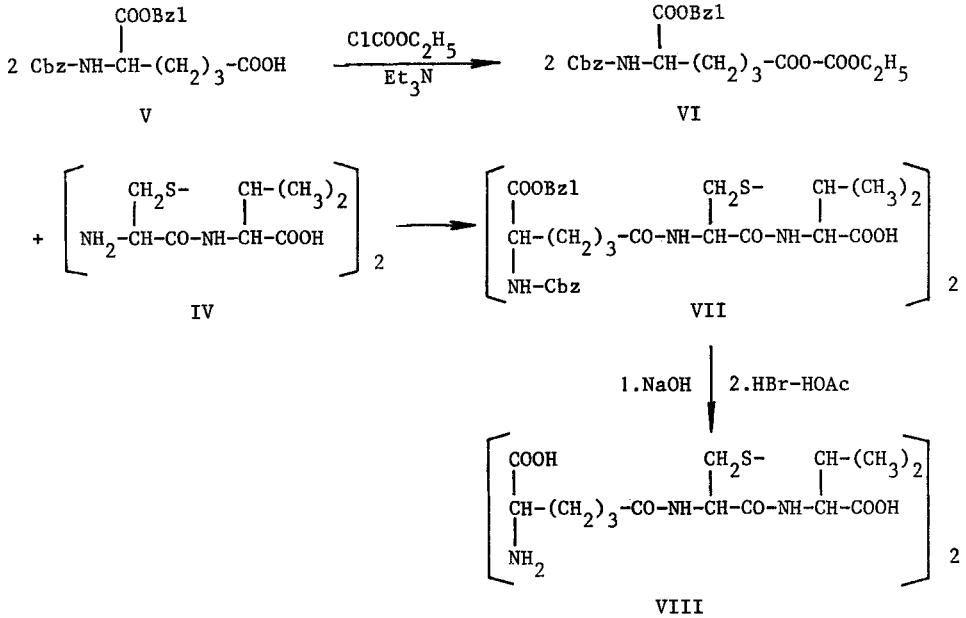
Although the dipeptide cysteinyl-valine has not been detected, it is a potential precursor of 6-aminopenicillanic acid (6-APA).

dipeptide 6-APA

Since incorporation studies with these labeled peptides could give information on the biosynthesis of penicillins and cephalosporins, their synthesis was carried out. Both peptides were separately labeled with tritium and carbon-14; in this way double labeled experiments and competition tests become possible. The dipeptides L-cystinyl-bis-L-valine-^{14}C(U) and L-cystinyl-3,3'-T-bis-L-valine were prepared by a modification of the procedure of Roeske (7,8,9). Because we obtained poor yields for the transformation of di-N-carbobenzyloxy-L-cystine (I) into its acid chloride with PCl_5, we coupled I with L-valine tert-butyl ester (II) in the presence of dicyclohexylcarbodiimide (DCC). By removal of the protecting groups of III with HBr in acetic acid, L-cystinyl-bis-L-valine (IV) was obtained.



For the synthesis of the tripeptide, L-2-carbobenzyloxyaminoadipic acid 1-benzyl ester (V) (10) was transformed into the mixed anhydride VI and condensed with L-cystinyl-bis-L-valine (IV). The protected tripeptide VII was then converted to bis-6-(L-2-aminoadipyl)-L-cystinyl-bis-L-valine (VIII) by hydrolysis with NaOH followed by HBr in acetic acid.



The same methods were used for the synthesis of L-cystinyl-bis-D-valine and bis-6-(L-2-aminoadipyl)-L-cystinyl-bis-D-valine.

EXPERIMENTAL

A. Peptides labeled with carbon-14

1. L-valine-^{14}C(U) tert-butyl ester (II). In a thick-walled bottle, about 450 μCi of L-valine-^{14}C(U) (The Radiochemical Centre, Amersham, England; specific activity 14.9 mCi/mmole) is diluted with 1.18 g (10 mmole) of L-valine and added to a solution of 3 ml of concentrated sulfuric acid in 40 ml of dioxane (redistilled from sodium). After cooling in ice, 60 ml of liquid isobutene is added and the closed bottle is allowed to stand for 3 days at room temperature. The contents are transferred to a cold stirred mixture of 120 ml of 2 N NaOH and 50 ml of ether. After separation of the organic layer, the aqueous layer is extracted three more times with 40 ml of ether. The ether solution is dried over anhydrous Na_2SO_4 and concentrated to 50 ml.
Yield 1.43 g (376 μCi), = 83 %. Specific activity 45.3 μCi/mmole.

2. Di-N-carbobenzyloxy-L-cystinyl-bis-L-valine-^{14}C(U) tert-butyl ester (III). Di-N-carbobenzyloxy-L-cystine (1.73 g, 3.4 mmole), prepared by the method of Du Vigneaud and Miller (11), is dissolved in 49.5 ml of ether, containing 8.3 mmole (372 μCi) of II. The solution is concentrated to 10 ml, mixed with 10 ml of dichloromethane and cooled to 0°C. After addition of 1.40 g (6.8 mmole) of dicyclohexylcarbodiimide, the mixture is stirred for 2 hours at 0°C and left to stand overnight at room temperature. The precipitated dicyclohexylurea is filtered off, the filtrate is diluted with 75 ml of ether and 75 ml of ethyl acetate and washed twice with 15 ml of 0.5 N H_2SO_4 and twice with 15 ml of 5 % $KHCO_3$. The organic layer is dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue is crystallized twice from ethyl acetate-petroleum ether 40-60°C; m.p. 171.5°-174°.
Yield 2.58 g = 92 %. Specific activity 87.3 μCi/mmole.
TLC on silicagel G with chloroform-acetone-acetic acid, 92:7:1, gives one spot, Rf = 0.80.

3. L-cystinyl-bis-L-valine-^{14}C(U) (IV). 2.46 g (3 mmole) of III is treated with 15 ml of 3 M HBr in acetic acid for 1 hour at room temperature and 1 minute at 100°C. The peptide, precipitated as the dihydrobromide by adding 150 ml of ether, is dissolved in 20 ml of water and washed twice with 15 ml of ether. After adjustement of the pH to 4.9 with 2 N LiOH the dipeptide crystallizes upon addition of 200 ml of ethanol.
Yield 1.23 g = 90 %; $[\alpha]_D^{20} = -22°$ (c = 1, HCl 2 N).
Specific activity 86.7 μCi/mmole.

The ^{14}C-dipeptide was radiochemically pure on testing by paper chromatography (Whatman no. 3 MM, descending; n-butyl alcohol-acetic acid-water, 4:1:1) and paper electrophoresis (Whatman no. 3 MM, 4 x 40 cm; acetic acid 20 % - formic acid 2 %, pH 1.85; 3.5 hours at 300 v) and scanning with a gasflow counter.

4. Bis-6-(L-1-benzyl-2-carbobenzyloxyaminoadipyl)-L-cystinyl-bis-L-valine-^{14}C(U) (VII).

a.- Mixed anhydride of L-1-benzyl-2-carbobenzyloxyaminoadipic acid (VI). 1.96 g (5.1 mmole) of V (10) is dissolved in 20 ml of dioxane-acetone, 1:1; after cooling in ice, 0.73 ml of triethylamine and 0.50 ml of ethyl chloroformate in 5 ml of acetone is added while stirring. The mixture is further stirred for 45 minutes at 0-2°C.

b.- Condensation with L-cystinyl-bis-L-valine-^{14}C(U). 663 mg (1.45 mmole, 125 µCi) of IV is suspended in 15 ml of water and dissolved with 5.8 ml of 0.5 N KOH, to a pH of 10; after addition of 10 ml of acetone, the solution is cooled to 0°C. The mixed anhydride VI is slowly added while stirring and cooling in ice; the pH is kept constant at 10 with 0.5 N KOH (pH-stat). The reaction is allowed to proceed for 30 minutes at 0-2°C and for a further hour at room temperature; the solution is diluted with 100 ml of water and washed with three 25 ml portions of ether. The water layer is mixed with 50 ml of ethyl acetate and acidified to a pH of 1.8; three more extractions with 30 ml of ethyl acetate are carried out. The combined extract is dried over anhydrous Na_2SO_4 and evaporated in vacuum; the residual oil solidifies after trituration with petroleum ether 40-60°C.

c.- Purification of VII. The mixture of VII and excess V is dissolved in 10 ml of chloroform and transferred to a column of 100 g of silicagel in chloroform. Elution is carried out with a gradient of acetone in chloroform plus 1 % acetic acid. The fractions are tested by TLC on silicagel G (chloroform-acetone-formic acid, 80:20:1 and charring with sulfuric acid at 170°C). The fractions which contain the pure protected tripeptide are combined and evaporated to dryness; the residue is crystallized from ethyl acetate-petroleum ether 40-60°C.

Yield 0.90 g = 53 %. Specific activity 87.5 µCi/mmole.

Non-radioactive compound VII was prepared in 57 % yield by the same reaction scheme. It was homogeneous on thin layer chromatography on silicagel with the system chloroform-acetone-formic acid, 80:20:1; $[\alpha]_D^{20} = +10°$ (c = 1, acetone).

Analysis. Calcd for $C_{58}H_{72}O_6N_{16}S_2$: C, 59.36; H, 6.18; N, 7.16.

Found : C, 59.21; H, 6.29; N, 7.24.

5. Bis-6-(L-2-aminoadipyl)-L-cystinyl-bis-L-valine-^{14}C(U) (VIII). 880 mg (0.75 mmole, 65.6 µCi) of VII is dissolved in 32 ml of 0.1 N KOH and allowed to react for 24 hours at room temperature. The alkaline solution is washed with three portions of ether and acidified to a pH of 1.7; four extractions with 25 ml of ethyl acetate are carried out. The solution in ethyl acetate is dried

with anhydrous Na_2SO_4 and evaporated to dryness. The residue is treated with 8 ml of 4 M HBr in acetic acid for 1.5 hours at room temperature. The peptide is precipitated as the dihydrobromide by adding 60 ml of ether.
Yield 600 mg = 90 %. Specific activity 87.3 µCi/mmole.
The ^{14}C-tripeptide was radiochemically pure; testing was done with paper chromatography (Whatman no. 3 MM, descending; n-butyl alcohol-acetic acid-water, 4:1:1 and n-butyl alcohol-pyridine-acetic acid-water, 4:1:1:5) and paper electrophoresis (Whatman no. 3 MM, 4 x 40 cm; 0.4 M pyridine-acetic acid-water, pH 4.5; 4.5 hours at 300 v) and scanning with a gasflow counter.
Non-radioactive VIII was prepared from VII by the same procedure and in the same yield.
Analysis. Calcd for $C_{28}H_{48}N_6O_{10}S_2.2\ HBr$: C, 37.88; H, 5.67; N, 9.46.
Found : C, 37.76; H, 5.78; N, 9.66.
The crystalline free peptide was obtained by dissolving the hydrobromide in ethanol, adding aniline and precipitation with acetone or acetone-ether.
$[\alpha]_D^{20} = -53°$ (c = 2, HCl 2 N).
Both the hydrobromide and the free peptide were homogenous upon paper chromatography and electrophoresis in the systems described for the ^{14}C-tripeptide.

B. Tritiated peptides

1. Di-N-carbobenzyloxy-L-cystine-3,3'-T (I). L-cystine (960 mg, 4 mmole) is mixed with about 3 mCi L-cystine-3,3'-T (The Radiochemical Centre, Amersham, England; specific activity 200 mCi/mmole) and dissolved in 1 N NaOH to obtain a pH of 11; after addition of 30 ml of acetone, the mixture is cooled to 0°C. A solution of carbobenzyloxychloride (12) in toluene (4.5 ml, 12 mmole) is added slowly while stirring and cooling in ice; the pH is kept constant at 11 with 1 N NaOH (pH-stat). After reaction for 1.5 hours at room temperature, the alkaline solution is diluted with water and washed twice with ether. The water layer is mixed with 60 ml of ethyl acetate and acidified to pH 1.8; four more extractions with 25 ml of ethyl acetate are carried out. The combined extracts are washed with 0.5 N H_2SO_4 and water and dried over anhydrous Na_2SO_4. Evaporation gives an oil which crystallizes from 10 ml of chloroform and 75 ml of petroleum ether 40-60°C; the product is recrystallized from acetonitrile-benzene.
Yield 1.58 g = 68 %.

2. L-cystinyl-3,3'-T-bis-L-valine (IV). The next steps are carried out as for the ^{14}C-dipeptide; the product is purified by a second recrystallization from water-ethanol, after dilution with pure non-radioactive dipeptide to a specific activity of 350 µCi/mmole.

3. Bis-6-(L-2-aminoadipyl)-L-cystinyl-3,3'-T-bis-L-valine (VIII). The procedure for the ^{14}C-tripeptide was also applied for the synthesis of the tritiated product; it was purified by recrystallization from ethanol-ether,

after dilution with pure inactive tripeptide to a specific activity of 465 μCi/mmole.

C. Bis-6-(L-2-aminoadipyl)-L-cystinyl-bis-D-valine

1. Di-N-carbobenzyloxy-L-cystinyl-bis-D-valine tert-butyl ester. Di-N-carbobenzyloxy-L-cystine was condensed with D-valine tert-butyl ester in the presence of dicyclohexylcarbodiimide as described above for the L-isomer. After column chromatography and recrystallization from ethyl acetate-petroleum ether, the protected peptide was obtained in 55 % yield; m.p. 138-140°; $[\alpha]_D^{20} = -70.5°$ (c = 2; dimethylformamide).

2. L-cystinyl-bis-D-valine. The protecting groups were removed with 3 M HBr in acetic acid, according to the procedure described for L-cystinyl-L-valine. The peptide, which was obtained in 97 % yield, had a m.p. > 300° and $[\alpha]_D^{20} = +55.5°$ (c = 2, HCL 2 N).
The Rf values on thin layer chromatography in the system n-butanol-acetic acid-water, 4:1:1, were 0.11 (silicagel) and 0.18 (cellulose), compared to 0.21 (silicagel) and 0.30 (cellulose) for L-cystinyl-bis-L-valine.

3. Bis-6-(L-2-aminoadipyl)-L-cystinyl-bis-D-valine. L-1-benzyl-2-carbobenzyloxyaminoadipic acid (10) was transformed into the mixed anhydride with ethyl chloroformate and condensed with L-cystinyl-D-valine dissolved in water at pH 10 (pH-stat). The protected peptide was purified by chromatography on a column of silicagel and elution with an acetone gradient in chloroform-acetic acid; yield 22 %. The protecting groups were removed with KOH 0.1 N and HBr-acetic acid as described for the LLL-peptide. The hydrobromide was obtained in 66 % yield. The free peptide was prepared by dissolving the hydrobromide in ethanol-acetone and adding pyridine; $[\alpha]_D^{22} = -9.5°$ (c = 2, HCl 2 N).

DISCUSSION

The synthesis of ^{14}C-cystinylvaline has already been described (13). In this method, the protecting group of cysteine was removed with sodium in liquid ammonia, which is a delicate manipulation and which can cause some racemization. For this reason, we have preferred the method of Roeske (9), which uses cystine instead of S-benzylcysteine. We, however, obtained poor yields in the condensation of I with II using PCl_5. Good results were obtained with dicyclohexylcarbodiimide.

The peptide, bis-6-(L-2-aminoadipyl)-L-cystinyl-bis-L-valine has been prepared by solid phase synthesis (14), but no physical constants were described. The synthesis of the same peptide by condensation of the chloride of the lactam of α-tosylaminoadipic acid with S-benzyl-L-cysteinyl-L-valine methyl ester is

mentioned in an abstract of a communication (15), but no experimental details are given.

We have prepared bis-6-(L-2-aminoadipyl)-L-cystinyl-bis-L-valine-^{14}C by coupling the mixed anhydride of L-1-benzyl-2-carbobenzyloxyaminoadipic acid (VI) with L-cystinyl-bis-L-valine-^{14}C (IV). After removal of the protecting groups, the yield was 47.7 %. The total radiochemical yield, based on valine-^{14}C (U) was 24 %.

Both the di- and the tripeptide containing L-cystine-3,3'-T were prepared by the same methods.

The peptides, L-cystinyl-bis-D-valine (IV) and bis-6-(L-2-aminoadipyl)-L-cystinyl-bis-D-valine (VIII) were prepared according to the same scheme.

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