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Note

Isolation and NMR studies of di-D-fructose anhydride III from *Lycoris radiata* Herbert by supercritical extraction with carbon dioxide

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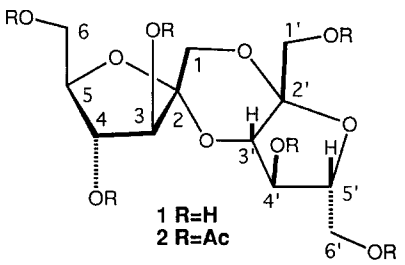
The genus *Lycoris* is widely distributed in Asia. The species of *Lycoris radiata* Herbert, commonly known as *Shi Shuan*, is used in China as a traditional folk medicine [1]. More than ten indole alkaloids have been isolated from this plant, and lycorine, a major pharmacological active constituent, was reported to have various physiological activities such as antiviral and antineoplastic activity, as well as inhibitory activities of ascorbic acid biosynthesis, cell division, and cell elongation [2].

During our extraction study of lycorine from *L. radiata* Herbert for pharmaceutical use, we isolated a disaccharide. Extensive NMR analyses indicated this compound to be di-D-fructofuranose 1,2′:2,3′ dianhydride (DFA III) **1**. Although DFA III **1** has been produced from inulin by pyrolysis [3], by digestion with a homogenate of *L. radiata* H [4], and also by an enzymatic intramolecular transfructosylation reaction [5,6], it has not been isolated directly from natural sources. Since DFA III **1** is a low calorie, non- or anti-tooth-decaying saccharide, it has been attracting a lot of attention as a novel sweetener [6]. For structural investigations of DFA III **1**, Taniguchi and Uchiyama reported X-ray structural analysis [7] and tentatively assigned ^{13}C NMR spectrum [8], which was reassigned by Richards et al. using a 2D NMR method [3]. Our research presented herein deals with isolation of DFA III (**1**) from nature for the first time, full assignment of all ^{1}H and ^{13}C signals, and conformational analysis of DFA III **1** and its hexaacetate **2** by extensive 2D NMR studies.



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The fresh bulbs of *L. radiata* Herbert were finely cut and extracted by supercritical CO_2 with ethanol as an entrainer to give brown residue. After acetylation, separation by medium-pressure liquid chromatography afforded hexaacetate **2**, which was hydrolyzed to give **1** as colorless syrup (see Experimental section). This is a rare example of the isolation of a highly polar disaccharide by supercritical extraction.

Both **1** and its hexaacetate **2** exhibited closely resonating NMR signals between $\delta_{H/C}$ 5.76/105.6 and 3.98/59.2 equivalent to two acetal/hemiacetals, six oxygenated methines, and four oxygenated methylenes, which suggested that **1** was a disaccharide. The molecular formula of **2** was established from the EIMS spectrum (*m/z* 576, M^+) and elemental analysis (calculated for $C_{24}H_{32}O_{16}$: C, 50.0; H, 5.6. Found: C, 50.1; H, 5.7). Therefore, compound **1** would have a molecular formula of $C_{12}H_{20}O_{10}$. Interpretation of the phase-sensitive DQF COSY spectrum [9] and the phase-sensitive TOCSY spectrum [10,11] of **1** in pyridine-d_5 revealed two structural units (C-3–C-6 and C-3′–C-6′) and two isolated methylene groups. The field-gradient HMBC [12] spectrum gave several conspicuous crosspeaks (H-1/C-2, H-3/C-2, H-1′/C-2′, and H-3′/C-2′) to assign TOCSY resonating structural units, quaternary carbons, and isolated methylene groups (Table 1) to each monosaccharide moiety, but the connection between each signal was not unequivocally solved. A modern NMR technique, decoupling HMBC, recently reported by Furihata et al. [13], was found to be suitable in solving this problem, because even a very small coupling interaction can be determined. The cross peaks (C-2/H-3′, C-2′/H-1, C-1′/H-1a, C-4′/H-1) led to the conclusion that the two monosaccharide units were coupled as in **1**.

The relative stereochemistry of **1** was deduced by ^1H–^1H coupling constants and NOESY [14] data (Table 1). The NOESY spectrum at 300 K gave no informative peaks, namely, some real NOESY effects were not determined, but several zero quanta transformed false peaks (TOCSY correlation) were observed (Fig. 1). These false peaks were very difficult to distinguish from real NOESY peaks. We therefore tried to determine the NOESY experiment at various conditions. The NOESY spectrum performed at 240 K in pyridine-d_6 with a short mixing time of 500 ms (t_1 900 ms) gave a clear stereostructure (Fig. 2), after NMR assignments were carried out from COSY, HMQC, and HMBC data. The intense NOESY cross peaks (δ 5.57/4.58, 5.57/4.28, and 5.57/4.17) indicated *syn*-relationships between H-3′ and H-5′ and between H-3′ and H-1′. H-4′ was assigned *trans* to H-3′ due to the large coupling constant (6.9 Hz) and the absence of a NOESY correlation between H-3′ and H-4′. The strong NOE correlation between H-4′ and H-1a revealed that the central six-membered ring exists as a skew-boat conformation in pyridine-d_5, which is in agreement with a conformation in the solid state (crystal) [7]. The central ring of hexaacetate **2** exists as a skew-boat conformation, also

Table 1
NMR data for **1** in pyridine-d_6 at 300 K

Position	^{13}C	^1H	HMBC	Decoupling HMBC
1a	59.6t	4.76 (1 H, d, *J* 12.5 Hz)		H-3
1b		4.55 (1 H, d, *J* 12.5 Hz)		
2	105.6s	–	H-3, H-3′, H-4, H-1a, H-1b	H-3, H-3′, H-4, H-1a, H-1b, H-6b
3	83.4d	4.98 (1 H, d, *J* 4.5 Hz)	H-4, H-1a, H-1b	H-4, H-1a, H-1b
4	77.7d	4.74 (1 H, dd, *J* 4.5, 6.4 Hz)	H-3, H-5, H-6a, H-6b	H-3, H-5, H-6a, H-6b
5	84.1d	4.67 (1 H, ddd, *J* 3.0, 4.3, 6.4 Hz)	H-3, H-6a, H-6b, H-4	H-3, H-6a, H-6b, H-4
6a	62.1t	4.15 (1 H, dd, *J* 3.0, 12.0 Hz)	H-4	H-4
6b		4.07 (1 H, dd, *J* 4.3, 12.0 Hz)		
1a′	65.3t	4.20 (1 H, d, *J* 11.6 Hz)	H-3′	H-3′, H-1a
1b′		4.14 (1 H, d, *J* 11.6 Hz)		
2′	103.1s	–	H-3′, H-1a′, H-1b′, H-1a, H-1b	H-3′, H-1a′, H-1b′, H-1a, H-1b, H-4′
3′	81.3d	5.38 (1 H, d, *J* 6.9 Hz)	H-4′, H-1a′, H-1b′	H-4′, H-1a′, H-1b′, H-5′, H-1a
4′	73.9d	5.67 (1 H, d, *J* 6.9, 8.0 Hz)	H-3′, H-6a′, H-6b′, H-5′	H-3′, H-6a′, H-6b′, H-5′, H-1a, H-1b
5′	82.9d	4.47 (1 H, ddd, *J* 2.8, 4.5, 8.0 Hz)	H-3′, H-6a′, H-6b′	H-3′, H-6a′, H-6b′
6a′	62.3	4.37 (1 H, dd, *J* 2.8, 11.8 Hz)	H-4′	H-4′, H-5′
6b′		4.22 (1 H, dd, *J* 4.5, 11.8 Hz)		

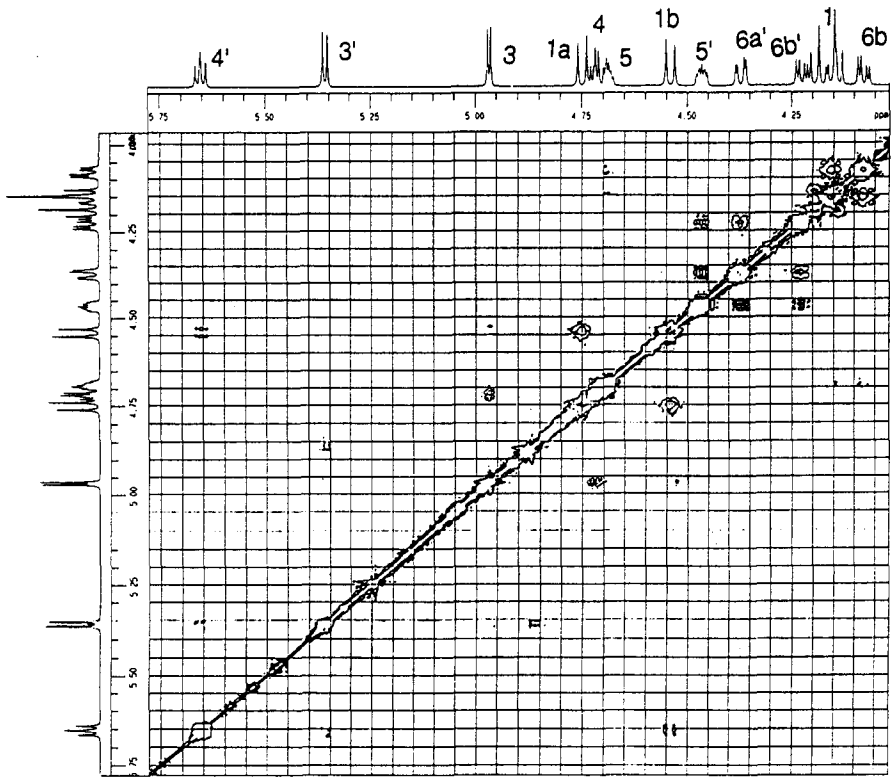


Fig. 1. The NOESY spectrum of **1** measured in pyridine-d_6 with mixing time of 1100 ms at 300 K.

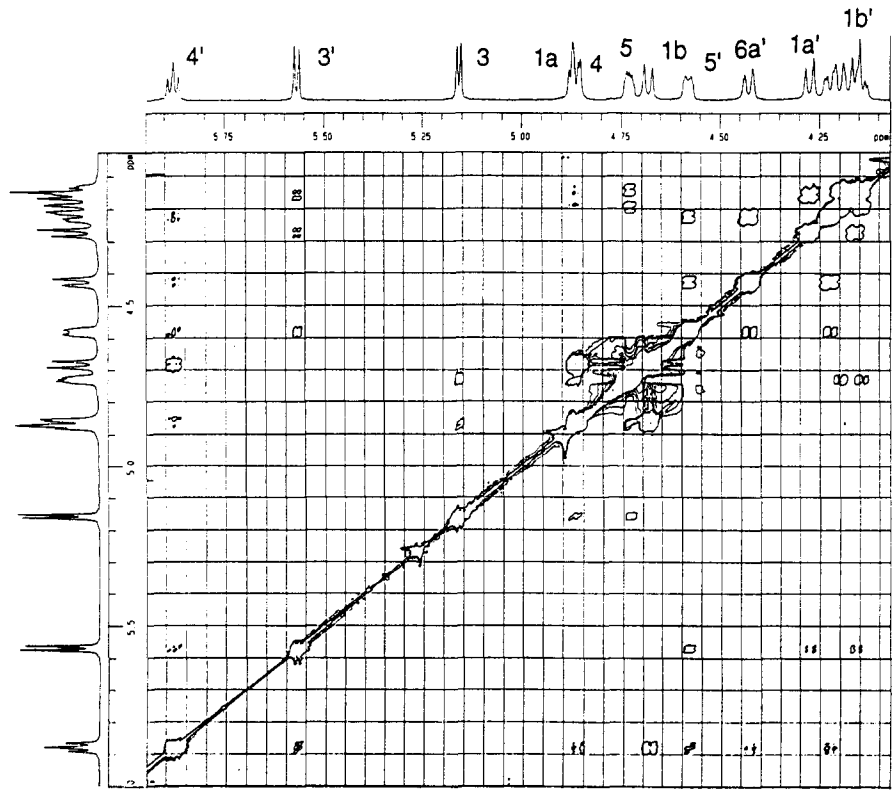


Fig. 2. The NOESY spectrum of **1** measured in pyridine-d_6 with mixing time of 500 ms at 240 K. The inclined cross peaks are zero quanta transformed false NOESY peaks.

Table 2
NMR data for **2** in C_6D_6 [a] at 300 K

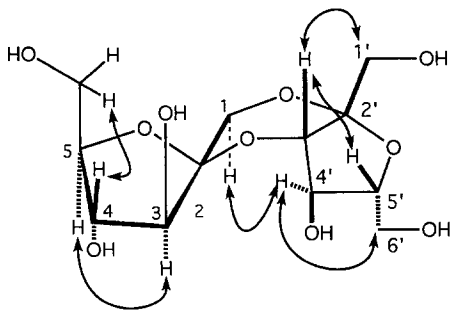
Position	^{13}C	^{1}H	HMBC	NOESY
1a	59.2	4.19 (1 H, d, *J* 12.2 Hz)		H-4′, H-6′
1b		3.98 (1 H, d, *J* 12.2 Hz)		
2	102.1s	–	H-3, H-3′, H-1a, H-1b	
3	78.4d	5.76 (1 H, d, *J* 2.2 Hz)	H-4, H-1a	H-4, H-3′
4	77.8d	5.13 (1 H, dd, *J* 2.1, 4.2 Hz)	H-3, H-5, H-6a	H-3, H-5, H-6b
5	80.4d	4.59 (1 H, ddd, *J* 3.7, 4.2, 6.6 Hz)	H-3, H-6a, H-6b	H-4
6a	63.2t	4.54 (1 H, dd, *J* 3.7, 11.9 Hz)	H-4	H-6b
6b		4.22 (1 H, dd, *J* 6.6, 11.9 Hz)		H-4, H-6a
1a′	64.5t	4.34 (1 H, d, *J* 11.6 Hz)	H-3′	H-1b′
1b′		4.31 (1 H, d, *J* 11.6 Hz)		H-1a′, H-6a
2′	103.3s	–	H-4′, H-3′, H-1a′, H-1b′, H-1a, H-1b, H-5′	
3′	77.2d	4.47 (1 H, d, *J* 3.4 Hz)	H-4′, H-1a′, H-1b′	H-4′, H-3, H-5′
4′	76.8d	5.64 (1 H, d, *J* 3.4, 4.6 Hz)	H-3′, H-6a′, H-6b′, H-5′	H-1a, H-5′, H-3′, H-6′
5′	79.4d	4.03 (1 H, ddd, *J* 4.3, 4.6, 6.1 Hz)	H-3′, H-6a′, H-6b′	H-3′, H-6′, H-4′
6a′	63.6t	4.48 (1 H, dd, *J* 6.1, 12.2 Hz)	H-4′	H-4′, H-5′, H-1a
6b′		4.37 (1 H, dd, *J* 4.3, 12.2 Hz)		

[a] ^{13}C NMR data at δ 170.1 (s), 170.0 (s), 169.9 (s), 169.8 (s), 169.4 (s), 168.9 (s), 20.3 (q), 20.2 (q), 20.1 (q, 2 ×), and 20.0 (q, 2 ×), ^{1}H NMR data at δ 1.81 (3 H, s), 1.80 (3 H, s), 1.75 (3 H, s), 1.67 (3 H, s), 1.64 (3 H, s), and 1.62 (3 H, s) were assigned for six acetyl groups.

(NOE data in Table 2). The stereochemistry of the other monosaccharide unit was established on the basis of the NOESY cross peaks of H-3/H-5 and H-4/H-6 (Scheme 1).

The 2D NMR coupling networks of **2** carried out in benzene-d_6 were essentially the same as those observed for **1**. The NMR assignments are shown in Table 2. The equivalence of the optical rotations for **2** (+81.4°) and authentic D-DFA III hexaacetate (+81.2°) [5] indicate that **2** is indeed D-DFA III hexaacetate. Isolation of D-DFA III from *L. radiata* Herbert could provide a new source to investigate this new type of sweetener further.

In summary, we have demonstrated the first isolation of DFA III from nature by supercritical CO_2 extraction, the first conformational analysis in solution, and full assignment of ^{1}H and ^{13}C NMR signals by modern 2D NMR techniques, including decoupling HMBC.



Scheme 1. Selected NOE correlation for **1**.

1. Experimental

Plant material.—Plant samples were collected at Kumamoto in April, 1991. The material was identified as *Lycoris radiata* Herbert. A reference specimen was deposited at Niigata University.

General experimental procedure.—The electron–ionization mass spectrum was run on a Jeol JMS DX-300 mass spectrometer at 70 eV. Specific rotations ($[\alpha]_D$) were determined on a JASCO DIP-1000 polarimeter. Medium-pressure liquid chromatography (MPLC) was carried out on a JASCO PRC-50 instrument with a silica gel packed column. Elemental analysis was carried out in the microanalytical laboratory of this institute. Thin-layer chromatography (TLC) was conducted on Silica Gel F_{254} (E. Merck), and the spots were detected by spraying the plates with Mn_2O_3–H_3PO_4–EtOH solution, followed by heating. Ion-exchange column chromatograpy was performed on Muromachi Dowex 50W-X8 (200–400 mesh) resin.

Extraction and isolation.—Fresh bulbs of *L. radiata* Herbert (300 g) were finely cut by a food processor and mixed well with ethanol (10 mL). The resulting sticky paste was placed in an extraction cell of the SCF apparatus. The apparatus for SCF extraction

was originally designed and assembled for the present purpose. Details of the experimental apparatus and procedure are described in Ref. [15]. Silica gel for flash chromatography (E. Merck, 10 g) was placed in a cylindrical filter paper in the absorbing cell. After introduction of carbon dioxide, the extraction cell was heated at 358 K and pressurized at 30 MPa by a high-pressure pump. Supercritical carbon dioxide was circulated for 8 h by a magnetic pump through the extraction and absorbing cells. After cooling to room temperature, the carbon dioxide was released, and the silica gel was removed with the cylindrical filter paper from the absorbing cell. Elution of the silica gel with ethanol (500 mL), followed by evaporation of the solvent, left a brown solid that was acetylated with pyridine (15 mL) and acetic anhydride (15 mL) at room temperature for 48 h. Excess acetic anhydride and pyridine were evaporated in vacuo, and the residue was dissolved in dichloromethane. The organic layer was washed with aq sodium hydrogencarbonate (2 ×), water, and brine. The aqueous layer was extracted with dichloromethane (2 ×). After evaporation of the solvent, the residue was passed through a short silica gel column with the aid of hexane–ethyl acetate. Evaporation of solvents left an oil (2.546 g) which was chromatographed on a medium-pressure liquid chromatography (silica gel column, 1:2 hexane–ethyl acetate as eluent) to give 660 mg of the acetate **2**.

The acetate **2** (120 mg) was treated with EtONa in MeOH at room temperature overnight. The reaction mixture was filtered through ion exchange resin, and washed with MeOH to give pure **1** (65 mg, quant.) as a colorless syrup.

Data for **2**: $[\alpha]_D$ +81.6° (*c* 1.661, $CHCl_3$); *m/z* 576 (M^+, 0.4%), 503 ($M^+ - CH_3CO_2CH_2$, 20%), 443 (*m/z* 503 − CH_3CO_2H, 4%), 347 (4%), 329 (4%), 323 (*m/z* 443 − $2CH_3CO_2H$, 5%), 227 (13%), 170 (37%), 169 (22%), 128 (22%), 110 (35%), and 43 (CH_3CO, 100%); Anal. Calcd for $C_{24}H_{32}O_{16}$: C, 50.00; H, 5.59. Found: C, 50.10; H, 5.74.

NMR experimental procedure.—NMR measurements were carried out on a Jeol JNM-LA 600 spectrometer. The HMQC, HMBC, and decoupling HMBC spectra were recorded in the absolute mode, while DQF–COSY, TOCSY, and NOESY were recorded in the pure phase-absorption mode. Residual (pyridine-d_5, δ 7.24/123.5 and benzene-d_6, 7.19/128.0) signals were used as internal standards. For the spectra measured in pyridine-d_6, the water resonance was suppressed by presaturation during the relaxation delay. Typically, a total of 512 increment of 2 K point was collected for each 2D NMR experiment. For decoupling HMBC experiment, a standard HMQC pulse sequence and wide decoupling mode MPF10 were used. The spectra were recorded at the spin-lock interval (Δ_2) of 120 ms (4 Hz) and 240 ms (2 Hz). NOESY spectra were acquired with mixing time of 500 and 1100 ms, while TOCSY spectra were acquired with a mixing time of 500 ms. The gradient strength value is G1:G2:G3 = 2:2:1 G cm^{-1}. A Nalorac inverse probe (5 mm) was used for all NMR experiments except for ^{13}C NMR measurement.

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