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The Biosynthesis of the *Streptomyces* Antibiotic Bicyclomycin

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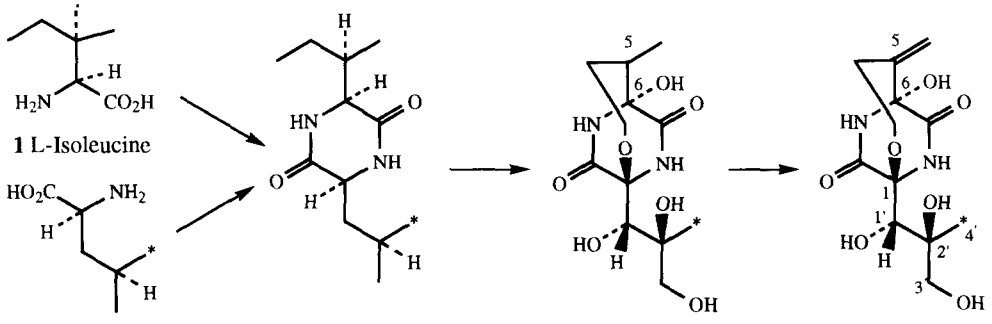
Abstract: Bicyclomycin **5** is biosynthesized in *Streptomyces sapporonensis* from L-leucine **2** and L-isoleucine **1** by way of the naturally occurring diketopiperazine **3** and dihydrobicyclomycin **4**. The mode of incorporation of (2*S*,4*R*)-[5,5,5-$^2H_3$]leucine into **5** (and **4**) shows that the entry of the hydroxy group at C-2' occurs with unusual inversion of configuration. The results of inhibition studies permit a useful working hypothesis to be advanced for bicyclomycin biosynthesis.Copyright © 1996 Elsevier Science Ltd

Bicyclomycin **5** (absolute configuration shown) is an antibiotic of intriguing structure which is elaborated by *Streptomyces sapporonensis*[1] and *S. aizunensis*.[2] It is active against Gram-negative, but not Gram-positive, organisms and its principal, indeed novel, mode of action is by inhibition of the RNA transcription termination factor rho.[3] We have found that resistance to the antibiotic can be induced in *Escherichia coli*, the mechanism of resistance being by the multiple expression of an intrinsic exporter protein which shows significant homology with other transporter proteins.[4]



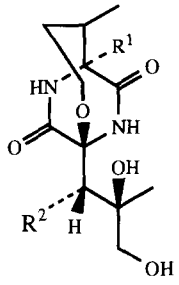
* denotes the labelling site in the ^{2}H-leucine & its fate

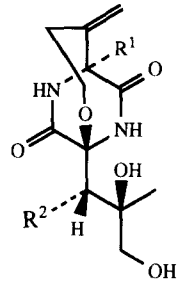
Consideration of the biosynthesis of bicyclomycin **5** suggests provenance in the amino acids L-leucine **2** and L-isoleucine **1**, and good incorporations of these amino acids have been recorded[5] using resting cells of *S. sapporonensis*. We were able to confirm these results, again using resting cells of this organism[6] (1.9%)

incorporation for L-[U-^{14}C]leucine and 2.4% for L-[U-^{14}C]isoleucine compared to < 0.02% for L-[U-^{14}C]serine chosen as a representative amino acid for incorporation through general amino acid metabolism. Incorporation was observed only with resting cells;[7] no incorporation was observed with normal cultures and resting cells were used solely in subsequent experiments.

Well-exemplified analogy[8] indicates that the diketopiperazine **3** should be an intermediate beyond the two amino acids **1** and **2** on the pathway to bicyclomycin. Indeed we find that **3** is present in the buffer solution associated with resting cells prepared from *S. sapporonensis* cultures early in growth and also in resting cells prepared as usual just after bicyclomycin production had begun when the oxidase inhibitor metyrapone was present. These observations fit nicely with a role for **3** as an early biosynthetic intermediate. Furthermore, *cyclo*-(L-isoleucyl-L-[U-^{14}C]leucyl) (as **3**) was a very efficient precursor for bicyclomycin **5** (31% incorporation). From this evidence we conclude that *cyclo*-(L-isoleucyl-L-leucyl) **3** is a normal biosynthetic intermediate *en route* to bicyclomycin.

Analysis of *S. sapporonensis* cultures (resting cells) revealed another prominent metabolite aside from bicyclomycin **5**. The metabolite was labelled by radioactive samples of L-leucine, L-isoleucine and the diketopiperazine **3**. It was isolated and from its mass spectrum and ^{1}H NMR it was deduced to be dihydrobicyclomycin **4**. (The n.m.r. data did not allow us to assign the configuration at C-5; this would in any case be difficult as **4** underwent isomerization in solution, reasonably through opening of the aminol system at C-6 to give a ketone with enolisation then resulting in racemisation at C-5; bicyclomycin is significantly more stable). Dihydrobicyclomycin, labelled biosynthetically with ^{14}C, was moreover found to be a very satisfactory precursor for **5** (6% incorporation); **5** was not incorporated into **4**. We conclude therefore that there is no *in vivo* conversion of **5** into **4**, and that dihydrobicyclomycin **4** is the ultimate, irreversible precursor for bicyclomycin **5**.





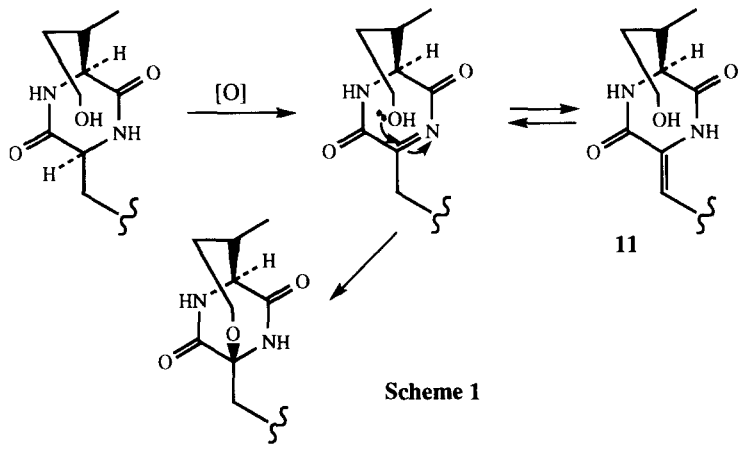
6 $R^1 = R^2 = H$ **9** $R^1 = OH, R^2 = H$

7 $R^1 = OH, R^2 = H$ **10** $R^1 = H, R^2 = OH$

8 $R^1 = H, R^2 = OH$

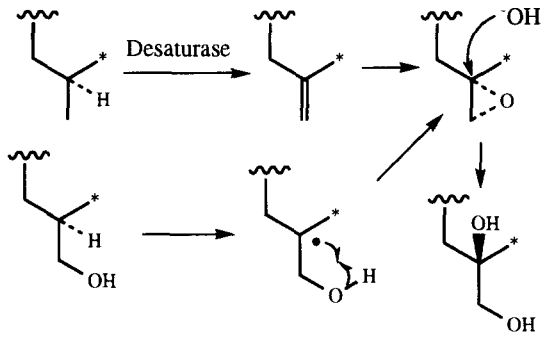
The bio-conversion of **3** into **5** involves oxidation of all but two of the sp^3 carbon atoms present in **3**. In order to delineate the biosynthetic pathway to **5**, a forbidding number of oxidised derivatives of **3** would have to be examined. We resolved on a different approach, namely the use of oxidase inhibitors with the aim of accumulating partially hydroxylated intermediates as pointers to the course of biosynthesis, and we report some success. The use by others of inhibitors in biosynthetic studies is noted.[9] The cytochrome P-450 inhibitors miconazole, prochloraz and metyrapone were examined for their ability to inhibit bicyclomycin biosynthesis. Of these only metyrapone was effective. At 10 & 20 millimolar concentrations we observed satisfactory inhibition and obtained small amounts of new metabolites (radioactive leucine and isoleucine were used to track possible

intermediates: only compounds on the pathway to bicyclomycin would have been labelled by both amino acids and to a similar degree). Through the use of LC-MS we tentatively identify compounds of interest as **7** through **10** (the molecular ions compared to **4** and **5** each showed a deficit of 16 and the major fragment ion (M^+ - 74) corresponds to loss of C-2' through C-4'). Unfortunately insufficient material was obtained for rigorous identification of these metabolites. However we take the deduced hydroxylation pattern in these compounds to provide a guide to what the course of biosynthesis is.



Scheme 1

The availability of a sample of (2*S*,4*R*)-[5,5,5-$^2H_3$]leucine[10] allowed us to probe the oxidation events in the side chain of the leucyl moiety in bicyclomycin **5** (L-[U-^{14}C]leucine was used as reference). The amino acid was incorporated satisfactorily into **5** (1.5% by radioactivity) and also into **4**. Each metabolite was examined by ^2H NMR and they both showed a single resonance at 1.3 p.p.m. corresponding to the C-methyl at C-2' (*i.e.* C-4') in the two metabolites. Thus it is 4-*pro-S* methyl group (C-6) of leucine which is hydroxylated. Given the absolute configuration of bicyclomycin, it follows that entry of the hydroxy group at C-2' of bicyclomycin **5** occurs with unusual, overall inversion of configuration



Scheme 2

It is manifest that entry of the hydroxy group at C-6 in **5** occurs with (overall) retention of configuration (compare the stereochemistry in **1** and **5**), and this is the stereochemistry normally observed for hydroxylation at

saturated carbon atoms.[11] Generally a different mechanism is implied in cases in which inversion occurs as must happen for C-1. We suggest a reasonable mechanism in this case (Scheme 1) which builds on the common involvement of compounds of type **11** in the biosynthesis of a number of diketopiperazine metabolites.[8] Possible routes incorporating the necessary inversion at C-2' in the leucyl side chain are illustrated in Scheme 2. The two conversions in Schemes 1 & 2 as well as the conversion of **4** into **5** may be grouped as desaturation reactions. The structures deduced for the inhibitor-generated metabolites **7** - **10** suggest that these putative desaturations are not inhibited by metyrapone whereas the straightforward hydroxylation at C-6 and (similarly we suppose) at C-1' are.

The foregoing evidence leads us to a working hypothesis for the biosynthesis of bicyclomycin, to be tested with judiciously chosen potential precursors:

3 → 6 → 7/8 → 4 → 5

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6. Production of **5** was quantitatively monitored by bioassay, measuring the zone of inhibition of *Escherichia coli* DH5 growth on agar plates. Confirmation of the presence of **5**, rather than another antibiotic, was achieved by observing overgrowth of *E. coli*[4] resistant to **5**. The outcome of each feeding experiment was determined by HPLC with radioactive counting of each column fraction. Bicyclomycin **5** was isolated according to a published method[12] and this was followed by HPLC purification (RP-Select B column, 220 nm, water). This method was also used for **4**. Resting cell cultures were generally prepared from growth cultures at the onset of bicyclomycin production. Compound **4**: δ 1.1 (3H, d, J 7Hz) & 2.4 (1H, m) ppm, replacing the olefinic signals at 5.4 & 5.1 ppm in the spectrum of **5**; LC-MS: 305 (MH^+), 287, 146; *i.e.* as for **5** plus 2 mass units in each case.
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