(M^+, 100), 236 (30), 210 (27), 209 (19), 208 (38), 194 (35), 166 (15), 154 (10), 140 (12), 139 (11).

1-Ethyl-3-(methoxycarbonyl)-β-carboline (25): pale golden needles, mp 209-211 °C; IR (KBr) 3340, 3050, 3940, 1705 (C=O, ester), 1490, 1425, 1345, 1250, 740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.22 (t, 3 H, J = 7 Hz), 3.07 (q, 2 H, J = 7 Hz), 3.98 (s, 3 H), 7.10-7.60 (m, 3 H), 3.13 (d, 1 H, J = 7 Hz), 8.68 (s, 1 H), 9.83 (1 H); mass spectrum (70 eV), m/e 254 (M^+, 100).

1-Acetyl-β-carboline (27): white crystals, mp 216-217 °C (lit.[25] mp 203-205 °C); IR (KBr) 3340, 1670 (C=O, ketone), 1495, 1210, 1170, 750 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.90 (s, 3 H), 7.30-7.60 (m, 4 H), 8.20 (d, 1 H, J = 5.0 Hz), 8.60 (d, 1 H, J = 5.0 Hz), 10.60 (1 H); mass spectrum (70 eV), m/e 210 (M^+, 92), 168 (100), 167 (70), 141 (24), 140 (84), 115 (28).

1-Ethyl-β-carboline (24): white crystals, mp 192-194 °C (lit.[35] mp 192-194 °C; IR (KBr) 3440, 3120, 2980, 2880, 2780, 1620, 1560, 1500, 1315, 1235, 1220, 820, 740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.50 (t, 3 H, J = 7.5 Hz), 3.20 (q, 2 H, J = 4.5 Hz), 7.20-7.70 (m, 3 H), 7.83 (d, 1 H, J = 6.0 Hz), 8.18 (d, 1 H, J = 8.0 Hz), 8.42 (d, 1 H, J = 6.0 Hz), 8.93 (br, 1 H); mass spectrum (70 eV), m/e 196 (M^+, 80), 195 (100), 168 (32), 154 (10), 140 (22), 127 (8), 115 (10).

3-Benzyl-1,2,3,3a,4,5-hexahydrocanthin-6-one (29a).[3] N_b-Benzyltryptamine hydrochloride (**28a**, 10 g, 35 mmol) was basified with aqueous NH_3 (14%) and extracted with chloroform. The solvent was evaporated to give an oil, which was dissolved in benzene (100 mL). α-Ketoglutaric acid (5.2 g, 35.6 mmol) was dissolved in dioxane (60 mL) and added dropwise to the refluxing solution of amine **28a**. The mixture was refluxed for 7 days. Water removal was accomplished via a Dean-Stark trap. The solvent was evaporated under reduced pressure and the residue then dissolved in $CHCl_3$. The $CHCl_3$ layer was washed with saturated aqueous $NaHCO_3$ solution and then chromatographed on Al_2O_3 to give 9.04 g (82%) of the lactam **29a**: mp 171-173 °C; IR (KBr) 1685 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.60-2.20 (m, 1 H), 2.20-2.95 (m, 6 H), 3.28 (d overlapping m, 3 H, J_d = 14 Hz), 4.10 (d, 1 H, J = 14 Hz), 7.15-7.40 (m, 8 H), 8.30 (m, 1 H); mass spectrum (70 eV), m/e (rel abundance), 316 (M^+, 15), 315 (12), 259 (10), 196 (100), 167 (30), 153 (10), 91 (55).

Anal. Calcd for $C_{21}H_{20}N_2$: C, 79.75; H, 6.33; N, 8.86. Found: C, 79.69; H, 6.46; N, 8.97.

The methylene protons of the benzyl group are diastereotopic and are split into doublets (J = 17 Hz).

Canthin-6-one (30a).[3] The amine **29a** (870 mg, 2.75 mmol) and SeO_2 (366 mg) were added to dioxane (80 mL). The mixture was heated to reflux for 1 day. An additional portion of SeO_2 (300 mg) was added and the mixture held at reflux for 2 days. The reaction was cooled to room temperature before each addition of SeO_2 was carried out. The solvent was evaporated under reduced pressure, and the residue was chromatographed on SiO_2 [eluent EtOAc/benzene, (1:3)] to provide canthin-6-one (**30a**), 200 mg, 33%: mp 152 °C (MeOH-H_2O, lit. mp 160-161 °C);[30-32] IR (KBr) 1670 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.78 (d, 1 H, J = 9 Hz), 7.20-8.10 (m, 5 H), 8.40 (dd, 1 H, J_1 = 9 Hz, J_2 = 2 Hz), 8.50 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 116.02 (d), 117.08 (d), 122.37 (d), 124.20 (s), 125.39 (d), 128.13 (s), 128.75 (d), 129.81 (s), 130.06 (s), 130.63 (d), 136.06 (s), 139.28 (d), 145.50 (d), 159.18 (s); mass spectrum (CI, NH_3), m/e 221 (M + 1, 100).

2-(Methoxycarbonyl)canthin-6-one (30b). The amine **29b**[29] (2.0 g, 5.35 mmol) and SeO_2 (4.1 g, 37 mmol) were added to dioxane (250 mL), and the mixture was heated to reflux for 34. The black selenium was removed by filtration over Celite, and the solvent was removed under reduced pressure. The resulting slurry was diluted with MeOH, and the solid that crystallized was removed by filtration to furnish **30b** (980 mg, 66%): mp 249-250 °C; IR (Nujol) 1670, 1725 cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.20 (s, 3 H), 7.10 (d, J = 10 Hz), 7.40-8.00 (m, 2 H), 8.00-8.50 (m, 2 H), 8.60-8.90 (m, 1 H), 9.00 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 53.14, 117.26, 118.33, 122.77, 124.03, 125.93, 129.89, 130.78, 131.26, 135.57, 139.48, 139.75, 144.15, 159.18, 165.54; mass spectrum, m/e 278.0684 ($C_{16}H_{10}N_2O_3$ requires 278.0691).

Registry No. 1a, 84133-23-3; **1b**, 84133-22-2; **2a**, 84133-24-4; **2b**, 84133-25-5; **2c**, 84133-26-6; **3**, 84133-27-7; **4**, 22282-00-4; **6**, 724-42-5; **6**·HCl, 888-17-5; **7**, 21469-60-3; **8**, 60702-96-7; **9**, 19171-89-2; **10**, 2738-25-2; **11a**, 75304-06-2; **12**, 75304-07-3; **15a**, 84133-29-9; **15b**, 5275-05-8; **16b**, 71491-91-3; **17**, 61364-26-9; **18**, 5580-44-9; **19**, 84133-32-4; **22**, 84133-30-2; **23**, 75304-03-9; **24**, 20127-61-1; **25**, 75304-04-0; **26**, 66154-37-8; **27**, 50892-83-6; **28a**, 15741-79-4; **29a**, 65284-99-3; **29b**, 60702-98-9; **30a**, 479-43-6; **30b**, 84133-31-3; 3-indolyl-2'-pyridylcarbinol, 51626-59-6; 3-indolyl-2'-*N*-(trimethylacetyl)piperidylmethane, 84133-28-8; 3-indolyl-2'-piperidylmethane, 5275-05-8; 2-oxoglutaric acid, 328-50-7; indole, 120-72-9; 2-pyridine carboxaldehyde, 1121-60-4; trimethylacetyl chloride, 3282-30-2; selenium dioxide, 7446-08-4; **5**, 73-22-3.

Nocardicin A: Biosynthetic Experiments with Amino Acid Precursors

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Abstract: In radioisotope and stable-isotope tracer studies using growing cells of *Nocardia uniformis* subsp. *tsuyamanensis* (ATCC 21806), L-methionine, L-serine, and L-(*p*-hydroxyphenyl)glycine were shown to be the most efficient amino acid precursors of the homoseryl, β-lactam, and aryl segments of the monocyclic β-lactam antibiotic nocardicin A (**1**). $^3H/^{14}C$ double-label experiments demonstrated (a) that β-lactam formation takes place at the β-carbon of serine substantially without loss of tritium label at this position and (b) that the α-hydrogens of both L- and D-(*p*-hydroxyphenyl)glycine are lost during incorporation at both sites in **1**. The data suggest a peptide origin for the antibiotic as is known for penicillin and that β-lactam formation is most simply interpreted as taking place by nucleophilic displacement of presumably activated seryl hydroxyl by amide nitrogen.

Nocardicin A (**1**) is the principal and most active member[1] of a family of β-lactam antibiotics isolated[2] from *Nocardia uniformis* subsp. *tsuyamanensis*. It shares with the very recently discovered sulfazecin (**2**)[3] and related monobactams[4] a monocyclic β-lactam ring but is additionally unusual with regard to its ether-linked homoseryl, oxime, and (*p*-hydroxyphenyl)glycine units. These structural features raise a number of questions of biosynthetic importance that have been the focus of efforts in this laboratory.[5]

[†] Research Fellow of the Alfred P. Sloan Foundation (1982-1984).

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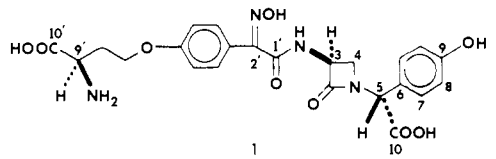
Presented in this paper are the details of studies that establish the primary precursors of **1** and that in the following paper address the fundamental issue of monocyclic β-lactam formation in vivo.



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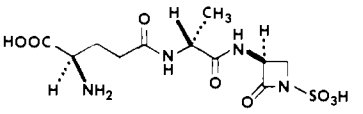

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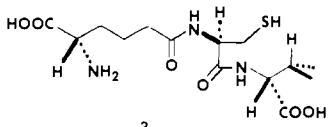

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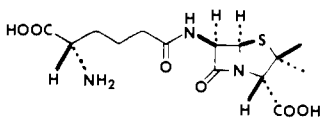

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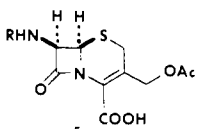

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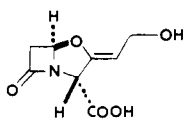

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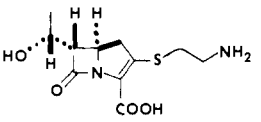

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These results reveal that nocardicin A (**1**), like penicillin N [**4**, side chain δ-(D-α-aminoadipyl)] and cephalosporin C [**5**, R = δ-(D-α-aminoadipyl)] with which it shares notable stereochemical similarities, is entirely amino acid derived. In contrast, the oxypenam clavulanic acid (**6**)[6] and the carbapenem thienamycin (**7**)[7] appear to be of mixed biogenetic origin. However, unlike the complex and poorly understood oxidative cyclizations that carry the Arnstein tripeptide (**3**) to isopenicillin N (**4**),[8] the evidence that can be brought to bear to date for the case of nocardicin A indicates a pathway of direct simplicity to the critical four-membered ring that may be general for the broader class of monocyclic β-lactam antibiotics of microbial origin.

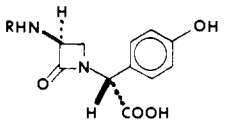
The seven known[1,2] nocardicins may be viewed as *N*-acyl derivatives of (−)-3-aminonocardicinic acid (**8**).[9] Nocardicins A–D contain a D-homoseryl segment, while E–G do not. Within these subgroups, differences are limited to the nature and oxidation state of the functional group at C-2′. Difficulties were encountered in initial attempts to produce in fermentation and to isolate nocardicin A according to procedures described in the literature,[10] evidently owing to inferior production by the available strain (ATCC 21806). However, by systematic modification of alternative procedures[11] and by monitoring production of **1** with the aid of a sensitive HPLC assay,[12] titers of 30–50 μg/mL were attained. These levels were at least 20 times lower than those of the commercial strain[11] and required the development of an ef-

Table I

amino acid[a]	^{14}C spec incorp/ unit	^{3}H/^{14}C amino acid	^{3}H/^{14}C **1**	% ^{3}H retained
L-[U-^{14}C]homoserine	1.5			
L-[1-^{14}C]-Met	19.6			
L-[U-^{14}C]-Tyr	15.0			
L-[1-^{14}C]-Tyr	0.03			
L-[U-^{14}C]-Phe	0.02			
L-[2-^{3}H,1-^{14}C]-PHPG[b]	49.6	4.44	0.06	1.3
D-[2-^{3}H,1-^{14}C]-PHPG[b]	6.3	4.45	0.04	0.9
L-[U-^{14}C]-Ala	0.07			
L-[U-^{14}C]-Cys	0.01			
[1-^{14}C]-Gly	2.6			
[2-^{14}C]-Gly	4.6			
L-[U-^{14}C]-Ser	7.8			
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

[a] Specific radioactivities and ^{3}H/^{14}C ratios were determined for the amino acids themselves and/or for the corresponding *N*-tosyl derivatives crystallized to constant specific activity. [b] PHPG = (*p*-hydroxyphenyl)glycine. Unlike the remaining experiments reported in this table, the enantiomers of doubly labled PHPG were fed in the presence of 0.4 mM L-methionine to maximize production of **1**, vide infra (The use of 1 mL L-methionine reported earlier[5] was in error).

(9) Structure **8**.



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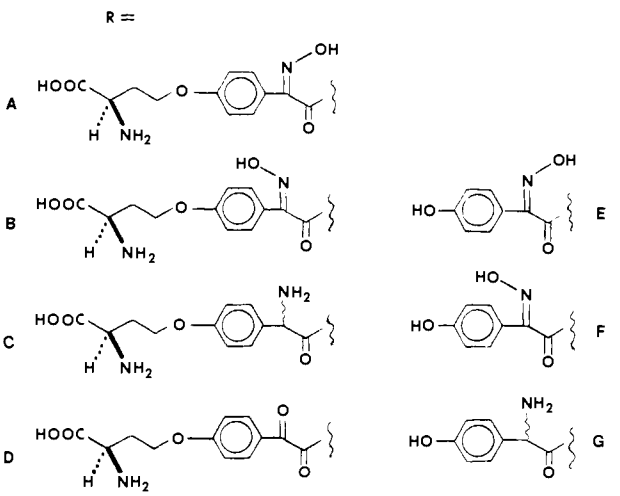

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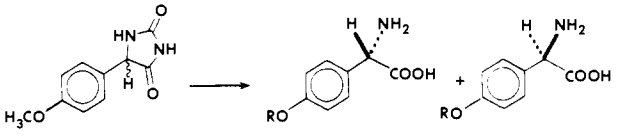
fective isolation scheme. This was ultimately accomplished by adsorption and partial purification on Amberlite XAD-4, concentration, and ion-exchange chromatography on DEAE Sephadex A-25. Following a desalting step on Biogel P-2, nocardicin A (**1**) was obtained as a colorless crystalline solid at pH 2.5 with an overall efficiency of 20–25%.

Radiolabeled potential precursors of **1** were crystallized to constant specific radioactivity as determined for the amino acids themselves and/or their *N*-tosyl derivatives and administered in sterile aqueous solutions to give the same initial concentration/precursor unit incorporated (0.4 mM) to vigorously shaken flasks of *N. uniformis* on the third day of growth, the point at which antibiotic production could be detected by analytical HPLC. Production of **1** reached a maximum on day 5 and remained unchanged through day 7. Therefore, the cells were harvested on day 6, and the metabolite was isolated, purified, and crystallized to constant specific activity as outlined above. The incorporation data obtained from these experiments are summarized in Table I.

The absence of the homoserine segment in the minor nocardicin metabolites E, F, and G[2,9] suggests, but does not in itself prove, that the non-peptide-linked amino acid is unnecessary for β-lactam formation. Apart from the timing of the attachment of this unit, it was found that while L-homoserine itself showed a positive incorporation into **1**, L-methionine was utilized with far greater efficiency (19.6%) and, moreover, antibiotic production was elevated by a factor of 2. These findings suggest that methionine or perhaps *S*-adenosylmethionine (AdoMet) may function in, for example, a pyridoxal phosphate-dependent γ-replacement reaction[13] or in a simple S_N2 displacement to form this ether linkage. While no stereochemical information presently exists, an example of the latter reaction path may be the utilization of AdoMet, with prior decarboxylation, by the aminopropyltransferases that act in the biosynthesis of the polyamines spermidine and spermine.[14] Similarly, intramolecular reactions of AdoMet have been demonstrated in the case of 1-aminocyclopropanecarboxylic acid,[15] the key intermediate in the biosynthesis of the plant hormone ethylene, and they have been proposed to be involved in the formation of azetidine-2-carboxylic acid[16a] and 2-amino-4-butyrolactone.[16b] These cases notwithstanding, the intact transfer of a 3-amino-3-carboxypropyl unit rather than methyl from AdoMet is a comparatively rare biochemical event, but nonetheless precedented in the modification of tRNA bases to form, for example, 3-(3-amino-3-carboxypropyl)uridine[17] and the Y base,[18] and in the biosynthesis of discadenine,[19] a spore germination inhibitor of *Dictyostelium discoideum*. On these bases, therefore, the aryl hydroxyl of a (*p*-hydroxyphenyl)glycine residue and AdoMet may serve as reaction partners to form the homoserine portion of nocardicin A. Striking an analogy to the known conversion of isopenicillin N (**4**) to penicillin N [**4**, side chain δ-(D-α-aminoadipyl)],[8,20] inversion at the amino terminus of **1** (C-9′) is presumably a late step in the biosynthesis.

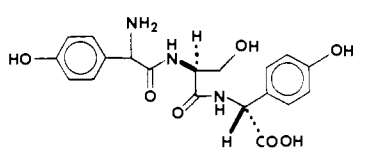
L-Tyrosine gave substantial incorporations into the two aromatic amino acid segments of nocardicin A (**1**), while L-phenylalanine did not. The carboxyl carbon of tyrosine was cleanly lost in the course of its incorporation, supporting the view that (*p*-hydroxyphenyl)glycine (PHPG) was, as expected, the true precursor. PHPG bearing ^{14}C- or ^{13}C-label at C-1 was prepared by condensation of the readily prepared bisulfite addition product of *p*-anisaldehyde with 1 equiv of labeled potassium cyanide in the presence of excess ammonium carbonate.[21] Alkaline hydrolysis of the resulting hydantoin **9** gave DL-(*p*-methoxyphenyl)glycine (PMPG) (**10/11**). Generation of the *N*-acetyl



9 **10** R=CH_3, **12** R=H **11** R=CH_3, **13** R=H

derivatives and resolution with hog kidney acylase I by a modification of the procedure of Crast[22] afforded L- and D-PMPG, **10** and **11**. Synthesis of the α-tritiated antipodes of PMPG was readily accomplished by using the method of Upson and Hruby[23] wherein the *N*-acetyl derivative of **10/11** was treated briefly with acetic anhydride and tritiated water at reflux. Resolution as above gave the α-tritiated enantiomers of PMPG which were mixed with ^{14}C-labeled amino acid of like optical purity. Demethylation in 48% hydrobromic acid[22] afforded L-[2-^{3}H,1-^{14}C]-PHPG (**12**) and D-[2-^{3}H,1-^{14}C]-PHPG (**13**) of 95% and 96% optical purity, respectively.

The intermediacy of this lower homologue of tyrosine was well supported by the high incorporation of carbon label (49.6%/unit) from doubly labeled L-PHPG. Utilization of the D enantiomer was nearly an order of magnitude less efficient, a positive incorporation that owes at least in part to small amounts of the L isomer present.[24] The specificity of labeling was established in an experiment with DL-[1-^{13}C]-PHPG that gave a sample of **1** showing marked enhancements in its ^{13}C NMR spectrum for signals at 176.5 and 166.9 ppm; resonances that had been reliably assigned[1,2] to C-10 and C-1′, respectively. The preferential processing of the L-amino acid parallels findings in penicillin biosynthesis for the antipodes of valine.[25] Furthermore, the essentially complete loss of tritium label from the α position of both enantiomers of PHPG mirrors precisely the fate of analogously labeled L- and D-valine in penicillin biosynthesis.[26] In summary, similarities are evident to what is known about the elaboration of the corresponding amino acid precursors to the Arnstein tripeptide (**3**) and isopenicillin N (**4**). Presuming formation of an analogous peptide precursor of **1**, e.g., **14** (R = H or homoseryl), its assembly may involve inversion at the carboxy terminus prior to β-lactam formation.



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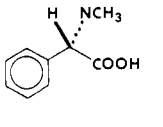

15

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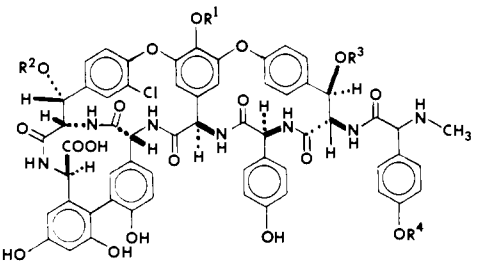
(*p*-Hydroxyphenyl)glycine is an amino acid encounted exceedingly rarely in secondary metabolism, and nothing is known of its biosynthesis. In considering possible routes for the conversion of L-tyrosine to L-PHPG, two are chemically feasible and indeed precedented: (a) carboxyl migration to the benzylic position is possible as has been demonstrated to occur in phenylalanine during the biosynthesis of both tenellin[27] and tropic acid[28] or (b) the sequence of catabolic steps may be initiated by tyrosine α,β-mutase, the isomeric β-amino acid having been observed in the peptide antibiotics edeine A and B.[29] A closer analogy, however, may be found in the work of Vining[30] on the peptide lactone etamycin produced by *Streptomyces griseoviridus*.[31] The L-α-phenylsarcosine (**15**) unit of this antibiotic was shown to be derived from L-phenylalanine with clean loss of the carboxyl carbon.[30] However, nothing further is known about this process.

As noted earlier, a similar loss of C-1 label was observed on incorporation of L-tyrosine into nocardicin A (**1**). Further catabolic pathways can be suggested: (c) transamination of tyrosine and oxidative decarboxylation to (*p*-hydroxyphenyl)acetic acid followed by hydroxylation to *p*-hydroxymandelic acid or (d) decarboxylation to tyramine, hydroxylation to β-hydroxytyramine (octopamine),[32] and subsequent oxidation to *p*-hydroxymandelate. *p*-Hydroxymandelic acid presumably can be oxidized to the 2-oxo acid, (*p*-hydroxyphenyl)glyoxylic acid, and finally transaminated to L-PHPG. Lastly, (e) hydroxylation may occur first to form either β-hydroxytyrosine or the corresponding β-hydroxy-α-keto acid, after transamination or amine oxidation, which may then be degraded to (*p*-hydroxyphenyl)mandelic acid and then to L-PHPG. Apart from the nocardicins, the only other known natural products that contain PHPG units (in some instances further modified) are the structurally similar group vancomycin,[33] isolated from *Streptomyces orientalis*, ristocetin A[34] from *Nocardia lurida*, and avoparcin (**16**).[35,36] An important clue to the possible identity of intermediates in the pathway to PHPG is contained in all of these structures, namely, the presence of both (2*S*,3*R*)- and (2*R*,3*R*)-3-hydroxytyrosine units. It is interesting to note further that a β-hydroxytyrosine unit (configurations not specified) has been found in one of the recently isolated monobactams.[4] Indeed DL-[2-^{13}C]tyrosine has been observed to label the expected methine positions of these residues in avoparcin (**16**) and most of the carboxyl carbons of the PHPG units[35] (enrichment was sufficiently low that incorporation at some carbonyl carbons could not be claimed beyond question and they may be otherwise derived). While these observations recapitulate the derivation of PHPG from tyrosine and demonstrate its expected precursor role for β-hydroxytyrosine, the biosynthetic relation of the latter to the former is not necessarily established. To that end, various possible intermediates have been prepared in radioactive form and tested for incorporation into nocardicin A. The outcome that has emerged so far from preliminary studies is that β-hydroxytyrosine and *p*-hydroxymandelic acid give positive incorporations into **1** whereas tyramine, octopamine, and *p*-hydroxyphenylacetic acid



16

R^1 = O-2- -(RISTOSAMINYL)-O- -O-GLUCOSE

R^2 = O- -RISTOSAMINE

R^3 = O- -O-MANNOSE

R^4 = O- -L-RHAMNOSE

do not.[37] While further experiments remain to be completed, these data tentatively support path e outlined above.

At this point the way was clear to examine the central question of the origin of the β-lactam ring itself in nocardicin A (**1**). Neither L-alanine nor L-cysteine was incorporated to any significant degree. While addition of cysteine at the concentration used did not appear to adversely affect the growth of the organism, levels of nocardicin A production were sharply diminished to about 10% of those observed in unsupplemented fermentations. L-Methionine, which had earlier been found to be stimulatory to production, did not reverse this effect.[38] Glycine and serine on the other hand gave good incorporations into **1**, the latter being the more efficient by a factor of 2 or 3. The comparatively higher specific incorporation of C-2-labeled glycine relative to C-1 (4.6% vs. 2.6%) supports the view that the incorporation of this amino acid takes place by way of its intermediate conversion to L-serine. It is commonly observed[39] in bacteria and fungi that serine hydroxymethyltransferase acts efficiently to carry out this conversion with the label from the C-2 position of glycine appearing directly at C-2 of serine and indirectly at C-3 by way of the C-1-pool and tetrahydrofolate. This interpretation was unambiguously borne out on ^{13}C NMR analysis of a specimen of nocardicin A (**1**) obtained by feeding [2-^{13}C]glycine in a fashion analogous to that employed in the radiochemical experiments summarized in Table I. Enhancement of resonances at 54.9 and 47.0 ppm were observed in a ratio of roughly 3:2 corresponding to C-3 and C-4, respectively.[40] In accord with expectation, the corollary experiment with DL-[3-^{13}C]serine gave enrichment at C-4 alone.

To examine the overall redox chemistry at the serine β-carbon through the course of β-lactam ring formation, we performed two double-label experiments. Wary of the substantial serine hydroxymethylase activity evident under the fermentation conditions, L-[3-^{3}H]serine was separately combined with L-[U-^{14}C]- and L-[3-^{14}C]serine. Upon incorporation into **1**, these doubly labeled specimens gave 86% and 98% retention of tritium, respectively. These results may be interpreted to show that β-lactam formation takes place without change in oxidation state at the β-carbon of serine in marked contrast to the complex oxidative chemistry acting in the cyclization of tripeptide **3** to isopenicillin N (**4**).

In conclusion, like penicillin and cephalosporin, with which it shares important stereochemical similarities, nocardicin A is entirely amino acid derived. The L isomers of methionine, serine,

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(37) Townsend, C. A.; Nguyen, L. T., unpublished results.

(38) The inhibitory effect of L-cysteine may be rationalized in a number of ways. One possibility could be that its structural similarity to L-serine (see text) may be adversely manifest at the assembly stage of a hypothetical peptide precursor or its cyclization to **1**. Neuss has observed peptide analogues of **3** in *P. chrysogenum* cultures: Neuss, N.; Miller, R. D.; Affolder, C. A. Nakatsukasa, W.; Mabe, J.; Huckstep, L. L.; De La Higuera, N.; Hunt, A. M.; Occolowitz, J. L.; Gilliam, J. H. *Helv. Chim. Acta* **1980**, *63*, 1119–1124.

(39) Prodigiosin is a similar case: Wasserman, H. H.; Sykes, R. J.; Peverada, P.; Shaw, C. K.; Cushley, R. J.; Lipsky, S. R. *J. Am. Chem. Soc.* **1973**, *95*, 6874–6875 and references cited.

(40) The published[1,2] ^{13}C assignments for the C-3 and C-5 methine carbons are incorrect and should be reversed. On the basis of single-frequency proton decoupling experiments, the correct assignments are C-3 (54.90 ppm) and C-5 (61.58 ppm).

and (*p*-hydroxyphenyl)glycine, which is in turn derived by degradation of tyrosine, serve as the most efficient amino acid precursors of the antibiotic. Presuming generation of a peptide intermediate, e.g., **14** (R = H or homoseryl), by analogy to the established role of tripeptide **3** in isopenicillin N (**4**) biosynthesis, it is proposed that the monocyclic β-lactam of nocardicin A is formed most simply and directly by nucleophilic displacement of a presumably activated seryl hydroxyl by amide nitrogen, a sequence requiring no change in oxidation state. Experiments that lend further support to this hypothesis are taken up in the accompanying paper.

Experimental Section

Melting points were determined in open capillaries with a Thomas-Hoover apparatus and are uncorrected. Spectral data were collected with use of the following spectrometers for solutions in solvents indicated in the text: IR, Perkin-Elmer Model 599B (band positions reported in reciprocal centimeters, cm^{-1}); ^1H NMR, Varian CFT-20 fitted with a proton probe to operate at 80 MHz (chemical shifts recorded in parts per million (ppm) relative to tetramethylsilane, as internal standard except for solutions in deuterium oxide where 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt hydrate (DSS) was used; coupling constants in hertz); ^{13}C NMR, Varian CFT-20 (20 MHz), 4.5-kHz spectral width, 8K data points, 0.9-s acquisition time (chemical shifts are recorded in ppm relative to Me_4Si with dioxane (δ 66.4) or deuteriochloroform (δ 77.0) as internal reference). Heteronuclear decoupling experiments observing ^{13}C were carried out at the Middle Atlantic Regional NMR Facility (University of Pennsylvania). Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter in a 1-dm quartz cell at the indicated temperature. Radioactivity determinations were made with a Beckman LS 7000 liquid scintillation counter for preweighed crystalline samples dissolved in 1 mL of 0.01 M phosphate buffer (pH 8.0) and 14 mL of Aquasol (New England Nuclear) liquid scintillation cocktail. Specific activities were computed in disintegrations per minute per millimole corrected for quenching factors and background counts. Orion Model 301 and Radiometer Model PHM62 (GK2321C electrode) pH meters were used to monitor the pH of aqueous solutions. Thin-layer chromatography (TLC) was carried out with Analtech glass plates coated (0.25 mm) with silica gel (GHLF Uniplate). Radiochemicals were purchased from Amersham or New England Nuclear. ^{13}C-Labeled substrates were obtained from Merck Sharp & Dohme, Canada, Ltd.

Approximate titers of nocardicin A were obtained by high-pressure liquid chromatography (HPLC) using a Varian 5020 microprocessor-controlled liquid chromatograph equpped with a fixed-wavelength ultraviolet detector (254 nm). Separations were carried out on a Whatman Partisil 10 PAC analytical column (4.6 × 250 mm) with 3:6:11:80 acetic acid/methanol/acetonitrile/degassed–deionized water as solvent,[12] flow rate 1–1.5 mL/min. All broths and slants were sterilized at 125 °C/20 psi for 20–30 min prior to inoculation. Liquid cultures were grown in cotton-stoppered Erlenmeyer flasks at 30 °C and 250 rpm in a New Brunswick Model G-25K gyrotory incubator shaker.

Incorporation of Labeled Precursors. *Nocardia uniformis* subsp. *tsuyamanensis* (ATCC 21806)[2,10] was maintained on yeast extract supplemented (Difco yeast extract 15 g/L) Czapex agar[41] slants and stored at 4 °C. Czapex broth[41] modified as above was inoculated with 2–3 mature colonies and incubated for 2–3 days. Transfers were then made to 125-mL seed flasks containing 25 mL of the same modified Czapex broth. After growing for 2–3 days or until a dense cell population was evident, the contents of each seed flask were used to inoculate 800 mL of fermentation broth[11] contained in a 4-L Erlenmeyer flask. Labeled precursors were administered at the same initial molar concentration (0.4 mM/unit site of incorporation) on the third day of growth in aqueous solutions after filtration through 0.2-μm filter units (Nalge/Sybron). ^{13}C-Labeled precursors were fed in the added presence of 0.4 mM L-methionine to maximize production of **1**. On the sixth day, the contents of the fermentation flasks were combined, a small amount of Celite was added, the pH was adjusted to 4.0 with 6 N hydrochloric acid, the solution was centrifuged (14 700 × *g*) for 15 min at 5 °C, and finally, the supernatants were filtered through Celite.

Isolation and Purification of Nocardicin A (1). The clarified broth was run onto a column of Amberlite XAD-4 (Rohm & Haas) essentially as previously described.[11] The column was washed with 6–7 bed volumes of distilled water, and elution of the metabolite was performed with 30% aqueous methanol. The eluate was concentrated in vacuo to $^1/_{15}$–$^1/_{20}$ of the original volume and run onto a column containing an equal volume of DEAE-Sephadex A-25 (chloride form).[10] After washing the column with 2–3 bed volumes of distilled water and 1 bed volume of 0.5% ammonium hydroxide, elution was carried out with 0.5 M aqueous sodium chloride, collecting 15-mL fractions. The fractions were assayed for nocardicin A content by analytical HPLC, the majority of the compound appearing in three or four tubes. These fractions were combined, concentrated to about 10 mL in vacuo, and desalted on Bio-Gel P-2 (Bio-Rad), collecting 13-mL fractions. The fractions were assayed by HPLC, those containing nocardicin A (if dark yellow) were recombined, and the desalting procedure was repeated. Acidification of the active fractions to pH 2.5 afforded, after refrigeration overnight, white crystals of nocardicin A (**1**), which were collected, washed with distilled water, absolute ethanol, and ether, and dried in vacuo.

Preparation of Radiolabeled Precursors. Of the radioactive amino acids shown in Table I, all but doubly labeled L- and D-(*p*-hydroxyphenyl)glycine were obtained commercially. The substrates were diluted with radioinactive material to give ^{14}C-specific activities of approximately 10^7 dpm/mmol and twice recrystallized from water or aqueous ethanol. Specific activities were determined for the amino acids themselves for L-methionine, L-tyrosine, and L-phenylalanine; for the *N*-tosyl derivatives recrystallized from aqueous ethanol for L-homoserine, L-alanine, glycine, and L-serine; and for cystine obtained by oxidation for L-cysteine. The latter was accomplished by dissolving L-[U-^{14}C]cysteine (75 mg, 0.62 mmol) in 10 mL of deionized water at pH 9 by the addition of 1 N sodium hydroxide and passing a stream of air through the solution overnight. The concentrated suspension was adjusted to pH 5.5 with 2 N hydrochloric acid and chilled in an ice bath for several hours. The white powder so obtained was washed with cold deionized water, absolute ethanol, and ether to afford 60 mg (80%) of [U-^{14}C]cystine; mp (sealed capillary) 258° C dec (lit.[42] mp 260° C dec).

Preparation of L- and D-[2-^3H,1-^{14}C](*p*-Hydroxyphenyl)glycine (12/13). (a) DL-[1-^{14}C](*p*-Methoxyphenyl)glycine (10/11). The method described is a modification of that reported by Bucherer and Steiner.[21] A 500-mL round-bottom flask fitted with a reflux condenser and containing a stirred suspension of the sodium bisulfite addition product of *p*-anisaldehyde (12.0 g, 50 mmol), ammonium carbonate (22.8 g, 200 mmol), and carrier potassium cyanide (3.30 g, 50 mmol) in 190 mL of 50% (v/v) aqueous ethanol was charged with a solution of 1 mCi of potassium [^{14}C]cyanide (61.2 mCi/mmol) dissolved in 1 mL of the above solvent. After heating the solution for 3–4 h at 50–60 °C, the temperature was raised slowly to and maintained at 80–90 °C for 1 h. Evaporation of the solvent afforded the crude known[43] hydantoin **9**, which was heated to reflux in 75 mL of 3 N sodium hydroxide for 24 h. Acidification to pH 5.5 with 4 N hydrochloric acid with constant swirling of the solution in an ice bath yielded DL-[1-^{14}C](*p*-methoxyphenyl)glycine (**10/11**). This crude material was collected by filtration, dissolved in 3 N sodium hydroxide, and treated three times with activated carbon (Norit A, Baker). The carbon was filtered on Celite, and an equal volume of 95% ethanol was added to the filtrate. Acidification to pH 5.5 of the ethanolic solution with 4 N hydrochloric acid afforded white crystals that were filtered, washed with cold absolute ethanol and ether, and dried in vacuo to give 4.59 g (51%); mp 266–268 °C dec (lit.[43] mp 284–285 °C dec, lit.[44] mp 264–265 °C dec); IR (KBr) 2915, 2650, 1580, 1515, 1455, 1425, 1400, 1355, 1255, 1185, 1030, 910, 825, 800 cm^{-1}; ^1H NMR (D_2O/DCl, pD ~ 1) δ 2.56 (s, 3 H, OMe), 5.79 (d, *J* = 9.0, 2 H), 6.18 (d, *J* = 9.0, 2 H).

(b) Resolution of DL-[1-^{14}C](*p*-Methoxyphenyl)glycine (10/11). The racemic [1-^{14}C]amino acid **10/11** was converted to its *N*-acetyl derivative: mp 210–212 °C (see below); IR (KBr) 3325, 2925, 2825, 1710, 1590, 1535, 1510, 1485, 1460, 1445, 1305, 1265, 1185, 1175, 825 cm^{-1}; ^1H NMR (Me_2SO-d_6/$CDCl_3$) δ 2.00 (s, 3 H, CH_3), 3.78 (s, 3 H, OMe), 5.42 (d, *J* = 7.2, 1 H, H-2), 6.84 (d, *J* = 8.8, 2 H), 7.34 (d, *J* = 8.8, 2 H), 7.65–7.90 (br d, *J* ~7.2, 1 H, NH). Resolution of *N*-acetyl-DL-(*p*-methoxyphenyl)glycine was carried out by a modification of the procedure of Crast[22] as follows. A portion of the labeled *N*-acetyl amino acid (1.50 g, 6.73 mmol) was diluted with carrier (7.50 g, 33.6 mmol) and dissolved in 180 mL of distilled water by adjustment of the pH to 7.8 with concentrated ammonium hydroxide. After passage through a 0.2-μm filter unit (Nalge/Sybron), 20-mL aliquots of the solution were dispensed in 250-mL Erlenmeyer flasks. Hog kidney acylase I (53 mg; Sigma, Grade I) was added to each flask, the pH of each was checked and readjusted to 7.8 with 6 N ammonium hydroxide as necessary, and they were incubated at 37 °C at 100 rpm. After 24 h, an additional 15 mg of the enzyme was added to each flask, and incubation was continued for

(41) "Difco Manual", 9th ed.; Difco Laboratories: Detroit, MI, 1953; p 245.

(42) "CRC Handbook of Tables for Organic Compound Identificiation", 3rd ed.; CRC Press: Cleveland, OH, 1967; p 287.

(43) Harvill, E. K.; Herbst, R. M. *J. Org. Chem.* **1944**, *9*, 21–30.

(44) Neims, A. H.; DeLuca, D. C.; Hellerman, L. *Biochemistry* **1966**, *5*, 203–212.

another 24 h. The flasks were refrigerated overnight before the solids containing crude L-[1-^{14}C](*p*-methoxyphenyl)glycine (PMPG) (**10**) were filtered and washed with cold water. Crude **10** was treated with 22.5 mL of hot 3 N hydrochloric acid and activated carbon (Norit A, Baker). Hot filtration and readjustment to pH 5.5 with 6 N ammonium hydroxide provided 2.29 g (68%) of L-[1-^{14}C]-PMPG (**10**); $[\alpha]^{25}_D$ 139.5° (*c* 1.0, 1 N HCl) [lit.[22] $[\alpha]^{25}_D$ 150.4° (*c* 1.0, 1 N HCl)]; specific activity ~1.2 × 10^7 dpm/mmol.

The filtrate above and water washes 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 water, and the resulting filtrate was adjusted to pH 2 with 40% phosphoric acid. After cooling the solution for 1 h at 0 °C, the crystalline *N*-acetyl-D-[1-^{14}C]-PMPG was collected, washed with water, and heated to reflux in 32 mL of 2 N hydrochloric acid for 1.5 h. The warm solution was treated with activated carbon, filtered, and cooled before adjusting to pH 5.5 with 6 N ammonium hydroxide. The resulting colorless crystalline product was filtered, washed with cold distilled water and ethanol, and dried under vacuum to afford 2.05 g (61%); $[\alpha]^{25}_D$ −138.6° (*c* 1.0, 1 N HCl) [lit.[22] $[\alpha]^{25}_D$ −149.9° (*c* 1.0, 1 N HCl)]; specific activity ~ 1.2 × 10^7 dpm/mmol.

(c) *N*-Acetyl-DL-[2-^{3}H](*p*-methoxyphenyl)glycine. The procedure of Upson and Hruby[23] was modified as follows. A 50-mL round-bottomed flask containing a stirred suspension of D,L-PMPG (**10**/**11**) (1.81 g, 10 mmol) in 21.7 mL of acetic anhydride was treated with 200 μL of tritiated water (100 mCi/g). The suspension was heated to vigorous reflux in a 170 °C silicon oil bath for 3–4 min. After cooling the solution to room temperature, 20 mL of distilled water was added and the solvents were removed in vacuo. The yellow residue was redissolved in 25 mL of distilled water, concentrated to dryness two times to remove readily exchangeable tritium, redissolved a third time, and extracted several times with 20-mL portions of ether. Removal of the water at the rotary evaporator gave an off-white solid that was dissolved in absolute ethanol, treated with activated carbon (Norit A, Baker), and filtered, and the solution was concentrated to 10–15 mL. Two or three volumes of distilled water were added, and the solution was refrigerated overnight, whereupon the colorless, fine crystalline product was filtered and washed with water and ether; 1.05 g (47%), mp 210–211 °C.

An analogous experiment conducted with DL-PMPG (**10**/**11**) that had been preequilibrated with deuterium oxide (99.8% D) and lyophilized but in the presence of deuterium oxide (2.5 mL) rather than tritiated water gave on workup a product whose ^{1}H NMR spectrum revealed no detectable hydrogen at C-2 and an exchange of about two-thirds of the acetyl methyl hydrogens.

(d) Resolution of DL-[2-^{3}H](*p*-Methoxyphenyl)glycine. As the acetyl group of *N*-acetyl-DL-[2-^{3}H]-PMPG contained tritium in unknown proportion to the label at C-2, resolution of the tritiated amino acid was carried out separately from the ^{14}C-labeled material but in the same manner as described above with the following results. L-[2-^{3}H]-PMPG (**10**): 210 mg (49%); $[\alpha]^{25}_D$ 141.4° (*c* 1.0, 1 N HCl); specific activity 5.74 × 10^7 dpm/mmol. D-[2-^{3}H]-PMPG (**11**): 116 mg (27%); $[\alpha]^{25}_D$ −141.0° (*c* 1.0, 1 N HCl); specific activity 5.79 × 10^7 dpm/mmol.

(e) D-[2-^{3}H,1-^{14}C](*p*-Hydroxyphenyl)glycine (13). D-[2-^{3}H]-PMPG (**11**) (101 mg, 0.558 mmol, 5.79 × 10^7 dpm/mmol), D-[1-^{14}C]-PMPG (**11**) (100 mg, 0.552 mmol, ~1.2 × 10^7 dpm/mmol), and carrier D-PMPG (800 mg, 4.42 mmol) were heated to reflux in 8 mL of 48% hydrobromic acid for 2 h.[22] The acid was removed in vacuo, 1.5 mL of distilled water was added to the residue, and 6 N ammonium hydroxide was added to pH 5 with cooling to afford a pink gel. Warming to 50 °C converted the gel into a crystalline form. After several hours of cooling the solution at 0 °C, the crystals were filtered, dissolved in the minimum amount of hot distilled water, treated two times with activated carbon, and crystallized by the addition of absolute ethanol. After cooling the solution overnight, the colorless product was filtered and washed with cold ethanol and ether to afford 715 mg (77%) of **13**: mp 224–228 °C dec (lit.[22] mp 223 °C dec, lit.[45] mp 223–225 °C dec, lit.[46] mp 225 °C dec); $[\alpha]^{25}_D$ −155.5° (*c* 1.0, 1 N HCl) [lit.[22] $[\alpha]^{25}_D$ −161.2°, lit.[45] $[\alpha]^{23}_D$ −158° (both *c* 1.0, 1 N HCl)]; specific activity (^{3}H) 5.69 × 10^6 dpm/mmol, (^{14}C) 1.28 × 10^6 dpm/mmol; ^{1}H NMR (D_2O/DCl pD ~ 1) δ 4.06 (s, 1 H, H-2), 5.86 (d, *J* = 8.8, 2 H), 6.26 (d, *J* = 8.8, 2 H); ^{13}C NMR (D_2O/NaOD, pD ~ 7.2) δ 58.8, 116.9, 126.8, 130.5, 157.4, 174.6.

(f) L-[2-^{3}H,1-^{14}C](*p*-Hydroxyphenyl)glycine (12). L-[2-^{3}H]-PMPG (**10**) (104 mg, 0.574 mmol, 5.74 × 10^7 dpm/mmol), L-[1-^{14}C]-PMPG (**10**) (103 mg, 0.568 mmol, ~1.2 × 10^7 dpm/mmol), and carrier L-PMPG (800 mg, 4.42 mmol) were transformed and purified as above to afford **12**: 820 mg (89%); mp 225–228 °C dec; $[\alpha]^{25}_D$ 153.2° (*c* 1.0, 1 N HCl) [lit.[22] $[\alpha]^{25}_D$ 161.2° (*c* 1.0, 1 N HCl)]; specific activity (^{3}H) 5.59 × 10^6 dpm/mmol, (^{14}C) 1.26 × 10^6 dpm/mmol.

Preparation of ^{13}C-Labeled Precursors. [2-^{13}C]Glycine and DL-[3-^{13}C]serine were used as received. Potassium [^{13}C]cyanide was converted to DL-[1-^{13}C](p-hydroxyphenyl)glycine without intermediate resolution as described above in 57% overall yield: mp (sealed capillary) 227–228 °C dec (lit.[44] mp 229–230 °C dec, lit.[43] mp 194–195 °C dec); ^{1}H NMR (D_2O/DCl, pD ~ 1) δ 4.30 (d, $^2J_{C-H}$ = 6.4, 1 H, H-2), 6.12 (d, *J* = 8.8, 2 H); ^{13}C NMR (D_2O/NaOD, pD ~ 7.6) δ 174.6.

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Registry No. 1, 39391-39-4; [^{14}C]-**9**, 83478-22-2; [1-^{14}C]-**10**, 83478-23-3; [2-^{3}H]-**10**, 83478-24-4; [1-^{14}C]-**11**, 83478-25-5; [2-^{3}H]-**11**, 83478-26-6; **12**, 32462-30-9; [2-^{3}H,1-^{14}C]-**12**, 83478-27-7; [2-^{3}H,1-^{14}C]-**13**, 83478-28-8; **14**, 83478-29-9; DL-PMPG, 19789-59-4; DL-[1-^{14}C]-PMPG, 83572-04-7; *N*-acetyl-DL-[1-^{14}C]-PMPG, 83478-30-2; *N*-acetyl-DL-[2-^{3}H]-PMPG, 83478-31-3; L-methionine, 63-68-3; L-tyrosine, 60-18-4; β-hydroxytyrosine, 6809-69-4; *p*-hydroxymandelic acid, 1198-84-1; bisulfite addition product of *p*-anisaldehyde, 33402-67-4; L-serine, 56-45-1.

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