Nupharanin, the first ellagitannin with 1,4-dehydrohexahydroxydiphenoyl-α-D-glucopyranose from *Nuphar japonicum*

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Nupharanin (**1**), the first ellagitannin with dehydrohexahydroxydiphenoyl esters at the 1,4-positions of α-D-glucose, was isolated from the fresh rhizome of *Nuphar japonicum*. Based on spectroscopic evidence, the structure was determined to be 1,4-(*S*)-dehydrohexahydroxydiphenoyl-2-galloyl-3,6-(*R*)-hexahydroxydiphenoyl-α-D-glucopyranose. The structure was supported by spectroscopic studies of acetonyl derivatives of **1** and its partial hydrolysate. In addition, unusual oxidative cleavage of the hydrated cyclohexenetrione ring of **1** was observed on moderate heating at 50˚C in pH 6 buffer.

Keywords: *Nuphar japonicum*; Nymphaceae; nupharanin; dehydrohexahydroxydiphenoyl; ellagitannin

1. Introduction

Dehydroellagitannins, a subgroup of hydrolyzable tannins with dehydrohexahydroxydiphenoyl (DHHDP) esters, are widely distributed in the plant kingdom.[1] Geraniin, 1-galloyl-2,4-(*R*)-DHHDP-3,6-(*R*)-hexahydroxydiphenoyl (HHDP)-β-D-glucopyranose is the most investigated dehydroellagitannin, displaying an array of biological activities.[2,3] The characteristic hydrated cyclohexenetrione structure of the DHHDP is presumed to be biogenetically related to other ellagitannin acyl groups.[4-8] The DHHDP esters in the reported dehydroellagitannins are located at the 1,3-,[9-11], 2,4-,[2,8] 3,6-,[12] and 4,6-positions of *β*-D-glucopyranose.[13] The plant of interest in this study, *Nuphar japonicum* DC (Nymphaceae), was previously shown to contain hydrolyzable tannins bearing only the α-D-glucopyranose core.[14-17] Moreover, both (*S*)- and (*R*)-HHDP esters were found to co-exist.[16,17] These characteristic features of this plant prompted us to reinvestigate ellagitannins. In the previous study, dried rhizome was used as it is an important traditional medicine in Eastern Asia.[15] In this study, fresh rhizome was used to avoid possible degradation during the drying process. Preliminary HPLC analysis of a 60% ethanol extract of the fresh rhizome showed peaks arising from known tannins identified in previous studies,[16,17] together with a characteristic broad peak (Fig. S1A, Supplementary data). The broad peak disappeared on treatment of the extract with 1,2-phenylenediamine, and a sharp peak with a characteristic UV absorption at 375 nm appeared (Fig. S1B).[2] The reaction indicated that the broad peak is attributable to a dehydroellagitannin. Herein, we elucidate the structure of the dehydroellagitannin (**1**).

2. Results and Discussion

2.1. Isolation and structure determination

Fresh rhizome was extracted with a mixture of acetone-H_2O (8:2) and the extract was fractionated using a Diaion HP20SS. The fraction containing the target compound was further subjected to Sephadex LH-20 and Chromatorex ODS column chromatography to yield **1** (0.016% from fresh rhizome). Separately, fresh rhizome was also extracted with aqueous acetone containing HCO_2NH_4, because the ammonium ion catalyzes addition of acetone molecule to the DHHDP esters to give stable derivatives of DHHDP esters without equilibrium between two hemiacetal structures.[8] Subsequent chromatographic separation afforded acetone adducts **1c** (0.004%) and **2** (0.003%), along with 1,2,3,4,6-penta-*O*-galloyl-α-D-glucose (0.020%), 1,2,4-tri-*O*-galloyl-α-D-glucose (0.013%), and nupharins A (0.027%) and C (0.006%) (Fig. S2).

Tannin **1** showed a $[M+Na]^+$ peak at *m/z* 975.0707 in the HRFABMS (calcd. for $C_{41}H_{28}O_{27}Na$, 975.0710), and the molecular formula suggested that **1** is a dehydroellagitannin composed of HHDP, DHHDP, galloyl groups and a fully acylated hexose core. The ^{1}H NMR spectrum of **1** (Table 1) displayed duplicate signals in a ratio of 2:1 arising from two tautomers. With the aid of HSQC and HMBC correlations, two singlet signals observed at δ_H 7.28 and 7.25 were assigned to H-2,6 of the galloyl group, while singlet signals at δ_H 6.73, 6.68, 6.97, and 6.68 were ascribed to the HHDP aromatic protons. As for the DHHDP group, mutually coupled doublets at δ_H 5.21 and 6.47 (J = 1.3 Hz) were attributed to the benzyl methine (H-1') and vinyl protons (H-3'), respectively, of the minor tautomer with a 5-membered hemiacetal ring (**1b**). Integration of the aromatic singlet at δ_H 7.32 indicated that this signal can be assigned to the H-3 of the DHHDP aromatic ring of **1b**.[2] The major tautomer with a 6-membered hemiacetal ring structure (**1a**) exhibited benzylic, vinyl and aromatic signals at δ_H 5.70, 5.97, and 7.42, respectively, as singlets. This was supported by the HSQC ^1J correlations with corresponding carbon signals observed at δ_C 46.0, 130.0, and 114.9. The methine proton at δ_H 5.70 of **1a** showed ^2J and ^3J correlations with hemiacetal carbons corresponding to C-6' (δ_C 92.7) and C-5' (δ_C 96.6) (Fig. 1), while the methine proton at δ_H 5.21 of **1b** was correlated to C-6' (δ_C 109.8) and C-5' (δ_C 92.4). The chemical shifts of acetal carbons were analogous to those observed for the tautomers of geraniin.[2]

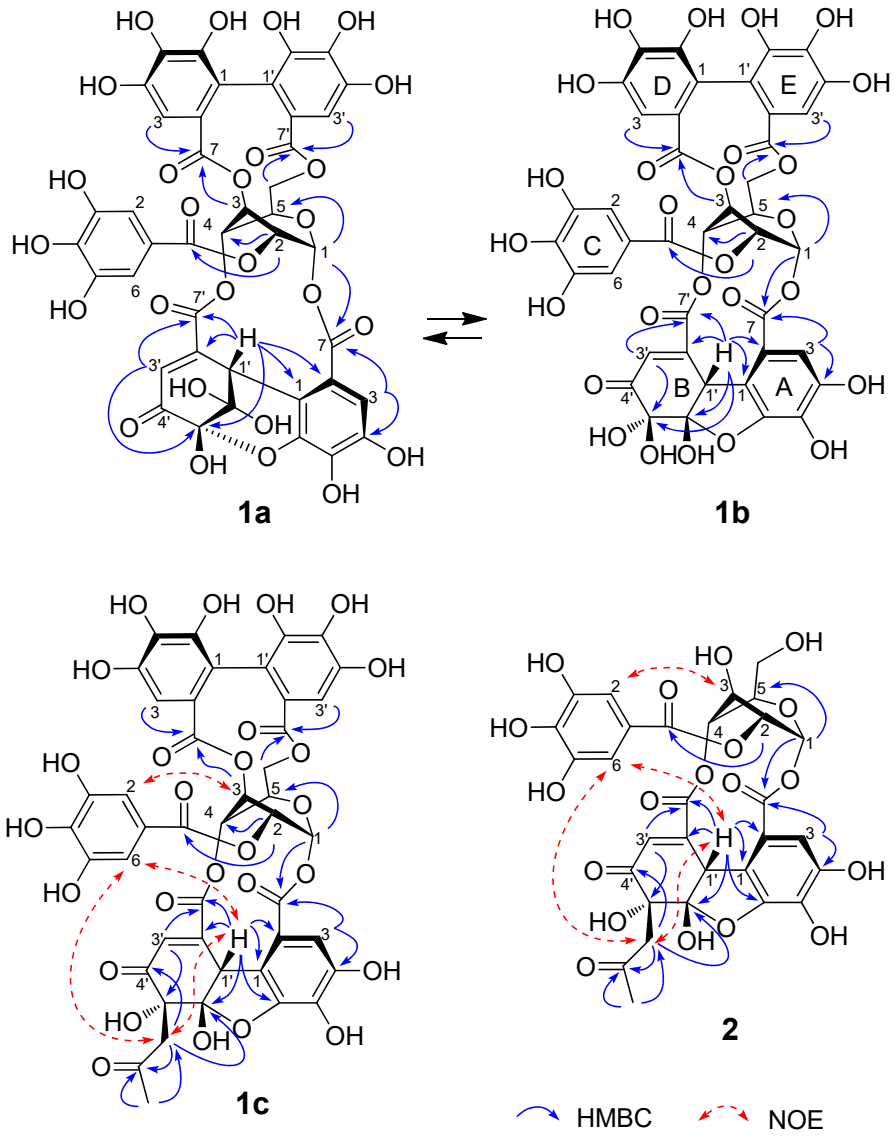


Fig. 1. Structures of tautomers **1a** and **1b**, acetone adduct **1c**, and **2** with selected HMBC and NOE correlations.

Table 1. ^{1}H (500 MHz) and ^{13}C (126 MHz) NMR data of **1a-c**, **2**, and **4** in acetone-*d* 6.

position		**1a**			**1b**			**1c**			**2**			**4**		
		δ_H	(*J* in Hz)	δ_C	δ_H	(*J* in Hz)	δ_C	δ_H	(*J* in Hz)	δ_C	δ_H	(*J* in Hz)	δ_C	δ_H	(*J* in Hz)	δ_C
Glc	1	6.95	d (7.0)	88.3	6.68	d (5.5)	89.0	6.62	d (6.4)	89.2	6.76	d (6.4)	90.4	6.99	d (6.8)	88.3
	2	5.74	d (6.5)	69.9	5.70	d (9.0)	69.6	5.46	d (6.6)	71.1	5.05	d (6.4)	74.0	5.78	d (6.8)	69.8
	3	5.16	brs	71.2	5.16	brs	70.9	5.27	d (3.9)	70.6	4.38	d (3.2)	69.2	5.12	d (3.4)	71.0
	4	5.58	d (3.5)	67.7	5.45	d (3.5)	68.1	5.43	d (3.9)	68.0	5.03	d (3.4)	74.4	5.75	d (3.7)	66.9
	5	4.85	t (7.5)	70.0	4.82	dd (4.5,8.5)	69.2	4.78	dd (4.9, 8.3)	69.4	4.23	t (4.0)	73.2	4.61	t (7.5)	71.8
	6	4.49	dd (11.8, 8.3)	64.8	4.59	dd (11.8, 8.3)	64.8	4.57	dd (8.3, 12.0)	64.8	3.86	t (3.8)	62.8	4.48	dd (8.3, 11.7)	65.0
		4.23	dd (6.5, 12.0)		4.20	dd (4.8, 12.3)		4.18	dd (4.9, 12.0)					4.21	dd (6.5, 11.6)	
A	1			115.1			117.9			117.3			117.1			119.5
	2			119.3			120.5			120.8			120.6			131.2
	3	7.42	s	114.9	7.32	s	113.7	7.31	s	113.6	7.30	s	113.5	7.26	s	115.4
	4			145.9			147.6			147.4			147.5			146.0
	5			137.7			139.5			137.5			137.5			140.4
	6			152.2			148.4			147.5			147.3			145.06[b]
	COO			165.3			163.6			163.5			163.7			165.1
B	1'	5.70	s	46.0	5.21	d (1.5)	51.6	4.84	d (1.5)	51.3	4.76	s	51.2			167.9
	2'			143.6			147.4			144.7			145.0			98.4
	3'	6.97	s	130.0	6.47	d (1.0)	127.5	6.46	d (1.5)	129.4	6.37	s	128.8	6.84	s	139.6
	C=O			190.4			193.8			197.3			197.4			135.7
	5'			96.6			92.4			80.6			80.7	6.47	s	43.5
	6'			92.7			109.8			110.7			110.6			175.9
	COO			164.9			165.8			165.7			165.9			163.8
C	1			120.2			120.1			120.3			120.4			120.0
	2,6	7.28		110.6	7.25		110.6	7.23	s	110.4	7.18	s	110.2	7.04	s	110.0
	3,5			145.9			145.9			146.1			146.0			146.0
	4			139.4			139.4			139.4			139.2			139.4
	COO			165.8			165.6			166.0			166.3			165.5
D	1			116.3			116.2			116.3						116.3
	2			124.6[a]			124.5[a]			124.6[a]						125.7[a]
	3	6.73		109.8	6.97		109.8	6.97	s	109.9				6.95	s	109.7
	4			145.1			145.1			145.1						145.4
	5			137.3			137.3			137.3						137.2
	6			145.0			145.0			145.01[b]						145.00[b]
	COO			166.7			166.5			166.6						166.6
E	1'			114.9			115.1			114.9						115.1
	2'			125.7[a]			125.6[a]			125.6[a]						124.7[a]
	3'	6.68		108.1	6.68		108.3	6.67	s	108.3				6.70	s	108.2
	4'			145.3			145.3			145.3						146.5
	5'			136.4			136.3			136.3						136.4
	6'			145.3			145.3			145.03[b]						145.09[b]
	COO			167.7			167.9			167.8						167.8
Acetonyl	CH_2							2.87	d (15.2)	49.1	2.75	d (14.9)	49.2			
								3.51	d (15.2)		3.57	d (14.7)				
	CH_3							2.16	s	32.2	2.22	s	32.5			
	C=O									206.0			207.1			

[a,b] interchangeable assignment

^{1}H NMR signals of **1c** (Table 1) were closely related to those of **1b**, except for the appearance of singlet methyl (δ_H 2.16) and geminal coupled methylene signals (δ_H 2.87, 3.51, each d, J = 15.2 Hz), indicating that **1c** is an acetone adduct of **1**.[8] Acid hydrolysis of **1c** and subsequent HPLC comparison of the thiazolidine derivative of the sugar with those of standard sugars confirmed that the hexose moiety of **1** was D-glucose (Fig. S3).[18,19] The glucose proton and carbon signals of **1c** were assigned by ^{1}H-^{1}H COSY and HSQC and HMBC correlations (Fig. 1), and those of **1a** and **1b** were also confirmed using similar techniques. The HMBC correlations of the anomeric proton (**1a**: δ_H 6.95, **1b**: δ_H 6.68, **1c**: δ_H 6.62) to glucose C-5 (**1a**: δ_C 70.0, **1b**: δ_C 69.2, **1c**: δ_C 69.4) confirmed the pyranose form of the glucose. The anomeric carbons (**1a**: δ_C 88.3, **1b**: δ_C 89.0, **1c**: δ_C 89.2) resonated at a higher field compared with those of ellagitannins with a β-configuration,[20] and the chemical shifts were similar to those of nupharins A (δ_C 89.5).[16] This suggests that the configuration of the anomeric center is α, which is the same as other hydrolysable tannins of this plant.[14-17]

The location of the esters on the glucopyranose was determined by HMBC correlations of **1** (Fig. 1): the galloyl H-2, 6 (δ_H 7.28 and 7.25) and glucose H-2 (δ_H 5.74, 5.70) protons were correlated to the same ester carbonyl carbons (δ_C 165.8, 165. 6), while glucose H-3 (δ_H 5.16) and H-6 methylene protons (δ_H 4.49, 4.23, 4.59, 4.20) were correlated to the HHDP C-7 and 7' carboxyl carbons (δ_C 166.7, 167.7, 166.5, 167.9). These results implied that the DHHDP group is attached to the 1, 4 positions of glucose. In fact, the glucose H-1 proton (δ_H 6.95, 6.68) showed HMBC cross peaks with the DHHDP C-7 carboxyl carbons (δ_C 165.3 and 163.6) that correlated to the aromatic H-3 (δ_H 6.97 and 6.47). Unfortunately, correlations from glucose H-4 to DHHDP ester carbonyls of **1a**, **1b** and **1c** were not observed. However, these results strongly suggest that the DHHDP is attached to the 1,4 positions of glucose as shown in Fig. 1. Compound **2** was

isolated together with **1c** from the aqueous acetone extract containing HCO_2NH_4, and spectroscopic data indicated that **2** is a partial hydrolysate of **1c** lacking the HHDP moiety. The coupling pattern of glucopyranose and HMBC correlations of **2** were similar to those of **1c** except for large up field shifts of glucose H-3 and H-6. This also supported the 1,4-DHHDP structure of **1**.

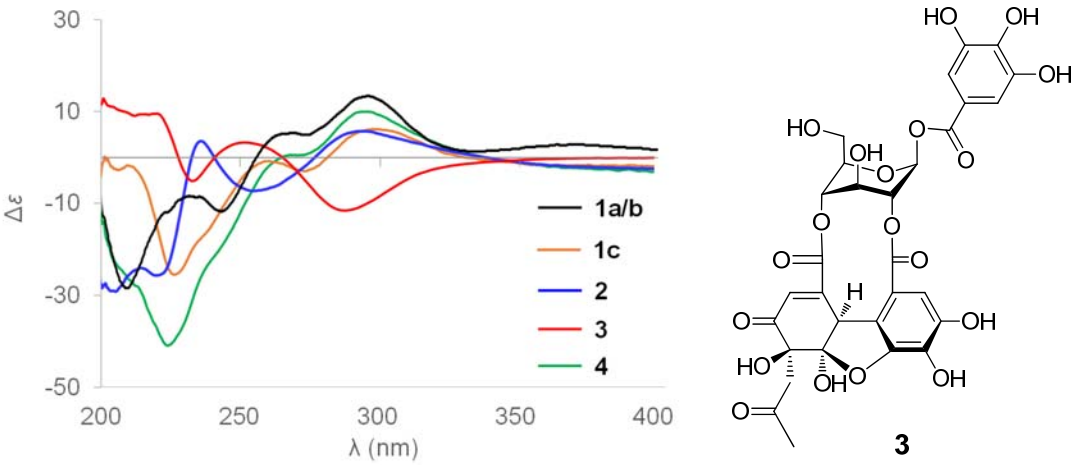
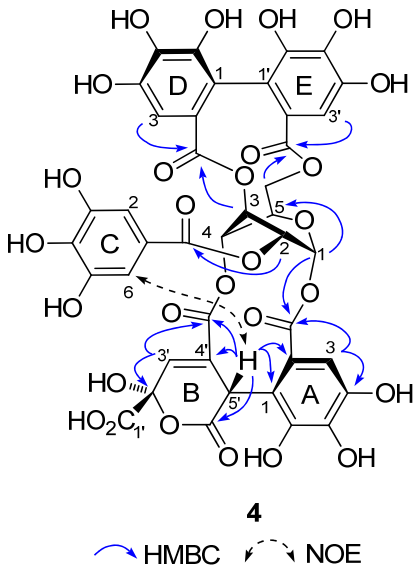


Fig. 2. Experimental CD spectra of **1a/b**, **1c**, **2**, **3**, and oxidation product **4**.

Atropisomerism of the HHDP moiety of **1** is assigned to have *R* configuration, based on the negative Cotton effect at 220-240 nm in the CD spectrum of **1** and at 225 nm in that of **1c** (Fig. 2).[21] The weak positive Cotton effect around 360 nm of **1** suggested that the DHHDP group is *S* configuration.[21] This was supported by NOESY correlations observed for **1c** and **2**: galloyl protons at the D-glucose C-2 showed NOE correlations with the acetonyl group and the DHHDP benzylic methane (H-1'), indicating that these are located at the same side of the molecule (Fig. 1). Furthermore, the configuration was confirmed by comparison of the CD spectra of **2** and

acetone adduct of furosin (**3**), 1-galloyl-2,4-(*R*)-acetonyl-DHHDP-β-D-glucopyranose[8] (Fig. 2). These compounds showed opposite Cotton effects around 220-350 nm,[21] confirming the *S*-configuration of the acetonyl DHHDP group of **2**. Thus, the novel ellagitannin was characterized to be 1,4-(*S*)-DHHDP-2-galloyl-3,6-(*R*)-HHDP-α-D-glucopyranose and was named nupharanin.



4

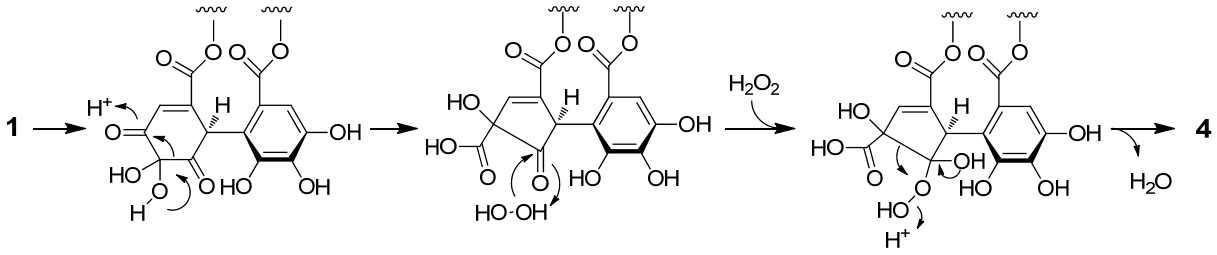
Fig. 3. Structure of **4** with key HMBC and NOE correlations**.**

2.2. Reaction of nupharanin

Nupharanin (**1**) was somewhat unstable under neutral conditions, and heating at 50°C in pH 6 McIlvaine buffer for 4.5 h afforded degradation product **4**. The ^{1}H and ^{13}C NMR spectra (Table 1) were related to those of **1a**; however, the signals of the hydrated cyclohexenetrione moiety were replaced by two carboxyl groups (δ_C 167.9, 175.9), a hemiacetal carbon (δ_C 98.4), a trisubstituted double bond (δ_C 139.6, 135.7, δ_H 6.84) and a aliphatic methine carbon (δ_C 43.5, δ_H 6.47). In the HMBC spectrum (Fig. 3), the aliphatic methine proton (δ_H 6.47) showed cross peaks

with aromatic carbons (A-1, 2), an olefinic carbon (B-4'), and conjugated (δ_C 167.9) and non-conjugated (δ_C 175.9) carboxyl carbons, indicating that this aliphatic proton signal is attributable to the benzylic methine. The vinyl proton (δ_H 6.84) was assigned to the B-ring H-3' and showed HMBC correlations with the conjugated carboxyl carbon (δ_C 167.9) and B-2' hemiacetal carbon (δ_C 98.4), suggesting the planar structure of the B-ring as shown in Fig. 3. This was in agreement with the molecular formula $C_{41}H_{28}O_{28}$ confirmed by the HRFABMS (*m/z* 969.0846 $[M+H]^+$; calcd for $C_{41}H_{29}O_{28}$, 969.0840). The molecular formula also suggested formation of a lactone ring between the B-6' carboxyl group and the B-2' acetal hydroxy group. The strong NOE correlation between the methine proton (B-5') and galloyl protons at δ_H 7.04 (2H) indicated that B-5' retained the configuration of **1** during the degradation reaction. The unusual low-field shift of the B-5' aliphatic proton at δ_H 6.47 was accounted for by strong deshielding from the galloyl group. The absolute configuration at B-2' could not be determined spectroscopic methods, hence, the experimental ^1H, ^{13}C NMR, and ^1H-^1H coupling constant data were compared to those generated through DFT calculation using GIAO method at mPW1PW91-SCRF/6 311+G(2d,p)//B3LYP-SCRF/6-31G (d,p) level in acetone (PCM) (Fig. S35). However, the coefficients of determination value (R^2) for B2'-*S* (δ_H, R^2 = 0.9824; δ_C, R^2 = 0.9938; $J_{H,H}$, R^2 = 0.9846) and B2'-*R* (δ_H, R^2 = 0.9753; δ_C, R^2 = 0.9934; $J_{H,H}$, R^2 = 0.9856) were too close to make reliable conclusion. Therefore, method based on DP4+ probability was employed.[22] Comparison of the scaled (*s*DP4+), unscaled (*u*DP4+) and summative (DP4+) (Table S1) revealed that the structure B2'-*R* was more consistent with that of, in terms of ^{13}C and combined $^1H + ^{13}C$ shift, both for the unscaled (88.52%, 76.90%) and summative (94.85%, 72.32%) DP4+ probability. The results suggested that *R* configuration at B-2' is more probable. The involvement of H_2O_2 in the mechanism was deduced from previous studies showing autooxidation of polyphenols

generates H_2O_2 by reduction of O_2 molecule (Scheme 1).[23] This type of degradation of DHHDP groups has not been observed previously.[5]



Scheme 1. Possible mechanism for the formation of **4** from **1**.

2.3. Confirmation of glucose core

The coupling constants of the pyranose ring protons of **1a**, **1b**, **1c**, **2**, and **4** were essentially the same (Table 3), and the value suggested that the glucose adopts a skewed boat conformation,[24] particularly with ($^3S_1$) conformation.[25] This was embodied by the most stable conformer obtained by DFT calculation with dihedral angles between the pyranose ring protons (Fig. 4, Table S8). This strained conformation is presumed to be a consequence of the more rigid DHHDP moiety linked at the α-1,4 position taking precedence over the 3,6-linked HHDP. This is further substantiated by the observed similarity in coupling pattern of vicinal glucose protons of **2**, despite the lack of 3,6-HHDP esters. In contrast, geraniin [1β-galloyl-2,4-(*R*)-DHHDP-3,6-(*R*)-HHDP][26] and granatin B [1β-galloyl-2,4-(*S*)-DHHDP-3,6-(*R*)-HHDP][27] adopt a $^1C_4$ conformation.[28,29]

Table 3. Coupling constants, $J_{H\text{-}H}$ (Hz) of glucose protons of **1a**, **1b**, **1c**, **2**, **4**, geraniin[26] and granatin B.[27]

	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{6,6}$
1a	6.8	< 1	3.5	< 1	7.5, 7.5	11.9
1b	5.5	< 1	4.0	< 1	4.4, 8.4	12.1
1c	6.5	< 1	3.9	< 1	4.9, 8.3	12.0
2	6.8	< 1	3.6	< 1	7.5, 7.5	11.7
4	6.9	< 1	3.6	< 1	7.5, 7.5	11.7
geraniin	1.2	2.4	3.8	1.6	8.2, 10.4	10.9
granatin B	1.5	2.6	2.7	1.9	8.8, 10.6	-

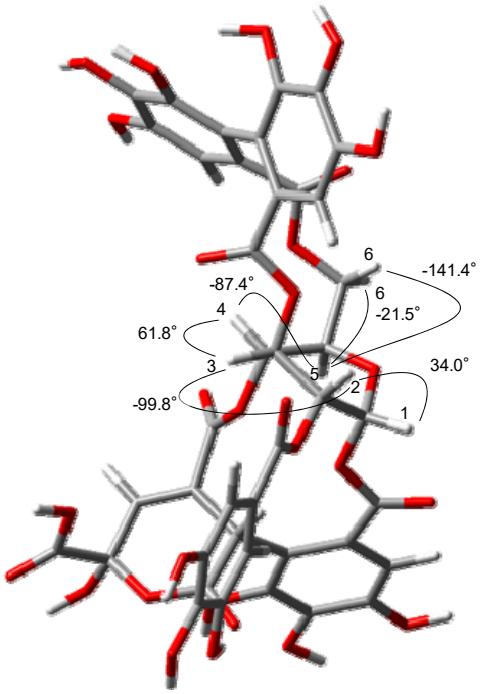


Fig. 4. The most stable conformer of **4** obtained by DFT calculation.

3. Conclusion

In summary, nupharanin (**1**) is the first dehydroellagitannin with an α-D-glucopyranose core, and is also the first ellagitannin with the DHHDP ester at the 1,4 positions of glucose. Structure elucidation of **1** and its semisynthetic derivatives (**1c**, **2**, and **4**) broadened our understanding of

how the substituents affect the conformation of the glucose core, as well as the reactivity of the DHHDP group. More significantly, the discovery of **1** will serve as a springboard for further exploration of the biosynthesis of ellagitannins containing an unconventional sugar moiety.

4. Experimental

4.1. General experimental procedures.

NMR spectra were recorded in acetone-d_6 (Wako Pure Chem. Ind. Ltd., Osaka, Japan), with a Varian Unity Plus 500 spectrometer (Palo Alto, CA, USA) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, and with a JEOL JNM-AL 400 spectrometer (JEOL Ltd, Tokyo, Japan) at 400 MHz for ^{1}H and 100 MHz for ^{13}C. HRFABMS spectra were recorded on a JMS 700N spectrometer (JEOL Ltd., Tokyo, Japan) in positive ion mode, with glycerol or *m*-nitrobenzyl alcohol, with or without NaCl, as the matrix. UV spectra were recorded in MeOH with a Jasco V-560 UV/Vis spectrometer (Jasco Co. Ltd., Tokyo, Japan). The same solvent was used for the CD spectroscopic analysis using a Jasco-725N spectrometer (Jasco Co. Ltd., Tokyo, Japan), and optical rotation measurement using a Jasco P-1020 polarimeter (Jasco Co. Ltd., Tokyo, Japan). IR spectra were recorded using a Jasco FT/IR-410K IR spectrometer (Jasco Co. Ltd., Tokyo, Japan). Column chromatography was performed using Sephadex LH-20 (25–100 mm, GE Healthcare UK Ltd., Buckinghamshire HP7 9NA, England), Diaion HP20SS (Mitsubishi Chemical Co., Tokyo, Japan), and Chromatorex ODS (Fuji Silysia Chemical Ltd., Kasugai, Japan). TLC was performed both on 0.25-mm thick, pre-coated silica gel 60 F_{254} (Merck, Darmstadt, Germany) and on 0.1-mm thick, pre-coated cellulose F (Merck, Darmstadt, Germany), with toluene–ethyl formate–formic acid (1:7:1, v/v) and 2% aqueous acetic acid, respectively, as solvent systems. Spots were detected by illumination under short wavelength UV

(254 nm) followed by spraying with 2% ethanolic $FeCl_3$. Analytical HPLC of the crude ethanolic extract of the leaves and fruits were of performed with gradient elution from 4–30% (39 min), 30–75% (15 min), 75–95% (6 min) acetonitrile (Kanto Chemical Co., Inc., Tokyo, Japan) in 50 mM phosphoric acid (Kishida Chemical Co., Osaka, Japan) on a Cosmosil 5C_{18}-ARII 4.6 × 250 mm column (Nacalai Tesque, Inc., Kyoto, Japan) at a flow rate of 0.8 mL/min, using an HPLC system composed of a Jasco DG-2080-53 Plus degasser, Jasco PU-2080 Plus pump, Jasco AS-2055 Plus autosampler, Jasco CO-2065 Plus column oven (maintained at 35°C), and Jasco MD-2018 Plus PDA detector (Jasco Co. Ltd., Tokyo, Japan). HPLC during fraction monitoring was performed with gradient elution from 4–27.3% (35 min), 27.3-90% (10 min), acetonitrile (Kanto Chemical Co., Inc., Tokyo, Japan) in 50 mM phosphoric acid (Kishida Chemical Co., Osaka, Japan) on a Cosmosil 5C_{18}-ARII 3 × 150 mm column (Nacalai Tesque, Inc., Kyoto, Japan) at a flow rate of 0.4 mL/min. The HPLC system is composed of a Jasco PU-4180 RHPLC pump, Jasco AS-4050 autosampler, Jasco CO-4061 column oven (maintained at 40°C), and Jasco MD-4017 PDA detector (Jasco Co. Ltd., Tokyo, Japan).

4.2. Plant material.

Fresh rhizome of *N. japonicum* was collected from a biotope of the Faculty of Education, Nagasaki University on November 11, 2018. A voucher specimen (Nj20181111) was deposited at the Nagasaki University Graduate School of Biomedical Sciences.

4.3. Extraction and isolation.

Fresh rhizome (1.0 kg) was crushed with 80% acetone (2 L) in Waring blender (4 L) and extracted overnight at r.t. After filtration, the plant debris was extracted again with 80% acetone in the same manner. The filtrate was combined and concentrated by rotary evaporator at 40°C

until acetone is removed. The resulting aqueous solution was applied to Diaion HP20SS column (5 cm i.d. × 30 cm) with gradient elution of 0–100% MeOH (10% stepwise, each 300 mL) to give Fr. 1 containing 1,2,4-tri-*O*-galloyl-α-D-glucose and nupharins A and C (5.45 g) and Fr. 2 containing **1** (1.79 g). Fr 2 was further separated by Sephadex LH-20 column chromatography (3 cm i.d. × 25 cm) with 80% MeOH (300 mL), 90% MeOH (200 mL) and then MeOH-acetone-H_2O (8:1:1, v/v, 300 mL), to yield crude crop of **1** (522 mg), a portion (400 mg) of which was further purified by Chromatorex ODS (3 cm i.d. × 30 cm) with 0 – 40% CH_3CN containing 0.05% trifluoroacetic acid (5% stepwise, each 100 mL) to give **1** (156 mg) as a lyophilized amorphous powder.

4.3.1. Nupharanin (**1a/b**).

Yellow amorphous powder; $[\alpha]_D^{19}$ +151.1 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 277 (4.57), 221 (4.96) nm; IR(film) ν_{max} 3410, 1713, 1615 cm^{-1}; ^1H NMR (acetone-d_6, 500 MHz) and ^{13}C NMR (acetone-d_6, 126 MHz) see Table 1; HRFABMS *m/z* 975.0707 $[M+Na]^+$ (calcd. for $C_{41}H_{28}O_{27}Na$, 975.0716).

4.4. Preparation of acetonyl condensate.

Fresh rhizome (2.0 kg) was homogenized with 80% acetone (5.0 L) containing ammonium formate (50 g) for 2 weeks at r.t. After filtration, the filtrate was concentrated by rotary evaporator at 40°C and resulting aqueous solution was subjected to Diaion HP20SS column chromatography (5 cm i.d. × 30 cm) with 0-100% MeOH (10% stepwise, each 300 mL) to give 5 fractions (Fr 1 – 5). Fr 4 (1.46 g) was further fractionated on Sephadex LH-20 (3 × 31 cm) with 60-100% MeOH (10% stepwise, each 100 mL) and then MeOH-acetone-H_2O (90:5:5, 8:1:1, 6:1:1, each 100 mL) to yield nine fractions (Fr 4-1 – 4-9) including 1,2,3,4,6-penta-*O*-galloyl-α-

D-glucose (Fr 4-8, 395.8 mg). Fr 3 (2.53 g) was separated by Sephadex LH-20 column chromatography (3 × 23 cm), in a similar manner to give 13 sub-fractions. Fr. 3-11 (395.7 mg) was further fractionated over Chromatorex ODS (2 × 15 cm), with 0-100% MeOH, furnishing two fractions. Purification of Fr 3-11-1 (279.0 mg) over Diaion HP20SS (2 × 22 cm) with 30-100% acetonitrile led to isolation of **1c** (81.5 mg), which was recrystallized from H_2O to give microcrystalline powder (41.3 mg). Fr 2 (2.52 g) was subjected to Sephadex LH-20 chromatography (3 × 23 cm) with 0-100% MeOH to yield **2** (60.6 mg), 1,2,4-tri-*O*-galloyl-α-D-glucose (259.9 mg), nupharin A (529.4 mg), and nupharin C (119.1 mg).

4.4.1. Acetonylnupharanin (**1c**).

Off-white microcrystalline powder (from H_2O); mp: 258-260°C (dec.); $[\alpha]_D^{17}$ -713.0 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε): 278 (4.64) 219 (5.02) nm; IR (film) ν_{max} 3403, 1719, 1617, 1213 cm^{-1}; ^1H NMR (acetone-d_6, 500 MHz) and ^{13}C NMR (acetone-d_6, 126 MHz) see Table 1; HRFABMS *m/z* 993.1207 $[M+H]^+$ (calcd for $C_{44}H_{33}O_{27}$, 993.1204).

4.4.2. 1,4-Acetonyl-DHHDP-2-O-galloyl-α-D-glucose (**2**).

Yellow amorphous powder; $[\alpha]_D^{17}$ -329.4 (*c* 0.7, MeOH); UV (MeOH) λ_{max} (log ε) 277 (4.32) 219 (4.73) nm; IR (film) ν_{max} 3419, 1725, 1621, 1217 cm^{-1}; ^1H NMR (acetone-d_6 + D_2O, 500 MHz) and ^{13}C NMR (acetone-d_6 + D_2O, 126 MHz) see Table 1; HRFABMS *m/z* 691.1146 $[M+H]^+$ (calcd for $C_{30}H_{27}O_{19}$, 691.1141).

4.4. Acid hydrolysis of acetonylnupharanin (1c).

Compound **1c** (5 mg) was heated in 2% TFA in H_2O (200 μL) at 90°C for 10 h in screw-capped tube and then lyophilized. The resulting solid was treated with a solution of L-cysteine in pyridine (10mg/mL, 200 μL) at 60°C for 1 h. To the solution *o*-tolylisothiocyanate (4 μL) was added and heated at 60°C for another 1 h. After cooling to r.t. the solution containing the thiazolidine derivative was directly analyzed by HPLC-DAD. Thiazolidine derivatives of D- and L-glucose was prepared in a similar manner. Retention time of the peak observed in the chromatogram of the hydrolysates coincided with that of the D-glucose thiazolidine derivative (t_R = 35.18 min) (L-glucose derivative (t_R = 35.76 min) (Fig. S3).

4.5. Oxidative degradation of 1 at pH 6.

A solution of **1** (50 mg) in pH 6 McIlvaine buffer (50 mL) was heated at 50°C for 4.5 h. After cooling, the solution was acidified by addition of a few drops of TFA and directly applied to Sephadex LH-20 column (2 cm ×15 cm) with H_2O. Elution of the column with H_2O containing increasing amounts of MeOH (20% stepwise, each 100 mL) and further purification by Chromatorex ODS column chromatography (2 cm ×10 cm, with 0-50% MeOH, 5% stepwise, each 50 mL) to give **4** (3.7 mg).

4.5.1 Reaction product **4**

Yellow amorphous powder; $[\alpha]_D^{17}$ +88.7 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 276 (4.38) 221 (4.81) nm; IR (film) ν_{max} 3387, 1720, 1610, 1224 cm^{-1}; ^1H NMR (acetone-d_6, 500 MHz) and ^{13}C NMR (acetone-d_6, 126 MHz) see Table 1; HRFABMS *m/z* 969.0846 $[M+H]^+$ (calcd for $C_{41}H_{29}O_{28}$ 969.0840).

4.6. DFT calculation

A conformational search was performed using the Monte Carlo method and the MMFF94 force field with Spartan '14 (Wavefunction, Irvine, CA, USA). The obtained low-energy conformers within a 6 kcal/mol window were optimized at the B3LYP/6-31G(d,p) level in acetone (PCM). The vibrational frequencies were calculated at the same level to confirm their stability, and no imaginary frequencies were found. The DFT-optimized conformers were classified and ^{1}H NMR coupling constants of the lowest-energy conformers in each classified group were calculated at the B3LYP/6-31G(d,p)u+1s (using only the Fermi contact term) level (PCM) and scaled using a slope parameter of 0.94. The calculated values were averaged using Boltzmann distribution theory at 298 K from their relative Gibbs free energies. All DFT calculations were performed using Gaussian 16.[30] Three-dimensional structures of the molecules were generated using GaussView.[31]

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