# The Biosynthesis of Carbocyclic Nucleosides

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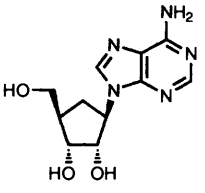
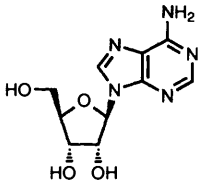
## 1 Introduction

### 1.1 Isolation and Biological Activity

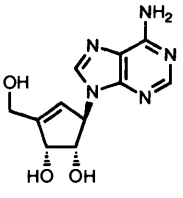
Carbocyclic nucleosides are naturally occurring secondary metabolites produced by certain prokaryotic organisms. Aristeromycin (1), the carbocyclic analogue of adenosine (2), was first isolated in 1967, from *Streptomyces citricolor*,[1,2] having been previously synthesized in racemic form.[3] Neplanocin A (3), and some closely related compounds, were subsequently identified in 1981 from cultures of *Ampullariella regularis*.[4,5] Recently, neplanocin A has been shown to be co-produced alongside aristeromycin by *Streptomyces citricolor*.[6] Other related carbocyclic nucleosides, *e.g.* adecypenol (4), have been isolated more recently from *Streptomyces* spp.[7]



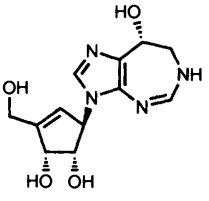

aristeromycin (1)




adenosine (2)




neplanocin A (3)




adecypenol (4)

As a result of their close structural relationship to natural nucleosides, aristeromycin and neplanocin A have been shown to possess potent biological activity. For example neplanocin A exhibits anti-viral[8] and anti-tumour[5] activity, and both aristeromycin and neplanocin A have been shown to inhibit the enzyme *S*-adenosyl homocysteine hydrolase.[9] Owing to their potential use as therapeutic agents, carbocyclic nucleosides, including aristeromycin and neplanocin have been the subject of considerable synthetic effort[10,11] including the reports of several total syntheses.[12–15]

The biosynthesis of aristeromycin and neplanocin A first received attention in the mid 1980's through the seminal work of Parry[16,17,6] who was the first to propose a route for their biosynthetic origin. Recently, in collaboration with the Natural Products Group at Glaxo Research and Development, we have carried out extensive studies using mutants of *S. citricolor*.[18,19] These studies have shed considerable light on the latter stages of the biosynthesis and have led to a significant revision of the proposed route by which these carbocyclic nucleosides are assembled. It is therefore timely to review the state of knowledge in this area, and in particular focus one aspect of the biosynthesis, namely the method that Nature uses for the conversion of carbohydrate building blocks into 5-membered rings, and to contrast this with the formation of 6-membered rings.

## 2 Origin of the Carbocyclic Ring

### 2.1 Preliminary Labelling Experiments

Early biosynthetic studies on aristeromycin and neplanocin A established that the carbocyclic skeleton was derived from D-glucose (Scheme 1).[16,6] From a biogenetic viewpoint, this places carbocyclic nucleosides in a small family of natural products, namely those that contain 5-membered carbocyclic rings that are derived from a carbohydrate precursor. Through a series of

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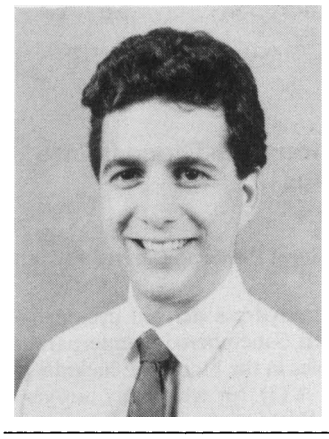
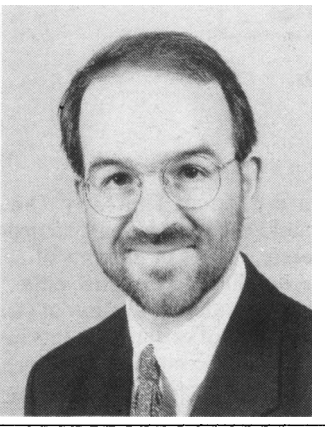
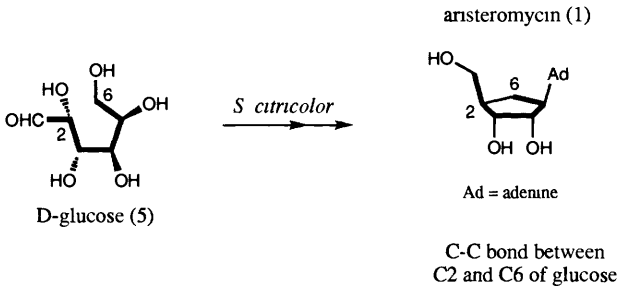


*Nick Turner was born in Kent and educated at the Universities of Bristol and Oxford. He obtained his D.Phil. from Oxford in 1985 working with Jack E. Baldwin on the biosynthesis of penicillins. Thereafter he gained a Royal Society Junior Research Fellowship spending an additional year at the Dyson Perrins Laboratory in Oxford, working on the application of NMR methods to the detection of low-level biosynthetic intermediates, followed by a year at Harvard University, U.S.A. with George M. Whitesides learning about biocatalysis. In 1987 he joined the Chemistry Department at the University of Exeter as a Lecturer and in June 1995 will be moving to the Chemistry Department at Edinburgh University as a Reader in Organic Chemistry. His research interests are in the field of bioorganic chemistry including the use of enzymes in synthesis, carbohydrate chemistry, and biosynthesis.*



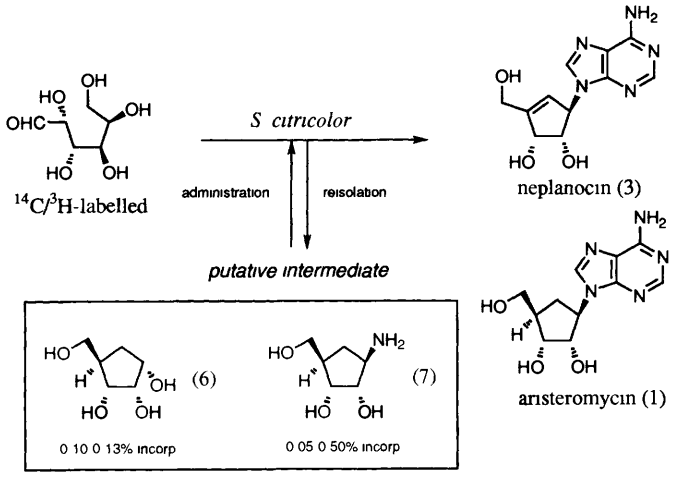



**Scheme 1** Origin of carbocyclic ring in aristeromycin

feeding experiments using isotopically labelled precursors, Parry established that formation of the carbocyclic ring in aristeromycin was achieved by union of the C2 and C6 carbon atoms of D-glucose [6] In addition, he deduced that the cyclization reaction proceeded with loss of the 6 *pro-S* hydrogen atom

### 2.2 Isotope Dilution Studies

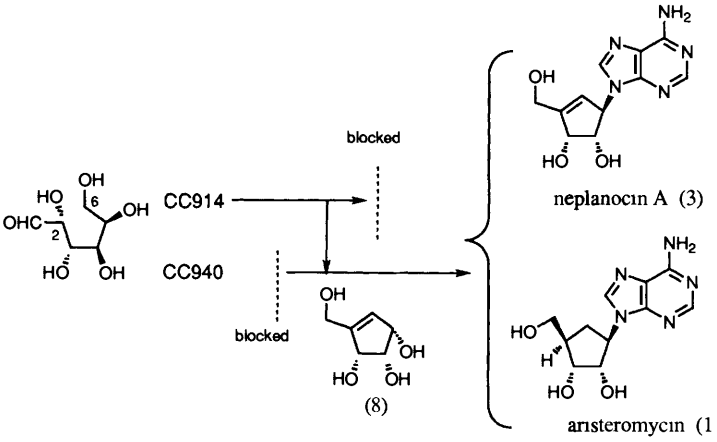
Subsequent to these initial feeding experiments using isotopically labelled precursors, Parry and Johnson reported the results of a series of isotope dilution studies that were designed to identify putative intermediates on the biosynthetic pathway (Scheme 2) These experiments were carried out as follows, a putative intermediate, that is believed to lie on the pathway, is synthesized and then added to a culture of the organism that has been administered with a radio-labelled precursor (in this case D-glucose) The intermediate is then reisolated, derivatized, and purified to constant radiochemical purity The level of incorporation of the radio-label is then assessed and compared with that of the precursor Using this approach Parry and Johnson concluded that the saturated tetrol (6)[20] and aminotriol (7)[21] were present in producing cultures and hence they proposed that they might be intermediates on the biosynthetic pathway




**Scheme 2** Isotope dilution experiments

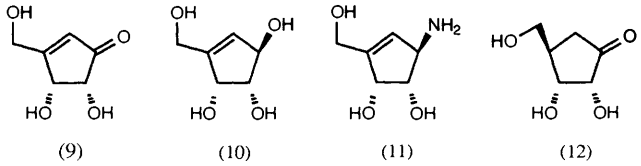
### 2.3 Cosynthesis Experiments

The work at Exeter relied upon a different approach Our collaborators at Glaxo Research and Development had carried out a programme of work to generate a series of mutants of *S citricolor* that were blocked in their ability to synthesize either neplanocin A or aristeromycin However, the production of (1) and (3) could be rescued by combinations of certain mutants in which the supernatant from cultures of one mutant (secretor) were added to a culture of a second mutant (converter) Such cosynthesis experiments led to the identification of a pairing in which mutant CC914 secreted a compound that supported the production of neplanocin A and aristeromycin in a second mutant CC940 (Scheme 3) The structure of this compound was shown to be the tetrol (8) [18] Convincing evidence that the tetrol (8) was an intermediate on the biosynthetic pathway was obtained by synthesis of 6-$^{13}$C-tetrol and feeding to CC940 This experiment yielded 6′-$^{13}$C-neplanocin and 6′-$^{13}$C-aristeromycin in which the $^{13}$C label was located entirely at C6′ in the carbocyclic ring of (1) and (3) [19]




**Scheme 3** Cosynthesis of neplanocin A and aristeromycin using CC914 (secretor) and CC940 (converter)

Furthermore, the identification of a mutant of *S citricolor* (CC940) that could act as an efficient converter suggested the possibility of synthesizing other putative intermediates and examining their conversion into (1) and (3) In this context, the addition of the enone (9)[22] to CC940 supported the production of neplanocin A and aristeromycin However, synthesis and feeding of the C1-*epi*-tetrol (10), aminotriol (11), and saturated tetrol (6) and aminotriol (7) gave no evidence of production of aristeromycin [19] Attempts to synthesize the ketone (2) were unsuccessful It thus seems probable, at this stage, that the enone (9) is the first-formed carbocyclic intermediate on the biosynthetic pathway, and that it undergoes reduction to the tetrol (8) Evidence will be presented below to account for the subsequent transformations of the enone (9) that lead to aristeromycin and neplanocin A These observations will suggest that, contrary to the previous proposal,[20 21] the saturated carbocycles (6) and (7) do not lie on the central biosynthetic pathway Before discussing this, it is relevant to examine what is known concerning the biosynthesis of 5-membered rings from carbohydrate precursors

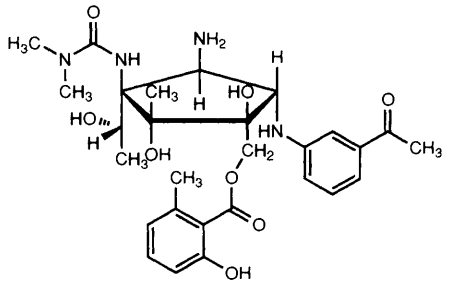



## 3 Mechanism of Conversion of D-Glucose into 5- and 6-Membered Rings

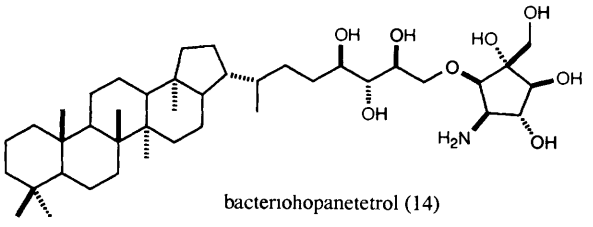
### 3.1 Related 5-Membered Ring Natural Products Derived From D-Glucose

Natural products containing carbohydrate derived cyclopentane rings are much rarer than their 6-membered counterparts Indeed, there are only four examples in the literature, including aristeromycin (1) and neplanocin A (3), for which any biosynthetic studies have been reported Pactamycin (13) was isolated from *Streptomyces pactum* var *pactum* in 1961[23] and consists of

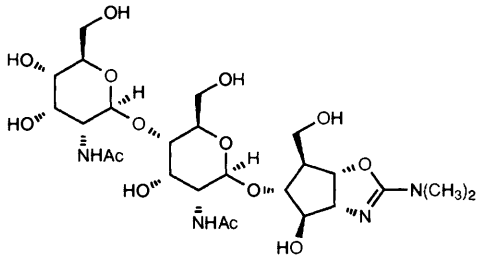
a multiply hydroxylated and aminated cyclopentane ring The bacteriohopanetetrol (14), a triterpenoid isolated from *Methylobacterium organophilum*,[24 25] *Rhodopseudomonas acidophila*,[26] and *Zymomonas mobilis*,[24 27] contains a multiply hydroxylated amino cyclopentane moiety linked to the steroidal bacteriohopane skeleton Allosamidin (15), an inhibitor of chitinase, was isolated in 1986 from a *Streptomyces* sp [28 29] This unusual disaccharide contains two *N*-acetyl-D-allosamine units linked to a carbocyclic ring named allosamizoline



pactamycin (13)



bacteriohopanetetrol (14)

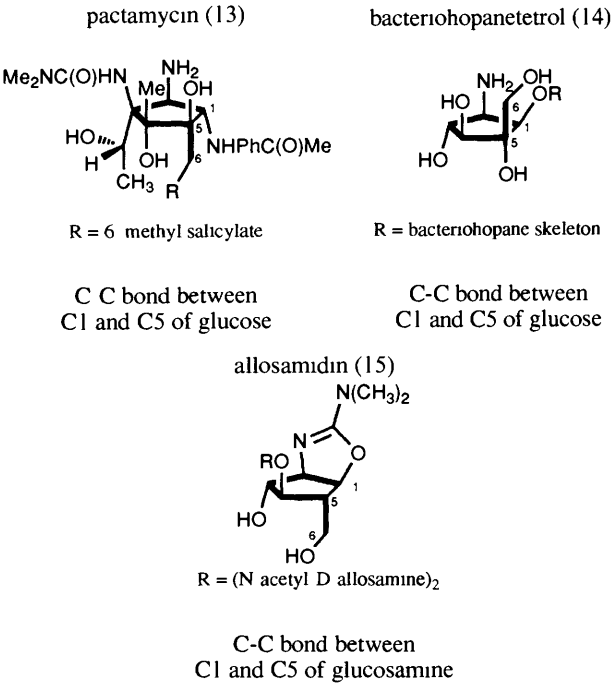


allosamidin (15)

The biosynthetic routes leading to the formation of pactamycin (13),[30 32] the bacteriohopanetetrol (14),[25 27] and allosamidin (15)[33 34] have been studied to varying extents For metabolites (13) and (14), the carbocyclic ring has been shown to be derived from D-glucose Preliminary feeding experiments, in both systems, with suitably labelled D-glucose precursors, have established the correlation between the carbon atoms of the starting material and products For allosamidin (15), both the *N*-acetyl-D-allosamine disaccharide and the carbocylic ring allosamizoline are derived from D-glucosamine (Scheme 4) However, it is significant that in contrast to the biosynthesis of neplanocin A and aristeromycin where the carbocylic ring is formed *between C-2 and C-6 of* D-*glucose*, in the case of pactamycin (13), the bacteriohopanetetrol (14) and allosamidin (15), the carbocyclic ring is derived by formation of a carbon–carbon bond *between C-1 and C-5 of* D-*glucose/glucosamine*

### 3.2 The Conversion of D-Glucose into 6-Membered Carbocyclic Rings

The two most extensively studied systems involving the biosynthesis of six-membered rings from carbohydrates are the formation of shikimic acid and inositols The shikimate pathway is responsible for the synthesis of aromatic amino acids whereas inositol phosphates are important as secondary messengers In the formation of six-membered carbocyclic rings from carbohydrate precursors, two distinct mechanisms for the biosynthesis of the ring have been shown to operate Both involve an

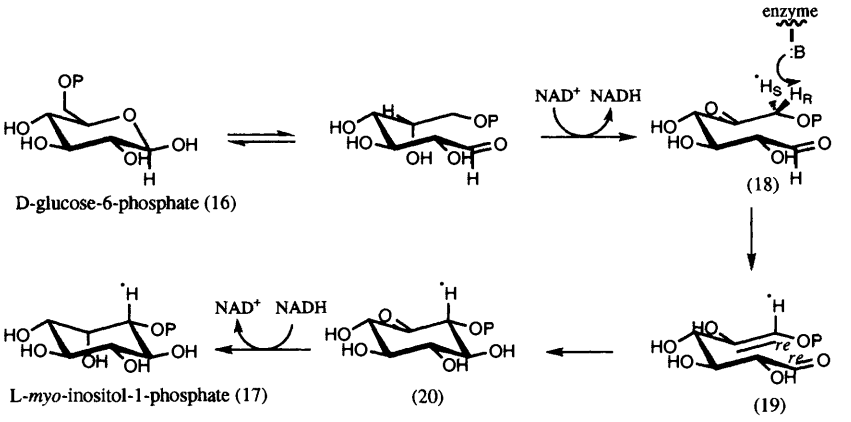



**Scheme 4** Origin of carbocyclic ring in (13), (14), and (15)

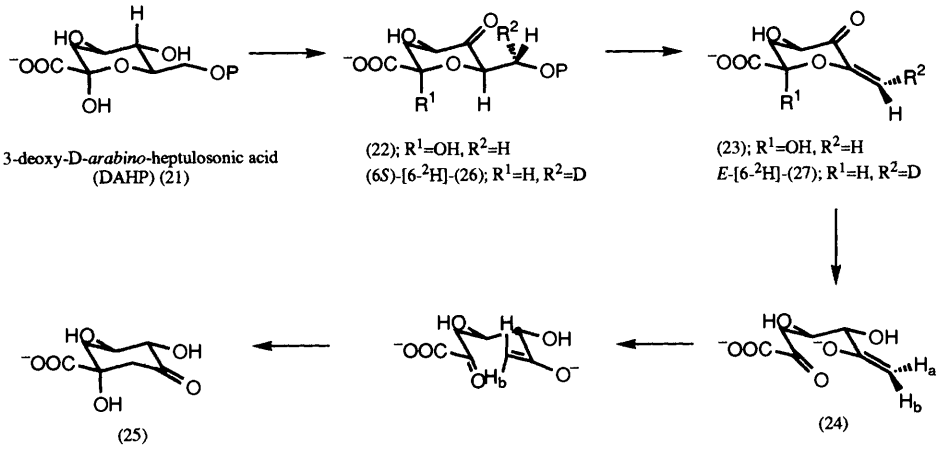
intramolecular aldol-type condensation to effect ring closure, the difference being in the method of generation of the required enol In the biosynthesis of *myo*-inositol, D-glucose-6-phosphate (16) is cyclized to give *myo*-inositol-1-phosphate (17) This reaction is catalysed by the enzyme *myo*-inositol-1-phosphate synthase (MIPS) and requires $NAD^+$ as a cofactor [35] The overall transformation is given in Scheme 5

Activation of the C-6 protons is achieved by oxidation of (16) at C-5 to give (18) which sets up an aldol condensation between C-6 and the aldehyde at C-1 to produce (10) Subsequent reduction produces L-*myo*-inositol-1-phosphate (17) The stereochemistry of the aldol condensation has been investigated by several groups using different systems The stereochemical considerations during this aldol condensation concern the stereospecific removal of either the *6-pro-R* or *6-pro-S* hydrogen atoms and reaction of the subsequent enolate from the *re* or *si* face with either the *re* or *si* face of the aldehyde Floss has investigated the stereochemistry of this reaction by using stereospecifically tritiated glucose substrates [35] By feeding (6*S*)-D-[4-$^2$H, 6-$^3$H]-glucose-6-phosphate to pollen or beef testes MIPS, conversion into *myo*-inositol-1-P occurred with virtually complete retention of the tritium Use of the 6*R* analogue resulted in predominant loss of tritium Therefore, the cyclization of D-glucose-6-P involves the loss of the *6-pro-R* hydrogen and retention of the *6-pro-S* hydrogen This steric course, demonstrated for both plant and mammalian enzymes, is also in accord with that deduced for microbial synthase in *S flacopersious* This indicates that the synthase reaction proceeds in retention mode at C-6 From the configuration of the product, the mode of addition of the two $\pi$-systems can be concluded to be from the *re* face of the enol to the *re* face of the carbonyl

Another system in which a carbohydrate precursor is cyclized to give a six-membered ring is that of shikimate biosynthesis (Scheme 6) In this case, a C-7 unit (3-deoxy-D-*arabino*-heptulosonic acid (DAHP) (21)), derived from condensation of erythrose-4-P and phosphoenol pyruvic acid, is activated towards cyclization by oxidation at C-4 to give (22) (using numbering analogous to that of D-glucose) (Scheme 6) This then allows elimination of the C-6 pyrophosphate group to form a masked enolate (23) which, after reduction of C-4 back to the hydroxy group (24), allows the aldol condensation producing dehydroquainate (DHQ) (25) Extensive studies by Knowles *et al*, have uncovered all of the stereochemical details of this cyclization [36]




**Scheme 5** Stereochemistry and mechanism of the *myo*-inositol-1-phosphate synthase reaction.




**Scheme 6** Mechanism of cyclization during shikimate biosynthesis.

The elimination of the pyrophosphate group from C-6 could occur by an *anti* or *syn* mechanism. By feeding (6*S*)-[6-$^2$H]-(26), a deoxy analogue of DAHP, isolation of intermediate (27) showed that only the *E*-[6-$^2$H]-(27) isomer was produced, indicating that the elimination had occurred by the *syn* mechanism.

### 3.3 Possible Mechanisms for the Formation of 5-Membered Rings

By analogy with the mechanisms of formation of 6-membered rings from D-glucose, it is possible to speculate on the formation of the 5-membered carbocycles. Preliminary feeding experiments described above indicate first that D-glucose [or D-glucosamine in the case of allosamidin(15)] is the source of the C-6 unit of all the metabolites and secondly that two different modes of cyclization operate: cyclization can either occur between C-1 and C-5 of glucose [*e.g.* for pactamycin (13) the bacteriohopanetetrol (14), and allosamidin(15)] or between C-2 and C-6, which is the mode adopted for the biosynthesis of aristeromycin (1) and neplanocin A (3). All the work reported has been carried out using intact cells and thus far there is no detail concerning the enzymes that are responsible for the conversions.

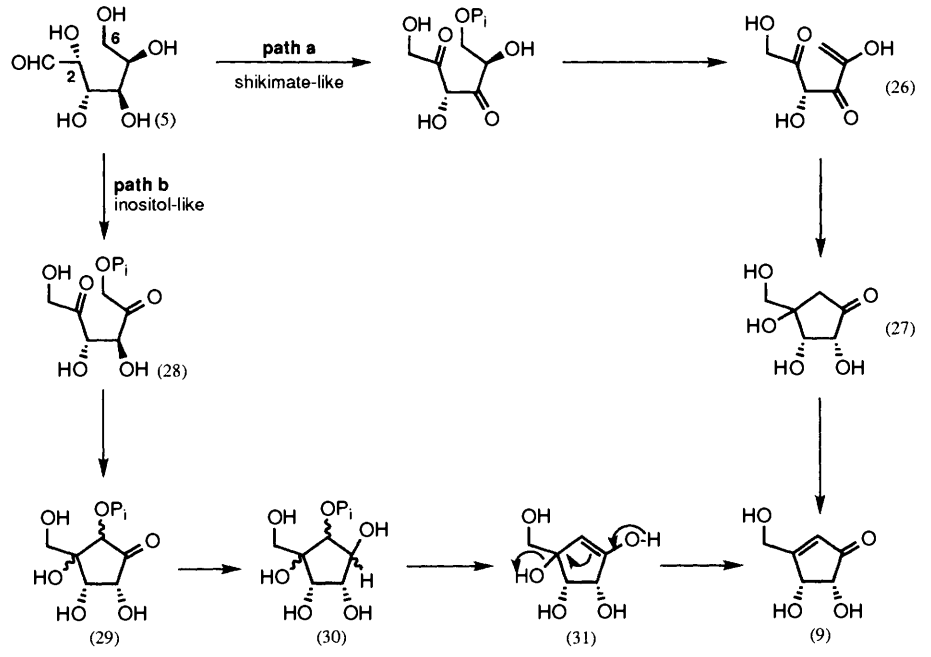
First, let us consider the possible modes of [2,6]-cyclization that lead to the biosynthesis of carbocyclic nucleosides. In order to reach the first known intermediate, enone (9), the following transformations must occur:

(i) isomerization of D-glucose to D-fructose
(ii) inversion of the C-4 hydroxyl group
(iii) loss of 6-*pro*-*S* hydrogen atom
(iv) introduction of a double bond between C-2 and C-6.

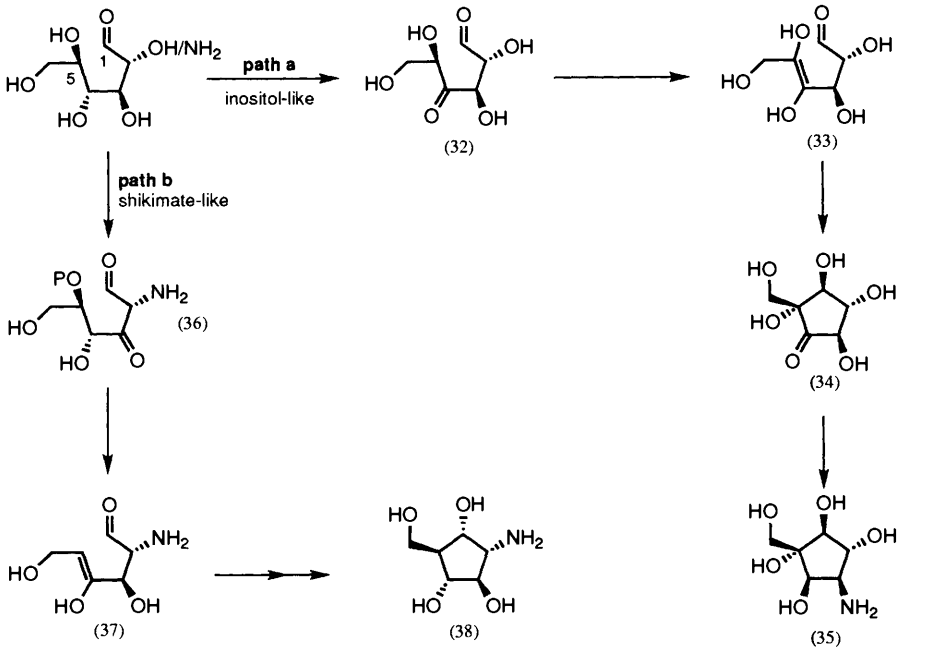
Cyclization *via* a shikimate-like pathway is shown in Scheme 7, path a. Thus, isomerization of fructose-6-phosphate followed by oxidation at C-4 and elimination of the phosphate group leads to the enol (26). This acyclic precursor can then undergo a 5-[enol-*endo*]-[*exo*-trig][37] cyclization followed by reduction of the keto group to yield the carbocycle (27). The ketone (27) would then undergo dehydration, with stereospecific removal of the *6-proS* hydrogen atom, to introduce the double bond, generating the enone (9).

The alternative inositol-like process is illustrated in path b. In this case isomerization of D-glucose to D-fructose is followed by oxidation at C-5 to give the diketone (28). Ring closure can then proceed by stereospecific loss of the C-6-*proS*-hydrogen atom (*NB* this is the opposite proton to that removed during the cyclization of glucose-1-P in inositol biosynthesis) followed by an aldol-type closure. Subsequent epimerization at C-4 would yield the carbocycle (29). Reduction of (29) followed by elimination of the phosphate would yield (30) which could undergo an extended elimination reaction to give the enone (9).

In the case of the [1,5]-cyclization, which operates for the other three carbohydrate-derived cyclopentanoids, there are again two possible pathways (Scheme 8). Path a, based on an analogy with inositol biosynthesis, required initial oxidation at C-4 to give the ketone (32). This oxidation activates the H-5 proton to allow generation of the enol (33). This can then undergo the aldol condensation producing a polyhydroxylated ketone (34). This ketone has most of the required functionality and would only need to undergo reduction of the ketone and introduction of the amino group to give (35) which is the carbocyclic ring found in bacteriohopanetetrol (14). It is notable that both pactamycin (13) and the bacteriohopane tetrol (14) contain cyclopentane rings which are hydroxylated at C-5 and therefore it seems more plausible that they are derived from path




**Scheme 7** Possible mechanisms for [2,6]-cyclization.




**Scheme 8** Possible mechanisms for [1,5]-cyclization.

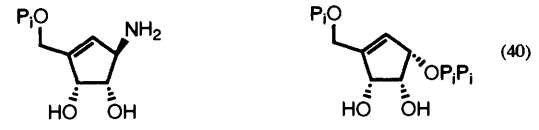
a since the alternative biosynthesis along path b requires re-introduction of a hydroxyl group at C-5 after cyclization (see below).

The alternative [1,5]-cyclization (analogous to shikimate cyclization) is also shown in Scheme 8 and may represent the pathway by which the allosamizoline ring of allosamidin (15) is biosynthesized. Phosphorylation of the C-5 hydroxyl group of D-glucosamine, followed by activation of the C-4 hydrogen atom by oxidation at C-3 would give (36) (Scheme 8, path b). After elimination of the phosphate, reduction at C-3 produces (37) ready for the aldol condensation to give (38) which has all of the required functionality for allosamizoline.

## 4 Origin of the Adenine Base

### 4.1 Preliminary Labelling Experiments

For the origin of the adenine base in neplanocin and aristeromycin, two possibilities were initially considered. First, the adenine ring could be biosynthesized by stepwise construction of the purine ring on a carbocyclic analogue of 5-phosphoribosyl pyrophosphate containing a pre-existing amino group at C-1 (*e.g.* 39) or secondly, the adenine ring might be added intact to a carbocyclic intermediate activated at C-1 by a pyrophosphate group (40).



By feeding labelled putative precursors it was established that the C-2, 4, 5, and 8 carbon atoms of the adenine ring of aristeromycin were derived from glycine and the C-6 atom from bicarbonate.[6] By contrast, formate proved to be a poor precursor. This pattern of labelling is consistent with previously published work on purine biosynthesis.[38] In a complementary experiment, some evidence was obtained for intact incorporation of adenine by administration of doubly-labelled [2-$^{3}$H, 8-$^{14}$C]-adenosine (0.39% incorporation).

### 4.2 Feeding Experiments with Auxotrophs

In order to probe further the origin of the adenine ring, work in Exeter focused on the isolation of mutants of *S. citricolor* that were defective in purine biosynthesis. These mutants required exogenous addition of adenine in order for normal growth to occur. One such mutant, CC268, was grown on a defined medium containing 8-$^{13}$C-adenine (41) as the only source of purines. The labelled aristeromycin that was produced contained the $^{13}$C label only at C-8 in the adenine ring (*ca.* 75% incorporation), thereby establishing the existence of a pathway in which adenine can be incorporated intact into aristeromycin (Scheme 9). However, it was also shown that 1-$^{13}$C glycine (42) could function as a precursor leading to the production of 4-$^{13}$C aristeromycin (*ca.* 20% incorporation). The latter observation may be due to some 'leakiness' of the adenine auxotroph and is consistent with either a *de novo* or salvage-type pathway. The weight of evidence certainly suggests that *direct incorporation of adenine is the major route to aristeromycin* whilst not eliminating the involvement of a *de novo* pathway.

## 5 Relationship of Neplanocin to Aristeromycin

### 5.1 Whole Cell Studies

The co-production of neplanocin A and aristeromycin in cultures of the wild-type *S. citricolor* suggested from the beginning that these two metabolites, differing only in presence of a double bond between C-4′ and C-6′, might be closely related on the biosynthetic pathway. Preliminary experiments had also shown that certain mutants which were unable to produce either aristeromycin or neplanocin A, were able to convert added neplanocin A into aristeromycin. However, the observations of Parry described above, in which evidence for the participation of the saturated intermediates (6) and (7) was obtained, suggested that neplanocin and aristeromycin might be biosynthesized along independent parallel biosynthetic routes. Work at Exeter was therefore directed at trying to resolve this conflict.

In his original study, Parry reported that the feeding of [6-$^{3}H_2$]-D-glucose to wild type *S. citricolor* resulted in a 50% loss of the tritium label in the aristeromycin isolated. Repetition with (*R*)-[6-$^{3}$H]-D-glucose and then (*S*)-[6-$^{3}$H]-D-glucose resulted in predominant retention of the 6-*pro-R* label and complete loss of the 6-*pro-S* label. Feeding with [6-$^{2}H_2$]-D-glucose (5a) and subsequent $^{2}$H NMR analysis of the isolated aristeromycin (1a) indicated that the retained deuterium atom resided in the 6′-*pro-S* position. Thus, the 6-*pro-R* proton of glucose becomes the 6′-*pro-S* proton in aristeromycin (in contrast to the *myo*-inositol cyclization discussed above). Repetition of this experiment in our hands using IFO 13005, a wild-type strain, gave an identical result (Scheme 10).

At Exeter, we then carried out an analogous experiment using a mutant of *S. citricolor* (CC1026) which produced neplanocin A but no aristeromycin. By administering [6-$^{2}H_2$]-D-glucose (5a), we were able to isolate the [6′-$^{2}$H]-neplanocin (3a). This material was then re-fed to a second mutant CC826 which was blocked in the production of both aristeromycin and neplanocin and was also able to act as an efficient converter of neplanocin A to aristeromycin. The 6′-$^{2}$H-aristeromycin isolated from this experiment was stereochemically identical to that derived from the 6-$^{2}H_2$-D-glucose experiment suggesting that *neplanocin A is the direct precursor of aristeromycin.*

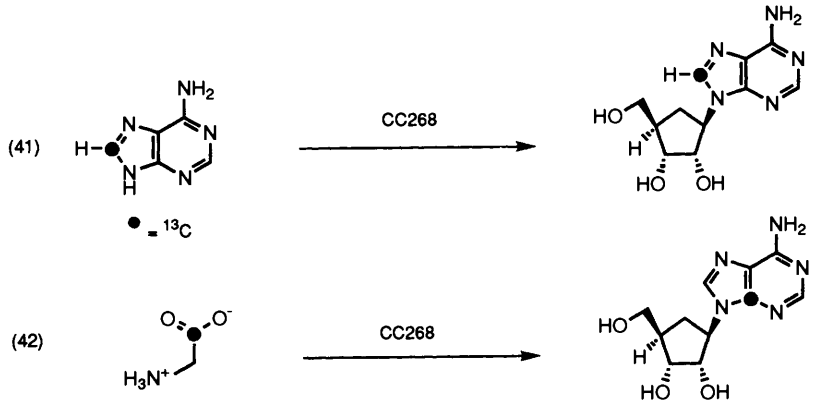
### 5.2 Cell-free Extracts

Supporting evidence for the direct conversion of neplanocin A into aristeromycin has recently been obtained by Parry using cell-free extracts of wild-type *S. citricolor.*[39] He demonstrated that a partially purified cell-free extract was able to catalyse the NADPH-dependent reduction of neplanocin A to aristeromycin. Moreover, it was shown that the reaction proceeds with *anti*-geometry and involves the transfer of the *pro-R* hydrogen atom of NADPH to the 6′β position of aristeromycin (Scheme 11). This result, which is consistent with the whole-cell studies described above, suggests that the mechanism of conversion of neplanocin A to aristeromycin may involve initial oxidation of the C-5 hydroxymethyl group to an aldehyde, followed by conjugate reduction, and then reduction of the aldehyde back to the alcohol.

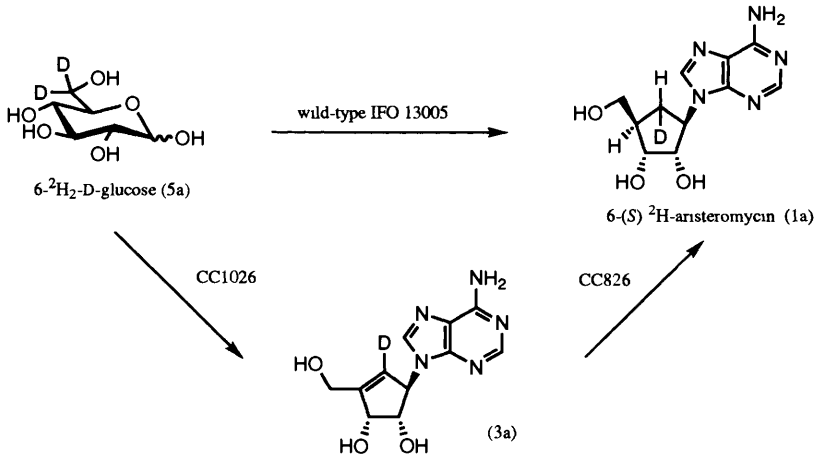
## 6 Proposed Biosynthetic Pathway

### 6.1 Current Proposal

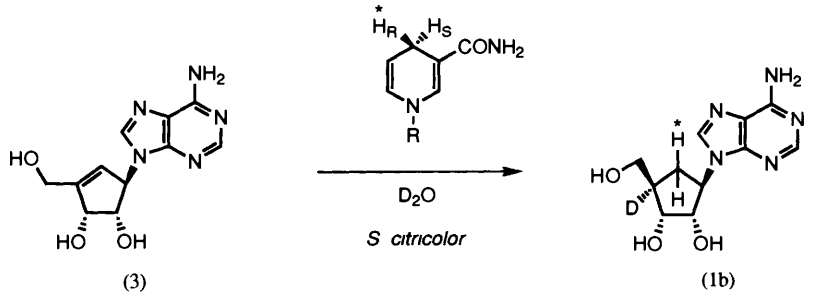
The weight of evidence outlined above suggests that, in contrast to earlier proposals,[6,20,21] the biosynthesis of aristeromycin and neplanocin A proceeds according to the route; D-glucose



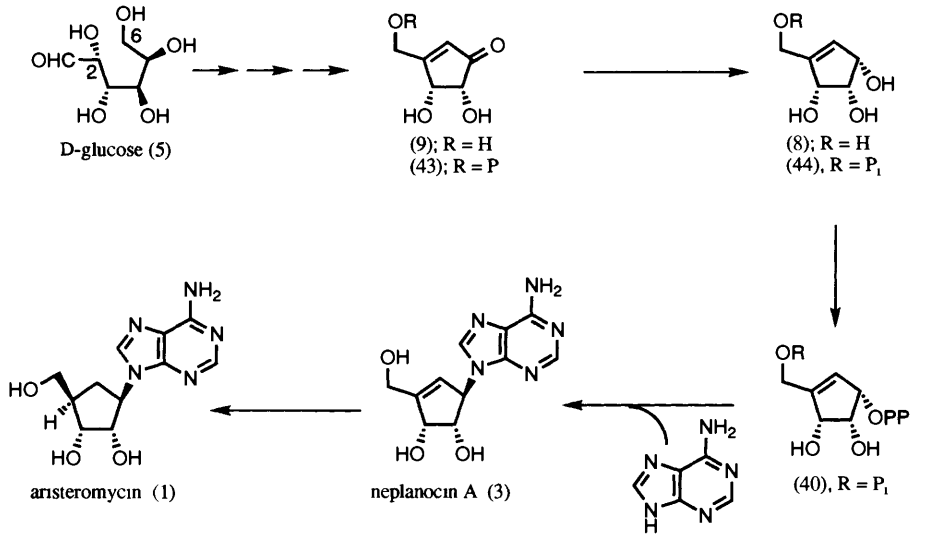
**Scheme 9** Origin of the adenine base in aristeromycin (1).




**Scheme 10** Stereochemistry of deuterium incorporation into (1a) and (3a)




**Scheme 11** Conversion of neplanocin A into aristeromycin using a cell free extract of *S citricolor*




**Scheme 12** Linear pathway for the biosynthesis of aristeromycin and neplanocin A

(5) → enone (9) → tetrol (8) → neplanocin A (3) → aristeromycin (1) (Scheme 12) [19] Although the enone (9) is likely to be the first-formed carbocyclic intermediate, there is a lack of information regarding the detail of the pathway from D-glucose to (9) Reduction of the enone (9) to the tetrol (8) followed by activation at C-1 *via* the pyrophosphate allows the introduction of the adenine base [it is possible that these transformations could occur on the corresponding phosphorylated congeners, (43), (44), and (40)] Introduction of the adenine base *via* this route would be favoured mechanistically on account of the displacement at an allylic centre It is interesting that the activation at C-1 which is normally provided by the ring-oxygen atom in natural nucleosides is partially compensated for by the C-4–C-6 double bond

The proposed reduction of neplanocin A to aristeromycin is supported by both the studies using intact cells[19] as well as experiments involving cell-free extracts [39] The mechanism for the reduction may well involve initial oxidation of the allylic alcohol of neplanocin A to the α,β-unsaturated aldehyde followed by conjugate addition of NADH Such a sequence has previously been observed in the reduction of allylic alcohols by baker's yeast [40 41]

### 6.2 Conclusions and Future Experiments

Many issues remain to be resolved concerning the detail of the biosynthesis of neplanocin A and aristeromycin First, very little evidence has been obtained on the identity of the intermediates

between D-glucose and the enone (9) The determination of the structure of the compounds will allow firm conclusions to be drawn regarding the mechanism of ring-closure as speculated above Such information is of fundamental importance regarding the biosynthesis of 5-membered rings Having identified the intermediates, it will then be possible to carry out studies using partially purified enzyme extracts in order to determine the detailed mechanism of the reactions

Secondly, the exact identity of the precursor that undergoes the addition of the adenine base needs to be established This reaction is central to the biosynthesis of carbocyclic nucleosides and could be exploited as a means of synthesizing unnatural nucleosides by the addition of analogues of adenine It is possible that the tetrol is phosphorylated, both at C-1 (pyrophosphate) and C-5 prior to addition of the adenine The synthesis and feeding of these putative intermediates is currently underway in our laboratories

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