methoxyindene, kindly provided by Prof. F. D. Lewis, was crystallized from ethanol [mp 79–80 °C (lit.[19] 78–80.5 °C)]. The preparations of 3-phenyl-, 2-phenyl-, and 1,2,3-triphenylindenes have been described earlier.[20] We thank Dr. Olle Matsson for samples of 3-methylindene and 5-nitro-1-methylindene.

3-*tert*-Butylindene. Reaction of 1-indanone with *tert*-butylmagnesium chloride and subsequent dehydration as described by Weidler[21] gave a mixture of 1-indanone and 3-*tert*-butylindene. Purification by column chromatography over silica gel gave 3-*tert*-butylindene, which was 97% pure by GLC analysis: ^{1}H NMR δ 7.75–7.10 (m, 4 H), 6.21 (m, 1 H), 3.29 (m, 2 H), 1.38 (s, 9 H); MS *m/e* (relative intensity) 173 (3), 172 (18), 157 (15), 142 (9), 116 (27), 57 (100).

3-Cyanoindene. Dehydration of 1*H*-indene-3-carboxamide to 3-cyanoindene was accomplished following the procedure described by Casini:[22] bp 120 °C (0.5 mm) (lit.[22] bp 135–137 °C (7 mm)).

3-Piperidinylindene. This enamine was prepared by azeotropic removal of water from a mixture of 1-indanone and piperidine (2 equiv) in benzene. The crude residue obtained after removal of solvent was distilled under reduced pressure. The 3-piperidinylindene was collected between 83–88 °C (0.05 mm): ^{1}H NMR δ 7.60–7.08 (m, 4 H), 5.51 (m, 1 H), 3.29 (m, 2 H), 3.06 (t, 4 H, 5 Hz), 1.69 (m, 6 H).

3-Morpholinylindene was prepared from 1-indanone and morpholine as described above: bp 100 °C (0.08 mm); ^{1}H NMR δ 7.52–7.06 (m, 4 H), 5.50 (t, 1 H, 2.5 Hz), 3.87 (t, 4 H, 5 Hz), 3.30 (m, 2 H), 3.05 (t, 4 H, 5 Hz). Enamines obtained from 1-indanone are extremely air sensitive.

2-Bromoindene. Indene was converted to indene bromohydrin and further dehydrated to 2-bromoindene following the procedure described by MacDowell:[23] bp 66 °C (0.08 mm) (lit.[23] bp 135 °C (18 mm); ^{1}H NMR δ 7.45–6.80 (m, 4 H), 6.73 (s, 1 H), 3.38 (s, 2 H); MS *m/e* (relative intensity) 196 (19), 194 (19.6), 116 (10), 115 (100).

Acknowledgment. We are grateful to the National Science Foundation for support of this research.

(19) Eliasson, B.; Edlund, U. *J. Chem. Soc., Perkin Trans. II* **1981**, 403–408.
(20) Bordwell, F. G.; Drucker, E. G. *J. Org. Chem.* **1980**, *45*, 3325–3328.
(21) Weidler, A.-M. *Acta Chem. Scand.* **1963**, 2724–2734.
(22) Campagna, F.; Carotti, A.; Casini, G. *Tetrahedron Lett.* **1977**, 1813–1816.
(23) Lindley, W. A.; MacDowell, D. W. H. *J. Org. Chem.* **1982**, *47*, 705–709.

Stereochemistry of C-3 Deoxygenation of Sugar Nucleosides: Formation of Pentopyranine C from [3-^{2}H]-D-Glucose by *Streptomyces griseochromogenes*

Steven J. Gould* and Jincan Guo

Contribution from the Department of Chemistry, Oregon State University, Corvallis, Oregon 97331-4003. *Received July 8, 1991. Revised Manuscript Received August 3, 1992*

Abstract: Cytosylglucuronic acid (CGA) has previously been shown to be the first intermediate in the biosynthesis of the antibiotic blasticidin S (BS), produced by *Streptomyces griseochromogenes*. Addition of aminooxyacetic acid (AOAA), an inhibitor of pyridoxal phosphate/pyridoxamine phosphate-dependent transaminases, to *S. griseochromogenes* fermentations led to substantial accumulations of CGA and pentopyranine C (PPNC, a shunt metabolite which has undergone decarboxylation at C-5′, deoxygenation at C-3′, and epimerization at C-4′) and to substantial reductions in the production of BS and *N*-demethylBS. In contrast, inhibitors of glutamine-dependent amidotransferases had little effect. [3-^{2}H]-D-Glucose was fed to a fermentation of *S. griseochromogenes* containing arginine hydroxamate, an inhibitor of arginine biosynthesis, and a large quantity of cytosine—currently the best conditions for maximum production of CGA and PPNC. This yielded cytosyl[3′-^{2}H]glucuronic acid, an 85:15 mixture of [3′-2H$_{axial}$]- and [3′-^{2}H$_{equatorial}$]PPNC, and a small amount of a 46:54 mixture of [3′-^{2}H$_{axial}$]- and [3′-^{2}H$_{equatorial}$]pentopyranone (the immediate precursor to PPNC). The relationship of this C-3 sugar deoxygenation to blasticidin S biosynthesis and to the pyridoxamine phosphate-dependent CDP-4-keto-6-deoxy-D-glucose-3-dehydrase reaction which is central to cell-wall lipopolysaccharide biosynthesis of Gram-negative bacteria is discussed.

Blasticidin S (**1**), an antifungal antibiotic produced by *Streptomyces griseochromogenes* that is used commercially for the control of *Piricularia oryzae* (rice blast), was first isolated by Takeuchi et al. in 1958.[1] Its structure and absolute stereochemistry were elucidated by chemical means[2-5] and confirmed by X-ray diffraction.[6,7] Seto et al.[8] established that **1** is biosynthesized from cytosine (**2**), D-glucose (**3**), L-α-arginine (**4**), and L-methionine (**5**). Isolation of a number of structurally related metabolites from *S. griseochromogenes* has also been reported.[9-14] We have shown that **4** is converted to L-β-arginine (**6**) and have established the stereochemistry of the arginine 2,3-aminomutase reaction.[15] We have also demonstrated,[16] using cell-free extracts

(1) Takeuchi, S.; Hirayama, K.; Ueda, K.; Sakai, H.; Yonehara, H. *J. Antibiot.* **1958**, *11*, 1.
(2) Fox, J. J.; Watanabe, K. A. *Tetrahedron Lett.* **1966**, 897.
(3) Yonehara, H.; Otake, N. *Tetrahedron Lett.* **1966**, 3785.
(4) Otake, N.; Takeuchi, S.; Endo, T.; Yonehara, H. *Agric. Biol. Chem.* **1966**, *30*, 126.
(5) Otake, N.; Takeuchi, S.; Endo, T.; Yonehara, H. *Agric. Biol. Chem.* **1966**, *30*, 132.
(6) Onuma, S.; Nawata, Y.; Saito, Y. *Bull. Chem. Soc. Jpn.* **1966**, *39*, 1091.
(7) Swaminathan, V.; Smith, J. L.; Sundaralingam, M.; Coutsogeorgopoulos, C.; Kartha, G. *Biochim. Biophys. Acta* **1981**, *655*, 335.
(8) Seto, H.; Yamaguchi, I.; Otake, N.; Yonehara, H. *Agric. Biol. Chem.* **1968**, *32*, 1292.
(9) Seto, H.; Yonehara, H. *J. Antibiot.* **1977**, *30*, 1019.
(10) Seto, H.; Yonehara, H. *J. Antibiot.* **1977**, *30*, 1022.
(11) Seto H.; Furihata, K.; Yonehara, H. *J. Antibiot.* **1976**, *29*, 595.
(12) Seto, H. *Agric. Biol. Chem.* **1973**, *37*, 2415.
(13) Seto, H.; Otake, N.; Yonehara, H. *Agric. Biol. Chem.* **1973**, *37*, 2421.
(14) Guo, J.; Gould, S. J. *Phytochemistry* **1992**, in press.

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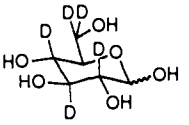
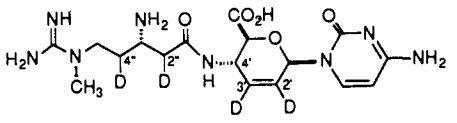
of *S. griseochromogenes*, that the first step in the nucleoside portion of the pathway is the reaction of UDP-glucuronic acid (**7**) and **2** to give cytosylglucuronic acid (**8**)[11] and that N-methylation of *N*-demethylblasticidin S (**9**)[10] is the last step in the pathway (Scheme I).[17] We now report studies of the stereochemistry of C-3 deoxygenation leading to a cometabolite, pentopyranine C (**10**),[12,13] and its relevance to blasticidin S biosynthesis and to cell-wall lipopolysaccharide biosynthesis in various Gram-negative bacteria.

Results and Discussion

Our previous studies[18] have shown that incorporation of [2,3,4,6,6-$^2H_5$]-D-glucose (**11a**) yielded **1a** with the apparent retention of deuterium at H-2′ and H-3′ but loss of the deuterium label at H-4′. To investigate the possible involvement of hydride



11a

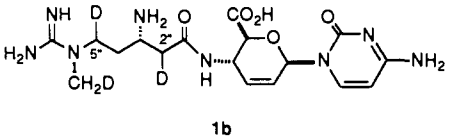


1a

transfers, such as those involved in the biosynthesis of swainsonine,[19] we subsequently performed fermentations in the presence of [1,1-$^2H_2$]ethanol (**12a**) as an in vivo source of NAD^{2}H and NADP^{2}H. The deuterium at C-1 of **12a** can only be utilized via the nicotinamide cofactors; therefore, no direct incorporation into primary precursors would be possible. This led to the labeling pattern shown in **1b**. The deuterium enrichments at H-2″, H-5″,

CH_3CD_2OH

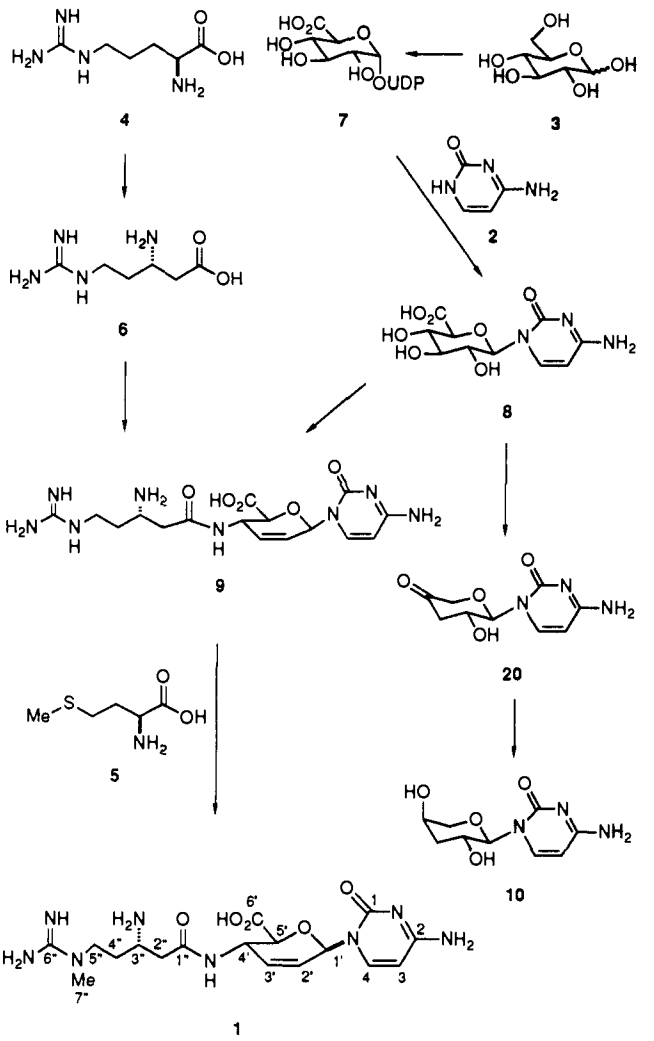
12a



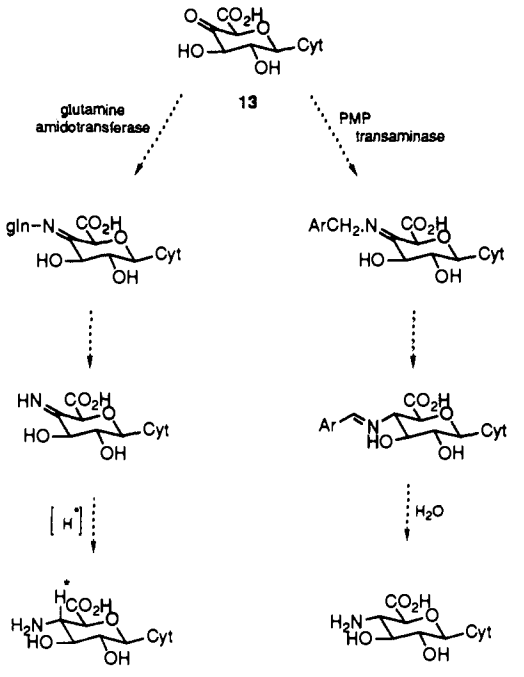
1b

and the *N*-methyl group are due to the biosynthesis of their primary precursors, **4** and **5**, which require NADPH,[20] and the subsequent action of arginine 2,3-aminomutase, in which the 3-*pro-R* hydrogen of **4** migrates to the 2-*pro-R* hydrogen of **7**.[15] These enrichments served as internal standards to show that the biological system was working properly. The lack of deuterium at H-2′ and H-3′ confirmed the intact incorporation of the C_2–D and C_3–D bonds of **11a**. More importantly, H-4′ was not labeled in this experiment, either.

Scheme 1



Scheme II



Cyt = cytosine

Since H-4′ of **1** comes neither intact from glucose nor via NAD(P)H, it should be derived from the solvent (water). This would indicate that the C-4′ amino group is introduced by trans-

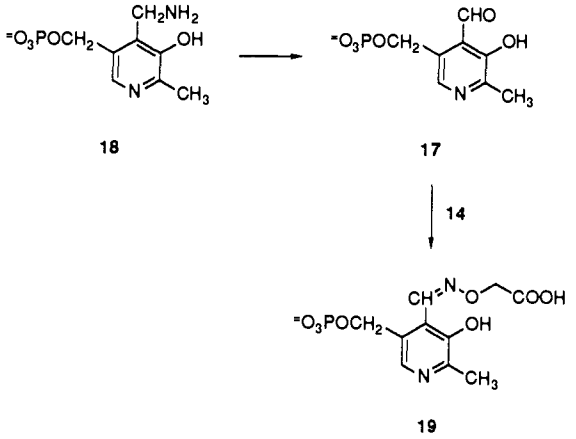
(15) Prabhakaran, P. C.; Woo, N-T.; Yorgey, P. S.; Gould, S. J. *J. Am. Chem. Soc.* **1988**, *110*, 5785.
(16) Guo, J.; Gould, S. J. *J. Am. Chem. Soc.* **1991**, *113*, 5988.
(17) Guo, J.; Gould, S. J. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 497.
(18) Gould, S. J.; Tann, C. H.; Prabhakaran, P. C.; Hillis, L. R. *Bioorg. Chem.* **1988**, *16*, 258.
(19) Schneider, M. J.; Ungemach, F. S.; Broquist, H. P.; Harris, T. M. *J. Am. Chem. Soc.* **1982**, *104*, 6863.
(20) Smith, E. L.; Hill, R. L.; Lehman, I. R.; Lefkowitz, R. J.; Handler, P.; White, A. In *Principles of Biochemistry*, 7th ed.; McGraw-Hill: New York, 1983; pp 577, 584, 610.

Table I. Metabolite Production by *S. griseochromogenes* in the Presence of Enzyme Inhibitors

compd fed	amount[c] fed,[c] mg	[8], μM	[10], μM	[9], μM	[1], μM
		4	15	83	2395
AOAA[a]	40	15	31	56	1774
AOAA	140	93	36	4	422
DON[b]	17	6	21	82	2224
azaserine	31	2	19	78	2061

[a] Aminooxyacetic acid. [b] 6-Diazo-5-oxo-L-norleucine. [c] For conditions, see Experimental Section.

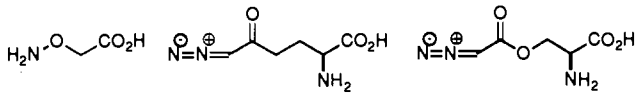
Scheme III



18 17 19

amination to a ketosugar, such as **13**. A glutamine-dependent amidotransferase would require a hydride transfer,[21] whereas a pyridoxamine phosphate (PMP) transaminase would require proton addition, as shown in Scheme II (the necessary modifications at C-2′ and C-3′ leading to **1** are not considered at this point).

Concurrently with the labeling studies, we had also adopted an approach of altering fermentation conditions to block potential biosynthetic steps.[22] This has allowed us to accumulate intermediates in the pathway to **1** by feeding inhibitors of specific types of biochemical reactions. To minimize the potentially lethal inhibitory effects on primary metabolism and to maximize the chance for selective inhibition of secondary metabolic processes, these inhibitors were added to fermentations in the middle of logrithmic growth of cell mass, which is the onset of antibiotic production. The experiments relevant to the present study included additions of aminooxyacetic acid (AOAA, **14**), which inhibits

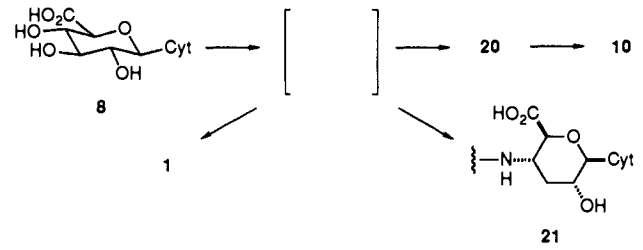


14 15 16

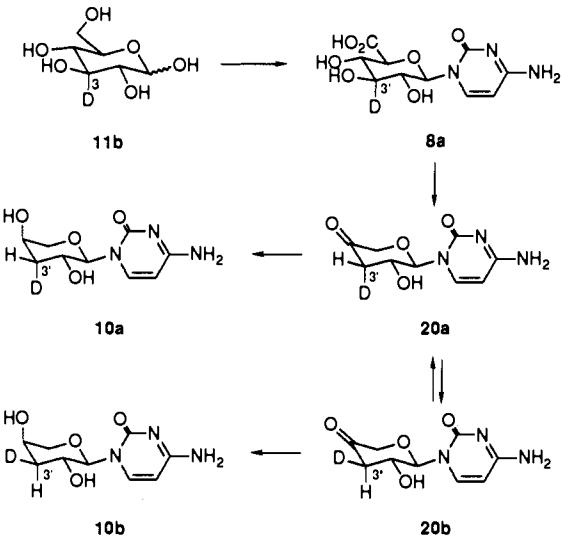
pyridoxal phosphate (PLP)/PMP-dependent enzymes,[23,24] and 6-diazo-5-oxo-L-norleucine (DON, **15**),[25] and azaserine (**16**),[26] which inhibit glutamine-dependent amidotransferases.

Addition of standard[25,26] amounts of DON or azaserine had little effect on the concentrations of the various cytosine glycosides produced by *S. griseochromogenes*. In contrast, addition of moderate amounts of AOAA to fermentations of *S. griseochromogenes* led to a 26% decrease in the maximum concentration of **1** and a 32% decrease in the concentration of **9**, while causing a 275% increase in the concentration of **8** and a 107% increase in the concentration of the cometabolite **10**. Addition of large

Scheme IV



Scheme V



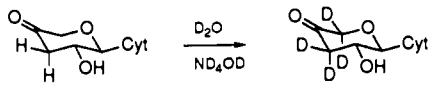
amounts of AOAA caused even more dramatic changes in the metabolite profile: an 83% decrease in the concentration of **1** + **9** and a 579% increase in the concentration of **8** + **10**. These results, summarized in Table I, were also consistent with involvement of a PLP/PMP transaminase for introduction of the C-4′ amino group. AOAA inhibits a broad spectrum of transaminases, presumably by reacting with PLP (**17**). Pyridoxal phosphate (**17**), generated from PMP (**18**), involved in the biosynthesis of **1** would be "drained off" by AOAA to give a very stable oxime (**19**) and would no longer be available for transamination back to **18** (Scheme III). With inhibition of the transamination necessary for **1** and **9**, a substantial accumulation of **8** occurs and some of this is converted to **10** by the shunt pathway. PMP is only catalytic for **8** to **10**, but is stoichiometric for **8** to **1**.

The retention of deuterium at H-2′ and H-3′ in **1a** revealed that the deoxygenations occurring in the formation of **1** were not simple dehydrations. Although our inhibitor feedings have not directly shed additional light on this portion of the pathway, we have recently characterized a new metabolite, pentopyranone (PPN, **20**) (PPN exists as the hydrate in solution).[14] Efforts to find additional cell-free enzyme activities to transform **8** have so far yielded only "PPN dehydrogenase"; in the shunt pathway this converts **20** to **10**.[27] The structural relationships of **10**, **1**, and blasticidin H (**21**)[9] indicate that these compounds should be all derived from the same 3′-deoxy intermediate (Scheme IV). Therefore, accumulation of **10** in the presence of **14** provided a method to explore the mechanism of C-3′ deoxygenation in this metabolic matrix.

To determine the stereochemistry of C-3′ deoxygenation, [3-^{2}H]-D-glucose (**11b**)[28] was fed to a 1-L fermentation,[15] and large quantities of **1** and arginine hydroxamate, an inhibitor of L-α-arginine biosynthesis,[29] were added 52 h later. The combination of these two additives has been found to be superior to **14** for maximum production of **10**, and it greatly enhances production

(21) Reference 20, p 452.
(22) A full description of this work will be published in detail elsewhere.
(23) Wallach, D. P. *Biochem. Pharmacol.* **1961**, *5*, 323.
(24) Brunk, D.; Rhodes, D. *Plant Physiol.* **1988**, *87*, 447.
(25) Kida, T.; Shibai, H. *Agric. Biol. Chem.* **1985**, *49*, 3231.
(26) Kurashashi, O.; Noda-Watanabe, M.; Toride, Y.; Takeonuchi, T.; Akashi, K.; Morinaga, Y.; Enei, H. *Agric. Biol. Chem.* **1987**, *51*, 1791.
(27) Gould, S. J.; Guo, J.; Klumpp, M. Unpublished work.
(28) Koch, H. J.; Perlin, A. S. *Carbohydr. Res.* **1970**, *15*, 403.
(29) Nakayama, K.; Yoshida, H. *Agric. Biol. Chem.* **1972**, *36*, 1675.

Scheme VI



20 20c

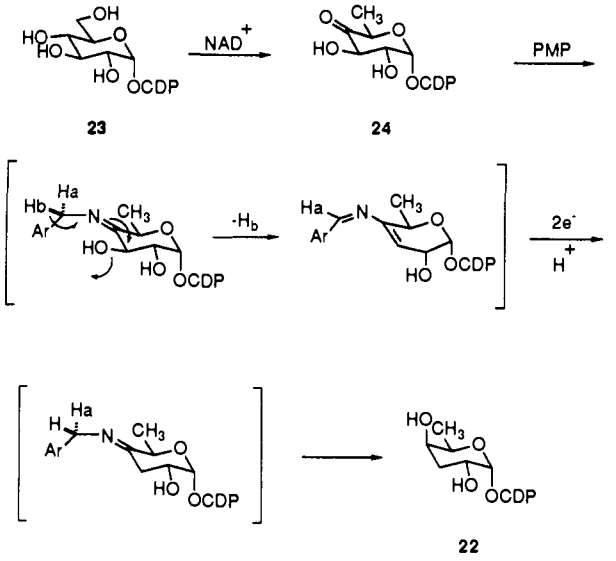
of **8**, as well.[22] In this experiment, 112 mg of **8a** and 170 mg of deuterated PPNC were produced (HPLC assay). Pure samples of each were obtained by ion-exchange chromatography followed by preparative HPLC.[14]

Deuterium NMR analysis of the labeled PPNC revealed that the deuterium label had been retained preferentially (85%) at H-3′$_{axial}$ (Scheme V, **10a**). A small amount of deuterium (15%) was present at H-3′$_{equatorial}$ (Scheme V, **10b**). As expected, the deuterium label was retained at H-3′ of **8a**. The deuterium enrichment in **10a,b** was 3.3%, and in **8a** it was 3.7%.

The presence of 15% of the deuterium at H-3′$_{equatorial}$ seemed to present a problem since enzyme-catalyzed reactions are usually stereospecific. However, the deuterium enrichments at H-3′$_{axial}$ and H-3′$_{equatorial}$ in [3′-2H]PPN, a small amount of which was isolated from the same experiment, were nearly indentical (54:46), indicating an approximately equal mixture of **20a** and **20b**. This suggested that H-3′$_{axial}$ had subsequently partially epimerized by a nonenzymatic chemical process to H-3′$_{equatorial}$ before **20a** was converted to **10a**, rather than the label distribution arising from a lack of enzyme stereospecificity. This was confirmed by a deuterium-exchange experiment in D_2O at pD 9.3, where ^1H and ^2H NMR analyses of the product revealed that both of the H-3′ hydrogens in **20** were completely exchanged at room temperature within 45 min, yielding **20c** (Scheme VI). In addition, the H-5′ hydrogens were exchanged to the extent of 18%. Therefore, the product from the biosynthetic experiment can most reasonably be viewed as a mixture of **10a** and **10b**, generated from **20a** and **20b**, respectively.

C-3′ deoxygenation is also an important biochemical transformation in the biosynthesis of a class of 3′,6′-dideoxy carbohydrates which are found solely in the lipopolysaccharide components of a number of Gram-negative bacterial cell envelopes.[30-32] These unusual sugars have been shown to contribute to the serological specificity of many immunologically active polysaccharides.[33-36] In the past two decades, substantial efforts have been devoted to exploring their biosynthesis. The nature of the biosynthetic precursor of four of the five known 3′,6′-dideoxyhexoses—paratose, abequose, tyvelose, and ascarylose— has been shown to be CDP-glucose,[37] while the fifth, colitose, is derived from GDP-glucose.[38] These 3′,6′-dideoxyhexoses are formed by three consecutive enzymes, two of which are NAD$^+$-dependent oxidoreductases.[39-41] The third step involves a PMP-linked enzyme, although there is no net transamination.[42,43] Recently, the enzymes involved in the biosynthesis of ascarylose (**22**) have been purified.[44-47] As shown in Scheme VII, a detailed mechanistic

Scheme VII



23 24 22

study revealed that 3′,6′-dideoxygenation of CDP-glucose (**23**) occurs through the sequential reactions of an NAD$^+$-mediated oxidoreduction to CDP-4′-keto-6′-deoxyglucose (**24**), followed by a novel PMP-mediated β-elimination[44,45] and then an NAD$^+$-dependent electron-transfer reaction ultimately leading to **22**.[46,47] Although it has been established that the *pro-R* hydrogen of PMP is lost and is subsequently replaced with a proton from the medium with retention of configuration,[45] the fate of the original C-3 carbinol hydrogen and the stereochemistry of the deoxygenation at this center has not been determined.

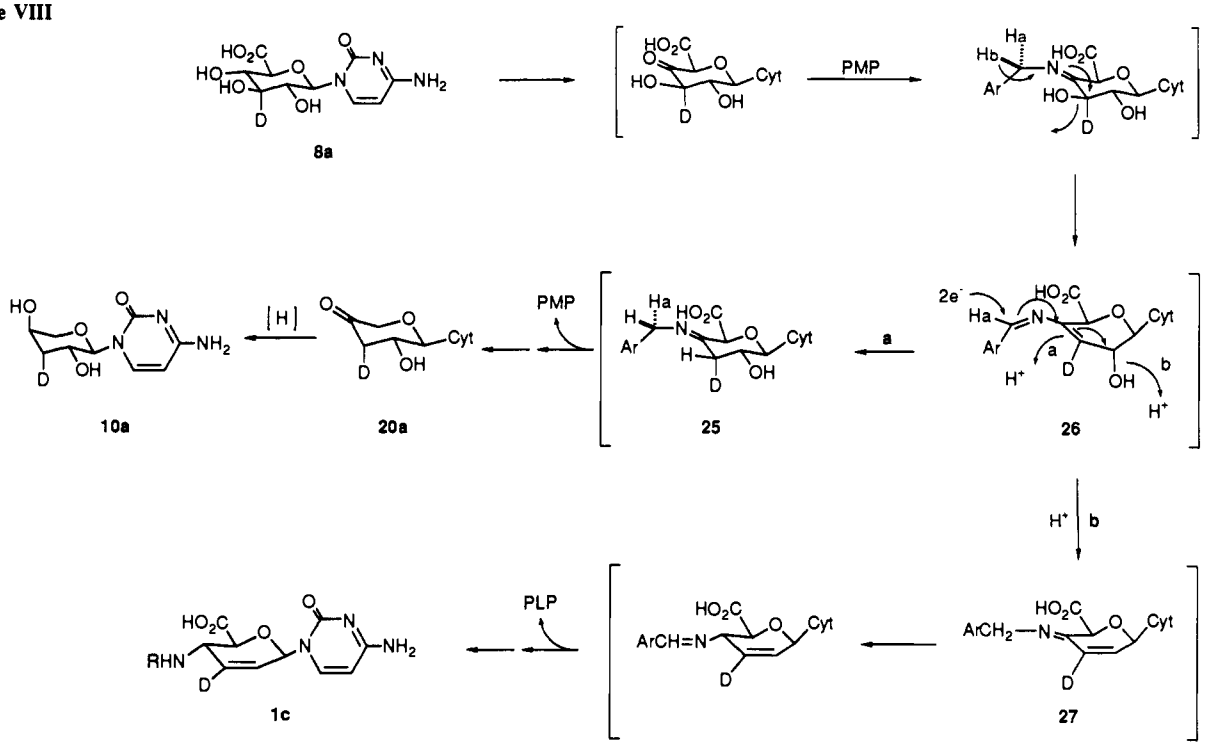
The net retention of configuration at C-3′ of **10a**, the numerous data indicating involvement of PMP, and the earlier finding[18] that H-3 of glucose **11a** is retained in **1a** lead us to propose the mechanism shown in Scheme VIII for the formation of **1c** and **10a** from **8a**. In route a, C-6′ decarboxylation of **25** leading to **10a** should be similar to that in UDP-xylose biosynthesis.[48] The mechanism for C-2′/C-3′ dideoxygenation in **1c** biosynthesis can be accommodated by elimination of the C-2′-OH from the proposed intermediate, **26**, rather than protonation at C-3′, now leading to intermediate **27** and finally to **1** (route b). Considering our results in combination with those on the biosynthesis of ascarylose,[45] the stereochemistry of the overall process of general C-3′ deoxygenation would be net retention of configuration at both centers with all bond-breaking and bond-forming events taking place on one face of the substrate–coenzyme complex. This would be consistent with the stereochemical course and facial specificity of all PLP/PMP-dependent enzyme-catalyzed β-elimination reactions,[49-52] with one exception.[48]

Experimental Section

General. [1,1-$^2H_2$]Ethanol was obtained from Cambridge Isotope Laboratories, and both D_2O and ND_4OD were obtained from Aldrich Chemical Co. Cytosine, AOAA, DON, and azaserine were obtained from Sigma Chemical Co.

(30) Hanessian, S. *Adv. Carbohydr. Chem. Biochem.* **1966**, *21*, 143.
(31) Butterworth, R. F.; Hanessian, S. *Adv. Carbohydr. Chem. Biochem.* **1971**, *26*, 279.
(32) Williams, N. R.; Wander, J. D. In *The Carbohydrates: Chemistry and Biochemistry*; Pigman, W., Horton, D., Eds.; Academic Press: Orlando, FL, 1980; Vol. 1B, p 761.
(33) Westphal, O.; Luderitz, O. *Angew. Chem.* **1960**, *72*, 881.
(34) Lüderitz, O.; Staub, A. M.; Westphal, O. *Bacteriol. Rev.* **1966**, *30*, 192.
(35) Bishop, C. T.; Jennings, H. J. In *The Polysaccharides*; Aspinall, G. O., Ed.; Academic Press: Orlando, FL, 1982; Vol. 1, p 291.
(36) Kennedy, J. F.; White, C. A. *Bioactive Carbohydrates in Chemistry and Biology*; Ellis Horwood: Chichester, U.K., 1983.
(37) Masuhashi, S.; Masuhashi, M.; Strominger, J. L. *J. Biol. Chem.* **1966**, *241*, 4267.
(38) Health, E. C.; Elbein, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **1962**, *48*, 1209.
(39) Masuhashi, S.; Masuhashi, M.; Brown, J. G.; Strominger, J. L. *J. Biol. Chem.* **1966**, *241*, 4283.
(40) Masuhashi, S.; Strominger, J. L. *J. Biol. Chem.* **1967**, *242*, 3494.
(41) Rubenstein, P. A.; Strominger, J. L. *J. Biol. Chem.* **1974**, *249*, 3782.
(42) Gonzalez-Porque, P.; Strominger, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 1625.
(43) Rubenstein, P. A.; Strominger, J. L. *J. Biol. Chem.* **1974**, *249*, 3776.
(44) Han, O.; Miller, V. P.; Liu, H-w. *J. Biol. Chem.* **1990**, *265*, 8033.
(45) Shih, Y.; Yang, D.-y.; Weigel, T. M.; Liu, H.-w. *J. Am. Chem. Soc.* **1990**, *112*, 9652.
(46) Han, O.; Liu, H-w. *J. Am. Chem. Soc.* **1988**, *110*, 7893.
(47) Vaughn, P.; Liu, H-w. *J. Am. Chem. Soc.* **1992**, *114*, 1880.
(48) Smith, E. L.; Hill, R. L.; Lehman, I. R.; Lefkowitz, R. J.; Handler, P.; White, A. In *Biochemistry, General Aspect*, 7th ed.; McGraw-Hill: New York, 1983; p 456.
(49) Dunathan, H. C. *Proc. Natl. Acad. Sci. U.S.A.* **1966**, *55*, 713.
(50) Vederas, J. C.; Floss, H. G. *Acc. Chem. Res.* **1980**, *13*, 455.
(51) Palcic, M. M.; Floss, H. G. In *Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley-Interscience: New York, 1986; Part A, p 25.
(52) Miles, E. W. In *Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley-Interscience: New York, 1986; Part B, p 253.

Scheme VIII



Culture Maintenance and Fermentation Conditions. Standard culture maintenance and fermentation conditions for seed cultures and production cultures in the complex medium have been described previously,[15] except that the fermentation broth was now harvested after 144 h instead of 120 h. The complex medium was used for the isotope labeling experiments. For the inhibitor feedings, a synthetic medium was prepared.[53] The broth to flask volume was 1:5 for both complex and synthetic media; however, the complex production medium was cultured in baffled flasks (Bellco Glass Co., Vineland, NJ), while the synthetic production medium was cultured in standard Erlenmyer flasks. Inhibitors were fed by addition of the solid compounds directly at the 52nd hour of incubation. Cytosine was fed by addition of a 3% sterile solution. Production of metabolites was quantified by HPLC. For the assay, a 0.5-mL aliquot was removed and centrifuged (Eppendorf Model 5414) for 10 min, and 10–20 μL of the supernatant was injected to HPLC.

HPLC Analysis. The conditions for analytical HPLC were as follows: Nova-Pak C_{18} Radial-Pak column, 0.8 cm × 10 cm (Waters Associates, 4-μm packing), eluted with 95% H_2O, 5% CH_3CN, 0.1% TFA, at a flow rate of 1.2 mL/min. The effluent was monitored with a Waters Model 990+ photodiode array detector. For preparative HPLC the conditions were as follows: Nova-Pak C_{18} Radial-Pak column, 2.5 cm × 10 cm (Water, 6 μm), eluted with 97% H_2O, 3% CH_3CN, 0.05% AcOH, at a flow rate of 6 mL/min, and monitored by UV detection at 275 nm.

Feeding [1,1-$^2H_2$]Ethanol (12a). [1,1-$^2H_2$]Ethanol (1.0 mL) was added to a 200 mL of complex production broth 38 h after inoculation and again 10 h later. After an additional 12 h, another 0.8 mL was added, and this was repeated 12 h later. Workup after a total of 120 h yielded 144 mg of **1b** (340 mg had been produced according to bioassay).[15] A portion of this (64 mg) was used for analysis by ^2H NMR at both 24 and 67 °C (deuterium depleted water + *t*-BuOH for chemical shift reference and deuterium quantitation): δ 2.75 (0.223 μmol, 0.16% enrichment), 3.08 (1.814 μmol, 1.30%), and 3.52 (0.363 μmol, 0.26%).

Isolation of Metabolites. Broth from five flasks (1 L total) was centrifuged at 10000*g* for 10 min to remove solids. The pellets were washed with a minimum amount of water and recentrifuged, and the washings were combined with the original supernatants. A sample was saved for analysis of production by analytical HPLC. The remainder of the broth was cooled to 4 °C, carefully adjusted to pH ca. 3.0 using 6 N HCl, and allowed to stand for 10 min. After removal of precipitated colloidal materials by centrifugation at 4 °C, the supernatant was neutralized to pH 6.0 using 1 N NaOH and loaded onto a cation-exchange column (Bio-Rad AG 50W-X2, H^+, 100–200 mesh, 3.5 cm × 60 cm) at a flow rate of ca. 6.0 mL/min. The column was washed with ca. 300 mL of water and then with 5% aqueous pyridine. The first 500 mL was discarded, and the next ca. 1 L was collected and saved ("pyridine eluate", see below). The cation-exchange column was next eluted with 1.2% NH_4OH (2.5 L). The eluate was monitored regularly by HPLC, and fractions were collected according to the appearance of the major components. All fractions were then lyophilized.

Isolation of 8. The pyridine eluate was loaded onto an anion-exchange column (Amberlite IRA-410, AcO^-, 2.5 cm × 30 cm) at ca. 3 mL/min. After loading, the column was washed with H_2O and then eluted with 1.0 M acetic acid. After lyophilization, the late fractions were combined to give crude **8**. This was dissolved in ca. 10 mL of 1% aqueous pyridine, divided into two parts, filtered through two C_{18} Sep-Pak columns (J. T. Baker, 3 mL bed volume), and washed with 2 column volumes of H_2O. The effluent and H_2O washings were combined and lyophilized. Recrystallization twice from hot H_2O yielded 70 mg of pure **8**: ^1H NMR (D_2O, 400 MHz) δ 7.92 (1 H, d, *J* = 8.1 Hz, H-5), 6.22 (1 H, d, *J* = 8.3 Hz, H-6), 5.66 (1 H, d, *J* = 8.6 Hz, H-1′), 3.91 (1 H, d, *J* = 9.4 Hz, H-5′), 3.64 (2 H, m, H-2′ and H-3′), 3.57 (1 H, t, *J* = 9.5 Hz, H-4′); ^{13}C NMR (D_2O, 100 MHz) δ 175.2 (C-6′), 160.6 (C-2), 150.4 (C-4), 144.6 (C-6), 96.6 (C-5), 83.4 (C-1′), 78.9 (C-5′), 76.2 (C-4′) 71.8 (C2′, C-3′); FAB-MS (H_2O–glycerol) 288 $(M + H)^+$.

Isolation of 10. After lyophilization, fractions from the water wash and early acetic acid elution of the cation-exchange column were combined and taken up in ca. 50 mL of H_2O and then filtered through a pad of C_{18} silica gel (40 μm, 10 cm × 5 cm). The C_{18} pad was washed thoroughly with H_2O, and the effluent was monitored by HPLC continually. Appropriate fractions were combined and lyophilized separately, and each was further purified by preparative HPLC. Pure **10** (40 mg) was obtained directly in this manner: ^1H NMR (D_2O, 400 MHz) δ 1.93 (1 H, m, H-3′$_{ax}$), 2.39 (1 H, m, H-3′$_{eq}$), 3.88 (1 H, d, *J* = 12.7 Hz, H-5′$_{ax}$), 3.92 (1 H, d, *J* = 12.7 Hz, H-5′$_{eq}$), 4.13 (1 H, m, H-2′), 4.17 (1 H, br s, H-4′), 5.53 (1 H, d, *J* = 9.2 Hz, H-1′), 6.33 (1 H, d, *J* = 7,8 Hz, H-3), 8.05 (1 H, d, *J* = 8.0 Hz, H-4); ^{13}C NMR (D_2O, 100 MHz) δ 158.8 (C-1), 148.4 (C-2), 144.0 (C-4), 95.5 (C-3), 85.3 (C-1′), 71.7 (C-4′), 64.9 (C-2′), 63.5 (C-5′), 36.7 (C-3′). The ^1H NMR spectrum was identical to that obtained with an authentic sample.

Isolation of 1 and 9. Fractions eluted from the original cation-exchange column with 1.2% NH_4OH were each further purified. Recrystallization of the appropriate fraction from H_2O–MeOH directly gave pure **1** (30 mg). The fraction containing **9** was redissolved in a minimum amount of H_2O and purified by preparative HPLC to give 5 mg.

Incorporation of [3-^2H]-D-Glucose (11b). Five 200-mL complex medium broths were inoculated with a seed culture and incubated in standard fashion.[15] Arginine hydroxamate (1.0 g) and cytosine (2.0 g) were added at 52 and 66 h, respectively. In the meantime, [3-^2H]-D-glucose[28] (500 mg) was added at the 54th h of incubation, and an additional 256 mg was added 14 h later. The broth was harvested and worked up after a total of 160 h to yield **8a** (70 mg), **10a,b** (40 mg), and **20a,b** (ca. 5 mg). ^2H NMR samples were prepared in ca. 400 μL of deuterium-depleted water with either *t*-BuOH (25 μL) or 1,4-dioxane (25

(53) Yonehara, H.; Otake, N. *Antimicrob. Agents Chemother.* **1965**, 855.

μL) as an internal reference for chemical shift and quantification. Proton-decoupled ^2H NMR spectra (61.4 MHz) were obtained with sweep width 1433 Hz, 4K data points zero-filled to 16K, 1.43 s acquisition time, 90° pulse width, and 1- or 3-Hz line broadening: **8a** (20 mg, *t*-BuOH reference, 20 μL of pyridine to improve the solubility) 35 837 scans, 3.0-Hz line broadening; **10a,b** (40 mg, 1,4-dioxane reference) 99226 scans, 3.0 Hz line broadening; **20a,b** (5 mg, 1,4-dioxane reference) 43 367 scans, 3.0-Hz line broadening; **20c** (5 mg, *t*-BuOH reference) 2774 scans, 1.0-Hz line broadening.

Deuteration of 20. Pentopyranone (5 mg, 0.02 mmol) was dissolved in D_2O (400 μL) in an NMR tube, and the ^1H NMR spectrum was acquired. The sample was then treated with ND_4OD (15 μL of a 28% solution, giving a final pD 9.3), and the ^1H NMR spectrum was immediately recorded. After 45 min at room temperature, the spectrum was again recorded, and the sample was then lyophilized. It was next triturated with H_2O and then lyophilized, and this procedure was repeated once again to yield **20c**. The H-3′ resonances were no longer detected in the ^1H NMR spectrum, and the H-5′ resonances were reduced in intensity; ^2H NMR (deuterium-depleted water) δ 1.23 (*t*-BuOH, added for chemical shift reference and deuterium quantitation), 1.98 (1 H, H-3′$_{ax}$), 2.48 (1 H, H-3′$_{eq}$), 3.72 (0.18 H, H-5′$_{ax}$), 3.81 (0.18 H, H-5′$_{eq}$); FAB-MS (glycerol) *m/z* (rel intensity) 246 ([M + H]$^+$, 45%), 228 (25%), 207 (50%), 204 (100%), 195 (35%); HR FAB-MS (glycerol) exact mass calculated for $C_9H_{12}D_2N_3O_5$ 246.10590 ([M + H]$^+$), found 246.10588.

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Total Synthesis of (-)-Nummularine F

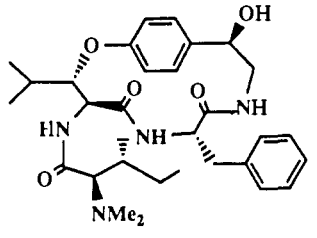
Robert J. Heffner, Jianjun Jiang, and Madeleine M. Joullié*

Contribution from the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323. *Received April 1, 1992*

Abstract: The first total synthesis of the 14-membered para ansa cyclopeptide alkaloid (-)-nummularine F is reported. The pivotal transformations of the synthetic strategy are (1) a stereocontrolled approach for introducing the absolute stereochemistry at the α- and β-carbons of the parent β-hydroxyproline utilizing D-serine as a source of chirality; (2) cyclization to a rigid 14-membered ring; and (3) introduction of the enamide double bond. The synthesis began with the conversion of D-serine to (2*S*,3*S*)-3-(4-cyanophenoxy)-1-[(1,1-dimethylethoxy)carbonyl]proline. After conversion of the cyano function to a formyl group, a Henry reaction between the 4-formylphenoxy group and the anion of nitromethane gave a mixture of epimeric benzyl alcohols containing a terminal nitro group. The nitro group was reduced to an amine and coupled to Z-protected L-isoleucine to afford the desired acyclic precursor. Cyclization was achieved by the coupling of the proline pentafluorophenyl ester and the amino group of the L-isoleucine. The enamide bond was introduced after cyclization, via thermal selenoxide elimination.

Introduction

Cyclopeptide alkaloids are a group of closely related polyamide bases of plant origin.[1-7] Although cyclopeptide alkaloids were mentioned in the literature as early as 1884,[8] the isolation and structural elucidation of pandamine (**1**) in 1966 marks the beginning of a widespread interest in these natural products.[9] Cyclopeptide alkaloids are particularly common in plants of the Rhamnacea family and also in more than 25 other species. Their widespread occurrence makes them an important class of natural products.



Pandamine (1)

Except for the 15-membered macrocycles, all cyclopeptide alkaloids contain one β-hydroxy-α-amino acid whose oxygen is contained in the macrocycle. This unit determines the family to which they belong, and the amino acids may be β-hydroxyproline, β-hydroxyleucine, or β-hydroxyphenylalanine. Cyclopeptide alkaloids may also be classified according to size: 13-, 14-, or 15-membered rings. In addition, cyclopeptide alkaloids contain another ring-bound amino acid which forms an enamide bond with another fragment, a phenethylamine. This moiety may be a hydroxyphenethylamine, its oxidation product, or, most commonly, its dehydration product. Finally, attached to the amino group of the β-hydroxy-α-amino acid is an acyl unit derived from another amino acid or from a di- or tripeptide which contains the basic monomethylated or dimethylated amino group. The main structural features and the numbering of a 14-membered macrocycle are shown in Figure 1.

Cyclopeptide alkaloids are usually present as complex mixtures isolated from various parts of the plant. Yields from dried plants vary from 0.01 to 1% depending on the plant source, location, method of isolation, and the plant maturity. Therefore, the biological profile of this class of natural products is not well defined.

(1) Warnhoff, E. W. *Fortschr. Chem. Org. Naturst.* **1970**, *28*, 162.
(2) Pais, M.; Jarreau, F. X. In *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*; Weinstein, B., Ed.; Dekker: New York, 1971; Vol. 1, p 127.
(3) Tschesche, R.; Kaussman, E. V. In *The Alkaloids*; Manske, R. H. F., Ed.; Academic Press: New York, 1975; Vol. 15, p 165.
(4) Ogihara, Y. *Ann. Rep. Fac. Pharm. Sci., Nagoya City Univ.* **1974**, *22*, 1.
(5) Tschesche, R. *Heterocycles* **1976**, *4*, 107.
(6) Joullié, M. M.; Nutt, R. F. In *The Alkaloids: Chemical and Biological Perspectives*; Pelletier, S. W., Ed.; Wiley: New York, 1985; Vol. 3, p 113.
(7) Schmidt, U.; Lieberknecht, A.; Haslinger, E. In *The Alkaloids*; Manske, R. H. F., Ed.; Academic Press: New York, 1985; Vol. 26, p 299.
(8) Clinch, J. H. M. *Am. J. Pharm.* **1884**, *56*, 131.
(9) Pais, M.; Jarreau, F. X.; Lusinchi, X.; Goutarel, R. *Ann. Chim. (Paris)* **1966**, *14*, 83.

0002-7863/92/1514-10181$03.00/0