NMR analyses and to Roger Dinallo, Keith McKellop, and Walter Davidson for mass spectral determinations. Many compounds in this series were originally prepared by Günther Schmidt at Dr. Karl Thomae GmbH.

**Registry No.** 1, 135810-41-2; 2, 14417-38-0; 3, 134894-43-2; 4, 134364-78-6; 5, 135810-44-5; 6, 140412-81-3; 7, 140412-82-4; 8, 3158-85-8; 9, 17296-47-8; 10, 17296-50-3; 11, 135810-46-7; 12, 135810-42-3; 13, 135810-43-4; 14, 140412-83-5; 15, 140412-84-6; 16, 140438-10-4; 17, 140412-85-7; 18, 140412-86-8; 19, 2474-62-6; 20, 23474-61-5; 21, 140412-87-9; 22, 135810-50-3; 23, 23474-52-4; 24, 23474-51-3; 25, 140412-88-0; 26, 140412-89-1; 27, 135810-45-6; 28, 140412-90-4; 29, 23474-58-0; 30, 140412-91-5; 31, 140412-92-6; 32, 140412-93-7; 33, 140412-94-8; 34, 140412-95-9; 35, 140412-96-0; 36, 140412-97-1; 37, 140412-98-2; 37·HCl, 140412-99-3; 38, 140413-00-9; 39, 140413-01-0; 40, 140413-02-1; 41, 140413-03-2; 42, 140413-04-3; 43, 140413-05-4; 44, 140413-06-5; 45, 14527-81-2; 46, 140413-07-6; 47, 140413-08-7; 48, 140413-09-8; 49, 134369-51-0; 50, 140413-10-1; 51, 134894-49-8; 52, 134894-50-1; 53, 134894-51-2; 54, 140413-11-2; 55, 134894-53-4; 56, 140413-12-3; 57, 140413-13-4; 58, 140413-14-5; 59, 140413-15-6; 60, 140413-16-7; 61, 140413-17-8; 62, 140413-18-9; 63, 140413-19-0; 64, 134894-56-7; 65, 134894-60-3; 66, 134894-45-4; 67, 140413-20-3; 68, 140413-21-4; 69, 140413-22-5; 70, 134894-46-5; 71, 134894-47-6; 72, 140413-23-6; 73, 140413-24-7; 74, 140413-25-8; 75, 140413-26-9; 76, 140413-27-0; 77, 140413-28-1; 78, 140413-29-2; 79, 140413-30-5; 80, 140413-31-6; 81, 140413-32-7; 82, 140413-33-8; 83, 140413-34-9; 84, 140413-35-0; 85, 140413-36-1; III ($R^1 = CH_3$, $R^2 = 7\text{-}CH_3$, $R^3 = 2\text{-}NO_2$), 135810-40-1; V ($R^2 = 9\text{-}CH_3$, $R^3 = 3\text{-}NO_2$), 134894-59-0; V ($R^1 = H$, $R^2 = 7\text{-}NO_2$, $9\text{-}CH_3$, $R^3 = H$), 140413-37-2; V ($R^1 = H$, $R^2 = 7\text{-}NH_2$, $9\text{-}CH_3$, $R^3 = H$), 140413-38-3; VIII ($R^2 = 4\text{-}CH_3$), 140413-39-4; IX ($R^2 = 7\text{-}CH_3$), 135810-39-8; XIII ($R^2 = 4,6\text{-}(CH_3)_2$, Hal = Cl), 140413-40-7; XVb ($R^2 = 4,6\text{-}(CH_3)_2$, Hal = Cl), 140413-41-8; XVI ($R^2 = 4,6\text{-}(CH_3)_2$), 140413-42-9; XIX ($R^2 = 4\text{-}CH_3$, $R^3 = 5\text{-}NO_2$), 134894-58-9; XIX ($R^2 = 4\text{-}CH_3$, $R^3 = H$), 140413-43-0; 2-chloro-5-nitrobenzoic acid, 2516-96-3; 6-amino-*m*-cresol, 2835-98-5; 3-cyano-4,6-dimethyl-2-hydroxypyridine, 769-28-8; 2-chloro-3-cyano-4,6-dimethylpyridine, 14237-71-9; 2-chloro-4,6-dimethylnicotinamide, 140413-44-1; salicyloyl chloride, 1441-87-8; 2,4-dimethyl-6-methoxypyrido-[2,3-*b*][1,4]benzoxazepine, 140413-45-2; 2-hydroxynicotinic acid, 609-71-2; 2-hydroxy-5-nitronicotinic acid, 42959-38-6; 2-chloronicotinoyl chloride, 49609-84-9.

# Substrate Specificity of Isopenicillin N Synthase

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Highly purified isopenicillin N synthase (IPNS) from two sources (naturally occurring in *Penicillium chrysogenum* and that expressed in *Escherichia coli* via a cloned gene derived from *Cephalosporium acremonium*) have been isolated and utilized in vitro to test synthetic modifications of the natural substrate, (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine (ACV). A very sensitive procedure utilizing the ability of β-lactams to induce the synthesis of β-lactamase was employed to determine whether an ACV analogue could serve as a substrate for IPNS. A wide variety of amino and carboxyl terminal tripeptide substitutions were examined and found to elicit positive β-lactamase induction profiles. However, none of these modifications were found to function as efficiently as a substrate as ACV. One of the β-lactam products which was formed from the reaction of IPNS and the tripeptide analogue was independently synthesized and evaluated for antibacterial activity. Modification of the L-cysteine residue in the second position of ACV resulted in tripeptides that were unable to serve as substrates. Conversion of the D-valine residue in the third position of ACV to an aromatic amino acid or to a highly electronegative residue such as trifluorovaline resulted in elimination of substrate activity and creation of an inhibitor of the enzyme.

Isopenicillin N synthase (IPNS) is the enzyme responsible for the oxidative conversion of the tripeptide (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine (ACV) (8) to the bicyclic β-lactam antibiotic, isopenicillin N (Scheme I). The recent cloning and expression of the enzymes responsible for the biosynthesis of the penicillin and cephalosporin antibiotics[1-7] has opened the way for a systematic investigation of substrate specificity of these novel enzymes. Use of synthetic analogues of the naturally occurring peptide substrate (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine [Aad(–Cys–D–Val) or ACV (8)] would allow us to evaluate the ability of IPNS to interact with and convert these analogues into β-lactam products. This information could be instrumental in understanding the mechanism of action of this unique biosynthetic enzyme. Furthermore, if IPNS proved to be flexible in its ability to accept modified substrates, we would be able to readily generate new β-lactam antibiotics which might exhibit novel antibacterial profiles.

## Chemistry

Tripeptide analogues of ACV can be prepared by a number of methods, several of which have been previously

(1) Queener, S. W. Molecular Biology of Penicillin and Cephalosporin Biosynthesis. *Antimicrob. Agents Chemother.* **1990**, *34*, 943-948.

(2) Samson, S. M.; Belagaje, R.; Blankenship, D. T.; Chapman, J. L.; Perry, D.; Skatrud, P. L.; VanFrank, R. M.; Abraham, E. P.; Baldwin, J. E.; Queener, S. W.; Ingolia, T. D. Isolation, Sequence Determination and Expression in *Escherichia coli* of the Isopenicillin N Synthetase Gene from *Cephalosporium acremonium*. *Nature (London)* **1985**, *318*, 191-194.

(3) Carr, L. G.; Skatrud, P. L.; Scheetz, M. E.; Queener, S. W.; Ingolia, T. D. Cloning and Expression of the Isopenicillin N Synthetase Gene from *Penicillium chrysogenum*. *Gene* **1986**, *48*, 257-266.

(4) Samson, S. M.; Dotzlaf, J. E.; Slisz, M. L.; Becker, G. W.; VanFrank, R. M.; Veal, L. E.; Yeh, W. K.; Miller, J. R.; Queener, S. W.; Ingolia, T. D. Cloning and Expression of the Fungal Expandase Hydroxylase Gene Involved in Cephalosporin Biosynthesis. *Bio/Technology* **1987**, *5*, 1207-1208, 1211, 1213-1214.

(5) Samson, S. M.; Chapman, J. L.; Belagaje, R.; Queener, S. W.; Ingolia, T. D. Analysis of the Role of Cysteine Residues in Isopenicillin N Synthetase Activity by Site-directed Mutagenesis. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5705-5709.

(6) Ingolia, T. D.; Queener, S. W. Beta-lactam Biosynthetic Genes. *Med. Res. Rev.* **1989**, *9*, 245-264.

(7) Chapman, J. L.; Skatrud, P. L.; Ingolia, T. D.; Samson, S. M.; Kaster, K. R.; Queener, S. W. Recombinant DNA Studies in *Cephalosporium acremonium*. *Dev. Ind. Microbiol.* **1987**, *27*, 165-174.

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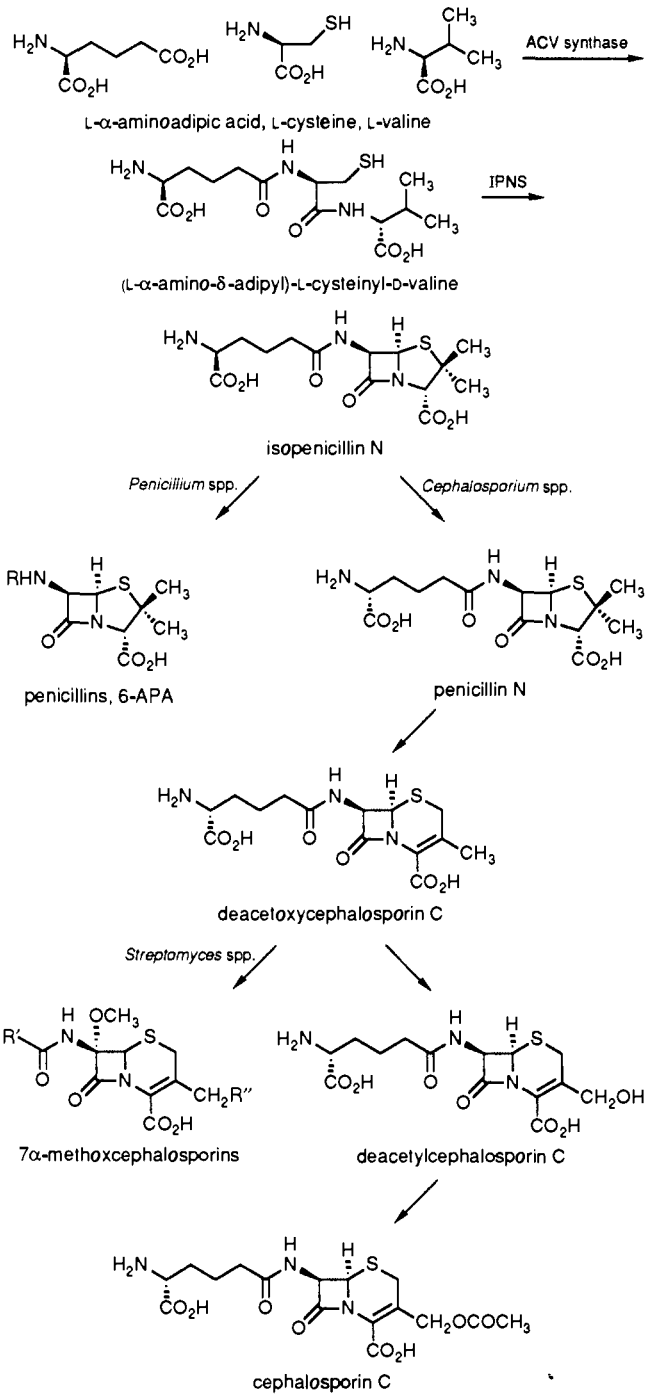
Scheme I. Penicillin/Cephalosporin Biosynthetic Pathways




described.[8-12] We began the preparation of our synthetic analogues of ACV (8) with the approach reported by Wolfe and Jokinen[10] wherein they used *S*-tritylcysteine in combination with benzhydryl esters. We verified their observation that saponification conditions were detrimental to the integrity of the final tripeptide. However, we were able to employ benzyl esters in our synthesis protocol because we used acidic (liquid HF) rather than basic conditions to remove the ester groups. In a related approach, tripeptide disulfides were prepared directly with the use of bis(Boc-cystine) instead of *S*-tritylcysteine. In this fashion, the tripeptide disulfide was generated directly without need for a separate oxidation step. This synthetic protocol required one less synthetic step and as such offered an improvement. Finally, several tripeptides were assembled using *p*-nitrobenzyl esters, *p*-nitro-CBZ groups, and *S*-(acetamidomethyl) protection on cysteine. Final deprotection was achieved by using catalytic hydrogenation or zinc in acetic acid hydrogenolysis, followed by mercuric acetate removal of the Acm group on sulfur. Unlike the previous two methods, this protocol resulted in generation of the tripeptide analogue in the reduced form. Although many of the ACV analogues were prepared by one of the above three methods, the chemical manipulations were time consuming and the overall yields were low.

Solid-phase peptide synthesis is a technique which has been used with increasing frequency over the last decade.[13] Generally, peptides of greater than 10 residues in length are now routinely prepared by this technique. The focus of the value of the technique for larger peptides has obscured the fact that the solid-phase peptide synthesis methodology is just as valuable for smaller peptides. To date none of the syntheses of ACV (8) or its analogues have been accomplished by solid-phase routes. Using solid-phase techniques, we were able to prepare tripeptide analogues in high yield and in a significantly shorter span of time than that required for the solution synthesis. Furthermore, the crude peptide as removed from the solid support (resin) was often of sufficient purity (>95%) to eliminate the requirement for chromatographic purification and allow immediate bioassay.

## Biology

In order to determine whether any of the synthetic peptides were functional substrates for (or inhibitors of) IPNS, a battery of biological assays were utilized. Following suitable reaction times in the presence of IPNS the reaction products were evaluated for their ability to inhibit growth of four different bacteria, *Bacillus subtilis* (strain X12), *E. coli* (strain X580), *Pseudomonas solanacearum* (strain X185), and *Micrococcus luteus* (strain ATCC 9341). A negative result in this test does not prove that the analogue was incapable of serving as a substrate for IPNS since conversion of the peptide to an antibacterial agent might be inefficient or the β-lactam product might not have antibiotic activity. In order to ascertain whether the peptide was being converted to a β-lactam a second test was employed wherein the reaction mixture was tested for its ability to induce the formation of β-lactamase. Very few β-lactams are unable to induce β-lactamase, and conversely non-β-lactam compounds rarely cause induction.[14,15]

(8) Adriaens, P.; Meesschaert, B.; Wuyts, W.; Vanderhaeghe, H.; Eyssen, H. Presence of δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine in Fermentations of *Penicillium chrysogenum*. *Antimicrob. Agents Chemother.* **1975**, *8*, 638–642.

(9) Fawcett, P. A.; Usher, J. J.; Huddleston, J. A.; Bleaney, R. C.; Nisbet, J. J.; Abraham, E. P. Synthesis of δ-(L-α-Aminiadipyl)-L-cysteinyl-D-valine and Its Role in Penicillin Biosynthesis. *Biochem. J.* **1976**, *157*, 651–660.

(10) Wolfe, S.; Jokinen, M. G. Total Synthesis of δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine (ACV), a Biosynthetic Precursor of Penicillins and Cephalosporins. *Can. J. Chem.* **1979**, *57*, 1388–1396.

(11) Baldwin, J. E.; Herchen, S. R.; Johnson, B. L.; Jung, M.; Usher, J. J.; Wan, T. Synthesis of δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine and Some Carbon-13 and Nitrogen-15 Labelled Isotopomers. *J. Chem. Soc., Perkin Trans.* **1981**, *1*, 2253–2257.

(12) Thomson, G. A.; Scott, I. A.; Baxter, R. L. Synthesis of δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine and δ-(L-α-aminoadipyl)-L-cysteinyl-D-valylglycine. *J. Chem. Soc., Perkin Trans.* **1983**, *1*, 941–944.

(13) Barany, G.; Kneib-Cordonier, N.; Mullen, D. G. Solid-phase Peptide Synthesis: A Silver Anniversary Report. *Int. J. Pept. Protein Res.* **1987**, *30*, 705–739.

(14) Parker, W. L.; Rathnum, M. L.; Liu, W. C. SQ 26,517 – A Beta-lactone Produced by a *Bacillus* Species. *J. Antibiotics* **1982**, *35*, 900–902.

Every product detected as a β-lactam by the β-lactamase induction assay and found to exhibit antibacterial activity by bioassay was also sensitive to penicillinase treatment; i.e., after treatment with penicillinase, the hydrolyzed product did not induce β-lactamase or exhibit antibacterial activity. Thus, failure to induce β-lactamase was considered good evidence that IPNS was incapable of catalyzing cyclization of the peptide to a β-lactam product. The ability of the synthetic peptides to act as inhibitors of IPNS was also evaluated by standard techniques of addition of peptide to a standardized ACV/IPNS reaction mixture.

## Results and Discussion

Examination of the substrate specificity of IPNS by use of altered substrates has been reported[10,16-40] (for reviews, see refs 39 and 40). In many of these previous reports IPNS was laboriously isolated from fermentation broths of *Penicillium chrysogenum*, *Streptomyces clavuligerus*, or *Cephalosporium acremonium*. In these instances it was not uncommon for the enzyme preparation to be contaminated with other enzymes in the β-lactam biosynthetic pathway, leading to the potential for misleading results.[37] By use of IPNS which had been generated by recombinant techniques, we were able to bypass these potentially complicating enzymatic impurities and generate data which were unequivocal.

### Amino Terminal Modifications

Previous reports indicate that very little can be done to the Aad residue and still retain the ability to serve as a substrate for IPNS.[16] We wanted to examine many of these previously reported modifications as well as to further explore the scope of the allowed substitutions using our cloned and expressed Cephalosporium IPNS[2] in order to compare the results with the previously used partially purified extracts from Penicillium,[17,18] Cephalosporium,[19-35] or Streptomyces[36-38] IPNS. Clearly the previous reports[16,35,41-43] indicate that the chirality of the Aad residue


(15) Sykes, R. B.; Wells, J. S. Screening for Betalactam Antibiotics in Nature. *J. Antibiotics* **1985**, *38*, 119–121.

(16) Baldwin, J. E.; Abraham, E. P.; Adlington, R. M.; Bahadur, G. A.; Chakravarti, B.; Domayne-Hayman, B. P.; Field, L. D.; Flitsch, S. L.; Jayatilake, G. S.; Spakovskis, A.; Ting, H.-H.; Turner, N. J.; White, R. L.; Usher, J. J. Penicillin Biosynthesis: Active Site Mapping with Aminoadipoylcysteinylvaline Variants. *J. Chem. Soc., Chem. Commun.* **1984**, 1225–1227.

(17) Ramos, F. R.; Lopez-Nieto, M. J.; Martin, J. F. Isopenicillin N Synthetase of *Penicillium chrysogenum*, an Enzyme That Converts δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine to Isopenicillin N. *Antimicrob. Agents Chemother.* **1985**, *27*, 380–387.

(18) Luengo, J. M.; Alemany, M. T.; Salto, F.; Ramos, F.; Lopez-Nieto, M. J.; Martin, J. F. Direct Enzymic Synthesis of Penicillin G Using Cyclases of *Penicillium chrysogenum* and *Acremonium chrysogenum*. *Bio/Technology* **1986**, *4*, 44–47.

(19) Abraham, E. P.; Huddlestone, J. A.; Jayatilake, G. S.; O'Sullivan, J.; White, R. *Recent Advances in the Chemistry of Beta-Lactam Antibiotics*; Royal Society of Chemistry: Oxford, 1981.

(20) Bahadur, G. A.; Baldwin, J. E.; Wan, T.; Jung, M.; Abraham, E. P.; Huddleston, J. A.; White, R. L. On the Proposed Intermediacy of β-Hydroxyvaline- and Thiazepinone-containing Peptides in Penicillin Biosynthesis. *J. Chem. Soc., Chem. Commun.* **1981**, 1146–1147.

(21) Bahadur, G. A.; Baldwin, J. E.; Usher, J. J.; Abraham, E. P.; Jayatilake, G.; White, R. L. Cell-free Biosynthesis of Penicillins. Conversion of Peptides into New β-Lactam Antibiotics. *J. Am. Chem. Soc.* **1981**, *103*, 7650–7651.

(22) Bahadur, G. A.; Baldwin, J. E.; Field, L. D.; Lehtonen, E. M. M.; Usher, J. J.; Vallejo, C. A.; Carlos, A.; Abraham, E. P.; White, R. L. Direct Proton NMR Observation of the Cell-free Conversion of δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine and δ-(L-α-Aminoadipyl)-L-cysteinyl-D-(−)-isoleucine into Penicillins. *J. Chem. Soc., Chem. Commun.* **1981**, 917–919.

(23) Baldwin, J. E.; Abraham, E. P.; Adlington, R. M.; Chakravarti, B.; Derome, A. E.; Murphy, J. A.; Field, L. D.; Green, N. B.; Ting, H. H.; Usher, J. J. Penicillin Biosynthesis. Dual Pathways from a Modified Substrate. *J. Chem. Soc., Chem. Commun.* **1983**, 1317–1319.

(24) Baldwin, J. E.; Abraham, E. P.; Adlington, R. M.; Murphy, J. A.; Green, N. B.; Ting, H. H.; Usher, J. J. Penicillin Biosynthesis. On the Stereochemistry of Carbon-sulfur Bond Formation with Modified Substrates. *J. Chem. Soc., Chem. Commun.* **1983**, 1319–1320.

(25) Baldwin, J. E.; Abraham, E. P.; Burge, G. L.; Ting, H. H. Penicillin Biosynthesis. Direct Biosynthetic Formation of Penicillin V and Penicillin G. *J. Chem. Soc., Chem. Commun.* **1985**, 1808–1809.

(26) Baldwin, J. E.; Adlington, R. M.; Basak, A.; Flitsch, S. L.; Forrest, A. K.; Ting, H. H. Penicillin Biosynthesis. Structure-reactivity Profile of Unsaturated Substrates for Isopenicillin N Synthetase. *J. Chem. Soc., Chem. Commun.* **1986**, 273–275.

(27) Baldwin, J. E.; Adlington, R. M.; Basak, A.; Flitsch, S. L.; Petursson, S.; Turner, N. J.; Ting, H. H. Enzymic Synthesis of a New Type of Penicillin. *J. Chem. Soc., Chem. Commun.* **1986**, 975–976.

(28) Baldwin, J. E.; Adlington, R. M.; Bohlmann, R. Biomimetic Synthesis of Penicillin. *J. Chem. Soc., Chem. Commun.* **1985**, 357–358.

(29) Baldwin, J. E.; Adlington, R. M.; Bradley, M.; Norris, W. J.; Turner, N. J.; Yoshida, A. Identification and Characterization of Shunt Metabolites from Isopenicillin N Synthase. *J. Chem. Soc., Chem. Commun.* **1988**, 1125–1128.

(30) Baldwin, J. E.; Adlington, R. M.; Turner, N. J.; Domayne-Hayman, B. P.; Ting, H. H.; Derome, A. E.; Murphy, J. A. Penicillin Biosynthesis: Enzymatic Synthesis of New Cephams. *J. Chem. Soc., Chem. Commun.* **1984**, 1167–1170.

(31) Baldwin, J. E.; Adlington, R. M.; Domayne-Hayman, B. P.; Ting, H. H.; Turner, N. J. Stereospecificity of Carbon-sulfur Bond Formation in Penicillin Biosynthesis. *J. Chem. Soc., Chem. Commun.* **1986**, 110–113.

(32) Baldwin, J. E.; Adlington, R. M.; Flitsch, S. L.; Ting, H. H.; Turner, N. J. Penicillin Biosynthesis: The Origin of Hydroxy Groups in β-Lactams Derived from Unsaturated Substrates. *J. Chem. Soc., Chem. Commun.* **1986**, 1305–1308.

(33) Baldwin, J. E.; Adlington, R. M.; Moroney, S. E.; Field, L. D.; Ting, H. H. Stepwise Ring Closure in Penicillin Biosynthesis. Initial β-Lactam Formation. *J. Chem. Soc., Chem. Commun.* **1984**, 984–986.

(34) Baldwin, J. E.; Chakravarti, B.; Field, L. D.; Murphy, J. A.; Whitten, K. R.; Abraham, E. P.; Jayatilake, G. The Synthesis of L-α-Aminoadipyl-L-cysteinyl-D-3,4-didehydrovaline, a Potent Inhibitor of Isopenicillin N Synthetase. *Tetrahedron* **1982**, *38*, 2773–2776.

(35) Demain, A. L.; Shen, Y. Q.; Jensen, S. E.; Westlake, D. W. S.; Wolfe, S. Further Studies on the Cyclization of the Unnatural Tripeptide δ-(D-α-Aminoadipyl)-L-cysteinyl-D-valine to Penicillin N. *J. Antibiotics* **1986**, *39*, 1007–1010.

(36) Castro, J. M.; Liras, P.; Cortes, J.; Martin, J. F. Conversion of Phenylacetyl-cysteinyl-valine in vitro into Penicillin G by Isopenicillin N Synthase of *Streptomyces lactamdurans*. *FEMS Microbiol. Lett.* **1986**, *34*, 349–353.

(37) Jensen, S. E.; Westlake, D. W. S.; Bowers, R. J.; Ingold, C. F.; Jouany, M.; Lyubechansky, L.; Wolfe, S. Penicillin Formation by Cell-free Extracts of *Streptomyces clavuligerus*. Behavior of Aminoadipyl-modified Analogs of the Natural Peptide Precursor δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine (ACV). *Can. J. Chem.* **1984**, *62*, 2712–2720.

(38) Jensen, S. E.; Westlake, W. S.; Bowers, R. J.; Lyubechansky, L.; Wolfe, S. Synthesis of Benzylpenicillin by Cell-free Extracts from *Streptomyces clavuligerus*. *J. Antibiotics* **1986**, *39*, 822–826.

(39) Baldwin, J. E.; Bradley, M. Isopenicillin N Synthase: Mechanistic Studies. *Chem. Rev.* **1990**, *90*, 1079–1088.

(40) Baldwin, J. E. The Biosynthesis of Penicillins and Cephalosporins. *J. Heterocycl. Chem.* **1990**, *27*, 71–78.

is important as is the length of the amino acid and the presence of the amino acid functional groups. Removal of the Aad residue (H-Cys-D-Val) (29) results in a completely inactive substrate[16] (Table I). Since the putative product would have been 6-aminopenicillanic acid which is a weak antibiotic, the enzymatic reaction mixture was treated with phenylacetyl chloride in an attempt to generate the more potent penicillin G; however, no antibacterial activity was observed. Preparation of the desamino derivative Adp-Cys-D-Val (21) did result in an active substrate, albeit of lower relative conversion ability than ACV (8). This indicated that the amino group was not required.

The ability of the isomeric analogue D-Ada(-Cys-D-Val) (20) to serve as a substrate, albeit weaker, indicated that the chiral specificity at this site was not absolute. In addition, since the enzyme was prepared by recombinant techniques instead of being isolated from natural sources, there was no doubt that the enzyme was converting the unnatural substrate to penicillin N rather than converting a small amount of ACV which had been produced by a contaminating amount of the racemase enzyme. We also verified the report[16,37] that if the distance between the $\alpha$-carboxylic acid group of Aad and the rest of the molecule is shortened to give Glu(-Cys-D-Val) (40), then no activity could be detected. Additionally, if the Glu was attached to the Cys-D-Val portion of the peptide via the $\alpha$-carboxylic acid group rather than via the side-chain carboxylic acid group to give Glu-Cys-D-Val (41), no bioactivity was observed. This points out the strict distance constraints for the substrate and supports the concept that the substrate binds to the enzyme in at least two areas, one near the amino terminus and the other most probably at the cysteine residue. Qualitative maintenance of the distance constraint but restriction of the allowable bond rotations led to the analogue Gly-Pro-Cys-D-Val (35). Although this analogue possesses in the $\alpha$-amino acid moiety the requisite number of bonds away from the remainder of the molecule, no substrate activity was observed. This again points out the strict spatial orientational requirement of a good substrate for this enzyme. The immediate precursor to the above analogue (Pro-Cys-D-Val) (34) was also found to be devoid of substrate activity.

Literature reports indicated that the deletion of the carboxylic acid residue of Aad to give a 5-aminopentanoyl (5-Ape) derivative[16] resulted in an inactive substrate. This led to the proposal that the carboxylic acid residue of Aad was required for enzymatic activity.[16] However, we found that the derivative containing the methyl ester of adipic acid (MeO-Adp-Cys-D-Val) (22) was a substrate for IPNS. This was our first indication that the carboxylic acid was not required and indicated that perhaps the carbonyl group itself was serving as one of the binding sites to the enzyme. The diester MeO-Adp-Cys-D-Val-OBzl (48) did not, however, exhibit any substrate ability and verified the concept that a free carboxyl terminus of the peptide was probably required for strong interaction with the enzyme. We then prepared the 5-Ape peptide 23 and discovered that it could act as a substrate (albeit a weak one) for the enzyme. The homologues 6-Ahx-Cys-D-Val (24) and 7-Ahp-Cys-D-Val (25) were prepared and found to increase in potency as substrates as the chain length was increased. The penicillin 76 corresponding to that which would have been derived from the 6-Ahx-Cys-D-Val (24) precursor was chemically synthesized and used to establish a conversion rate of approximately 0.3% when compared to ACV (8). Thus, although this substrate was found to react with IPNS, the relative conversion rate was low. The antibacterial profile for this pencillin analogue was determined to be a typical Pen G-like antibiotic. Confirming earlier reports,[18,26,37,39,44,45] we verified that Pen G and Pen V could be prepared directly from the appropriate substituted dipeptide precursors (phenylacetyl)-Cys-D-Val (27) and (phenoxyacetyl)-Cys-D-Val (26); however, in our assays the conversion rates were found to be less than 0.1%. This reinforced the concept that neither the free amino group nor the free amino terminal carboxyl groups were required for interaction at the IPNS active site and subsequent conversion to a $\beta$-lactam antibiotic. Interestingly enough, when we prepared an isosteric analogue of the Pen V precursor, namely Cbz-Cys-D-Val (33), we found it exhibited strong $\beta$-lactamase induction without any indication of antibacterial activity. This is in contrast to the Pen V precursor, PhOAc-Cys-D-Val (26) which generates an active antibiotic.

Reports that *m*-carboxypenicillin G could be prepared from (*m*-carboxyphenyl)acetyl-Cys-D-Val were exciting because the conversion rate was implied to be approximately equivalent to that for ACV itself.[46] This meant that a conformationally restricted analogue of Aad could be incorporated without severely compromising the ability to interact with IPNS. Our preparation of this analogue 31 resulted in a reasonable conversion by IPNS. We prepared the isomeric analogue Bz(3-$CH_2CO_2H$)-Cys-D-Val (32) and found no ability to interact with IPNS. Examination of molecular models of these two analogues indicate that the latter compound is unable to attain some of the conformations that are attainable by either the PhAc(3-$CO_2H$)-Cys-D-Val (31) or the Adp-Cys-D-Val (21) analogues. We then attempted to add a second binding site back to this structure by preparation of the DL-Phg(3-$CO_2H$)-Cys-D-Val (36) derivative. This analogue demonstrated weak conversion based on the $\beta$-lactamase induction assay. Reversal of the connectivity lead to the DL-Phg(3-CO-Cys-D-Val) (37) analogue. This derivative can be viewed as a rigid form of the Aad side chain, and it was found to be converted by IPNS into a product with antibacterial properties. This is in sharp contrast to all other benzoic acid structures which have been made to date and all of which failed to interact with IPNS. Apparently the $\alpha$-amino acid moiety is capable of vastly increasing the binding of a formerly impotent substrate. Finally, the

(41) Baldwin, J. E.; Adlington, R. M.; Crabbe, M. J. C.; Knight, G. C.; Nomoto, T.; Schofield, C. J. Effective Substitute for $\alpha$-Aminoadipic Acid in the Enzymic Synthesis of Penicillins. *J. Chem. Soc., Chem. Commun.* **1987**, 806–807.

(42) Baldwin, J. E.; Adlington, R. M.; Crabbe, M. J. C.; Nomoto, T.; Schofield, C. J. An Efficient Substitute for the $\alpha$-Aminoadipoyl Moiety of $\delta$-(L-$\alpha$-Aminoadipoyl)-L-cysteinyl-D-valine in the Enzymatic Synthesis of Penicillins. *Tetrahedron* **1987**, *43*, 4217–4220.

(43) Baldwin, J. E.; Adlington, R. M.; Moss, N.; Robinson, N. G. Penicillin Biosynthesis: Active Site Mapping with L-$\alpha$-Aminoadipoyl-(C-methyl-L-cysteinyl)-D-valine Variants. *J. Chem. Soc., Chem. Commun.* **1987**, 1664–1667.

(44) Baldwin, J. E.; Pratt, A. J.; Moloney, M. G. The Synthesis of Aryl Substituted Analogs of (Phenoxyacetyl)-L-cysteinyl-D-valine and (Phenylacetyl)-L-cysteinyl-D-valine. Application to the Photoaffinity Labeling of Isopenicillin N Synthetase. *Tetrahedron* **1987**, *43*, 2565–2575.

(45) Luengo, J. M.; Lopez-Nieto, M. J.; Salto, F. Cyclization of Phenylacetyl-L-cysteinyl-D-valine to Benzylpenicillin Using Cell-free Extracts of *Streptomyces clavuligerus*. *J. Antibiotics* **1986**, *39*, 1144–1147.

(46) Baldwin, J. E.; Adlington, R. M.; Domayne-Hayman, B. P.; Knight, G.; Ting, H. H. Use of the Cyclopropylcarbinyl Test to Detect a Radical-like Intermediate in Penicillin Biosynthesis. *J. Chem. Soc., Chem. Commun.* **1987**, 1661–1663.

preparation of the three non-carboxylic acid analogues PhAc(3-$NH_2$)-Cys-D-Val (38), PhAc(4-OH)-Cys-D-Val (42), and PhPr(3-OH)-Cys-D-Val (39) was accomplised to determine if the carboxylic acid moiety could be replaced by other residues with differing $pK_a$'s. The poor conversion profiles by IPNS indicate that the carboxylic acid residue is superior.

Thus it appears from the data generated with these analogues, that although the Aad moiety is not strictly required for a substrate to be converted into a β-lactam structure by IPNS, analogues without the α-amino acid feature are generally inferior with regard to overall conversion rates. Deletion of one or the other of the two moieities of the amino acid (e.g., the carboxylic acid or the amino group) leads to reduced conversions. From these data it appears that the deletion of the amino group is better tolerated than deletion of the carboxylic acid group. If both moieties are deleted then the conversion rates become almost insignificant (300–1000-fold lower) or nonexistent when compared to that of ACV itself.

### Cysteine Modifications

Modification of the cysteine residue represented in our minds the most interesting and potentially important changes in the natural substrate. Literature reports indicated that no substitutions could be made at this position without complete loss of activity.[16] The synthesis of Aad(-Val-D-Val) (104) was undertaken as a result of the isolation of this tripeptide as a minor natural product from penicillin fermentation cultures (A57406) in our laboratories. In fact, alanine, valine, and serine analogues have all been isolated from fermentation broths.[47,48] Although we now speculate that these peptides (104, 44) exist as shunt products of attempted enzymatic conversion of ACV to IPN or shunt products in the earlier steps in the biosynthesis of ACV, we originally thought that they might have served as IPNS substrates for an as yet uncharacterized β-lactam-like product. Deletion of the sulfhydryl function of the cysteine residue in the Ala analogue 44 failed to produce any detectable enzymatic product (Table II). Masking of the sulfhydryl with an acetamidomethyl protecting group gave an analogue (43) which initially resulted in antibacterial activity; however, this activity was found to arise from a small amount of ACV (8) which probably had been generated during the final deprotection of the peptide. It is also conceivable that the enzyme may have catalyzed premature removal of the Acm protecting group. In any event the protected derivative was devoid of substrate activity which again highlights the importance of the sulfhydryl group.

The serine analogue Aad(-Ser-D-Val) (19) did generate initial excitement as the reaction mixture which was obtained following standard incubation with IPNS exhibited strong β-lactamase induction activity. However, all attempts to isolate and identify the active product failed, thus no conclusions can be made at this point. The synthesis of Aad(-hCys-D-Val) (99) and subsequent testing with IPNS in our hands revealed neither biologically active products nor β-lactamase induction potential. Baldwin et al. however, have reported isolation of a λ-lactam enzymatic product from this substrate.[49] We interpret this discrepancy by noting that the product is neither a β-lactam nor does it possess antibiotic activity which would preclude its detection in our assays. Replacement of the cysteine residue with an allylglycine residue also resulted in a completely inactive substrate Aad(-Alg-D-Val) (96). In an intriguing atomic rearrangement of the substrate, the sulfhydryl group was relocated to the C-terminal position of the tripeptide substrate of ACV to give the isomeric analogue, Aad(-Ala-D-Pen) (45) (Table IV). The putative enzymatic product in this instance could have been the natural iospencillin N, however this analogue was also inactive as an IPNS substrate.

Several published mechanisms of binding for ACV with IPNS have the sulfhydryl moiety binding to the ferrous atom at the active site of the enzyme.[49-51] We rationalized that the imidazole group of histidine might also function as an iron coordinating residue and thus we prepared Aad(-His-D-Val) (112). This tripeptide did not act as a substrate for IPNS although possible binding to the active site has been suggested.[52] Therefore, it appears that a sulfhydryl residue in the substrate and in one exact orientation is an absolute requirement for IPNS reactivity. We speculate that the sulfhydryl residues activates the iron–oxygen–enzyme complex via donation of electron(s) from an electron-rich thiolate residue. Further work to explore this rationale is underway.

### Carboxyl Terminus Modification

By far the most interesting of the successful modifications of the natural substrate to date have been the valine substitutions. Baldwin et al. have successuflly modified the valine residue to provide numerous enzymatic products.[32,46] These modifications have implicated the closure of the thiazolidione ring via a radical-mediated mechanism.[46] The identification of several five-, six-, and seven-membered fused β-lactam ring systems from modified substrates reveals the latitude of the enzyme for terminal modifications. In no situation, however, has any product resulted in biological activity greater than that of IPN, nor has any modification resulted in a better substrate for conversion (higher $K_m$ or $V_{max}$) compared to ACV (Table III). On the basis of the exciting report by Baldwin et al.[27] of the ready cyclization of Aad(-Cys-D-alloThr(Me)) to a β-lactam product, we undertook the synthesis and testing of numerous C-terminally modified ACV analogues with several goals in mind, not the least of which was the desire to further define the structural requirements for facile interaction of peptide substrate with IPNS. We also hoped to identify modified ACV analogues which would lead to new and unusual β-lactam antibiotics which might otherwise not have been prepared by conventional synthesis. Finally, we wished to be able to utilize IPNS to create β-lactams which might serve as intermediates for additional synthetic elaborations to give complex β-lactam antibiotics.

(47) Neuss, N.; Miller, R. D.; Affolder, C. A.; Nakatsukasa, W.; Mabe, J.; Hückstep, L. L.; De La Higuera, N.; Hunt, A. H.; Occolowitz, J. L.; and Gilliam, J. H. High Performance Liquid Chromatography (HPLC) of Natural Products. III[1]. Isolation of New Tripeptides from the Fermentation Broth of *P. chrysogenum*. *Helv. Chim. Acta* **1980**, *63*, 1119–1129.

(48) Shields, J. E.; Campbell, C. S.; Doyle, S.; Miller, R. D.; Neuss, N. Synthesis of L-α-Aminoadipyl-L-serinyl-D-valine, a Naturally Occurring Tripeptide from the Fermentation of *Penicillium chrysogenum*. *Helv. Chim. Acta* **1981**, *64*, 2587–2591.

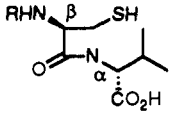
(49) Baldwin, J. E.; Norris, W. J.; Freeman, R. T.; Bradley, M.; Adlington, R. M.; Long-Fox, S.; Schofield, C. γ-Lactam Formation from Tripeptides with Isopenicillin N Synthase. *J. Chem. Soc., Chem. Commun.* **1988**, 1128–1130.

(50) Ming, L. J.; Que, L. J.; Kriauciunas, A.; Frolik, C. A.; Chen, V. J. Coordination Chemistry of the Metal Binding Site of Isopenicillin N Synthase. *Inorg. Chem.* **1990**, *29*, 1111–1112.

(51) Chen, V. J.; Orville, A. M.; Harpel, M. R.; Frolik, C. A.; Surerus, K. K.; Munck, E.; Lipscomb, J. D. Spectroscopic Studies of Isopenicillin N Synthase. A Mononuclear Nonheme Iron(2+) Oxidase with Metal Coordination Sites for Small Molecules and Substrate. *J. Biol. Chem.* **1989**, *264*, 21677–21681.

(52) Chen, V. J. Personal communication.

**Table I.** IPNS Substrate Results. α-Aminoadipic Modifications to ACV



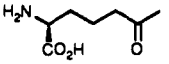
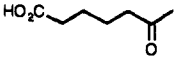
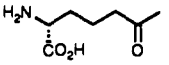
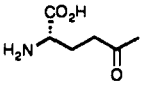
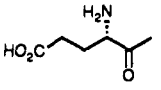
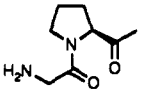
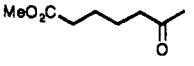
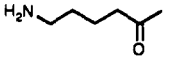
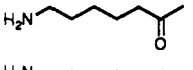
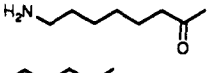
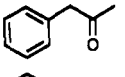
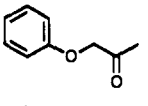
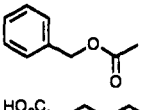
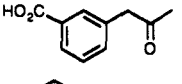
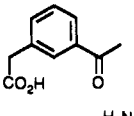
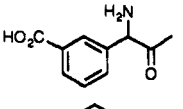
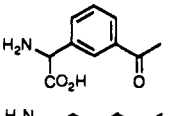
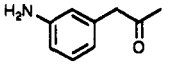
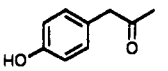
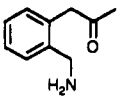
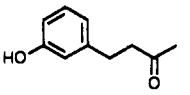
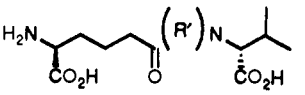
| compd | name (salt and oxidation state) | structure of R (disregarding salt) | β-lactamase induction | antibiotic activity | IPNS inhibitor |
|---|---|---|---|---|---|
| 8 | bis[Ada(-Cys-D-Val)] |  | +++ | yes | no |
| 29 | bis[H-Cys-D-Val] | H– | – | no | yes |
| 21 | bis[Adp-Cys-D-Val] |  | NT | yes | NT[a] |
| 20 | D-Aad(-Cys-D-Val) |  | +++ | yes | no |
| 40 | Glu(-Cys-D-Val) |  | ++ | no | no |
| 41 | Glu-Cys-D-Val |  | – | no | no |
| 35 | bis[Gly-Pro-Cys-D-Val] |  | + | no | no |
| 34 | bis[Pro-Cys-D-Val] |  | + | no | no |
| 22 | bis[MeO-Adp-Cys-D-Val] |  | NT | yes | NT |
| 23 | bis[5-Ape-Cys-D-Val] |  | +++ | yes | no |
| 24 | bis[6-Ahx-Cys-D-Val] |  | +++ | yes | no |
| 25 | bis[7-Ahp-Cys-D-Val] |  | +++ | yes | no |
| 27 | bis[PhAc-Cys-D-Val] |  | NT | yes | NT |
| 26 | bis[PhOAc-Cys-D-Val] |  | NT | yes | NT |
| 33 | bis[Cbz-Cys-D-Val] |  | +++ | no | no |
| 31 | bis[PhAc(3-$CO_2H$)-Cys-D-Val] |  | + | yes | NT |
| 32 | bis[Bz(3-$CH_2CO_2H$-Cys-D-Val)] |  | – | no | no |
| 36 | vis[DL-Phg(3-$CO_2H$)-Cys-D-Val] |  | +++ | no | no |
| 37 | bis[DL-Phg(3-CO-Cys-D-Val)] |  | +++ | yes | no |
| 38 | bis[PhAc(3-$NH_2$)-Cys-D-Val] |  | +++ | yes | no |

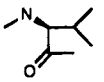
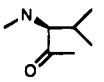
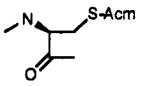
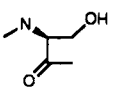
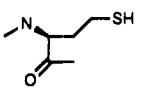
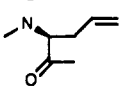
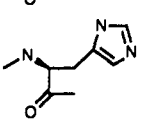
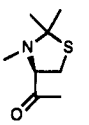
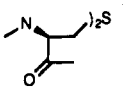
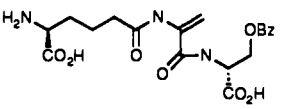
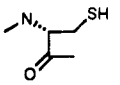
**Table I** (Continued)

| compd | name (salt and oxidation state) | structure of R (disregarding salt) | β-lactamase induction | antibiotic activity | IPNS inhibitor |
|---|---|---|---|---|---|
| **42** | PhAc(4-OH)-Cys-D-Val |  | +++ | no | no |
| **108** | PhAc(2-$NH_2CH_2$)-Cys-D-Val |  | NT | yes | NT |
| **39** | bis[PhPr(3-OH)-Cys-D-Val] |  | + | yes | no |

[a] NT = not tested.

**Table II.** IPNS Substrate Results. Cysteine modifications to ACV

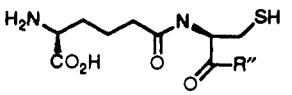


| compd | name (salt and oxidation state) | structure of R′ | β-lactamase induction | antibiotic activity | IPNS inhibitor |
|---|---|---|---|---|---|
| **104** | Aad(-Val-D-Val) |  | - | no | NT[a] |
| **44** | Aad(-Ala-D-Val) |  | +++ | no | no |
| **43** | Aad(-Cys(Acm)-D-Val) |  | + | yes | no |
| **19** | Aad(-Ser-D-Val)·HF |  | +++ | no | no |
| **99** | Aad(-hCys-D-Val) |  | - | no | NT |
| **96** | Aad(-Alg-D-Val) |  | - | no | no |
| **112** | Aad(-His-D-Val) |  | NT | no | ? |
| **101** | Aad(-4-Thp(2,2-$Me_2$)-D-Val) |  | - | no | no |
| **47** | Bis[Aad(-Lan-D-Ser(Bzl))] |  | + | no | no |
| **48** | Aad(-ΔAla-D-Ser(Bzl)) |  | - | no | no |
| **46** | Aad(-D-Cys-D-Val) |  | +++ | no | no |

[a] NT = not tested.

Carboxyl modification of the terminal residue revealed a much more strigent enzyme requirement than was predicted by earlier publications. Verifying earlier reports,[20] we found that replacement of the *gem*-dimethyl-D-valine residue of ACV with a threonine residue led to an inactive substrate (**50**). Other β-gem-disubstituted analogues were prepared such as γ,γ′-dimethoxyvaline **80**, cyclohexylglycine **61**, 3-chloroaminobutyric acid **71**, and didehydrovaline **87**. The latter three analogues gave products with a very weak β-lactamase induction; however, no antibacterial activity was found. Therefore, any conversion was extremely low. Preparation of the unsubstituted serine

**Table III.** IPNS Substrate Results. Valine Modifications to ACV



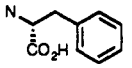
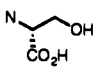
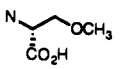
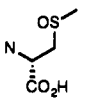
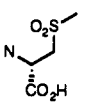
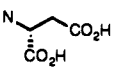
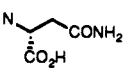
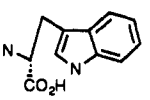
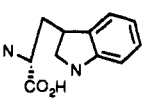
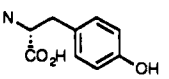
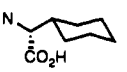
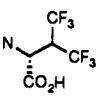
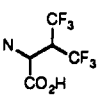
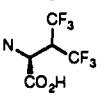
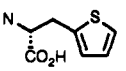
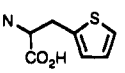
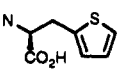
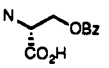
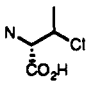
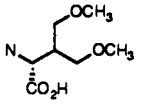
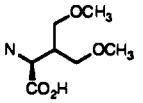
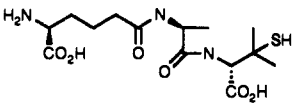
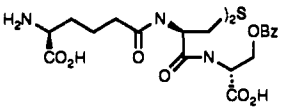
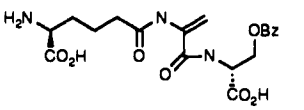
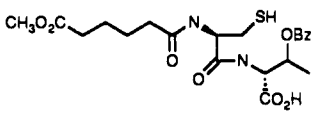
| | | | | | |
|---|---|---|---|---|---|
| 16 | bis[Aad(-Cys-D-Phe)] |  | + | no | yes |
| 50 | bis[Aad(-Cys-D-Thr)] |  | NT | no | no |
| 51 | bis[Aad(-Cys-D-Ser)] |  | NT | yes | NT[a] |
| 52 | bis[Aad(-Cys-D-Ser(OMe))] |  | NT | yes | NT |
| 53 | bis[Aad(-Cys-D-Cys(Me))] |  | + | no | NT |
| 54 | bis[Aad(-Cys-D-Cys(SOMe)] |  | NT | no | NT |
| 55 | bis[Aad(-Cys-D-Cys($SO_2$Me)] |  | NT | no | NT |
| 56 | bis[Aad(-Cys-D-Asp)] |  | NT | no | NT |
| 57 | bis[Aad(-Cys-D-Asn)] |  | NT | no | NT |
| 58 | bis[Aad(-Cys-D-Trp)] |  | + | no | yes |
| 59 | bis[Aad(-Cys-D-Trp($H_2$))] |  | | no | NT |
| 60 | bis[Aad(-Cys-D-Tyr)] |  | NT | no | yes |
| 61 | bis[Aad(-Cys-D-Chg)] |  | + | no | no |
| 62 | bis[Aad(-Cys-D-Val($F_6$))] |  | - | no | yes |
| 63 | bis[Aad(-Cys-DL-Val($F_6$))] |  | - | no | yes |
| 64 | bis[Aad(-Cys-Val($F_6$))] |  | - | no | yes |
| 65 | bis[Aad(-Cys-D-Ala(Thn))] |  | - | no | NT |
| 66 | bis[Aad(-Cys-DL-Ala(Thn))] |  | - | no | NT |
| 67 | bis[Aad(-Cys-Ala(Thn))] |  | - | no | NT |
| 68 | bis[Aad(-Cys-D-Ser(Bzl))] |  | - | no | no |
| 69 | bis[Aad(-Cys-D-Ala(Cl))] |  | - | no | yes |

**Table III.** (Continued)

| | | | | | |
|---|---|---|---|---|---|
| 70 | bis[Aad(-Cys-cLeu)] |  | - | no | no |
| 71 | bis[Aad(-Cys-D-Abu(3-Cl))] |  | + | no | no |
| 72 | Aad(-Cys-Gly) |  | + | no | yes |
| 73 | Aad(-Cys-D-Val-$NH_2$) |  | +++ | no | no |
| 92 | Aad(-Cys-DL-α-aminoisobutanesulfonic acid) |  | - | no | NT |
| 79 | bis[Aad(-Cys-D-Val($OMe)_2$)] |  | - | no | no |
| 80 | bis[Aad(-Cys-Val($OMe)_2$)] |  | - | no | no |
| 87 | Aad(-Cys-ΔVal) |  | + | no | NT |

[a] NT = not tested.

**Table IV.** IPNS Substrate Results. Multiple Modifications to ACV

| compd | name | structure | β-lactamase induction | antibiotic activity | IPNS inhibitor |
|---|---|---|---|---|---|
| 45 | Aad(-Ala-D-Pen) |  | - | - | no |
| 47 | Aad(-Lan-D-Ser(Bzl)) |  | + | no | no |
| 48 | Aad(-ΔAla-D-Ser(Bzl)) |  | - | no | no |
| 49 | bis[MeO-Adp-Cys-D-Val-OBzl] |  | - | no | NT[a] |

[a] NT = not tested.

analogue **51** gave tantalizing results in that very weak antibiotic activity was seen against *M. luteus*. This weak zone of activity was reproducible, but again no β-lactam product could be isolated from the crude reaction mixture. The weak activity with this analogue led us to prepare the methyl ether **52** and benzyl ether analogues **68** on the basis that the methyl ether analogue of D-allothreonine was active while the unsubstituted threonine was not. Perhaps hydrogen bonding of the hydroxyl group with the IPNS active site sequesters efficient bioconversion. By masking or protecting this hydroxyl group then, the H-bonding does not occur and the enzyme can function normally. Although the benzyl ether analogue was totally devoid of substrate activity, the methyl ether analogue was quite active and generated a product with potent antibacterial activity against several bacterial stains. This demonstrated that β-gem disubstitution was not required for conversion by IPNS and led us to generate a series of β-monosubstituted derivatives as potential IPNS substrates.

Our first disappointment in this series was when it was shown that the *S*-methylcysteine derivative **53** was not a substrate. Although this derivative is more or less isosteric with the *O*-methylserine analogue **52**, the sulfur moiety is larger than the oxygen and this might explain the negative result. One also might speculate that the sulfur of the cysteine derivative is interfering with the binding of the cysteine sulfur to the ferrous atom. To test this hypothesis the sulfur of the *S*-methylcysteine residue was oxidized to either the sulfoxide or the sulfone. Neither of these analogues (**54**, **55**) would be expected to serve as an electron donor for hydrogen bonding, but neither analogue possessed any ability to serve as an IPNS substrate. Neither aspartic acid nor asparagine derivatives (**56**, **57**) were capable of bioconversion by IPNS. The fact that the latter analogue was inactive reduces the possibility that the charge on the acid was responsible for interfering with

proper binding to the IPNS active site. The cycloleucine analogue **70** was prepared with the hopes that conversion would generate a tricyclic fused ring system; however, no β-lactam product was detected. Since at least one of the methyl hydrogens on the valine residue is removed during ring expansion from a penicillin to a cephalosporin, we decided to attempt to prepare hexafluoro-IPN from the corresponding tripeptide precursor **62** and then test this penicillin as a substrate or inhibitor of the expandase enzyme. Unexpectedly however, this peptide did not serve as a substrate for IPNS but rather turned out to be a potent inhibitor of IPNS. We speculate that possibly the peptide retains all of the structure information required for binding to the active site, but the high electron density at the C-terminus prohibits some critical step in the enzymatic mechanism. Thus, the tightly bound and spatially correct intermediate serves as an inhibitor of IPNS. Other analogues were then also found to be inhibitors. When valine was replaced with phenylalanine, the peptide **16** did not serve as a substrate but rather was found to be a relatively potent inhibitor. In fact, all other aromatic substitutions (Tyr, Trp, and thienylalanine) (**60**, **58**, **65**) for the valine residue were found to be IPNS inhibitors. Interestingly enough, the *O*-benzylserine derivative **68** did not prove to be an inhibitor. Therefore, the spatial locus of the aromatic group appears to be critical for inhibitory activity. The β-chloroalanine derivative **69** was prepared with the hopes of generating a chlorinated β-lactam nucleus which could be used for later synthetic modifications. Again, unexpectedly, the tripeptide proved to be a potent inhibitor of IPNS rather than a substrate. As preincubation did not increase the inhibition, it does not appear as if this inhibition is of an irreversible nature.

Although amide derivatives have not been reported to serve as substrates for the enzyme, we found a positive result with the β-lactamase induction assay when ACV-$NH_2$ (**73**) was tested as a substrate for IPNS. As mentioned earlier, the benzyl ester derivative of the methoxyadipyl substrate **49** did not elicit a positive response in this assay (Table IV). This may point out that steric and charge requirements cannot both be tampered with at the binding site. The synthesis of the sulfonic acid analogue **92** of ACV was accomplished in the hopes of obtaining a sulfonic acid analogue of penicillin. The predicted increase in acidity of this penicillin might enhance the biological spectrum. In our hands however, IPNS did not appear to accept this modified analogue for cyclization.

## Experimental Section

The following abbreviations are used: Acm, acetamidomethyl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Cbz, carbobenzyloxy; Trt, trityl; ONb, 4-nitrobenzyl ester; Z($NO_2$), (4-nitrobenzyloxy)-carbonyl; HOTs, *p*-toluenesulfonic acid; HCl, hydrochloric acid; DCC, *N,N'*-dicyclohexylcarbodiimide; DIC, *N,N'*-diisopropylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; EEDQ, 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; HOBt, 1-hydroxybenzotriazole; DCHA, dicyclohexylamine; DIEA, diisopropylethylamine; TEA, triethylamine; HOAc, acetic acid; HF, hydrofluoric acid; TFA, trifluoroacetic acid; $Et_3SiH$, triethylsilane; DMF, dimethylformamide; $Et_2O$, diethyl ether; EtOAc, ethyl acetate; THF, tetrahydrofuran; MeCN, acetonitrile; $CH_2Cl_2$, methylene chloride; $CHCl_3$, chloroform; MeOH, methanol; EtOH, ethanol; BuOH, 1-butanol; Aad, α-aminoadipic acid; Adp, adipic acid; 5-Ape, 5-aminopentanoic acid; 6-Ahx, 6-aminohexanoic acid; 7-Ahp, 7-aminoheptanoic acid; hCys, homocysteine; Pen, penicillamine; Val($F_6$), hexafluorovaline; ΔVal, α,β-didehydrovaline; Val($F_3$), γ,γ,γ-trifluorovaline; Val($OMe)_2$, γ,γ'-dimethoxyvaline; Ala(Thn), β-thienylalanine; Ala(Cl), β-chloroalanine; Abu(3-Cl), α-amino-β-chlorobutyric acid; Alg, allylglycine; Ser(Me), serine methyl ether; Chg, cyclohexylglycine; cLeu, cycloleucine; Phg, phenylglycine; Lan, lanthionine. Unless otherwise indicated, all amino acids were of the natural L configuration. Other abbreviations used were those recommended by the IUPAC-IUB Commission (*Biochem. J.* 1984, *219*, 345).

**Physical Methods.** Melting points were determined in open glass capillaries on a Thomas-Hoover melting point apparatus and are uncorrected. NMR spectra were recorded on a General Electric QE 300 spectrometer. Coupling constants (*J*) are listed in hertz. IR spectra were recorded with a Nicolet DX 10 spectrophotometer, and optical rotations were recorded on a Perkin-Elmer Model 241 polarimeter. Mass spectra were taken on VG ZAB-3 or Varian-MAT Model 731 mass spectrometers. Elemental analyses were performed by the physical chemistry department of The Lilly Research Laboratories. Thin-layer chromatography (TLC) experiments were performed on Merck silica gel 60 $F_{254}$ plates (5 × 20 cm). The following TLC solvent systems were used: A = $CHCl_3$/$CH_3OH$/HOAc (90:30:5), B = $CHCl_3$/$CH_3OH$/HOAc (135:15:1), C = EtOH/HOAc/EtOAc (9:1:1), D = BuOH/HOAc/$H_2O$/EtOAc (1:1:1:1), E = BuOH/HOAc/$H_2O$ (4:1:1), F = $CHCl_3$/MeOH (9:1), G = EtOAc/MeCN/HOAc/$H_2O$ (21:7:7:9), H = $CHCl_3$/MeOH/HOAc (90:5:5), I = toluene/EtOAc (7:3).

**Biochemical Methods. Microorganisms and Growth Conditions.** *Bacillus licheniformis* (strain ATCC 14580) was cultured in ABB medium.[15] Spore suspensions of *B. licheniformis* were prepared,[53,54] washed, suspended in freezing solution (1% lactose, 5% glycerol), and stored at −20 °C until used. Bacterial strains from the Lilly culture collection (*B. subtilis* X12, *Escherichia coli* X580, *Pseudomonas solanacearum* X185), and *Micrococcus luteus* (strain ATCC 9341) were grown in nutrient agar for antibiotic assays.

**Isopenicillin N Synthase Reaction Conditions.** Peptides were assayed at a concentration of 2 mg/mL under conditions as described,[55] with the exception that MOPS buffer (pH 8.0) was used in placed of Tris (pH 7.4). The reactions were begun by the addition of purified IPNS, derived from *E. coli*,[2] or in some cases, with IPNS purified from *Penicillium acremonium.* and assayed for biological activity. For β-lactamase susceptibility and induction assays, a reaction mixture of 500 microliters contained the following reagents at the indicated concentrations: 500 mM Tris-HCl, pH 8.0; KCl, 100 mM; $MgSO_4$, 100 mM; $FeSO_4$, 2.0 mM, ascorbic acid, 6.7 mM; dithiothreitol [DTT], 0.75 mM; peptide, 0.3 mM; enzyme, 5 or 50 milliunits. In a second reaction, substrate and DTT were added after reacting in water for 20 min so that thiol-containing substrates were reduced. Reaction temperature was 25 °C. Each reaction mixture was run in quadruplicate. Enzyme reactions were terminated by the addition of 100 mL of ethanol. Sister reactions were allowed to proceed for 2, 5, 10, and 20 min. Two samples (100 mL) were removed from the ethanol-treated mixtures. To one sample 200 units of penicillinase A in 10 mL of water was added. After 10 min at room temperature, both samples were tested for the presence of β-lactam compounds using a β-lactamase induction assay and/or an antibacterial assay.

**Isopenicillin N Synthase Assay.** β-Lactamase induction: IPNS reaction mixtures were assayed for β-lactams by accessing the ability of the reaction products to induce synthesis of β-lactamase[56] in *B. licheniformis*. Cells of *B. licheniformis* were prepared for the induction assay by the following procedure. A primary culture (50 mL of ABB medium in a 250 mL Erlenmeyer flask inoculated with 1 × $10^5$/mL *B. licheniformis* spores) was incubated at 30 °C for 16 h at which time 1 mL was withdrawn and used as inoculum for a second 25-mL culture in the same

(53) Sherratt, D. J.; Collins, J. F. Analysis by Transformation of the Penicillinase System in *Bacillus licheniformis*. *J. Gen. Microbiol.* **1973**, *76*, 217–230.
(54) Collins, J. F.; Kornberg, H. L. The Metabolism of $C_2$ Compounds in Micro-organisms 4. Synthesis of Cell Materials from Acetate by *Aspergillus niger*. *Biochem. J.* **1960**, *77*, 430–438.
(55) Shen, Y. Q.; Wolfe, S.; Demain, A. L. Enzymatic Conversion of the Unnatural Tripeptide δ-(D-α-Aminoadipyl)-L-cysteinyl-D-valine to β-Lactam Antibiotics. *J. Antibiotics* **1984**, *37*, 1044–1048.
(56) Duthie, E. The Production of Stable Potent Preparations of Penicillinase. *J. Gen. Microbiol.* **1947**, *1*, 370–377.

medium. The secondary culture was incubated at 30 °C until it reached a density of 50 Klett units (log phase growth), then diluted to 30 Klett units with fresh medium for use in the assay. The induction assay mixture consisted of 0.05 mL of the IPNS reaction and 0.05 mL of the log phase *B. licheniformis* cell suspension. This mixture was incubated for 2 h at 30 °C, at which time tylosin (0.01 mL of a 1 mg/mL solution) was added to stop protein synthesis. The reaction was incubated for 15 min, and nitrocefin[57] (0.05 mL of a 0.25 mg/mL solution) was added. The optical density was read immediately with a microtiter plate reader (492 nm) and at 10-min intervals thereafter for a total of 30 min. The amount of β-lactam formed in the IPNS reaction was determined by the amount of β-lactamase synthesized during the induction period relative to the amount synthesized in response to penicillin G. This procedure assumes that all β-lactams are equal in the ability to induce β-lactamase in this organism,[14,15] and although there are quantitative differences among various compounds in the ability to induce β-lactamase synthesis, this procedure provides a very sensitive method for detection of β-lactams independent of their antibiotic activity.

**Antibiotic Assays.** When testing for presence of products with antibiotic activity against *M. luteus*, the penicillinase-treated sample and the untreated sample were separately placed in wells (9 mm diameter) of bioassay plates seeded with *Micrococcus luteus* (strain ATCC 9341). Plates were incubated overnight at 37 °C, and zones of inhibition of bacterial growth in lawns of *M. luteus* were read. To check the amount of IPNS present in reaction mixtures authentic ACV was used as substrate at 0.3 mM, and zones in bacterial lawns were compared to standard curve prepared with authentic isopenicillin N in the same volume of water. When a substrate was not converted to a product with anti-*Micrococcus luteus* activity, it was retested with a reaction time of 1 h and (a) peptide concentration at 3 mM and DTT at 3.75 mM and (b) with 10 mU of enzyme. Samples from incubated reactions containing IPNS and synthetic peptide were also tested for antimicrobial activity by standard agar diffusion assays using *Bacillus subtilis* (strain X12), *E. coli* (strain X580), and *Pseudomonas solanacearum* (strain X185). Each compound was also tested alone and with IPN against the test bacteria to insure the compounds did not inhibit the test organism or enhance the antibacterial activity of isopenicillin N against the test microbe.

**Inhibition of IPNS by Substrate Analogues.** Each synthetic peptide that was not converted to a product with anti-*Micrococcus luteus* activity was tested for inhibition of IPNS. A known amount of the peptide was added to a standard IPNS reaction mixture (see above), prior to initiation of the reaction by addition of enzyme. Percent inhibition was determined by dividing the rate obtained in a standard reaction mixture with ACV as substrate in the absence of the synthetic peptide by the rate in a standard reaction mixture with ACV as substrate in the absence of the synthetic peptide.

**Chemistry.** Tripeptides or tripeptide disulfides were prepared by one of four general methods: method A, solution synthesis of the disulfide peptide using Boc-Cys(Trt or Acm) and iodine oxidation; method B, solution synthesis of the disulfide peptide using bis(Boc-cystine); method C, solid-phase peptide synthesis of the reduced peptide; method D, solution synthesis of sulfhydryl peptide using Boc-Cys(Acm).

**Boc-Aad (1).** Prepared by the method of Ramsamy.[58] Recrystallized from $Et_2O$ and petroleum ether: 2.59 g (80%); mp 125–126 °C; MS (FD) $m/z$ 262 ($MH^+$); TLC $R_f$ (A) 0.63, (B) 0.22, (C) 0.65; $^1$H-NMR ($CDCl_3$) δ 1.42 (s, 9 H), 1.70–1.80 (m, 4 H), 2.36–2.44 (m, 2 H), 4.28–4.32 (m, 1 H). Anal. ($C_{11}H_{19}NO_6$) C, H, N.

**Boc-Aad-OBzl·DCHA (2).** To 2.5 g (9.56 mmol) of 1 in DMF (50 mL) were added DCHA (1.9 mL, 9.56 mmol) and benzyl bromide (1.15 mL, 9.66 mmol), and the suspension was allowed to stir 24 h at 70 °C. The reaction mixture was cooled (0 °C) and filtered. The filtrate was concentrated in vacuo, dissolved in EtOAc (100 mL), and washed with $NaHCO_3$ (2 × 50 mL). The aqueous layers were extracted with EtOAc (50 mL), and the organic layers were combined, dried over $Na_2SO_4$, filtered, washed with EtOAc (2 × 50 mL), and concentrated in vacuo to give an oil. The oil was dissolved in EtOH (35 mL) and $Et_2O$ (140 mL), and DCHA (2.1 mL, 10.6 mmol) was added with stirring (2 h). The precipitate was filtered, washed with $Et_2O$ (2 × 50 mL), and dried in vacuo to give 2.739 g (5.15 mmol, 54%) of the salt **2** as a white solid: mp 153–154 °C, MS (FD) $m/z$ 352 ($MH^+$); TLC $R_f$ (A) 0.72, (B) 0.50, (C) 0.75; $^1$H-NMR ($CDCl_3$) δ 1.38–1.43 (s, 9 H), 1.1–1.4 (m, 12 H), 1.6–2.0 (m, 12 H), 2.18–2.24 (m, 2 H), 2.78–2.90 (m, 2 H), 4.25–4.35 (m, 1 H), 5.17 (AB, 2 H), 5.22 (d, 1 H), 7.24–7.40 (m, 5 H). Anal. ($C_{30}H_{48}N_2O_6$) C, H, N.

**Boc-Cys(Trt)-D-Val-OBzl (3).** To a stirred solution of Boc-Cys(Trt)-OH (21.5 g, 46.4 mmol), D-Val-OBzl (17.6 g, 46.4 mmol), and HOBt (6.27 g, 46.4 mmol) in cold (−10 °C) DMF (150 mL) was added TEA (6.5 mL, 46.4 mmol) followed by 9.57 g (46.4 mmol) of DCC in cold (0 °C) DMF. The mixture was stirred at 0 °C for 3 h followed by 18 h at room temperature. The reaction mixture was cooled (0 °C) and filtered. The filtrate was concentrated in vacuo and dissolved in EtOAc (400 mL), and 100 mL of 1 N $KHCO_3$ was added slowly with stirring. The layers were separated, and the EtOAc layer was washed with 1 N $KHCO_3$ (2 × 100 mL), 10% NaCl solution (100 mL), 1 N HCl (2 × 100 mL), and 10% NaCl solution (2 × 100 mL). The aqueous washes were reextracted with 200 mL of EtOAc, and the combined EtOAc layers were dried over $Na_2SO_4$ (18 h), filtered, and concentrated in vacuo to give an oil. The oil was dissolved in $Et_2O$, filtered to remove residual DCU, and concentrated in vacuo to give the dipeptide **3** as an oil (29.8 g, 98.4%): TLC $R_f$ (B) 0.75, (C) 0.77; $[\alpha]^{25}_D$ + 13.8° (*c* 1.0, EtOH). Anal. ($C_{39}H_{44}N_2O_5S$) C, H, N, S.

**Cys(Trt)-D-Val-OBzl·HOTs (4).** To a stirred solution of **3** (28.4 g, 43.5 mmol) in MeCN (435 mL) were added $CHCl_3$ (16.3 mL), $Et_3SiH$ (16.3 mL), and HOTs (24.8 g, 130 mmol). This mixture was allowed to stir at room temperature. After 1.5 h, the product began to crystallize out of solution. The reaction mixture was allowed to stir for an additional 2.5 h whereupon $Et_2O$ (350 mL) was added and stirring was continued for 18 h. The precipitate was filtered, washed with $Et_2O$ (2 × 50 mL), and dried in vacuo to give **4** (22.4 g, 70.9%) as a white solid: mp 213–217 °C; TLC $R_f$ (A) 0.58, (E) 0.57, $[\alpha]^{25}_D$ + 20.3° (*c* 1.0, EtOH). Anal. ($C_{41}H_{44}H_2O_6S_2$) C, H, N, S.

**Boc-Aad(-Cys(Trt)-D-Val-OBzl)-OBzl (5).** To a chilled (0 °C) suspension of **2** (0.50 g, 0.95 mmol) in DMF (25 mL) was added **4** (0.68 g, 0.94 mmol), and the mixture was allowed to stir for 0.5 h. HOBt (0.13 g, 0.94 mmol) and DCC (0.12 g, 0.94 mmol) were added, and stirring was continued in the melting ice bath (16 h). The reaction mixture was concentrated in vacuo, and the residue was partitioned between EtOAc (100 mL) and $H_2O$ (50 mL). The layers were separated, and the organic layer was washed with 0.1 N HCl (3 × 50 mL), $H_2O$ (50 mL), 5% $NaHCO_3$ (3 × 50 mL), and $H_2O$ (50 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo to an oil. This oil was purified by chromatography over silica gel using a Waters Prep 500 instrument with a step gradient from 100% $CHCl_3$ to 5% MeOH in $CHCl_3$. Appropriate fractions were pooled and concentrated in vacuo to give the title compound (660 mg, 79%). This material was used without further purification or characterization.

**Bis[Boc-Aad(-Cys-D-Val-OBzl)-OBzl] (6).** To a solution of **5** (0.63 g, 0.71 mmol) in MeOH (10 mL) was added 0.06 mL of pyridine (0.71 mmol) and 0.18 g of iodine (0.71 mmol). This mixture was stirred at room temperature (4 h) and then added to $CHCl_3$ (85 mL). The organic solution was washed with 10% $NaHSO_3$ (2 × 40 mL) and $H_2O$ (3 × 40 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo to an oil (0.44 g). This material (0.435 g) was purified on a prep TLC plate (eluting with EtOAc). The final disulfide **6** was isolated as an oil (0.408 g, 90%) and was used without further purification or characterization.

**Bis[Aad(-Cys-D-Val)·HF] (7).** To a mixture of **6** (0.408 g, 0.31 mmol) and anisole (1.0 mL) was added 10.0 mL of liquid HF in a teflon/Kel-F apparatus. This mixture was stirred at 0 °C for 1 h, and then the HF was removed in vacuo. The residue was triturated with $Et_2O$ (100 mL) to give a white precipitate. This solid was filtered, washed with $Et_2O$ (2 × 50 mL), dissolved in

(57) O'Callaghan, C. H.; Morris, A.; Kirby, S. M.; Shingler, A. H. Novel Method for Detection of β-Lactamases by Using a Chromogenic Cephalosporin Substrate. *Antimicrob. Agents Chemother.* 1972, *1*, 283–288.

(58) Ramsamy, K.; Olsen, R. K.; Emery, T. Synthesis of *N*-*t*-Boc-L-α-Aminoadipic Acid 1-*t*-Butyl 6-Ethyl Ester from L-Aspartic Acid: A New Route to L-α-Aminoadipic Acid. *Synthesis* 1982, *1*, 42–43.

$H_2O$, and lyophilized to give **7** as an amorphous white solid (0.156 g, 68%): TLC $R_f$ (D) 0.43, (E) 0.44; amino acid analysis, Aad 1.00, Cys 0.84, Val 0.77, $^1$H-NMR ($D_2O$) $\delta$ 0.97 (dd, 12 H), 1.65–1.80 (m, 4 H), 1.84–2.00 (m, 4 H), 2.16–2.25 (m, 4 H), 2.38–2.50 (t, 4 H), 3.10 (d of AB, 4 H), 3.86 (t, 2 H), 4.24 (d, 2 H), 4.80–4.85 (m, 2 H); HPLC Col W-1 1.3 × 59 cm, C18 silica, 10% MeCN/0.1 N $NH_4OAc$, eluted at 45 min.

**Bis[Aad(-Cys-D-Val)] (8).** Compound **7** (0.214 g, 0.280 mmol) was suspended in $H_2O$ (1.0 mL), and 20% aqueous pyridine was added dropwise to give a yellow solution. The pH was adjusted to 2.9 with 1 N HCl, and then 95% EtOH (5.0 mL) was added with stirring at 0 °C (2 h). The solid was filtered, washed with EtOH (2.0 mL), $Et_2O$ (3 × 5.0 mL), and dried in vacuo. This solid (0.118 g) was dissolved in 8% MeCN/0.1 N $NH_4OAc$ (10 mL) and applied to a C18 silica gel column (3.8 × 58 cm). The pure compound was obtained by isocratic elution with the loading solvent at a rate of 7.0 mL/min. The fractions were monitored at 220 nm, and appropriate fractions were pooled and lyophilized to give **8** as an amorphous white solid (0.091 g, 45%): amino acid analysis, Aad 1.11, Cys 0.95, Val 1.05; MS (FAB) $m/z$ 725 ($MH^+$); HPLC Col W-1, 8% MeCN/0.1 N $NH_4OAc$, eluted at 90 min.

**Boc-D-Phe-OBzl (9).** To a well-stirred solution of Boc-D-Phe (20.0 g, 75.4 mmol) in DMF (100 mL) were added DCHA (15.0 mL, 75.4 mmol) and benzylbromide (9.9 mL, 82.9 mmol). This reaction mixture was allowed to stir at room temperature overnight (20 h). The reaction was filtered, and the filtrate was concentrated in vacuo to an oil. This oil was partitioned between EtOAc (200 mL) and $H_2O$ (100 mL). The layers were separated, and the organic layer was washed with 0.1 N HCl (3 × 100 mL), $H_2O$ (100 mL), 5% $NaHCO_3$ (3 × 100 mL), and $H_2O$ (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo to give a yellow oil. This oil was recrystallized from petroleum ether (100 mL) to give **9** (9.22 g, 34%): MS (FD) $m/z$ 355 ($M^+$); $^1$H-NMR ($CDCl_3$) $\delta$ 1.32–1.48 (s, 9 H), 3.02–3.18 (m, 2 H), 4.60–4.68 (dd, 1 H), 4.92–5.02 (m, 1 H), 5.08–5.22 (AB, 2 H), 7.00–7.12 (m, 2 H), 7.20–7.40 (m, 8 H). Anal. ($C_{21}H_{25}NO_4$) C, H, N.

**D-Phe-OBzl·HCl (10).** A solution of 1.7 N HCl in glacial HOAc (75.3 mL, 128 mmol) and anisole (7.53 mL) was added to **9** (9.1 g, 25.6 mmol) and allowed to stir at room temperature (45 min). $Et_2O$ (1.0 L) was added, and the white precipitate was filtered, washed with $Et_2O$ (2 × 200 mL), and dried in vacuo to give **10** as a white solid (7.35 g, 98%): mp 208–210 °C; TLC $R_f$ (A) 0.70, (B) 0.58, (C) 0.27; MS (FD) $m/z$ 256 ($MH^+$); $^1$H-NMR (DMSO-$d_6$) $\delta$ 3.06–3.26 (d of AB, 2 H), 4.34–4.39 (t, 1 H), 5.10–5.20 (AB, 2 H), 7.18–7.40 (m, 10 H).

**Bis[Boc-Cys-D-Phe-OBzl] (11).** To a chilled (0 °C) solution of **10** (5.3 g, 18.2 mmol) and DIEA (3.14 mL, 18.2 mmol) in DMF (40 mL) were added solid HOBt (2.46 g, 18.2 mmol), a chilled (0 °C) solution of Boc-cystine (4.0 g, 9.1 mmol) in DMF (10.0 mL), and a chilled (0 °C) solution of DCC (3.76 g, 18.2 mmol) in DMF (5.0 mL). This reaction mixture was allowed to stir in a melting ice bath overnight (24 h). The reaction mixture was cooled (0 °C) and filtered to remove DCU, and the filtrate was concentrated in vacuo to an oily residue. This material was dissolved in $CHCl_3$ (200 mL) and washed with 5% $NaHCO_3$ (3 × 100 mL), $H_2O$ (100 mL), 0.1 N HCl (3 × 100 mL), and $H_2O$ (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo to give **11** as a white solid (8.31 g, 100%): TLC $R_f$ (A) 0.88, (B) 0.79, (C) 0.80, MS (FAB) $m/z$ 915 ($M^+$), 1H-NMR ($CDCl_3$) $\delta$ 1.40–1.48 (s, 18 H), 2.6–2.9 (m, 4 H), 2.9–3.3 (m, 4 H), 4.64–4.80 (m, 2 H), 4.92–5.02 (dd, 2 H), 5.12 (AB, 4 H), 5.46–5.54 (br d, 2 H), 7.08–7.36 (m, 20 H), 7.70–7.80 (br d, 2 H). Anal. ($C_{48}H_{58}N_4O_{10}S_2$) C, H, N, S.

**Bis[Cys-D-Phe-OBzl·HCl] (12).** A solution of 1.75 N HCl in glacial HOAc (24.9 mL, 43.7 mmol) and thioanisole (2.5 mL) was added to **11** (8.0 g, 8.74 mmol) and allowed to stir at room temperature (30 min). $Et_2O$ (500 mL) was added, and the white precipitate was filtered, washed with $Et_2O$ (2 × 200 mL), and dried in vacuo to give **12** as a white solid (6.37 g, 92%): TLC $R_f$ (A) 0.36, (B) 0.43, (C) 0.20; MS (FD) $m/z$ 715 ($MH^+$), $^1$H-NMR ($CDCl_3$) $\delta$ signals too broad to interpret? Anal. ($C_{38}H_{44}N_4O_6S_2Cl_2$) C, H, N, S, Cl.

**Bis[Boc-Aad(-Cys-D-Phe-OBzl)-OBzl] (13).** To a chilled (0 °C) solution of **12** (2.3 g, 2.9 mmol) and DIEA (1.0 mL, 5.8 mmol) in DMF (30 mL) were added solid HOBt (0.78 g, 5.8 mmol), solid Boc-Aad-OBzl·DCHA (3.09 g, 5.8 mmol), and a chilled (0 °C) solution of DCC (1.2 g, 5.8 mmol) in DMF (5.0 mL). This reaction mixture was allowed to stir in a melting ice bath overnight (26 h). The reaction mixture was filtered to remove DCU, and the filtrate was concentrated in vacuo to a light brown oily residue. This material was dissolved in EtOAc (100 mL) and washed with 5% $NaHCO_3$ (3 × 50 mL), $H_2O$ (50 mL), 0.1 N HCl (3 × 50 mL), and $H_2O$ (50 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo to give **13** as a tan solid (3.93 g, 98%). TLC $R_f$ (A) 0.87, (B) 0.78, (C) 0.77; MS (FAB) $m/z$ 1382 ($MH^+$).

**Bis[Aad(-Cys-D-Phe-OBzl)-OBzl·TFA] (14).** A solution of TFA (20.0 mL) and thioanisole (2.0 mL) was added to **13** (1.9 g, 1.37 mmol) and allowed to stir at room temperature (45 min). The TFA was removed in vacuo, and $Et_2O$ (500 mL) was added. The resulting precipitate was filtered, washed with $Et_2O$ (2 × 200 mL), and dried in vacuo to give crude **6** as a tan solid (1.45 g, 75%). This solid (1.40 g) was dissolved in $CHCl_3$ (50 mL) and chromatographed over silica gel using a step gradient from 0 to 10% MeOH in $CHCl_3$ on a Waters Prep 500 instrument. The eluant was monitored at 254 nm, and the appropriate fractions were pooled and concentrated in vacuo to give pure **14** as a white solid (0.70 g, 36%): TLC $R_f$ (F) 0.40; MS (FAB) $m/z$ 1183 ($MH^+$ + 1); amino acid analysis, Aad 0.91, Cys 0.91, Phe 1.00; $^1$H-NMR ($CDCl_3$) $\delta$ 1.5–1.8 (m, 8 H?), 2.18 (t, 4 H), 2.48–2.74 (m, 4 H), 2.92–3.24 (d of AB, 4 H), 3.42 (dd, 2 H), 4.82–4.94 (m, 2 H), 5.06–5.18 (m, 6 H), 5.30–5.40 (m, 2 H), 6.50–6.58 (d, 2 H), 7.10–7.40 (m, 30 H), 8.56–8.62 (d, 2 H).

**Bis[Aad(-Cys-D-Phe)·HF] (15).** To a mixture of **14** (0.675 g, 0.48 mmol) and thioanisole (1.0 mL) was added 10.0 mL of liquid HF in a teflon/Kel-F apparatus. This mixture was stirred at 0 °C for 1 hour, and then the HF was removed in vacuo. The residue was triturated with $Et_2O$ (100 mL) to give a white precipitate. This solid was filtered and washed with $Et_2O$ (3 × 30 mL) and dried in vacuo to give **15** (0.266 g, 65%): TLC $R_f$ (D) 0.5, (E) 0.23, HPLC Col W-1, 25% MeCN/0.1 N $NH_4OAc$, eluted at 73.5 min.

**Bis[Aad(-Cys-D-Phe)] (16).** The HF salt **15** (0.220 g, 0.256 mmol) was dissolved in 25% MeCN/0.1 N $NaHCO_3$ (40.0 mL) and applied to a C18 silica gel column (3.8 × 58 cm). The pure compound was obtained by isocratic elution with the loading solvent at a rate of 4.0 mL/min. The fractions were monitored at 220 nm, and appropriate fractions were pooled and lyophilized to give **16** as an amorphous white solid (0.073 g, 33%): MS (FAB) $m/z$ 821 ($MH^+$), amino acid analysis, Aad 1.07, Cys 0.92, Phe 1.00; $^1$H-NMR ($D_2O$/DCl 1.4 wt% solution) $\delta$ 1.55–1.80 (m, 4 H), 1.84–2.02 (m, 4 H), 2.34 (dd, 4 H), 2.72 (m, 4 H), 3.18 (d of AB, 4 H), 4.17 (t, 2 H), 4.56 (dd, 2 H), 4.70 (dd, 2 H), 7.17–7.42 (m, 10 H); TLC $R_f$ (D) 0.46 (E) 0.15.

**Boc-D-Val-OCH$_2$-resin (17).** Prepared by the method of Roeske and Gesellchen.[59] In this fashion 27.3 g of **17** was obtained with an incorporation level calculated to be 0.69 mmol/g-resin from amino acid analysis and elemental analysis.

**Aad(-Ser(Bzl)-D-Val-OCH$_2$-resin)-OBzl·TFA (18).** The peptide-resin **18** was assembled by automated solid-phase peptide synthesis in a Beckman 990 peptide synthesizer using **17** (2.0 g, 1.38 mmol). The peptide-resin was assembled by successive incorporation of Boc-Ser(Bzl) and Boc-Aad-OBzl. Initial coupling was performed according to programs A, B, or C, and subsequent recoupling of the same amino acid was performed according to programs D or E[64]. Each amino acid was coupled to the growing

(59) Roeske, R. W.; Gesellchen, P. D. Reaction of Potassium Salts of Boc Amino Acids with Chloromethyl Polystyrene Catalyzed by 18-Crown-6. *Tetrahedron Lett.* **1976**, *38*, 3369–3372.
(60) Huffman, G. W. Improved Preparation of Penicillin N and Isopenicillin N. *Org. Prep. Proced. Int.* **1988**, *20*, 497–503.
(61) Araki, Y.; Nagasawa, J.; and Ishido, Y. Synthetic Studies of Carbohydrate Derivatives by Photochemical Reactions. Part 16. Synthesis of DL-Apiose Derivatives by Photochemical Cycloaddition of 1,3-Dihydroxypropan-2-one Derivatives with Ethanediol or Ethanol Derivatives. *J. Chem. Soc., Perkin Trans.* **1981**, *1*, 12–33.
(62) Frankel, M.; Moses, P. Synthesis of Amino Alkyl Sulfonic Acids and Their Peptide Analogues. *Tetrahedron* **1960**, *9*, 289–294.
(63) Still, W. C.; Kahn, M.; Mitra, A. Rapid Chromatographic Technique for Preparative Separations with Moderate Resolution. *J. Org. Chem.* **1978**, *43*, 2923–2925.

peptide-resin via its symmetric anhydride or HOBt ester. The symmetric anhydride was performed in the synthesizer-metering vessel by reaction (10 min) of 4 equiv of the suitably protected amino acid with 2 equiv of DIC (0.432 mL, 2.76 mmol). In the case of HOBt ester formation, 4 equiv of the amino acid was reacted with 4 equiv of DIC and 4 equiv of HOBt (0.75 g, 5.52 mmol). The use of DIC prevents the formation of an insoluble urea. Following attachment of the final amino acid, the amino-terminal Boc group was removed by treatment with TFA (steps 1–4 of program A). The peptide-resin was dried in vacuo to give 18 (2.53 g, 90%).

**Program A.** (1) Wash with $CH_2Cl_2$ (3 × 1 min). (2) Treat with 50% TFA in $CH_2Cl_2$ (1 × 2 min). (3) Treat with 70% TFA in $CH_2Cl_2$ (1 × 20 min). (4) Wash with $CH_2Cl_2$ (4 × 1 min). (5) Treat with 12% DIEA in $CH_2Cl_2$ (2 × 2 min). (6) Wash with DMF (6 × 1 min). (7) Preform symmetric anhydride by reaction of 4.0 equiv of Boc-amino acid and 2.0 equiv of DIC in meter vessel (1 × 10 min). (8) Treat with the aminoacyl symmetric anhydride (1 × 20 min). (9) Add DMF (10 mL) and continue reaction (2 × 5 min). (10) Wash with $CH_2Cl_2$ (7 × 1 min).

**Program B.** Identical to program A with the following exception: (9) Add DMF (10 mL) and continue reaction (2 × 20 min).

**Program C.** (1) Wash with $CH_2Cl_2$ (3 × 1 min). (2) Treat with 50% TFA in $CH_2Cl_2$ (1 × 2 min). (3) Treat with 70% TFA in $CH_2Cl_2$ (1 × 20 min). (4) Wash with $CH_2Cl_2$ (4 × 1 min). (5) Treat with 12% DIEA in $CH_2Cl_2$ (2 × 2 min). (6) Wash with DMF (5 × 1 min). (7) Preform HOBt ester by reaction of 4.0 equiv of Boc-amino acid, 4.0 equiv of DIC, and 4.0 equiv of HOBt in meter vessel (1 × 40 min). (8) Treat with the HOBt ester (1 × 40 min). (9) Wash with DMF (4 × 1 min).

**Program D.** (1) Wash with DMF (2 × 1 min). (2) Treat with 12% DIEA in $CH_2Cl_2$ (2 × 2 min). (3) Wash with DMF (5 × 1 min). (4) Preform symmetric anhydride by reaction of 4.0 equiv of Boc-amino acid, 4.0 equiv of DIC, and 4.0 equiv of HOBt in meter vessel (1 × 40 min). (5) Treat with the aminoacyl symmetric anhydride (1 × 20 min). (6) Add DMF (10 mL) and continue reaction (2 × 20 min). (7) Wash with $CH_2Cl_2$ (7 × 1 min).

**Program E.** (1) Wash with DMF (1 × 1 min). (2) Treat with 12% DIEA in $CH_2Cl_2$ (2 × 2 min). (3) Wash with DMF (5 × 1 min). (4) Preform HOBt ester by reaction of 4.0 equiv of Boc-amino acid, 4.0 equiv of DIC, and 4.0 equiv of HOBt in meter vessel (1 × 40 min). (5) Treat with the HOBt ester (1 × 40 min). (6) Wash with DMF (2 × 1 min). (7) Wash with $CH_2Cl_2$ (7 × 1 min).

**Aad(-Ser-D-Val)·HF (19).** To a mixture of the peptide-resin 18 (2.53 g) and *m*-cresol (2.5 mL) was added 25.0 mL of liquid HF in a teflon/Kel-F apparatus. This mixture was stirred at 0 °C for 1 h, and then the HF was removed in vacuo. The residue was triturated with $Et_2O$ (100 mL), filtered, and washed with $Et_2O$ (3 × 30 mL). The residue was triturated with $Et_2O$ (100 mL), filtered, and washed with $Et_2O$ (3 × 30 mL). The residue was triturated with 10% HOAc (100 mL), filtered, and washed with additional 10% HOAc (3 × 30 mL). The filtrate was lyophilized to give 19 as a white amorphous solid (0.375 g, 74%) with a purity calculated at greater than 98% as judged by reversed-phase HPLC: MS (FD) *m/z* 348 ($MH^+$), amino acid analysis, Aad 1.00, Ser 0.74, Val 0.81; $^1$H-NMR ($D_2O$) δ 0.97 (dd, 6 H), 1.64–1.80 (m, 2 H), 1.86–2.00 (m, 2 H), 2.16–2.28 (m, 1 H), 2.43 (t, 2 H), 3.83 (t, 1 H), 3.88 (d, 2 H), 4.27 (d, 1 H), 4.54 (t, 1 H).

**Substitutions at the Aad Residue.** **D-Aad(-Cys-D-Val) (20).** Prepared by method C: MS (FD) *m/z* 364 ($MH^+$), amino acid analysis, Aad 1.00, Cys 0.75, Val 0.77; $^1$H-NMR ($D_2O$) δ 0.98 (dd, 6 H), 1.66–1.84 (m, 2 H), 1.91–2.03 (m, 2 H), 2.17–2.30 (m, 1 H), 2.46 (t, 2 H), 2.86–3.02 (m, 2 H), 3.94 (t, 1 H), 4.30 (d, 1 H), 4.60 (t, 1 H).

**Bis[Adp-Cys-D-Val] (21).** Prepared by method B: purified by method 1 on column W-1 using an isocratic elution with 8% MeCN/0.1 N $NH_4OAc$; MS (FD) *m/z* 695 ($MH^+$), amino acid analysis, Cys 0.98, Val 1.02; TLC $R_f$ (A) 0.28, (E) 0.65, (D) 0.80.

**Bis[MeO-Adp-Cys-D-Val] (22).** Prepared by method B: MS (FAB) *m/z* 723 ($MH^+$); amino acid analysis, Cys 0.94, Val 1.00; TLC $R_f$ (C) 0.52; $^1$H-NMR (DMSO-$d_6$) δ 0.87 (dd, 12 H), 1.46–1.56 (m, 8 H), 2.00–2.13 (m, 2 H), 2.12–2.22 (m, 4 H), 2.26–2.34 (m, 4 H), 2.95 (d of AB, 4 H), 3.57 (s, 6 H), 4.14 (dd, 2 H), 4.70–4.82 (m, 2 H), 8.13 (d, 2 H), 8.17 (d, 2 H), 12.4–12.8 (br s, 2 H).

**Bis[5-Ape-Cys-D-Val] (23).** Prepared by method B: FAB-MS 637 ($MH^+$); amino acid analysis, 5-Ape 1.09, Cys 0.95, Val 1.00; $^1$H-NMR ($D_2O$) δ 0.92 (dd, 12 H), 1.65–1.86 (m, 8 H), 2.12 (m, 2 H), 2.42 (t, 4 H), 3.02 (m, 4 H), 3.12 (d of AB, 4 H), 4.10 (d, 2 H), 4.75 (m, 2 H).

**Bis[6-Ahx-Cys-D-Val] (24).** Prepared by method B: MS (FAB) *m/z* 665 ($MH^+$); amino acid analysis, 6-Ahx 1.07, Cys 0.94, Val 0.99; $^1$H-NMR ($D_2O$/DCl 1.4 wt% solution) δ 0.98 (dd, 12 H), 1.34–1.48 (m, 4 H), 1.60–1.76 (m, 8 H), 2.17–2.30 (m, 2 H), 2.37 (t, 4 H), 3.01 (t, 4 H), 3.12 (d of AB, 4 H), 4.29 (d, 2 H), 4.73–4.80 (m, 2 H).

**Bis[7-Ahp-Cys-D-Val] (25).** Prepared by method C: MS (FD) *m/z* 347 ($MH^+$); amino acid analysis, 7-Ahp 0.94, Cys 1.01, Val 1.00; $^1$H-NMR ($D_2O$) δ 0.93 (dd, 6 H), 1.30–1.50 (m, 4 H), 1.60–1.75 (m, 4 H), 2.18 (dq, 1 H), 2.39 (t, 2 H), 2.85–3.06 (m, 4 H), 4.18 (d, 1 H), 4.58 (dd, 1 H).

**Bis[PhOAc-Cys-D-Val] (26).** Prepared by method B: MS (FAB) *m/z* 707 ($MH^+$); amino acid analysis, Cys 0.95, Val 1.00, $NH_3$ 0.83?; TLC $R_f$ (C) 0.63, (B) 0.18, (A) 0.65; $^1$H-NMR ($D_2O$) δ 0.89 (dd, 12 H), 2.05–2.20 (m, 2 H), 3.13 (d of AB, 4 H), 4.10 (d, 2 H), 4.60–4.80 (m, 6 H), 6.97–7.14 (m, 8 H), 7.33–7.43 (m, 2 H).

**Bis[PhAc-Cys-D-Val] (27).** Prepared by method B: MS (FAB) *m/z* 675 ($MH^+$); amino acid analysis, Cys 0.95, Val 1.00; $^1$H-NMR (DMSO-$d_6$) δ 0.81 (t, 12 H), 2.03 (m, 2 H), 3.04 (d of AB, 4 H), 2.52 (s, 4 H), 4.02 (dd, 2 H), 4.63 (m, 2 H), 7.04 (m, 10 H), 7.95 (d, 2 H), 8.52 (d, 2 H).

**Bis[Boc-Cys-D-Val] (28).** Prepared by method B: MS (FD) *m/z* 639 ($MH^+$); $[\alpha]^{25}_D$ −71.8° (*c* 0.5, MeOH); $^1$H-NMR ($CDCl_3$) δ 0.98 (dd, 12 H), 1.46 (s, 18 H), 2.15–2.28 (m, 2 H), 3.02–3.10 (m, 4 H), 4.53 (dd, 2 H), 4.68–4.82 (br s, 2 H), 5.72–5.84 (br s, 2 H), 7.48–7.56 (br s, 2 H). Anal. ($C_{26}H_{48}N_4O_{10}S_2$) C, H, N.

**Bis[H-Cys-D-Val] (29).** Prepared by method B: MS (FD) *m/z* 439 ($MH^+$); amino acid analysis, Cys 0.96, Val 1.04; $^1$H-NMR (DMSO-$d_6$/$D_2O$) δ 0.88 (dd, 12 H), 2.06–2.22 (m, 2 H), 3.13 (d of AB, 4 H), 3.95–4.10 (m, 4 H).

**Bis[Cys-D-Tyr] (30).** Prepared by method B: MS (FAB) *m/z* 567 ($MH^+$); amino acid analysis, Cys 0.94, Tyr 1.00; $^1$H-NMR ($D_2O$) δ 2.80–2.92 (m, 6 H), 3.20–3.30 (m, 2 H), 4.20–4.28 (t, 2 H), 4.46–4.56 (dd, 2 H), 7.00 (AB, 8 H).

**Bis[PhAc(3-$CO_2H$)-Cys-D-Val] (31).** Prepared by method B: assignment based on HPLC retention time; MS (FAB) *m/z* 763 ($MH^+$); $^1$H-NMR ($D_2O$) δ 0.82 (dd, 12 H), 2.06 (m, 2 H), 3.10 (d of AB, 4 H), 3.72 (s, 4 H), 4.12 (d, 2 H), 4.86 (m, 2 H), 7.42–7.60 (m, 4 H), 7.83–7.93 (m, 4 H).

**Bis[Bz(3-$CH_2CO_2H$)-Cys-D-Val] (32).** Prepared by method B: assignment based on HPLC retention time; MS (FAB) *m/z* 763 ($MH^+$); $^1$H-NMR ($D_2O$) δ 0.8 (m, 12 H), 2.1 (m, 2 H), 3.1 (d of AB, 4 H), 3.1 (s, 4 H), 4.1 (d, 2 H), 4.9 (m, 2 H), 7.4–7.6 (m, 4 H), 7.8–7.9 (m, 4 H).

**Bis[Cbz-Cys-D-Val] (33).** Prepared by method B: MS (FAB) *m/z* 707 ($MH^+$); amino acid analysis, Cys 0.71, Val 1.00; $^1$H-NMR ($CDCl_3$) δ 0.98 (dd, 12 H), 2.12–2.26 (m, 2 H), 3.00 (d of AB, 4 H), 4.48 (dd, 2 H), 5.10 (AB, 4 H), 5.14 (m, 2 H), 5.94 (d, 2 H), 7.24–7.40 (m, 10 H), 7.90 (d, 2 H); 94.5% pure by anal. HPLC.

**Bis[Pro-Cys-D-Val] (34).** Prepared by method B: MS (FAB) *m/z* 633 ($MH^+$); amino acid analysis, Pro 1.01, Cys 0.97, Val 1.00; $^1$H-NMR ($D_2O$) δ 0.92 (dd, 12 H), 2.06–2.20 (m, 8 H), 2.44–2.58 (m, 2 H), 3.18 (d of AB, 4 H), 3.36–3.56 (m, 4 H), 4.10 (d, 2 H), 4.46–4.56 (m, 2 H), 4.82 (m, 2 H).

**Bis[Gly-Pro-Cys-D-Val] (35).** Prepared by method B: MS (FAB) *m/z* 747 ($MH^+$); amino acid analysis, Gly 1.00, Pro 1.01, Cys 0.94, Val 0.99; $^1$H-NMR ($D_2O$) δ 0.92 (t, 12 H), 2.04–2.22 (m, 10 H), 3.20 (d of AB, 4 H), 3.54–3.74 (m, 4 H), 4.06 (AB, 4 H), 4.14 (d, 2 H), 4.48 (m, 2 H), 4.82 (m, 2 H).

**Bis[DL-Phg(3-$CO_2H$)-Cys-D-Val] (36).** Prepared by method B: MS (FAB) *m/z* 793 ($MH^+$); amino acid analysis, Cys 0.88, Val 1.00; $^1$H-NMR ($D_2O$) δ 0.74 (d, 3 H), 0.82 (d, 3 H), 0.96 (dd, 6 H),

(64) Program A is used to couple the amino acids Aad, Ala, Asp, Glu, Gly, Ser, and Tyr. Program C is used to couple the amino acids Arg, Asn, and Gln, and program B is used to couple the remaining naturally occurring amino acids. Program E is used to recouple the amino acids which were coupled using program C while the remaining amino acids are recoupled using program D.

1.96–2.08 (m, 1 H), 2.12–2.28 (m, 1 H), 2.62–2.74 (dd, 1 H), 2.92–3.02 (dd, 1 H), 3.12 (d of AB, 2 H), 4.16 (d, 1 H), 4.24 (d, 1 H), 4.84 (m, 2 H), 5.28 (s, 1 H), 5.36 (s, 1 H), 7.55–7.80 (complex, 4 H), 8.06–8.18 (m, 4 H).

**Bis[DL-Phg(3-CO-Cys-D-Val)] (37).** Prepared by method B: MS (FAB) $m/z$ 793 ($MH^+$); amino acid analysis, Cys 0.80, Val 1.00; $^1$H-NMR ($D_2O$) $\delta$ 0.98 (t, 12 H), 2.16–2.30 (m, 2 H), 3.26 (d of AB, 4 H), 4.32 (d, 2 H), 5.02 (m, 2 H), 7.45–7.84 (complex, 8 H).

**Bis[PhAc(3-$NH_2$)-Cys-D-Val] (38).** Prepared by method B: MS (FAB) $m/z$ 705 ($MH^+$); amino acid analysis, Cys 0.93, Val 1.00; $^1$H-NMR ($D_2O$) $\delta$ 0.84 (d, 6 H), 0.92 (d, 6 H), 2.08–2.22 (m, 2 H), 3.10 (d of AB, 4 H), 3.72 (AB, 4 H), 4.24 (d, 2 H), 4.72 (m, 2 H), 7.30–7.55 (complex, 8 H).

**Bis[PhPr(3-OH)-Cys-D-Val] (39).** Prepared by method B: MS (FAB) $m/z$ 735 ($MH^+$); amino acid analysis, Cys 0.89, Val 1.00; $^1$H-NMR (DMSO-$d_6$) $\delta$ 0.88 (d, 12 H), 2.00–2.16 (m, 2 H), 2.36–2.46 (m, 4 H), 2.66–2.78 (m, 4 H), 2.98 (d of AB, 4 H), 4.18 (dd, 2 H), 4.77–4.85 (m, 2 H), 6.52–6.64 (m, 6 H), 6.98–7.08 (m, 2 H), 8.18 (d, 2 H), 8.17 (d, 2 H), 9.15–9.30 (br s, 2 H), 12.4–12.9 (br s, 2 H).

**Glu(-Cys-D-Val) (40).** Prepared by method C: MS (FAB) $m/z$ 350 ($MH^+$); amino acid analysis, Glu 1.00, Cys 0.93, Val 0.99; $^1$H-NMR ($D_2O$) $\delta$ 0.98 (dd, 6 H), 2.13–2.25 (m, 3 H), 2.51–2.60 (dt, 2 H), 2.90–3.00 (d of AB, 2 H), 3.85 (t, 1 H), 4.28 (d, 1 H), 4.60 (t, 1 H).

**Glu-Cys-D-Val (41).** Prepared by method C: MS (FAB) $m/z$ 350 ($MH^+$); amino acid analysis, Glu 1.00, Cys 1.01, Val 0.98, $^1$H-NMR ($D_2O$) $\delta$ 0.98 (dd, 6 H), 2.14–2.30 (m, 3 H), 2.56 (t, 2 H), 2.94 (d of AB, 2 H), 4.17 (t, 1 H), 4.32 (d, 1 H), 4.68 (t, 1 H).

**PhAc(4-OH)-Cys-D-Val (42).** Prepared by method C: MS (FD) $m/z$ 355 ($MH^+$); amino acid analysis, Val 1.00, Cys 0.87; $^1$H-NMR (DMSO-$d_6$) $\delta$ 0.86 (dd, 6 H), 1.98–2.10 (m, 1 H), 2.14 (t, 1 H), 2.62–2.84 (m, 2 H), 3.38 (s, 2 H), 4.18 (dd, 1 H), 4.52 (dt, 1 H), 6.85 (AB, 4 H), 8.13 (d, 2 H), 9.20 (s, 1 H), 12.6–12.8 (br s, 1 H).

**Substitutions at the Cys Residue. Aad(-Cys(Acm)-D-Val) (43).** Prepared by method C: MS (FD) $m/z$ 435 ($MH^+$); amino acid analysis, Aad 1.03, Cys 0.81, Val 1.00; $^1$H-NMR (DMSO-$d_6$) $\delta$ 0.78 (d, 6 H), 1.5–1.6 (m, 1 H), 1.6–1.8 (m, 2 H), 1.84 (s, 3 H), 1.9–2.1 (m, 2 H), 2.1–2.2 (m, 2 H), 2.78 (d of AB, 2 H), 3.20 (dd, 1 H), 3.82 (dd, 1 H), 4.18 (d of AB, 2 H), 4.60 (dd, 1 H), 7.6–7.8 (br m, 3 H), 8.45 (d, 1 H), 8.92 (dd, 1 H).

**Aad(-Ala-D-Val) (44).** Prepared by method C: MS (FD) $m/z$ 332 ($MH^+$); amino acid analysis, Aad 1.00, Ala 0.81, Val 0.81, $^1$H-NMR ($D_2O$) $\delta$ 0.90 (dd, 6 H), 1.42 (d, 3 H), 1.62–1.78 (m, 2 H), 1.85–1.95 (m, 2 H), 2.08–2.20 (m, 1 H), 2.40 (t, 2 H), 3.75 (t, 1 H), 4.11 (t, 1 H), 4.37 (q, 1 H).

**Aad(-Ala-D-Pen) (45).** Prepared by method C: MS (FD) $m/z$ 364 ($MH^+$); amino acid analysis, Aad 1.00, Pen 1.00, Ala 0.76; $^1$H-NMR ($D_2O$) $\delta$ 1.41 (s, 3 H), 1.43 (d, 3 H), 1.53 (s, 3 H), 1.68–1.82 (m, 2 H), 1.86–2.04 (m, 2 H), 2.41 (t, 2 H), 3.98 (t, 1 H), 4.02 (q, 1 H), 4.52 (s, 1 H).

**Aad(-D-Cys-D-Val) (46).** Prepared by method C: MS (FD) $m/z$ 364 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.93, Val 0.93; $^1$H-NMR ($D_2O$) $\delta$ 0.99 (dd, 6 H), 1.67–1.85 (m, 2 H), 1.90–2.04 (m, 2 H), 2.15–2.30 (m, 1 H), 2.43 (t, 2 H), 2.92 (d of AB, 2 H), 3.96 (t, 1 H), 4.30 (d, 1 H), 4.56 (t, 1 H).

**Aad(-Lan-D-Ser(Bzl)) (47).** Prepared by method B; MS (FAB) $m/z$ 849 ($MH^+$); amino acid analysis, Aad 1.00, Ser 0.90; TLC $R_f$ (D) 0.51, (E) 0.56.

**Aad(-ΔAla-D-Ser(Bzl)) (48).** Prepared by method B: MS (FAB) $m/z$ 408 ($MH^+$); TLC $R_f$ (D) 0.50, (E) 0.38; $^1$H-NMR ($D_2O$) $\delta$ 1.68–1.80 (m, 2 H), 1.88–1.98 (m, 2 H), 2.45 (t, 2 H), 3.82 (t, 1 H), 3.90–4.02 (m, 2 H), 4.62 (s, 2 H), 4.68 (t, 1 H), 5.65 (s, 2 H), 7.35–7.50 (m, 5 H).

**Substitutions at the D-Val Residue. Bis[MeO-Adp-Cys-D-Val-OBzl] (49).** Prepared by method B: MS (FAB) $m/z$ 903 ($MH^+$); amino acid analysis, Cys 0.93, Val 1.00; TLC $R_f$ (C) 0.75; $^1$H-NMR (DMSO-$d_6$) $\delta$ 0.84 (d, 12 H), 1.44–1.57 (m, 8 H), 2.02–2.14 (m, 2 H), 2.12–2.18 (m, 4 H), 2.24–2.34 (m, 4 H), 2.94 (d of AB, 4 H), 3.57 (s, 6 H), 4.22 (dd, 2 H), 4.70–4.83 (m, 2 H), 5.12 (AB, 4 H), 7.30–7.40 (m, 10 H), 8.16 (d, 2 H), 8.37 (d, 2 H).

**Bis[Aad(-Cys-D-Phe)] (16).** Prepared by method B. See example.

**Bis[Aad(-Cys-D-Thr)] (50).** Prepared by method B: MS (FAB) $m/z$ 729 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.84, Thr 0.85, $^1$H-NMR (TFA) $\delta$ 1.50 (d, 6 H), 2.0–2.2 (m, 4 H), 2.2–2.4 (m, 4 H), 2.6–2.8 (m, 4 H), 3.28 (d of AB, 4 H), 4.43 (m, 2 H), 4.88 (m, 4 H), 5.25 (m, 2 H).

**Bis[Aad(-Cys-D-Ser)] (51).** Prepared by method B: MS (FAB) $m/z$ 701 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.98, Ser, 0.90; $^1$H-NMR ($D_2O$) $\delta$ 1.64–1.82 (m, 4 H), 1.86–1.96 (m, 4 H), 2.44 (t, 4 H), 3.15 (d of AB, 4 H), 3.82 (t, 2 H), 3.94 (d of AB, 4 H), 4.50 (t, 2 H), 4.80 (m, 2 H). Anal. ($C_{24}H_{40}N_6O_{14}S_2$) C, H, N, S.

**Bis[Aad(-Cys-D-Ser(OMe))] (52).** Prepared by method B: MS (FAB) $m/z$ 728 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.75, Ser(OMe) 0.40, Ser 0.44; $^1$H-NMR ($D_2O$) $\delta$ 1.65–1.82 (m, 4 H), 1.88–2.00 (m, 4 H), 2.22 (t, 4 H), 3.13 (d of AB, 4 H), 3.39 (s, 3 H), 3.77 (dd, 2 H), 3.87 (m, 4 H), 4.58 (t, 2 H), 4.75 (m, 2 H). Anal. ($C_{26}H_{44}N_6O_{14}S_2$) C, H, N, S.

**Bis[Aad(-Cys-D-Cys(Me))] (53).** Prepared by method B: MS (FAB) $m/z$ 761 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.89, Cys(Me) 1.05; $^1$H-NMR ($D_2O$) $\delta$ 1.66–1.80 (m, 4 H), 1.85–2.00 (m, 4 H), 2.13 (s, 6 H), 2.45 (t, 4 H), 3.94 (d of AB, 4 H), 3.11 (d of AB, 4 H), 3.75 (t, 2 H), 4.42 (dd, 2 H), 4.71 (m, 2 H).

**Bis[Aad(-Cys-D-Cys(SOMe))] (54).** Prepared by method B: MS (FAB) $m/z$ 793 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.81, $^1$H-NMR ($D_2O$) $\delta$ 1.66–1.82 (m, 4 H), 1.86–1.98 (m, 4 H), 2.45 (t, 4 H), 2.84–3.08 (complex, 4 H), 3.15 (s, 6 H), 3.26–3.35 (complex, 2 H), 3.66–3.88 (complex, 4 H), 4.42 (dd, 2 H), 4.80 (m, 2 H).

**Bis[Aad(-Cys-D-Cys($SO_2$Me))] (55).** Prepared by method B: MS (FAB) $m/z$ 825 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.79, $^1$H-NMR ($D_2O$) $\delta$ 1.70–1.84 (m, 4 H), 1.88–2.02 (m, 4 H), 2.44 (t, 4 H), 3.14 (d of AB, 4 H), 3.16 (s, 6 H), 3.70–3.84 (m, 2 H), 3.90–4.00 (m, 4 H), 4.82 (m, 2 H), 4.94 (dd, 2 H).

**Bis[Aad(-Cys-D-Asp)] (56).** Prepared by method B: MS (FAB) $m/z$ 757 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.84, Asp 0.91; $^1$H-NMR ($D_2O$) $\delta$ 1.62–1.82 (m, 4 H), 1.84–1.96 (m, 4 H), 2.42 (t, 4 H), 2.91 (m, 4 H), 3.10 (d of AB, 4 H), 3.80 (t, 2 H), 4.63 (t, 2 H), 4.74 (dd, 4 H).

**Bis[Aad(-Cys-D-Asn)] (57).** Prepared by method B: MS (FAB) $m/z$ 755 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.88, Asp 1.00, $NH_3$ 1.00; $^1$H-NMR ($D_2O$) $\delta$ 1.60–1.80 (m, 4 H), 1.84–2.10 (m, 4 H), 2.40 (t, 4 H), 2.90 (m, 4 H), 3.10 (d of AB, 4 H), 3.80 (t, 2 H), 4.18 (t, 2 H), 4.70 (dd, 4 H).

**Bis[Aad(-Cys-D-Trp)] (58).** Prepared by method B: MS (FAB) $m/z$ 900 ($MH^+$), amino acid analysis, Aad 1.00, Cys 0.85, Trp 0.95; NH3 0.98; $^1$H-NMR ($D_2O$/DCl 1.4 wt% solution) $\delta$ 1.56–1.76 (m, 4 H), 1.82–1.96 (m, 4 H), 2.22–2.32 (m, 4 H), 2.46–2.54 (m, 4 H), 3.34 (d of AB, 4 H), 4.03 (t, 2 H), 4.50 (dd, 2 H), 4.76–4.85 (m, 4 H), 7.07–7.25 (m, 6 H), 7.45 (d, 2 H), 7.64 (d, 2 H).

**Bis[Aad(-Cys-D-Trp($H_2$))] (59).** Prepared by method B: MS (FAB) $m/z$ 904 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.84, Trp($H_2$) 0.99; $^1$H-NMR ($D_2O$/DCl 1.4 wt% solution) $\delta$ 1.64–1.84 (m, 4 H), 1.90–2.07 (m, 4 H), 2.14–2.28 (m, 2 H), 2.35–2.54 (m, 6 H), 3.14 (d of AB, 4 H), 3.63–3.78 (m, 4 H), 3.98–4.17 (m, 4 H), 4.52–4.64 (m, 2 H), 4.69–4.78 (m, 2 H), 4.83 (s, 2 H), 7.42–7.6 (m, 8 H).

**Bis[Aad(-Cys-D-Tyr)] (60).** Prepared by method B: MS (FAB) $m/z$ 853 ($MH^+$), amino acid analysis, Aad 1.00, Cys 0.86, Tyr 0.88; $^1$H-NMR ($D_2O$) $\delta$ 1.60–1.80 (m, 4 H), 1.80–2.00 (m, 4 H), 2.35 (t, 4 H), 2.63–3.30 (complex, 4 H), 3.81 (t, 2 H), 4.58–4.70 (m, 4 H), 7.00 (AB, 8 H).

**Bis[Aad(-Cys-D-Chg)] (61).** Prepared by method B: MS (FAB) $m/z$ 804 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.81, Chg 0.83; $^1$H-NMR ($D_2O$) $\delta$ 0.92–1.32 (m, 12 H), 1.48–1.80 (m, 16 H), 1.82–1.98 (m, 2 H), 2.41 (t, 4 H), 3.12 (d of AB, 4 H), 3.73 (t, 2 H), 4.12 (d, 2 H), 4.75 (m, 2 H).

**Bis[Aad(-Cys-D-Val($F_6$))] (62).** Prepared by method B: MS (FAB) $m/z$ 941 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.85, Val($F_6$) 0.95; $^1$H-NMR ($D_2O$) $\delta$ 1.64–1.82 (m, 4 H), 1.86–2.04 (m, 4 H), 2.10 (d, 2 H), 2.42 (t, 4 H), 3.18 (d of AB, 4 H), 3.78 (m, 2 H), 4.31 (m, 2 H), 4.93 (m, 2 H).

**Bis[Aad(-Cys-DL-Val($F_6$))] (63).** Prepared by method B: MS (FAB) $m/z$ 941 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.84, Val($F_6$) 0.95; $^1$H-NMR ($D_2O$) $\delta$ 1.64–1.78 (m, 4 H), 1.82–1.96 (m, 4 H), 2.04 (m, 2 H), 2.41 (dd, 2 H), 3.10 (d of AB, 4 H), 3.74 (m, 2 H), 4.28 (m, 2 H), 5.08 (d, 2 H).

**Bis[Aad(-Cys-Val($F_6$))] (64).** Prepared by method B: MS (FAB) $m/z$ 941 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.84, Val($F_6$) 0.97; $^1$H-NMR ($D_2O$) $\delta$ 1.68–1.82 (m, 4 H), 1.84–1.98 (m, 4 H), 2.04 (d, 2 H), 2.41 (dd, 2 H), 3.05 (d of AB, 4 H), 3.75 (m, 2 H), 4.32 (m, 2 H), 5.08 (m, 2 H).

**Bis[Aad(-Cys-D-Ala(Thn))] (65).** Prepared by method B: MS (FAB) $m/z$ 833 ($MH^+$); Amino acid analysis, Aad 1.00, Cys 0.77, Ala(Thn) 0.81; $^1$H-NMR ($D_2O$) $\delta$ 1.60–1.78 (m, 4 H), 1.80–1.92 (m, 4 H), 2.38 (t, 4 H), 2.88 (dd, 2 H), 3.22 (dd, 2 H), 3.36 (d of AB, 4 H), 3.72 (t, 2 H), 4.48 (dd, 2 H), 4.68 (dd, 2 H), 6.91 (d, 2 H), 7.03 (dd, 2 H), 7.32 (d, 2 H).

**Bis[Aad(-Cys-DL-Ala(Thn))] (66).** Prepared by method B: MS (FAB) $m/z$ 833 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.74, Ala(Thn) 0.84; $^1$H-NMR ($D_2O$) $\delta$ 1.62–1.78 (m, 4 H), 1.80–1.94 (m, 4 H), 2.36 (t, 4 H), 2.70–3.04 (complex, 2 H), 3.12–3.48 (complex, 6 H), 3.72 (dd, 2 H), 4.42–4.52 (m, 2 H), 4.64–4.74 (m, 2 H), 6.90 (d, 2 H), 7.01 (dd, 2 H), 7.32 (dd, 2 H).

**Bis[Aad(-Cys-Ala(Thn))] (67).** Prepared by method B: MS (FAB) $m/z$ 833 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.71, Ala(Thn) 0.87; $^1$H-NMR ($D_2O$) $\delta$ 1.60–1.80 (m, 4 H), 1.80–1.96 (m, 4 H), 2.39 (t, 4 H), 2.74–3.04 (complex, 4 H), 3.24–3.52 (complex, 4 H), 3.76 (t, 2 H), 4.54 (dd, 2 H), 4.70 (dd, 2 H), 6.91 (d, 2 H), 7.02 (dd, 2 H), 7.32 (dd, 2 H).

**Bis[Aad(-Cys-D-Ser(Bzl))] (68).** Prepared by method B: MS (FAB) $m/z$ 881 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.76, Ser 0.71; $^1$H-NMR ($D_2O$) $\delta$ 0.62–0.78 (m, 4 H), 0.82–0.94 (m, 4 H), 2.40 (t, 4 H), 3.02 (d of AB, 4 H), 3.72 (t, 2 H), 3.85 (m, 2 H), 4.44 (t, 2 H), 4.56 (AB, 4 H), 4.72 (m, 2 H), 7.36–7.52 (m, 10 H).

**Bis[Aad(-Cys-D-Ala(Cl))] (69).** Prepared by method B: MS (FAB) $m/z$ 737/739 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.79, $NH_3$ 1.43, plus unknown peak; $^1$H-NMR ($D_2O$/DCl 1.4 wt% solution) $\delta$ 1.68–1.86 (m, 4 H), 1.90–2.02 (m, 4 H), 2.30 (t, 4 H), 2.95 (d of AB, 4 H), 3.78 (d of AB, 4 H), 3.92 (t, 2 H), 4.54–4.62 (m, 2 H), 4.70 (dd, 2 H).

**Bis[Aad(-Cys-cLeu)] (70).** Prepared by method B: MS (FAB) $m/z$ 749 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.74, cLeu 0.90; $^1$H-NMR ($D_2O$) $\delta$ 1.70–1.84 (m, 6 H), 1.91–2.03 (m, 4 H), 2.13–2.25 (m, 2 H), 2.42 (t, 2 H), 3.09 (d of AB, 2 H), 4.13 (t, 1 H), 4.68 (dd, 1 H).

**Bis[Aad(-Cys-D-Abu(3-Cl))] (71).** Prepared by method B: MS (FAB) $m/z$ 766 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.86, Thr 0.59; $^1$H-NMR ($D_2O$) $\delta$ 1.54 (d, 6 H), 1.64–1.80 (m, 4 H), 1.84–1.96 (m, 4 H), 2.44 (dd, 4 H), 3.15 (d of AB, 4 H), 3.76 (t, 2 H), 4.48–4.56 (m, 2 H), 4.56–4.62 (m, 2 H).

**Aad(-Cys-Gly) (72).** Prepared by method C: MS (FD) $m/z$ 322 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.77, Gly 0.80; $^1$H-NMR ($D_2O$) $\delta$ 1.68–1.84 (m, 2 H), 1.90–2.04 (m, 2 H), 2.46 (t, 2 H), 2.94 (d of AB, 2 H), 3.96 (t, 1 H), 4.04 (s, 2 H), 4.57 (t, 1 H).

**Aad(-Cys-D-Val-$NH_2$) (73).** Prepared by method C: MS (FD) $m/z$ 363 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.83, Val 0.81, $NH_3$ 0.87; $^1$H-NMR ($D_2O$) $\delta$ 0.97 (dd, 6 H), 1.65–1.80 (m, 2 H), 1.90–2.02 (m, 2 H), 2.10–2.22 (dq, 1 H), 2.44 (t, 2 H), 2.92 (d of AB, 2 H), 3.95 (t, 1 H), 4.17 (d, 1 H), 4.53 (t, 1 H).

**Z($NO_2$)-Ahx (74).** 6-Aminohexanoic acid (8.03 g, 61 mmol) was charged into a 500 mL three-necked roundbottom flask equipped with a magnetic stirring bar and two addition funnels. $p$-Dioxane (26.6 mL) and 2 N NaOH (33.25 mL, 66.5 mmol) were added, and the mixture was stirred. Upon solution, 2 N NaOH (66.5 mL, 133 mmol) and 4-nitrobenzyl chloroformate (20.0 g, 92.7 mmol) dissolved in $p$-dioxane (102 mL) were added simultaneously from the two separate addition funnels over a 30-min period, maintaining the temperature between 20–25 °C. The reaction mixture was subsequently stirred for 18 h at room temperature. The mixture was diluted with water (200 mL), and the pH was adjusted to 2.0 with 1 N HCl. The aqueous solution was extracted with EtOAc (4 × 150 mL). The combined organic extracts were washed with 200 mL of saline, dried over $MgSO_4$, filtered, and reduced in vacuo to provide a thick oil. The oil was crystallized from EtOAc/$Et_2O$ to provide 74: 12.5 g (65%); MS $m/z$ 136 (M – $C_7H_{12}NO_3$), 173 (M – $C_7H_6NO_2$); $^1$H-NMR ($CDCl_3$) $\delta$ 1.3–1.7 (m, 6 H), 2.36 (t, 2 H), 3.22 (q, 2 H), 4.86 (b s, 1 H), 5.2 (s, 2 H), 7.4–8.3 (AB, 4 H).

**Z($NO_2$)-Ahx-penicillin-ONb (75).** 6-Aminopenicillin-ONb-HOTs salt (prepared according to the method of Huffman[60]) (6.8 g, 13 mmol) was sprung to the free base in EtOAc with saturated $NaHCO_3$ solution. After washing with water and drying over $MgSO_4$, a yellow foam ((3.52 g, 10 mmol) was obtained. This was dissolved in $CH_2Cl_2$ (80 mL) along with 72 (3.10 g, 10 mmol). To this solution was added EEDQ (2.50 g, 10 mmol), and the resulting solution was stirred 18 h at room temperature. The reaction mixture was diluted with $CH_2Cl_2$, washed with cold saturated $NaHCO_3$ solution, 1 N HCl, and saline, dried over $MgSO_4$, filtered, and reduced in vacuo to provide 5.0 g of a white foam. This material was deposited on 15 g of silica gel ($CH_2Cl_2$) and chromatographed (flash column) over 50 g of hexane-packed silica gel utilizing a toluene/EtOAc gradient (0–100%), collecting 2.0 L of eluent in 100 20-mL fractions at a flow rate of about 15 mL/min. The desired component was identified and pooled, and solvent was removed in vacuo to provide 75 (4.0 g, 62%) as a white foam: TLC $R_f$ (I) 0.37; IR ($CHCl_3$) 1787, 1725 cm$^{-1}$; MS (FD) $m/z$ 644 ($MH^+$); $^1$H-NMR ($CDCl_3$) $\delta$ 1.18–1.74 (m, 8 H), 1.46 (s, 3 H), 1.68 (s, 3 H), 2.27 (t, 2 H, $J$ = 5), 3.22 (m, 2 H), 4.50 (s, 1 H), 4.86–5.00 (b s, 1 H), 5.18 (s, 2 H), 5.31 (m, 2 H), 5.54 (d, 1 H), 5.75 (dd, 1 H), 6.13 (d, 1 H), 7.18 (d, 1 H), 7.26 (d, 1 H), 7.25–8.32 (AB, 8 H).

**6-Ahx-penicillanic Acid (76).** Compound 75 (3.81 g, 5.9 mmol) was dissolved in 50% aqueous THF (100 mL) and hydrogenated over 10% Pd/C (1 g) at 4.21 kg/cm$^2$ for 2 h at room temperature. The catalyst was removed by filtration, and the filtrate was extracted with EtOAc twice. The aqueous phase was shell frozen and lyophilized to provide 76 as a white powder: 750 mg (39%); MS (FD) $m/z$ 330 ($MH^+$); $^1$H-NMR ($D_2O$) $\delta$ 1.2–1.75 (m + 2 s, 14 H), 2.38 (t, 2 H), 3.00 (t, 2 H), 4.25 (s, 1 H), 5.50 (d, 1 H), 5.58 (d, 1 H).

**2-Phenyl-4-(2-methoxy-1-methoxyethylidene)-2-oxazolin-5-one (77).** 1,3-Dimethoxyacetone (prepared according to the method of Araki[61]) (21.24 g, 18 mmol), hippuric acid (26.92 g, 15 mmol), lead(II) acetate trihydrate (24.37 g, 6.4 mmol), and acetic anhydride (45.07 g, 44 mmol) were refluxed in THF (345 mL) under a $N_2$ atmosphere for 18 h. The reaction mixture was cooled and filtered. The filtrate was reduced in vacuo to dryness and taken up in benzene (200 mL), and $H_2S$ was bubbled through the solution for 15 min. The resulting mixture was filtered through Celite and reduced in vacuo to low volume whereupon crystallization occurred. The solid mass was recrystallized from 2-propanol to provide 77 (26.11 g, 67%): IR (KBr) 1792, 1569, 1451, 1327, 1162 cm$^{-1}$; MS (FAB) $m/z$ 262 ($MH^+$); UV $\lambda_{max}$ (EtOH) nm ($\epsilon$) 304 (21264), 237 (11531); $^1$H-NMR ($CDCl_3$) $\delta$ 3.44 (s, 6 H), 4.62 (s, 2 H), 4.72 (s, 2 H), 7.45–8.13 (m, 5 H).

**Z($NO_2$)-DL-Val($OMe)_2$ (78).** Compound 77 (10.44 g, 40 mmol) was charged into a pressure bottle containing 10% Pd/C (300 mg) and $p$-dioxane (100 mL). The substrate was hydrogenated at 4.21 kg/cm$^2$ for 6 h at room temperature. The reaction mixture was filtered through talc and reduced in vacuo to provide a yellow oil which was taken up in 5 N HCl (82 mL) and refluxed for 4 h. The reaction mixture was cooled and filtered to remove benzoic acid and reduced in vacuo to low volume. The residue was taken up in a minimal amount of $H_2O$ and chromatographed over Dowex 50Wx8 ($H^+$) (170 mL). The column was washed with $H_2O$, and the desired product was eluted with 2 N $NH_4OH$ (1 L). The eluent was reduced in vacuo, and $Et_2O$ was added to provide 5.22 g of a yellow-white solid. This material was suspended in DMF (30 mL) and cooled to 0 °C, and tetramethylguanidine (3.69 mL, 30 mmol) was added and stirred for 30 min. Ethyl acetoacetate (3.81 mL, 29.9 mmol) was added, and the mixture was stirred for 18 h. To this solution was added 4-nitrobenzyl chloroformate (6.50 g, 30 mmol), and the resulting mixture was stirred for 24 h at room temperature. The solution was diluted with saturated $NaHCO_3$ (150 mL) and EtOAc (210 mL). The organic phase was separated and washed with saturated $NaHCO_3$ solution (150 mL) and $H_2O$ (150 mL), dried over $MgSO_4$, filtered, and reduced in vacuo to a gum which crystallized from EtOAc/$Et_2O$ to provide 78 (7.14 g, 57%) as white crystals: IR (KBr) 1345, 1520, 1750, 1790 cm$^{-1}$; UV $\lambda_{max}$ (EtOH) nm ($\epsilon$) 264 (8123); MS (FD) $m/z$ 313 ($MH^+$); $^1$H-NMR (DMSO-$d_6$) $\delta$ 2.50–2.62 (m, 1 H) 3.20 (s, 3 H), 3.23 (s, 3 H), 3.44 (m, 4 H), 4.09–4.18 (m, 1 H), 5.37 (m, 2 H), 7.70–8.30 (AB, 4 H, AB), 8.6–8.9 (b s, 1 H).

**Bis[Aad(-Cys-D-Val($OMe)_2$)] (79).** Prepared by method C: MS (FD) $m/z$ 424 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.79; $^1$H-NMR ($D_2O$) $\delta$ 1.68–1.82 (m, 2 H), 1.91–2.02 (m, 2 H), 2.45 (t, 2 H), 2.56–2.65 (m, 1 H), 2.95 (d of AB, 2 H), 3.35 (s, 6 H), 3.42–3.62

(m, 4 H), 3.95 (t, 1 H), 4.55 (t, 1 H), 4.68–4.73 (m, 1 H).

**Bis[Aad(-Cys-Val(OMe)$_2$)] (80).** Prepared by method C: MS (FD) $m/z$ 424 ($MH^+$); amino acid analysis, Aad 1.00, Cys 0.76; $^1$H-NMR ($D_2O$) $\delta$ 1.67–1.83 (m, 2 H), 1.90–2.05 (m, 2 H), 2.48 (dd, 2 H), 2.58–2.64 (m, 1 H), 2.95 (d, 2 H), 3.35 (s, 6 H), 3.42–3.60 (m, 4 H), 3.98 (t, 1 H), 4.55 (t, 1 H), 4.70 (d, 1 H).

**Boc-ΔVal-ONb (81).** Boc-ΔVal (2.39 g, 11.1 mmol) was dissolved in hot DMF (20 mL), and DCHA (2.3 mL, 11.1 mmol) was added. 4-Nitrobenzyl bromide (2.59 g, 12 mmol) was added, and the mixture was stirred for 3 h, filtered, and the filtrate washed six times with 1 N HCl, saturated $NaHCO_3$ solution, and brine. The organic phase was dried over $MgSO_4$, filtered, and reduced in vacuo to provide a crystalline mass which was recrystallized from $Et_2O$ to provide 3.31 g (85%) of 81 as white crystals which was used without further purification or characterization.

**ΔVal-ONb·HCl (82).** Compound 81 (3.31 g, 9.94 mmol) was slurried in $Et_2O$ (200 mL), cooled to 0 °C, and flushed with $N_2$. Dry HCl gas was bubbled into the cold slurry until solution was achieved. The solution was warmed to room temperature whereupon crystals formed. The excess HCl was removed in vacuo, and the crystalline mass reslurried in fresh $Et_2O$ and filtered to provide 1.74 g (61%) of 82: IR (KBr) 1732, 1517, 1346, 1302, 1229 cm$^{-1}$; UV $\lambda_{max}$ (EtOH) nm ($\epsilon$) 266 (10738); MS (FD) $m/z$ 250 ($MH^+$); $^1$H-NMR (DMSO-$d_6$) $\delta$ 1.90 (s, 3 H), 2.05 (s, 3 H), 5.25 (m, 2 H), 7.40–8.20 (AB, 4 H, AB), 7.3 (b s, 1 H).

**Boc-Cys(Acm)-ΔVal-ONb (83).** Compound 82 (1.07 g, 3.7 mmol) was dissolved in THF (100 mL) and chilled to 0 °C. Diisopropylamine (0.65 mL, 3.7 mmol) was added and subsequently Boc-Cys(Acm) (1.09 g, 3.7 mmol) and DCC (770 mg, 3.7 mmol) were added. The mixture was stirred at room temperature for 20 h. The white precipitate was removed by filtration, and the filtrate was reduced in vacuo and redissolved in EtOAc. The organic solution was washed with saturated $NaHCO_3$ solution (100 mL), 1 N HCl (100 mL), and $H_2O$ (100 mL), dried over $MgSO_4$, filtered, and reduced in vacuo to low volume whereupon crystallization occurred. Addition of $Et_2O$ and filtration afforded 1.47 g (75%) of 83: $^1$H-NMR ($CDCl_3$) $\delta$ 1.45 (s, 9 H), 1.90 (s, 3 H), 2.01 (s, 3 H), 2.18 (s, 3 H), 2.72–2.92 (m, 2 H), 4.28–4.38 (m, 1 H), 4.50–4.66 (m, 2 H), 5.27 (s, 2 H), 5.66 (d, 1 H, $J$ = 9), 6.92 (b s, 1 H), 7.5–8.25 (AB, 4 H), 8.0 (s, 1 H).

**Cys(Acm)-ΔVal-ONb·HCl (84).** Compound 83 (1.43 g, 2.8 mmol) was dissolved in cold TFA (25 mL) and stirred for 30 min. The solvent was removed in vacuo, and the residue was redissolved in EtOAc and reduced again in vacuo. The residue was taken up in EtOAc, and dry HCl was bubbled into the solution which resulted in crystallization of 84 (938 mg, 73%); MS (FD) $m/z$ 425 ($MH^+$); $^1$H-NMR (DMSO-$d_6$) $\delta$ 1.80 (s, 3 H), 1.83 (s, 3 H), 2.20 (s, 3 H), 2.1–2.2 (m, 2 H), 2.8–2.9 (m, 2 H), 4.18–4.28 (m, 1 H), 5.25 (m, 2 H), 7.55–8.25 (AB, 4 H).

**Z(NO$_2$)-Aad(-Cys(Acm)-ΔVal-ONb)-ONb (85).** Compound 84 (920 mg, 2 mmol), diisopropylamine (0.35 mL, 2 mmol), and Z(NO$_2$)-Aad-ONb (951 mg, 2 mmol) were slurried in THF (50 mL). To this was added DCC (412 mg, 2 mmol), and the resulting mixture was stirred for 18 h. The reaction mixture was filtered, and the filtrate was reduced in vacuo and redissolved in EtOAc. The organic solution was washed with saturated $NaHCO_3$ solution (2 × 100 mL), 1 N HCl (2 × 100 mL), and saline (100 mL), dried over $MgSO_4$, filtered, and reduced in vacuo to low volume. Crystallization occurred and was completed by addition of $Et_2O$. Filtration afforded 85 (1.76 g, 99%): MS (FD) $m/z$ 882 ($MH^+$), $^1$H-NMR (DMSO-$d_6$) $\delta$ 1.5–1.8 (m, 4 H), 1.80 (s, 3 H), 1.83 (s, 3 H), 2.20 (s, 3 H), 2.1–2.2 (m, 2 H), 2.8–2.9 (m, 2 H), 4.18–4.28 (m, 1 H), 4.62 (m, 1 H), 5.19–5.30 (m, 4 H), 7.55–8.25 (AB, 8 H), 8.00 (d, 1 H), 8.55 (m), 9.35 (d, 1 H).

**Aad(-Cys(Acm)-ΔVal) (86).** Compound 85 (972 mg, 1.1 mmol) was dissolved in DMF (100 mL) containing 10% Pd/C (500 mg). The substrate was hydrogenated at 4.21 kg/cm$^2$ for 2 h at room temperature. The reaction mixture was filtered through talc and reduced in vacuo to a gum. Trituration with acetone resulted in a white solid (86): MS (FD) $m/z$ 433 ($MH^+$).

**Aad(-Cys-ΔVal) (87).** Compound 86 was dissolved in $H_2O$ (25 mL) and the pH was adjusted to 4.0 with HOAc. To this solution was added $Hg(OAc)_2$ (200 mg), and the resulting mixture was stirred at room temperature for 1 h. The mixture was then saturated with $H_2S$ for 2 h and filtered through talc, and the black precipitate was washed with water. A small amount of copper wire was added to the filtrate, and air was bubbled through the solution for 18 h. The aqueous solution was reduced in vacuo to a clear glass which was taken up in MeOH. Addition of $Et_2O$ resulted in formation of a white precipitate (87): MS (FAB) $m/z$ 721 ($MH^+$); $^1$H-NMR ($D_2O$/DMSO-$d_6$/DCl) $\delta$ 1.50–1.88 (m, 4 H), 1.74 (s, 3 H), 2.02 (s, 3 H), 2.15–2.26 (m, 2 H), 2.82–3.18 (m, 2 H), 3.9 (m, 1 H), 4.58–4.68 (m, 1 H).

**Boc-Cys(Acm)-DL-α-aminoisobutanesulfonic acid (88).** Boc-Cys(Acm) (7.30 g, 25 mmol) was dissolved in THF (100 mL) and TEA (3.48 mL, 25 mmol) and chilled to −10 °C. To this solution was added isobutyl chloroformate (3.25 mL, 25 mmol), and the mixture was stirred cold for 20 min. To the mixed anhydride was then added α-aminoisobutanesulfonic acid[62] (3.83 g, 25 mmol) dissolved in water (25 mL) containing $K_2CO_3$ (3.45 g, 25 mmol). The resulting mixture was stirred and permitted to warm slowly to 10 °C over a 2-h period. The mixture was reduced in vacuo to low volume and extracted three times with $Et_2O$, and the aqueous portion was then adjusted to pH 2.0 with concentrated HCl, reduced in vacuo to a syrup, taken up in MeOH, and filtered free of KCl. The filtrate was again reduced in vacuo to a syrup (88): $^1$H-NMR ($CDCl_3$) $\delta$ 1.35 (t, 6 H), 1.43 (s, 9 H), 2.03 (s, 3 H), 2.5 (m, 1 H), 2.90 (m, 2 H), 4.3–4.6 (m, 4 H), 5.29 (d, 1 H).

**Cys(Acm)-DL-α-aminoisobutanesulfonic acid (89).** Compound 88 was dissolved in TFA (20 mL) and stirred at room temperature for 1 h. The solution was repeatedly reduced in vacuo to a gum and taken up in EtOAc. Addition of $Et_2O$ resulted in precipitation of a solid. The solid material was slurried in EtOH, chilled, and filtered to provide 882 mg of 89 as a white solid: TLC $R_f$ (G) 0.39; $^1$H-NMR ($D_2O$) $\delta$ 1.06 (m, 6 H), 2.06 (s, 3 H), 2.43 (m, 1 H), 3.10–3.27 (m, 2 H), 4.35–4.70 (m, 4 H).

**Boc-Aad(-Cys(Acm)-DL-α-aminoisobutanesulfonic acid)-ONb (90).** Boc-Aad-ONb (1.06 g, 2.68 mmol) was dissolved in THF (15 mL) containing TEA (0.374 mL, 2.68 mmol). The solution was chilled to −10 °C, and isobutyl chloroformate (0.348 mL, 2.68 mmol) was added and stirred cold for 20 min. In another flask was dissolved 85 (880 mg, 2.68 mmol) in $H_2O$ containing $K_2CO_3$ (371 mg, 2.68 mmol). This solution was added to the mixed anhydride at 0 °C and stirred for 3 days at room temperature. The mixture was reduced in vacuo to remove THF, additional $H_2O$ was added, and the solution was extracted twice with $Et_2O$. The aqueous portion was adjusted to pH 2.0 with concentrated HCl and extracted twice with EtOAc. The aqueous portion was reduced in vacuo to a syrup, taken up in MeOH, filtered, and reduced in vacuo to a foam (90) (1.46 g, 73%): MS (FD) $m/z$ 606 (M − $(CH_3)_3CCO$); $^1$H-NMR ($D_2O$) $\delta$ 1.30 (t, 6 H), 1.43 (s, 9 H), 1.60–2.05 (m, 4 H), 2.01 (s, 3 H), 2.39 (m, 3 H), 2.82–3.18 (m, 2 H), 3.22 (m, 1 H), 3.67 (m, 1 H), 4.10–4.40 (m, 4 H), 5.35 (m, 2 H), 7.63–8.31 (AB, 4 H).

**Aad(-Cys(Acm)-DL-α-aminoisobutanesulfonic acid) (91).** Compound 90 (1.46 g, 1.96 mmol) was dissolved in 50% aqueous EtOH and hydrogenated over 10% Pd/C (250 mg) at 4.21 kg/cm$^2$ for 3 h. The catalyst was removed by filtration, and the filtrate was reduced in vacuo to a yellow foam. This foam was dissolved in TFA (10 mL) and stirred at room temperature for 45 min. The TFA was removed in vacuo, and the residue was repeatedly taken up in EtOAc and reduced in vacuo, and taken up again in EtOAc to provide a white precipitate (91) (956 mg, 70%): TLC $R_f$ (G) 0.33; $^1$H-NMR ($D_2O$) $\delta$ 1.02 (m, 6 H), 1.71–2.00 (m, 4 H), 2.03 (s, 3 H), 2.35–2.46 (m, 3 H), 2.89–3.15 (m, 2 H), 3.36 (s, 1 H), 4.04 (t, 1 H), 4.30–4.35 (m, 2 H), 4.65–4.69 (m, ?), 8.16 (t, 1 H), 8.67 (t, 1 H), 8.81 (d, 1 H).

**Aad(-Cys-DL-α-aminoisobutanesulfonic acid) (92).** Compound 91 (762 mg, 1.5 mmol) was dissolved in $H_2O$ (50 mL), and $Hg(OAc)_2$ (500 mg) was added and stirred for 2 h. $H_2S$ was then bubbled into the thick mass for 2.5 h. The black mixture was filtered through talc, and the filtrate was lyophilized to provide 741 mg of a white powder. HPLC analysis revealed the presence of starting material in addition to a new product. Chromatography of the powder provided starting material and 157 mg (24%) of the desired product (92) after relyophilization: MS (FD) $m/z$ 399 ($M^+$); 318 (M − $SO_3H$); $^1$H-NMR ($D_2O$) $\delta$ 1.01 (m, 6 H), 1.67–2.08 (m, 4 H), 2.31–2.50 (m, 3 H), 2.45 (t, 2 H), 2.88–3.01 (m, 2 H), 3.35 (s, 1 H), 4.05 (t, 2 H), 4.59–4.69 (m, ? H).

**Boc-Alg (93).** Allylglycine (3.0 g, 26 mmol) was slurried in $H_2O$ (40 mL) and TEA (11.0 mL, 79 mmol). To this mixture was

added dropwise Boc-azide (4.43 mL, 3 mmol) in *p*-dioxane (40 mL) over a 30-min period while maintaining the temperature at 5–15 °C. The reaction was then stirred at room temperature (24 h). The mixture was reduced in vacuo and extracted with $Et_2O$ (2 × 50 mL). The aqueous phase was layered with EtOAc (50 mL) and pH was adjusted to 2.0 with N HCl. The layers were separated, the aqueous layer washed with EtOAc (2 × 50 mL). The combined organic layers were washed with saline, dried over $MgSO_4$, filtered, and reduced in vacuo to an oil (**93**) (5.37 g, 95%): $^1$H-NMR (60 MHz, $CDCl_3$) δ 1.50 (s, 9 H), 2.65 (m, 2 H), 2.45–2.80 (m, 2 H), 4.2–4.7 (m, 1 H), 5.1–5.5 (m, 2 H), 5.6–6.0 (m, 1 H), 6.0–6.7 (b s, 1 H).

**Boc-Alg-D-Val-ONb (94).** D-Val-ONb·HCl (2.88 g, 10 mmol) was dissolved in THF (50 mL) and TEA (1.39 mL, 10 mmol). The precipitated triethylamine hydrochloride was removed by filtration and **91** (2.15 g, 10 mmol) and DCC (2.27 g, 11 mmol) were added and stirred (24 h). The precipitated DCU was removed by filtration, and the filtrate was reduced in vacuo to low volume and taken up in EtOAc (100 mL). The organic solution was washed with saturated $NaHCO_3$ solution (2 × 100 mL), 1 N HCL (2 × 100 mL), and saline (2 × 100 mL), dried over $MgSO_4$, filtered, and reduced in vacuo to an oil (5.0 g). The oil was taken up in $CH_2Cl_2$ and deposited on 15 g of silica gel. The solvent was carefully removed by evaporation, and the silica gel was deposited on top of a column of 50 g of hexane-packed silica gel. The column was flash chromatographed[63] using a toluene/EtOAc (0–100%) gradient. Three liters of eluent was collected in 200 15-mL fractions; the desired derivative was identified and pooled to provide 2.55 g (56%) of **94**: UV $\lambda_{max}$ (EtOH) nm (ε) 265 (10818); $^1$H-NMR ($CDCl_3$) δ 0.88, 0.96 (dd, 6 H, *J* = 7), 1.47 (s, 9 H), 2.12–2.28 (m, 1 H), 2.53 (t, 2 H, *J* = 7), 4.2 (m, 1 H), 4.60 (dd, 1 H, *J* = 6, 5), 4.92 (b s, 1 H), 5.15–5.32 (m, 4 H), 5.66–5.84 (m, 1 H), 6.70 (b d, 1 H, *J* = 8), 7.50–8.26 (AB, 4 H).

**Z(NO$_2$)-Aad(-Alg-D-Val-ONb)-ONb (95).** Compound **94** (2.55 g, 5.68 mmol) was dissolved in cold TFA (10 mL) and stirred for 30 min. The TFA was removed in vacuo, the residue was taken up in EtOAc (20 mL), and the solution was saturated with dry HCl gas. The solvent and excess HCl were removed in vacuo, and the residue was triturated with $Et_2O$ and hexane to provide a white solid (2.2 g). This material (849 mg, 2.2 mmol) was dissolved in THF (20 mL) and diisopropylamine (0.348 mL, 2.2 mmol) and stirred 1 h. To this solution was added Z(NO$_2$)-Aad-ONb (951 mg, 2 mmol) and DCC (430 mg, 2.1 mmol). The reaction mixture was stirred three days at room temperature. The solvent was removed in vacuo, the residue was taken up in hot EtOAc (50 mL) and washed with saturated $NaHCO_3$ solution and with 1 N HCl, dried over $MgSO_4$, filtered, and reduced in vacuo to low volume whereupon crystallization occurred. The crystals were removed by filtration and washed with EtOAc and $Et_2O$ to give **95** (1.27 g, 79%): MS (FAB) *m/z* 807 ($MH^+$); $^1$H-NMR ($CDCl_3$) δ 0.88, 0.94 (dd, 6 H, *J* = 9), 1.65–2.00 (m, 4 H), 2.15–2.35 (m, 1 H), 2.45–2.60 (m, 1 H), 3.4–3.55 (m, 1 H), 4.0–4.1 (b d, 1 H, *J* = 9), 4.35–4.45 (m, 1 H) 4.5–4.6 (m, 1 H), 5.1–5.3 (m, 8 H), 5.7–5.85 (m, 2 H), 6.09 (d, 1 H, *J* = 10), 6.62 (d, 1 H, *J* = 9), 7.4–8.3 (AB, 12 H).

**Aad(-Alg-D-Val) (96).** Compound **95** (1.26 g, 1.5 mmol) was dissolved in HOAc (5 mL) and THF (20 mL) and chilled to 0 °C. To this stirred solution was added zinc dust (2.0 g) portionwise over a 30-min period at 0 °C. The mixture was stirred 6 h, gradually warming to room temperature. The zinc dust and salts were removed by filtration, the filtrate was reduced in vacuo to low volume, EtOAc and $H_2O$ were added, and the aqueous portion was extracted twice with fresh EtOAc, saturated with $H_2S$, stirred for 2 h, filtered free of any precipitate, and reduced in vacuo to a clear gum which crystalized. Addition of acetone completed the crystallization; the crystals were filtered and dried in vacuo to give **96** (302 mg, 54%): TLC $R_f$ (E) 0.26; MS (FAB) *m/z* 358 ($MH^+$); $^1$H-NMR ($D_2O$) δ 0.94, 0.96 (dd, 6 H, *J* = 7, $CH_3$), 1.6–1.8 (m, 2 H, $CH_2$), 1.8–2.0 (m, 2 H, $CH_2$), 2.40 (m, 2 H, $CH_2$), 2.44–2.62 (m, 2 H, $CH_2$), 3.78 (m, 1 H, CH), 4.19 (d, 1 H, *J* = 9, CH), 4.48 (m, 1 H, CH), 5.16–5.26 (m, 2 H), 5.72–5.90 (m, 1 H).

**Boc-hCys-D-Val-ONb (97).** Boc-hCys (2.23 g, 5 mmol) and D-Val-ONb·HCl (2.88 g, 10 mmol) were dissolved in THF (50 mL) and TEA (1.39 mL, 10 mmol). To this mixture was added DCC (2.26 g, 11 mmol), and the resulting mixture was stirred at room temperature for 24 h. The precipitate was removed by filtration, and the filtrate was reduced in vacuo to a low volume, taken up in EtOAc, washed twice with saturated $NaHCO_3$ solution, twice with 1 N HCl, and once with saline, dried over $MgSO_4$, filtered, and reduced in vacuo to a solid which was flash chromatographed over 50 g of silica gel using a toluene/EtOAc (0–100%) gradient. Three liters of eluent was collected in 200 15-mL fractions; the desired derivative was identified and pooled to provide 2.65 g (56%) of **97**: UV $\lambda_{max}$ (EtOH) nm (ε) 265 (14005); MS (FAB) *m/z* 937 ($MH^+$); $^1$H-NMR (DMSO-$d_6$) δ 1.89 (dd, 6 H, *J* = 9), 1.37 (s, 9 H), 1.8–2.0 (m, 2 H), 2.0–2.1 (m, 1 H), 2.60–2.72 (m, 2 H), 4.10–4.18 (m, 1 H), 4.26 (t, 1 H, *J* = 9), 5.30 (m, 2 H), 7.04 (d, 1 H, *J* = 10), 7.8–8.28 (AB, 4 H), 8.18 (d, 1 H, *J* = 10).

**Z(NO$_2$)-Aad(-hCys-D-Val-ONb)-ONb (98).** Compound **97** (2.64 g, 3.26 mmol) was dissolved in TFA (10 mL) and stirred at room temperature for 1 h. The TFA was removed in vacuo, and the residue was dissolved in EtOAc and saturated with dry HCl gas resulting in formation of a white precipitate (2.31 g). This solid (889 mg, 1.1 mmol) was dissolved in THF (20 mL) and diisopropylamine (0.35 mL, 1.1 mmol). To this solution was added Z(NO$_2$)-Aad-ONb (950 mg, 2 mmol) and DCC (430 mg, 2.1 mmol). The reaction mixture was stirred at room temperature for 3 days. The solvent was removed in vacuo, and the residue taken up in EtOAc (50 mL) and washed with saturated $NaHCO_3$ solution and with 1 N HCl, dried over $MgSO_4$, filtered, and reduced in volume under a $N_2$ stream resulting in crystallization of **98** (1.07 g, 65%): MS (FAB) *m/z* 1194 (monoacylated deriv + H); $^1$H-NMR (DMSO-$d_6$) δ 1.89 (dd, 6 H, *J* = 9), 1.37 (s, 9 H), 1.80–2.0 (m, 2 H), 2.0–2.1 (m, 1 H), 2.60–2.72 (m, 2 H), 4.10–4.20 (m, 1 H), 4.26 (t, 1 H, *J* = 9), 5.30 (d, 2 H, *J* = 4), 7.4 (d, 1 H, *J* = 10), 7.8–8.28 (AB, 4 H), 8.18 (d, 1 H, *J* = 10).

**Aad(-hCys-D-Val) (99).** Compound **97** (1.06 g, 0.65 mmol) was dissolved in HOAc (5 mL) and THF (20 mL) and chilled to 0 °C. To this stirred solution was added zinc dust (2.0 g) portionwise over a 30-min period at 0 °C. The mixture was stirred 18 h, gradually warming to room temperature. The zinc dust and salts were removed by filtration, the filtrate was reduced in vacuo to low volume, EtOAc and $H_2O$ were added, and the aqueous portion was extracted twice with fresh EtOAc, saturated with $H_2S$, stirred for 2 h, filtered free of any precipitate, and reduced in vacuo to low volume. Addition of acetone resulted in formation of 73.3 mg (15%) of **99** as a white precipitate: MS (FAB) *m/z* 753 ($MH^+$).

**Z(NO$_2$)-Aad[(-2,2-dimethylthiazolidin-4-yl)carbonyl]-D-Val-ONb]-ONb (100).** Z(NO$_2$)-Aad-ONb (950 mg, 2 mmol), HOBt (270 mg, 2 mmol), and DCC (430 mg, 2.1 mmol) were dissolved in THF (25 mL) and stirred at room temperature for 1 h. To this mixture was added 4-carboxy-2,2-dimethylthiazolidine hydrochloride (395 mg, 2 mmol) dissolved in THF (25 mL) and TEA (0.55 mL, 4 mmol). The resulting mixture was stirred at room temperature for 18 h, filtered, reduced in vacuo to low volume, dissolved in EtOAc, washed with 1 N HCl, dried over $MgSO_4$, filtered, and reduced in vacuo to a clear gum. This gum was dissolved in THF (20 mL) and added to D-Val-ONb·HCl (577 mg, 2 mmol) dissolved in THF (25 mL) and TEA (0.28 mL, 2 mmol). DCC (417 mg, 2 mmol) was added to the mixture and stirred for 3 h. The mixture was filtered, and the filtrate was reduced in volume in vacuo, taken up in EtOAc, washed with saturated $NaHCO_3$ solution, 1 N HCl, and saline, dried over $MgSO_4$, filtered, and reduced in vacuo to a foam (1.714 g). This foam was flash chromatographed over 50 g of silica gel using a toluene/EtOAc (0–100%) gradient. Three liters of eluent was collected in 200 15-mL fractions; the desired derivative was identified, pooled, and reduced in vacuo to dryness to provide 1.41 g (83%) of **100** as a foam: MS (FD) *m/z* 853 ($MH^+$); $^1$H-NMR ($CDCl_3$) δ 0.82–0.96 (m, 6 H), 1.56–2.06 (m, 4 H), 1.62 (s, 3 H), 1.83 (s, 3 H), 2.15–2.32 (m, 1 H), 2.36 (m, 2 H), 3.1–3.42 (m, 2 H), 4.04 (m, 1 H), 4.38–4.45 (m, 1 H), 4.52–4.75 (m, 1 H), 5.20–5.36 (m, 6 H), 5.62 (d, 1 H, *J* = 8), 6.83 (d, 1 H, *J* = 8), 7.45–8.32 (AB, 12 H).

**Aad[(-2,2-dimethylthiazolidin-4-yl)-D-Val] (101).** Compound **100** (1.37 g, 1.6 mmol) was dissolved in 50% aqueous THF (50 mL) and hydrogenated over 10% Pd/C (500 mg) at 4.21 kg/cm$^2$ for 2 h at room temperature. The catalyst was removed by filtration, the filtrate was reduced in vacuo to low volume and extracted with EtOAc, and the aqueous portion was reduced in vacuo to a low volume. Addition of acetone precipitated a white solid (443 mg) which was collected by filtration. Prep HPLC gave

101 (239 mg, 37%): MS (FD) $m/z$ 404 ($MH^+$); $^1$H-NMR ($D_2O$) $\delta$ 0.90 and 0.94 (dd, 6 H, $J$ = 8), 1.57–2.05 (m, 4 H), 1.83 (s, 3 H), 1.90 (s, 3 H), 2.07–2.28 (m, 2 H), 2.4–2.55 (m, 1 H), 3.12 (m, 1 H), 3.55–3.67 (m, 1 H), 3.67–3.76 (m, 1 H), 4.4.11 (d, 1 H, $J$ = 6), 4.62–? (m, ?).

**Boc-Val-D-Val-ONb (102).** Boc-Val (2.17 g, 10 mmol), D-Val-ONb·HCl (2.88 g, 10 mmol), TEA (1.39 mL, 10 mmol), and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-$p$-toluenesulfonate (4.89 g, 11 mmol) were dissolved in $CH_2Cl_2$ (50 mL) and stirred 18 h at room temperature. The mixture was reduced in vacuo to low volume, taken up in EtOAc, washed twice each with saturated $NaHCO_3$ solution, 1 N HCl, and saline, dried over $MgSO_4$, filtered, and reduced in vacuo to give **102** as a foam (2.52 g, 55%): $^1$H-NMR ($CDCl_3$) $\delta$ 0.85–1.02 (m, 12 H), 1.42 (s, 9 H), 2.18–2.30 (m, 2 H), 3.90–4.02 (m, 1 H), 4.54–4.64 (m, 1 H), 4.96 (b s, 1 H), 5.23 (s, 2 H), 6.46 (d, 1 H, $J$ = 6), 7.48–8.28 (AB, 4 H).

**Z(NO$_2$)-Aad(-Val-D-Val-ONb)-ONb (103).** Compound **102** (4.64 g, 10.3 mmol) was dissolved in TFA (10 mL) and stirred for 30 min at room temperature. The TFA was removed in vacuo, the residue was dissolved in EtOAc, washed twice each with saturated $NaHCO_3$ solution and saline, dried over $Na_2SO_4$, filtered, and reduced in volume in vacuo to a crystalline mass (1.27 g). The crystalline mass was added to a solution of Z($NO_2$)-Aad-ONb (1.72 g, 3.62 mmol), HOBt (489 mg, 3.62 mmol), and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-$p$-toluenesulfonate (1.77 g, 4 mmol) in $CH_2Cl_2$ (20 mL) and stirred at room temperature for 24 h. The mixture was set up into a thick crystalline mass which was filtered, and the precipitate was washed with $CH_2Cl_2$ and dried to yield 2.68 g (91%) of **103**. This compound was used without further purification: $^1$H-NMR ($CDCl_3$) $\delta$ 0.85–1.00 (m, 12 H), 1.60–1.88 (m, 4 H), 2.00–2.20 (m, 2 H), 3.50–3.60 (m, 1 H), 3.70–3.80 (m, 2 H), 3.90–3.98 (m, 2 H), 4.25–4.50 (m, 2 H), 5.15–5.30 (m, 6 H), 7.1–8.2 (m, 12 H).

**Aad(-Val-D-Val) (104).** Compound **103** (2.42 g, 3 mmol) was dissolved in 50% THF (50 mL) and hydrogenated over 10% Pd/C (500 mg) at 4.21 kg/cm$^2$ for 4 h at room temperature. The catalyst was removed by filtration, and the filtrate was reduced in vacuo to low volume. The residue was dissolved in a minimal amount of MeOH, and acetone was added resulting in a thick precipitate. The precipitate was removed by filtration, washed with acetone, and dried in vacuo to provide **104** (981 mg, 91%): TLC $R_f$ (G) 0.66; $[\alpha]_D^{25}$ 21° ($c$ 1, $H_2O$); IR (KBr) 1718, 1704, 1693, 1640, 1592 cm$^{-1}$; MS (FD) $m/z$ 360 ($MH^+$); $^1$H-NMR ($D_2O$) $\delta$ 0.88–0.94 (m, 12 H), 1.66–1.87 (m, 4 H), 2.05–2.13 (m, 2 H), 2.34–2.40 (m, 2 H), 3.71–3.75 (t, 1 H, $J$ = 5), 4.11–4.18 (m, 2 H).

**Boc-Cys(Acm)-D-Val-ONb (105).** Boc-Cys(Acm) (4.0 g, 13.7 mmol), D-Val-ONb·HCl (3.95 g, 13.7 mmol), TEA (1.91 mL, 13.7 mmol), and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-$p$-toluenesulfonate (6.10 g, 14.4 mmol) were dissolved in $CH_2Cl_2$ (200 mL) and refluxed for 18 h. The mixture was cooled, washed twice each with saturated $NaHCO_3$ solution, 1 N HCl, and $H_2O$, dried over $MgSO_4$, filtered, and reduced in volume in vacuo to provide **105** as a foam (7.19 g, 99%): TLC $R_f$ (H) 0.46; MS (FD) $m/z$ 527 ($MH^+$); $^1$H-NMR ($CDCl_3$) $\delta$ 0.88–1.00 (m, 6 H), 1.46 (s, 9 H), 2.03 (s, 3 H), 2.20–2.32 (m, 1 H), 2.90 (d, 2 H, $J$ = 6), 4.30–4.40 (m, 1 H), 4.46–4.65 (m, 3 H), 5.60 (s, 2 H), 5.66 (d, 1 H), 6.93 (b s, 1 H), 7.17 (d, 1 H, $J$ = 8), 7.80–8.28 (AB, 4 H). Anal. ($C_{23}H_{34}N_4O_8S$) C, H, N.

**Cys(Acm)-D-Val-ONb·HCl (106).** Compound **105** (7.19 g, 13.6 mmol) was dissolved in TFA (20 mL) and stirred at room temperature for 30 minutes. The TFA was removed in vacuo, the residue was dissolved in EtOAc, washed twice each with saturated $NaHCO_3$ solution and saline, dried over $Na_2SO_4$, filtered, and reduced in volume in vacuo to a foam (**106**) (4.85 g, 83%): MS (FD) $m/z$ 427 ($MH^+$).

**Boc-$o$-(aminomethyl)phenylacetyl-Cys(Acm)-D-Val-ONb (107).** Compound **106** (852 mg, 2 mmol), Boc-$o$-(aminomethyl)phenylacetic acid (530 mg, 2 mmol) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-$p$-toluenesulfonate (892 mg, 2.1 mmol) were refluxed in THF (100 mL) for 18 h. The mixture was cooled, washed twice each with $H_2O$, saturated $NaHCO_3$ solution, and 1 N HCl, dried over $MgSO_4$, filtered, and reduced in volume in vacuo to a white foam (**107**) (1.19 g, 88%): $^1$H-NMR ($CDCl_3$) $\delta$ 0.82–1.00 (m, 6 H), 1.48 (s, 9 H), 2.08 (s, 3 H), 2.16–2.26 (s, 3 H), 2.70–3.02 (m, 2 H), 3.70 (s, 2 H), 4.10–4.58 (m, 5 H), 4.84 (q, 1 H), 5.28 (s, 2 H), 6.98 (b s, 1 H), 7.2 (b s, 1 H), 7.2–7.5 (m, 4 H), 7.50–8.25 (AB, 4 H).

**$o$-(Aminomethyl)phenylacetyl-Cys-D-Val (108).** Compound **107** (1.19 g, 1.7 mmol) was dissolved in 40% aqueous THF (50 mL). $Hg(OAc)_2$ (565 mg, 1.7 mmol) was added, and the mixture was stirred for 1 h. The mixture was then saturated with $H_2S$ for 30 min and filtered through talc. The filtrate was washed twice each with $H_2O$ and saline, and reduced in volume in vacuo to a foam (1.03 g). The foam was dissolved in THF (100 mL) and hydrogenated over 10% Pd/C (500 mg) at 4.21 kg/cm$^2$ for 18 h at room temperature. The catalyst was removed by filtration, and the filtrate was reduced in volume in vacuo to a foam (946 mg). The foam was dissolved in TFA (20 mL) and stirred 30 min at room temperature. The TFA was removed in vacuo, and the residue was taken up in EtOAc and $H_2O$; the aqueous phase was separated and lyophilized to provide **108** as a yellow powder (527 mg, 81%): $^1$H-NMR ($D_2O$) $\delta$ 0.75–0.92 (m, 3 H), 2.05–2.2 (m, 1 H), 2.76–3.20 (m, 2 H), 3.94 (s, 2 H), 4.27 (s, 2 H), 7.18–7.56 (m, 4 H).

**Boc-His-D-Val-ONb (109).** Boc-His (1.27 g, 5 mmol), D-Val-ONb·HCl (1.44 g, 5 mmol), TEA (0.69 mL, 5 mmol), and DCC (1.13 g, 5.5 mmol) were dissolved in DMF (20 mL) and warmed gently to achieve solution. The mixture was stirred at room temperature for 24 h. The mixture was chilled and filtered, and the resulting filtrate was reduced in vacuo to low volume, diluted with EtOAc (100 mL), and washed repeatedly with $H_2O$ and saturated $NaHCO_3$ solution. The organic layer was dried over $MgSO_4$, filtered, and reduced in vacuo to low volume whereupon crystallization occurred. Filtration afforded the product **109** as white crystals (1.56 g, 64%): MS (FD) $m/z$ 490 ($MH^+$); $^1$H-NMR ($CDCl_3$) $\delta$ 0.85 (d, 3 H), 0.93 (d, 3 H), 1.42 (s, 9 H), 2.2 (m, 1 H), 2.9–3.3 (m, 2 H), 4.2 (m, 1 H), 4.52 (m, 1 H), 5.23 (s, 2 H), 6.87 (s, 1 H), 7.55 (s, 1 H), 7.25–8.23 (AB q, 4 H); IR ($CHCl_3$) 1742, 1707, 1676, 1525, 1496 cm$^{-1}$.

**His-D-Val-ONb·HCl (110).** Compound **109** (1.19 g, 2.43 mmol) was dissolved in TFA (10 mL) and stirred at room temperature for 30 min. The TFA was removed in vacuo, the residue was dissolved in EtOAc, reduced in volume in vacuo, and taken up again in EtOAc, and HCl gas was bubbled into the solution whereupon crystallization occurred. Filtration afforded **110** (1.15 g, 99%): MS (FD) $m/z$ 390 ($MH^+$).

**Z(NO$_2$)-Aad(-His-D-Val-ONb)-ONb (111).** Z($NO_2$)-Aad-ONb (475 mg, 1 mmol) and EEDQ (255 mg, 1 mmol) were dissolved in $CH_2Cl_2$ (10 mL) and stirred at room temperature for 1 h. To this mixture was added **110** in $CH_2Cl_2$ (10 mL) containing $Et_3N$ (0.28 mL, 2 mmol). The resulting mixture was stirred at room temperature for 24 h. The solvent was removed in vacuo, and the residue was dissolved in EtOAc and washed with $H_2O$, saturated $NaHCO_3$, and cold dilute HOAc. The organic layer was dried over $MgSO_4$, filtered, and reduced in volume in vacuo, and the residue was redissolved in $CH_2Cl_2$ whereupon crystallization occurred. The crystalline mass was filtered (655 mg, 78%) and identified as the desired product **111**: MS (FD) $m/z$ 847 ($MH^+$).

**Aad(-His-D-Val) (112).** Compound **111** (600 mg, 0.71 mmol) was hydrogenated over 10% Pd/C (300 mg) in 50% aqueous THF (40 mL) at 4.21 kg/cm$^2$ for 2 h at room temperature. The catalyst was removed, and the filtrate was reduced in vacuo to an oil which was precipitated by the addition of $Et_2O$. The precipitate was filtered and purified by HPLC to provide 79 mg of **112**: MS (FD) $m/z$ 398 ($MH^+$).

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