3-TREHALOSAMINE, A NEW DISACCHARIDE ANTIBIOTIC

L. A. DOLAK, T. M. CASTLE and A. L. LABORDE

The Upjohn Company, Kalamazoo, MI 49001, U.S.A.

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3-Amino-3-deoxy-α-D-glucopyranosyl-α-D-glucopyranoside (α,α-3-trehalosamine) was isolated from a culture of *Nocardiopsis trehalosei* sp. nov. (NRRL 12026). The structure was determined using a combination of spectroscopic techniques on derivatives of the component sugars, especially gas chromatography-mass spectrometry. The compound exhibited antibiotic activity against Gram-positive organisms at levels similar to what was found for the 2- and 4-trehalosamines.

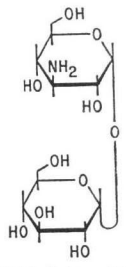
Herein we report the isolation and characterization of 3-trehalosamine (Fig. 1). 3-Trehalosamine represents a novel structure in a series of aminodeoxytrehalose analogs, each of which possesses only weak Gram-positive activity. The compound has recently been synthesized[17] but this is the first report of the isolation of 3-trehalosamine from a biological source.

Fermentation and Isolation

The antibiotic was produced by *Nocardiopsis trehalosei* sp. nov. (NRRL 12026) when the organism was grown in a medium that contained 1% Cerelose, 2% dextrin, 0.25% steepwater, 0.3% ammonium nitrate, 0.2% sodium chloride, and 0.5% calcium carbonate. Prior to sterilization, the medium was adjusted to pH 7.2 with 5 N NaOH. The fermentation was conducted at 28°C and 3-trehalosamine production was detected by antibacterial activity against *Bacillus subtilis* UC® 564. When grown as described above, peak titers equalled 1 mg/ml after three days.

The beer was filtered using Dicalite 4200 filter aid as a slurry in the beer and as a bed in a filter funnel. The filtrate was adjusted to pH 6 with sulfuric acid and was then percolated over 1/20 volume of Dowex 50W×2 (H^+) at 5 bed volume/hour. The column was washed with deionized water and eluted with a water to 1 M ammonium sulfate gradient. The active fractions were pooled and the activity was adsorbed onto a column of Amberlite XE-348 (Rohm & Haas) equal in size to the Dowex column. The resin bed was washed with deionized water and the activity was eluted with 1:1 (v/v) acetone - water. The acetone was removed on a rotary evaporator and the light yellow solid obtained after lyophilization was suspended in 30 ml 0.01 N HCl. After filtration (Whatman #2 paper) the solution was injected onto a silica gel column and the activity was eluted with the upper phase from 2:1:1 (v/v) chloroform - methanol - 17% NH_4OH containing 150 ml/liter butanol. The fractions containing the antibiotic were pooled and concentrated to an aqueous solution on a rotary evaporator. The tacky solid obtained after lyophilization was chromatographed over Amberlite IR-45 (OH^-) with deionized water to give the pure antibiotic.

Fig. 1.



3-Trehalosamine

The product was crystallized as the hydrochloride by dissolving the hydrochloride in a minimal amount of water at 50°C. The clear solution was decanted from the oil that separated when the solution had reached room temperature. Ethanol was added until the solution became cloudy and the solution was cooled

to −20°C. The white crystals decomposed at 200°C. The elemental analyses (after drying at 110°C) were in agreement with those obtained by BAER[17].

Table 1. Specific rotation of trehalose analogs

Compound	$[\alpha]_D$	Reference
2-Trehalosamine	+176°(2.4, H_2O)	3
4-Trehalosamine	+179°(0.5, H_2O)	4
Altro 3-trehalosamine	+170°(1.0, MeOH)	15
6-Azido-6-deoxytrehalose	+178°(0.19, MeOH)	7
Mannosyl glucosaminide	+91.3°(2.0, H_2O)	5
3-Trehalosamine	+161°(13.7, H_2O)	this work

3-Trehalosamine has recently been synthesized and has been reported to be a weak competitive inhibitor of cockchafer trehalase.[17]

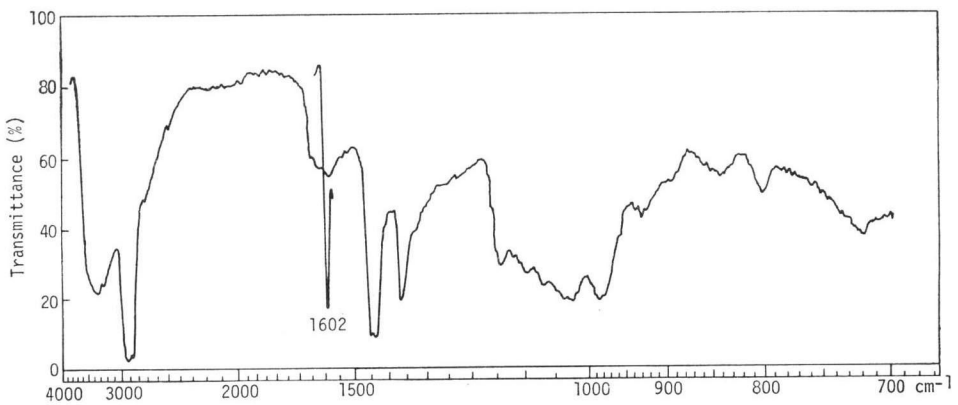
Characterization

The new antibiotic had an Rf of 0.8 when upper phase from 2: 1: 1 chloroform - methanol - 17% ammonium hydroxide (v/v) was used as developing solvent on Analtech silica gel plates. Visualization was accomplished *via* bioautography on *B. subtilis* inoculated agar trays. The Rf on Ionex SA tlc sheets according to PAUNCZ[1] was 0.92 (ninhydrin). The mobility relative to streptothricin by high voltage electrophoresis at pH 1.8 on Whatman #1 sheets with a formic acid - acetic acid - water (22: 75: 100, v/v) buffer[2] at 55 v/cm for 1 hour was 0.57 toward the cathode. Streptothricin migrated about 30 cm under these conditions.

The UV spectrum had only end absorption. The CMR spectrum (in D_2O to pH 2 with 6 N HCl, external TMS) showed twelve lines: δ 94.8 (D=doublet), δ 93.8 (D), δ 74.0, δ 73.8, δ 73.6, δ 72.5, δ 71.2, δ 70.0 (D), δ 68.1 (D), δ 62.0 (T=triplet), δ 61.6 (T), and δ 56.2 (D). The IR spectrum possessed strong OH/NH bands (Fig. 2). The 100 MHz PMR spectrum in D_2O with internal SDSS showed an unresolved band between δ 3.2 and δ 4.0 along with two doublets near δ 5.2 (J=3.0 and 3.5 cps). The antibiotic was ninhydrin-positive and did not reduce aniline phthalate solution. The optical rotation is given in Table 1.

These data are consistent with a mono-amino, mono-deoxy disaccharide such as 2-trehalosamine[3], 4-trehalosamine[4] or mannosyl glucosaminide[5]. Structures similar to nojirimycin[6] or 6-trehalosamine[7] are excluded by the data.

Fig. 2. Infrared spectrum of 3-trehalosamine (Nujol mull).



Biological Activity

3-Trehalosamine exhibited antibacterial activity against *B. subtilis* UC® 564 and *Staphylococcus*

aureus UC® 80. Application of a 12.7-mm disc containing 0.08 mcg of the antibiotic on seeded agar trays of these organisms gave zones of inhibition of 39 mm and 32 mm, respectively. The MIC *versus* *Diplococcus pneumoniae* I UC® 41 was 62.5 mcg/ml, whereas neamine gave an MIC of 7.8 mcg/ml. In addition, 3-trehalosamine was inactive at the highest concentration tested against a variety of other Gram-positive and Gram-negative organisms against which neamine was active. It cured *D. pneumoniae* I-infected mice at 650 mg/kg and was well-tolerated at 800 mg/kg.

Structure Determination

About 10 mg of the new antibiotic was refluxed in 5 ml of methanol for 16 hours with 2.0 g Dowex 50×2 (H^+) resin. The methanol was removed on a rotary evaporator and the resin was washed with three 5-ml portions of deionized water. The resin was collected by filtration. The aqueous filtrate was

Fig. 3. Partial mass spectrum of methyl 3-deoxy-3-trifluoroacetamido-2,4,6-tri-TMS-α-D-glucopyranoside

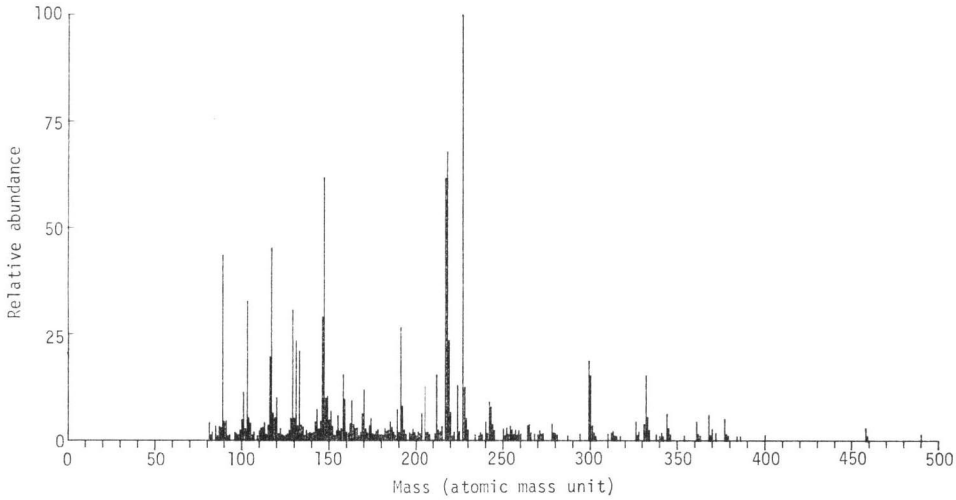
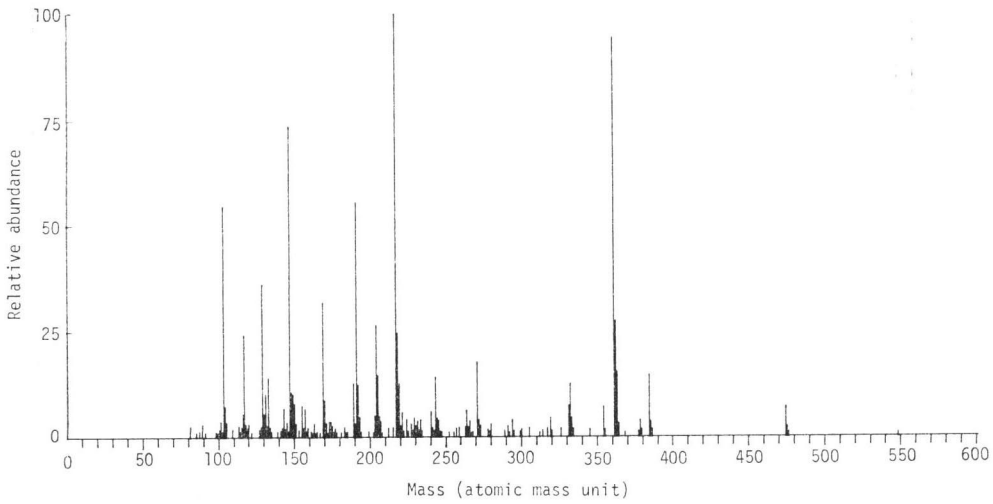


Fig. 4. Partial mass spectrum of 3-trehalosamine (N-trifluoroacetyl-per-O-TMS derivative).



lyophilized and the residue was derivatized with an excess of trimethylsilyl imidazole (TMSI) in a sealed vial at 60°C for 10 minutes. The resin was eluted by slurrying it with three 15-ml portions of 1 N NH_4OH. The basic eluates were lyophilized and the residue was treated first with TMSI as above and then with an excess of trifluoroacetic anhydride.

Each sample was then analyzed on a Hewlett-Packard Model 5992 desktop GC-MS instrument. The column measured 4 mm × 80 cm and was packed with 3% OV-17 on high efficiency Chromosorb W. The starting temperature was 140°C and the thermal program went to 250°C at 5°C/min. The total ion abundance between *m/e* 80 and 510 was recorded.

The neutral sugar derivative gave a major peak at 7.9 minutes which corresponded in retention time to methyl α-D-glucopyranoside. The fragmentation pattern was identical to that recorded[8] for the TMS derivative of methyl α-D-glucopyranoside. These conditions separated the methyl O-TMS-glycosides of galactose, mannose and several five-carbon sugars.

The basic sugar derivative gave peaks at 8.4 and 8.9 minutes with base peaks at *m/e* 227 (Fig. 3). The fragmentation pattern indicated these were α and β anomers of a methyl aminodeoxyhexopyranoside. These peaks separated from that of the neutral sugar derivative when run as a mix. The N-TFA, O-TMS derivative of methyl 4-amino-4-deoxyglucopyranoside (obtained from the methanolysis of 4-trehalosamine and apramycin) gave a base peak at *m/e* 204 and was also separable from the TMS derivative of methyl α-D-glucopyranoside under these conditions.

Methyl 3-deoxy-3-trifluoroacetamido-α-D-glucopyranoside was prepared from 3-amino-3-deoxyglucose obtained from fermentation.[9] Its retention time and mass fragmentation were identical to that observed for the unknown aminodeoxy sugar. The significant loss of 60 mass units (elements of methylformate) from the molecular ion at *m/e* 505 (not observed), followed by the loss of another 113 mass units (elements of trifluoroacetamide) to give a peak at *m/e* 332 (8.1% abundance) differentiate this from the 2-amino-2-deoxy isomer which should first lose the elements of trifluoroacetamide from the molecular ion to give an expected peak at *m/e* 392[8].

Under these conditions the retention time of the N-TFA, O-TMS derivative of 3-trehalosamine was 29 minutes. The fragmentation pattern (Fig. 4) was the sum of that of both the glucose and 3-amino-3-deoxyglucose components and therefore differed little from that of the corresponding derivative of 4-trehalosamine.

The location of the amino group at the 3-position was further substantiated by noting that its chemical shift (δ 56.3) differed from that of the C-2′ carbon of neamine[10] (δ 57.7) and the C-4 carbon of apramycin[12] (δ 52.3) but was very similar to that of the C-3″ carbon of kanamycin[11] (δ 56.1).

The α, α linkage was deduced from the coupling constants of the 1, 1′ protons[13] and from the CMR chemical shift of the 1, 1′ carbons.[14] The gluco-gluco configuration was deduced from the coincidence of retention time in the gas chromatograph with derivatives of known configuration noted above and from a comparison of the optical rotations observed from the analogous compounds noted in Table 1.

Acknowledgements

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