Nocardicin A: Stereochemical and Biomimetic Studies of Monocyclic β-Lactam Formation

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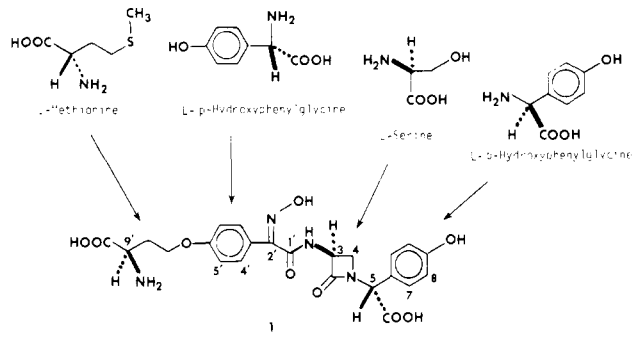
Abstract: Using $^3H/^{14}C$ doubly labeled specimens of L- and D-serine bearing tritium label at C-2, biosynthetic studies of nocardicin A (**1**) produced by whole cells of *Nocardia uniformis* subsp. *tsuyamanensis* (ATCC 21806) are reported that indicate (a) that D-serine is very much more poorly incorporated than L-serine and (b) that utilization of the latter apparently takes place in β-lactam formation without transient generation of an α,β-dehydroalanyl intermediate. Incorporations of diastereotopically labeled [3-^{2}H]serines establish on ^{2}H NMR spectroscopic analysis of the enriched nocardicins that β-lactam ring closure takes place substantially, if not completely, with stereochemical inversion. A chemical model for such a cyclization process is presented in which optically pure serine-containing peptide **22** is converted in the presence of triethyl phosphite and diethyl azodicarboxylate to **23** as the sole β-lactam product. This model formally affords an efficient, asymmetric synthesis of (−)-3-aminonocardicinic acid, the structural element common to all the known nocardicins. Extensive analogous cyclizations in the presence of varying amounts of diethyl azodicarboxylate and triethyl phosphite or triphenylphosphine are described that yield mechanistic insights into the ring-closure reaction.

The primary biosynthetic precursors of the homoseryl, aryl, and β-lactam segments of nocardicin A (**1**) have been defined in extensive incorporation studies to be the L isomers of methionine, (*p*-hydroxyphenyl)glycine, and serine, respectively.[1] The obvious and important stereochemical similarities among **1**, penicillin N



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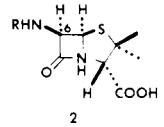
(**2**) [R = δ-(D-α-aminoadipyl)], and cephalosporin C (**3**), therefore, are further manifest from a biosynthetic point of view wherein each of these structural types is entirely amino acid derived.[1,2] The β-lactam families exemplified by the oxypenam clavulanic acid (**4**) and the carbapenem thienamycin (**5**), on the other hand, appear to be of mixed biogenetic origin.[3]



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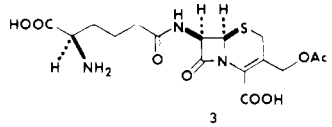

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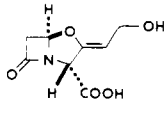

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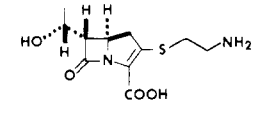

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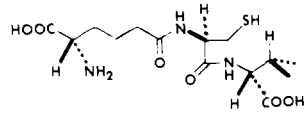
While several strong parallels may be drawn between nocardicin and penicillin with regard to the stereochemical details of amino acid utilization,[1,2] a fundamental difference is evident in that the assembly of these precursors takes place at the same oxidation level as the ultimate β-lactam ring itself for nocardicin A (**1**) alone. Moreover, this oxidation state is maintained during the overall transformation of L-serine to nocardicin as revealed in double-label experiments where tritium was situated at the β-carbon (Table I).[1] In contrast, the known direct cyclization[4] of the Arnstein tripeptide (**6**) to isopenicillin N (**2**) [R = δ-(L-α-aminoadipyl)],

Table I

amino acid[a]	^{14}C spec incorp	$^3H/^{14}C$ amino acid	$^3H/^{14}C$ 1	$^3H/^{14}C$ (% ^3H retained)
L-[3-^{3}H,U-^{14}C]-Ser	8.2	4.87	4.19	(86)
L-[3-^{3}H,3-^{14}C]-Ser	5.8	3.74	3.67	(98)
L-[2-^{3}H,1-^{14}C]-Ser	5.2	4.96	0.93	(19)
D-[2-^{3}H,1-^{14}C]-Ser	0.24	4.94		[b]

[a] Specific activities and $^3H/^{14}C$ ratios were determined for the respective *N*-tosylserines. The last two experiments were conducted in the presence of 0.4 mM L-methionine whereas the first two were not. [b] The level of activity was too low to obtain a meaningful $^3H/^{14}C$ ratio.



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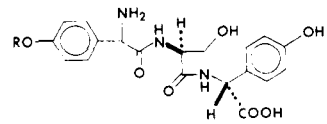

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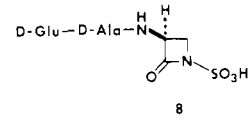

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the immediate precursor of penicillin N (**2**) [R = δ-(D-α-aminoadipyl)][5] and subsequently cephalosporin C (**3**),[6] requires

† Research Fellow of the Alfred P. Sloan Foundation (1982–1984).

(1) Townsend, C. A.; Brown, A. M. *J. Am. Chem. Soc.*, preceding paper in this issue. Townsend, C. A.; Brown, A. M. *Ibid.* **1981**, *103*, 2873–2874. See also: Hosoda, J.; Tani, N.; Konomi, T.; Ohsawa, S.; Aoki, H.; Imanaka, H. *Agric. Biol. Chem.* **1977**, *41*, 2007–2012.

(2) For reviews of penicillin and cephalosporin biosynthesis, see: O'Sullivan, J.; Abraham, E. P. In "Antibiotics"; Cocoran, J. W. Ed.; Springer-Verlag: West Berlin, 1981, Vol. 4; pp 101–122. Aberhart, D. J. *Tetrahedron* **1977**, *33*, 1545–1559.

(3) Clavulanic acid: Elson, S. W.; Oliver, R. S. *J. Antibiot.* **1978**, *31*, 586–592. Sterling, I.; Elson, S. W. *Ibid.* **1979**, *32*, 1125–1129. Elson, S. W. *Spec. Publ.-Chem. Soc.* **1980**, *No. 38*, 142–150. Townsend, C. A.; Ho, M.-F., unpublished results. Thienamycin: Albers-Schönberg, G.; Arison, B. H.; Kaczka, E.; Kahan, F. M.; Kahan, J. S.; Lago, B.; Maise, W. H.; Rhodes, R. E.; Smith, J. L. "Abstracts of Papers", 16th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, 1976; American Society for Microbiology: Washington, D.C., 1976; No. 229.

(4) O'Sullivan, J.; Bleaney, R. C.; Huddleston, J. A.; Abraham, E. P. *Biochem. J.* **1979**, *184*, 421–426. Konomi, T.; Herchen, S.; Baldwin, J. E.; Yoshida, M.; Hunt, N. A.; Demain, A. L. *Ibid.* **1979**, *184*, 427–430.

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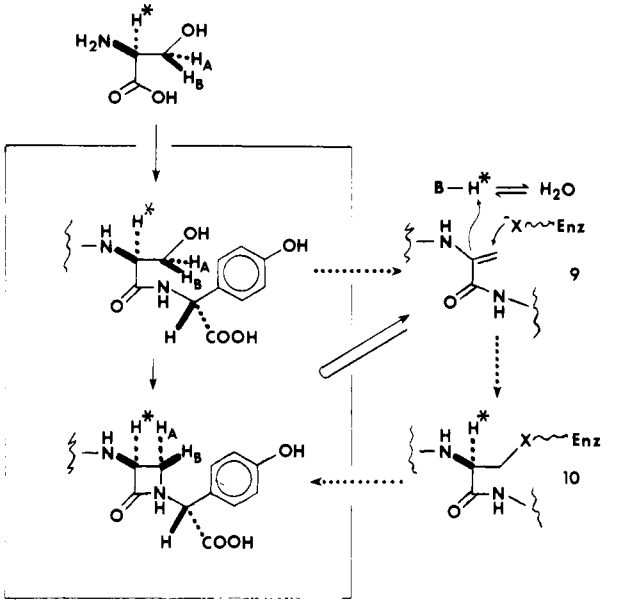
a net change of two oxidation states. Presuming an analogous peptide precursor of **1**, e.g., **7** (R = H or homoseryl), the most direct mechanistic rationale for monocyclic β-lactam formation is nucleophilic displacement by amide nitrogen of presumably activated seryl hydroxyl. In this connection, very recently reported results for sulfazecin (**8**) indicate that its four-membered ring is also derived from L-serine[7,8] and, further, that like **1**, no change in oxidation state occurs at C-3 during the course of β-lactam biosynthesis.[8] The proposed role of amide nitrogen as the sole nucleophile in β-lactam formation carries with it stereochemical and synthetic implications that are subject to experimental test. Described in this paper are studies that specifically address the mechanistic questions of four-membered ring formation by tracing the stereochemical fate of diastereotopic [3-^{2}H]-labeled serines into **1** by ^{2}H NMR spectroscopy. In addition, phosphorylation, while not the only potential means of serine hydroxyl activation in vivo, is examined in an asymmetric, biogenetically modeled synthesis of (−)-3-aminonocardicinic acid (**25**) from a suitably protected serine-containing peptide. It will be seen that these various lines of investigation converge in support of the simple mechanistic model advanced above, which, on the basis of the evidence accumulated to date in this and other laboratories,[7,8] suggests a common biosynthetic solution to monocyclic β-lactam formation among the nocardicins, sulfazecin and related monobactams.

In both nocardicin A (**1**) and penicillin N (**2**) [R = δ-(D-α-aminoadipyl)], the only asymmetric centers that remain of unchanged configuration from the progenitor L-amino acids are C-3 in **1** and C-6 in **2**; that is, those amino acids that make up the carbon skeletons of the respective β-lactam rings. We undertook to examine the fate of the serine α-hydrogen in the biosynthesis of **1**. DL-[2-^{3}H]Serine was prepared by the method of Miles and McPhie[9] in tritiated water in the presence of pyridoxal and aluminum sulfate. Reaction was allowed to proceed for 2.5 days at room temperature. Control experiments in deuterium oxide indicated essentially complete exchange at the α position within 24 h. The tritium-labeled serine was isolated, crystallized, converted to its *N*-acetyl derivative, and combined with *N*-acetyl-DL-[1-^{14}C]serine. Resolution with hog kidney acylase[10] afforded L- and D-[2-^{3}H,1-^{14}C]serines of 96% and 85% optical purity, respectively. The doubly labeled amino acids were administered to growing cultures of *Nocardia uniformis* subsp. *tsuyamanensis* (ATCC 21806) as before[1] in the presence of 0.4 mM L-methionine to maximize production of **1**.[1] As shown in Table I, the incorporation of carbon label was at least 20 times more efficient for the L isomer; the low but positive incorporation of the D isomer owing almost certainly to the L-serine present. Extensive but not complete loss of tritium (81%) from the L antipode was observed, most probably resulting from reversible transamination to hydroxypyruvate prior to incorporation into the antibiotic. For comparison, an analogous experiment conducted with a high-producing strain of *Penicillium chrysogenum* showed remarkably only a 16% loss of tritium from L-[2-^{3}H,U-^{14}C]cysteine on incorporation into penicillin G (**2**, R = $PhCH_2CO$).[11] The relatively greater loss of α label from serine in the case of nocardicin A could be interpreted alternatively in terms of a β elimination to a dehydroalanyl intermediate **9** and either (a) direct cyclization to β-lactam



in violation of Baldwin's rules (4-Endo-Trig)[12] or (b) through the transitory β addition of an enzyme nucleophile (X) to **10** followed by its displacement to form the β-lactam ring. By either route a or b, tritium originally residing at the seryl α position would have to be partially recovered in **1** to be in accord with the experimental observations. While final resolution of these mechanistic possibilities will likely have to await experiments at the peptide level, we view retention of about one-fifth of the α label in an amino acid in close proximity to the intermediates of primary metabolism as provisionally sufficient to rule out a dehydroalanyl intermediate enroute to nocardicin; a contention in keeping with the widely held noninvolvement of an α,β-dehydrocysteinyl intermediate in penicillin biosynthesis.[2,11]

Before proceeding to the synthesis and incorporation of the diastereomeric [3-^{2}H]serines, the ^{1}H NMR assignments of nocardicin were determined on the basis of both comparisons with model compounds and extensive homonuclear decoupling experiments at 300 MHz. The resulting assignments are completely in accord with those made by workers at Fujisawa[13] with the exception that the published chemical shifts for H-4A and H-9′ should be reversed. The revised chemical shift assignments are shown in Table II. Of central importance to the envisioned

Table II. ^{1}H NMR (300 MHz) Assignments of Nocardicin A (**1**) in D_2O, pD 8, 25 °C Relative to DSS

assign	chem shift, δ	mult	coupling const, Hz
H-3	4.99	d × d	2.2, 5.1
H-4A	3.83	~t (d × d)	~5.5
H-4B	3.18	d × d	2.2, 5.9
H-5	5.32	s	
H_2-7	7.24	d	8.8
H_2-8	6.90	d	8.8
H_2-4′	7.48	d	8.8
H_2-5′	7.01	d	8.8
H_2-7′	4.22	br t	
H_2-8′	2.26–2.46	sym m	
H-9′	3.94	d × d	4.8, 7.4

(5) Jayatilake, G. S.; Huddleston, J. A.; Abraham, E. P. *Biochem. J.* **1981**, *194*, 645–647. Baldwin, J. E.; Keeping, J. W.; Singh, P. D.; Vallejo, C. A. *Ibid.* **1981**, *194*, 649–651.

(6) Baldwin, J. E.; Singh, P. D.; Yoshida, M.; Sawada, Y.; Demain, A. L. *Biochem. J.* **1980**, *186*, 889–895.

(7) Imada, A. Paper presented at the 21st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, Nov 1981; American Society for Microbiology: Washington, D.C. 1981. See also: Imada, A.; Kitano, K.; Kinotaka, K.; Muroi, M.; Asai, M. *Nature (London)* **1981**, *289*, 590–591.

(8) O'Sullivan, J.; Aklonis, C. A.; Gillum, A. M.; Souser, M. L.; Sykes, R. B. "Abstracts of Papers", 21st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, Nov. 1981; American Society for Microbiology: Washington, D.C., 1981; No. 877. See also: Sykes, R. B.; Cimarusti, C. M.; Bonner, D. P.; Bush, K.; Floyd, D. M.; Georgopapadakou, N. H.; Koster, W. H.; Liu, W. C.; Parker, W. L.; Principe, P. A.; Rathnum, M. L.; Slusarchyk, W. A.; Trejo, W. H.; Wells, J. S. *Nature (London)* **1981**, *291*, 489–491.

(9) Miles, E. W.; McPhie, P. *J. Biol. Chem.* **1974**, *249*, 2852–2857.

(10) Greenstein, J. P.; Winitz, M. "Chemistry of the Amino Acids"; Wiley: New York, 1961; pp 2229–2234. Greenstein, J. P. *Methods Enzymol.* **1957**, *3*, 554–570.

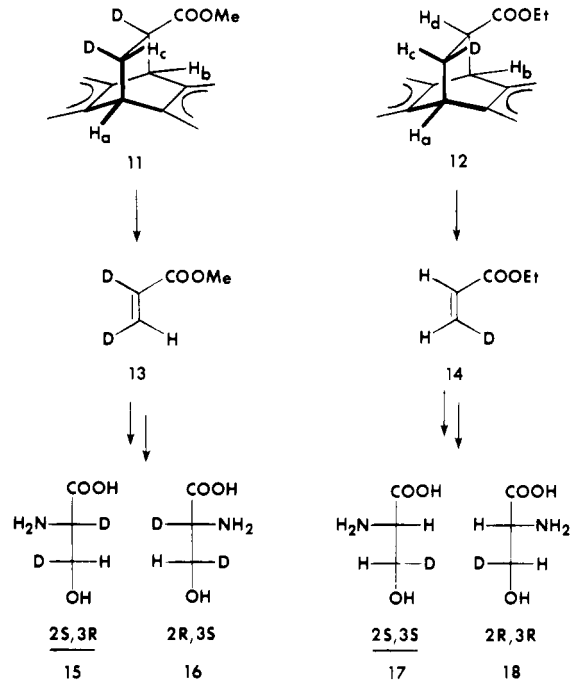
(11) Bycroft, B. W.; Wels, C. M.; Corbett, K.; Lowe, D. A. *J. Chem. Soc., Chem. Commun.* **1975**, 123–125. See also: Arnstein, H. R. V.; Crawhall, J. C. *Biochem. J.* **1957**, *67*, 180–187.

(12) Baldwin, J. E. *J. Chem. Soc., Chem. Commun.* **1976**, 734–736.

(13) Hashimoto, M.; Komori, T.; Kamiya, T. *J. Antibiot.* **1976**, *29*, 890–901.

stereochemical experiment is the fact that the three hydrogens bound to the β-lactam of **1** form an AMX spin system, H-3, H-4A, and H-4B having chemical shifts in deuterium oxide of δ 4.99, 3.83, and 3.18, respectively. These fortuitously well-spaced resonances were ideally suited to the planned application of ^2H NMR spectroscopy.[14]

Methyl (*E*)-[2,3-$^2H_2$]acrylate (**13**) and ethyl (*Z*)-[3-^2H]acrylate (**14**), the key intermediates of the chiral serine synthesis, were prepared in gram quantities using the method of Hill and Newkome.[15] Catalytic deuteration over thoroughly preequilibrated catalyst of the α,β-unsaturated ester arising from Diels–Alder reaction of methyl propiolate and anthracene gave the dideuterated adduct **11**. Pyrolysis at 290–300 °C of the latter gave **13**. Treatment of ethyl propiolate with deuterium oxide and a trace of sodium deuteroxide at room temperature for 96 h gave the corresponding [3-^2H]propiolate ester with greater than 90% deuterium enrichment as judged by integration of its ^1H NMR spectrum. Formation of the analogous anthracene adduct and catalytic hydrogenation afforded **12**, which on pyrolysis gave the corresponding (*Z*)-[3-^2H]acrylate ester (**14**). The deuterated esters were separately converted largely[16] to racemic pairs of the diastereomeric deuterium-labeled serines **15**/**16** and **17**/**18** by the Walsh–Cheung procedure[17] as improved by Benkovic and Slieker.[18] Averaged mass spectral scans of intermediates **11** and **12** revealed deuterium contents of 99 ± 1% d_2 and 92 ± 2% d_1, respectively. These data taken in tandem with 300-MHz spectra of their derived racemic serines at pD 12.4[19] indicated diastereomeric purities at the respective deuterium-labeled β-carbons of 86 ± 3% for both **15**/**16** and **17**/**18**.[16]



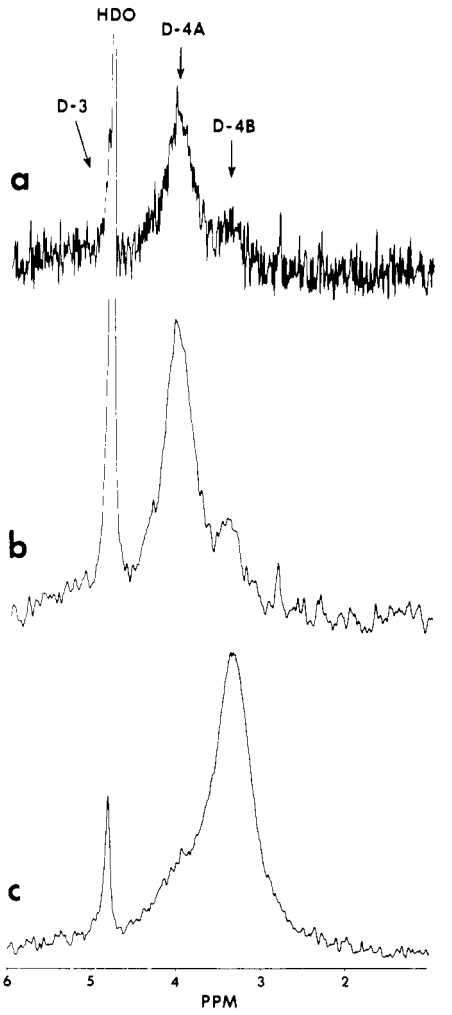


Figure 1. ^2H NMR spectra of nocardicin A (**1**) obtained from incorporations of diastereotopically ^2H-labeled serines **15**/**16** and **17**/**18** acquired under the following conditions: Bruker WM-300, 46.1 MHz; spectral width 2000 Hz, 4K points, acquisition time 1.024 s; 90° pulse. (a) 55 mg in 2.5 mL of deuterium-depleted water (3.3 × 10^{-3} times natural abundance, Aldrich); 52 250 transients, zero filling the FID by successive transfers into 8 and 16K of zeros prior to Fourier transformation. (b) As in (a), 51 715 transients, sensitivity enhancement achieved by treatment of FID with 1.5-Hz line broadening. (c) 150 mg in 2.5 mL of deuterium-depleted water (3.3 × 10^{-3} times natural abundance, Aldrich); 23 505 transients, FID treated as in (a) and (b) but with 1.0-Hz line broadening.

The substantial processing of glycine to serine that had been observed in earlier precursor screening experiments[1] coupled with the high degree of retention of tritium label at the serine β-carbon on incorporation into nocardicin A (**1**) (Table I) suggested that under the fermentation conditions used the flux through serine hydroxymethyltransferase was principally toward serine synthesis rather than its degradation. Therefore, with respect to the planned stereochemical experiment, any conceivable dissipation of serine diastereomeric purity by reversible traverse of the C-3 carbon to the C-1-pool was unlikely to significantly exercise its deleterious effects. Second, the markedly lower specific incorporation of D-serine (Table I) made it possible to administer the racemates **15**/**16** and **17**/**18** without prior resolution, a technical simplification.

Therefore, separate administrations of the racemic ^2H labeled serines containing the 2*S*,3*R* and 2*S*,3*S* diastereomers **15** and **17** were carried out with growing cultures of *N. uniformis*. The nocardicin A produced was isolated as previously described,[1] and the sites of deuterium enrichment were determined by ^2H NMR spectroscopy at 46.1 MHz in deuterium-depleted water (3.3 × 10^{-3} times natural abundance, Aldrich) at pH 7.6. A spectrum

(14) It has been known for some time that deuterium and hydrogen chemical shifts correlate very closely: Diehl, P.; Leipert, T. *Helv. Chim. Acta* **1964**, *47*, 545–557. Diehl, P. In "Nuclear Magnetic Resonance Spectroscopy of Nuclei Other Than Protons"; Axenrod, T., Webb, G. A., Eds.; Wiley: New York, 1974; pp 275–285.

(15) Hill, R. K.; Newkome, G. R. *J. Org. Chem.* **1969**, *34*, 740–741.

(16) The initial addition of the elements of HOBr to the acrylates takes place substantially in a trans fashion. The degree to which this mode of addition is observed is one of two major determinants of the diastereomeric purity at C-3 in the final chiral serines. The other was revealed in the 300-MHz ^1H NMR spectrum of the pair **15**/**16** where a clean doublet integrating to less than 0.1 hydrogen was observed for the α-position. This observation would indicate an 8–10% epimerization at C-2 through the steps subsequent to the HOBr addition since mass spectral analysis of intermediate **11** was found to bear two deuteria to the extent of 99 ± 1%.

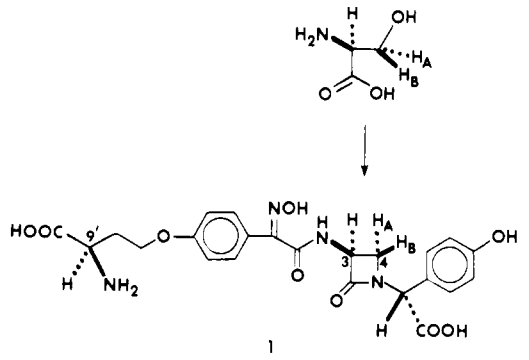
(17) Cheung, Y.-F.; Walsh, C. *J. Am. Chem. Soc.* **1976**, *98*, 3397–3398.

(18) Slieker, L.; Benkovic, S. J. *J. Labelled Compd. Radiopharm.* **1982**, *19*, 647–657.

(19) Ogura, H.; Arata, Y.; Fujiwara, S. *J. Mol. Spectrosc.* **1967**, *23*, 76–85.

of (2S,3S)-[3-^{2}H]serine-derived **1** recorded at close to saturation (0.12 M) at 25 °C gave line widths at half-height of nearly 30 Hz. With the hope of reducing intermolecular association and hence reducing rotational correlation times,[20] samples were heated to 45 °C during the acquisition of spectral data. The spectra so obtained under conditions of broad-band proton decoupling are displayed in Figure 1 and show widths at half-height of about 15 Hz.

A sample of nocardicin A derived from fermentation in the presence of (2S,3R)/(2R,3S)-serines **15**/**16** gave spectrum a. Despite a presumably lower isotope effect for deuterium loss by transamination than for tritium (cf. Table I), a degree of deuterium enrichment at C-3 was detectable at 5.0 ppm as a weak signal on the downfield side of the HDO resonance. Application of 1.5-Hz line broadening to the FID that gave rise to spectrum a generated spectrum b. The D-3 resonance was now merged with the comparatively intense HDO signal, but the distribution of deuterium label at C-4 was clearly discernible, the A position (4α) bearing approximately 85% of the heavy isotope. The wholly



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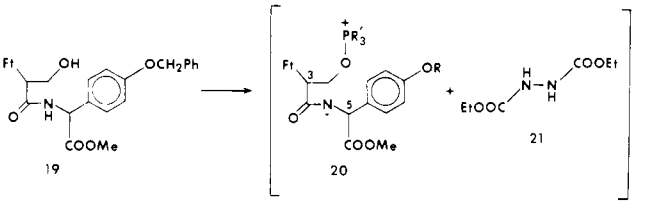
complementary result was obtained from a second specimen of nocardicin A derived from incorporation of the diastereomeric (2S,3S)/(2R,3R)-serines **17**/**18**, whose ^{2}H NMR analysis is presented as spectrum c (Figure 1). In summary, therefore, L-serine bearing label at the 3R locus gave rise to enhanced deuterium content at position 4A with a selectivity reflecting the diastereomeric purity of the substrate administered, while 3S-label specifically enriched position 4B. Within narrow confines of accuracy, this method defines the stereochemical course of monocyclic β-lactam formation in vivo to be very largely, if not exclusively, inversion.[21]

The cumulative force of the biosynthetic findings, namely, the retention of [3-^{3}H]serine label on incorporation into nocardicin A, the apparent noninvolvement of an α,β-dehydroalanyl intermediate in monocyclic β-lactam formation, and the observation of clean stereochemical inversion at the critical four-membered ring, strongly supports the mechanistic rationale that amide nitrogen of a hypothetical peptide intermediate, e.g., **7**, functions as the sole nucleophile involved in β-lactam formation displacing presumably activated seryl hydroxyl in a classic S_N2 sense.

In considering the possible modes of serine activation in vivo, phosphorylation (or the corresponding pyrophosphate), while not the only potential means,[22] is mechanistically attractive and precedented inter alia in L-3-phosphoserine, the immediate precursor of L-serine from the intermediates of glycolysis. Important chemical analogies for such a process of β-lactam formation can be readily identified in the work of Kishi,[23] Baldwin,[24] Koppel,[25] and Wasserman,[26] where intramolecular reactions have been carried out of primary and secondary halides with amide anions generated typically by sodium hydride in dimethylformamide–methylene chloride. However, to more closely mimic the hypothetical in vivo cyclization to the four-membered ring, we sought a milder reaction system to generate the desired O-phosphorylated intermediate and deprotonate the amide in situ, that is, to form a reactive species as **20** (Ft = phthalimido). The Mitsunobu



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reaction[27] held promise to fulfill these two requirements. This choice was motivated by the successful application of this reaction for alcohol inversion in a then ongoing synthesis of chiral-methyl valine,[28] an interesting report by DiNinno[29] of a rearrangement in the penam series to form a new four-membered, sulfur-containing ring, and, more recently, by the germane efforts of Miller,[30] where serine and threonine O-benzylhydroxamates were converted to the corresponding N-oxidized β-lactams. Miller pointed out quite reasonably[30] that on the basis of the apparent pK_a of the Mitsunobu reagent itself,[31] the acidic component of the reactant(s) should have a p$K \leq 13$. Therefore rather than the amide itself, the O-benzylhydroxamate was chosen to lower the pK_a of the N–H to 9–10[30] and, hence, to enable the desired reaction to proceed in an amino acid based reactant. However, while hydroxamates have demonstrated biochemical relevance in other contexts,[32] presuming the obligate intermediacy of peptide precursors in penicillin[2,4] and nocardicin biosynthesis, their direct involvement in β-lactam formation in vivo is so far without experimental support. In the event, we have found that the enhancement of amide hydrogen acidity afforded by oxidation to the corresponding hydroxamate is unnecessary for the sake of cyclization as treatment of serine-containing peptides as **19** under Mitsunobu conditions proceeds rapidly at room temperature to yield β-lactam products.[33]

Selection of protected-dipeptide **19** was made on a number of counts. First, it would serve as a close biogenetic model of the proposed in vivo cyclization. Second, in optically active form, **19** could function in a highly efficient, asymmetric synthesis of (−)-3-aminonocardicinic acid (**25**), the common structural element of the known nocardicins,[13,34] by generating in a more direct fashion a key relay compound in a synthesis of **25** and nocardicin A (**1**) published by workers at Fujisawa.[35] Therefore, the racemic

(20) For a demonstrated case of aggregation in a species of similar molecular weight investigated by ^{2}H NMR, see: Egan, W. *J. Am. Chem. Soc.* **1976**, *98*, 4091–4093.

(21) Preliminary communication: Townsend, C. A.; Brown, A. M. *J. Am. Chem. Soc.* **1982**, *104*, 1748–1750.

(22) In view of the structures of the various isolated monobactams,[8] sulfation is an interesting possibility as well for hydroxyl activation and inter alia protonation or acylation cannot be ignored.

(23) Nakatsuka, S.; Tanino, H.; Kishi, Y. *J. Am. Chem. Soc.* **1975**, *97*, 5008–5010; 5010–5012.

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(27) Mitsunobu, O.; Wada, M. Sano, T. *J. Am. Chem. Soc.* **1972**, *94*, 679–680. Loibner, H.; Zbiral, E. *Helv. Chim. Acta* **1976**, *59*, 2100–2113. Mitsunobu, O. *Synthesis* **1981**, 1–28.

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(29) DiNinno, F. *J. Am. Chem. Soc.* **1978**, *100*, 3251–3252.

(30) Mattingly, P. G.; Kerwin, J. F., Jr.; Miller, M. J. *J. Am. Chem. Soc.* **1979**, *101*, 3983–3985. Miller, M. J.; Mattingly, P. G.; Morrison, M. A.; Kerwin, J. F., Jr. *Ibid.* **1980**, *102*, 7026–7032, correction **1981**, *103*, 2909. Mattingly, P. G.; Miller, M. J. *J. Org. Chem.* **1981**, *46*, 1557–1564. See also: Bose, A. K.; Sahu, D. P.; Manhas, M. S. *Ibid.* **1981**, *46*, 1229–1230.

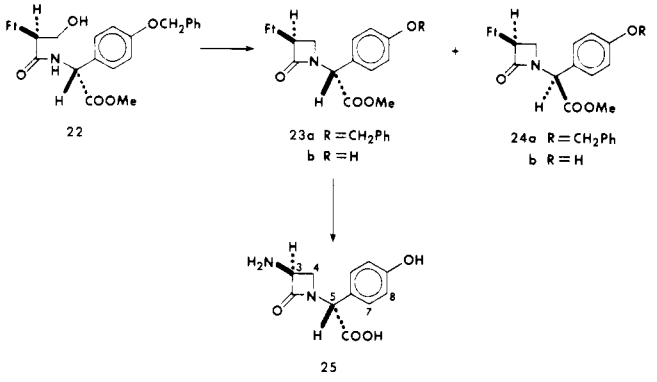
(31) Wada, M.; Mitsunobu, O. *Tetrahedron Lett.* **1972**, 1279–1282.

(32) Weisburger, J. H.; Weisburger, E. K. *Pharmacol. Rev.* **1973**, *25*, 1–66. Maehr, H. *Pure Appl. Chem.* **1971**, *28*, 603–636 and references cited.

(33) Preliminary communication: Townsend, C. A.; Nguyen, L. T. *J. Am. Chem. Soc.* **1981**, *103*, 4582–4583.

(34) Hashimoto, M.; Komori, T.; Kamiya, T. *J. Am. Chem. Soc.* **1976**, *98*, 3023–3025. Hosoda, J.; Konomi, T.; Tani, N.; Aoki, H.; Imanaka, H. *Agric. Biol. Chem.* **1977**, *41*, 2013–2020. Kamiya, T. In "Bioactive Peptides Produced by Microorganisms"; Umezawa, H., Takita, T., Shiba, T., Eds.; Halsted: New York, 1978; pp 87–104.

(35) Kamiya, T.; Hashimoto, M.; Nakaguchi, O.; Oku, T. *Tetrahedron* **1979**, *35*, 323–328. See also ref 53 and 54.



22 23a R = CH_2Ph, b R = H 24a R = CH_2Ph, b R = H 25

dipeptide **19** was prepared by reaction of *N*-phthaloyl-DL-serine[36] and DL-(*p*-benzyloxyphenyl)glycine methyl ester[35] under standard conditions with dicicyclohexylcarbodiimide in the presence of 2 equiv of 1-hydroxybenzotriazole hydrate.[37] After crystallization from ethyl acetate–hexanes, **19** was obtained (83%, mp 126.5–135 °C) as an approximately 10:1 mixture of diastereomers (favoring **22** and its enantiomer, vide infra) as judged by ^{1}H NMR spectroscopy at 300 MHz and analytical HPLC. Treatment of **19** with 2.5 equiv each of triphenylphosphine and diethyl azodicarboxylate in dry tetrahydrofuran at room temperature gave within 15 min, as monitored by TLC, complete disappearance of starting material and appearance of a single, less polar spot. Water was added to destroy the excess Mitsunobu reagent, and a mixture of **23a** (and enantiomer) and **24a** (and enantiomer) was isolated by chromatography on silica gel. Coeluting diethyl hydrazinedicarboxylate (**21**) coproduct was removed by fractional crystallization from chloroform–hexanes, and debenzylation of the diastereomeric mixture of β-lactams gave an approximately 2:1 mixture of racemic **23b** and **24b**, whose ^{1}H NMR spectra were identical with published data.[35] The change in diastereomeric composition from roughly 10:1 in dipeptide **19** to approximately 2:1 for the products **23** and **24** indicated that epimerization had taken place at least one of the asymmetric centers.

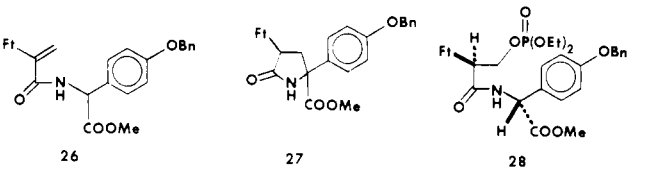
The success of this model reaction, apart from providing the sought for in vitro analogy to the proposed biosynthetic pathway, opened the way to a short, asymmetric synthesis of (−)-3-aminonocardicinic acid (**25**).[25,26,30,35,38] Condensation of *N*-phthaloyl-L-serine[39] with methyl D-(*p*-benzyloxyphenyl)glycinate[35] as above gave **22**: mp 189–191 °C; $[\alpha]^{25}_D$ −118°. A ^{1}H NMR spectrum at 300 MHz of this material indicated no detectable epimerization and made trivial the assignment of relative configurations noted earlier for the principal diastereomer of the racemic dipeptide **19**. Cyclization and workup as previously executed afforded a 2:1 mixture of the diastereomers **23a** and **24a**. Hydrogenation of this mixture gave **23b** and **24b**, which upon crystallization from absolute ethanol readily afforded pure **23b**: 43%; mp 169–170 °C; $[\alpha]^{25}_D$ −239°. Obtention of optically pure β-lactam established, as hoped, that the stereochemical integrity of the serine α position had remained intact throughout the reaction. Epimerization was therefore limited to C-5, a position whose base sensitivity had been noted earlier by workers at Fujisawa[35] and indeed had been used to advantage in the Lilly synthesis[25] of **25**. Sequential deprotection of **23b** may be carried out as previously described to yield (−)-3-aminonocardicinic acid (**25**) identical in all respects with material produced by degradation of nocardicin A (**1**).[35]

Contrary to our initial impressions described in a preliminary communication,[33] when the β-lactam product mixture was examined by FT NMR after silica gel chromatography, a small amount (7% under the conditions originally reported; see Table III) of inseparable dehydroalanyl peptide **26** was detected. An

Table III. Proportion of Dehydropeptide **26** Relative to β-Lactams **23** and **24** on Reaction of Dipeptide **22** (50 mM) with Varying Amounts of Triphenylphosphine/DEAD in Dry Tetrahydrofuran at Room Temperature

equiv PPh_3/DEAD	extent of reactn, %	% **26** in totl prod mixt[a]	β-lactam ratio **23**:**24**[a]
1.0	90–95	23	2.1:1
1.5	100	16	1.9:1
1.8	100	12	2.0:1
2.5	100	7	2.0:1
4.0	100	3	1.8:1
5.0	100	<2	2.0:1

[a] Determined by integration of ^{1}H NMR spectra in $CDCl_3$ at 80 MHz.



26 27 28

authentic sample of the latter was provided by mesylation of **22** and elimination in the presence of triethylamine. Highly diagnostic resonances in the ^{1}H NMR spectrum were observed for the C-3 vinyl hydrogens at δ 6.24 and 5.85 (^2J = 1.4 Hz). Given the obtention of optically pure **23** above, it is evident that when to a very limited extent proton abstraction does occur at C-3, elimination to **26** is the immediate result.

The proportion of **26** in the cyclization mixture was found to be dependent upon the relative excess of Mitsunobu reagent used. A series of 15-min reactions was carried out under comparable conditions, and the relative amount of **26** formed was determined by ^{1}H NMR spectroscopy. The results are summarized in Table III and indicate an essentially unchanged integrated ratio of β-lactam products **23** and **24** but sharply increasing amounts of dehydropeptide **26** as the quantity of triphenylphosphine/diethyl azodicarboxylate (DEAD) was reduced. Reactions were complete in 15 min as monitored by the disappearance of peptide **22** on TLC except for the case of 1.0 equiv of Mitsunobu reagent.

To gain some insight into the mechanism of the cyclization reaction, we treated 300 mg of **22** with 1.0 equiv of triphenylphosphine/DEAD and quenched the reaction with water after 15 min. Approximately 10 mg of peptide reactant was recovered, crystallized once, and found to have melting point and ^{1}H NMR spectrum identical with starting **22** itself. In another experiment, **22** was reacted with 2.5 equiv of Mitsunobu reagent, and after 15 min the reaction was quenched with deuterium oxide. No deuterium incorporation was detectable at C-3 or C-5 in **23** or **24** on ^{1}H NMR analysis. These observations suggest that epimerization that takes place at C-5 to give the diastereomers **23** and **24** occurs either (or both) in an intermediate that is not in reversible equilibrium with **22** or in **23** and **24** themselves after β-lactam formation (vide supra[25,35]). The absence of incorporated deuterium implies that the composition of the product mixture is established before workup and isolation. Further observations in this connection will be described below.

We were struck by the ease with which the Mitsunobu reaction had taken place to form β-lactam products. The success of this intramolecular reaction stands in marked contrast to earlier experiments of Mitsunobu[31] where benzamide (pK_a = 13–14) and *p*-nitrobenzamide failed to produce N-alkylated products from intermolecular reaction with *n*-propyl alcohol in the presence of triphenylphosphine/DEAD. To mimic possible phosphorylation even more closely in the in vitro cyclization, we tested triethyl phosphite in place of triphenylphosphine (R′ = OEt rather than Ph in **20**). Under otherwise identical conditions of concentration and temperature, the reaction of **22** with 2.5 equiv of triethyl phosphite/DEAD took significantly longer to go to completion

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(37) König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 2034–2040.
(38) Other approaches to the synthesis of **25**: Kamiya, T.; Oku, T.; Nakaguchi, O.; Takeno, H.; Hashimoto, M. *Tetrahedron Lett.* **1978**, 5119–5122. Foglio, M.; Francheschi, G.; Lombardi, P.; Scarfile, C.; Arcamone, F. *J. Chem. Soc., Chem. Commun.* **1978**, 1101–1102. Chiba, K.; Mori, M.; Ban, Y. *Ibid.* **1980**, 770–772.
(39) Hodges, R. S.; Merrifield, R. B. *J. Org. Chem.* **1974**, *39*, 1870–1872.

(ca. 45 min) but interestingly gave **23** and **24** in a ratio of 6.8:1 instead of 2:1. No dehydropeptide **26** was detected, but, inter alia, a weak, broadened triplet at δ 5.95 ($J \sim 5$ Hz) and a multiplet at δ 1.9–2.1 were observed. These resonances were tentatively assigned to γ-lactam **27** (probably a mixture of diastereomers) derived from anion formation at C-5 comprising 9% or less of the product mixture. Parallel reactions run for 16 h and 161 h (6.7 days) gave ratios of **23**/**24** of 6.5:1 and 3.1:1, respectively (Table IV). To our great pleasure, an analogous reaction in the presence of 1.0 equiv of triethyl phosphite/DEAD proceeded slowly over 4 h to afford **23a** as the exclusive β-lactam product (diastereomeric purity of >50:1), a very small quantity of starting material **22**, and less than 3% of **27**. An identical reaction run overnight led to the virtually identical outcome with only the barest detectable trace of **24b** present (from epimerization, vide infra). Hydrogenation of **23a** so generated and crystallization as before gave optically pure[33] **23b**; mp 170–171 °C, $[\alpha]^{25}_{D}$ −242°.

The fact that both racemic dipeptide **19** containing an approximately 10:1 mixture of diastereomeric *d,l* pairs and optically pure diastereomer **22** gave the same 2:1 mixture of β-lactams **23** and **24** under a variety of conditions with triphenylphosphine/DEAD (Table III) suggested that this mixture represented the thermodynamic equilibrium of these products in the presence of base. That this supposition was correct was established by separate treatments of pure **23a** obtained above with a trace of triethylamine in $CDCl_3$ for 1 week and with 2.5 equiv of triphenylphosphine/DEAD in dry tetrahydrofuran for 15 min at room temperature, conditions comparable to the reactions in Table III. In both instances the expected 2:1 ratio was obtained. However, in the case of the triethyl phosphite reactions (Table IV), the extent of epimerization at C-5 is significantly less, and for the series with 2.5 equiv of triethyl phosphite/DEAD, it is clear that further epimerization of the initially formed 6.8:1 ratio toward equilibrium in the presence of excess reagent is rather a slow process. The 6.8:1 mixture formed at 1 h cannot be accounted for solely on the basis of epimerization of the preformed β-lactam by presuming the approach to equilibrium is kinetically first order;[40] i.e., there must be an intermediate that, as discussed above, is not in reversible equilibrium with starting peptide **22**, whose optical integrity remains unassailed, but which is significantly prone to epimerization prior to β-lactam formation. We present data in the following that indicate that this intermediate in all likelihood is **20** (R′ = OEt, amide protonated).

As noted above, when **22** was treated with 1.0 equiv of triethyl phosphite/DEAD, the cyclization proceeded quite slowly. When monitored by TLC, a low-R_f spot was observed early in the reaction but grew fainter as starting material disappeared. Rapid removal of solvent in vacuo after 15 min of reaction time and preparative TLC of the residue afforded a small sample of the transient species. ^{1}H NMR analysis at 300 MHz of this material revealed a single diastereomer whose spectrum was similar to that of the protected dipeptide **22** but most notably showed the ABX spin system for the C-3 and C-4 hydrogens of the seryl residue to be shifted significantly to lower field. The AB system gave 14 lines rather than the expected maximum of 8. Extraction of the coupling constants disclosed interaction with a fourth spin, $J = 7.9$ Hz,[41] which in tandem with the required ethoxy signals upfield led to structure **28**. This assignment was substantiated further by the $^{13}C\{^1H\}$ NMR spectrum of **28** where coupling to phosphorus was manifest in the ethyl phosphate resonances (methyl δ 16.0 ($^3J_{P-C} = 6.4$ Hz) and methylene δ 63.5 ($^2J_{P-C} = 5.9$ Hz) and in the signal to C-4 (δ 64.1 ($^2J_{P-C} = 6.4$ Hz)).[41] It is proposed that this phosphate triester is derived by an Arbuzov-like reaction of the true reactive intermediate **20** on exposure to silica gel.

Attempts to prepare **28** by the reaction of dipeptide **22** with diethyl chlorophosphate in the presence of triethylamine or 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), conditions that had been used earlier to prepare the corresponding mesylate, gave no detectable **28** but cleanly afforded the elimination product **26**. However, treatment of **22** at −23 °C in methylene chloride with 0.9 equiv of sodium hydride followed by 1 equiv of diethyl chlorophosphate provided a specimen of **28** whose chromatographic behavior and 300-MHz ^{1}H NMR spectrum were identical with the intermediate isolated above. About 10% of the diastereomeric phosphate triester was also produced in this reaction, evidently by epimerization at C-5.

To summarize, reaction of protected dipeptide **22** under classical Mitsunobu conditions with triphenylphosphine/DEAD proceed rapidly to afford a 2:1 mixture of **23** and **24** accompanied by decreasing amounts of dehydroalanyl peptide **26** as larger excesses of reagent were used. A thermodynamic mixture of the diastereomeric β-lactams was obtained. Whether epimerization at C-5 takes place before or after four-membered ring formation, i.e., in **20** (R′ = Ph) or in **23**/**24** alone, cannot be determined from the information available. Most probably it is rapid in both. Once formed, **20** apparently proceeds to product(s) without reversion to starting peptide **22**, as the latter is recovered at partial reaction optically pure. Cyclization alternatively in the presence of triethyl phosphite/DEAD takes place with significantly less epimerization at C-5, and exposure to excess reagent catalyzes the approach to the equilibrium mixture of β-lactam products only very slowly. To account for the observations summarized in Table IV, epimerization at C-5 is apparently faster prior to β-lactam closure in intermediate **20** (R′ = OEt, deprotonation at C-5 rather than at amide nitrogen), which is zwitterionic, rather than after. The intermediacy of **20** is, therefore, inferred kinetically and is indirectly demonstrated by the isolation of diethyl phosphate **28**. The comparatively lower nucleophilicity of triethyl phosphite and hence a lesser tendency to form the initial diethyl azodicarboxylate adduct could account for the overall slower rate of these reactions and the markedly reduced rates of epimerization of the β-lactam products in the presence of excess reagent. The diminished extent of C-5 epimerization, however, may additionally reflect the relative leaving group abilities of the two positively charged phosphorus species in **20**, that is, $-OP^+(OEt)_3$ is better, and hence there is less reversible deprotonation at C-5 with respect to the rate of β-lactam formation.[42] Other interpretations of these observations are, of course, possible and cannot be discarded on the basis of the data presented. In the presence of 1.0 equiv of triethyl phosphite/DEAD, epimerization very nearly ceases and ring-closure proceeds in the synthetically most useful manner to afford **23**, having diastereomeric purity of >50:1.

In conclusion, while the naturally occurring penams, cephems, and nocardicin A are each amino acid derived[1,2] and share common stereochemical features, the details of the oxidative cyclization of the Arnstein tripeptide (**6**) to isopenicillin N (**2**) [R = δ-(L-α-aminoadipyl)] remains an unresolved problem in bioorganic chemistry. Mechanistic proposals wherein amide nitrogen serves as a nucleophile have spanned additions to a thioaldehyde,[43] a

(40) Frost, A. A.; Pearson, R. G. "Kinetics and Mechanism", 2nd ed.; Wiley: New York, 1961; pp 185–186.

(41) Observed spectral parameters were in accord with literature precedent: Jackman, L. M.; Sternhell, S. "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", 2nd ed.; Pergamon Press: Oxford, England, 1969; pp 351–356. Stothers, J. B. "Carbon-13 NMR Spectroscopy"; Academic Press: New York, 1972; pp 376–385.

(42) The fact that this intramolecular cyclization does occur while a comparable intermolecular case fails[31] deserves some further comment. One interpretation is that the Mitsunobu base generated in the reaction is sufficiently strong to generate the pair **20**/**21**. The amide of the peptide would have a $pK_a = 14$–16, which evidently can be abstracted in preference to H-3 and H-5 to give β-lactam products(s). Generation of a zwitterionic intermediate in **20** may further assist proton removal, and a kinetic preference for four-membered vs. five-membered ring formation may lead to the observed β-lactam product. Alternatively, the course of the reaction may proceed initially by S_N1 ionization at C-4 with partial departure of $O{=}PR_3'$ to significantly weaken the C-4–O bond and hence lower σ^*_{C-O}. Provided the energy of this orbital could be decreased sufficiently that interaction with the amide lone pair could take place orthogonal to and compete energetically with normal amide resonance, the result of incipient N–C-4 bond formation would be to reduce the pK_a of the amide hydrogen for preferential abstraction by the Mitsunobu base.

The ability of amide nitrogen to add intramolecularly to a nearby electrophile is well-known for the case of carbonyls. Some recent examples include: Girota, N. N.; Wendler, N. L. *Tetrahedron Lett.* **1979**, 4793–4796. Bodanszky, M.; Martinez, J. *J. Org. Chem.* **1978**, *43*, 3071–3073.

Table IV. Product Composition of β-Lactams 23 and 24 on Reaction of Dipeptide 22 (50 mM) with Triethyl Phosphite/DEAD in Dry Tetrahydrofuran for the Times Indicated

equiv $P(OEt)_3$/DEAD	duration of reactn, h	extent of reactn, %	β-lactam ratio 23:24[a]
2.5	1	100	6.8:1
2.5	16	100	6.5:1
2.5	161	100	3.1:1
1.0	4	90–95	>50:1
1.0	15	90–95	~50:1[b]

[a] Determined by integration of ^{1}H NMR spectra in $CDCl_3$ at 80 MHz. [b] See text.

thioaldehyde equivalent,[44] and transannular additions in seven-membered cyclic intermediates.[45] Conversion of the amide nitrogen to an electrophilic center through oxidation to an hydroxamate has been suggested to allow β-lactam ring formation by closure of a sulfur-stabilized anion.[46] Finally, radical intermediates have been proposed.[47] For the case of nocardicin, however, the evidence that has been brought to bear on the question of β-lactam formation in this paper and its companion turns on the maintenace of a constant oxidation state from the amino acid serine to the monocyclic β-lactam of the antibiotic. Interpretation of these data restricts mechanistic proposals for the biosynthetic task to the simplest of solutions.

Serine double-label experiments showed retention in nocardicin A of about one-fifth of the radiolabel originally residing at C-2, which we interpret as disfavoring the intervention of an α,β-dehydroalanyl intermediate. The essentially complete retention of tritium from C-3 of serine in **1** is best accommodated by a mechanism of β-lactam formation requiring no change in oxidation level at C-3. The observation of clean stereochemical inversion at this center on incorporation of diastereomeric [3-^{2}H]serines supports the view that displacement in the classic S_N2 sense of a presumably activated seryl hydroxyl takes place, with the amide nitrogen of an at present hypothetical peptide precursor serving as the sole nucleophile. An in vitro model for such a process has been provided in the cyclization of the protected dipeptide **22** under Mitsunobu conditions. This reaction has been widely observed to occur with inversion of configuration at primary and secondary carbinol centers[27,28,30] in accord with the observed stereochemical course of four-membered ring formation in vivo. In contrast, during the biosynthesis of penicillin, the β-lactam ring is formed from cysteine with stereochemical retention.[48] The apparently similar derivation of sulfazecin (**8**) from L-serine with retention of both of its β-hydrogens suggests that the simple and direct proposal that has emerged for nocardicin may well be general for the known monocyclic β-lactam antibiotics of microbial origin.

Experimental Section

The instrumentation used and the data format are the same as described in the preceding paper with the following additions. ^{1}H NMR spectra at 300.0 MHz and ^{2}H NMR spectra at 46.1 MHz were obtained with a Bruker WM-300 spectrometer; the former are so indicated in this section. Electron-impact mass spectra at 70 eV were obtained on a Finnigan Model 7000 spectrometer. Analytical HPLC separations of the diastereomers of dipeptide **19** were carried out on a 4 × 300 mm Varian MicroPak Si5 column: solvent, 4:1 methylene chloride/acetonitrile; flow rate, 1.5 mL/min.

Incorporation of labeled precursors was carried out as described in the preceding paper with the exception that L- and D-[2-^{3}H,1-^{14}C]serine were administered in the presence of 0.4 mM L-methionine to maximize production of nocardicin A (**1**). For the case of the chiral deuterium-labeled serines, the identical concentration of L-methionine was added to the fermentation medium prior to inoculation. Isolation and purification of the nocardicin A (**1**) produced was carried out as previously described.[1]

Microanalyses were performed by Galbraith Laboratories, Knoxville, TN. Dimethylformamide was distilled from calcium hydride. Tetrahydrofuran was distilled from sodium benzophenone ketyl immediately before use. *p*-Xylene was distilled from and stored over clean sodium metal.

Preparation of L- and D-[2-^{3}H,1-^{14}C]Serine. (a) DL-[2-^{3}H]Serine. The method of Miles and McPhie[9] was adapted as follows. Pyridoxal hydrochloride (796 mg, 3.9 mmol; Sigma) in 3 mL of distilled water was converted to its free base on a 1.5 × 14 cm Dowex 50-H$^+$ column by eluting with 200 mL of 2 N ammonium hydroxide. The yellow solution was concentrated in vacuo and added to a 55-mL aqueous solution of DL-serine (4.1 g, 3.9 mmol) and aluminum sulfate octadecahydrate (796 mg, 1.19 mmol) contained in a 500-mL round-bottom flask. The pH was adjusted to 9.4–9.6 with 5 N potassium hydroxide, and 300 mCi of tritiated water (New England Nuclear) were added to the rapidly stirred solution. After stirring the solution for 62 h in the dark, 300 mL of absolute ethanol were added, and the solution was stored in the freezer overnight. The crystalline product obtained was collected, redissolved in distilled water, treated several times with activated carbon (Norit A, Baker), and crystallized by the addition of 10 volumes of absolute ethanol to afford 2.47 g (60%) of DL-[2-^{3}H]serine as a very pale yellow solid after washing with cold absolute ethanol and ether and drying under vacuum.

(b) *N*-Acetyl-DL-[2-^{3}H]serine. The procedure of Greenstein and Winitz[10] was applied. Acetic anhydride (6.95 mL, 7.50 g, 73.5 mmol) and 2 N sodium hydroxide (63.9 mL) were added alternately to a vigorously stirred solution of DL-[2-^{3}H]serine (2.36 g, 23.4 mmol) dissolved in 12.3 mL of 2 N sodium hydroxide at 0 °C. Stirring was continued overnight at room temperature whereupon the reaction mixture was run onto a 4.2 × 35 cm Dowex 50-H$^+$ column. Elution with 600 mL of distilled water, collecting 100-mL fractions, gave the product in fractions 4–6. Concentration under vacuum gave a yellow oil that was dissolved in the minimum amount of absolute ethanol, treated two times with activated carbon (Norit A, Baker), and triturated with ether to yield 1.68 g (49%) of crude *N*-acetyl amino acid. Redissolution in absolute ethanol followed by a further two treatments with activated carbon and addition of ether at 0 °C provided 1.38 g (40%) of *N*-acetyl-DL-[2-^{3}H]serine: mp 132–133 °C lit.[10] 131–132 °C, lit.[49] 129–130 °C; specific activity ~1.3 × 10^8 dpm/mmol.

(c) *N*-acetyl-DL-[1-^{14}C]serine. DL-[1-^{14}C]Serine (250 μCi, ICN Pharmaceuticals) was diluted with carrier DL-serine (2.10 g, 20.0 mmol) and converted to its *N*-acetyl derivative as above, but no decolorizing treatments were necessary: 82%; mp 130–131 °C; specific activity ~2.4 × 10^7 dpm/mmol.

(d) Resolution of N-Acetyl-DL-[2-^{3}H, 1-^{14}C]serine. The procedure of Greenstein and Winitz was modified[10] such that *N*-acetyl-D,L-[2-^{3}H]-serine (1.375 g, 9.35 mmol, ~1.3 × 10^8 dpm/mmol) and *N*-acetyl-D,-L-[1-^{14}C]serine (1.530 g, 10.4 mmol, ~2.4 × 10^7 dpm/mmol) were dissolved in 160 mL of distilled water and adjusted to pH 7.2 with 6 N ammonium hydroxide. After the solution was filtered through a 0.2-μm filter unit (Nalge/Sybron), 23.8 mg of hog kidney acylase I (Sigma, Grade I) was added. The total volume was brought to 200 mL and the pH checked and readjusted to 7.2 as necessary. The solution was divided into 50-mL fractions in 125-mL Erlenmeyer flasks and incubated at 37 °C and 150 rpm. After 24 h, the contents of all flasks were pooled, acidified to pH 4 with glacial acetic acid, and warmed on a steam bath for 30 min with activated carbon. The carbon was filtered and washed with 10 mL of distilled water, and the filtrate and water washes were run onto a 2.8 × 16.2 cm Bio-Rad AG 50-H$^+$ column. *N*-Acetyl-D-[2-^{3}H,1-^{14}C]serine was eluted with 450 mL of distilled water (pH <3) and was concentrated in vacuo to give a yellow oil.

The doubly labeled L-serine was eluted with 450 mL of 2 N hydrochloric acid; elution was monitored by analytical TLC (silica gel, 4:1:1 *n*-butyl alcohol/water/acetic acid; ninhydrin visualization). The acidic solution was concentrated to 5 mL and precipitated with aniline (pH 5.5)

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to yield 880 mg (86%) of crude product. This material was redissolved in 5 mL of distilled water, treated twice with activated carbon, triturated with absolute ethanol, and refrigerated overnight. The crystalline product was collected, washed with cold absolute ethanol and ether, and air-dried to yield 683 mg (66%); $[\alpha]^{25}_D$ 13.85° (*c* 1.2, 1 N HCl) [lit.[50] $[\alpha]^{25}_D$ 14.45° (*C* ~9, 1 N HCl)]; specific activity (as *N*-tosyl derivative, mp 234–235 °C dec. (lit.[51] mp 235–236 °C dec for unlabeled *N*-tosyl-L-serine)) (^{3}H) 7.67 × 10^7 dpm/mmol, (^{14}C) 1.55 × 10^7 dpm/mmol.

The yellow oil containing the *N*-acetyl D antipode was dissolved in 5 mL of 2 N hydrochloric acid and refluxed for 2.5 h in a 25-mL round-bottom flask. After the solvent was removed at the rotary evaporator, the residue was dissolved in 3 mL of deionized water and adjusted to pH 5 with aniline. Upon cooling overnight, the crude crystalline product that formed, 718 mg (59%), was filtered and recrystallized from water–ethanol to afford 545 mg (53%) of D-[2-^{3}H,1-^{14}C]serine: $[\alpha]^{25}_D$ 12.22° (*c* 1.2, 1 N HCl); specific activity (as *N*-tosyl derivative, mp 236–238 °C) (^{3}H) 7.69 × 10^7 dpm/mmol, (^{14}C) 1.55 × 10^7 dpm/mmol.

Preparation of Chiral Serines 15/16 and 17/18. (a) Dideuterated Adduct 11. The Vaughan–Milton[52] and Hill–Newkome[15] procedures were used to condense methyl propiolate (5.64 g, 67.1 mmol) and anthracene (12.0 g, 67.3 mmol) in dry xylene at reflux. The resulting crude unsaturated Diels–Alder adduct was purified by column chromatography on silica gel (5 × 45 cm; Baker 40–140 mesh) by eluting with benzene, two treatments with activated carbon (Norit A, Baker), and crystallization from 95% ethanol to give 11.0 g (62%) of **11** mp 177–178 °C (lit.[15] mp 177–178 °C); ^{1}H NMR ($CDCl_3$) identical with that reported.[15]

A three-neck 1-L flame-dried round-bottom flask fitted with three gas inlet adapters connected to a water aspirator, a cylinder of 99.9% D deuterium gas (Matheson), and a deuterium-gas-filled balloon was charged with 161 mg of 5% palladium on calcium carbonate (Pfaltz and Bauer) and then evacuated for 2 h. Dry ethyl acetate (575 mL, distilled from phosphorus pentoxide under nitrogen) was added, and the catalyst was equilibrated with deuterium gas by alternately evacuating and filling the system with deuterium over a 24-h period with sonication. The unsaturated Diels–Alder adduct (23 g, 88 mmol) was added quickly to the catalyst suspension, and the flask was evacuated and then filled with deuterium gas. Reduction was allowed to continue for 24 h (alternating between stirring and sonication). The catalyst was filtered and washed with ethyl acetate, and the washes and filtrate were pooled and concentrated in vacuo to yield compound **11** quantitatively as a clean white solid, which was recrystallized from 95% ethanol: mp 114–116 °C (lit.[15] mp 117–118 °C); ^{1}H NMR ($CDCl_3$) δ 2.13 (br s, 1 H, H_c), 3.58 (s, 3 H, OCH_3), 4.32 (d, J = 2.6, 1 H, H_a), 4.66 (s, 1 H, H_b), 6.85–7.45 (c m, 8 H); MS (70 eV), *m/e* 268 (0.03), 267 (0.61), 266 (3.52), 178 (100) deuterium estimation 99 ± 1% d_2.

(b) Ethyl [3-^{2}H]Propiolate. A flame-dried 100-mL round-bottom flask fitted with a calcium chloride drying tube was charged with 9.48 g (96.7 mmol) of ethyl propiolate (Aldrich), 30 mL of deuterium oxide (99.8 %D), and three drops of 1 N sodium deuteroxide. After 96 h of rapid stirring at room temperature, four drops of 5 N deuterium chloride were added and the suspension was extracted four times with 20-mL portions of methylene chloride. The organic layers were pooled, washed twice with saturated brine, and dried over anhydrous magnesium sulfate. Filtration and rotary evaporation at 0 °C provided 8.4 g (88%) of the deuterated ester, which was used without further purification. ^{1}H NMR spectroscopy revealed that 90–95% of the acetylenic hydrogen had been replaced by deuterium.

(c) Monodeuterated Adduct 12. The deuterated ethyl propiolate was converted to its anthracene Diels–Alder adduct as above and hydrogenated to afford **12**, which was recrystallized from 95% ethanol: mp 97–99 °C; IR ($CHCl_3$) 3080, 3000, 2960, 1725, 1465, 1455, 1080, 865 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.18 (t, J = 7.2, 3 H), 1.75–2.15 (c m, 1 H, H_c), 2.83 (d × d, J = 2.5, 10.3, 1 H, H_d), 4.04 (q, J = 7.2, 2 H), 4.32 (d, J = 2.5, 1 H, H_a), 4.68 (d, J = 2.5, 1 H, H_b), 6.80–7.50 (c m, 8 H); MS (70 eV), *m/e* 281 (0.02), 280 (0.46), 279 (2.61), 278 (0.21), 178 (100) deuterium estimation 92 ± 2% d_1, 8 ± 2% d_0.

(d) Methyl (*E*)-[2,3-$^2H_2$]Acrylate (13) and Ethyl (*Z*)-[3-^{2}H]Acrylate (14). Pyrolysis at 290–300 °C of the deuterated adducts **11** and **12** as described by Hill[15] gave **13** and **14**, respectively.

(e) (2S,3R)/(2R,3S)-[2,3-$^2H_2$]Serine 15/16 and (2S,3S)/(2R,3R)-[3-^{2}H]Serine 17/18. The (*E*)-dideuteroacylate **13** and the (*Z*)-monodeuteroacylate **14** were converted to racemic mixtures of largely[16] (2*S*,3*R*)/(2*R*,3*S*)-serines **15/16** and (2*S*,3*S*)/(2*R*/3*R*)-serines **17/18**, respectively, by the procedure of Slieker and Benkovic.[18]

^{1}H NMR (D_2O/NaOD, 300 MHz) spectra of the chiral serines were recorded at pD ~ 12.5 to obtain a useful dispersion[19] of the diastereotopic serine β-hydrogen resonances: **15/16** δ 3.31 (br d, J = 5.6, ~0.08 H, H-2), 3.66 (br s, ~0.86 H, H-3), 3.71 (br s, ~0.14 H). Mass spectral analysis of precursor adduct **11** indicated a deuterium content of 99 ± 1% d_2. Therefore, the diastereotopic purity at C-3 is 86:14 (±2%) as determined by direct proton integration and triangulation, and about 8% exchange has taken place at C-2 in the transformations of **11** to **15/16**. For the diastereomeric serines **17/18**, δ 3.31 (br d, J = 4.4, 1 H, H-2), 3.71 (br d, J ~ 4.4 superimposed on *AB*X of unlabeled serine and an upfield doublet, total integration ~1.1 H, ratio of lowfield:highfield signals ~4.9, H-3). Recalling that precursor adduct **12** bore 92 ± 2% d_1 and 8 ± 2% d_0 on mass spectral analysis, 0.04 H may be distributed to the integrals of the low and upfield signals which gives a diastereomeric purity for the *deuterium*-labeled species of 86:14 (±3%).

***N*-Phthaloyl-DL-(*p*-benzyloxyphenyl)glycine Methyl Ester (19).** To a solution of 60 mg (0.25 mmol) of *N*-phthaloyl-DL-serine[36] in 2 mL of dry dimethylformamide were added 100 mg (0.37 mmol) of methyl DL-(*p*-benzyloxyphenyl)glycinate[35] and 67 mg (0.50 mmol) of 1-hydroxybenzotriazole hydrate.[37] The mixture was stirred for 15 min in an ice bath, and 57 mg (0.27 mmol) of dicyclohexylcarbodiimide was added. Stirring was continued at 0 °C for 1 h and at room temperature for 2 h. A few drops of 20% aqueous acetic acid were added, and precipitated dicyclohexyl urea was removed by filtration. The filtrate was concentrated in vacuo, and the residue dissolved in ethyl acetate was washed three times each with 1 N hydrochloric acid, 1 N potassium bicarbonate, and water. After drying the solution over anhydrous magnesium sulfate, the volume was reduced, and three crystallizations from ethyl acetate removed the remaining 1-hydroxybenzotriazole. Dipeptide **19** was then crystallized from ethyl acetate–hexanes to provide 100.6 mg (83%); mp 126.5–135 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.04 (br s, 1 H, OH), 3.70 (s, 3 H, OMe), 4.09 (d × d, J = 11.4, 5.2, 1 H, H-4), 4.39 (d × d, J = 11.4, 6.6, 1 H, H-4), 5.00 (d × d, J = 6.6, 5.2, 1 H, H-3), 5.04 (s, 2 H, CH_2Ph), 5.51 (d, J = 6.7, 1 H, H-5), 6.95 (d, J = 8.8, 2 H, PHPG), 7.26–7.44 (c m, 7 H), 7.53, (d, J = 6.5, NH?), 7.75 (c m, 2 H, phthaloyl), 7.87 (c m, 2 H, phthaloyl).

The ^{1}H NMR spectrum above revealed that the major diastereomer formed was the 3*S*,5*R*/3*R*,5*S* pair as was subsequently determined when the 3*S*,5*R* stereoisomer was prepared optically pure (compound **22**). Present also in **19** was the minor 3*S*,5*S*/3*R*,5*R* pair as revealed by HPLC analysis (MicroPak Si5) and integration of one of the H-4 resonances which was the only signal well separated from the major isomers: δ 4.48 (d × d, J = 11.6, 7.2). The minor stereoisomers were present to the extent of 8–10%.

***N*-Phthaloyl-L-seryl-D-(*p*-benzyloxyphenyl)glycine Methyl Ester (22).** The optically active dipeptide was prepared in the same fashion as **19** except that the room-temperature phase of the condensation reaction was carried out for 1 h: 48%; mp 189–191 °C; $[\alpha]^{25}_D$ −118° (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) 3420, 3020, 2925, 1780, 1760 (sh), 1720, 1685, 1610, 1510, 1390, 1180 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) identical with major isomer in **19**. Anal. ($C_{27}H_{24}N_2O_7$): C, H, N.

Preparation of (−)-3-Phthalimidonocardicinic Acid Methyl Ester (23b) Using Triphenylphosphine. Diethyl azodicarboxylate (400 μL, 435 mg, 2.5 mmol) was added to a solution of 500 mg (1.02 mmol) of optically active dipeptide **22** and 670 mg (2.5 mmol) of triphenylphosphine in 20 mL of dry tetrahydrofuran, and the solution was stirred under a dry nitrogen atmosphere for 15 min. Reaction was quenched by the addition of 3 mL of water, and the mixture was stirred for 2 h more. The solvent was removed at the rotary evaporator, and the oily residue was chromatographed on a 1.8 × 31 cm column of silica gel by eluting with 1:1 ethyl acetate–hexanes. Fractions containing the desired product were combined, the solvents were removed in vacuo, and residual diethyl hydrazinedicarboxylate (**21**) was removed by fractional crystallization from chloroform–hexanes. The yellow oily product (**23a** and **24a**) was maintained under vacuum overnight to give 560 mg of crude material. Of this, 470 mg were dissolved in 20 mL of 1:1 methanol–acetic acid and hydrogenated at atmospheric pressure with sonication over 250 mg of 5% palladium on carbon.[35] After 2 h, the catalyst was removed by filtration through Celite and the solvents were removed under vacuum. ^{1}H NMR analysis revealed that the known[35] β-lactams **23b** and **24b** had been generated in a ratio of 2:1. Fractional crystallization of the mixture of diastereomers gave 119 mg of pure **23b**: (43%); mp 169–170 °C (lit.[35,53]

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(51) Reference 10, p 889.
(52) Vaughan, W. R.; Milton, K. M. *J. Am. Chem. Soc.* **1952**, *54*, 5623–5630.

(53) The melting point observed for **23b** does not agree with that cited in ref 35 and may represent an isomorph. However, with respect to all spectral data and specific rotation, agreement is exact.
(54) The chemical shift cited in ref 35 for H-3 in **23b** (δ 4.89) is in error and should be δ 5.49 as shown above. This assignment has been kindly confirmed by Dr. M. Hashimoto (Fujisawa) in a personal cummunication to Professor M. Koreeda (University of Michigan), whom we thank.

mp 203–204 °C); $[\alpha]^{25}_D$ −239° (*c* 0.03, MeOH) [lit.[35] $[\alpha]_D$ −236° (*c* 0.025, MeOH]; IR ($CHCl_3$) 3410, 3020, 1760, 1740, 1720, 1610, 1595, 1515, 1390, 1220, 1180 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.44 (d × d, *J* = 5.5, 2.9, 1 H, H-4B), 3.80 (s, 3 H, OMe, 3.93 (t, *J* = 5.5, 1 H, H-4A), 5.49 (d × d, *J* = 5.5, 2.9, 1 H, H-3),[54] 5.71 (s, 2 H, CH_2Ph), 6.08 (br s, 1 H, OH), 6.82 (d, *J* = 8.8, 2 H), 7.25 (d, *J* = 8.8, 2 H), 7.76 (sym m, 4 H, phthaloyl).

The diastereomeric β-lactam **24b** could be not crystallized from any one of a number of solvents tried. Its presence in the original debenzylation mixture was clearly discernible and gave chemical shifts completely in accord with those reported.[35]

Reactions in Table III. Each reaction in this series was run with 50 mg (0.1 mmol) of dipeptide **22** in 2 mL of dry tetrahydrofuran and the indicated number of equivalents of triphenylphosphine/DEAD for 15 min at room temperature. After being quenched with water, workup and ^1H NMR analysis was carried out as above.

***N*-Phthaloyldehydroalanyl(*p*-benzyloxyphenyl)glycine Methyl Ester (26).** Protected dipeptide **22** (100 mg, 0.2 mmol) in 1 mL of dry methylene chloride was treated with triethylamine (145 mg, 1.4 mmol, 200 μL) at 0 °C in an ice bath and 0.2 mL of a 13.0 M solution of methanesulfonyl chloride in methylene chloride. After 1 h of stirring, TLC (silica, 3:2 ethyl acetate–hexanes) showed the complete disappearance of starting material and the presence of two new spots (diastereomers), R_f 0.55 and 0.63. Water was added to the reaction mixture, and the aqueous layer was extracted three times with methylene chloride. The combined organic extracts were dried over anhydrous magnesium sulfate, the solvent was removed in vacuo, and the residue was dried in a vacuum desiccator overnight, 116 mg (quantitative): ^1H NMR ($CDCl_3$) δ 2.96 and 3.13 (both s, 3 H, diastereomeric OSO_2Me, ratio ~2:3), 3.67 (s, 3 H), 4.8–5.2 (ABX, 3 H, H-3 and H-4), 5.04 (s, 2 H, CH_2Ph), 5.42 (d, *J* = 7.0, 1 H, H-5), 6.94 (br d, *J* ~ 8.5, 2 H), 7.22 (br d, *J* ~8.5, 2 H), 7.38 (br s, 5 H), 7.84 (sym m, 4 H, phthaloyl).

The so-obtained mesylated dipeptide was dissolved in 1 mL of methylene chloride and treated with triethylamine (109 mg, 108 mmol, 150 μL) for 15 h at room temperature. TLC (silica, 3:2 ethyl acetate–hexanes) showed formation of a single product, R_f 0.57. Chloroform and water were added to the reaction mixture, and the organic layer was washed twice with 2 N hydrochloric acid and then with 5% sodium bicarbonate and water. The solvents were removed, and the oily product was dried overnight under vacuum: 70 mg, (74%); ^1H NMR ($CDCl_3$) 3.74 (s, 3 H), 5.04 (s, 2 H, CH_2Ph), 5.58 (d, *J* = 6.7, 1 H, H-5), 5.85 (d, *J* = 1.4, 1 H, vinyl), 6.24 (d, *J* = 1.4, 1 H, vinyl), 6.95 (d, *J* = 8.9, 2 H), 7.32 (d, *J* = 8.9, 2 H), 7.38 (br s, 5 H), 7.82 (sym m, 4 H, phthaloyl).

Reactions in Table IV. These reactions were carried out in a fashion analogous to those in Table III but substituting triethyl phosphite for triphenylphosphine.

Preparation of (−)-3-Phthalimidonocardicinic Acid Methyl Ester (23b) Using Triethyl Phosphite. Protected dipeptide **22** (400 mg, 0.8 mmol) in 16 mL of dry tetrahydrofuran was treated with triethyl phosphite (133 mg, 0.8 mmol, 140 μL) and diethyl azodicarboxylate (139 mg, 0.8 mmol, 125 μL). The solution was stirred at room temperature under nitrogen for 4 h. Water was added, and stirring was continued for 1 h. The solvents were removed in vacuo, and the residue was chromatographed as before (40 g silica gel, 1.8 × 30 cm column) to afford 336 mg (87%) of **23a** as an oil. Debenzylation and crystallization from ethanol as earlier yielded optically pure **23b**: 143.7 mg (58%); mp 170–171 °C; $[\alpha]^{25}_D$ −242° (*c* 0.038, MeOH).

Isolation of Phosphate Triester 28. Dipeptide **22** (100 mg, 0.2 mmol) in 4 mL of dry tetrahydrofuran was treated with 1.0 equiv of triethyl phosphite/diethyl azodicarboxylate as above. After 15 min, TLC (silica, 3:2 ethyl acetate–hexanes) revealed the presence of the intermediate, R_f 0.21. The solvent was partially removed in vacuo (bath temperature ca. 25 °C), and the residue was applied to a 20 × 20 cm PLC plate (2.0 mm, E. Merck) and eluted with 5:2 ethyl acetate–hexanes. Isolation of the polar product afforded 8 mg of oil: ^1H NMR ($CDCl_3$, 300 MHz) δ 1.25 (br q, *J* ~ 6.5, 6 H), 3.69 (s, 3 H), 4.02 (br quintet, *J* ~ 7.5, 4 H), 4.65–4.81 (14 lines centered at 4.71, *AB*X-P, J_{AX} = 6.1, J_{BX} = 8.2, J_{AB} = 11.2, $^3J_{P-H}$ = 7.9, 2 H, H-4), 5.04 (s, 2 H, CH_2Ph), 5.17 (AB*X* as d × d, *J* = 6.1, 8.2, 1 H, H-3), 5.49 (d, *J* = 7.0, 1 H, H-5), 6.94 (*AA'*XX', J_{app} = 8.5, 2 H, H-8), 7.27 (AA'*XX'*, J_{app} = 8.5, 2 H, H-7), 7.3–7.45 (br m, 5 H), 7.75–7.77 (4 lines, 2 H, phthaloyl), 7.88–7.91 (4 lines, 2 H, phthaloyl).

Preparation of Phosphate Triester 28. Sodium hydride (4.4 mg, 0.092 mmol) was washed free of mineral oil with pentane and suspended in 1 mL of dry tetrahydrofuran. Peptide **22** (50 mg, 0.1 mmol) was added. Under nitrogen at −23 °C, diethyl chlorophosphate (35.6 mg, 2.0 mmol, 30 μL) was added. After 2.5 h of stirring, the reaction mixture was allowed to come to room temperature. TLC (silica, as above) indicated the presence of **28**, starting peptide **22**, and a very small amount of dehydropeptide **26**. PLC as above afforded 5.4 mg of **28**, whose 300-MHz ^1H NMR spectrum was identical with that isolated above.

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Registry No. 1, 839391-39-4; **11**, 31460-49-8; **12**, 79833-59-3; **13**, 3321-61-7; **14**, 83160-20-7; **15**, 80612-42-6; **17**, 83212-43-5; **19**, 83212-44-6; **22**, 78246-50-1; mesylated **22**, isomer 1, 83160-21-8; mesylated **22**, isomer 2, 83160-22-9; **23a**, 64232-07-1; **23b**, 71336-84-0; **24a**, 78246-51-2; **24b**, 78246-52-3; **25**, 67509-41-5; **26**, 83160-23-0; **28**, 83160-24-1; DL-[2-^{3}H]serine, 83160-15-0; *N*-acetyl-DL-[2-^{3}H]serine, 52757-34-3; *N*-acetyl-DL-[1-^{14}C]serine, 83160-16-1; *N*-acetyl-D-[2-^{3}H,1-^{14}C]serine, 83160-17-2; L-[2-^{3}H,1-^{14}C]serine, 83160-18-3; *N*-tosyl-L-[2-^{3}H,1-^{14}C]-serine, 83174-75-8; D-[2-^{3}H,1-^{14}C]serine, 83160-19-4; *N*-tosyl-D-[2-^{3}H,1-^{14}C]serine, 83174-76-9; ethyl [3-^{2}H]propiolate, 59938-67-9; L-[3-^{3}H,V-^{14}C]serine, 83160-25-2; L-[3-^{3}H,3-^{14}C]serine, 83212-45-7; DL-serine, 302-84-1; DL-[1-^{14}C]serine, 867-86-7; methyl propiolate, 922-67-8; anthracene, 120-12-7; ethyl propiolate, 623-47-2; *N*-phthaloyl-DL-serine, 65391-10-8; methyl DL-(p-benzyloxyphenyl)glycinate, 71829-83-9; *N*-phthaloyl-L-serine, 29588-89-4; methyl D-(p-benzoyloxyphenyl)glycinate, 71336-83-9; diethyl azo dicarboxylate, 1972-28-7; methanesulfonyl chloride, 124-63-0; triethylphosphite, 122-52-1; diethyl chlorophosphate, 814-49-3.