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Glycosylation of tocopherols by cultured cells of *Eucalyptus perriniana*

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Abstract

Cultured suspension cells of *Eucalyptus perriniana* converted exogenously administered α-tocopherol into α-tocopheryl 6-*O*-β-D-glucopyranoside (46 μg/g fr. wt of cells) and two biotransformation products: α-tocopheryl 6-*O*-(6-*O*-β-D-glucopyranosyl)-β-D-glucopyranoside (19 μg/g fr. wt of cells) and α-tocopheryl 6-*O*-(6-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside (6 μg/g fr. wt of cells). On the other hand, two other compounds, i.e., δ-tocopheryl 6-*O*-(6-*O*-β-D-glucopyranosyl)-β-D-glucopyranoside (27 μg/g fr. wt of cells) and δ-tocopheryl 6-*O*-(6-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside (12 μg/g fr. wt of cells), together with δ-tocopheryl 6-*O*-β-D-glucopyranoside (63 μg/g fr. wt of cells) were isolated from suspension cells following the administration of δ-tocopherol.
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1. Introduction

Higher plants accumulate a wide range of glycosides as secondary metabolites. Plant cells can conjugate sugar residues not only to endogenous metabolic intermediates but also to xenobiotics, since many plant UDP-glycosyltransferases have broad substrate specificity. In plant cells, glycosylation activates biosynthetic intermediates and detoxifies compounds that originate in the environment (Tabata et al., 1976; Kamel et al., 1992). Cultured plant cells should be useful for the preparation of glycosides as biocatalysts, due to the convenient one-step enzymatic glycosylation. Many studies have been reported on the glycosylation of exogenous phenolic compounds by cultured plant cells (Tabata et al., 1976, 1988; Furuya et al., 1987a, 1988, 1989; Moyer and Gustine, 1987; Upmeier et al., 1988; Ushiyama and Furuya, 1989; Suga and Hirata, 1990; Lewinson et al., 1996; Ishihara et al., 2003).

Vitamin E is the term used for a group of tocopherols and tocotrienols, and has been known to be an essential nutrient for reproduction since 1922 (Evans and Bishop, 1922). Vitamin E functions as a chain-breaking antioxidant that prevents the propagation of free radical reactions (Packer, 1994; Kamal-Eldin and Appelqvist, 1996). Recently, vitamin E has attracted much attention clinically because of its potential to be a very useful drug, and has been widely studied for its anti-aging, anticancer, anti-atherosclerosis, and anticarcinogenesis effects (Rimm et al., 1993; Stampfer et al., 1993; Brigelius-Flohe and Traber, 1999). On the other hand, vitamin E is a lipophilic compound that is poorly absorbed after oral administration (Satoh et al., 2001). Glycosylation is a characteristic reaction which converts water-insoluble and unstable aromatic

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compounds into the corresponding water-soluble and stable compounds to improve their bioavailability and pharmacological properties, e.g., the glycosides of aromatic compounds have been used in folk medicines and the glycosides of drugs have been used as potent prodrugs (Barua and Olson, 1992). To overcome the shortcomings of vitamin E, considerable effort has been made to synthesize vitamin E glycosides, water-soluble vitamin E derivatives, by chemical methods (Uhrig et al., 2000; Satoh et al., 2001). However, little attention has been paid to the biological glycosylation of vitamin E by cultured plant cells.

We report here the biotransformation of α- and δ-tocopherols into the corresponding β-glucosides, β-gentiobiosides, and β-rutinosides, which are more water-soluble, by cultured plant cells of *Eucalyptus perriniana*.

2. Results and discussion

Incubation of cultured suspension cells of *E. perriniana* with α-tocopherol (**1**) for five days followed by extraction of the cells with MeOH gave three glycoside products **3**, **4**, and **5**. No other products were detected in both medium and MeOH-extracts of the cells by HPLC analyses. Spectroscopic data including HRFABMS, ^{1}H and ^{13}C NMR (Table 1), H–H COSY, C–H COSY, and HMBC spectra identified **3** as α-tocopheryl β-D-glucopyranoside (46 μg/g fr. wt of cells) (Satoh et al., 2001), **4** as α-tocopheryl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (β-gentiobioside, 19 μg/g fr. wt of cells), and **5** as α-tocopheryl 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside (β-rutinoside, 6 μg/g fr. wt of cells). The products **4** and **5** have not been identified before.

The HRFABMS spectrum of **4** included a pseudomolecular ion $[M+Na]^{+}$ peak at *m/z* 777.4770, consistent with a molecular formula of $C_{41}H_{70}O_{12}$ (calcd. 777.4765 for $C_{41}H_{70}O_{12}Na$). The ^{1}H NMR spectrum of **4** included proton signals at δ 4.24 (1H, *d*, $J = 8.4$ Hz) and 4.52 (1H, *d*, $J = 7.6$ Hz), indicating the presence of two β-anomers in the sugar moiety. The ^{1}H and ^{13}C NMR spectra of **4** indicated that it was a β-gentiobiosyl analogue of **1** (Ushiyama and Furuya, 1989). Furthermore, the HMBC spectrum included correlations between the anomeric proton signal at δ 4.52 (H-1′) and the carbon signal at δ 147.0 (C-6), as well as between the anomeric proton signal at δ 4.24 (H-1″) and the carbon signal at δ 70.0 (C-6′). These findings confirm that the inner β-D-glucopyranosyl residue was attached to the phenolic hydroxyl group of α-tocopherol (**1**) and that the pair of β-D-glucopyranosyl residues were 1,6-linked. Thus, the product **4** was identified as α-tocopheryl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside.

The HRFABMS spectrum of **5** included an ion attributed to $[M+Na]^{+}$ pseudomolecular ion (*m/z* 761.4827), suggesting a molecular formula of $C_{41}H_{70}O_{11}$ (calcd. 761.4816 for $C_{41}H_{70}O_{11}Na$). Two proton signals at δ 4.38 (1H, *d*, $J = 1.6$ Hz) and 4.50 (1H, *d*, $J = 8.0$ Hz) were observed in the ^{1}H NMR spectrum of **5**, indicating that the glucoside linkage in **5** had α- and β-orientations. The ^{13}C NMR spectroscopic data of the sugar moiety of **5** was in good agreement with that of β-rutinose (Ishimaru et al., 2003). HMBC correlations were observed between the anomeric proton signal at δ 4.50 (H-1′) and the carbon resonance at δ 147.1 (C-6), and between the anomeric proton signal at δ 4.38 (H-1″) and the carbon resonance at δ 68.2 (C-6′), confirming that the inner β-D-glucopyranosyl residue was attached to the 6-hydroxyl group of α-tocopherol (**1**) whereas the second α-L-rhamnopyranosyl and inner β-D-glucopyranosyl residues were 1,6-linked. Thus, compound **5** was determined to be α-tocopheryl 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside.

The biotransformation pathway of **1** in the *E. perriniana* cells was investigated (Fig. 1). Substrate **1** was first converted into β-glucoside **3**, whereas **4** and **5** were not

Table 1
^{13}C chemical shifts of the biotransformation products **3–8** in CD_3OD

Product		**3**	**4**	**5**	**6**	**7**	**8**
Aglycone	1	75.5	75.6	75.6	76.6	76.6	76.7
	2	32.6	32.6	32.6	32.5	32.5	32.5
	3	21.6	21.6	21.6	23.5	23.5	23.6
	4	118.2	118.2	118.4	122.0	122.2	122.2
	5	123.1	123.3	123.2	116.0	116.1	116.1
	6	147.0	147.0	147.1	151.5	151.5	151.5
	7	127.7	127.7	127.7	118.7	118.7	118.7
	8	129.5	129.7	129.6	127.6	127.6	127.7
	9	149.0	149.1	149.1	148.4	148.5	148.5
	10	40.8	40.8	40.8	40.9	40.9	40.8
	11	22.0	22.0	22.1	22.0	22.0	22.0
	12	38.3	38.2	38.2	38.3	38.3	38.3
	13	33.8	33.8	33.8	33.8	33.8	33.8
	14	38.3	38.2	38.2	38.3	38.3	38.3
	15	25.3	25.3	25.3	25.4	25.4	25.4
	16	38.3	38.2	38.2	38.3	38.3	38.3
	17	33.8	33.8	33.8	33.8	33.8	33.8
	18	38.3	38.2	38.2	38.3	38.3	38.3
	19	25.8	25.8	25.8	25.8	25.7	25.8
	20	40.4	40.5	40.5	40.5	40.5	40.5
	21	29.0	29.1	29.1	29.1	29.0	29.1
	22	23.0	23.0	23.1	23.1	23.1	23.1
	1-Me	24.1	24.1	24.0	24.4	24.4	24.3
	5-Me	12.1	12.1	12.1			
	7-Me	14.1	14.1	14.1			
	8-Me	13.2	13.2	13.2	16.4	16.4	16.4
	13-Me	20.2	20.1	20.2	20.2	20.2	20.1
	17-Me	20.2	20.1	20.2	20.2	20.2	20.1
	21-Me	23.0	23.0	23.1	23.1	23.1	23.1
Glc	1′	105.8	104.4	103.9	103.5	103.4	103.0
	2′	75.6	74.9	74.8	74.9	74.8	74.8
	3′	77.8	77.7	77.9	77.9	77.8	78.0
	4′	71.6	71.5	71.5	71.4	71.5	71.4
	5′	77.5	76.6	77.0	77.9	77.1	77.1
	6′	62.8	70.0	68.2	62.5	69.6	67.7
	1″		105.7	104.5		104.6	103.8
	2″		75.6	71.8		75.1	72.1
	3″		77.7	72.2		77.8	72.4
	4″		71.4	73.7		71.5	73.9
	5″		77.6	69.9		77.7	70.0
	6″		62.6	18.0		62.7	18.1

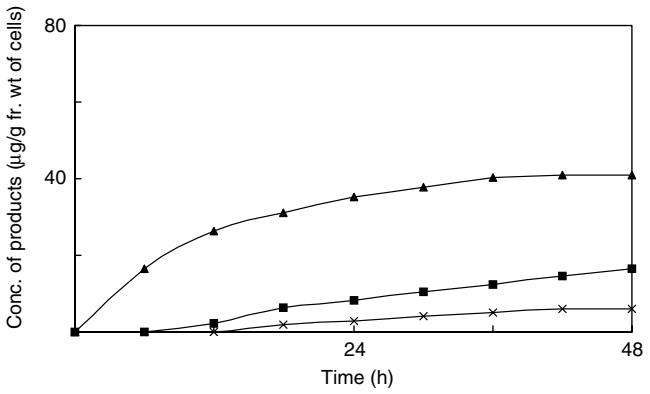


Fig. 1. Time course of the biotransformation of α-tocopherol (**1**) by cultured cells of *E. perriniana*. Concentrations of **3** (▲), **4** (■), and **5** (×) are plotted.

detected at an early stage of incubation. The products **4** and **5,** however, predominantly accumulated in the cells after 12 h of incubation. The biotransformation pathway of **1** is shown in Scheme 1.

The substrate δ-tocopherol (**2**) was incubated with cultured suspension cells of *E. perriniana* in the same method as for the biotransformation of **1**. After five days, products **6–8** were isolated from the MeOH-extracts of the cells. The products were identified as δ-tocopheryl β-D-glucopyranoside (**6**, 63 μg/g fr. wt of cells) (Satoh et al., 2001), δ-tocopheryl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (β-gentiobioside, **7**, 27 μg/g fr. wt of cells), and δ-tocopheryl 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside (β-rutinoside, **8**, 12 μg/g fr. wt of cells). The disaccharide products **7** and **8** have not been identified before.

The HRFABMS spectrum of **7** showed a pseudomolecular ion $[M+Na]^+$ peak (*m*/*z* 749.4459), which established a molecular formula of $C_{39}H_{66}O_{12}$ (calcd. 749.4452 for $C_{39}H_{66}O_{12}Na$). The ^1H NMR spectrum of **7** showed two proton signals at δ 4.35 (1H, *d*, $J = 8.0$ Hz) and 4.72 (1H, *d*, $J = 7.6$ Hz), which indicated the presence of two β-anomers in the sugar moiety. The ^{13}C NMR spectroscopic data of the sugar moiety of **7** were in good agreement with those of **4** (β-gentiobioside). Correlations were observed in the HMBC spectrum between the anomeric proton signal at δ 4.72 (H-1′) and the carbon resonance at δ 151.5 (C-6) as well as between the anomeric proton signal at δ 4.35 (H-1″) and the carbon resonance at δ 69.6 (C-6′). These data confirmed that the inner β-D-glucopyranosyl residue was attached to the phenolic hydroxyl group of δ-tocopherol (**2**) and that the pair of β-D-glucopyranosyl residues were 1,6-linked. Thus, compound **7** was identified as δ-tocopheryl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside.

The HRFABMS spectrum of **8** ($[M+Na]^+$ peak at *m*/*z* 733.4511) suggested a molecular formula of $C_{39}H_{66}O_{11}$ (calcd. 733.4503 for $C_{39}H_{66}O_{11}Na$). The ^1H NMR spectrum of **8** included two proton signals at δ 4.45 (1H, *d*, $J = 1.7$ Hz) and 4.71 (1H, *d*, $J = 7.6$ Hz), which indicated the presence of α- and β-anomers in the sugar moiety. The ^{13}C NMR spectroscopic data of the sugar moiety of **8** were in good agreement with those of **5**, i.e., β-rutinose. Correlations between the anomeric proton signal at δ 4.71 (H-1′) and the carbon resonance at δ 151.5 (C-6), as well as between the anomeric proton signal at δ 4.45 (H-1″) and the carbon resonance at δ 67.7 (C-6′) were included in the HMBC spectrum. These results confirm that the inner β-D-glucopyranosyl residue was attached to the 6-hydroxyl group of δ-tocopherol (**2**) whereas the second α-L-rhamnopyranosyl and inner β-D-glucopyranosyl residues were 1,6-linked. Thus, the structure of compound **8** was determined to be δ-tocopheryl 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside.

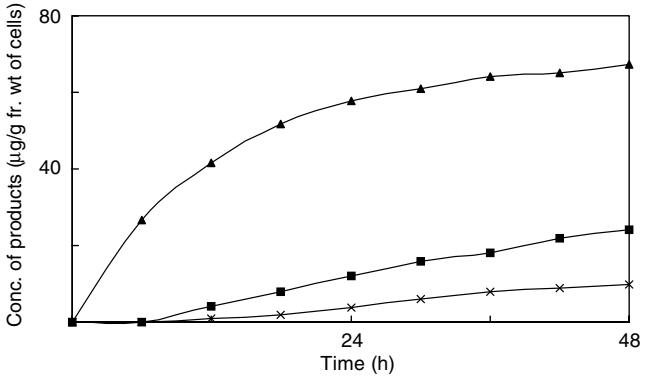
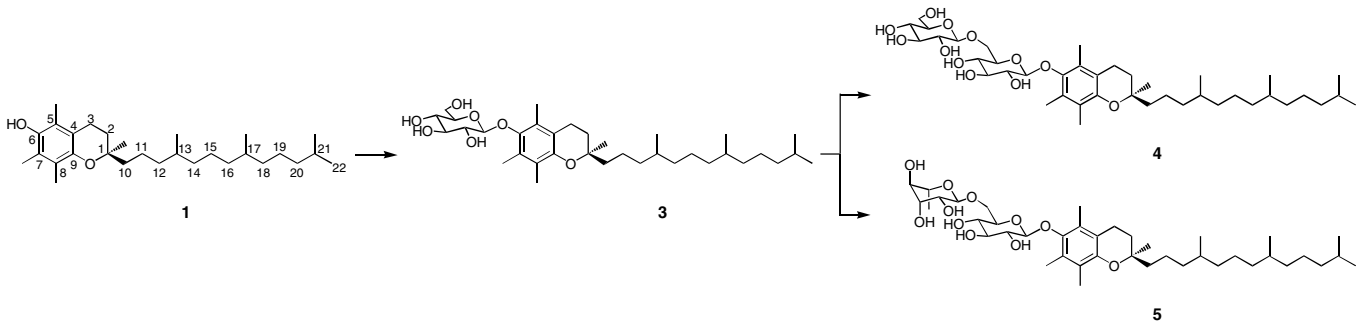
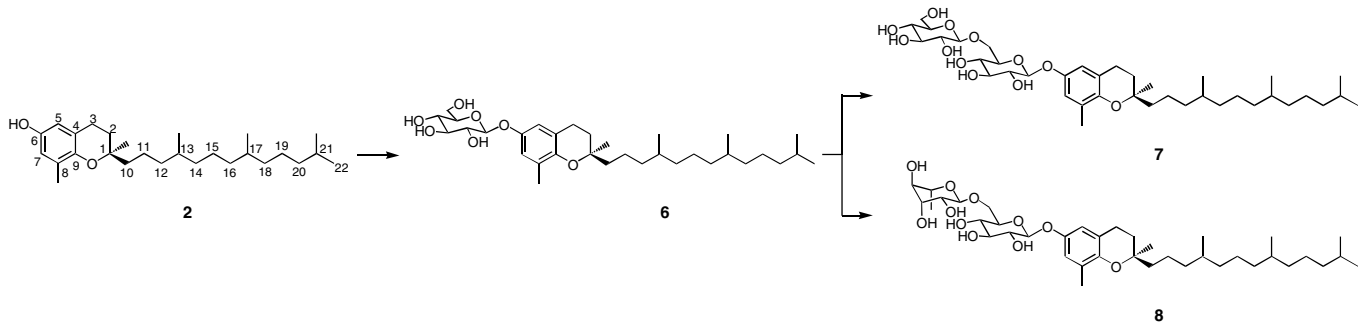


Fig. 2. Time course of the biotransformation of δ-tocopherol (**2**) by cultured cells of *E. perriniana*. Concentrations of **6** (▲), **7** (■), and **8** (×) are plotted.



Scheme 1. Glycosylation of α-tocopherol (**1**) by cultured cells of *E. perriniana*.



Scheme 2. Glycosylation of δ-tocopherol (**2**) by cultured cells of *E. perriniana*.

The time course in the conversion of **2** (Fig. 2) showed that disaccharide products **7** and **8** predominantly accumulated following the formation of glucoside **6** (Scheme 2).

3. Conclusion

This study demonstrated that cultured cells of *E. perriniana* can convert α- and δ-tocopherols into the corresponding β-glucosides, β-gentiobiosides, and β-rutinosides, i.e., water-soluble vitamin E derivatives. This is the first description of the biological production of tocopheryl disaccharides, such as β-gentiobioside and β-rutinoside, by biotransformation using cultured plant cells. In the time course experiments, the concentration of glycosides of δ-tocopherol at 48 h was much higher than that of α-tocopherol. This is probably due to the difference in the relative incorporation rates into the cells between α- and δ-tocopherols, i.e., the relative incorporation rate of δ-tocopherol was about 1.7 times higher than that of α-tocopherol at 48 h.

Recently, Satoh et al. reported the synthesis of α-tocopheryl β-glucoside and δ-tocopheryl β-glucoside by chemosynthetic methods (Satoh et al., 2001). These glucosides were obtained by condensing each tocopherol with β-D-pentaacetylglucose followed by deacetylation: 54% yield for α-tocopheryl β-glucoside and 23% yield for δ-tocopheryl β-glucoside. Cultured cells of *E. perriniana* appear to have high potential for the production of tocopheryl disaccharides, i.e., α-tocopheryl β-gentiobioside, α-tocopheryl β-rutinoside, δ-tocopheryl β-gentiobioside, and δ-tocopheryl β-rutinoside, as well as the corresponding monoglucosides, i.e., α-tocopheryl β-glucoside and δ-tocopheryl β-glucoside. It has been reported that vitamin E glycosides such as α-tocopheryl β-glucoside and α-tocopheryl α-mannoside, which have been synthesized chemically, had excellent anti-allergic and anti-inflammatory activities, i.e., these glycosides strongly inhibited histamine release from mast cells and suppressed IgE antibody formation (Uhrig et al., 2000; Satoh et al., 2001). The new tocopheryl glycosides obtained here may act as potent anti-allergic agents. It is well known that sugar conjugation of drugs reduces their toxicity and enhances their oral absorption (Barua and Olson, 1992). The plant UDP-glycosyltransferases responsible for these biotransformations should be useful for the preparation of water-soluble prodrugs of vitamin E. Studies on the physiological activities of the glycosides are now in progress.

4. Experimental

4.1. Substrates

The substrates α-tocopherol (**1**) and δ-tocopherol (**2**) were purchased from Aldrich Chemical Co. and purified by silica gel column chromatography before use.

4.2. Cell line and cell suspension culture

Cultured suspension cells of *E. perriniana* were prepared as described previously (Furuya et al., 1987b). Callus tissues (40 g) were added to a 300-ml conical flask containing MS medium (100 ml, pH 5.7, 3% sucrose) and cultivated with continuous shaking for 2 weeks on a rotary shaker (120 rpm) at 25 °C in the dark.

4.3. Biotransformation conditions

Substrate (34 mg) was added to each of ten 300-ml conical flasks containing suspension cultured cells of *E. perriniana*. The cultures were incubated at 25 °C for five days on a rotary shaker (120 rpm) in the dark. Glycosylation products were extracted and isolated according to the previously reported procedures (Shimoda et al., 2006, 2007). Time course experiments were carried out by a method similar to the normal biotransformation experiments except that substrate (43 mg) was individually administered to each flask (Shimoda et al., 2006, 2007).

The relative incorporation rates of substrates into the cells were calculated as follows: %Incorporation $= 100 \times T_c/(T_m + T_c)$, where T_c is the total amount (mol) of substrate and products in cultured *E. perriniana* cells and T_m is the amount (mol) of substrate that remained in the medium after an incubation period of 48 h.

4.4. Analysis

^{1}H and ^{13}C NMR, H–H COSY, C–H COSY, NOE, and HMBC spectra were recorded using a Varian XL-400 spectrometer in CD_3OD solution. HRFABMS spectra were obtained using a JEOL MStation JMS-700 spectrometer. The structures of the products were determined on the basis of their HRFABMS, ^{1}H and ^{13}C NMR, H–H COSY, C-H COSY, NOE, and HMBC spectra.

α-Tocopheryl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (**4**): HRFABMS: *m/z* 777.4770 $[M+Na]^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 0.86 (6H, *d*, $J = 6.0$ Hz, 13-, 17-CH_3), 0.87 (6H, *d*, $J = 6.8$ Hz, 21-CH_3, H-22), 1.02–1.46 (18H, *m*, H-11, 12, 13, 14, 15, 16, 17, 18, 19, 20), 1.21 (3H, *s*, 1-CH_3), 1.48–1.61 (3H, *m*, H-10, 21), 1.78 (2H, *m*, H-2), 2.04 (3H, *s*, 5-CH_3), 2.19 (3H, *s*, 8-CH_3), 2.22 (3H, *s*, 7-CH_3), 2.59 (2H, *m*, H-3), 3.10–3.54 (8H, *m*, H-2′, 2″, 3′, 3″, 4′, 4″, 5′, 5″), 3.65 (1H, *dd*, $J = 12.0$, 5.2 Hz, H-6a″), 3.73 (1H, *dd*, $J = 11.6$, 5.2 Hz, H-6a′), 3.86 (1H, *dd*, $J = 12.0$, 2.4 Hz, H-6b″), 4.05 (1H, *dd*, $J = 11.6$, 3.2 Hz, H-6b′), 4.24 (1H, *d*, $J = 8.4$ Hz, H-1″), 4.52 (1H, *d*, $J = 7.6$ Hz, H-1′); ^{13}C NMR (100 MHz, CD_3OD) see Table 1.

α-Tocopheryl 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside (**5**): HRFABMS: *m/z* 761.4827 $[M+Na]^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 0.86 (6H, *d*, $J = 6.0$ Hz, 13-, 17-CH_3), 0.87 (6H, *d*, $J = 6.8$ Hz, 21-CH_3, H-22), 1.04–1.46 (18H, *m*, H-11, 12, 13, 14, 15, 16, 17, 18, 19, 20), 1.19 (3H, *d*, $J = 6.0$ Hz, H-6″), 1.21 (3H, *s*, 1-CH_3), 1.48–1.62 (3H, *m*, H-10, 21), 1.78 (2H, *m*, H-2), 2.04 (3H, *s*, 5-CH_3), 2.18 (3H, *s*, 8-CH_3), 2.22 (3H, *s*, 7-CH_3), 2.59 (2H, *m*, H-3), 3.08–3.56 (8H, *m*, H-2′, 2″, 3′, 3″, 4′, 4″, 5′, 5″), 3.67 (1H, *dd*, $J = 12.0$, 5.4 Hz, H-6a′), 3.93 (1H, *dd*, $J = 11.6$, 2.0 Hz, H-6b′), 4.38 (1H, *d*, $J = 1.6$ Hz, H-1″), 4.50 (1H, *d*, $J = 8.0$ Hz, H-1′); ^{13}C NMR (100 MHz, CD_3OD) see Table 1.

δ-Tocopheryl 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside (**7**): HRFABMS: *m/z* 749.4459 $[M+Na]^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 0.86 (6H, *d*, $J = 6.8$ Hz, 13-, 17-CH_3), 0.87 (6H, *d*, $J = 6.8$ Hz, 21-CH_3, H-22), 1.02–1.48 (18H, *m*, H-11, 12, 13, 14, 15, 16, 17, 18, 19, 20), 1.24 (3H, *s*, 1-CH_3), 1.49–1.57 (3H, *m*, H-10, 21), 2.76 (2H, *m*, H-2), 2.09 (3H, *s*, 8-CH_3), 2.73 (2H, t, $J = 6.4$ Hz, H-3), 3.12–3.53 (8H, *m*, H-2′, 2″, 3′, 3″, 4′, 4″, 5′, 5″), 3.69 (1H, *dd*, $J = 12.0$, 5.2 Hz, H-6a″), 3.84 (1H, *dd*, $J = 11.6$, 5.2 Hz, H-6a′), 3.86 (1H, *dd*, $J = 12.0$, 2.4 Hz, H-6b″), 4.04 (1H, *dd*, $J = 11.6$, 3.2 Hz, H-6b′), 4.35 (1H, *d*, $J = 8.0$ Hz, H-1″), 4.72 (1H, *d*, $J = 7.6$ Hz, H-1′), 6.68 (1H, *d*, $J = 2.8$ Hz, H-5), 6.73 (1H, *d*, $J = 2.8$ Hz, H-7); ^{13}C NMR (100 MHz, CD_3OD) see Table 1.

δ-Tocopheryl 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside (**8**): HRFABMS: *m/z* 733.4511 $[M+Na]^+$; ^{1}H NMR (400 MHz, CD_3OD): δ 0.86 (6H, *d*, $J = 6.8$ Hz, 13-, 17-CH_3), 0.87 (6H, *d*, $J = 6.8$ Hz, 21-CH_3, H-22), 1.02–1.47 (18H, *m*, H-11, 12, 13, 14, 15, 16, 17, 18, 19, 20), 1.20 (3H, *d*, $J = 6.0$ Hz, H-6″), 1.23 (3H, *s*, 1-CH_3), 1.48–1.59 (3H, *m*, H-10, 21), 2.75 (2H, *m*, H-2), 2.09 (3H, *s*, 8-CH_3), 2.73 (2H, t, $J = 6.4$ Hz, H-3), 3.12–3.53 (8H, *m*, H-2′, 2″, 3′, 3″, 4′, 4″, 5′, 5″), 3.74 (1H, *dd*, $J = 11.6$, 5.2 Hz, H-6a′), 3.95 (1H, *dd*, $J = 11.6$, 3.2 Hz, H-6b′), 4.45 (1H, *d*, $J = 1.7$ Hz, H-1″), 4.71 (1H, *d*, $J = 7.6$ Hz, H-1′), 6.67 (1H, *d*, $J = 2.9$ Hz, H-5), 6.74 (1H, *d*, $J = 2.9$ Hz, H-7); ^{13}C NMR (100 MHz, CD_3OD) see Table 1.

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