Biosynthesis of the Nucleoside Antibiotic Aristeromycin

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Abstract: The biosynthesis of the nucleoside antibiotic aristeromycin (**1**) was investigated by administration of radioactive and ^{13}C-labeled precursors to *Streptomyces citricolor*. The results of these studies indicate that the adenine ring of **1** is formed in the manner expected for prokaryotes and that the cyclopentane ring of the antibiotic is generated by C–C bond formation between C-2 and C-6 of D-glucose. Additional incorporation experiments with specifically tritiated and deuterated forms of D-glucose suggest that the cyclization of glucose proceeds by oxidation at C-5 or C-4 of the hexose followed by formation of a cyclopentenone derivative. This conclusion is supported by the isolation of the related antibiotic neplanocin A (**2**) from the fermentation broth of *S. citricolor*. A stereochemical analysis of the cyclization reaction was carried out by administration of (6*R*)- and (6*S*)-[6-^{3}H]-D-glucose to the fermentation. These experiments revealed that the reaction proceeds with loss of the 6 *pro-S* hydrogen atom, a stereochemical outcome that is the opposite to that observed in the cyclization of glucose 6-phosphate to *myo*-inositol 1-phosphate.

Aristeromycin (**1**) (Figure 1) is a carbocyclic analogue of adenosine which was first synthesized in racemic form in 1966.[1] Shortly thereafter, the compound was isolated from the fermentation broth of *Streptomyces citricolor*, its structure was elucidated, and its absolute configuration was determined.[2] Aristeromycin exhibits a variety of interesting biological activities,[3,4] including the inhibition of AMP synthesis in mammalian cells, inhibition of cell division and elongation in rice plants, and inhibition of the enzyme *S*-adenosylhomocysteine hydrolase.[5]

For 12 years after its isolation, aristeromycin appeared to be the only naturally occurring carbocyclic adenosine analogue. This situation changed in 1980 with the isolation of neplanocin A (**2**) (Figure 1) and its congeners from the fermentation broth of *Ampullariella regularis*.[6,7] Neplanocin A exhibits potent antitumor activity[6] and antiviral activity,[8] and it is a powerful inhibitor of *S*-adenosylhomocysteine hydrolase.[8] An additional member of this family of nucleoside antibiotics, adecypenol (**3**), was isolated in 1986 from *Streptomyces* sp. OM-3223.[9] Adecypenol bears a structural resemblance to both neplanocin A and to the nucleoside antibiotic coformycin.[10] Like coformycin, adecypenol is a potent inhibitor of adenosine deaminase.

The unusual structures and interesting biological activities of these nucleoside antibiotics prompted us to carry out an investigation of the biosynthesis of aristeromycin. The results of these studies will now be reported.[11]

Results

Origin of the Adenine Ring. Our investigations of the biosynthesis of the adenine ring of aristeromycin were guided by the extensive studies of purine biosynthesis previously conducted in a variety of living systems.[12] As the result of these studies, it is generally accepted that the carbon atoms of adenine are derived from formate (C-2, C-8), bicarbonate (C-6), and the carboxyl and methylene group of glycine (C-4 and C-5, respectively). The N-7 nitrogen is also derived from the nitrogen atom of glycine. A preliminary evaluation of this pathway in *S. citricolor* was carried out by administration of [^{14}C]formate and [1-^{14}C]glycine. Radioactive aristeromycin was obtained in both experiments, but glycine was clearly the more efficient precursor (Table I, expts 1, 2). These experiments were followed by evaluation of (^{13}C)-formate and (1,2-$^{13}C_2$)glycine as aristeromycin precursors. No visible enrichment was apparent in the aristeromycin derived from (^{13}C)formate, while the aristeromycin produced from doubly ^{13}C-labeled glycine showed high enrichment at C-2 and C-8 as well as lower enrichment at C-4 and C-5 (expts 3, 4). The coupling between C-4 and C-5 observed in experiment 4 confirms the expected intact incorporation of glycine into this position of the purine nucleus. The high levels of enrichment observed at C-2 and C-8 are consistent with the reported derivation of C-2 and C-8 of the purine nucleus from C-2 of glycine in other bacterial systems.[13] The mode of glycine incorporation was more completely defined by administration of (2-^{13}C,^{15}N)glycine to *S. citricolor*. The resulting aristeromycin exhibited enrichment at C-2, C-8, and C-5 (Table I, expt 5), thereby confirming both the derivation of C-2 and C-8 from C-2 of glycine and the orientation of glycine incorporation into C-4 and C-5 of the adenine ring. However, no coupling was observed for the labeled C-5 carbon atom, suggesting complete loss of the ^{15}N label of glycine by

Table I. Administration of Labeled Precursors to *S. citricolor*

expt	precursor ($^{3}H/^{14}C$)	% incorpn or enrichment ($^{3}H/^{14}C$)	labeling pattern
1	sodium [^{14}C]formate	0.04	
2	[1-^{14}C]glycine	0.24	
3	sodium (^{13}C)formate	0	
4	(1,2-$^{13}C_2$)glycine	7	C-4, C-5 $^{1}J_{CC}$ = 65 Hz
		13	C-2, C-8
5	(^{15}N,2-^{13}C)glycine	10	C-5
		9	C-2, C-8
6	sodium (^{13}C)bicarbonate	0.5	C-6
7	[2-^{3}H,8-^{14}C]adenosine (5.98)	0.39 (4.90)	

(1) Shealy, Y. F.; Clayton, J. D. *J. Am. Chem. Soc.* **1966**, *88*, 3885.
(2) (a) Kusaka, T.; Yamomoto, H.; Shibata, M.; Muroi, M.; Kishi, T.; Mizuno, K. *J. Antibiot.* **1967**, *21*, 255. (b) Kishi, T.; Muroi, M.; Kusaka, T.; Nishikawa, M.; Kamiya, K.; Mizuno, K. *Chem. Pharm. Bull.* **1972**, *20*, 940.
(3) Suhadolnik, R. J. *Nucleosides as Biological Probes*; Wiley: New York, 1979; p 147.
(4) Suhadolnik, R. J. *Nucleoside Antibiotics*; Wiley-Interscience: New York, 1970; p 236.
(5) Guranowski, A.; Montgomery, J. A.; Cantoni, G. L.; Chiang, P. K. *Biochemistry* **1981**, *20*, 110.
(6) Yaginuma, S.; Muto, N.; Tsujino, M.; Sudate, Y.; Hayashi, M.; Otani, M. *J. Antibiot.* **1981**, *34*, 359.
(7) Hayashi, M.; Yaginuma, S.; Yoshioka, H.; Nakatsu, K. *J. Antibiot.* **1981**, *34*, 675.
(8) Borchardt, R. T.; Keller, B. T.; Patel-Thombre, U. *J. Biol. Chem.* **1984**, *259*, 4353.
(9) Omura, S.; Ishikawa, H.; Kuga, H.; Imamura, N.; Taga, S.; Takahashi, Y.; Tanaka, H. *J. Antibiot.* **1986**, *39*, 1219.
(10) Nakamura, H.; Koyama, G.; Iitaka, Y.; Ohno, M.; Yagisawa, N.; Kondo, S.; Maeda, K.; Umezawa, H. *J. Am. Chem. Soc.* **1974**, *96*, 4828.
(11) Portions of this work have been the subject of a preliminary communication: Parry, R. J.; Bornemann, V. *J. Am. Chem. Soc.* **1985**, *107*, 6402.
(12) Hartman, S. C. *Metabolic Pathways*, 3rd ed.; Greenberg, D. M., Ed.; Academic Press: New York, 1970; Vol. IV.
(13) Kozluk, T.; Spenser, I. D. *J. Am. Chem. Soc.* **1987**, *109*, 4698 and references cited therein. For a possible mechanism, see: Kochi, H.; Kikuchi, G. *J. Biochem.* **1974**, *75*, 1113.

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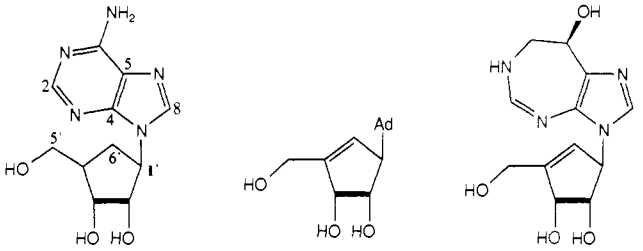


1 2 3

Figure 1. The structures of naturally occurring carbocyclic nucleosides.

Table II. Administration of Labeled Carbohydrates to *S. citricolor*

expt	precursor	% incorpn or enrichment	labeling pattern
1	[1-^{14}C]-D-ribose[a]	0.0003	
2	[U-^{14}C]-D-glucose[a]	0.01	
3	(1-^{13}C)-D-glucose[b]	4.0	C-5′
4	(6-^{13}C)-D-glucose[b]	2.0	C-6′
5	(2-^{13}C)-D-fructose[b]	0.74	C-4′

[a] Normal fermentation conditions. [b] Replacement cultures.

transamination in vivo. Finally, addition of (^{13}C)bicarbonate to the aristeromycin-producing fermentation yielded antibiotic labeled at C-6 (Table I, expt 6), an observation in harmony with previous studies.

While the preceding investigations established that the adenine ring of aristeromycin is biosynthesized from the expected building blocks, they did not determine whether the adenine ring is biosynthesized by stepwise assembly of the purine ring on a carbocyclic analogue of 5-phosphoribosyl pyrophosphate (PRPP) or whether the adenine ring of **1** is derived from free adenine, which would in turn be derived by catabolism[14] of adenosine. Some insight regarding this question was gained by administration of [2-^{3}H,8-^{14}C]adenosine to *S. citricolor*. The tritium to carbon-14 ratio of the resulting aristeromycin (Table I, expt 7) indicated that the adenine ring had been incorporated largely intact (82% ^{3}H retention). This observation demonstrates that **1** can be biosynthesized via the purine-salvage pathway, but it does not rule out the simultaneous operation of the stepwise assembly process for the adenine ring.

Origin of the Cyclopentane Ring. The structure of aristeromycin suggested that the carbohydrate pool would be a plausible origin for the cyclopentane ring. Accordingly, [1-^{14}C]-D-ribose and [U-^{14}C]-D-glucose were initially evaluated as precursors. Very low incorporations were observed in each instance (Table II, expts 1 and 2). An incorporation experiment with (1-^{13}C)-D-glucose under these same conditions yielded antibiotic that exhibited no enrichment in its ^{13}C NMR spectrum (data not shown). Since these experiments were carried out with a production medium that is rich in glucose,[2a] it seemed likely that the low incorporation observed for glucose in experiment 2 could be attributed to dilution of the labeled precursor. A possible solution to this problem appeared to be the use of replacement cultures. A series of experiments was therefore carried out to measure and optimize aristeromycin production in various replacement media. After many trials, it was discovered that adequate production of the antibiotic could be obtained by fermentation in the usual medium for 48 h followed by fermentation in a replacement medium lacking both glucose and soluble starch. When [1-^{14}C]glucose was administered under replacement conditions, there was a 6-fold increase in the incorporation figure compared to experiment 2 (data not shown). This encouraging observation was followed up by administration of (1-^{13}C)glucose under the same conditions. Examination of the ^{13}C NMR spectrum of the resulting aristeromycin revealed a 4-fold enrichment in the signal due to C-5′ (Table II, expt 3).

(14) (a) Lawrence, N. L. *J. Bacteriol.* **1955**, *70*, 577. (b) Hurwitz, J.; Heppel, L. A.; Horecker, B. L. *J. Biol. Chem.* **1957**, *226*, 525.

Table III. Administration of Labeled D-Glucose to *S. citricolor*[a]

expt	precursor (^{3}H/^{14}C)	^{3}H/^{14}C in product	% ^{3}H retention
1	(6*RS*)-[6-^{3}H,6-^{14}C] (5.70)	2.71[b]	47.5
		2.57[c]	45.1
2	[5-^{3}H,6-^{14}C] (5.71)	0.07[b]	1.2
		0.02[c]	0.4
3	[4-^{3}H,6-^{14}C] (2.41)	0.28[b]	11.6
		0.72[c]	29.9
4	[3-^{3}H,1-^{14}C] (4.47)	4.25[b]	95.1
		4.35[c]	97.3
5	[2-^{3}H,2-^{14}C] (6.14)	0.68[b]	11.1
		0.59[c]	9.6
6	[1-^{3}H,1-^{14}C] (5.07)	2.19[b]	43.2
		2.18[c]	43.0
7	[1-^{3}H,1-^{14}C] (5.28)	3.09[b]	58.5
8	(6*R*)-[6-^{3}H,6-^{14}C] (2.26)	1.74[b]	77.0
		1.49[c]	65.9
9	(6*S*)-[6-^{3}H,6-^{14}C] (4.98)	0.16[b]	3.2

[a] Replacement cultures. [b] Ratio for aristeromycin. [c] Ratio for neplanocin A.

If D-glucose is incorporated intact into the cyclopentane moiety of aristeromycin, then the discovery that C-1 of glucose corresponds to C-5′ of the antibiotic indicates that C-6 of glucose should reside at either C-3′ or C-6′ of aristeromycin. In order to differentiate between these two possibilities, (6-^{13}C)-D-glucose was synthesized[15] and evaluated as an aristeromycin precursor. The antibiotic produced from this form of labeled glucose exhibited clear enrichment at C-6′ (Table II, expt 4). It follows that the cyclopentane moiety of aristeromycin is created by C–C bond formation between C-2 and C-6 of glucose. A precursor incorporation experiment with (2-^{13}C)-D-fructose demonstrated that this ketose can also serve as a specific precursor of aristeromycin (Table II, expt 5).

Mechanism of Formation of the Cyclopentane Ring. The origin of the cyclopentane ring of aristeromycin having been established, investigations of the mechanism of cyclopentane ring formation were initiated. Insight into the nature of the C–C bond forming process was gained in several ways. First, examination of the fermentation broth of *S. citricolor* by HPLC revealed the presence of a substance with the same retention time as neplanocin A. This compound was isolated by preparative HPLC and found to be identical with neplanocin A. It therefore seems likely that a double bond is formed between C-2 and C-6 of glucose as a result of the cyclization process leading to aristeromycin. Second, a series of incorporation experiments were carried out using specifically tritiated forms of D-glucose mixed with [^{14}C]-D-glucose as an internal reference. In these experiments both aristeromycin and neplanocin A were isolated and their tritium to carbon-14 ratios were determined. The [4-^{3}H]-D-glucose used in these experiments was prepared by the method of Kohn and Kohn;[16] all the other tritiated forms of glucose were commercially available. The results of these experiments (Table III, experiments 1–7) can be summarized as follows. Administration of [6-^{3}H]glucose yielded both aristeromycin and neplanocin A that had lost ca. 50% of the tritium label, suggesting a stereospecific loss of one hydrogen atom from C-6 of glucose. Incorporation of [5-^{3}H]glucose gave samples of the two nucleosides that had lost all of the tritium label. This observation suggests that C-5 of glucose is oxidized to a ketone as part of the cyclization process. Administration of [4-^{3}H]glucose yielded aristeromycin and neplanocin A that had lost most of the tritium label; the loss is presumably related to the inversion of configuration at C-4 of glucose which must transpire at some point in the biosynthetic pathway (see the Discussion section). The incorporation of [3-^{3}H]glucose into the two antibiotics is accompanied by complete tritium retention; this result is consistent with the noninvolvement of C-3 in the cyclization reaction. Incorporation of [2-^{3}H]glucose into aristeromycin and neplanocin A

(15) Williams, D. L.; Whaley, T. W. *J. Labelled Compds. Radiopharm.* **1981**, *19*, 669.
(16) Kohn, B.; Kohn, P. *J. Org. Chem.* **1963**, *28*, 1037.

proceeds with virtually complete tritium loss, within experimental error (about ±5%); this is consistent with the formation of a double bond between C-2 and C-6 of glucose during the biosynthesis. Lastly, administration of [1-^{3}H]glucose produced the two antibiotics that exhibited a 43% retention of tritium. Since this value was difficult to interpret, the feeding experiment was repeated. The tritium to carbon-14 ratio observed for aristeromycin in the second experiment corresponded to 59% tritium retention. While the average of the two values is about 50%, this degree of tritium loss from C-1 is difficult to interpret mechanistically (see the Discussion section).

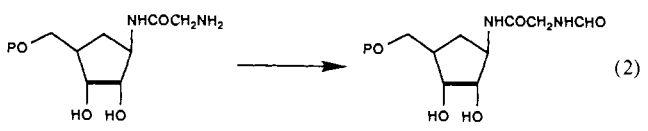
The degree of tritium loss observed from (6*RS*)-[6-^{3}H]-D-glucose during aristeromycin biosynthesis suggests that there is a stereospecific removal of one hydrogen atom from this position of the hexose. Additional insight into the cyclization mechanism was therefore sought by using (6*R*)- and (6*S*)-[6-^{3}H]-D-glucose as aristeromycin precursors. The stereospecifically tritiated glucoses were prepared enzymatically[17] and administered to *S. citricolor* growing in replacement culture. The results of these two experiments (Table III, expts 8, 9) clearly show that D-glucose is converted into aristeromycin and neplanocin A with loss of the 6 *pro-S* hydrogen atom.[18]

The preceding observations indicate that the 6 *pro-R* hydrogen atom of glucose is retained in aristeromycin. This hydrogen atom should reside at C-6′ of the antibiotic and occupy either the 6′ *pro-R* or 6′ *pro-S* position. The configuration of the 6 *pro-R* hydrogen atom of glucose in aristeromycin was determined by administration of commercial (6-^{2}H$_2$)-D-glucose to *S. citricolor* and examination of the resulting antibiotic by ^{2}H NMR spectrometry. The deuterium NMR spectrum of the labeled nucleoside exhibited a strong enrichment at 2.6 ppm. The two hydrogens present at C-6′ of aristeromycin resonate at 1.78 and 2.53 ppm. The configuration of these two hydrogen atoms was determined by NOE experiments which clearly show that the signal at 2.53 ppm corresponds to the 6′ *pro-S* hydrogen atom of the antibiotic. One can conclude, therefore, that the 6 *pro-R* hydrogen of glucose resides at the 6′ *pro-S* position of aristeromycin (eq 1).

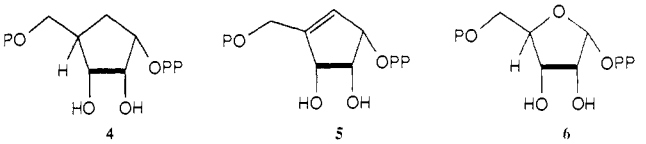
(1)

Discussion

Our investigations of the biosynthesis of the adenine ring of aristeromycin (Table I expts 1–6) establish that the purine nucleus is assembled in a manner that is well precedented for bacteria. The results of an incorporation experiment with doubly labeled adenosine (Table I, expt 7) indicate that *S. citricolor* can biosynthesize aristeromycin via the purine-salvage pathway, but it does not rule out the simultaneous operation of a stepwise assembly process for the adenine ring. If such a stepwise assembly takes place, it is possible that the same enzymes that catalyze the biosynthesis of the adenine ring of adenosine in *S. citricolor* are employed to catalyze the assembly of the adenine ring on a carbocyclic analogue of PRPP. Evidence in support of this possibility is provided by a recent report that mammalian glycinamide ribonucleotide transformylase, which catalyzes the second step in purine biosynthesis, will utilize the carbocyclic analogue of glycinamide ribonucleotide as a substrate (eq 2).[19] Nevertheless, a crucial question remains unanswered regarding the biosynthesis of aristeromycin via either the purine-salvage route or the stepwise-assembly route. This question concerns the nature of the carbocylic substrate utilized in either of these pathways. The co-occurrence of aristeromycin and neplanocin A in the same fermentation suggests that the carbocyclic substrate could be either the cyclopentyl analogue of PRPP, **4**, or the cyclopentenyl analogue **5**. Since the allylic pyrophosphate **5** would more closely approximate the chemical reactivity of PRPP (**6**), **5** might be a more likely substrate for introduction of the purine ring than **4**.

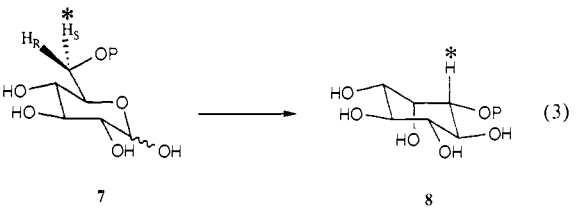


(2)



The precursor incorporation experiments carried out with ^{13}C-labeled glucose and fructose (Table II expts 3–5) establish that the cyclopentane ring of aristeromycin is biosynthesized by C–C bond formation between C-2 and C-6 of glucose. This mode of cyclopentane ring formation may be widespread in nature, since it has recently been reported that the biosynthesis of the carbocyclic pentose analogue linked to bacteriohopanetetrol in *Methylobacterium organophilum* also involves bond formation between C-2 and C-6 of glucose.[20]

The incorporation experiments with tritiated forms of glucose (Table III, expts 1–9) place constraints upon the number of possible mechanisms that can be envisioned for the cyclization of glucose to the cyclopentyl moiety of aristeromycin and neplanocin A. The closest analogy to the cyclization process may be the cyclization of D-glucose 6-phosphate (**7**) to *myo*-inositol 1-phosphate (**8**) (eq 3). However, this transformation has been



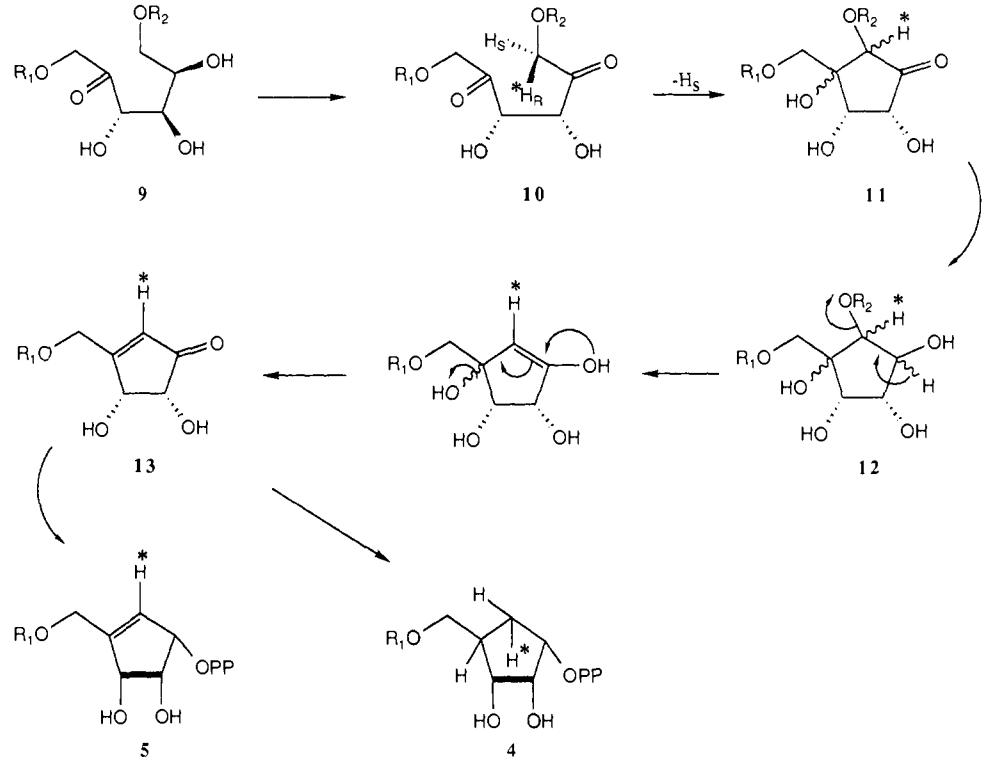
(3)

found to proceed with the stereospecific loss of the 6 *pro-R* hydrogen atom of glucose,[21] while the cyclization associated with aristeromycin and neplanocin A biosynthesis leads to loss of the 6 *pro-S* hydrogen atom of glucose (Table III, expts 8 and 9). The significance of this stereochemical difference is not clear at the present time.

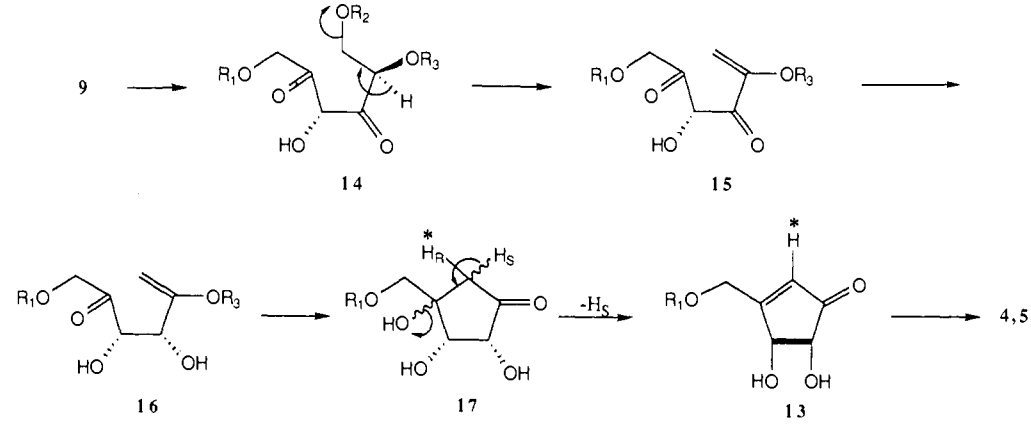
The conversion of D-glucose into aristeromycin must involve an inversion of the C-4 hydroxyl group at some stage in the biosynthesis. One might therefore predict that D-galactose would be an intermediate in the biosynthesis. Administration of [1-^{14}C]-D-galactose to *S. citricolor* yields antibiotic exhibiting an exceedingly low incorporation figure (0.000005%), a result that is probably due to catabolite repression.[22] A more informative result is obtained from the administration of [4-^{3}H]-D-glucose (Table III, expt 3), which is incorporated into aristeromycin with complete tritium loss. Studies of the enzymatic conversion of D-glucose to D-galactose[23] have shown that the reaction proceeds via reversible oxidation of the C-4 hydroxyl group by enzyme-bound NAD$^+$ and that the transformation proceeds with no introduction of solvent protons into the substrate. It therefore appears unlikely that galactose is an intermediate in aristeromycin biosynthesis. If the preceding observations are combined with the results from the incorporation experiments utilizing [5-^{3}H]-, [3-^{3}H]-, and [2-^{3}H]-D-glucose (Table III expts 2, 4, and 5), it is

(17) Snipes, C. E.; Brillinger, G.-U.; Sellers, L.; Mascaro, L.; Floss, H. G. *J. Biol. Chem.* **1977**, *252*, 8113.
(18) There appears to be some excess tritium loss from both the 6*S* and 6*R* isomers (compare with ref 21). The reason for this is presently unknown.
(19) Caperelli, C. A.; Price, M. F. *Arch. Biochem. Biophys.* **1988**, *264*, 340.
(20) Flesch, G.; Rohmer, M. J. *Chem. Soc., Chem. Commun.* **1988**, 869.
(21) Loewus, M. W.; Loewus, F. A.; Brillinger, G.-U.; Otsuka, H.; Floss, H. G. *J. Biol. Chem.* **1980**, *255*, 11710.
(22) Lewin, B. *Genes II*; Wiley: New York, 1985; p 249.
(23) Walsh, C. *Enzymatic Reaction Mechanisms*; Freeman: San Francisco, 1979; p 347.

Scheme I



Scheme II



possible to formulate two plausible mechanisms for the formation of the carbocyclic rings present in aristeromycin and neplanocin A. Because of the analogy provided by *myo*-inositol phosphate formation, the cyclization reaction in both mechanisms is presumed to proceed via a fructose derivative (**9**) (R_1, R_2 = H or P). The first mechanism (Scheme I) begins with oxidation of the C-5 hydroxyl of **9** to a ketone. Epimerization at C-4 would then yield intermediate **10**.[24] Cyclization of **10** would proceed with loss of H_S to generate the cyclopentane derivative **11**. Reduction of **11** to **12** followed by two eliminations would produce the cyclopentenone **13**. Reduction of the ketone function of **13** with subsequent phosphorylation could then give the carbocyclic PRPP analogue **5**. Alternatively, the double bond in cyclopentenone **13** could be reduced in an anti fashion with subsequent reduction of the carbonyl group and phosphorylation to produce the carbocyclic PRPP analogue **4**.

The second mechanism (Scheme II) proceeds from intermediate **9** by oxidation of the C-4 hydroxyl group to give the keto derivative **14**, followed by dehydration to an enol or enol phosphate, **15** (R_1, R_3 = H or P). Reduction of **15** to **16** could then be succeeded by cyclization to the cyclopentanone **17**. The latter compound could undergo dehydration with loss of H_S to yield the cyclopentenone **13**, an intermediate that also appears in the first cyclization mechanism. This second cyclization mechanism bears some resemblance to the reaction catalyzed by the enzyme dehydroquinate synthase.[25]

The hypothetical cyclization mechanisms shown in Schemes I and II are consistent with all of the data listed in Table III, except for the results obtained with [1-^{3}H]glucose (expt 6 and 7). Both of the cyclization mechanisms predict that [1-^{3}H]glucose should be incorporated into aristeromycin and neplanocin A with complete tritium retention. The values observed in two experiments were 43 and 59%, giving an average value of about 50%. At present we suspect that this loss of tritium is due to some exchange process that is unrelated to aristeromycin biosynthesis. The exchange process might be chemical in nature or it might result from the operation of one or more reversible enzymatic reactions. For example, an initial stage in the biosynthesis of aristeromycin may involve the isomerization of [1-^{3}H]glucose 6-phosphate to [1-^{3}H]fructose 6-phosphate by glucose-6-phosphate isomerase. This

(24) Epimerization at C-4 is arbitrarily shown as taking place at an early stage in the pathway.

(25) See: Widlanski, T. S.; Bender, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 1873, and references cited therein.

transformation will yield [1-^{3}H]fructose 6-phosphate that carries tritium in the 1 *pro-S* position.[26] If the labeled [1-^{3}H]fructose 6-phosphate is then reversibly isomerized to [1-^{3}H]mannose 6-phosphate by mannose-6-phosphate isomerase, the migrating 1 *pro-S* hydrogen could undergo partial exchange with the medium.[27,28]

Summary

Investigations using the aristeromycin producing organism *S. citricolor* have established that the primary building blocks for the adenine ring of this antibiotic are the same as those utilized in other bacterial species. An experiment with adenosine doubly labeled in the adenine ring demonstrated that the adenine ring of aristeromycin can arise via the purine-salvage pathway, but the simultaneous operation of the de novo pathway for purine ring assembly was not ruled out. Neplanocin A was isolated from the fermentation broth of *S. citricolor*, suggesting a close relationship between the biosynthetic pathways leading to these two nucleoside antibiotics. Experiments with 11 labeled forms of D-glucose demonstrated that the cyclopentyl moiety of both nucleosides is created by C–C bond formation between C-2 and C-6 of glucose and revealed several critical features of the cyclization mechanism. A more complete understanding of the mechanism of cyclopentane ring formation will undoubtedly require access to cell-free extracts that catalyze the cyclization reaction.

Experimental Section

General Methods. ^{1}H and ^{13}C NMR spectra were recorded on either a JEOL FX-90Q (90 and 22.5 MHz, respectively) or on an IBM AF300 (300 and 75.5 MHz, respectively) spectrometer. Chemical shifts are given in parts per million downfield from tetramethylsilane for proton spectra taken in $CDCl_3$. For proton spectra taken in D_2O, sodium (2,2,3,3-$^2H_4$)-3-(trimethylsilyl)propionate was used as an internal standard. ^{13}C NMR spectra in D_2O utilized either methanol or *p*-dioxane as an internal reference. All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Ultraviolet spectra were measured on a Varian DMS 80 UV/visible spectrophotometer. Infrared spectra were obtained with a Perkin-Elmer 1320 IR spectrophotometer or a Beckman 4230 spectrophotometer. Mass spectra were run on Finnigan 3300 and CEC 111021-110B mass spectrometers. High-performance liquid chromatography was carried out with either an Altex 110A pump or a Spectra Physics SP8700 Solvent Delivery System. The monitors used were a Hitachi Model 100-40 variable-wavelength spectrophotometer, an ISCO Model UA-5 absorbance monitor set at 254 nm, an ISCO Model V4 variable-wavelength monitor, or an Altex refractive-index detector. For the analysis of all nucleosides and their analogues, Altex or Whatman ODS (C18) reverse-phase columns, 4.6 mm × 150 mm, were used. MPLC was carried out with an Eldex B-100S pump. Carbohydrates were analyzed with a BioRad Aminex HPX-87C column, 4 mm × 250 mm. Samples for liquid scintillation counting were weighed out on a Perkin-Elmer AD-2 autobalance and counted on either a Beckman LS 100C or LS 3801 liquid scintillation counter. Aquasol 2 from New England Nuclear was used as the scintillation cocktail. The radiochemical purity of radioactive precursors was evaluated using a Berthold LB 2832 TLC Linear Analyzer interfaced with an Apple IIe microcomputer. Centrifugation was carried out with a DuPont Sorvall RC-5B refrigerated centrifuge. Measurements of pH were made with a Corning Model 12 pH meter or an Orion Model 611 pH meter. Lyophilization was carried out with a Labline benchtop lyophilizer or an FTS Maxi-Dry lyophilizer. Fermentations were conducted in a New Brunswick Scientific G-25 rotary shaker. Analytical thin-layer chromatography was performed with precoated Merck silica gel type 60, F-254 (0.25-mm layers on glass plates) and Merck precoated cellulose F (0.1-mm layers on glass plates). Column chromatography was performed with Baker silica gel (60–200 mesh or 200–400 mesh), Baker reversed-phase octadecyl silane bonded to silica gel (40 μm), Sephadex G-10 and SP-Sephadex C-25 (Pharmacia Corp.), and Dowex 50X8 ion-exchange resin (Bio-Rad).

Materials. Authentic aristeromycin was obtained from Wako Pure Chemical Industries., Ltd. and as a gift from Schering Corp., courtesy of Dr. T. L. Nagabhushan. Authentic neplanocin A was a gift of Toyo Jozo Co., Ltd., courtesy of Dr. S. Yaginuma, and a gift of the National Cancer Institute, courtesy of Dr. M. Suffness. Radioactive precursors were purchased from New England Nuclear or Amersham/Searle Corp. Precursors labeled with stable isotopes were obtained from Merck and Co., Cambridge Isotopes, KOR Isotopes, and from Omicron Biochemicals. Enzymes were obtained from Sigma Chemical Co. and Boehringer Mannheim Biochemicals.

Organism and Fermentation. *S. citricolor* IFO 13005 was obtained from The Institute for Fermentation, Osaka, Japan. The organism was maintained on yeast malt extract agar slants[26] at 28 °C and preserved by gas-phase liquid nitrogen storage in 20% glycerol–water, 10% glycerol-5% lactose–water,[27] or lyophilization in "mist. desiccans."[28] The fermentation medium used for "normal" fermentations was that described by Kusaka et al.[2a] A typical fermentation was carried out on a 2-L scale with the medium distributed equally in 10 1-L Erlenmeyer flasks closed with foam plugs. Each flask was inoculated with 2 mL of a spore suspension prepared by adding 10 mL of sterile water to each of two yeast malt agar slants. The inoculated flasks were then incubated at 160 rpm and 28 °C. Under these conditions, antibiotic production began at 48 h and peaked at approximately 144 h. Under optimum conditions, the yield of isolated antibiotic was ca. 15 mg/L.

Isolation of Aristeromycin. In our hands, the isolation procedure described by Kusaka et al.[2a] proved unsatisfactory. The following is a typical procedure used for the isolation of aristeromycin from 2 L of fermentation broth.

The mycelium was removed from the fermentation broth by centrifugation in 500 mL centrifuge bottles (5000 rpm, 10 min). The pH of the combined supernatants was adjusted to 8.0 with 1 N NaOH. Then 100 g of coarse, water-washed activated charcoal (12 × 40 U.S. mesh, Calgon Corp.) was added and the mixture was stirred for 1 h. The charcoal was allowed to settle and the supernatant was discarded. The charcoal was washed several times with deionized water and then loaded into a 25 × 500 mm glass column. It was first eluted with 1.5 L of acetone–water, 12:3, followed by 1.5 L of acetone–water, 3:12. The acetone was removed from the yellow eluate in vacuo and the resulting aqueous solution was lyophilized. The residue was dissolved in 20 mL of water and applied to a 25 × 500 mm glass column containing 50 mL of Dowex 50X8, 20–50 mesh. The column was washed with water until the eluate was no longer yellow (800–1000 mL). The column was then eluted with 1.5 L of 4 N NH_4OH. Ammonia was removed from the eluate in vacuo and the resulting solution was lyophilized.

The residue was dissolved in 2–3 mL of water, with heating if necessary, and the solution was applied to a 15 × 800 mm Sephadex G-10 column which was then eluted with water at a flow rate of ca. 0.5 mL/min. Fractions (12 mL) were collected and monitored by HPLC with a Whatman Partisphere 110 × 4.7 mm reverse-phase column and water–methanol (92:8) as the solvent. With a flow rate of 1.5 mL/min, the retention times for aristeromycin and neplanocin A were ca. 12 and 7 min, respectively. The appropriate fractions were combined and lyophilized. If the resulting aristeromycin could not be easily crystallized from water, further purification was carried out by MPLC with an Altex, glass 9 × 500 mm reverse-phase column with water–methanol (92:8) as a solvent and a flow rate of 1.5 mL/min.

Administration of Labeled Compounds to *S. Citricolor*. The labeled precursor was dissolved in 10 mL of distilled water and 0.5 mL of the solution was added to each of the 10 1-L flasks through a sterile Millipore filter after 36 h of fermentation time. The remainder of the precursor solution was added in 0.5-mL aliquots to the fermentation broth after 48 h.

Fermentation under Replacement Conditions. For precursor incorporation experiments with labeled D-glucose, D-galactose, and D-fructose, the following general procedure was utilized. A 2-L fermentation was initiated in the manner described under Organism and Fermentation. After 36 h, the fermentation broth was transferred to several sterile centrifuge bottles (500 mL) and the mixture was centrifuged at ca. 9000 rpm and 10 °C for 20 min. Subsequent operations were conducted in a laminar-flow hood. The supernatant was decanted and the solid material was combined into two portions. Each portion was suspended in 250 mL of 0.2 M NaCl and then centrifuged at 9000 rpm for 20 min at 10 °C. The sequence of centrifugation, decantation, and resuspension in 0.2 M NaCl was carried out three times by which point the supernatant was clear. The washed mycelium was then suspended in enough 0.2 M NaCl to give a thick, pourable slurry. This slurry was distributed

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approximately equally between 10 1-L Erlenmeyer flasks, each of which contained 200 mL of sterile replacement medium. The replacement medium was identical to the "normal" fermentation medium described by Kusaka et al.[2a] except that it lacked both glucose and soluble starch. After replacement, the fermentation was carried out in the usual fashion. Labeled precursors were administered at ca. 40 and 48 h after the start of the original fermentation. The fermentation was usually terminated at about 115 h. The yield of aristeromycin under replacement conditions was typically less than half that produced under "normal" fermentation conditions.

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Registry No. 1, 19186-33-5; **2**, 72877-50-0; sodium formate, 141-53-7; glycine, 56-40-6; sodium bicarbonate, 144-55-8; adenosine, 58-61-7; D-ribose, 50-69-1; D-glucose, 50-99-7; D-fructose, 57-48-7.

Mechanism and Synthetic Applications of the Photochemical Generation and X–H Insertion Reactions of Oxacarbenes

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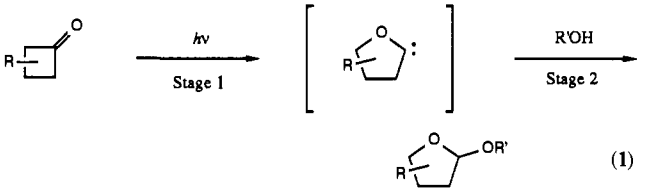
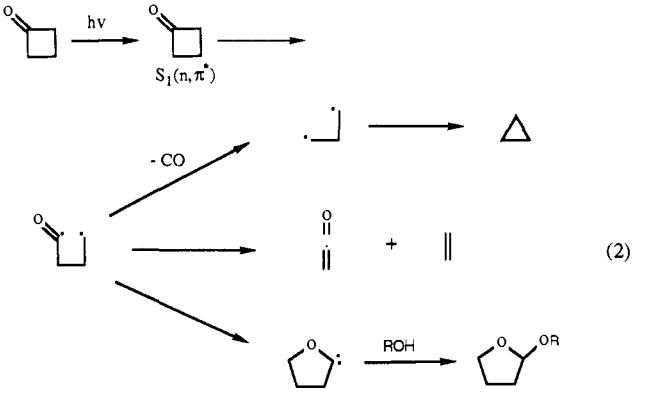
Abstract: A kinetic study of the photochemical ring expansion of cyclobutanones to oxacarbenes has shown that, in the presence of alcohol, the oxacarbene is generated irreversibly. Substrates for the intramolecular versions of the oxacarbene generation–insertion sequence (heteroalkylcyclobutanones) were prepared by an intramolecular [2 + 2]-Baeyer–Villiger sequence. These have been used to form 5-, 6-, 7-, and 8-membered nitrogen, oxygen, and sulfur bicyclic ring systems.

The production of tetrahydrofuryl ethers via irradiation of cyclobutanones in the presence of alcohols has been postulated to proceed as a two-stage process: the first is generation of a 2-tetrahydrofuranylidene; the second is insertion into the O–H bond (eq 1). Much of the previous work on this reaction has



(1)

focused on stage 1. The mechanistic postulate of Yates[2] for the first reported oxacarbene[3] generation, namely α-cleavage to a biradical followed by rebonding on oxygen (eq 2), has been adopted



(2)

widely.[4] This mechanistic view is strongly supported by the following factors: that ring expansion is highly regioselective in unsymmetrical cyclobutanones, that byproducts include cyclopropanes from decarbonylation and ketene-derived esters from β-cleavage, and that alkyl substitution enhances the yield of ring expansion products.[5] This latter point reflects the requirement for a nucleophilic alkyl radical for attack at the oxygen of the acyl radical.[6] One impediment to acceptance of the hypothesis is the retention of stereochemistry observed when the cyclobutanone possesses an α-stereogenic center.[7,8] This has led Quinkert to propose, based on qualitative and quantitative MO arguments, that conversion of S_1 (n,π^*) cyclobutanone to the oxacarbene may be a concerted process.[9] However, stereospecific reactions (β-cleavage or ring formation) involving 1,4-biradicals are well-known.[10] This seeming conflict was resolved by Miller,[11] who independently generated 1,4-acylalkyl biradicals from the corresponding 1,1-diazenes. When the biradicals are produced in methanol, the tetrahydrofuryl ethers derived from them show retention of configuration. Thus, the α-cleavage mechanism has

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