BIOCHEMICAL STUDIES ON 2-DEOXY-SCYLL0-INOSOSE, 
AN EARLY INTERMEDIATE IN THE 
BIOSYNTHESIS OF 2-DEOXYSTREPTAMINE

IV. A CLUE TO THE SIMILARITY OF 
2-DEOXY-SCYLL0-INOSOSE SYNTHASE 
TO DEHYDROQUINATE SYNTHASE

Sir:

2-Deoxystreptamine (1), is a common aminocyclitol aglycon in a major group of clinically important aminoglycoside antibiotics. The crucial step in the biosynthesis of 1 is the formation of the precursor, 2-deoxy-scyllo-inosose (2), from D-glucose (3) via the intramolecular C-C bond formation between C-1 and C-6.1,2) The transformation of 3 into 2 was proposed by us to involve a multi-step mechanism as shown in Scheme 1, the chemistry of which was suggested to be similar to the dehydroquinate synthase in the shikimate pathway,3'4) and an enzyme responsible of the intramolecular cyclization was named "2-deoxy-scyllo-inosose synthase"5~8) Akhtar then reported that the C-4 hydrogen of 3 was lost during the biosynthesis of 1 in the whole cells of Streptomyces fradiae (producing neomycins) and suggested involvement of oxide-redaction at C-4 of the substrate.7'8) However, nothing has so far been clarified as to whether a single enzyme is involved or certain dissociable enzymes cooperate to form 2. This communication is an approach to this problem.

Recently, we established a cell-free system from Streptomyces fradiae IFO 13147 cells and successfully observed the production of 2 from D-glucose-6-phosphate (4) in the presence of NAD.9) Using this system, we studied the closer insight into the reaction mechanism of the aforementioned 2-deoxy-scyllo-inosose synthase, focusing on the fate of the C-4 hydrogen of 4.

A partially purified enzyme was prepared from the (NH₄)₂SO₄ saturation precipitate derived from the 10,000 x g supernatant of the sonicate of S. fradiae cells.9) After dialysis of the precipitate, the enzyme fraction (20 ml, 15 mg protein/ml) was chromatographed over a DEAE-Cellulofine A-800 column (i.d. 1 cm x 15 cm, buffer: 50 mM Tris-HCl, pH 7.5, containing 0.2 mM of Ca²⁺ and Mg²⁺) with a linear gradient of the NaCl (0 to 0.4 M) concentration. The enzyme activity was assayed by the HPLC method described previously.9) Appropriate enzyme fractions were collected and used for the isotope-tracer experiments.

D-[4-2H]-3 (>95% enriched), which had been synthesized by a literature-cited procedure with slight modifications,10) was chemically converted to D-[4-2H]-4. The enzyme reaction was carried out at 37°C for 2 hours with a mixture of the enzyme fraction, 2 mM of NAD and 1 mM of either the labeled or the non-labeled substrate. D-[6,6-2H₂]-4 was used as a positive reference.5'6) Production of 2 was quantitated by the aforementioned HPLC analysis.9) The yields usually ranged around 10%. No endogenous or residual 2 was observed in the control experiment. Significant reduction (~40%) of the production of 2 was observed in the experiments with the D-[4-2H]-4 substrate compared with the non-labeled or D-[6,6-2H₂]-4 substrate (duplicate, data not shown). These observations may be suggestive of the primary kinetic isotope effect.

Scheme 1. The proposed mechanism of 2-deoxy-scyllo-inosose (2) synthase reaction in the biosynthesis of 2-deoxystreptamine (1).
Table 1. Relative intensities of the mass spectra of 6 obtained from the enzyme reaction products and synthetic standard.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>d-Glucose-6-phosphate</th>
<th>d-[4-2H]Glucose-6-phosphate</th>
<th>Chemically synthesized [6-\textsuperscript{3}H]-2-deoxy-scyllalininosose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
<td>Run 1</td>
</tr>
<tr>
<td>m/z 599</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>600</td>
<td>100</td>
<td>100</td>
<td>56.6</td>
</tr>
<tr>
<td>601</td>
<td>50.3</td>
<td>49.0</td>
<td>100</td>
</tr>
<tr>
<td>602</td>
<td>26.3</td>
<td>26.4</td>
<td>50.6</td>
</tr>
<tr>
<td>603</td>
<td>10.5</td>
<td>9.1</td>
<td>26.3</td>
</tr>
</tbody>
</table>

\* Mass spectral scanning was performed in triplicate for each case.

in the oxidoreduction during the 2-deoxy-scyllalininosose synthase reaction.

The deuterium enrichment of the enzyme reaction product was determined by mass spectrometry. The \(\text{O-(4-nitrobenzyl)oxime derivatives 5, prepared from each enzyme reaction, were further separately silylated to the corresponding trimethylsilyl (TMS) ethers 6. Each reaction mixture was diluted, and then subjected to GC-MS (Shimadzu-LKB 9020 DF spectrometer; OV-1, 12 m).

The relative signal intensities of the molecular ion region of 6 obtained from each enzyme reaction are shown in Table 1. The non-labeled control showed the \(\text{M}^+\) ion at \(m/z\) 600, but nothing at \(m/z\) 599, and only the \(\text{M}^+\) ion (\(m/z\) 601) was observed in the spectrum of chemically synthesized [6-\textsuperscript{3}H]-5 from d-[4-2H]-3 as well.\textsuperscript{11*} In contrast, the enzyme reaction product from d-[4-2H]-4 showed the ions at \(m/z\) 600 and 601 (\(\text{M}^+\)), suggesting the formation of both non-labeled and monodeuterated-2. Calculation allowed us to estimate the deuterium content in the product from the d-[4-2H]-4 substrate to be 56%. Based upon the previously proposed reaction mechanism, the deuterium of d-[4-2H]-4 was expected to be incorporated into C-6 of 2, which has ultimately been proved as follows.

The location of deuterium in 5 obtained from the d-[4-2H]-4 substrate was determined unambiguously by \(^2\text{H}\) NMR spectra as shown in Fig. 1. The \(^2\text{H}\) chemical shifts were unequivocally assigned by comparison with the corresponding \(^1\text{H}\) NMR spectrum of the non-labeled oxime 5. A positive control, [2,2-\textsuperscript{2}H\textsubscript{2}]-3 formed enzymatically from d-[6,6-\textsuperscript{2}H\textsubscript{2}]-4, clearly showed the signals at \(\delta\) 2.54 and 3.81 to be due to the C-2 methylene group (\(^1\text{H}\) NMR, \(\delta\) 2.58 and 3.89).\textsuperscript{5,6} Most crucial is that the derivative 5 obtained from d-[4-2H]-4 showed a single deuterium signal at \(\delta\) 4.65, which was attributed to the C-6 position (\(^1\text{H}\) NMR, \(\delta\) 4.71). Thus, the deuterium of the d-[4-2H]-4 substrate appears to be incorporated into the C-6 position of 2.

Of significance is that the deuterium at the C-4 position of d-[4-2H]-4 is retained in the product during the in vitro reaction of the NAD-assisted 2-deoxy-scyllalininosose synthase reaction. Thus, the deuterium may be held in the vicinity of the substrate during the NAD-assisted oxidation and reduction. The observed enrichment ratio can be explained as follows. The non-deuterated 2 was probably derived from a minute amount of non-labeled 4 in the d-[4-2H]-4 specimen. Accordingly, the low transfor-
formation efficiency (~10% chemical yield) and a possible kinetic isotope effect in the oxidoreduction at C-4 seem to effect preferential conversion of the non-labeled substrate, resulting in significant formation of the non-labeled 2. The aforementioned Akhtar’s in vivo results can be explained similarly.\(^7\)\(^8\)

From the present in vitro experiments using a partially purified enzyme, it appears that the formation of 2 from 4 indeed requires NAD and the hydrogen at C-4 of the substrate is retained in the product, thereby suggesting that a series of reactions of 2-deoxy-scyll0-inosose formation may be performed by a single enzyme, “2-deoxy-scyll0-inosose synthase”, with catalytic turn over of the NAD cofactor.

As to the mechanism of dehydroquinate synthase with respect to the 2-deoxy-scyll0-inosose synthase reaction, two features may be emphasized: 1) The C-5 hydrogen (at the \(\beta\)-position to the phosphate group, synonymous to the C-4 hydrogen of 4) of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate is retained in the cyclization reaction,\(^1\)\(^2\) and 2) the NAD requirement is only catalytic and NAD is tightly bound to the enzyme.\(^3\) While it is not clear that the 2-deoxy-scyll0-inosose synthase catalyzes only the oxidoreduction and/or cyclization reaction, as has been discussed for the dehydroquininate synthase,\(^4\) the present results suggest close similarity of 2-deoxy-scyll0-inosose synthase, functioning in microbial “secondary metabolism”, to dehydroquininate synthase in respect to the mechanism. The more detailed comparison must await closer analysis of the former enzyme.

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