Biosynthesis of Streptothricin F. Part 6.[1] Formation and Intermediacy of D-Glucosamine in *Streptomyces* L-1689-23

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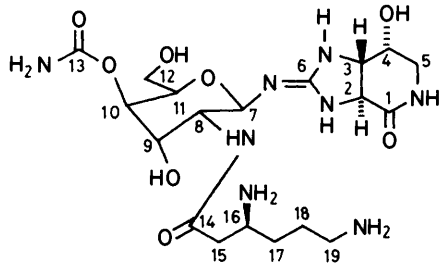
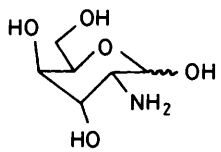
Aspects of the biosynthesis of the D-gulosamine moiety of the antibiotic streptothricin F have been studied with stable isotopes. After having obtained substantial incorporation (*ca.* 9%) of [1-^{14}C]-D-glucosamine into the total compound, [2-^{13}C, ^{15}N]-D-glucosamine was synthesized and fed to *Streptomyces* L-1689-23. Analysis of the ^{13}C n.m.r. spectrum of the derived antibiotic reveals a doublet (J_{CN} 11.4 Hz) for the C-8 resonance (3.1% enrichment in both ^{13}C and ^{15}N), indicating the intact incorporation of the material fed and establishing D-glucosamine as a direct precursor to streptothricin. [1-^{2}H]-D-Glucose has been synthesized and incorporated into streptothricin with retention of the deuterium label at 7-H (1.8% enrichment) and incorporation of deuterium at 17-H/18-H (0.4% enrichment); data from the incorporation of [1-^{14}C]-D-glucose that had been co-fed indicate extensive metabolism into other parts of the structure. Incorporation of [2-^{2}H]-D-glucose leads only to deuterium enrichment at 17-H/18-H (0.09% enrichment), indicating that 2-H is apparently lost in the phosphoglucose isomerase reaction of this organism.

As part of our continuing studies[1] on the biosynthesis of streptothricin F (**1**)[2,3] representative of a ubiquitous family of broad spectrum antibiotics,[4] we are investigating the formation of the D-gulosamine unit (**2**) in *Streptomyces* L-1689-23. All members of the streptothricin family contain this unit as the central moiety, with other substituents appended to it.[4] Remarkably, these antibiotics are the only instances in which (**2**) has been found in Nature.

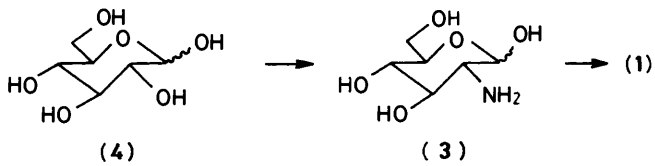


(**1**)



(**2**)

Sawada, *et al.*[5,6] have carried out preliminary experiments aimed at locating the origin of the gulosamine moiety of racemomycin A (streptothricin F) in *Streptomyces lavendulae*. [U-^{14}C]-D-Glucose and [1-^{14}C]-D-glucosamine were each fed to cultures of *S. lavendulae* and these yielded incorporations of 0.71% and 4.46% into the total antibiotic, respectively. This suggested that glucosamine (**4**) might be a more direct precursor than glucose (**3**) to (**2**). Partial degradations localized much of the radioactivity in the gulosamine portion, although significant radioactivity was also found in the β-lysine and streptolidine portions, as well. This ambiguity could have resulted from the extensive metabolism expected for glucose if transamination of glucosamine were also taking place, and emphasizes the need to clearly identify specifically labelled sites, which we have now done using stable isotopes and high field n.m.r. spectroscopic analysis. We report herein on the formation of (**3**) in *S.* L-1689-23 and its role in the biosynthesis of (**1**).



(**4**) (**3**)

Results and Discussion

Our initial investigation was focused on whether D-glucosamine (**3**) was a direct precursor to the D-gulosamine moiety of streptothricin F, planning subsequently to investigate the formation of (**3**) in *S.* L-1689-23, if warranted. A synthetic medium with glycerol and acetate as the primary carbon sources was developed to minimize the extent to which glucose and glucosamine would label other portions of (**1**). Based on time course studies, the sterile addition of sugars 24 h after inoculation of production cultures (the onset of antibiotic production) and harvesting of the cultures 50 h later were chosen as standard conditions for feeding experiments. The antibiotic was isolated *via* a series of chromatographic steps, further purified by recrystallization of its helianthate salt, and then converted into the hydrochloride salt for n.m.r. analysis.[1]

[1-^{14}C]-D-Glucose (**4a**) and [1-^{14}C]-D-glucosamine (**3a**) were each fed a number of times to production broths. The former yielded a 1.3—2.0% total incorporation into (**1**), while an 8.9—9.4% incorporation was obtained with the latter. No chemical degradations of these samples of (**1**) were undertaken. It was then necessary to establish whether (**3**) was a direct intermediate to the gulosamine moiety and, if so, to what extent general metabolic degradation had led to random re-incorporation of its carbon atoms into other portions of

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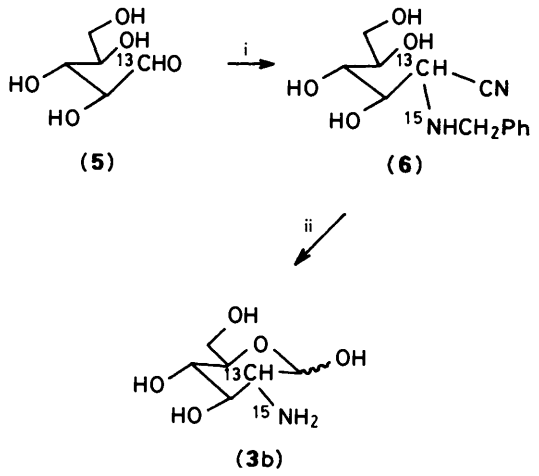
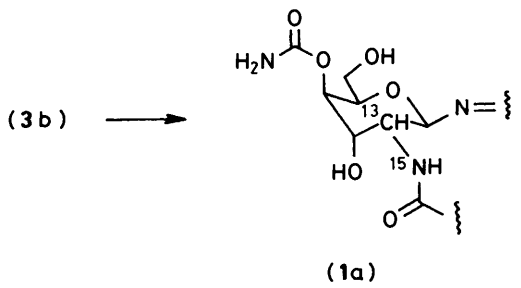
(**1**). [2-^{13}C,^{15}N]-D-Glucosamine (**3b**) was synthesized[7] from [1-^{13}C]arabinose (**5**) and [^{15}N]benzylamine[7,8] in 40% overall yield from the labelled nitrile (**6**), as shown in Scheme 1, and was then fed to *S.* L-1689-23.



Scheme 1. *Reagents:* i, HCN–PhCH$_2$^{15}NH$_2$–EtOH; ii, H$_2$–Pd–BaSO$_4$–0.5M HCl

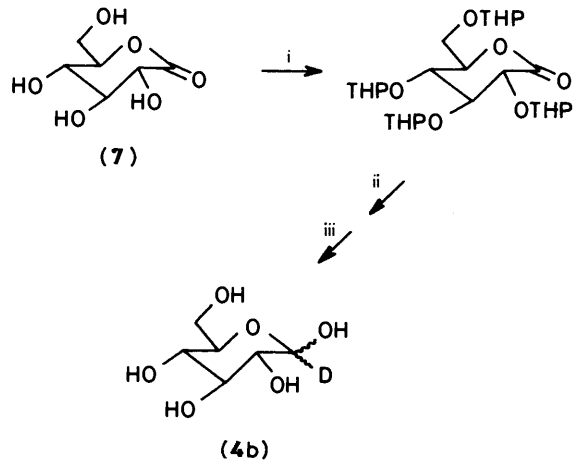
Administration of (**3b**) (0.14 mmol), admixed with (**3a**) (13.54 μCi), to three 250 ml production broths of the modified medium afforded 73 mg of pure (**1a**). The 100.6 MHz ^{13}C n.m.r. spectrum of (**1a**) showed a doublet due to ^{13}C–^{15}N heteronuclear spin coupling[9,10] (J_{CN} 11.4 Hz) flanking the singlet for C-8 at 46.4 p.p.m. By comparison of the C-8 singlet with various other resonances, normalized to those of a natural abundance spectrum, it was determined that there was no measurable ^{13}C enrichment in the C-8 singlet. Thus, the ^{15}N label had been fully retained with the ^{13}C label (2.3% enrichment measured from the normalized resonances), and D-glucosamine was unequivocally established as a direct precursor to (**1**). Furthermore, there was no detectable ^{13}C enrichment at any other individual carbon atom. Based on the total incorporation of ^{14}C (9.4%), a 3.1% enrichment in ^{13}C was calculated if (**3**) had been incorporated exclusively in the aminosugar portion. Thus, comparison with the n.m.r. results suggested that as much as 25% of the glucosamine may have been degraded and the ^{14}C randomly re-incorporated.



Cell-free studies in other laboratories have shown that (**3**) is derived from fructose 6-phosphate (F-6-P) and glutamine in eukaryotic and prokaryotic organisms.[11,12] The initial step, when catalysed by glucosamine 6-phosphate synthetase (EC 5.3.1.19) from rats, is irreversible.[11] This contrasts with *E. coli*, which contains an enzyme that converts glucosamine 6-phosphate to F-6-P and NH_3.[12,13] Of more direct significance, during neomycin biosynthesis in *S. fradiae*, [1-^{13}C]-D-glucosamine was specifically incorporated into not only the neosamine B and C units, but also into the D-ribose and the deoxystreptamine moieties; these latter two require loss of the amino group, presumably by conversion back into an (activated) glucose.[14] The specific enrichments at the two relevant carbon atoms in the ribose and deoxystreptamine units were fully comparable to the atoms specifically enriched by [6-^{13}C]-D-glucose (1.60 and 6.32% compared with 2.28 and 1.94%, respectively). Furthermore, in the biosynthesis of the kanamycin antibiotics in *S. kanamycetius* radioactivity from [1-^{14}C]-D-glucosamine was well incorporated into the 6-amino-6-deoxy-D-glucose moiety of kanamycin A, again requiring the loss of the amino group.[15] Thus, the complete retention of the ^{15}N label from (**3b**) in streptothricin biosynthesis, indicating that glucosamine-6-phosphate synthetase in this organism is irreversible, too, is particularly noteworthy.

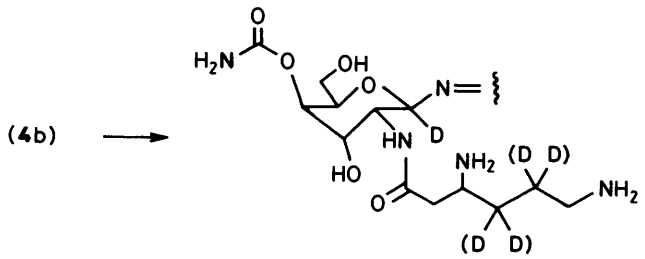
We next sought information regarding the mechanism of conversion of glucose into glucosamine in *S.* L-1689-23. A key step would be the conversion of glucose 6-phosphate (G-6-P) to F-6-P, catalysed by phosphoglucose isomerase (EC 5.3.1.9). The rabbit muscle enzyme reaction has been shown to involve a *cis*-enediol intermediate with transfer of hydrogen from C-2 to the 1-*pro-R* position *via* a shielded, or 'sticky,' base.[16] As much as 80% of the original 2-H was found in the derived F-6-P.

In order to ensure that the hydrogens of glucose would behave reliably, we first fed [1-^{2}H]-D-glucose (**4b**). This was synthesized in 40% overall yield from D-gluconolactone (**7**) as shown in Scheme 2.[17] When 300 mg of (**4b**), mixed with (**4a**),

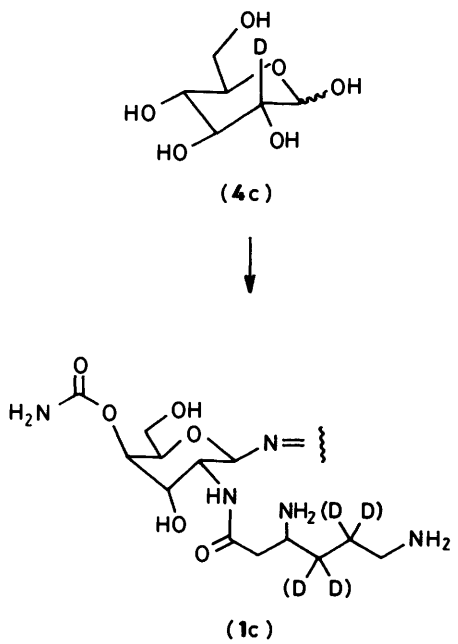


Scheme 2. *Reagents:* i, DHP–TsOH–DMF; ii, LiAlD$_4$–AlCl$_3$–Et$_2$O; iii, Dowex 50Wx4

was fed to three 250 ml production broths of *S.* L-1689-23, 235 mg of (**1b**) were produced and a 2.0% incorporation of ^{14}C was obtained. This should have yielded a 8.7% enrichment in deuterium at 7-H of (**1b**). In fact, a signal was observed at δ 5.2 in the 61.4 MHz ^{2}H n.m.r. spectrum. However, in order to observe this, it was necessary to obtain the spectrum at 303 K in order to shift upfield the signal from residual HOD in the deuterium depleted water used as the solvent. By comparison with the signal at δ 1.27 for t-butyl alcohol added as a chemical shift and quantitation reference, a much reduced 1.8% enrichment at 7-H was obtained. In a second experiment, in addition to the signal at δ 5.2, another signal a quarter of its size was observed at δ 1.8. This latter signal would correspond to the hydrogens at C-17 and /or C-18. Even with our modified medium a considerable amount of the glucose fed had been diverted into less direct pathways.



[2-^{2}H]-D-Glucose (**4c**) was next fed to the same size fermentation. Again, a good production (256 mg) of streptothricin F was obtained. In this fermentation, (**4a**) had not been included. A lower limit of 1.3% incorporation was assumed based on the majority of previous feedings; on this basis a ^{2}H enrichment of 4.0% at 8-H would have been expected. This time, however, when the sample (**1c**) was subjected to ^{2}H n.m.r. analysis, the only signals that could be seen were those from the added t-butyl alcohol, the residual HOD, and a small resonance at δ 1.8—again, presumably at 17-H/18-H—that integrated for 2.3% of the expected molar equivalence of deuterium. Observation of the latter signal gave assurance that had deuterium been retained at C-8, it would have been observable.



The loss of 2-H of glucose was an unexpected result. In addition to the work reported for phosphoglucose isomerase, D-xylose isomerase (EC 5.3.1.5) has been reported to catalyse a reaction that results in almost complete transfer to C-1 of the hydrogen that had been at C-2.[18] In contrast, however, in the reaction catalysed by phosphomannose (M-6-P) isomerase (EC 5.3.1.8), which interconverts M-6-P and F-6-P, only 5—10% of the original hydrogen originally at C-2 was 'retained' in the transfer to the 1-*pro-S* position of the ketose,[19] and in the triose phosphate isomerase reaction only 3—6% of 1-H from dihydroxyacetone phosphate was retained at 2-H of glyceraldehyde 3-phosphate.[20]

Since all four of these sugar phosphate isomerases do transfer at least some of the original carbinol hydrogen, it is possible that the *Streptomyces* phosphoglucose isomerase does too, but the proof must await cell-free studies and a lower limit of detection. At this point it appears that this enzyme will be significantly different from the mammalian enzyme. We are continuing work on the further steps between D-glucosamine and the D-gulosamine moiety of (**1**).

Experimental

General.—^{1}H N.m.r. spectra were taken on a Varian FT 80A or Bruker AM 400 spectrometer; ^{13}C n.m.r. spectra were taken at 20 MHz and 100.6 MHz on Varian FT 80A and Bruker AM 400 spectrometers, respectively; ^{2}H n.m.r. spectra were obtained at 61.4 MHz on a Bruker AM 400 spectrometer. All ^{13}C n.m.r. were broadband proton decoupled and ^{2}H n.m.r. spectra were proton decoupled and run unlocked. Five mm n.m.r. tubes were used for all n.m.r. measurements; ^{1}H and ^{13}C n.m.r. samples were prepared in $CDCl_3$, $(CD_3)_2SO$, or D_2O, and TMS or Bu^{t}OH were used as internal references. ^{2}H N.m.r. samples were prepared in ^{2}H-depleted water and 25 μl of Bu^{t}OH was used as reference for chemical shift (1.27 p.p.m.) and quantitation (0.38 μmol ^{2}H).

All radioactivity measurements were carried out using a Beckman LS 7800 liquid scintillation counter with automatic quench correction to yield disintegrations per minute (d.p.m.). Melting points were taken on a Buchi melting point apparatus and are uncorrected. Analytical thin layer chromatography (t.l.c.) was carried out on precoated Kieselgel 60 F_{254}, visualized by short wave u.v. and by spraying with 0.1% ninhydrin.

Materials.—All solvents were reagent grade and were used directly as purchased. [1-^{13}C]-D-Arabinose (99 atom% ^{13}C) and $^{15}NH_4NO_3$ (99 atom% ^{15}N) were brought from Cambridge Isotopes, Boston, MA. [2-^{2}H]-D-Glucose (96 atom% ^{2}H) was purchased from MSD Isotopes, St. Louis, MO. [1-^{14}C]-D-Glucose was purchased from New England Nuclear Corp.

Production Broth and Culture Conditions.—A seed culture[1] of *Streptomyces* L-1689-23 was used to inoculate (5% v/v) production broths of O'Brien's medium[21] modified to include $CaCO_3$ and additional carbon sources. O'Brien's medium (0.2% glycine, 0.23% sodium acetate trihydrate, 0.02% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.0016% $CuSO_4 \cdot 5H_2O$, 0.0013% $MnSO_4 \cdot 4H_2O$, 0.0025% $FeSO_4 \cdot 7H_2O$, 0.0066% $CaCl_2 \cdot 2H_2O$, and 0.003% $ZnSO_4 \cdot 7H_2O$ in distilled water) was autoclaved. The equivalent of 1% (w/v) of powdered $CaCO_3$ was autoclaved separately and then added to the broth. Finally, 2.0% glycerol and 0.25% glucose (w/v) were dissolved in a minimum volume of distilled water and was added by syringe in a sterile manner *via* a Millipore filter.

250 ml Cultures in 1 l Erlenmeyer flasks were incubated at 29 °C in a gyratory shaker at 200 r.p.m. Solutions of labelled glucose or glucosamine were added in a sterile manner *via* a Millipore filter 24 h after inoculation of the production broth. Fermentations were continued for an additional 26 h and then worked up as previously described.[1]

2-Benzyl[^{15}N]*amino-2-deoxy*[2-^{13}C]-D-*glucononitrile* (**6**).—A saturated solution of KCN [prepared by dissolving KCN (55 g) in water (100 ml)] was slowly added 1 cm below the surface of H_2SO_4 (50%; 100 ml) contained in a 3-necked flask equipped with a stopper, and a gas outlet tube. The tube led to an empty 250 ml 3-necked flask fitted with an N_2 inlet and an outlet tube that was connected to two U-tubes in series filled with Drierite. The U-tubes and the empty flask were immersed in a warm water bath to avoid condensation of HCN. The outlet from the

U-tubes was connected to a 100 ml 3-necked flask immersed in a solid CO_2–acetone bath and equipped with a solid CO_2 condenser and a rubber septum. The final exit, from the top of the condenser, was fitted with a drying tube filled with NaOH pellets. When enough of the HCN thus generated had been condensed, the addition of KCN was stopped and the system was flushed with N_2 to remove residual HCN. The dry ice bath was removed, the HCN allowed to melt, and the solid CO_2 condenser replaced with a stopper.

[1-^{13}C]-D-Arabinose (250 mg, 1.66 mmol), absolute ethanol (3 ml), and 250 μl [^{15}N]benzylamine (245 mg, 2.29 mmol) were heated at reflux with stirring for 15 min. The resulting yellow solution was cooled to room temperature and injected into the flask containing the anhydrous HCN. The mixture was stirred overnight at room temperature. The precipitate that had formed was filtered, washed with a small volume of cold ethanol, and recrystallized from the same solvent to give (**6**) (243 mg, 55%), m.p. 132—134 °C (for unlabelled material lit.,[7] 131—132 °C, lit.,[8] 124—126 °C); δ_H[$(CD_3)_2SO$; 80 MHz] 7.25 (5 H, s), 4.95 (1 H, d, *J* 7 Hz), 4.20—4.75 (3 H, br m), 3.55—4.00 (8 H, br m), and 2.70—3.10 (1 H, br s); δ_C[$(CD_3)_2SO$; 100.6 MHz] 139.00, 139.07, 128.19, 128.09, 128.88, 127.89, 126.91, 126.78, 120.35, 119.20 (d, J_{CC} 54.6 Hz, C-1), 70.99, 70.93, 70.75, 69.54, 69.46, 69.30 (d, J_{CC} 41.3 Hz, C-3), 63.50, 63.21, 53.11 (d, J_{CN} 2.3 Hz, PhCH_2), 52.76 (d, J_{CN} 3.2 Hz, C-2), 50.68, and 50.53.

[^{15}N,2-^{13}C]-2-*Amino*-2-*deoxy*-D-*glucose Hydrochloride* (**3b**).—2-Benzyl[^{15}N]amino-2-deoxy[2-^{13}C]-D-glucononitrile (0.16 g, 0.597 mmol) was hydrogenated in HCl (0.5M; 3.6 ml) in the presence of Pd–$BaSO_4$ (29 mg). The reaction was monitored by t.l.c. [EtOAc–pyridine–MeOH (5:2:1)], and after 18 h the starting material could no longer be detected. The catalyst was removed by filtration and washed with water and a semicrystalline solid was obtained when the combined filtrates were concentrated. The solid was dissolved in distilled water (2 ml), neutralized with AG3x4 (OH^-) resin, and filtered. This solution was loaded onto a Dowex 50Wx8 (H^+) resin (5 ml) and the column was washed with water until the washings were neutral. The amino sugar was then eluted with 1M HCl. The fractions collected were analysed by t.l.c. and those containing (**3b**) were pooled and concentrated under reduced pressure to afford a semicrystalline solid. Finally, this was washed with cold methanol and filtered, to yield pure (**3b**) (69.8 mg, 54%) δ_H (D_2O; 80 MHz) identical with authentic glucosamine except for the 2-H resonance that was additionally split by ^{13}C ($^1J_{CH}$ 140 Hz), and a general lack of sharp resolution due to long range C–H couplings.

Feedings.—(a) [^{15}N,2-^{13}C]-2-*Amino*-2-*deoxy*-D-*glucose hydrochloride* (**3b**). Three 250 ml production broths were fed compound (**3b**) (total of 30 mg, 0.139 mmol) mixed with (**3a**) (29.8×10^6 d.p.m.). Bioassay after fermentation indicated that (**1a**) (209 mg) had been produced, and helianthate (175 mg) was subsequently obtained (6.77×10^6 d.p.m. mmol^{-1}, 9.4% total incorporation).[1] Based on the ^{13}C n.m.r. spectrum of the hydrochloride of (**1a**), a 2.3% enrichment at C-8 and the attached amino nitrogen were obtained.

(b) [1-^{2}H]-D-*Glucose* (**4b**). Three 250 ml production broths were fed compound (**4b**) (total of 300 mg, 1.67 mmol) mixed with (**4a**) (10.9×10^6 d.p.m.). Bioassay after fermentation indicated that (**1b**) (238 mg) had been produced, and helianthate (125 mg) was subsequently obtained (9.43×10^5 d.p.m. mmol^{-1}, 2.0% total incorporation). Based on the ^{2}H n.m.r. spectrum of the hydrochloride of (**1b**), a 1.8% enrichment at 7-H was obtained.

(c) [2-^{2}H]-D-*Glucose* (**4c**). Three 250 ml production broths were fed compound (**4c**) (total of 204 mg, 1.13 mmol). Bioassay after fermentation indicated that (**1c**) (256 mg) had been produced, and 94 mg of the antibiotic was subsequently obtained. Based on ^{2}H n.m.r. spectroscopy a 0.092% enrichment at 17-H/18-H was obtained.

Acknowledgements

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