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Biosynthesis of the Trehalase Inhibitor Trehazolin

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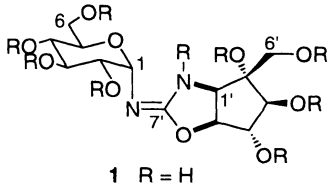
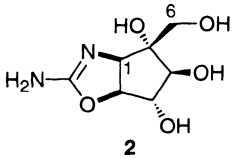
Trehazolin (**1**) is a trehalase inhibitor produced by *Micromonospora coriacea*. Biosynthesis of **1** was studied by feeding experiments with a variety of labeled precursors. Feeding experiments with [1-^{13}C]- and [6-^{13}C]-D-glucose revealed that the carbon skeletons of both a glucose residue and a cyclopentane ring moiety in **1** were each derived from glucose, and that C–C bond formation between C-1 and C-5 of glucose occurred during the cyclopentane ring formation. Furthermore, an experiment with [guanidino-^{13}C, $^{15}N_2$]-L-arginine revealed that two nitrogen atoms and a quaternary carbon atom involved in the aminooxazoline moiety of **1** originated from an amidino group of arginine. Further feeding experiments with [1-^{2}H]-, [2-^{2}H]-, [4-^{2}H]-, [6,6-$^{2}H_2$]- and [1,2,3,4,5,6,6-$^{2}H_7$]-D-glucose as well as [1-^{13}C]-D-fructose showed that deuteriums on C-1, C-3, C-4 and C-6 of glucose were retained during the formation of the cyclopentane ring moiety of **1**.

Trehazolin (**1**) was isolated from a culture broth of *Micromonospora* sp. SANK 62192 by ANDO *et al.* as a potent trehalase inhibitor[1~3]. It has a unique pseudodisaccharide structure with *N*-glycosidic linkage between a glucose residue and an aminocyclitol derivative, trehalamine (**2**). Trehalamine has a novel cyclopentanoid structure fused with an aminooxazoline ring[4]. The chemistry and biochemistry of **1** have been studied well[5,6], but its biosynthesis has not been studied yet. A cyclopentanoid skeleton biosynthesized from carbohydrate is uncommon in natural products compared with a cyclohexanoid skeleton, but several important compounds with a cyclopentanoid skeleton to which a hydroxymethyl group is attached are known[7]. The cyclopentane rings of pactamycin[8], bacteriohopane[9], aristeromycin[10] and allosamidin[11] have been proved to be biosynthesized from glucose. In the formation of the cyclopentane ring of pactamycin, bacteriohopane and allosamidin, C–C bond formation occurs between C-5 and C-1 of glucose. On the other hand, C–C bond formation in the cyclopentane ring of aristeromycin occurs between C-2 and C-6 of glucose. The mechanism of formation of the cyclopentane ring in aristeromycin has been studied with specifically ^{3}H-labeled glucoses.[10] We have been studying the biosynthesis of allosamidin[11] and recently reported the results of feeding experiments with specifically ^{2}H-labeled glucosamines and glucoses to investigate the mechanism of the cyclopentane ring formation of allosamizoline (**3**), an aminocyclitol component of allosamidin[12]. While the structures of **2** and **3** resemble each other, there are some critical differences



1 R = H
1a R = Ac



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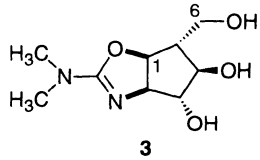

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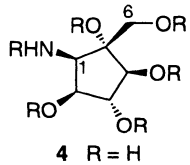

4 R = H
4a R = Ac

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between them. Trehalamine has a nitrogen atom at C-1 to form an oxazoline ring, while **3** has a nitrogen atom at C-2 derived from a glucosamine molecule. A hydroxyl group is present at C-5 in **2**, but not present at C-5 in **3**. Therefore, the biosynthetic mechanism to form **2** may be very different from that to form **3**. In this paper, we describe the elucidation of the origin of the carbon and nitrogen atoms of **1** by means of feeding experiments with ^{13}C- and ^{15}N-labeled precursors. The results of feeding experiments with a variety of specifically ^{2}H-labeled glucoses to investigate the mechanism of the cyclopentane ring formation of **2** are also described.

Micromonospora sp. SANK 62192 was used throughout this study. Strain SANK 62192 was cultivated in a 500-ml Erlenmeyer flask containing 100 ml medium on a rotary shaker. We used a medium without glucose for incorporation experiments to avoid dilution of the labeled glucose. Production of trehazolin (**1**) started around the 120th hour of cultivation under the culture conditions, and *ca.* 1.5 mg of **1** was obtained from 100 ml of the culture after 240 hours cultivation.

Since **1** has a glucose residue and the absolute configurations at C-2, 3 and 4 of the cyclopentane ring moiety are identical with those of D-glucose, feeding experiments with ^{13}C-labeled D-glucoses were carried out first. [1-^{13}C]- or [6-^{13}C]-D-Glucose was added in one portion to the culture at the 96th hour of cultivation. The ^{13}C NMR spectra of ^{13}C-labeled **1** obtained showed high enrichment at C-1 and C-1′, and C-6 and C-6′, respectively (Table 1). These results indicated that the carbon skeletons of both the glucose residue and the cyclopentane ring moiety were each derived from glucose, and the cyclopentane ring of **1** was biosynthesized by C–C bond formation between C-1 and C-5 of glucose. In the ^{13}C NMR spectrum of **1** derived from [1-^{13}C]-D-glucose, C-6 and C-6′ signals were also enriched to some extent probably due to metabolism of [1-^{13}C]-D-glucose to [6-^{13}C]-D-glucose and incorporation of the latter into **1** during the cultivation (Table 1).

Next, [guanidino-^{13}C, $^{15}N_2$]-L-arginine was fed to the culture to elucidate the biosynthetic origin of the aminooxazoline moiety of **1**. Labeled **1** obtained was acetylated to afford *N*-acetyltrehazolin octaacetate (**1a**) and the ^{13}C NMR spectrum of labeled **1a** was measured. In the spectrum, clear enrichment was observed at C-7′. The enriched C-7′ showed mainly double-doublet signals (signals 1, 4, 5, 7 in Figure 1) with coupling constants of 22.1 and 12.9 Hz. In the double-doublet signals, signal 4 is higher than the other three signals (1, 5, and 7) due to overlapping with non-labeled C-7′ signal. The ESI-MS spectrum of the labeled **1a** indicated that mono-, di- and tri-labeled molecules increased by 1.1, 1.7 and 6.2%, respectively. The coupling pattern of C-7′ signal and increased ratio of tri-labeled molecules in labeled **1a** derived from [guanidino-^{13}C, $^{15}N_2$]-L-arginine showed that the amidino group of the multiple labeled arginine was incorporated into a quaternary carbon and two nitrogen atoms involved in the aminooxazoline moiety of **1** without

Table 1. ^{13}C abundance in **1** obtained from the feeding experiments with ^{13}C labeled precursors.

Carbon no.	δ_C [a]	Relative ^{13}C abundance		δ_C [b]	Relative ^{13}C abundance
		[1-^{13}C]-D-glucose	[1-^{13}C]-D-fructose		[6-^{13}C]-D-glucose
1	80.53	7.6	7.1	81.37	c
2	69.03	0.8	0.9	70.98	1.3
3	72.93	1.0	1.0	72.97	1.0
4	69.25	0.9	0.7	70.69	0.9
5	73.30	0.8	0.8	74.03	d
6	60.47	1.9	2.1	61.77	8.2
1'	66.25	8.6	7.9	74.03	d
2'	91.49	1.1	0.9	88.12	0.7
3'	79.74	1.0	0.9	81.37	c
4'	79.36	1.0	1.0	81.21	0.9
5'	82.94	1.3	0.7	83.77	1.2
6'	60.77	1.7	1.9	63.11	6.8
7'	163.31	1.0	0.8	161.99	1.1

[a] In D_2O + 3% CD_3COOD.
[b] In D_2O.
[c,d] Signal overlappings.

Fig. 1. The C-7′ signal of ^{13}C NMR spectrum of labeled **1a** derived from [guanidino-^{13}C, $^{15}N_2$]-L-arginine (125 MHz, $CDCl_3$).

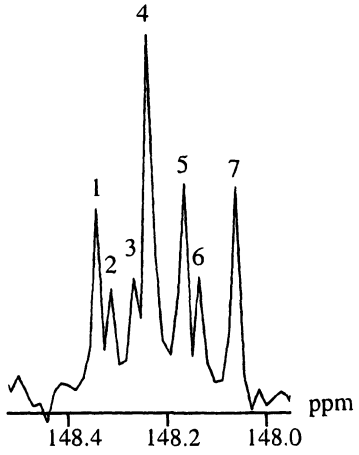
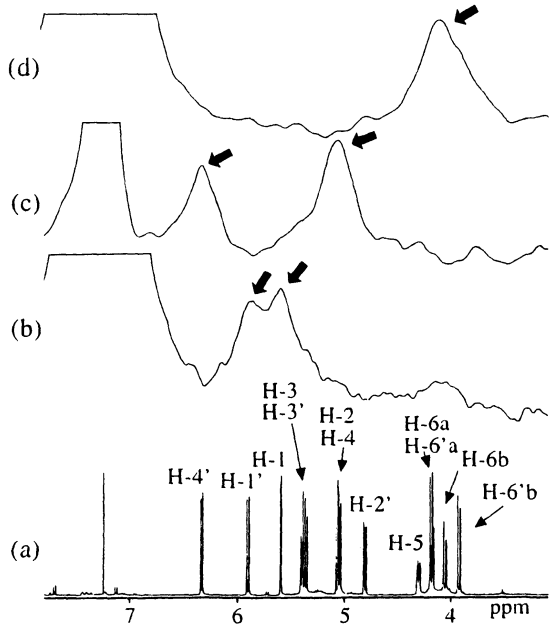


Fig. 2.

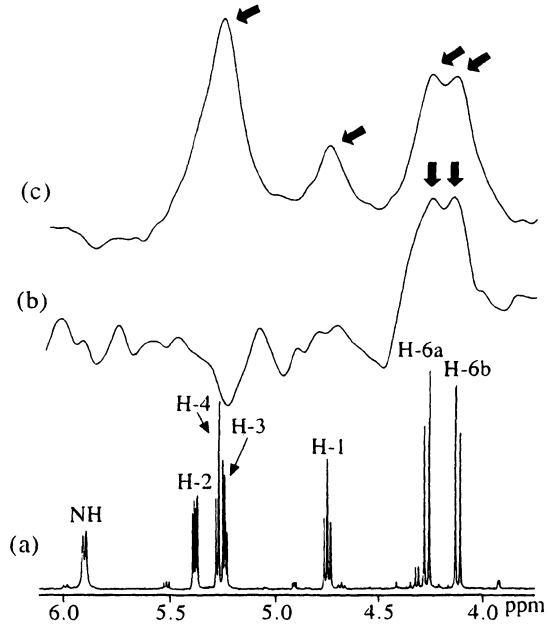


(a) ^1H NMR spectrum of natural **1a** (500 MHz, $CDCl_3$).

(b)~(d) ^2H NMR spectra of labeled **1a** (76.65 MHz, $CHCl_3$) derived from [1-^2H]-D-glucose (b), [4-^2H]-D-glucose (c), [6,6-$^2H_2$]-D-glucose (d).

cleavage of the ^{15}N–^{13}C–^{15}N bond. Three small signals (2, 3 and 6 in Figure 1) observed at C-7′ in the ^{13}C NMR spectrum of the labeled **1a** can be assigned as parts of

Fig. 3.



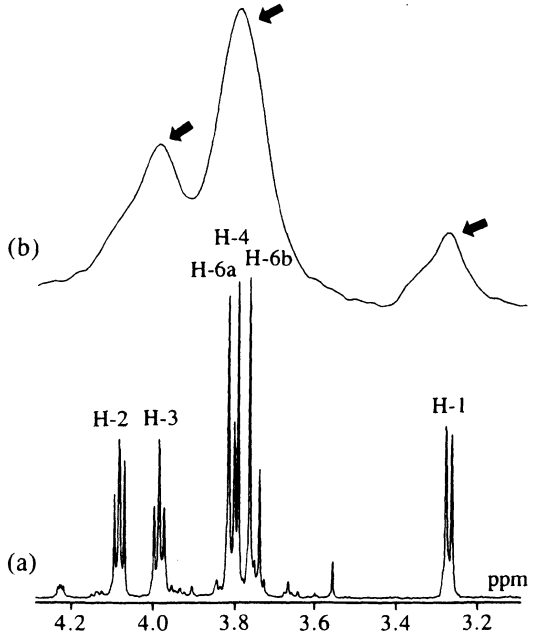
(a) ^1H NMR spectrum of natural **4a** (500 MHz, $CDCl_3$).

(b), (c) ^2H NMR spectrum of labeled **4a** (76.65 MHz, $CHCl_3$) derived from [6,6-$^2H_2$]-D-glucose (b), [1,2,3,4,5,6,6-$^2H_7$]-D-glucose (c).

two pairs of doublet signals (2 and 6, and 3 and 5) with J values of 22.0 and 12.9 Hz, respectively, indicating that metabolism of triply labeled [guanidino-^{13}C, $^{15}N_2$]-L-arginine to doubly labeled [guanidino-^{13}C, ^{15}N]-L-arginine and its incorporation into **1** occurred during the feeding experiment.

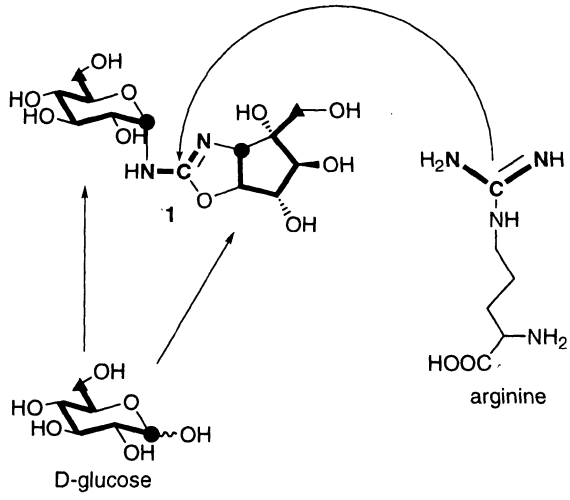
Feeding experiments with each of [1-^2H]-, [2-^2H]-, [4-^2H]-, [6,6-$^2H_2$]- and [1,2,3,4,5,6,6-$^2H_7$]-D-glucose were next carried out to investigate the mechanism of the cyclopentane ring formation of **1**. The ^2H NMR spectra of the labeled samples obtained were measured to evaluate the incorporation of deuterium and to determine the position of the incorporated deuterium. In the feeding experiment with [1-^2H]-D-glucose, deuterium incorporation was observed at C-1′ and C-1 in the ^2H NMR spectrum of labeled **1a** (Figure 2b). Deuterium incorporation was observed at C-4′ and at C-4 and/or C-2 in the experiment with [4-^2H]-D-glucose (Figure 2c). In the ^2H NMR spectrum of labeled **1a** derived from [6,6-$^2H_2$]-D-glucose (Figure 2d), deuterium incorporation at C-6′ was not confirmed because of signal overlapping. Therefore, labeled *N*-acetyltrehazolamine

Fig. 4.



(a) ^{1}H NMR spectrum of natural **4** (500 MHz, D_2O).
(b) ^{2}H NMR spectrum of labeled **4** derived from [1,2,3,4,5,6,6-$^{2}H_7$]-D-glucose (76.65 MHz, H_2O).

Fig. 5. Biosynthetic origin of trehazolin.



pentaacetate (**4a**) was prepared from labeled **1a** by hydrolysis and acetylation and its NMR spectrum was measured. In the spectrum (Figure 3b), two deuterium signals, whose chemical shifts correspond to those of the methylene protons on C-6 of **4a**, were observed, indicating that both of the deuterium atoms on C-6 of glucose were not lost during the biosynthesis of the cyclopentane ring moiety. This was supported by ESI-MS analysis of the labeled **4a**, in which mono- and di-labeled molecules increased by 0 and 2.9%, respectively.

In the feeding experiment with [2-^{2}H]-D-glucose, no deuterium incorporation into **1** was detected at either C-2′ or C-2. The ^{2}H NMR spectra of labeled **4a** and **4** derived from [1,2,3,4,5,6,6-$^{2}H_7$]-D-glucose (Figures 3c and 4b) showed clear deuterium incorporation at C-3 in addition to that at C-1 and C-6 of the cyclopentane ring moiety. Altogether, we could confirm that deuteriums on C-1, C-3, C-4 and C-6 of glucose were not lost during the formation of the cyclopentane ring moiety of **1** from the feeding experiments with the ^{2}H-labeled glucoses we used.

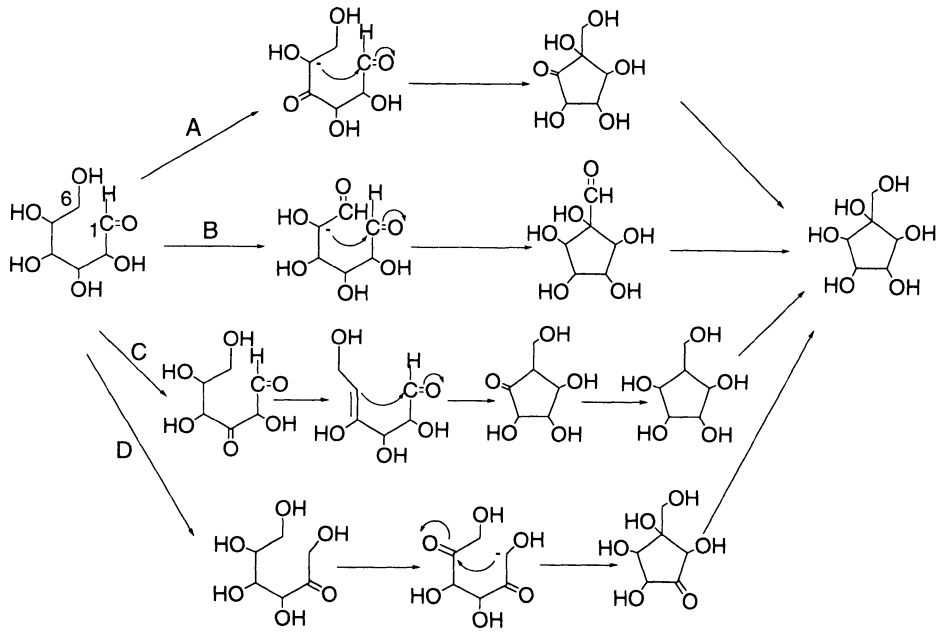
Finally, incorporation experiment with [1-^{13}C]-D-fructose was carried out. The ^{13}C NMR spectrum of labeled **1** obtained showed enrichment at C-1 and C-1′ (Table 1). In the spectrum, the ^{13}C enrichment rate at C-1′ was almost the same as that at C-1 similarly to the case of the labeled **1** derived from [1-^{13}C]-D-glucose above-mentioned (Table 1).

Discussion

The basic building blocks of **1** clarified in this study are summarized in Figure 5. Two glucose molecules and an amidino group are the origin of the carbon and nitrogen atoms of **1**. The origin of the dimethylaminooxazoline ring of allosamizoline (**3**) has been elucidated.[11] In the case of **3**, however, a carbon atom and one of the two nitrogen atoms of the amidino group of arginine was incorporated into a quaternary carbon and the nitrogen atom of the dimethylamino group without cleavage of the C–N bond. Therefore, the biosynthesis of the aminooxazoline moiety of **1** is unique, in that the amidino group was incorporated intact into this unit. We have little information about the mechanism of the aminooxazoline ring formation of **1**. Since deuterium was incorporated at C-1′ in the feeding experiment with [1-^{2}H]-D-glucose, the C–N bond at C-1′ of **1** should be formed without loss of H-1 of glucose.

By analogy to the biosynthetic mechanisms of natural compounds with cyclohexane rings, we may presume that the cyclization to form the cyclopentane ring of **1** proceeds *via* a 4-keto or 6-aldehyde glucose derivative, or a fructose derivative (or their enol equivalents), which would undergo an aldol condensation of C-5 with C-1. Therefore, four

Fig. 6. Plausible mechanism of cyclization of the cyclopentane ring of **1**.



Glucose is used as a tentative substrate in the cyclization reaction.

basic pathways to form the cyclopentane ring of **1** are possible (Figure 6). Pathways A, B and D are analogous to mechanism of cyclization in *myo*-inositol biosynthesis.[7] Pathway C is analogous to the mechanism of cyclization in the biosynthesis of dehydroquinate[7] and 2-deoxy-*scyllo*-inososе.[13]

In the feeding experiments with [4-^{2}H]- or [6,6-$^2H_2$]-D-glucose, deuterium loss at C-4′ or C-6′ of **1**, which would be evidence for the operation of pathway A or B, was not observed. But, this result can not rule out the possibility of pathway A or B since loss of deuterium from C-4 or C-6 may not occur if the cyclization reaction involves an enzyme-bound NAD(NADP)$^+$ for oxidation and subsequent reduction at C-4 or C-6 in the pathway A or B, similar to the case of *myo*-inositol 1-phosphate synthase.[14] Deuterium incorporation at C-4′ of **1** observed in the feeding experiment with [4-^{2}H]-D-glucose may rule out pathway C. To evaluate the possibility of pathway D, we carried out feeding experiments with [2-^{2}H]-D-glucose and [1-^{13}C]-D-fructose. But, deuterium loss was observed at both C-2′ and C-2 of **1** in the former experiment, and the result of the latter experiment was almost the same as that with [1-^{13}C]-D-glucose. These facts suggest that rapid interconversion of glucose and fructose may occur in the culture by the action of glucose isomerase,[15] which may lead to deuterium loss from [2-^{2}H]-D-glucose and equilibrium of [1-^{13}C]-D-fructose and [1-^{13}C]-D-glucose before incorporation of the labeled precursor added into **1**.

In conclusion, we have clarified the biosynthetic origin of all the carbon and nitrogen atoms involved in **1** and ruled out pathway C among four possible pathways to form the cyclopentane ring of **1**. Further work to clarify the mechanism of the cyclopentane ring formation in the biosynthesis of **1** is now in progress using a cell-free system.

Experimental

General Methods

^{13}C NMR spectra were recorded at 125 MHz on a JEOL JMN-A500 spectrometer, using acetone δ_C 29.2 as a standard. ^{1}H and ^{2}H NMR spectra were recorded at 500 MHz and 76.65 MHz on a JEOL JMN-A500 spectrometer. Acetone $\delta_{H(D)}$ 2.225 and chloroform $\delta_{H(D)}$ 7.24 were used as a standard for $D_2O(H_2O)$ and $CDCl_3(CHCl_3)$ solutions, respectively. ESI mass spectra were recorded on a JEOL JMS-SX102 spectrometer.

Culture

Micromonospora coriacea SANK 62192, a high producer of trehazolin, was kindly provided from Sankyo Co., Ltd. Spores of the strain maintained on slants were inoculated into a medium (60 ml), which consisted of glucose 1%, yeast extract 0.5%, polypeptone 0.5%, soluble starch 0.25%, potato starch 1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.1%, KH_2PO_4 0.02%, K_2HPO_4 0.04% and carboxymethylcellulose 0.2%, pH 7.2 for preculture in a 300-ml Erlenmeyer flask. The flask was incubated at 28°C and 210 rpm on a rotary shaker for 150 hours. This culture (2 ml) was transferred into a medium (100 ml), which consisted of yeast extract 0.5%, polypeptone 1%, soluble starch 2%, meat extract 1%, dry yeast 1.8% and carboxymethylcellulose 0.2%, pH 7.2 for the main culture in a 500-ml Erlenmeyer flask. Incubation was carried out for 240 hours under the same conditions as that of the preculture.

Isolation of Trehazolin

Isolation of trehazolin was performed according to Ando's method[1] with slight modifications. The culture broth (5×100 ml) was filtered, and the filtrate was adjusted to pH 5 and applied to a column of Dowex 50W (200 ml, H^+ form). The column was washed with water (600 ml) and then eluted with 0.5 M NH_4OH (1000 ml). The 0.5 M NH_4OH solution was concentrated under reduced pressure, and the concentrated solution was adsorbed onto a charcoal column (150 ml, Charcoal, Activated, Wako Pure Chemical Ind.). After being washed with water (450 ml), the column was eluted with 30% methanol (750 ml). The 30% methanol solution was concentrated and lyophilized, and acetic anhydride (10 ml) and dry pyridine (20 ml) were added to the residue. The reaction solution was stirred overnight. The reaction was stopped by adding water (20 ml) and the reaction mixture was applied to a Sep-pak C_{18} cartridge (35 ml, Waters). After being washed with water (110 ml), the cartridge was eluted with 60% CH_3CN (175 ml). The 60% CH_3CN solution was evaporated to dryness *in vacuo* and the residue was finally purified by HPLC (column: Capcell-Pak C_{18}, 4.6×250 mm, Shiseido; mobile phase: linear gradient elution of 40~80% CH_3CN in 10 mM $AcONH_4$ pH 8.9 in 40 minutes; flow rate; 1 ml/minute) to afford 20 mg of *N*-acetyltrehazolin octaacetate. **1a**: δ_H ($CDCl_3$, 500 MHz) 6.36 (1H, d, J=8.4 Hz, H-4′), 5.93 (1H, d, J=10.0 Hz, H-1′), 5.62 (1H, d, J=4.3 Hz, H-1), 5.43 (1H, dd, J=8.4, 3.6 Hz, H-3′), 5.40 (1H, dd, J=10.0, 10.0 Hz, H-3), 5.09 (1H, dd, J=10.1, 10.1 Hz, H-4), 5.08 (1H, dd, J=10.0, 4.3 Hz, H-2), 4.83 (1H, dd, J=10.0, 3.6 Hz, H-2′), 4.33 (1H, ddd, J=10.1, 4.7, 2.2 Hz, H-5), 4.21 (1H, dd, J=12.2, 4.7 Hz, H-6), 4.21 (1H, d, J=11.5 Hz, H-6′), 4.08 (1H, dd, J=12.2, 2.2 Hz, H-6), 3.95 (1H, d, J=11.5 Hz, H-6′), 2.64 (3H, s, Ac), 2.12 (6H, s, 2Ac), 2.11 (3H, s, Ac), 2.10 (3H, s, Ac), 2.08 (3H, s, Ac), 2.03 (3H, s, Ac), 2.00 (3H, s, Ac), 1.97 (3H, s, Ac).

To prepare **1** from **1a**, 20 mg of **1** was dissolved in ethanolic 0.2 M NaOH solution (2.2 ml) and the reaction solution was stirred for 30 minites at room temperature. After adding water (25 ml), pH of the solution was adjusted to 5, and the solution was applied to a column of Dowex 50W (H^+ form, 30 ml). After being washed with water (60 ml), the column was eluted with 0.5 M NH_4OH. Eight mg of **1** was obtained from the 0.5 M NH_4OH solution after lyophilization.

Acid Hydrolysis of Trehazolin

Acid hydrolysis of trehazolin and preparation of trehazolamine (**4**) and **4a** were performed according to Ando's method.[4] **4**: δ_H (D_2O, 500 MHz) 3.90 (1H, dd, J=7.1, 5.5 Hz, H-2), 3.78 (1H, dd, J=6.8, 5.5 Hz, H-3), 3.62 (1H, d, J=11.9 Hz, H-6a), 3.61 (1H, d, J=6.8 Hz, H-4), 3.56 (1H, d, J=11.9 Hz, H-6b), 3.12 (1H, d, J=7.1 Hz, H-1). **4a**: δ_H ($CDCl_3$, 500 MHz) 5.88 (1H, d, J=7.3 Hz, NH), 5.40 (1H, dd, J=8.1, 4.0 Hz, H-2), 5.29 (1H, d, J=6.4 Hz, H-4), 5.25 (1H, dd, J=6.4, 4.0 Hz, H-3), 4.76 (1H, dd, J=8.1, 7.3 Hz, H-1), 4.28 (1H, d, J=12.0 Hz, H-6a), 4.15 (1H, d, J=12.0 Hz, H-6b), 1.9~2.1 (18H, Ac).

Labeled Compounds

[1-^{13}C]-D-Glucose (99 atm% ^{13}C), [6-^{13}C]-D-glucose (99 atm% ^{13}C), [1-^{13}C]-D-fructose (99 atm% ^{13}C) and [1-^{2}H]-D-glucose (97 atm% ^{2}H) were purchased from Aldrich and [1,2,3,4,5,6,6-$^{2}H_7$]-D-glucose (98 atm% ^{2}H) was obtained from EURISO-TOP. [Guanidino-^{13}C, $^{15}N_2$]-L-arginine was synthesized from ornithine and [^{13}C, $^{15}N_2$]-urea (99 atom% ^{13}C, 99 atom% ^{15}N, ISOTEC INC.) *via* *O*-methyl isoureatosylate as described by MARTINKUS *et al.*[16] δ_C (125 MHz; D_2O) 156.3 (t, $^1J_{NC}$ 21.1 Hz, guanidino-C). [2-^{2}H]-D-Glucose, [4-^{2}H]-D-glucose and [6,6-$^{2}H_2$]-D-glucose were prepared by the procedures of HARDICK *et al.*,[17] YU *et al.*[18] and MOSS *et al.*,[19] respectively.

Administration of Labeled Compounds to *Micromonospora coriacea* SANK 62192

The labeled precursor was dissolved in water, and the solution was passed through a sterile Millipore filter before administration. The solution (1 ml) was added to each 500-ml flask containing the medium (100 ml). In the feeding experiments with [1-^{13}C]-D-glucose, [6-^{13}C]-D-glucose, [1-^{13}C]-D-fructose or [1,2,3,4,5,6,6-$^{2}H_7$]-D-glucose, the solution

of the labeled precursor was added once at the 96th hour of cultivation. In this manner, 9.0, 10.0 and 8.7 mg of **1** and 23.0 mg of **1a** were obtained from broth (5×100 ml) in feeding experiments in which each flask received [1-^{13}C]-D-glucose (100 mg), [6-^{13}C]-D-glucose (100 mg), [1-^{13}C]-D-fructose (100 mg) and [1,2,3,4,5,6,6-$^{2}H_7$]-D-glucose (100 mg). In the feeding experiment with [guanidino-^{13}C, $^{15}N_2$]-L-arginine, the solution of the labeled arginine (70 mg/ml) was added into the culture once at the 96th hour of cultivation and non-labeled glucose (100 mg) was also added twice into the same culture at 72nd and 120th hours of cultivation. In this manner, 25.1 mg of **1a** was obtained from culture broth (5×100 ml). In the feeding experiments with [1-^{2}H]-, [2-^{2}H]-, [4-^{2}H]- or [6,6-$^{2}H_2$]-D-glucose, the solution of the labeled glucose (100 mg/ml) was added into the culture twice at 72nd and 120th hours of cultivation. After cultivation and work-up, 24.1, 24.3, 22.1 and 20.1 mg of **1a** was obtained from broth (5×100 ml) in the experiments with [1-^{2}H]-, [2-^{2}H]-, [4-^{2}H]- and [6,6-$^{2}H_2$]-D-glucose, respectively.

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