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

## II. QUANTITATIVE ANALYSIS OF 2-DEOXY-*SCYLLO*-INOSOSE

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A practical quantitative analysis of 2-deoxy-*scyllo*-inosose by means of GC-MS selected-ion monitoring (SIM) was exploited in order to assay the enzymatic 2-deoxy-*scyllo*-inosose formation, the first stage of the 2-deoxystreptamine biosynthesis. Mass spectral fragmentations of 2-deoxy-*scyllo*-inosose tetra-*O*-trimethylsilyl (TMS) ether was also investigated by the use of [2,2-$^2H_2$]-2-deoxy-*scyllo*-inosose.

A major interest in the biosynthesis of clinically important aminoglycoside antibiotics is the formation of 2-deoxystreptamine, the central aminocyclitol constituent of a series of these antibiotics. The formation of 2-deoxystreptamine from D-glucose has been well established in terms of chemical mechanisms.[1,2] The biosynthetically crucial chemistry involves the cyclization of D-glucose into 2-deoxy-*scyllo*-inosose, the earliest precursor of 2-deoxystreptamine. We pointed out previously that this cyclization reaction has some similarity to the reaction of dehydroquinate synthase in the shikimate pathway, rather than the formation of *myo*-inositol from D-glucose.[3~6] While dehydroquinate synthase has been well studied in terms of the enzymic as well as the genetic level,[7~10] essentially no enzymic study of 2-deoxy-*scyllo*-inosose formation has been reported. What has been known so far as to the involvement of 2-deoxy-*scyllo*-inosose in the biosynthesis of 2-deoxystreptamine is that; (1) 2-deoxy-*scyllo*-inosose-requiring idiotrophic mutants of the antibiotic-producing strains can utilize supplemented 2-deoxy-*scyllo*-inosose to form the corresponding 2-deoxystreptamine-containing antibiotics,[11] and (2) the accumulation of 2-deoxy-*scyllo*-inosose in the cultures of some idiotrophic secretory mutants derived from the butirosin-producing *Bacillus circulans* was indirectly detected by measuring antibiotic activity after fermenting their cultured broths with another idiotrophic converter mutant of *Bacillus circulans*.[12]

As mentioned in the preceding paper, a major difficulty involved in the enzymatic studies of the 2-deoxy-*scyllo*-inosose formation from D-glucose may rest on the quantitative assay of the conversion. Specific analysis of 2-deoxy-*scyllo*-inosose without derivatization seemed inappropriate because of its high polarity as well as lack of characteristic UV absorbance or other spectrophotometric properties. We attempted to use GC-MS selected-ion monitoring (SIM) methodology to circumvent this problem. This methodology was also applied to quantitative analysis of 2-deoxy-*scyllo*-inosose in the fermentation broths of the antibiotic-producing microorganisms. Furthermore, in view of *in vitro* transformation of D-glucose into 2-deoxy-*scyllo*-inosose, we anticipated a difficulty with differentiation of endogenous 2-deoxy-*scyllo*-inosose and newly-formed 2-deoxy-*scyllo*-inosose. A deuterium-labeling technique was attempted to pursue a differential analysis, since we demonstrated previously that the hydroxymethyl hydrogens of D-glucose are stereospecifically incorporated into 2-deoxystreptamine at its C-2 position

(by chiral deuterium labeling and deuterium NMR experiments).[3,4] Thus, D-[6,6-$^2H_2$]glucose or its phosphate seemed to be appropriate as the starting material for the cell-free experiments, and as a consequence of the analysis of the expected product [2,2-$^2H_2$]-2-deoxy-*scyllo*-inosose should be crucial. Described here also is the mass fragmentation analysis of [2,2-$^2H_2$]-2-deoxy-*scyllo*-inosose.

## Materials and Methods

GC-MS spectra were obtained by a Shimadzu LKB 9020DF mass spectrometer. All the chemicals were those commercially available and were used without further purification.

### Analysis of 2-Deoxy-*scyllo*-inosose TMS Derivative

The trimethylsilyl ether (TMS) derivatives of D-glucose, D-ribose, *myo*-inositol and 2-deoxy-*scyllo*-inosose were prepared as follows: Each compound was dissolved in water to form a solution of 1 mg/ml (glucose, ribose, *myo*-inositol) or 3 mg/ml (2-deoxy-*scyllo*-inosose). An aliquot (10 $\mu$l) of each solution was separately dried by flushing $N_2$ gas and further dried under vacuum. Pyridine (40 $\mu$l each) was added to the dried samples, and then 10 $\mu$l of hexamethyldisilazane and 10 $\mu$l of chlorotrimethylsilane were successively added. The resulting mixture was warmed with a heat-gun for several minutes and allowed to stand at room temperature for 30 minutes. The yield of 2-deoxy-*scyllo*-inosose derivatization was approximately in the range of 50 to 55%. The each mixture was separately diluted with hexane (140 $\mu$l each), from which 1 $\mu$l each was injected into the GC-MS apparatus.

The GC conditions were as follows; the column being a glass-capillary (8 meter length) of Shimadzu CBP-1 (OV-1 equivalent), the injection port temperature being 200°C, the grating column temperature starting from 140°C with increase of 8°C per minute. The retention time and mass spectra of each standard compound were determined for identification. The amount of tetra-*O*-TMS-2-deoxy-*scyllo*-inosose was quantitatively estimated by comparing the corresponding GC peak height with that of a standard specimen prepared as described in the preceding paper.[13] A standard curve for quantitative analysis was prepared similarly.

### Analysis of 2-Deoxy-*scyllo*-inosose in the Cultures of Antibiotic-producing Organisms

The microorganisms producing 2-deoxystreptamine-containing antibiotics, *Streptomyces fradiae* IFO 13147, *Streptomyces ribosidificus* KCC S0923, *Streptoalloteichus hindstanus* KCC A0268, *Streptomyces kanamyceticus* JCM 4413, were maintained on Bennett agar slants consisting of (per liter of distilled water, pH 7.3); glucose 10 g, yeast extract 1 g (Oxoid), Ehrlich meat extract 1 g (Kyokuto Seiyaku), NZ-amine type A (Humco Sheffield Chemical) 2 g, and agar 20 g. Fermentation of each strain was carried out as follows. Cells on a slant were suspended in 2 ml of 0.9% aqueous NaCl solution and transferred aseptically for inoculation into 100 ml of the ISP No. 2 medium (per liter of distilled water, pH 7.3); glucose 4 g, yeast extract 4 g (Oxoid), malt extract 10 g (Oxoid), in a 500-ml Erlenmeyer flask. Cultivation was carried out on a rotary shaker at 28 to 29°C. An aliquot (5 ml) was dispensed at every 24 hours after inoculation, and centrifuged (2,500 rpm, 15 minutes). The cell precipitate was weighed and the supernatant was kept at −20°C until use for the aforementioned GC-MS analysis.

A portion (0.2 ml) of the melted supernatant was dried first by flashing with $N_2$ gas and then under vacuum (at 50°C) for 1 hour. The dried sample was derivatized to its tetra-*O*-TMS ether and analyzed by the selected ion monitoring method described above. The diagnostic ions were *m/z* 450, *m/z* 360, and *m/z* 270. The antibiotic activity of the supernatant was determined by the standard paper disk assay against *Bacillus subtillis* using ribostamycin sulfate as a standard.

## Results

### Quantitative Analysis of 2-Deoxy-*scyllo*-inosose

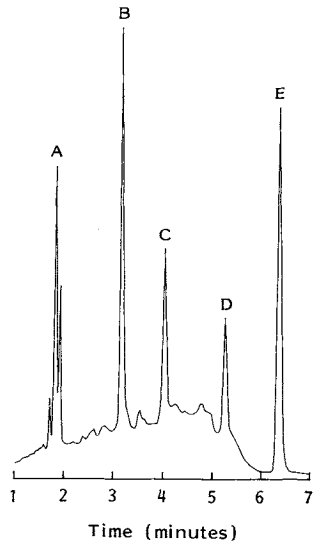
First, quantitative analysis of 2-deoxy-*scyllo*-inosose was explored by the GC-MS selected-ion monitoring method of 2-deoxy-*scyllo*-inosose tetra-*O*-TMS ether, because (1) this method generally

shows high sensitivity, and (2) derivatization of the hydroxyl groups is straightforward and easy.

The preparation of the standard 2-deoxy-*scyllo*-inosose from *myo*-inositol was described in the preceding paper.[13] The derivatization to the tetra-*O*-TMS ether was accomplished by pyridine-hexamethyldisilazane-TMSCl.

A problem to be assessed was the separation of the 2-deoxy-*scyllo*-inosose TMS ether from glucose, ribose, and *myo*-inositol, because the latter should be contained as contaminants in the biological samples such as cell-free extracts and the cultured broths. To circumvent this problem, the analysis conditions were researched and established as described in the methods section. Fig. 1 shows the GC-MS total-ion chromatogram of a mixture of these four derivatives. This result clearly indicated that the 2-deoxy-*scyllo*-inosose tetra-*O*-TMS ether can be completely separated from D-ribose, D-glucose, and *myo*-inositol derivatives under these conditions.

Fig. 1. Gas chromatographic separation of the TMS derivatives of D-ribose (A), 2-deoxy-*scyllo*-inosose (B), D-glucose (C and D), *myo*-inositol (E).




The ions to be monitored for the analysis of 2-deoxy-*scyllo*-inosose were determined from the mass spectrum. The molecular ion peak was observed at *m/z* 450 and the intensity of this peak was sufficient for the SIM. Other characteristic ions such as *m/z* 360 $(M-TMSOH)^+$, *m/z* 270 $(M-2\times TMSOH)^+$ were also observed. The intensities of these ions were also high enough to carry out the SIM.

Quantitative analysis of 2-deoxy-*scyllo*-inosose was next investigated by monitoring the signal intensities at *m/z* 450 and *m/z* 360 under the SIM conditions. The standard solution of 2-deoxy-*scyllo*-inosose was diluted into various concentrations. Aliquots (400 $\mu$l) of each solution was dispensed,

Fig. 2. Quantitative analysis of 2-deoxy-*scyllo*-inosose TMS ether by GC-MS SIM.
□ *m/z* 450, ■ *m/z* 360.

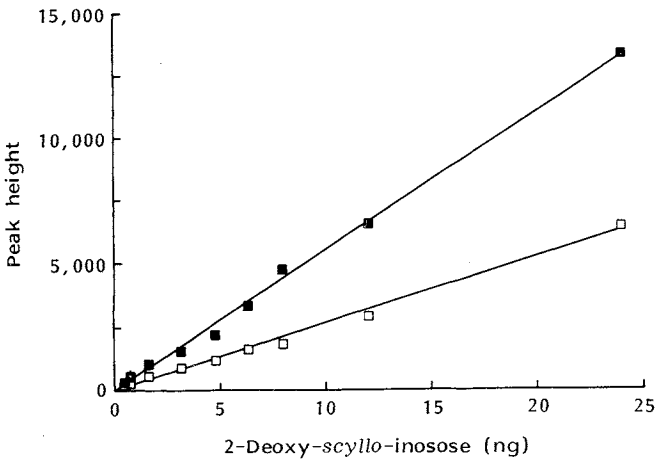
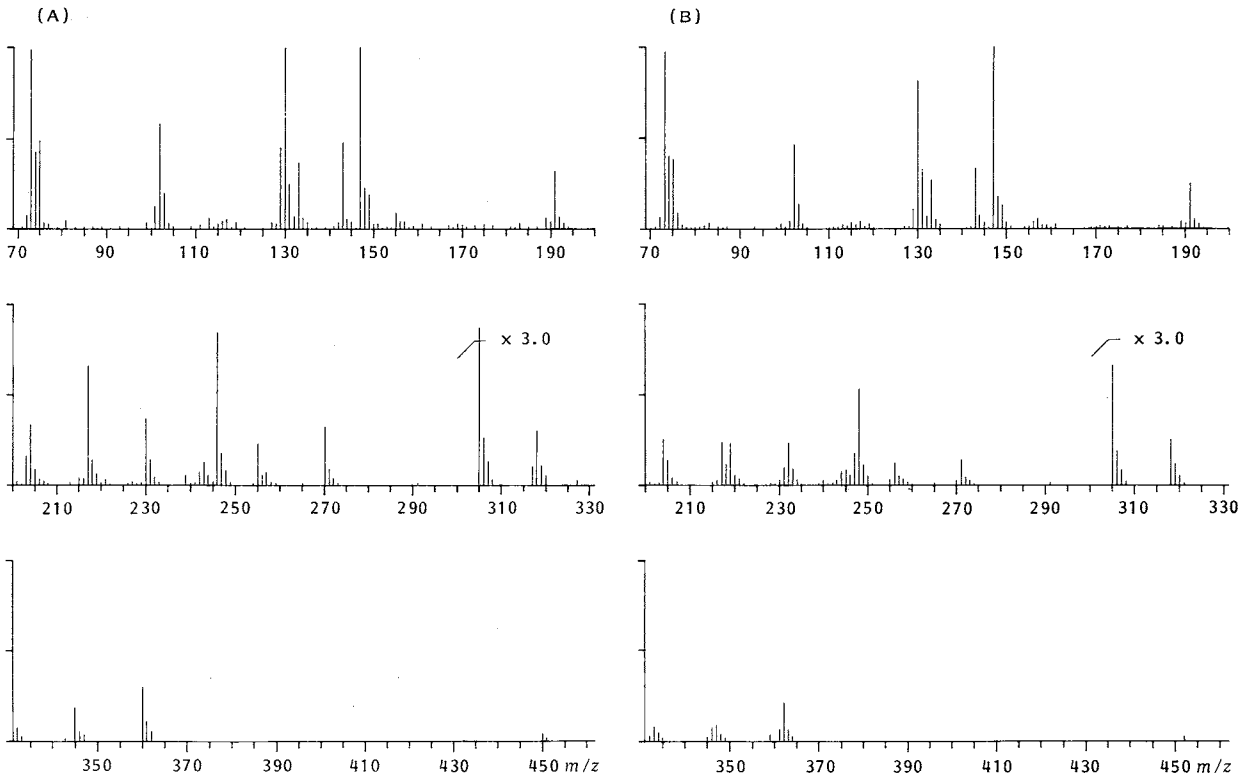


Fig. 3. Mass spectra of 2-deoxy-*scyllo*-inosose and [2,2-$^2H_2$]-2-deoxy-*scyllo*-inosose TMS derivatives.

Spectrum A: 2-Deoxy-*scyllo*-inosose; Spectrum B: [2,2-$^2H_2$]-2-deoxy-*scyllo*-inosose.



dried by flashing nitrogen gas, and then derivatized. The resulting mixtures were directly subjected to the GC-MS analyzer.

Fig. 2 shows the correlation of the amount of 2-deoxy-*scyllo*-inosose and the peak heights deduced from the GC-MS analyses. From this curve, the minimum amount of this quantitative analysis was estimated to be about 1 ng of 2-deoxy-*scyllo*-inosose.

The mass fragmentation pattern of the [2,2-$^2H_2$]-2-deoxy-*scyllo*-inosose tetra-*O*-TMS ether was studied by comparing with the non-labeled 2-deoxy-*scyllo*-inosose TMS derivative, and the results are illustrated in Fig. 3. The molecular ion peak was observed at *m/z* 452, 2 mass units higher than the non-labeled material. The fragment ions formed from the elimination of one molecule of TMSOH were observed at *m/z* 362 and *m/z* 361. The corresponding non-labeled counterpart showed only at *m/z* 360. Thus, two pathways were obviously involved in the fragmentations. Naturally, the ion of *m/z* 362 was formed by elimination of TMSOH and the ion of *m/z* 361 was formed by elimination of TMSO$^2$H. Under the experimental conditions, the major fragment peak was *m/z* 362, and the ion at *m/z* 361 was the minor. It thus seemed appropriate to monitor the ions of *m/z* 452 and 362 for the quantitative analysis.

## Direct Detection of 2-Deoxy-*scyllo*-inosose in the Culture of the Organisms Producing 2-Deoxystreptamine-containing Antibiotics

To evaluate the above-mentioned SIM analysis of 2-deoxy-*scyllo*-inosose, direct detection of 2-deoxy-*scyllo*-inosose in the cultured broths was attempted. Four microorganisms producing 2-deoxystreptamine-containing antibiotics were chosen. Each fermentation was carried out in the ISP No. 2 medium at 29°C, and aliquots of these broths were collected every 24 hours. After centrifugation at 1,500 rpm, the supernatant of each sample was dried and derivatized to the TMS ether. The analysis was performed at the SIM mode as described above. The cell growth and the total antibiotic activity in the supernatant were also determined.

Fig. 4. GC-MS SIM analyses of the 4-days culture of *Streptomyces fradiae* IFO 13147.

Top: Standard 2-deoxy-*scyllo*-inosose sample; Bottom: the 4-days culture.

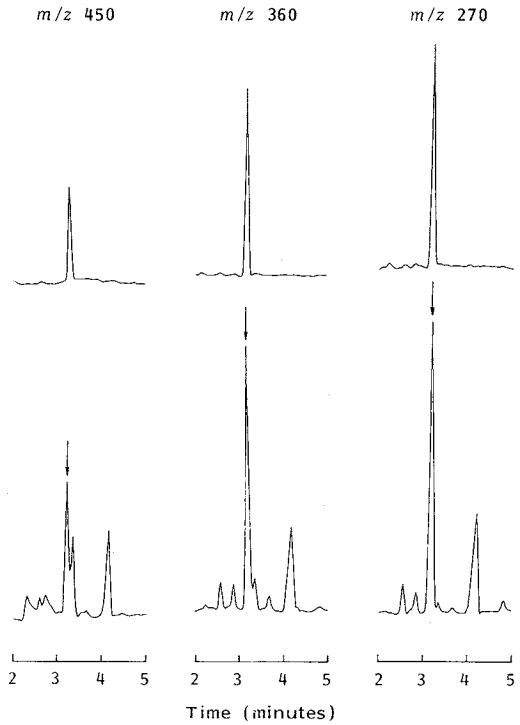
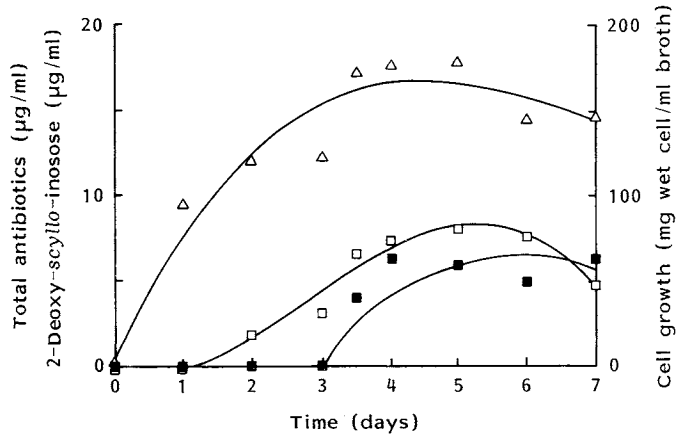



Fig. 4 shows the SIM trace of the 4-days culture of *Streptomyces fradiae* IFO 13147. The arrowed peak observed by the monitoring at *m/z* 450, 360, 270 was identical in retention time with the standard 2-deoxy-*scyllo*-inosose tetra-*O*-TMS ether. This result clearly indicated that 2-deoxy-*scyllo*-inosose exists in 7 μg/ml in the cultured broth of this strain. The same peak was observed similarly in the fermentation broths of other three strains (in *Streptoalloteichus hindstanus* 4-days' culture, *ca.* 4 μg/ml; in *Streptomyces ribosidificus*, *Streptomyces kanamyceticus* cultures, only detectable amount). Thus, 2-deoxy-*scyllo*-inosose was shown to be

Fig. 5. Time course of the 2-deoxy-*scyllo*-inosose formation in the cultured broth of *Streptomyces fradiae* IFO 13147.

□ 2-Deoxy-*scyllo*-inosose, ■ total antibiotics, △ cell grow.




biosynthesized by *Streptomyces* strains which produce 2-deoxystreptamine-containing antibiotics.

The time courses of the formation of 2-deoxy-*scyllo*-inosose, the cell growth, and the antibiotic activity during the culture of *S. fradiae* IFO 13147 are illustrated in Fig. 5. Under these conditions, the formation of 2-deoxy-*scyllo*-inosose was observed at the late log phase of growth. The amount gradually increased at the stationary phase, and then decreased somewhat. The antibiotic activity followed after the increase of 2-deoxy-*scyllo*-inosose.

## Discussion

As described in the Results section, the GC-MS spectra of the TMS ether derivatives of 2-deoxy-*scyllo*-inosose and [2,2-$^2H_2$]-2-deoxy-*scyllo*-inosose clearly demonstrated the molecular ions and the major fragmentation pathway $(M-TMSOH)^+$. When [2,2-$^2H_2$]-2-deoxy-*scyllo*-inosose was used, the major fragmentation ion was *m/z* 362. Thus, the elimination of TMSOH preferentially occurred through the loss of hydrogen at the oxygenated carbon, rather than at the methylene group. These results suggested that the SIM tracing should focus on the molecular ion as well as the fragmentation ion due to an elimination of TMSOH and that the dideuterated sample can be clearly differentiated from the non-labeled sample. This means that, under *in vitro* conditions, [2,2-$^2H_2$]-2-deoxy-*scyllo*-inosose can be chased even in the presence of endogenous non-labeled 2-deoxy-*scyllo*-inosose.

This analytical method was applied to identify 2-deoxy-*scyllo*-inosose in the biological samples. Previously, the intermediacy of 2-deoxy-*scyllo*-inosose in the 2-deoxystreptamine biosynthesis was shown by experiments using idiotrophic mutants.[11,12] In the present study, 2-deoxy-*scyllo*-inosose was physically and directly detected in the fermentation broths of some *Streptomyces* strains. The fate of 2-deoxy-*scyllo*-inosose formed in the cultured broths was also traced. The prior accumulation of 2-deoxy-*scyllo*-inosose in the antibiotic production was observed and the amount gradually decreased with the formation of antibiotics. This deviation of 2-deoxy-*scyllo*-inosose accumulation in the cultured broth seems to be typical for the early intermediates of secondary metabolism. These observations were not made previously because of the lack of the direct method of analysis of 2-deoxy-*scyllo*-inosose and may serve an additional indirect support for the intermediacy of 2-deoxy-*scyllo*-inosose in the biosynthesis of 2-deoxystreptamine.[11,12] The remaining crucial problem in the 2-deoxystreptamine includes an unequivocal detection of the 2-deoxy-*scyllo*-inosose formation at the cell-free level.

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