Structure of Trehalostatin: A Potent and Specific Inhibitor of Trehalase

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A structural study of trehalostatin **1**, a specific inhibitor of blowfly trehalase, revealed that **1** contains an unusual five-membered pseudocyclitol.

Trehalostatin **1**, a potent and specific inhibitor against blowfly (*Aldrichna grahami*) trehalase was purified from the culture broth of *Amycolatopsis trehalostatica*[1] as a white amorphous powder: $[\alpha]_D^{25}$ + 115° (*c*. 1.0, H_2O), IC_{50} 0.68 ng ml^{-1} (for blowfly trehalase). Several colour reactions‡ provided the evidence that **1** should contain a saccharide moiety and a C–N linkage, but neither a reducing terminal nor a free amino group. A characteristic absorption band at 1610 cm^{-1} among typical saccharide absorptions in its IR spectrum (film),§ as well as a UV *endo*-absorption (in H_2O) suggestd that **1** was a saccharide with amide or ureido functions. The liquid SIMS (secondary ion mass spectrometry) spectrum of **1** furnished the $[M + H]^+$ species at *m/z* 367, indicating a molecular formula of $C_{13}H_{22}O_{10}N_2$. A B/E linked scan on this molecular ion species yielded a daughter ion at *m/z* 205 [M +

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‡ Trehalostatin is positive to chlorine-tolidine and vanillin-sulphate reagents, while negative to *ninhydrin*, 2,3,5-triphenyltetrazolium chloride, Elson–Morgan, and Tollens reagents.

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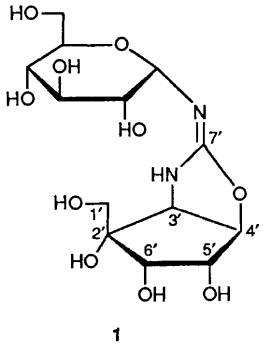

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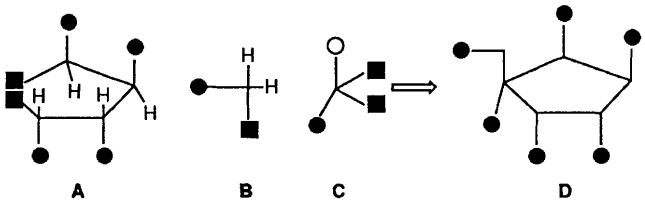


A B C D

Fig. 1 Fragments A–C and substructure D. ● indicates heteroatoms and ■ indicates carbon atoms, while ○ indicates heteroatom or carbon atom.

$H-C_6H_{10}O_5]^+$, thus the presence of a hexose moiety was indicated¶ and, therefore, the non-hexose moiety was $C_7H_{11}O_5N_2$ with three degrees of unsaturation. A ^{13}C NMR study of **1** revealed the presence of either a carbonyl or a C=N carbon at δ 163.8 (in D_2O), indicating that the non-hexose moiety was bicyclic. Acetylation of **1** with Ac_2O–pyridine at room temperature yielded two isomeric acetates, **2** and **3**,‖ in *ca.* 1 : 1 ratio.

Intensive NMR studies (500 MHz) of the acetates **2** and **3** (in $CDCl_3$) allowed us to obtain the structure of trehalostatin **1** as follows: H,H-COSY experiments disclosed three isolated proton spin systems in the acetates as well as in **1** itself, one being of an α-glucopyranosyl residue, and the rest, fragments A and B (Fig. 1). The chemical shift of the anomeric carbon of the α-glucopyranosyl residue appeared further up-field (δ_C 81.1 nd 81.0, respectively, for **2** and **3**) than would be expected for an *O*-glucoside suggesting that the glucopyranosyl residue was connected to nitrogen. Two protons (3′- and 4′-Hs) in fragment A with a large coupling constant ($J_{3',4}$ 8.6 for **1** and 9.8 Hz for **2** and **3**) showed an NOE to each other confirming their eclipsed configuration in a five-membered ring system. This was corroborated with down-field chemical shifts of three contiguous carbons (by ^1H-detected heteronuclear multiple-quantum coherence, HMQC) in the same fragment [δ_C 80.2(C-4′), 78.2(C-5′) and 78.1(C-6′)] of **2**.[2] The involvement of fragment B, an sp^3 quaternary carbon (δ_c 86.5 and 79.1 for **2** and **3**, respectively) in the five-membered ring system was obtained because two terminal protons of fragment A gave cross peaks with fragment C carbon in the ^1H-detected multiple-bond heteronuclear multiple-quantum coherence (HMBC) experiment. Both protons of the isolated methylene (fragment B) also showed cross peaks with the same quaternary carbons of fragment C. Thus, the connectivity of these fragments was obtained as substructure D.

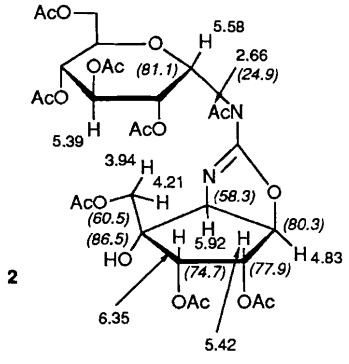
¶ The methyl glucopyranosides were identified as peracetates by 500 MHz ^1H NMR after methanolysis of **1** followed by acetylation. The absolute chirality of glucose was confirmed by converting the methyl glucopyranoside peracetates to methyl α-D-glucopyranoside tetra-*p*-bromobenzoate, which showed a positively split CD centred at 244 nm. For references, see H.-W. Liu and K. Nakanishi, *J. Am. Chem. Soc.*, 1981, **103**, 559 and 1982, **104**, 1178.

‖ These are the positional isomers regarding *N*-acetates derived from two possible tautomers at the isoureido group.

Table 1 ^1H and ^{13}C NMR data of trehalostatin **1**[a]

Carbon Number	δ_C (*J*/Hz)		δ_H (*J*/Hz)
1	83.4 (CH)[b,c]		5.23 (br d, $J_{1,2}$ 5.0)
2	72.8 (CH)[b]		3.62 (dd, $J_{1,2}$ 5.0; $J_{2,3}$ 9.0)
3	75.8 (CH)[b]		3.54 (t, $J_{2,3}$ $J_{3,4}$ 9.0)
4	72.4 (CH)[b]		3.30 (dd, $J_{3,4}$ 9.0; $J_{4,5}$ 10.0)
5	74.7 (CH)		3.46 (ddd, $J_{4,5}$ 10.0; $J_{5,6a}$ 5.2; $J_{5,6b}$ 2.5)
6	64.8 (CH_2)[b]	a	3.61 (dd, $J_{5,6}$ 5.2; $J_{6a,6b}$ 12.3)
		b	3.71 (dd, $J_{5,6b}$ 2.5; $J_{6a,6b}$ 12.3)
1′	63.5 (CH_2)	a	3.62 (d, $J_{1'a,1'b}$ 12.3)
		b	3.70 (d, $J_{1'a,1'b}$ 12.3)
2′	85.6 (C)[b,c]		
3′	76.5 (CH)[b]		4.24 (br d, $J_{3',4'}$ 8.6; $J_{3',6'}$ 0.1)
4′	89.9 (CH)		4.82 (ddd, $J_{3',4'}$ 8.6; $J_{4',5'}$ 2.7; $J_{4',6'}$ 0.1)
5′	83.2 (CH)[b,c]		4.09 (dd, $J_{4',5'}$ 2.7; $J_{5',6'}$ 4.8)
6′	83.0 (CH)[b]		3.84 (dt, $J_{5',6'}$ 4.8; $J_{3',6'}$ $J_{4',6'}$ 0.1)
7′	163.8 (C)		

[a] Chemical shifts are referenced to external TSP at 25 °C, pD 8.9 (pD value is not corrected for the deuterium effect). [b] Indicates the presence of DIS. [c] Small DIS. (DIS = differential isotope shifts.)



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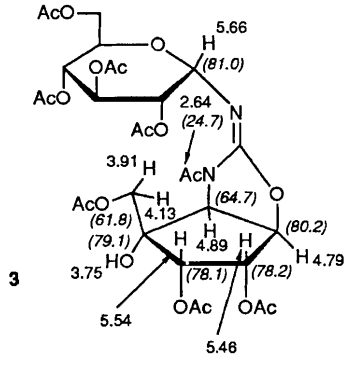

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Fig. 2 Structures of **2** and **3**, and their NMR data (in $CDCl_3$). Numbers in parentheses denote δ_C.

The up-field resonance of C-3′ (δ_C 58.5) of **2** proved that C-3′ bears nitrogen. Deuterium-induced differential isotope shifts (DIS;[3] in D_2O and in H_2O/D_2O 9 : 1 mixture) observed on C-3′ (δ_C 76.5) of **1** was evidence for the presence of an NH group on C-3′. Large down-field shift upon acetylation of 1′-H (δ_H 3.62/3.70 **1** to 3.94/4.21 **2** and 3.91/4.13 **2**′, 5′-H (δ_H 4.09 to 5.42 and 5.46) and 6′-H (δ_H 3.84 to 6.35 and 5.54), indicated that **1** bears free hydroxy groups at C-1′, -5′ and -6′ positions. This was in good agreement with the DIS experiment result obtained for **1** (Table 1).

The 4′-H proton attached to C-4′ on which no DIS was observed, showed no down-field shift upon acetylation. Therefore, the oxygen linked to C-4′ (δ_C 89.9 for **1**, 80.3 for **2** and 80.2 for **3**) is involved in further linkage. The substructure

D was, therefore, extended to the planar structure of **1** with a cyclic isoureido function, which is connected to the α-D-glucopyranosyl residue, when the nature of the remaining sp^2 carbon was taken into consideration.

The relative configuration of the pseudo cyclitol portion was deduced by a phase sensitive NOESY [(two dimensional) nuclear Overhauser effect spectroscopy] experiment. NOEs observed between 3'-H/4'-H (as was noted previously) and between 5'-H/6'-H ($J_{5',6}$ 4.8 Hz for **1** and 8.5 Hz for **2** and **3**) confirmed their *syn* configuration. The absence of NOEs between 4'-H/5'-H as well as small coupling constants ($J_{4',5}$ 2.7 Hz for **1** and 3.6 Hz for **2** and **3**) suggested their *anti* relationship. The observed NOE between 2'-OH (a singlet at δ_H 3.75) and 3'-H of **2** was good evidence for their *syn* relationship. An NOE observed between the *N*-acetate methyl group at δ_H 2.66 (of **2**) and 3-H of the glucose residue at δ_H 5.39 showed that this isomer, **2**, possessed an *N*-acetate on the nitrogen atom attached to the glucopyranosyl residue, while the other isomer, **3**, was an $N^{3'}$-acetate.

On the basis of these spectroscopic data, the structure of trehalostatin acetates were obtained as shown in structures **2** and **3** (Fig. 2). By comparing the NMR data of **1** with those of **2** and **3**, the structure of trehalostatin itself was thus proposed as **1**.**

It should be noted that known trehalase inhibitors[4] have a more basic imine function, in contrast to trehalostatin **1**, which contains a glycosyl isoureido functional group that can tautomerize, thereby showing acid/base properties. It has been suggested that insect trehalase has a catalytically important carboxylic acid/carboxylate 'flip-flop' couple at the active site,[5] and the carboxylic acid participates in protonation of glycosidic oxygens. The high selectivity of trehalostain **1** to insect trehalase is probably due to the ambident nature of the isoureido structure,†† which behaves in a very similar manner except that it neutralizes the effect of the carboxylic acid/carboxylate 'flip-flop' system.

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** In order to confirm the absolute configuration of the pseudocyclitol portion, synthetic studies are now in progress.

†† From the chromatographic behaviour of **1** on a cation exchange resin, **1** should have a pK_a value between lysine (pI 9.74) and histidine (pI 7.59).