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THREE GLUCOSIDES FROM *LYCOPODIUM CLAVATUM*

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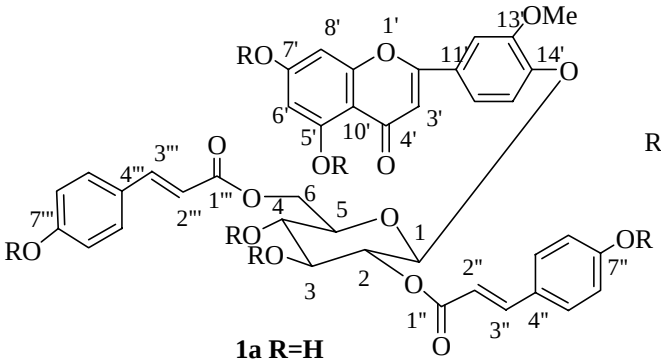
Abstract – Three new glucosides were isolated as their acetates from Japanese *Lycopodium clavatum* and the structures of acetates were determined based on extensive 2D NMR spectra. Two of them had the apigenin moiety attached to the anomeric position of glucose. The third one had a benzoate unit at the anomeric position, a *p*-coumarate at the 6-position, and a ferulate at the 2-position of the glucosyl unit.

INTRODUCTION

In the continual search for physiologically active substance in plants, we have studied the chemical constituents of *Lycopodium clavatum* collected in Japan. Studies of *Lycopodium* plants to date have resulted in isolation of triterpenoids,[1] glucosides,[2-4] $C_{30}N_3$ alkaloids,[5,6] and other alkaloids.[7] Quite recently, many reports about the chemical constituents of *Lycopodium* plants have appeared.[8-19] Some of them possess very unusual type of skeleton related to lycopodane and other types. We have previously studied the basic constituents of *L. lucidulum* collected in Canada and reported the structures of oxolucidine A and lucidine B as well as lucidulinone.[5,6] Thus, *Lycopodium* plants are still interesting sources for many people working in this area. In the present study, we have investigated Japanese *L. clavatum*, and have found three new glucosides. Here, we report the details of structure elucidation.

RESULTS AND DISCUSSION

The ethyl acetate-soluble fraction of an ethanol extract of *L. clavatum* was repeatedly separated by silica gel and Sephadex LH-20 column chromatography. Because the polar fraction was not easy to separate, the residue was acetylated after examination by NMR spectrum, which indicated the absence of acetyl group. The mixture was then separated by column chromatography in combination with HPLC to afford three new glucoside acetates (**1b**, **2b**, **3b**) as well as previously known **4b**[3] and **5b**.[4]



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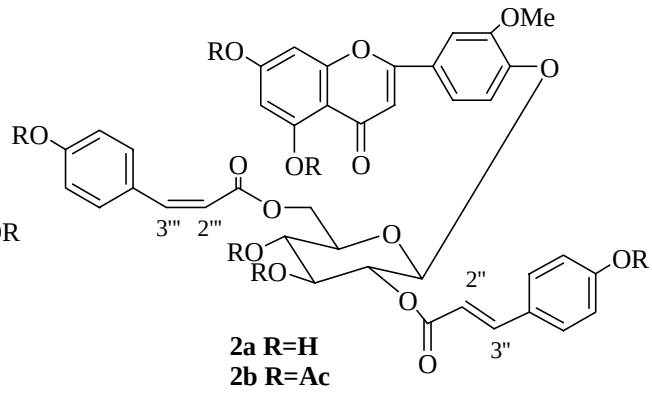
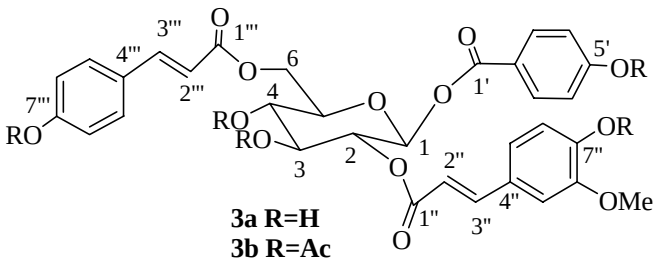
1b R=Ac



2a R=H
2b R=Ac



3a R=H
3b R=Ac

Compound (**1b**) gave a quasi-molecular ion peak at *m/z* 1007 $[M+H]^+$ by FABMS spectrum and its molecular formula was calculated as $C_{52}H_{46}O_{21}$. The ^{1}H NMR spectrum indicated the presence of six acetyl groups, a methoxy group, two α,β-unsaturated ester groups as well as the acylated glucosyl moiety. These groups were further supported by the ^{13}C NMR spectrum and 2D NMR spectral analysis. The signal at δ 5.17 (d, J = 7.4 Hz) was assigned as an anomeric proton, and the coupling network from H-1 to H-6, through H-2, H-3, H-4, and H-5, for the glucosyl unit was revealed by the COSY spectrum (Table 1). The signals at δ 6.39 and 7.73 coupled each other, whose J value (15.9 Hz) suggested the *trans* geometry of this double bond. Protons observed at δ 6.40 and 7.68 also coupled each other (J = 15.9 Hz) and this double bond should be *trans*, as well. These protons were due to the *p*-coumarate groups, because the HMBC (heteronuclear multiple bond connectivity) spectrum clearly indicated the correlation peaks between the proton at δ 7.73 (H-3" of 2-*O*-*p*-coumarate) and the carbon at δ 129.3 (C-5" and 9" of 2-*O*-*p*-coumarate), and also between the proton at δ 6.39 (H-2" of 2-*O*-*p*-coumarate) and the carbon at δ 131.8 (C-4" of 2-*O*-*p*-coumarate). This moiety was connected to the 2-position of the glucosyl unit, because the HMBC spectrum showed ^3J coupling between the proton at δ 5.49 (H-2 of glucose) and the carbon at δ 165.1 (C-1" of 2-*O*-*p*-coumarate). Similar analysis for the other *p*-coumarate lead to the conclusion that it is attached to the 6-position of the glucosyl unit. Other aromatic protons were due to the flavone unit indicating the presence of an apigenin unit, revealed by the HMBC spectrum. The oxygen atom, at the 14'-position of apigenin, was connected to the anomeric carbon of the glucosyl moiety.

Table 1. ^{1}H and ^{13}C NMR Spectral Data of Compounds (**1b–3b**) (600 MHz, $CDCl_3$)

position	**1b**		**2b**		**3b**	
glucose						
1	5.17 (d, 7.4)	100.4	5.12 (d, 7.4)	100.3	6.02 (d, 8.0)	92.5
2	5.49 (dd, 9.2, 7.4)	71.1	5.45 (dd, 9.0, 7.4)	71.1	5.51 (dd, 9.7, 8.0)	70.1
3	5.43 (dd, 9.6, 9.2)	72.3	5.41 (dd, 9.5, 9.0)	72.4	5.46 (dd, 9.7, 9.1)	72.6
4	5.28 (dd, 9.6, 9.6)	68.5	5.17 (dd, 9.5, 9.5)	68.7	5.29 (dd, 10.3, 9.1	68.2
5	3.97 (ddd, 9.6, 5.0, 2.7)	72.4	3.88 (ddd, 9.5, 6.9, 3.0)	72.2	4.07 (ddd, 10.3, 3.9, 2.6)	73.0
6	4.39 (dd, 9.6, 5.0)	62.1	4.32 (dd, 12.0, 6.9)	62.0	4.38 (m)	61.8
	4.43 (dd, 5.0, 2.7)		4.28 (dd, 12.0, 3.0)		0.0	
3-OAc	2.03[a]	20.6[b]	2.31[a]	20.6[b]	2.02[a]	21.1[b]
		170.3[c]		170.2[c]		170.2[c]
4-OAc	2.08[a]	20.6[b]	2.29[a]	20.6[b]	2.08[a]	20.6[b]
		169.4[c]		169.4[c]		169.4[c]
flavone						
2'	-	161.9	-	162.0	-	-
3'	6.52 (s)	108.2	6.55 (s)	108.2	-	-
4'	-	176.2	-	176.3	-	-
5'	-	150.2[d]	-	150.2[d]	-	-
6'	6.83 (d, 2.2)	113.6	6.83 (d, 2.2)	113.6	-	-
7'	-	153.9[d]	-	153.9[d]	-	-
8'	7.27 (d, 2.2)	109.0	7.33 (d, 2.2)	109.0	-	-
9'	-	157.5	-	157.6	-	-
10'	-	114.9	-	114.9	-	-
11'	-	127.2	-	127.2	-	-
12'	7.27 (d, 2.2)	110.2	7.28 (d, 2.2)	110.2	-	-
13'	-	151.0	-	150.9	-	-
14'	-	148.9	-	148.9	-	-
15'	7.23 (d, 8.4)	119.7	7.18 (d, 8.5)	119.7	-	-
16'	7.35 (dd, 8.4, 2.2)	119.4	7.34 (dd, 8.5, 2.2)	119.5	-	-
5'-OAc	2.44[a]	21.1[b]	2.43[a]	20.6[b]	-	-
		169.4[c]		169.4[c]	-	-
7'-OAc	2.30[a]	21.1[b]	2.29[a]	21.2[b]	-	-
		169.1[c]		169.2[c]	-	-
13'-OMe	3.80 (s)	56.3	3.79 (s)	56.3	-	-
1-*O*-benzoate						
1'	-	-	-	-	-	163.8
2'	-	-	-	-	-	125.9
3',7'	-	-	-	-	8.07 (d, 8.8)	131.9
4',6'	-	-	-	-	7.16 (d, 8.8)	122.1
5'	-	-	-	-	-	155.0
5'-OAc	-	-	-		2.30[a]	21.1[b]
	-	-	-	-	-	169.1[c]
2-*O*-*p*-coumarate						
1"	-	165.1	-	165.2	-	-
2"	6.39 (d, 15.9)	117.2	6.39 (d, 15.9)	117.0	-	-
3"	7.73 (d, 15.9)	145.0	7.73 (d, 15.9)	144.9	-	-
4"	-	131.8	-	131.7	-	-
5",9"	7.55 (d, 8.9)	129.3	7.55 (d, 8.5)	129.3	-	-
6",8"	7.14 (d, 8.9)	122.3	7.13 (d, 8.5)	122.3	-	-
7"	-	152.4	-	152.4	-	-
7"-OAc	2.32[a]	21.2[b]	2.31[a]	21.2[b]	-	-
	-	169.0[c]	-	169.1[c]	-	-
2-*O*-ferulate						
1"	-	-	-	-	-	165.1
2"	-	-	-	-	6.28 (d, 15.9)	116.4

3"	-	-	-	-	7.62 (d, 15.9)	146.0
4"	-	-	-	-	-	132.7
5"	-	-	-	-	7.06 (d, 1.6)	123.3
6"	-	-	-	-	-	151.4
7"	-	-	-	-	-	141.8
8"	-	-	-	-	7.03 (d, 7.5)	121.7
9"	-	-	-	-	7.08 (dd, 1.6, 7.5)	111.2
6"-OMe	-	-	-	-	3.85 (s)	55.9
7"-OAc	-	-	-	-	2.31[a]	21.1[b]
	-	-	-	-	-	168.7[c]
6-*O*-*p*-coumarate						
1"'	-	166.2	-	165.1	-	166.3
2"'	6.40 (d, 15.9)	116.9	5.97 (d, 12.6)	118.5	6.42 (d, 15.9)	117.3
3"'	7.68 (d, 15.9)	144.6	6.99 (d, 12.6)	144.0	7.69 (d, 15.9)	144.6
4"'	-	131.8	-	132.1	-	130.8
5"',9"'	7.54 (d, 8.7)	129.3	7.66 (d, 8.5)	131.4	7.56 (d, 8.5)	129.4
6"',8"'	7.11 (d, 8.7)	122.2	7.07 (d, 8.5)	121.2	7.13 (d, 8.5)	121.9
7"'	-	152.4	-	151.3	-	152.2
7"'-OAc	2.35[a]	21.2[b]	2.35[a]	21.2[b]	2.32[a]	21.1[b]
	-	168.0[c]	-	168.0[c]	-	168.7[c]

[a,b,c,d] assignment may be interchanged in each vertical column.

The stereochemistry of the anomeric position was β-configuration as indicated by the coupling constant (J = 7.4 Hz). A methoxy group was observed in the ^{1}H and ^{13}C NMR spectra (Table 1) and should be present at C-13' position of the flavone unit, because the correlation between the singlet peak (3H) at δ_H 3.80 and δ_C 151.0 (C-13' of apigenin) was observed in the HMBC spectrum. Thus, the structure of compound (**1b**) was established as depicted in the figure.

The FAB-MS spectrum of compound (**2b**) showed a quasi-molecular ion peak at *m/z* 1007 and its molecular formula was calculated as $C_{52}H_{46}O_{21}$. Most of the proton signals were very similar to those of compound (**1b**). However, the signals due to an α,β-unsaturated ester were slightly different from those of **1b**. The doublet peaks at δ 6.39 and 7.73 (each J=15.9 Hz) exactly the same as those of **1b** and were assigned for the *trans*-*p*-coumarate moiety at the 2-position of glucose by extensive 2D NMR spectral analysis. While those at δ 5.97 and 6.99 (each J=12.6 Hz) were slightly shifted higher and lower field, respectively, as compared to those of the *trans*-*p*-coumarate unit, and were assigned as the *cis*-*p*-coumarate group. Since two acetate groups were revealed to be at the 3- and 4-position of glucose by 2D NMR analysis, this *cis*-*p*-coumarate moiety should be at the 6-position of glucose, although the correlation of H-6 and C-1" was not detected in the HMBC spectrum. The chemical shift of the 6-position of glucose was observed at δ 62.0 (Table2), which was almost the same as those of other compounds (**1b**, **3b**, **4b**, and **5b**). The spectral data (Tables 1 and 2) indicated the presence of the same flavone unit, apigenin, which was connected to the anomeric carbon of glucose with the ether oxygen at the 14'-position of apigenin. Therefore, the structure of compound (**2b**) was established as depicted in the formula.

The third compound (**3b**) did not contain the flavone unit; rather it had a benzoate group. The molecular formula was determined as $C_{42}H_{40}O_{18}$ by HRFABMS spectrometry. Since the anomeric proton at δ 6.02 was slightly shifted down field and had a correlation peak to the carbon at δ 163.8, which was assigned as the carbonyl group of the benzoate (C-1'), it must be attached to the anomeric position of glucose. The presence of the *trans*-*p*-coumarate group was suggested by the HMBC spectrum as discussed before. The other ester carbonyl (δ 165.1) belonged to the ferulate unit. The proton at δ 7.62 (H-3"), which had a large coupling (15.9 Hz) with δ 6.28 (H-2") corresponding to *trans* geometry, correlated to the same carbonyl carbon (δ 165.1) (C-1") and also to the methine carbon at δ 111.2 (C-9"), which was one of the aromatic carbons of the same benzene ring possessing both the methoxy and acetoxy groups. Therefore, the ferulate unit was connected to the C-2 position of the glucosyl unit. The *trans*-*p*-coumarate unit was attached to the 6-position of glucose, because the correlation between H-6 of glucose and C-1'" carbonyl carbon of the ester was observed in the HMBC spectrum. Thus, the whole structure was established as depicted in the formula **3b**.

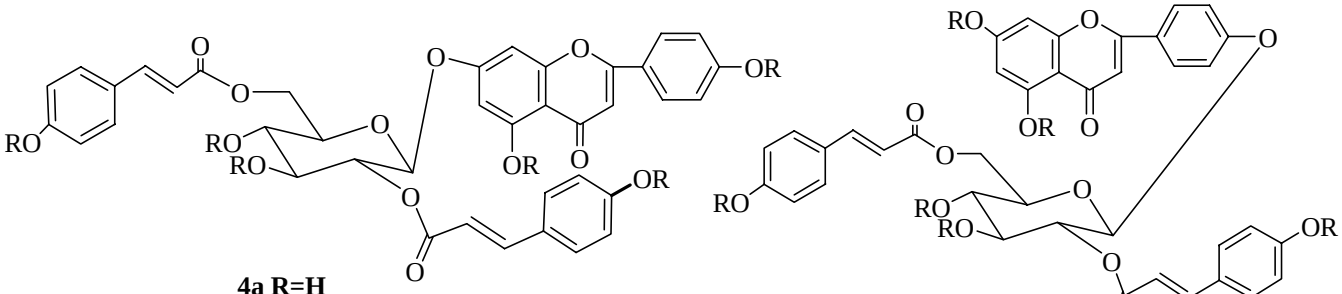


Table 2. ^{1}H and ^{13}C NMR Spectral Data of Compounds (**4b**) and (**5b**) (600 MHz, $CDCl_3$)

position	**4b**		**5b**	
glucose				
1	5.35 (d, 7.4)	98.1	5.29 (d, 7.2)	98.5
2	5.50 (dd, 9.1, 7.4)	70.8	5.49 (dd, 9.3, 7.2)	71.0
3	5.46 (dd, 9.3, 9.1)	72.4	5.45 (dd, 9.3, 9.0)	72.5
4	5.26 (dd, 9.6, 9.3)	68.6	5.29 (dd, 9.0, 10.0)	68.5
5	4.14 (ddd, 9.6, 6.4, 2.4)	72.5	4.06 (ddd, 10.0, 5.5, 2.7)	72.4
6	4.52 (dd, 12.0, 2.4)	62.2	4.46 (dd, 12.4, 2.7)	62.1
	4.32 (dd, 12.0, 6.4)	-	4.39 (dd, 12.4, 5.5)	-
3-OAc	2.04[a]	20.6[b]	2.03[a]	20.6[b]
	-	170.1[c]	-	170.2[c]
4-OAc	2.10[a]	20.6[b]	2.09[a]	20.6[b]
	-	169.6[c]	-	169.4[c]
flavone				
2'	-	161.3	-	161.8
3'	6.49 (s)	108.4	6.49 (s)	107.8
4'	-	176.1	-	176.2
5'	-	150.7[d]	-	150.1[d]

6'	6.71 (d, 2.5)	109.5	6.83 (d, 2.3)	113.6
7'	-	160.0[d]	-	153.8[d]
8'	7.00 (d, 2.5)	102.6	7.24 (d, 2.3)	108.9
9'	-	158.2	-	157.5
10'	-	112.8	-	114.8
11'	-	128.5	-	125.8
12'	7.79 (d, 8.8)	127.4	7.74 (d, 9.1)	127.9
13'	7.19 (d, 8.8)	122.3	7.11 (d, 9.1)	117.3
14'	-	153.2	-	159.3
15'	7.19 (d, 8.8)	122.3	7.11 (d, 9.1)	117.3
16'	7.79 (d, 8.8)	127.4	7.74 (d, 9.1)	127.9
5'-OAc	2.36[a]	21.1[b]	2.44[a]	21.2[b]
	-	169.6[c]	-	169.4[c]
7'-OAc	-	-	2.29[a]	21.1[b]
	-	-	-	169.0[c]
14'-OAc	2.30[a]	21.1[b]	-	-
	-	169.4[c]	-	-
2-*O*-*p*-coumarate				
1"		165.0	-	165.1
2"	6.36 (d, 15.9)	116.3	6.36 (d, 15.8)	117.1
3"	7.72 (d, 15.9)	145.7	7.72 (d, 15.8)	145.5
4"	-	131.6	-	131.7
5",9"	7.55 (d, 8.6)	129.5	7.54 (d, 8.5)	129.5
6",8"	7.13 (d, 8.6)	122.2	7.12 (d, 8.5)	122.2
7"	-	152.5	-	152.4
7"-OAc	2.31[a]	21.1[b]	2.31[a]	21.2[b]
	-	169.0[c]	-	169.0[c]
6-*O*-*p*-coumarate				
1"'	-	166.2	-	166.2
2"'	6.34 (d,15.9)	116.9	6.38 (d, 15.9)	117.1
3"'	7.62 (d,15.9)	144.9	7.66 (d, 15.9)	144.7
4"'	-	131.6	-	131.6
5"',9"'	7.37 (d, 8.6)	129.3	7.51 (d, 8.5)	129.3
6"',8"'	7.01 (d, 8.6)	122.1	7.10 (d, 8.5)	122.2
7"'	-	152.2	-	152.3
7"'-OAc	2.34[a]	21.1[b]	2.35[a]	21.2[b]
	-	168.9[c]	-	168.0[c]

[a, b, c]: Assignments may be interchanged in each vertical column.

Spectral data of compounds (**4b**) and (**5b**) were also analyzed (Table 2), and it was revealed that **4a** was anisfolin B, isolated by Rao *et al*. in 1983,[3] and that **5a** was the isomer of **4a** reported by Ansari *et al*. in 1979.[4]

Compound (**1b**) was, therefore, a methoxylated derivative of **5b**, and compound (**2b**) was the geometrical isomer of **1b**. Compound (**3b**) was a new class of glucoside isolated from *Lycopodium* species so far. None of the new compounds showed superoxide anion release inhibition activity determined by ESR spectrum.[20,21]

EXPERIMENTAL

GENERAL

The specific rotations and the CD spectra were taken on a JASCO DIP-1000 and J-725 polarimeter, respectively. IR spectra were measured on a JASCO FT/IR-5300 spectrophotometer. The ^{1}H and ^{13}C NMR spectra were taken on a Varian Unity 600 (600 MHz) spectrometer. MS spectra including high resolution mass spectra were recorded on a JEOL JMS AX-500 spectrometer. Chemcopak Nucleosil 50-5 (10×250 mm or 4.6×250 mm) was used for HPLC (JASCO pump system). Silica gel 60 (70-230 mesh, Fuji Sylisia) was used for column chromatography and silica gel 60 F_{254} plates (Merck) were used for TLC.

PLANT MATERIAL

The leaves and stems of *L. clavatum* (5.5 kg) were collected in Tokushima, in May, 2000. Voucher specimen (TBU-MT-200001) was deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Tokushima Bunri University.

EXTRACTION AND ISOLATION

Partially dried *L. clavatum* (5.5 kg) was cut into pieces and extracted with EtOH (60 L) at rt for *ca.* a month. A part of the EtOH extract (167 g) was partitioned between hexane and water. The water layer was further extracted with EtOAc. The EtOAc soluble fractions (12.3 g) were separated with silica gel column chromatography (elution with $CHCl_3$-MeOH, in gradient) to afford 6 fractions. The 6th fraction (3.5 g) was subjected to Sephadex LH-20 (MeOH) to give 5 fractions (A-E). The fraction E (1.0 g) was again purified by silica gel column chromatography (elution with $CHCl_3$-MeOH, in gradient) to give 4 fractions (E1-E4). The fraction E2 (689 mg) was acetylated (with 2 mL of acetic anhydride in 2 mL of pyridine at rt overnight) to give a crude fraction (668 mg). The acetylated residue was again purified by silica gel column chromatography ($CHCl_3$-EtOAc, in gradient) to afford 5 fractions (fr.1; 117.5 mg, fr.2; 24.0 mg, fr.3; 280.3 mg, fr.4; 46.8 mg, fr.5; 237.2 mg). The 1st fraction (117.5 mg) was further purified by HPLC (Nucleosil 50-5, 4.6×250 mm; hexane-$CHCl_3$=1:1; 2 mL/min) to give **3b** (6.0 mg). The 3rd fraction (280.3 mg) was subjected to HPLC (Nucleosil 50-5, 10×250 mm; $CHCl_3$-EtOAc=7:3; 2 mL/min) to afford 6 fractions (fr.1; 13.3 mg; fr.2; 14.0 mg; fr.3; 46.5 mg; fr.4; 28.9 mg; fr.5; 13.3 mg; fr.6; 137 mg). The 1st and 2nd fractions were further purified by HPLC (Nucleosil 50-5, 4.6×250 mm; hexane-$CHCl_3$=3:7; 2 mL/min) to give **2b** (2.2 mg) and **4b** (1.7 mg), respectively. The 3rd fraction was purified by HPLC (Nucleosil 50-5 10×250 mm; $CHCl_3$-EtOAc=8:2; 2 mL/min) to give **1b** (5.1 mg). The 4th fraction was purified by HPLC (Nucleosil 50-5; 4.6×250 mm; hexane-$CHCl_3$=3:7; 2 mL/min) to give **5b** (7.2 mg).

Compound (1b). colorless gum. ^{1}H- and ^{13}C-NMR spectra, see Tables 1 and 2. UV ($CHCl_3$) λmax 288 nm (ε 1.80×10^5). FTIR cm^{-1}:1760, 1720, and 1640. FABMS *m/z* 1007 $[M+H]^+$. HRFAB MS *m/z* 1007.2634 $[M+H]^+$ (calcd for $C_{52}H_{47}O_{21}$: 1007.2610). $[\alpha]_D^{22}$ -5.0° (*c* = 0.43, $CHCl_3$). CD ($CHCl_3$) Δε -4.9 (312 nm), +14.6 (276 nm), and +13.4 (259 nm).

Compound (2b). colorless gum. ^{1}H- and ^{13}C-NMR spectra, see Tables 1 and 2. UV ($CHCl_3$) λmax 288 nm (ε 1.74×10^5). FTIR cm^{-1}:1760,1730, and 1640. FABMS *m/z* 1007 $[M+H]^+$. HRFABMS *m/z* 1007.2590 $[M+H]^+$ (calcd for $C_{52}H_{47}O_{21}$: 1007.2610). $[\alpha]_D^{22}$ -1.4° (*c* = 0.29, $CHCl_3$). CD ($CHCl_3$) Δε -2.7 (307.7 nm), +3.0 (281.2 nm), and +0.9 (260 nm).

Compound (3b). colorless gum. ^{1}H- and ^{13}C-NMR spectra, see Tables 1 and 2. UV ($CHCl_3$) λmax 283.8 nm (ε 9.8×10^4). FTIR cm^{-1}:1760, 1720, and 1640. FABMS *m/z* 855 $[M+Na]^+$. HRFABMS *m/z* 855.2124 $[M+Na]^+$ (calcd for $C_{42}H_{40}O_{18}Na$: 855.2112). $[\alpha]_D^{22}$ -2.4° (*c* = 0.57, $CHCl_3$). CD ($CHCl_3$) Δε -4.9 (281 nm), +4.7 (246.1 nm).

Compound (4b).[3] colorless gum. ^{1}H- and ^{13}C-NMR spectra, see Tables 2 and 3. UV ($CHCl_3$) λmax 293.5 nm (ε 1.02×10^5). FTIR cm^{-1}:1760, 1720, and 1640. FABMS *m/z* 977 $[M+H]^+$. HRFABMS *m/z* 977.2519 $[M+H]^+$ (calcd for $C_{51}H_{45}O_{20}$: 977.2504). $[\alpha]_D^{22}$ -5.4° (*c* = 0.17, $CHCl_3$). CD ($CHCl_3$) Δε -11.6 (299.9 nm) and +6.3 (251.3 nm).

Compound (5b).[4] colorless gum. ^{1}H- and ^{13}C-NMR spectra, see Tables 2 and 3. UV ($CHCl_3$) λmax 293.6 nm (ε 1.30×10^5). FTIR cm^{-1}:1760, 1720, and 1640. FABMS *m/z* 977 $[M+H]^+$. HRFABMS *m/z* 977.2496 $[M+H]^+$ (calcd for $C_{51}H_{45}O_{20}$: 977.2504). $[\alpha]_D^{22}$ –3.9° (*c* = 0.71, $CHCl_3$). CD ($CHCl_3$) Δε +13.6 (278.2 nm) and +11.7 (260.5 nm).

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