Communications to the Editor

TREHAZOLIN, A NEW TREHALASE INHIBITOR

Sir:

During the course of screening for inhibitors of trehalase, we found the pseudodisaccharide termed trehazolin† (**1**) in a culture broth of *Micromonospora*, strain SANK 62390††. This paper describes the isolation, structure determination, and inhibitory activity of trehazolin.

One loopful of the producing organism was aseptically transferred from an agar slant into baffled 500-ml Erlenmeyer flasks each containing 80 ml of a primary seed medium. This medium was composed of glucose 1%, glycerol 1%, oatmeal 0.5%, sucrose 1%, soybean meal 2%, Casamino acids 0.5%, pressed yeast 1%, $CaCO_3$ 0.1%, and Disfoam CB-442 (Nippon Yushi Co.) 0.01%. After formulation, the pH was adjusted to 7.0 with aq NaOH, and the medium was sterilized. The incubation was performed on a rotary shaker at 220 rpm at 28°C for 216 hours. Forty ml of the primary seed culture were aseptically transferred into baffled 2-liter Erlenmeyer flasks each containing 800 ml of a secondary seed/production medium which was composed of glucose 2%, soluble starch 1%, pressed yeast 0.9%, meat extract 0.5%, Polypepton 0.5%, NaCl 0.5%, $CaCO_3$ 0.3%, and Disfoam CB-442 0.01%. The medium was then adjusted to pH 7.2 with aq NaOH before sterilization. The fermentation was continued under the previously described conditions for 96 hours. The secondary seed cultures (1.5 liters each) were then used as inocula for two jar fermenters containing 15 liters of the secondary seed/production medium. The organism was then cultured for 96 hours at 28°C with agitation at a rate of 100 rpm and aeration at a rate of 15 liters/minute.

The culture broth (30 liters) was filtered, and the filtrate was passed through a column of Dowex SBR-P (Cl^-). The effluent was adjusted to pH 5.0 and applied to a column of Dowex 50WX4 (H^+). After washing with deionized water, the active fraction was eluted with 0.5 N NH_4OH. The eluate was concentrated under reduced pressure and lyophilized to give 43.4 g of crude powder. This powder was dissolved in 20 mM ammonium formate buffer (pH 6.0), and was adsorbed on a column of Dowex 50WX4 (1.5 liters) equilibrated with the same buffer. The column was washed with the buffer and deionized water, and eluted with 0.2 N NH_4OH. Active fractions were combined, concentrated under reduced pressure, and lyophilized to yield 658 mg of solid. The material was then dissolved in water at pH 6.0, and adsorbed on a column of Amberlite CG-50 (H^+ - NH_4^+ (2:3), 300 ml). The column was washed with deionized water, and eluted with 0.1 N NH_4OH. Then the fraction was concentrated and lyophilized to afford 102.8 mg of powder. The powder was dissolved in 2 mM ammonium formate buffer (pH 6.0), and was chromatographed on a column of Diaion CHP20P (400 ml) equilibrated with the same buffer. The column was then eluted with the same buffer, and the active effluent was concentrated and lyophilized. This treatment was repeated twice to give 6.2 mg of crude trehazolin. This material was further purified by preparative TLC on silica gel (Merck Art. No. 5715) which was developed with CH_3CN-H_2O-AcOH (6:3:1). Trehazolin had an Rf value of about 0.45 using this system. The silica gel powder containing the compound was eluted with water. The contaminating silica gel was removed from the aqueous solution by a small column of Dowex 50WX4 (H^+, 5 ml) eluting with 0.5 N NH_4OH. After concentration and lyophilization 5.1 mg of trehazolin was obtained as an amorphous powder.

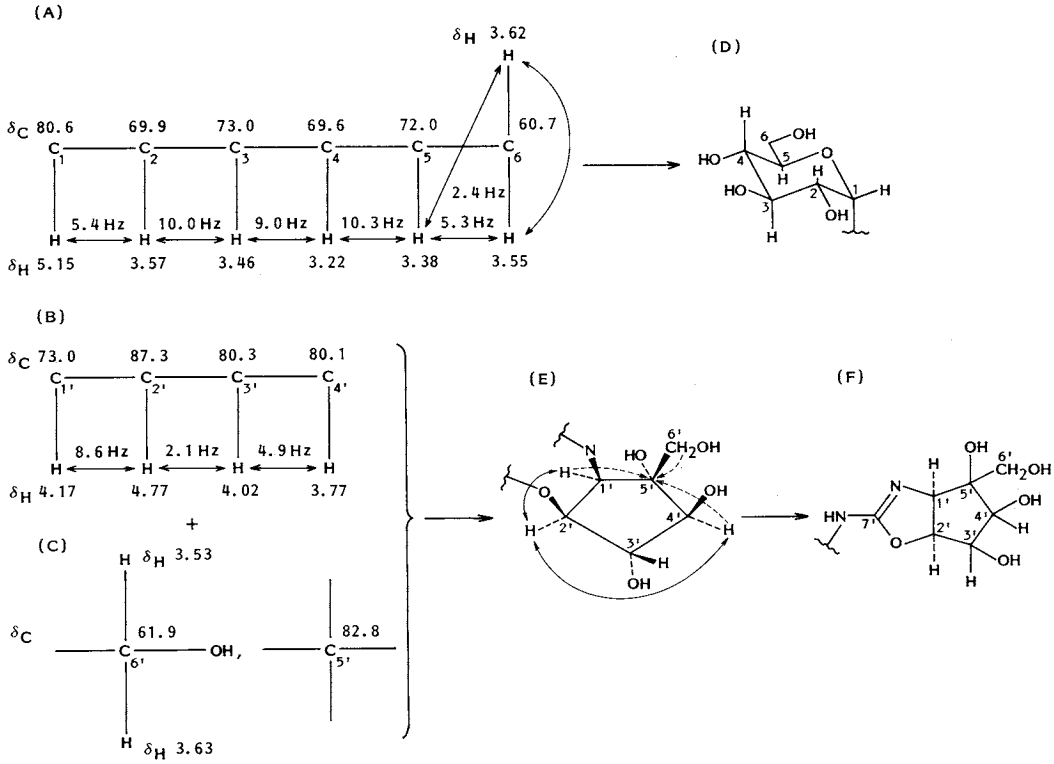
Trehazolin is a basic white powder with following physical and chemical properties: $[\alpha]_D^{25}$ +99.5° (*c* 0.41, H_2O); UV end absorption; IR ν_{max} (KBr) cm^{-1} 3368, 1663, 1056; soluble in water and MeOH, insoluble in Me_2CO and $CHCl_3$.

FAB-MS of trehazolin showed the $(M+H)^+$ ion

† A patent application for trehazolin with its chemical structure was filed on Aug. 20, 1990 (Application No. 217,104 ('90)). The structure of trehalostatin, which seems to be the same compound as trehazolin, was presented by the research group of Suntory Ltd. and Kumamoto Institute of Technology at the Annual Meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry, Kyoto, Apr. 1, 1991[1]. Their assignment of the configuration at C-4′, however, was opposite to our structure.

†† The strain was deposited with the Fermentation Research Institute of Agency of Industrial Science and Technology as FERM P-11631.

Fig. 1. Partial structures of trehazolin derived from ^{1}H and ^{13}C NMR data.



The ^{1}H and ^{13}C chemical shifts in D_2O referenced to the external TMS are given in parentheses and square brackets, respectively. The ^{1}H-^{1}H scalar coupling constants, ^{1}H-^{1}H NOEs (⟷), and ^{1}H-^{13}C long range couplings (– – →) are also shown.

at 367.1372, indicating the molecular formula $C_{13}H_{22}N_2O_{10}$. The partial structures depicted in Fig. 1 could be derived from ^{1}H-^{1}H connectivities by double quantum filter COSY spectrum. The assignments of ^{13}C signals directly bonded to those protons were made straightforwardly from the heteronuclear multiple-quantum correlation spectrum[2].

The partial structure (A) could be attributed to an α-glucose moiety on the observation that chemical shifts and the scalar coupling constants of (A) exhibit close similarity to those of α-glucopyranose. The sole exceptions were the ^{13}C chemical shifts of C-1 and C-2, which could be explained by the replacement of the hydroxyl group at C-1 with the NR group ($R=C_7H_{11}NO_5$).

Thus, the remaining part of the molecule must be constructed in a way satisfying the partial molecular formula $C_7H_{11}N_2O_5$ with the unsaturation number of three. A structure which satisfies the vicinal coupling constants and NOEs observed in the partial structure (B) (Fig. 1) was proposed to be the five membered ring (E) with its quarternary carbon C-5′ at 82.8 ppm linked to the hydroxy methyl group (C). This partial structure was proposed to be linked to (B) by virtue of the ^{1}H-^{13}C long range couplings shown in Fig. 1. The ^{1}H-^{13}C long range connections were obtained from the heteronuclear multiple-bond correlation (HMBC) experiment[3]. The structural information concerning the two nitrogens came from the pH titration experiments, in which the chemical shifts of C-1′, C-2′ and the remaining unassigned signal at 161.1 ppm exhibited the protonation shifts ($\Delta\delta$) of 7.3 (downfield), −4.6 (upfield) and −1.4 ppm, respectively. The resulting pKa value of 6.3 was not explained by the protonation of aliphatic amines[4], but was considered to be relevant to the protonation of the HN–C=N group[5].

Considering the large chemical shift differences between C-1′ and C-2′ and their significant pH-dependences, it is possible that the HN–C=N group is connected with the cyclopentane ring moiety giving the unique partial structure (F), where the signal at 161.1 ppm was assigned to C-7′.

Table 1. ^{1}H NMR assignment of **1** and **2**.

	1 in D_2O	**2** in CD_3Cl
1-H	5.15 d (5.4)	5.59 d (4.2)
2-H	3.57 dd (5.4, 10.0)	5.07 ddd (4.2, 10.0, 3.7*)
3-H	3.46 dd (10.0, 9.0)	5.40 td (10.0, 10.0, 3.8*)
4-H	3.22 dd (9.0, 10.3)	5.07 td (10.0, 10.0, 3.8*)
5-H	3.38 ddd (10.3, 5.3, 2.4)	4.32 ddd (10.0, 4.9, 2.5)
6-H	3.55 dd (12.2, 5.3),	4.10 dt (12.2, 2.3, 2.3*),
	3.62 dd (12.2, 2.4)	4.19 ddd (12.2, 4.9, 3.1*)
1′-H	4.17 d (8.6)	4.89 d (10.0, <1.0*)[a]
2′-H	4.77 dd (8.6, 2.1)	4.79 dd (10.0, 3.4)
3′-H	4.02 dd (2.1, 4.9)	5.47 dt (8.6, 3.4, 3.4*)
4′-H	3.77 d (4.9)	5.54 dd (8.6, 3.9*)
6′-H	3.53 d (12.0), 3.63 d (12.0)	3.92 dd (11.6, 2.3*),
		4.14 dd (11.6, 2.0*)
5′-OH		3.74 s
Ac[b]		1.98 d, 1.99 d, 2.03 d, 2.08 d,
		2.09 d, 2.10 d, 2.10 d, 2.66 d

Chemical shifts of **1** and **2** are given relative to external and internal TMS as references, respectively. Coupling constants in J=Hz are given in parentheses. In **2**, $^3J_{CH}$ couplings with ^{13}C-labeled acetyl carbonyl carbons are distinguished by asterisks.

[a] The $^3J_{CH}$ coupling between 1′-H and acetyl carbonyl carbon could not be observed directly in the ^{1}H NMR spectrum, but evidenced by the HMBC spectrum of **2**.

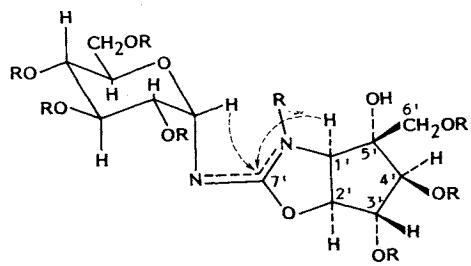
[b] Methyl protons of acetyl groups were observed as doublets due to $^2J_{CH}$ coupling with carbonyl carbons.

To identify the site at which the partial structure (F) is connected with the glucose moiety, trehazolin was acylated with 1, 1′-^{13}C labeled acetic anhydride in pyridine. In the ^{1}H NMR spectrum of the resulting peracetate (**2**), all non-exchangeable protons except 1-H, 5-H, and 2′-H showed additional couplings due to ^{1}H-^{13}C long range couplings with carbonyl carbons of the acetyl groups introduced (Table 1). The introduction of eight acetyl groups was consistent with mass spectral analysis. Since the observable ^{1}H-^{13}C long range couplings are practically limited to three bonds, the carbonyl carbon which exhibits the long range coupling with 1′-H could be uniquely assigned to that of the acetyl group introduced at the site adjacent to C-1′. Thus the partial structure (F) and the glucose moiety could be combined as shown in Fig. 2. The HMBC results are also consistent with the proposed structure, since long range coupling of C-7′ with 1-H and 1′-H was observed. The upfield shift of C-7′ from 161.1 ppm to 148.1 ppm was observed after the acetylation. An exchangeable singlet proton at 3.74 ppm was assigned to an acylation-resistant tertiary hydroxyl proton at C-5′ based on the presence of the long range coupling between C-1′ and this exchangeable proton in the HMBC spectrum of **2**.

Based upon the NOEs shown in Fig. 1 and the

Fig. 2. The structure of trehazolin.

The ^{1}H-^{13}C long range couplings connecting the glucose part and the aminocyclitol part are exhibited (⟶).



1 R=H
2 R=Ac

NOE observed between 1′-H and 5′-OH (δ 3.74 ppm), the relative configuration of the cyclopentane ring of trehazolin can be determined with the exception of C-3′. However, it can be concluded that 3′-H should be *trans* to 4′-H in view of the observed large vicinal coupling constants around 3′-H. Thus the structure of trehazolin was established as that shown in Fig. 2.

Effect of trehazolin on activity of silkworm and porcine trehalases was investigated. Porcine trehalase was purchased from Sigma, and silkworm trehalase was prepared as follows. Ten larvae of fifth

instar were homogenized in 20 mM citrate-40 mM phosphate disodium buffer (pH 5.6), and the homogenate was centrifuged at 6,000 rpm for 10 minutes. Cold acetone (240 ml) was added to the supernatant (120 ml) with stirring followed by centrifugation (9,000 rpm). The precipitate was dried to give 1.0 g of a powder which was used as crude trehalase. The trehalase catalyzed reaction was carried out in 20 mM citrate-40 mM phosphate disodium buffer at pH 5.6 for silkworm trehalase and at pH 6.2 for porcine trehalase. One hundred and eighty μl of trehalase solution (0.017 units/ml) were added to 50 μl of the experimental sample solution (2.5~80 ng/ml), and incubated at 37°C for 15 minutes. Then 20 μl of 250 mM trehalose were added, and the reaction was stopped after 15 minutes by heating on a boiling water bath for 3 minutes. Precipitates were removed by centrifugation, and the glucose concentration of the supernatant was measured by the glucose oxidase-peroxidase method using the Wako-C glucose assay kit.

I_{50} values of trehazolin for silkworm and porcine trehalases were 5.5×10^{-9} M and 3.7×10^{-9} M, respectively. Despite the extremely strong inhibition of these trehalases, trehazolin showed no activity with other glycosidases tested such as rat maltase, fungi amyloglucosidase, yeast invertase, almond β-glucosidase, and yeast α-glucosidase in comparable concentration (data not shown). Preliminary data revealed that trehazolin is a highly specific and an apparent irreversible inhibitor of trehalase.

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(Received April 6, 1991)

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