97. Synthesis of a *S*-(Carboxymethyl)cysteine Analog of (L-2-Amino-6-adipyl)-L-cysteinyl-D-valine and its Cell Free Biosynthetic Conversion into 6-[2-((D-2-Amino-2-carboxyethyl)thio)acetamido]penicillanic Acid

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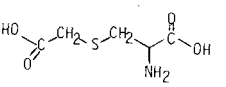
(3.I.84)

Summary

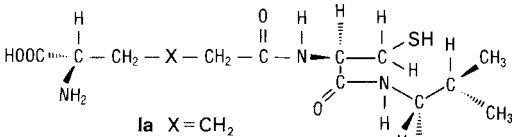
A new tripeptide (dimer), bis[(L-cysteine-*S*-acetyl)-L-hemicystinyl($S^2 \rightarrow S^{2'}$)-D-valine] (**6**) was synthesized by coupling *N*-(*tert*-butoxycarbonyl)-*S*-carboxymethyl-L-cysteine benzyl ester (**1**) with *S*-trityl-L-cysteinyl-D-valine benzyl ester and subsequent removal of the protecting groups. After reduction of the disulfide, the free tripeptide Cys(CH_2CO-Cys-D-Val) (**Ib**) was used as a substrate of isopenicillin-N synthetase in a cell-free conversion to 6-[2-((D-2-amino-2-carboxyethyl)thio)acetamido]penicillanic acid (**IIa**).

The first successful cell-free conversion of (L-2-amino-6-adipyl)-L-cysteinyl-D-valine (Aad(Cys-D-Val); **Ia**) to isopenicillin N(**IIa** with $X = CH_2$) by *Demain et al.* [1] prompted our studies of this biosynthetic step [2] using HPLC [3].

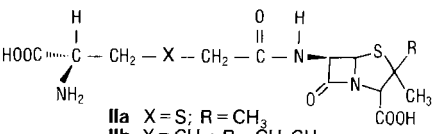


A

Troonen et al. [4] have used a lysine auxotroph of *Cephalosporium acremonium* and substituted the necessary side chain precursor DL-2-aminoadipic acid with *S*-carboxymethyl-L-cysteine (Cys(CH_2CO_2H); **A**) to produce the corresponding penicillin **IIa**. Therefore, we have attempted a cell-free conversion of (L-cysteine-*S*-acetyl)-L-cysteinyl-D-valine (Cys(CH_2CO-Cys-D-Val); **Ib**) to penicillin **IIa**. The conversion turned out to be quite efficient and could be followed by HPLC as in our previous studies [3].

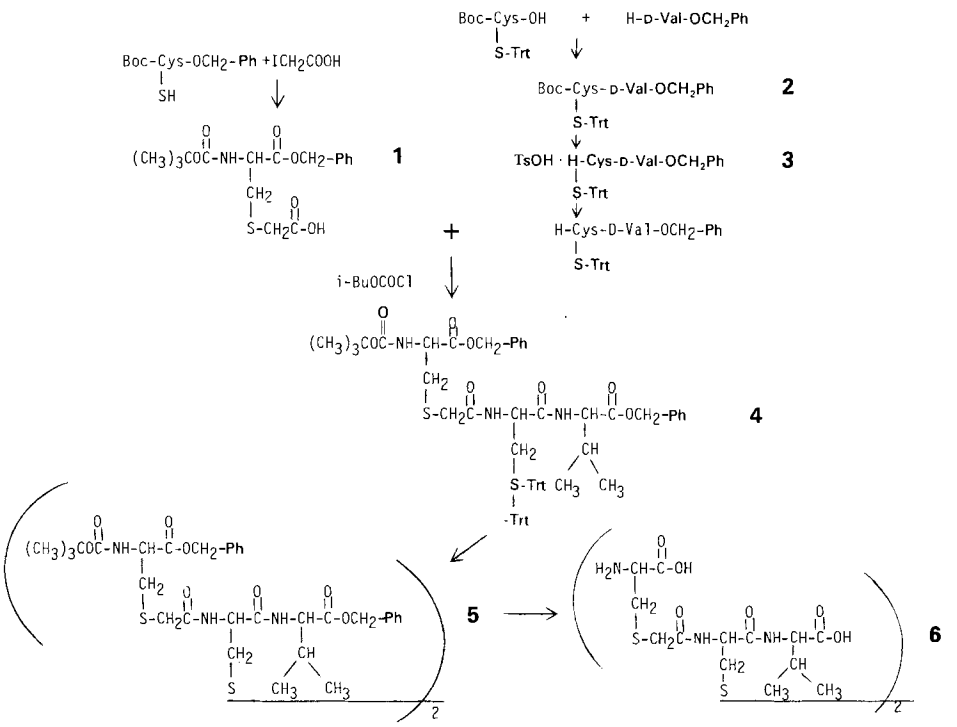


Ia X = CH_2
Ib X = S



IIa X = S; R = CH_3
IIb X = CH_2; R = CH_3CH_2

Scheme



Structural analogues of Aad(Cys-D-Val) (**Ia**) have been tested by another group [5] in order to evaluate the substrate specificity of the conversion of Aad(Cys-D-Val) to isopenicillin N. This was the first study to demonstrate that the cyclase enzyme system responsible for this conversion is able to accept and transform modified substrates into new penicillins. However, in that study the conversion yield of (L-2-amino-6-adipyl)-L-cysteinyl-D-isoleucine Aad(Cys-D-Ile) to the corresponding isopenicillin **IIb** was estimated at 36% while other derivatives gave considerably lower yields or failed to convert to isopenicillin. In our conversion of the *S*-carboxymethyl-cystein derivative Cys(CH_2CO-Cys-D-Val) (**Ib**) to **IIa**, the yield was comparable to that of the Aad(Cys-D-Val) conversion.

Because the proposed tripeptide substrate analog **Ib** for the cyclase system would contain two cysteine residues, we introduced the carboxymethyl group into a derivative of cysteine protected at its amino and carboxyl groups. The latter was treated with iodoacetic acid to generate a compound with only one reactive carboxyl group, *N*-(*tert*-butoxycarbonyl)-*S*-carboxymethyl-L-cysteine *α*-benzyl ester (**1**). The dipeptide moiety was synthesized by a standard dicyclohexylcarbodiimide (DCC) coupling of *N*-(*tert*-butoxycarbonyl)-*S*-trityl-L-cysteine with D-valine benzyl ester *p*-toluenesulfonate salt in DMF. The product **2** was a syrup, but of sufficient purity for use without further purification. This syrup was selectively deblocked with *p*-toluenesulfonic acid in $MeCN/CHCl_3$ to give a solid crystalline salt **3**; the overall yield of dipeptide was better than 69% at this point.

The *S*-carboxymethyl-cysteine derivative **1** was coupled to the dipeptide by the mixed anhydride procedure. An extensive purification by preparative HPLC was required to obtain the tripeptide, ((*N*-(*tert*-butoxycarbonyl)-L-cysteine benzyl ester)-*S*-acetyl)-(*S*-trityl-L-cysteinyl)-D-valine benzyl ester (**4**); its yield was 24.6%. The *S*-trityl group was removed and the peptide converted to the crystalline disulfide dimer **5** in one step by treating with I_2 in pyridine (76.3% yield). The dimer **5** was deblocked by treatment in liquid HF at 0° for one four in the presence of anisole, and the final solid product, bis[(L-cysteine-*S*-acetyl)-L-hemicystinyl($S^2 \rightarrow S^{2'}$)-D-valine] (**6**) was obtained after lyophilization. By analytical HPLC, **6** showed a purity of 94% at 254 nm and over 96% at 214 nm. The amino-acid analysis was satisfactory and showed that the solid contained 69% of peptide by weight; the balance was judged to be volatiles such as H_2O (see *Exper. Part*).

Experimental Part

General Remarks. ^{1}H-NMR spectra were recorded using a *Bruker WH360* NMR spectrometer. Optical rotations were measured using a *Perkin Elmer* polarimeter, model *241*. Mass spectra were recorded using FAB (fast atomic bombardment) on a *Varian-MAT* mass spectrometer, model *731*.

HPLC Conditions. All chromatograms were obtained using a *M6000A* pump, *U6K* septumless injector, differential refractometer *R401*, all from *Waters Associates*, Milford, Massachusetts. In addition, a *Fisher Omniscribe* strip chart recorder (*Fisher Scientific*, Cincinnati, Ohio) was used. Spectrally pure solvents were obtained from *Burdick E. Jackson*, Muskegon, Michigan or *J. T. Baker*. Only deionized and glass-distilled H_2O was used. Conditions for HPLC chromatography were as follows: attenuation *A RI* (2×); chart speed 5 mm/min; column size 4 × 300 mm, *C-18 μ Bondapak, Waters Associates;* solvent: H_2O/AcOH/pyridine 99.2:0.4:0.4.

Preparation of Partially Purified Isopenicillin-N Synthetase (IPNS) and its Assay. Cell extracts were prepared and fractionated by $(NH_4)_2SO_4$ and desalted [3]. The reaction mixture contained 425 μl of the fractionated extract from stain *M8650-4*, 50 μl of a substrate solution containing 1 mg of **6** (see below; for ^{1}H-NMR see *Fig. 1*), 1 mg of DTT (= 1,4-dithio-DL-threitol) and 1 ml of tris-HCl (= tris(hydroxymethyl)methylammonium chloride; pH 7.2 buffer), 25 μl of a solution containing the following cofactors: 10 mg of $FeSO_4$, 28 mg of

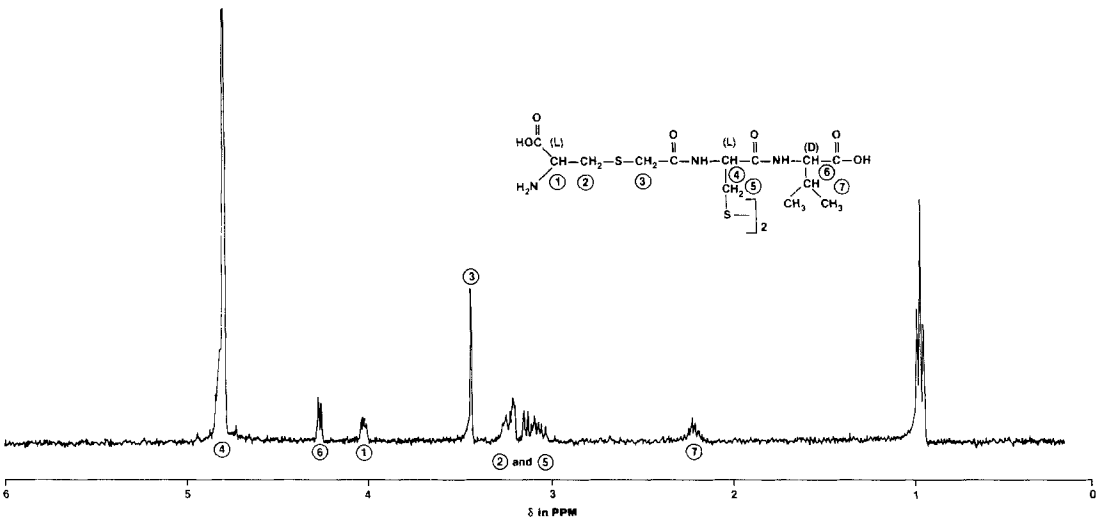


Fig. 1. *^{1}H-NMR Spectrum of Bis[(*L-cysteine-S-acetyl)-L-hemicystinyl($S^2 \rightarrow S^{2'}$)-D-valine] (**6**) in D_2O*

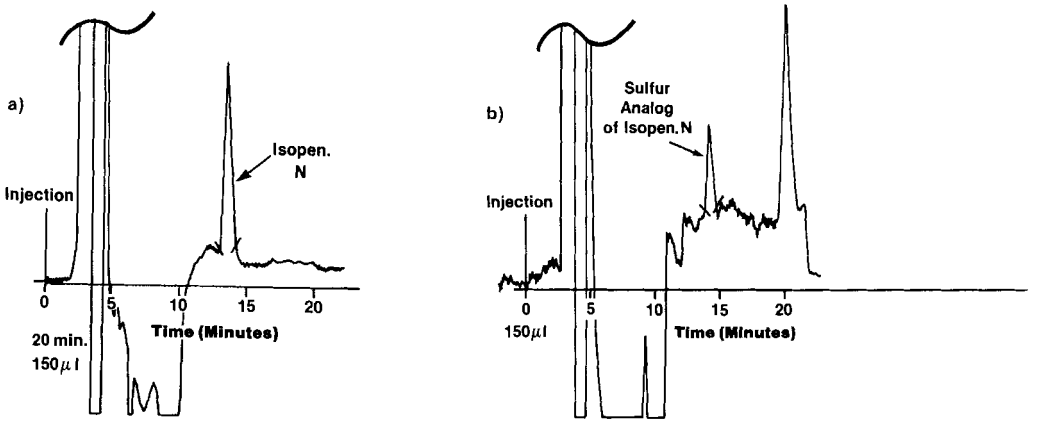


Fig. 2 *HPLC of* a) *Isopenicillin N and* b) *its Sulfur Analog* **IIa**. Conditions were as follows: attenuation *ΔRI* (2×); chart speed 5 mm/min; column size 4 × 300 mm; solvent H_2O/AcOH/pyridine 99.2:0.4:0.4; flow rate 1.0 ml/min; waters packing: *C-18 μ-Bondapak;* p.s.i. 1000.

ascorbic acid, 10 ml of tris-HCl, pH 7.2. Substrate solution was prepared 15 min prior to assays to reduce the disulfide bond of **6**. Reactions were initiated by adding the substrate solution. Each reaction mixture was incubated at 250 r/min and 25°C. Duplicate 150 μl aliquots were withdrawn at 0, 20, 40, and 60 min after addition of substrate and checked by HPLC. The retention time was approximately 13 min *(Fig. 2b)*. After checking the quality of material by 360-MHz NMR *(Fig. 3)*, samples were collected from 60 runs to accumulate approximately 680 μg of material (after lyophilization). In some runs, samples diluted by 30 μl of MeOH were added to a sensitivity disc (7 mm diameter). Discs were placed on an agar inoculated with *Sarcina lutea*. After incubation at 37°, zones of growth inhibition were compared to those produced by standard penicillin at known concentration, and were found to be comparable in activity.

In a control experiment, the HPLC retention time of isopenicillin N was also approximately 13 min *(Fig. 2a)*.

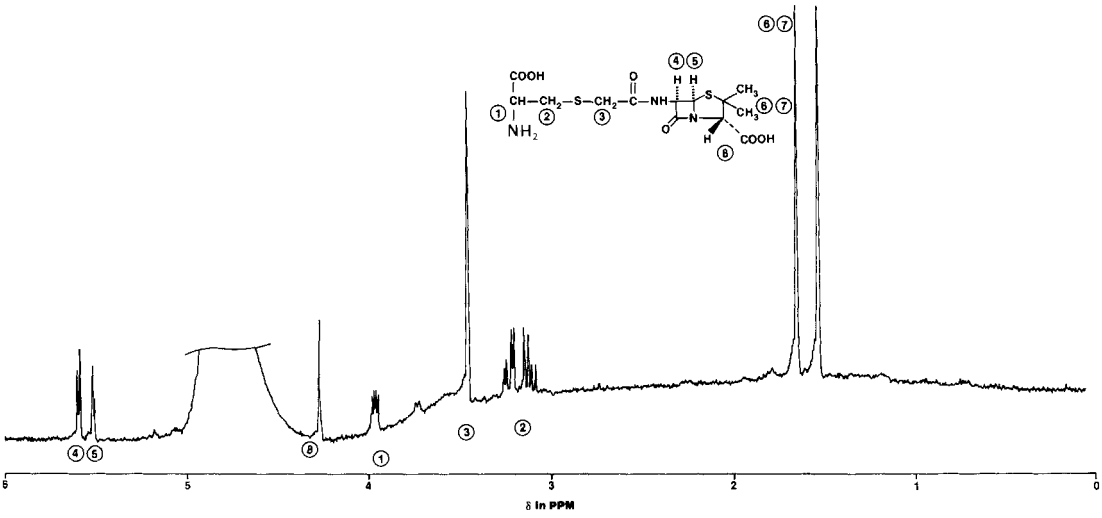


Fig. 3. *^{1}H-NMR Spectrum of 6-[2-((D-2-Amino-2-carboxyethyl)thio)acetamido]penicillanic Acid* (**IIa**)

N-(*tert*-*Butoxycarbonyl*)-L-*cysteine Benzyl Ester* (**1**). Bis(*N*-(*tert*-butoxycarbonyl))-L-cystine bis(benzyl ester) (33.5 g, 54.0 mmol) was treated with 20 g of Zn dust in 600 ml 85% aq. AcOH at r.t. for 1 h. The Zn was filtered from the mixture, and the solution was evaporated. The residue was dissolved in H_2O/AcOEt. The org. phase was separated, washed 4 times with H_2O, dried over $MgSO_4$, filtered, and evaporated. The thiol intermediate was dissolved in 200 ml of 95% EtOH and treated with 28.5 g (107.8 mmol) of 18-crown-6, 10.8 g (107.5 mmol) of $KHCO_3$, and 20.0 g (107.5 mmol) of ICH_2COOH. The mixture was stirred for 3 days at r.t. The EtOH was evaporated, the residue dissolved in H_2O/AcOEt, the aq. phase separated, and the org. phase washed 3 times with aq. $KHCO_3$. The aq. phase and $KHCO_3$ washes were combined and acidified with citric acid. This acidified aq. phase was extracted with AcOEt, the extract washed 3 times with aq. KCl and 3 times with H_2O, dried over $MgSO_4$, filtered, and evaporated. The syrupy residue (37.7 g) was extracted several times with hot pentane. The remaining oil was dissolved in 95% EtOH and precipitated by adding H_2O. After the addition of seed crystals, the precipitated oil crystallized overnight. The crystalline material was recrystallized from AcOEt/cyclohexane: 18.7 g (46.9%) of **1**, m.p. 75–76°, $[\alpha]_D^{25} = -35.4°$ ($c = 1$, MeOH), $[\alpha]_D^{25} = -41.0$ ($c = 1$, DMF).

Additional pure product was recovered by two recrystallizations of the residue from the filtrate of the first crop: 2.2 g (5.5%) of **1** m.p. 75–76°; homogeneous by TLC in $CHCl_3$/MeOH/AcOH 135:15:1, R_f 0.34. Anal. calc. for $C_{17}H_{23}NO_6S$ (369.436): C 55.27, H 6.28, N 3.79, S 8.68; found: C 55.32, H 6.48, N 3.53. S 8.48.

(N-(*tert*-*Butoxycarbonyl*)S-*trityl*-L-*cysteinyl*)-D-*valine Benzyl Ester* (**2**) was synthesized from *N*-(*tert*-butoxycarbonyl)-*S*-trityl-L-cysteine [6] (21.5 g) and D-valine benzyl ester *p*-toluenesulfonate [7] (17.6 g) by the DCC method [8] [9] in the presence of 1 equiv. of Et_3N in DMF (46.37 mmol in 200 ml): 29.8 g (98.4%) of oil $[\alpha]_D^{25} = +13.8°$ ($c = 1$, EtOH); homogeneous by TLC in BuOH/AcOH/H_2O 4:1:1, R_f 0.66. Anal. calc. for $C_{39}H_{44}N_2O_5S$ (652.864): C 71.75, H 6.79, N 4.29, S 4.91; found: C 71.47, H 6.99, N 4.54, S 4.65.

(S-*Trityl*-L-*cysteinyl*)-D-*valine Benzyl Ester* p-*Toluenesulfonate* (**3**) was prepared by treating **2** (28.4 g, 43.5 mmol) with TsOH (24.8 g, 130.5 mmol) in MeCN (435 ml)/$CHCl_3$ (16 ml)/triethylsilane (16 ml) with stirring for 4 h at r.t. Et_2O (350 ml) was added and the mixture allowed to stand overnight at r.t. Yield: 22.37 g (70.9%) of **3**, m.p. 213–217°, $[\alpha]_D^{25} = +20.3°$ ($c = 1$, EtOH); homogeneous by TLC in BuOH/AcOH/H_2O 4:1:1, R_f 0.68. Anal. calc. for $C_{41}H_{44}N_2O_6S_2$ (724.939): C 67.93, H 6.12, N 3.86, S 8.85; found: C 67.83, H 6.05, N 3.90, S 9.02.

((N-(*tert*-*Butoxycarbonyl*)-L-*cysteine benzyl ester*)-S-*acetyl*)-(S-*trityl*-L-*cysteinyl*)-D-*valine Benzyl Ester* (= (L-O^1-*Benzyl*-2-(N-(*tert*-*butoxycarbonyl*)*amino*)4-*thia*-6-*adipyl*)-(S-*trityl*-L-*cysteinyl*)-D-*valine Benzyl Ester*; **4**). Compound **1** (5.54 g, 15 mmol), dissolved in 15 ml DMF, was converted to the mixed anhydride by treatment with *N*-methylmorpholine (1.68 ml, 15 mmol) and dropwise addition of isobutyl chloroformate (1.96 ml, 15 mmol) at −20°. The solution was stirred for 15 min. Dipeptide **3** (10.87 g, 15 mmol), dissolved in 15 ml DMF, was converted to the free base by treatment with *N*-methylmorpholine (1.68 ml, 15 mmol) at −20°. The free base of **3** was added dropwise to the mixed anhydride of **1**. The mixture was stirred for 4 h at 0° and then allowed to warm to r.t. overnight. After the addition of 5 ml of H_2O, the mixture was evaporated. The residue was dissolved in H_2O/AcOEt plus a little BuOH. The org. phase was washed twice with 1N $KHCO_3$, once with H_2O, twice with 1N HCl, and 3 times with 5% NaCl, dried over Na_2SO_4, filtered, and evaporated. The residual syrup was purified by prep. HPLC using a *Waters Prep LC system 500A* equipped with a Prep PAK-500 silica column. The syrup was applied as $CHCl_3$ solution and eluted at a flow rate of 250 ml/min with the following sequence of solvents: $CHCl_3$, 10% AcOEt/$CHCl_3$, 18% AcOEt/$CHCl_3$, MeOH. The product-containing fractions were eluted with $CHCl_3$ and were located by TLC ($CHCl_3$/AcOEt 3:1). The fractions with the highest levels of product were rechromatographed using the following sequence of solvents: 50% $CHCl_3$/petroleum ether, 75% $CHCl_3$/petroleum ether, $CHCl_3$, MeOH. The product was recovered from the 75% $CHCl_3$/petroleum ether eluent as an amorphous solid: 3.24 g (24.6%) of **4** $[\alpha]_D^{25} = -2.4°$ ($c = 1$, MeOH), $[\alpha]_D^{25} = -15.4°$ ($c = 1$, DMF); homogeneous by TLC in $CHCl_3$/AcOEt/petroleum ether 3:1:1, R_f 0.43. Anal. calc. for $C_{51}H_{57}N_3O_8S_2$ (904.022): C 67.75, H 6.36, N 4.65, S 7.08; found: C 68.29, H 6.33, N 4.53, S 7.12.

Bis[((N-(*tert*-*butoxycarbonyl*)-L-*cysteine benzyl ester*)-S-*acetyl*)-L-*hemicystinyl*($S^2 \rightarrow S^{2'}$)-D-*valine Benzyl Ester*] (= *Bis*[(L-O^1-*benzyl*-2-(N-(*tert*-*butoxycarbonyl*)*amino*)-4-*thia*-6-*adipyl*)-L-*hemicystinyl*($S^2 \rightarrow S^{2'}$)-D-*valine Benzyl Ester*]; **5**) was prepared by treatment of **4** (2.19 g, 2.50 mmol) in 35 ml of MeOH with 0.81 ml (10.0 mmol) of pyridine and 0.63 g (2.50 mmol) of I_2 at r.t. for 4.5 h. The mixture was added to 300 ml of $CHCl_3$, the org. phase washed twice with 10% $NaHSO_3$ and 3 times with H_2O, dried over $MgSO_4$, filtered, and evaporated. The oil was triturated twice with hexane. The residue was crystallized from MeOH: 1.26 g (76.3%) of **5**, m.p. 141–143°. The product from this preparation was combined with 0.23 g of **5** from another preparation and was recrystallized from MeOH: 1.38 g, m.p. 142–143°, $[\alpha]_D^{25} = +24.0°$ ($c = 1$, $CHCl_3$). Anal. calc. for $C_{64}H_{84}N_6O_{16}S_4$ (1321.658): C 58.16, H 6.41, N 6.36, S 9.70; found: C 58.23, H 6.33, N 6.33, S 9.55.

Bis[(L-cysteine-S-acetyl)-L-hemicystinyl($S^2 \rightarrow S^{2'}$)-D-valine] (= *Bis[(L-2-amino-4-thia-6-adipyl)-L-hemicystinyl($S^2 \rightarrow S^{2'}$)-D-valine]*; **6**) was prepared by treatment of **5** (1.07 g, 0.81 mmol) with liquid HF (20 ml) in the presence of 10% anisole at 0° for 1 h. After removal of the HF by distillation, the residue was treated with Et_2O to precipitate the peptide, which was filtered, washed with Et_2O, and air-dried briefly. The residual solid was dissolved in H_2O and lyophilized: 0.69 g. TLC of the peptide in BuOH/AcOH/H_2O 4:1:1 showed small amounts of impurities. Amino-acid analysis showed the solid to be 69.0% peptide by weight. Amino-acid analysis found (ratios to average of (CH_2COOH) Cys + Val): 1/2 $Cys_{0.86}$ $Cys(CH_2COOH)_{1.02}$ $Val_{0.98}$. The purity on the basis of HPLC data, using 254 nm and 214 nm as detection system, was estimated at 94.14 and 96.83%, respectively. We used a 4 × 150-mm column with *Zorbax C-8 MS 853 (Dupont)* packing, and 15% of MeCN with 0.1% of H_3PO_4 and 84.9% of H_2O. MS (FAB): 761 (M + H; calc. for $C_{26}H_{44}N_6O_{12}S_4$ + H: 761). ^{1}H-NMR: see *Fig. 1*.

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REFERENCES

[1] *T. Konomi, S. Herchen, J.E. Baldwin, M. Yoshida, N.A. Hunt & A.L. Demain,* Biochem. J. *184,* 427 (1979).
[2] *S.W. Queener & N. Neuss,* 'The Biosynthesis of β-Lactam Antibiotics', in 'Chemistry and Biology of β-Lactam Antibiotics', Vol. *3,* ed. R.B. Morin and M. Gorman, Academic Press, New York, 1982, pp. 1–81.
[3] *N. Neuss, D.M. Berry, J. Kupka, A.L. Demain, S.W. Queener, D.C. Duckworth & L.L. Huckstep,* J. Antibiot. *35,* 580 (1982).
[4] *H. Troonen, P. Roelants & B. Boon,* J. Antibiot. *29,* 1258 (1976).
[5] *G.A. Bahadu, J.E. Baldwin, J.L. Usher, E.P. Abraham, G.S. Jayatilake & R.L. White,* J. Am. Chem. Soc. *103,* 7650 (1981).
[6] *S. Wolfe & M.G. Jokinen,* Can. J. Chem. *57,* 1388 (1979).
[7] *J.E. Shields, C.S. Campbell, K.S. Doyle, R.D. Miller & N. Neuss,* Helv. Chim Acta *64,* 2587 (1981).
[8] *J.C. Sheehan & G.P. Hess,* J. Am. Chem. Soc. *77,* 1067 (1955).
[9] *H.G. Korona,* Chem. Ind. *1955,* 1087.

[1]) Deceased June 14, 1983.