Biosynthetic Studies of Nocardicin A

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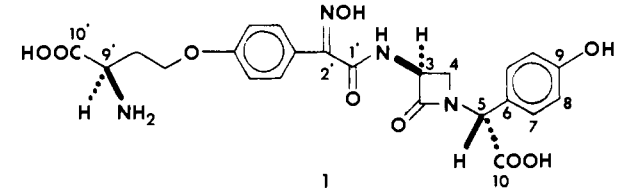
Nocardicin A (**1**) is the prototype[1] of one of three recently discovered families of β-lactam-containing compounds of microbial origin, the others being represented by the oxypenam clavulanic acid (**2**)[2] and the carbapenem thienamycin **3**.[3] The unusual ether-linked homoserine unit, oxime, and (*p*-hydroxyphenyl)glycine segments in **1** present a number of problems of biosynthetic interest, but in particular the structural simplicity of the monocyclic β-lactam offers a well-defined opportunity to approach the important general question of β-lactam formation in vivo. We wish to report our findings in this latter regard for nocardicin A (**1**)[4] and to record results that in certain details reveal marked similarities to penicillin biosynthesis and which in sum suggest an analogous peptide origin for **1**.



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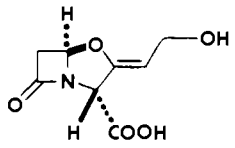

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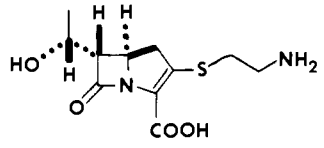

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Radiolabeled potential precursors of **1** were administered at the same initial molar concentration/precursor unit incorporated to vigorously shaken flasks of *Nocardia uniformis tsuyamanensis* (ATCC 21806) on the third day of growth. On the sixth day the mycelia were harvested, and the metabolite was isolated from the clarified broths by successive chromatography on Amberlite XAD-4 and DEAE Sephadex A-25, desalted on Biogel P-2, and crystallized at pH 2.5. The incorporation data obtained are summarized in Table I for specimens of nocardicin A (**1**) recrystallized to constant specific radioactivity.

L-Tyrosine gave substantial incorporations into the two aromatic amino acid segments (vide infra) of nocardicin A (**1**), whereas L-phenylalanine did not. The clean loss of C-1 from tyrosine in the course of these events supports the view that, as expected, the true precursor is (*p*-hydroxyphenyl)glycine (**4**) (PHPG). The intermediacy of this lower homologue is well supported by the high incorporation of carbon label, 49.6% per unit, from doubly labeled L-(*p*-hydroxyphenyl)glycine.[6] The lower rate of incorporation of the corresponding D-(*p*-hydroxyphenyl)glycine mirrors findings in penicillin biosynthesis for the antipodes of valine.[7,8] Similarly the essentially complete loss of tritium label from the α position of both enantiomers of (*p*-hydroxyphenyl)glycine exactly parallels the fate of analogously labeled L- and D-valine in penicillin biosynthesis.[9] In keeping with what is now known[10] about the formation of the Arnstein tripeptide **5** [R′ = δ-(L-α-aminoadipyl)], biosynthesis of a hypothetical peptide precursor, e.g., **7** (R = H or homoseryl), of **1** may involve inversion of configuration at the carboxy terminus during peptide assembly and prior to β-lactam formation.

Table I.

amino acid[a]	^{14}C spec incorp/ unit	$^{3}H/^{14}C$ amino acid	**1**	($\%$ ^{3}H retained)
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]homoserine	1.5			
L-[1-^{14}C]Met	19.6			
[1-^{14}C]Gly	2.6			
[2-^{14}C]Gly	4.6			
L-[U-^{14}C]Ser	7.8			
L-[U-^{14}C]Ala	0.07			
L-[U-^{14}C]Cys	0.01			
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	3.70	(99)

[a] Specific radioactivities and $^{3}H/^{14}C$ ratios were determined for the amino acids themselves and/or appropriate derivatives crystallized to constant specific activity. All precursors were fed at the level of 0.4 mmol/L/site of incorporation to fermentations grown at 30 °C. [b] PHPG = (*p*-hydroxyphenyl)glycine. The optical purities[5] of the doubly labeled L- and D-PHPG were 95% and 96%, respectively. Unlike the other experiments reported in Table I, the enantiomers of PHPG were fed in the presence of 1 mM L-methionine to maximize the production of **1**, vide infra.

Some minor metabolites[1] of the nocardicin family, e.g., nocardicin G (**8**) (R = H), do not contain the homoserine unit which implies, but in no way proves, that the nonpeptide-linked amino acid is unnecessary for β-lactam formation. In the event, although L-homoserine itself showed a positive incorporation into nocardicin A (**1**), L-methionine was incorporated at a far higher level and, moreover, showed a twofold stimulatory effect on production of the antibiotic. These data suggest that methionine or *S*-adenosylmethionine, in perhaps a pyridoxal phosphate dependent γ-replacement reaction[11] or in a simple S_N2 displacement,[12] serves

(1) Hashimoto, M.; Komori, T.; Kamiya, T. *J. Am. Chem. Soc.* **1976**, *98*, 3023–3025. *J. Antibiot.* **1976**, *29*, 890–901. Hosoda, J.; Konomi, T.; Tani, N.; Aoki, H.; Imanaka, H. *Agric. Biol. Chem.* **1977**, *41*, 2013–2020.

(2) Howarth, T. T.; Brown, A. G.; King, T. J. *J. Chem. Soc., Chem. Commun.* **1976**, 266–267.

(3) Albers-Schönberg, G.; Arison, B. H.; Hensens, O. D.; Hirschfield, J.; Hoogsteen, K.; Kaczka, E. A.; Rhodes, R. E.; Kahan, J. S.; Kahan, F. M.; Ratcliffe, R. W.; Walton, E.; Ruswinkle, L. J.; Morin, R. B.; Christensen, B. G. *J. Am. Chem. Soc.* **1978**, *100*, 6491–6499.

(4) These results largely confirm and substantially extend preliminary findings of workers at Fujisawa Pharmaceutical Co., Ltd.: Hosoda, J.; Tani, N.; Konomi, T.; Ohsawa, S.; Aoki, H.; Imanaka, H. *Agric. Biol. Chem.* **1977**, *41*, 2007–2012.

(5) Crast, L. B., Jr. (Bristol-Myers) U.S. Patent 3 489 750.

(6) The specificity of labeling was established in a subsequent experiment with D,L-[1-^{13}C]PHPG which gave a sample of nocardicin A (**1**) showing marked enhancements in its ^{13}C NMR spectrum for signals at 176.5 and 166.9 ppm, resonances which have been reliably assigned to carbons 10 and 1′, respectively.

(7) Stevens, L. M.; Inamine, E.; DeLong, C. W. *J. Biol. Chem.* **1956**, *219*, 405–409. Stevens, C. M.; DeLong, C. W. *Ibid.* **1958**, *230*, 991–999. Arnstein, H. R. V.; Clubb, M. E. *Biochem. J.* **1957**, *65*, 618–627. Arnstein, H. R. V.; Margreiter, H. *Ibid.* **1958**, *68*, 339–348.

(8) The low but positive incorporation of D-PHPG is almost certainly due in part to incorporation of small amounts of the L isomer present (see footnote b, Table I), but in any event this result indicates a low rate of enzymic racemization and/or transport in marked contrast to facile valine racemization observed in *Penicillium* species.[7,9]

(9) Bycroft, B. W.; Wels, C. M.; Corbett, K.; Maloney, A. P.; Lowe, D. A. *J. Chem. Soc., Chem. Commun.* **1975**, 923–924. See also: Adriaens, P.; Vanderhaeghe, H.; Meesschaert, B.; Eyssen, H. *Antimicrob. Agents Chemother.* **1975**, *8*, 15–17.

(10) Fawcett, P. A.; Usher, J. J.; Huddleston, J. A.; Bleaney, R. C.; Nisbet, J. J.; Abraham, E. P. *Biochem. J.* **1976**, *157*, 651–660. O'Sullivan, J.; Bleaney, R. C.; Huddleston, J. A.; Abraham, E. P. *Ibid.* **1979**, *184*, 421–426. Konomi, T.; Herchen, S.; Baldwin, J. E.; Yoshida, M.; Hunt, N. A.; Demain, A. L. *Ibid.* **1979**, *184*, 427–430. Sawada, Y.; Baldwin, J. E.; Singh, P. D.; Solomon, N. A.; Demain, A. L. *Antimicrob. Agents Chemother.* **1980**, *18*, 465–470. Adriaens, P.; Vanderhaeghe, H. *FEMS Microbiol. Lett.* **1978**, *4*, 19–21. Meesschaert, B.; Adriaens, P.; Eyssen, H. *J. Antibiot.* **1980**, *33*, 722–730.

(11) For example, the γ-replacement process catalyzed by β-cystathionase has been recently shown to occur with retention of stereochemistry at the γ carbon: Chang, M. N. T.; Walsh, C. T. *J. Am. Chem. Soc.* **1980**, *102*, 7368–7370.

(12) Although no stereochemical information presently exists, such a mechanism has been suggested for spermidine/spermine biosynthesis: Tabor, C. W.; Tabor, H. *Ann. Rev. Biochem.* **1976**, *45*, 285–306.

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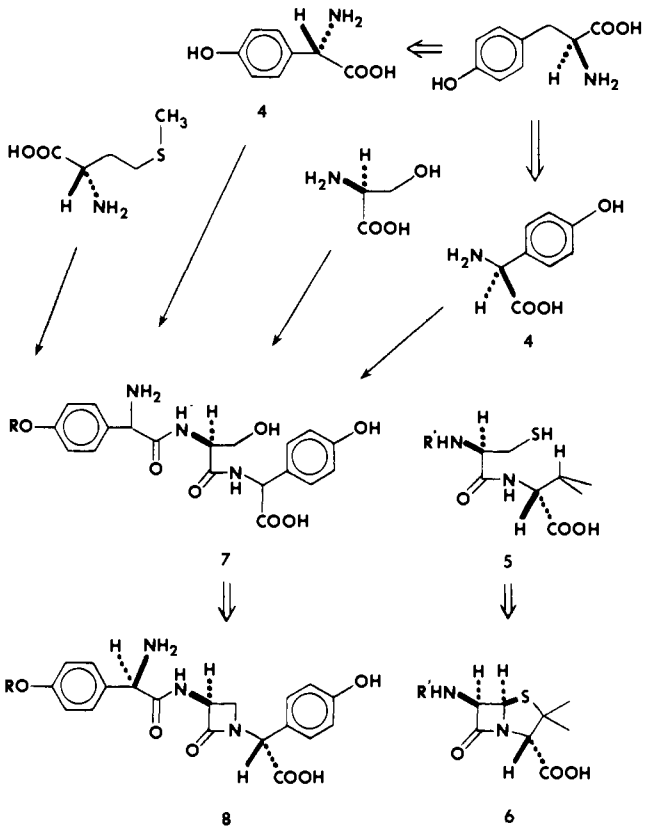

as the reaction partner to form this ether linkage. Inversion at the amino terminus (C-9′) in **1**, as is the case in the conversion of isopenicillin N (**6**) [R′ = δ-(L-α-aminoadipyl)] to penicillin N (**6**) [R′ = δ-(D-α-aminoadiphyl)], is presumably a late step in the pathway.

Neither L-alanine nor L-cysteine were incorporated to any significant degree into nocardicin A (**1**). However, it is interesting to note that the level of production of **1** was markedly reduced in the presence of cysteine to about 10% of that in the unsupplemented fermentation, an effect that was not reversed by added methionine. Glycine and L-serine on the other hand gave good incorporations into **1**, the latter being two to three times more efficient than the former. The positive incorporation of glycine almost certainly takes place by way of intermediate conversion to serine. This interpretation is supported by the relatively higher rate of incorporation of C-2 vs. C-1 labeled glycine where it is well-known[13] that radiolabel from C-2 will enrich the C_1 pool and through the action of serine hydroxymethyltransferase lead to the de novo synthesis of labeled serine in addition to that derived directly from labeled glycine. This ready conversion was unmistakably 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 for the radiochemical studies summarized in Table I.[14] Appreciable enhancements of two resonances at 54.9 and 47.0 ppm were observed in the ^{13}C NMR in a ratio of roughly 3:2 corresponding to C-3 and C-4, respectively.[15] As expected, the corollary experiment with D,L-[3-^{13}C]serine[14] gave enrichment at C-4 alone.

Bearing in mind the apparently substantial serine hydroxymethylase activity present under the fermentation conditions, two double label experiments were carried out to investigate the overall redox chemistry at the serine β carbon during β-lactam ring formation. L-[3-^{3}H]Serine admixed separately with L-[U-^{14}C]- and L-[3-^{14}C]serine gave, respectively, 86% and 99% retention of tritium label on incorporation into nocardicin A (**1**). These results indicate that β-lactam formation takes place *without* change in oxidation state at the β carbon in sharp contrast to the complex oxidative chemistry acting in penicillin biosynthesis, *cf.* **5** → **6** and **7** → **8**.

In conclusion, like penicillin and cephalosporin with which it shares important stereochemical similarities, nocardicin A is entirely amino acid derived, the L-enantiomers of methionine, serine, and (*p*-hydroxyphenyl)glycine serving as the most direct precursors. The monocyclic β-lactam in nocardicin A is apparently formed simply and directly by nucleophilic displacement of a presumably activated seryl hydroxyl[16] by amide nitrogen, a sequence requiring no change in oxidation state. The roles of nucleophile and electrophile may by played again in more complex fashion in the corresponding cyclizations of **5** to penicillin **6**.

Acknowledgment. We are indebted to L. T. Nguyen for several preparations and resolution of the doubly labeled specimens of (*p*-hydroxyphenyl)glycine used in this work and Eli Lilly and Co. and Fujisawa Pharmaceuticals, Ltd., for analytical samples of nocardicin A sodium salt. We gratefully acknowledge the financial support of The Natural Institutes of Health (5 R01 AI14937 and 5 S07 RR07041), the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the National Fellowship Fund (AMB). Single-frequency proton decoupling experiments were carried out at the Middle Atlantic Regional NMR Facility.

(13) 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.

(14) This experiment was additionally carried out in the presence of 1 mM L-methionine to maximize the production of nocardicin A (**1**) (see footnote b, Table I).

(15) The published[1] ^{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).

(16) Phosphorylation is an attractive possibility for hydroxyl activation which has received support in these laboratories in a biomimetic synthesis of 3-aminonocardicinic acid (Townsend, C. A.; Nguyen, L. T., unpublished results).

Trapping and Chirality as Evidence for an Allene Structure for 2,3,6-Bicyclo[3.2.1]octatriene

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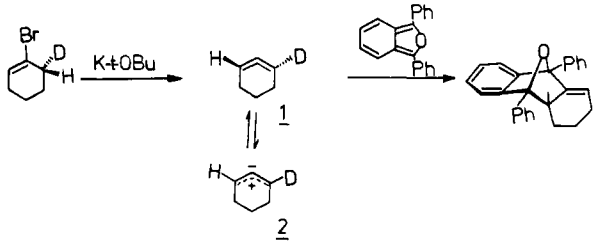
We have recently reported[1] evidence that has led us to the conclusion that, contrary to expectation,[2] at 53 °C the preferred structure of the intermediate formed from base-induced dehydrobromination of 1-bromocyclohexene is better represented by the twisted allene **1** than the less strained dipolar structure **2**. This



conclusion was based on the formation of optically active adduct from dehydrobromination of optically active 1-bromocyclo-

(1) Balci, M.; Jones, W. M. *J. Am. Chem. Soc.* **1980**, *102*, 7607.

(2) Greenberg, A.; Liebman, J. F. "Strained Organic Molecules"; Academic Press: New York, 1978; pp 126-130. Dillon, P. W.; Underwood, G. R. *J. Am. Chem. Soc.* **1974**, *92*, 779-787. Moore, W. R.; Moser, W. R. *J. Am. Chem. Soc.* **1970**, *92*, 5469-5474. See also: Bottini, A. T.; Cabral, L. J.; Dev, V. *Tetrahedron Lett.* **1977**, 165-168 and references cited therein.

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