Revised Pathway for the Biosynthesis of Aristeromycin and Neplanocin A from D-Glucose in *Streptomyces citricolor*

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Aristeromycin (**1**) and neplanocin A (**2**) are two naturally occurring carbocyclic nucleosides produced by *Streptomyces citricolor*. Aristeromycin was first isolated in 1967,[1] having been previously prepared in racemic form in 1966;[2] neplanocin A was first reported in 1981[3] from *Ampullariella regularis* and subsequently shown to be coproduced at low levels alongside aristeromycin in *S. citricolor*.[4] Neplanocin A has been shown to possess both antiviral[5] and antitumor activity.[3] Structurally, **1** and **2** are closely related, differing only in the presence in **2** of a double bond between C4′ and C6′. Both contain a carbocyclic ribose ring to which is attached an adenine ring at C1′. Herein we describe studies that have led us to propose a revised pathway for the biosynthesis of **1** and **2** in *S. citricolor*.

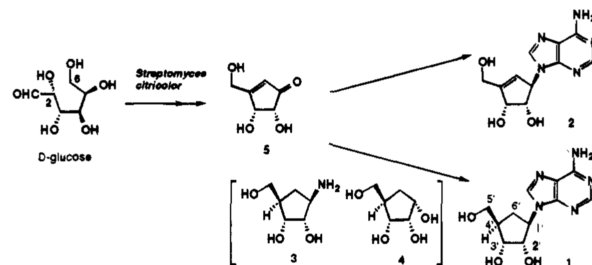
Seminal work by Parry's group in the late 1980s established a number of important facts concerning the biosynthesis of **1** and **2**.[4,6] Using the wild-type *S. citricolor*, Parry was able to show that (i) the carbocyclic ring is derived from D-glucose, (ii) cyclization occurs between C2 and C6 of D-glucose, (iii) feeding of labeled glycine and bicarbonate led to aristeromycin in which the adenine was labeled, and (iv) feeding of labeled adenosine led to intact incorporation of adenine, albeit at low efficiency (0.39%). Later, Parry and Johnson reported the preparation of the putative intermediates **3**[7] and **4**[8] and, by isotope dilution studies, concluded that **3** and **4** were intermediates on the biosynthetic pathway, leading them to propose a biosynthetic pathway in which D-glucose is converted to the enone **5**, followed by bifurcation at an early stage (Scheme 1).

The starting point for our work involved the generation of a number of mutants of *S. citricolor* that were blocked in their ability to synthesize either **1** or **2**. From a series of cosynthesis experiments in which pairs of mutants were selected in turn and examined for their ability to cosynthesize **1** and **2**, it became possible to identify secretor/convertor pairings in which the production of aristeromycin and neplanocin A could be recovered. In this context, it was shown that a mutant CC914 secreted a compound that supported production of **1** and **2** in mutant CC940 (Scheme 2).

We have previously determined the structure of this compound and shown it to be the unsaturated tetrol **6**.[9] The isolation of **6** led us to propose,[9] for the first time, that *contrary to the pathway shown in Scheme 1, neplanocin A may be a precursor of aristeromycin*. The next step was to establish unequivocally that **6** was an intermediate on the biosynthetic pathway. This was achieved by preparing 6-^{13}C-labeled tetrol **6a** from D-ribose,[10,11] feeding **6a** to the convertor CC940, and purifying the ^{13}C-labeled aristeromycin (35% yield) and neplanocin A (8%). ^{1}H and ^{13}C NMR spectroscopy clearly established that the ^{13}C label had been incorporated at >95% and was located entirely at C6′ in the carbocyclic ring of **1** and **2**.

We decided next to prepare other putative intermediates in an attempt to map out the biosynthetic pathway. Thus, the enone **5**,[12] C1-*epi*-tetrol **7**,[13] and enamine **8**[14] were synthesized and fed to the convertor CC940. Significantly, the addition of enone **5** supported the production of **1** and **2** by CC940, whereas under identical conditions, the C1-*epi*-tetrol **7** and the enamine **8** did not. When the saturated tetrol **4**[15] and aminotriol **3**[16] were likewise prepared and fed to CC940, *there was no evidence of production of aristeromycin 1*, despite the evidence from isotope dilution studies, reported by Parry and Johnson,[7,8] that **3** and **4** were present in producing cultures.

Scheme 1. Parry−Johnson Proposal for the Biosynthesis of **1** and **2** from D-Glucose in *Streptomyces citricolor*[7,8]



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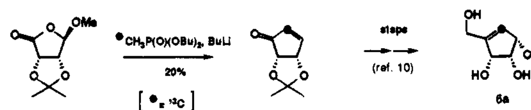
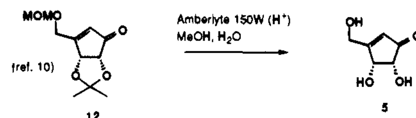
(1) Kusaka, T.; Yamamoto, H.; Shibata, M.; Muroi, M.; Kishi, T.; Mizuno, K. *J. Antibiot.* **1967**, *21*, 255.
(2) Shealy, Y. F.; Clayton, J. D. *J. Am. Chem. Soc.* **1966**, *88*, 3885.
(3) Yaginuma, S.; Muto, N.; Tsujino, M.; Sudate, Y.; Hayashi, M.; Otani, M. *J. Antibiot.* **1981**, *34*, 359.
(4) Parry, R. J.; Bornemann, V.; Subramanian, R. *J. Am. Chem. Soc.* **1989**, *111*, 5819.
(5) Borchardt, R. T.; Keller, B. T.; Patel-Thombre, U. *J. Biol. Chem.* **1984**, *259*, 4353.
(6) Parry, R. J.; Bornemann, V. *J. Am. Chem. Soc.* **1985**, *107*, 6402.
(7) Parry, R. J.; Haridas, K.; De Jong, R.; Johnson, C. R. *J. Chem. Soc., Chem. Commun.* **1991**, 740.
(8) Parry, R. J.; Haridas, K.; De Jong, R.; Johnson, C. R. *Tetrahedron Lett.* **1990**, *31*, 7549.
(9) Roberts, S. M.; Thorpe, A. J.; Turner, N. J.; Blows, W. M.; Buss, A. D.; Dawson, M. J.; Noble, D.; Rudd, B. A. M.; Sidebottom, P. J.; Wall, W. F. *Tetrahedron Lett.* **1993**, *34*, 4083.
(10) Hill, J. M.; LeGrand, D.; Roberts, S. M.; Thorpe, A. J.; Turner, N. J. *J. Chem. Soc., Perkin Trans. 1* **1994**, 1483.

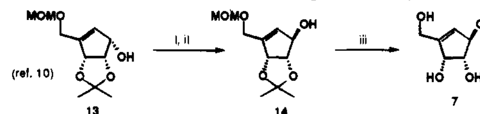
(11) 6-^{13}C-Labeled tetrol **6a** was prepared as follows:



(12) Enone **5** was prepared from protected enone **12**:

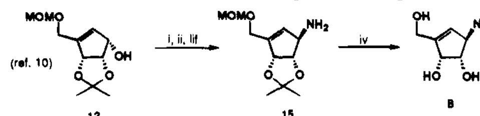


(13) C1-*epi*-Tetrol **7** was prepared from protected cyclopentenol **13**:



Reagents: (i) DEAD, PPh_3, AcOH (95%); (ii) K_2CO_3, MeOH (73%); (iii) Amberlyte 150W (H^+), MeOH, H_2O.

(14) Unsaturated aminotriol **8** was prepared from protected amine **15**:



Reagents: (i) MsCl, Et_3N (98%); (ii) NaN_3, 15-C-5 (89%); (iii) Ph_3P, H_2O (77%); (v) Amberlyte 150W (H^+).

(15) Prepared according to the published procedure: Bestmann, H. J.; Roth, D. *Synlett* **1990**, 751.
(16) Prepared in single enantiomer form according to a modification of the published procedure: Cermak, R. C.; Vince, R. *Tetrahedron Lett.* **1981**, *22*, 2331.

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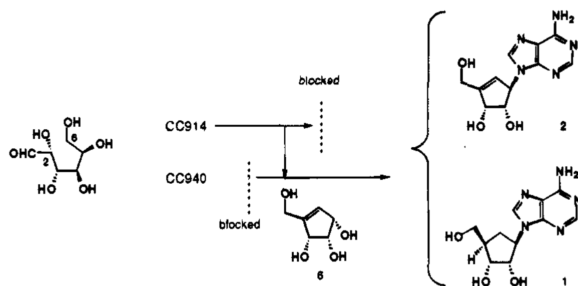
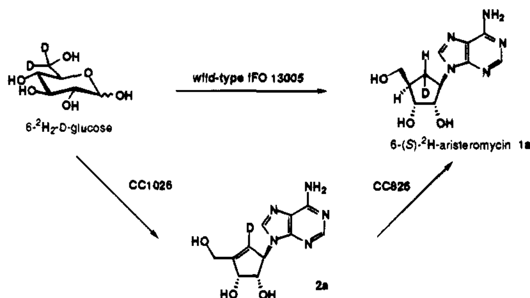
Scheme 2. Cosynthesis of Neoplanocin A and Aristeromycin Using CC914 (Secretor) and CC940 (Convertor)



Scheme 3. Stereochemistry of Deuterium Incorporation into **1a** and **2a**



These results began to suggest a biosynthetic pathway in which the flux of intermediates was D-glucose → enone **5** → tetrol **6** → neplanocin A (**2**) → aristeromycin (**1**). The suggestion that neplanocin A was a direct precursor of aristeromycin became a key feature of this proposal, and we therefore decided to direct our attention to experiments that might support this thesis.

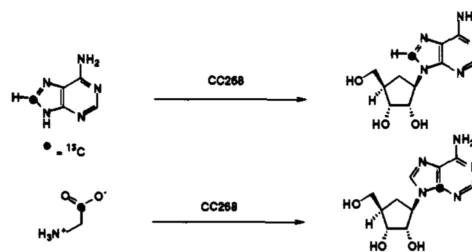
The wild-type *S. citricolor* IFO 13005 produces low levels of **2** in addition to **1**. Furthermore, we have isolated mutant strains which produce increased levels of **2** relative to **1**. Preliminary experiments[9] had already shown that a number of mutants, which were unable to produce either neplanocin A or aristeromycin, were able to convert added neplanocin A to aristeromycin. This observation subsequently led Parry and Jiang[17] to clearly demonstrate that the wild-type *S. citricolor* possessed an activity that could convert neplanocin A to aristeromycin, and they proposed a mechanism for the transformation. In attempts to further establish whether or not the normal biosynthetic route to aristeromycin in *S. citricolor* proceeded via neplanocin A by saturation of the double bond, we were stimulated by an earlier experiment reported by Parry.[6] Using wild-type *S. citricolor*, he showed that [6-$^2H_2$]D-glucose was converted to [6′-^{2}H]aristeromycin (**1a**) in which the deuterium atom was located only in the *pro-S* site (Scheme 3). Repetition of this experiment in our laboratory using IFO 13005, a wild-type strain, gave an identical result.

We then carried out the analogous experiment using the mutant strain CC1026, which produces neplanocin A but no aristeromycin. We were able to isolate [6′-^{2}H]neplanocin A (**2a**) (~50% incorporation of ^{2}H). This material was fed to a second mutant strain, CC826, which was blocked in the production of both carbocyclic nucleosides. The purified **1a** contained deuterium label only in the *pro-S* site. Thus, the **1a** obtained from [6-$^2H_2$]D-glucose was stereochemically identical to that derived from **2a**, providing further evidence that *neplanocin A is the direct precursor of aristeromycin.*

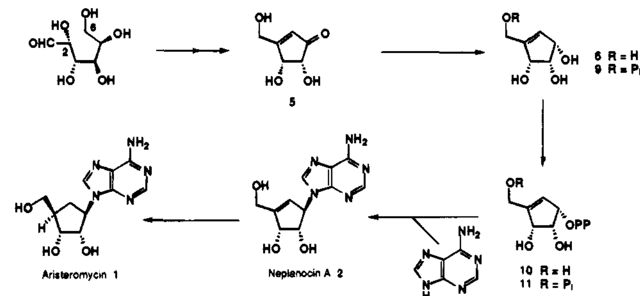
Finally, we turned our attention to the origin of the adenine base. The work of Parry's group[6] suggested that the adenine ring is derived from *de novo* purine biosynthesis (from glycine and formate) although some evidence was presented for the operation of a salvage pathway in which the adenine ring is incorporated intact. In order to further probe the latter pathway,

(17) Parry, R. J.; Jiang, Y. *Tetrahedron Lett.* **1994**, *35*, 9665.

Scheme 4. Origin of the Adenine Base in Aristeromycin



Scheme 5. Revised Pathway for the Biosynthesis of Aristeromycin and Neplanocin A



a number of mutants defective in purine biosynthesis and requiring adenine supplementation for normal growth in defined media were isolated. One of these, CC268, was grown in a defined medium in which the only source of purines was represented by 400 μg mL^{-1} of [8-^{13}C]adenine. The ^{13}C-labeled aristeromycin that was produced (27% yield) was found to contain the ^{13}C label solely at C8 in the adenine ring (~75% incorporation) (Scheme 4). This establishes the existence of a pathway whereby adenine can be incorporated intact into aristeromycin. In a complementary experiment, CC268 was administered with [1-^{13}C]glycine, which led to the isolation of aristeromycin (5% yield) containing the ^{13}C label at C4 in the adenine ring (~20% incorporation). We believe that this incorporation is due to the auxotroph being slightly "leaky", especially in liquid media, and is consistent with either a parallel pathway involving *de novo* biosynthesis of the adenine base upon a pre-existing amino group (*e.g.*, in enamine **8**) or synthesis of adenine and intact incorporation as described above. Taken together, these results strongly support the direct incorporation of adenine as a major route to aristeromycin in at least some conditions, while they do not eliminate the possibility of a *de novo* route playing a significant role in other circumstances.

Careful consideration of the results outlined above has encouraged us to propose a revised pathway for the biosynthesis of **1** and **2** (Scheme 5). The central feature of this pathway is a linear route from the enone **5** to **1** without bifurcation. Reduction of the enone **5** to the tetrol **6** followed by activation at C1 would lead, for example, to the pyrophosphate **10** (these conversions may proceed via the phosphates **9** and **11**). Allylic displacement at C1 of **10** or **11** introduces the adenine base, yielding **2**, which undergoes reduction to **1**. It is noteworthy that, according to this scheme, incorporation of the adenine base occurs via displacement at an allylic center and hence is favored on mechanistic grounds.

Examination of Scheme 5 also reveals many unresolved questions concerning the detailed biosynthetic pathway, in particular: (i) the identity of the intermediates between D-glucose and the enone **5**, especially the timing of the inversion of the C4-hydroxyl group of D-glucose; (ii) the exact nature of the intermediates **6**, **9**, **10**, and **11**; and (iii) the mechanism of reduction of **2** to **1**.[17] Experiments aimed at probing some of these problems are currently underway in our laboratories.

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