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A trinorsesterterpene glycoside from the North American fern *Woodwardia virginica* (L.) Smith

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Abstract

A new trinorsesterterpene glycoside was isolated from the ethanol extract of the American fern *Woodwardia virginica* having a 3-[6-(4,8-dimethyl-nona-1,3,7-trienyl)-4-hydroxy-2,6-dimethyl-cyclohex-1-enyl]-3-hydroxypropionic acid, as the aglycone and a saccharide moiety linked at C-4 to glucoses, xylose or arabinofuranose. The structure was elucidated using extensive spectroscopic analysis (1D and 2D NMR, MS, IR and UV) including determination of absolute stereochemistry by means of the MTPA and PGME derivatives and also by chemical methods.

Keywords: Trinorsesterterpene glycoside; American fern; *Woodwardia virginica*

1. Introduction

Ferns are a very ancient family of plants: early fern fossils predate the beginning of the Mesozoic era, 360 million years ago. They thrived on Earth for 200 million years before the flowering plants evolved (Hallowell and Hallowell, 2001). Many types of natural compounds have been discovered from different fern species during the last decade. Some ferns contain bioactive flavonoids such as chalcones, dihydrochalcones, avanones, dihydro-flavonols, flavones, flavonols, and bisflavonoids which have recently been reviewed (Wollenweber and Schneider, 2000, and references cited). Five lipophilic components, hopane type triterpenes, have been isolated from fronds of the fern *Lophosoria quadripinnata* (Lophosoriaceae, Pteridophyta) (Tanaka et al., 1991). Unusual betaine ether-linked lipids, phospholipids, and fatty acids have also been studied in some fern species (Rozentsvet et al., 2001; Dembitsky, 1996). Biological active terpenoids, such as gibberellins, were identified from ferns belonging to genus *Lygodium*: *L. japonicum* (Wynne et al., 1998), *L. flexuosum* and *L. circinnatum* (Takeno et al., 1989). The protoilludane sesquiterpene glycoside, pteridanoside, was isolated from fern *Pteridium aquilinum* var. *caudatum* (Castillio et al., 1999).

In the present paper, we describe the identification of a new trinorsesterterpene glycoside isolated from American fern *Woodwardia virginica*.

2. Results and discussion

The leaves of fern *W. virginica* were extracted by ethyl alcohol and the extract was subsequently separated on Sephadex LH-20. The fractions were further purified by RP-HPLC to give glycoside (**1**)—see Fig. 1, which was identified by IR, UV, MS and ^{1}H- and ^{13}C NMR spectral data and chemical degradation.

Woodwardinoside (**1**) was obtained as a major component from *W. virginica*. The molecular formula was deduced as $C_{50}H_{80}O_{27}$ from a quasi-molecular ion observed at m/z 1113.4969 $(M+H)^+$ (Δ 4 mmu) in the FABMS and from the ^{13}C NMR spectrum. The IR spectrum showed absorbance at 3450 and 1681 cm^{-1}, that indicated the presence of a hydroxyl and carbonyl groups in the molecule, and the UV absorption band at 252 nm (conjugated double bonds).

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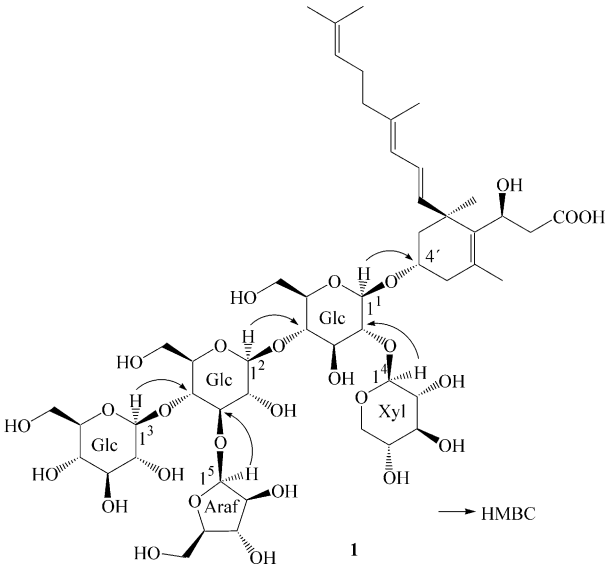

Fig. 1. Structure of woodwardinoside **1**.

The negative-ion FABMS of **1** showed fragment ion peaks at 979 $[M-H-132]^-$ and 949 $[M-H-162]^-$; the former was assignable to the loss of a pentosyl group from the parent ion at m/z 1111, and the latter corresponded to the loss of a glucosyl group. Furthermore, three prominent fragment ion peaks at m/z 847 $[M-H-2\times132]^-$, 817 $[M-H-162-132]^-$, 685 $[M-H-162-2\times132]^-$, were due to the elimination of two pentosyl, glucosyl and pentosyl, and the pentosyl and two glucosyl groups, respectively. This was suggestive of a branched pentaglycoside. The severe overlapping of the proton signals for the pentaglycoside moiety excluded the possibility of a complete assignment in a straightforward way using a conventional heteronuclear COSY spectrum in **1**. An HSQC-TOCSY technique was applied to solve the sugar sequence of **1**, which correlates the anomeric protons with their respective skeleton carbons atoms (Mimaki et al., 1996). This allowed identification of ^{13}C NMR signals for all the monosaccharides and the substituted positions of the inner glycosyl moieties, with only little knowledge of the sequential ^1H NMR assignment.

The glycoside was acidic hydrolyzed and the aglycone woodwardine (**2**) was isolated from the reaction mixture as colorless needles, with HRMS establishing its molecular formula as $C_{22}H_{34}O_4$ (Fig. 2). The ^{13}C NMR spectrum showed the signals of five methyls, five methylenes, six methines, and six quaternary carbons. The IR absorption at 3450 and 1681 cm^{-1} indicated the presence of a hydroxyl and carbonyl groups in the molecule. The ^1H NMR spectrum of **2** showed the occurrence of a methyl group at δ_H 1.07 and a geranyl side chain. The ^1H NMR spectrum in combination with the ^1H–^1H COSY NMR spectrum (Fig. 3) indicated the presence of the partial structure $-CH_2CH(OH)CH_2-$ from the multiplet signal at δ_H 3.54 (*ddt*, $J=12.0$, 9.2, 6.0 Hz), which coupled with two pairs of methylene protons at δ_H 2.31 (*dd*, $J=17.2$, 6.0 Hz), 1.94 (*dd*, $J=17.2$, 9.2 Hz), 1.76 (*dd*, $J=12.0$, 6.0 Hz), and 1.36 (*t*, $J=12.0$ Hz). Another partial structure, $-CH(OH)CH_2$-COOH, was accounted for by the observation of the signals at δ_C 60.7, 42.2, and 171.4 in the ^{13}C NMR spectrum and the signals at δ_H 3.64 (*dd*, $J=6.0$, 5.0 Hz), 3.27 (*dd*, $J=10.8$, 5.0 Hz), and 3.50 (*dd*, $J=10.8$, 6.0 Hz) in the ^1H NMR spectrum.

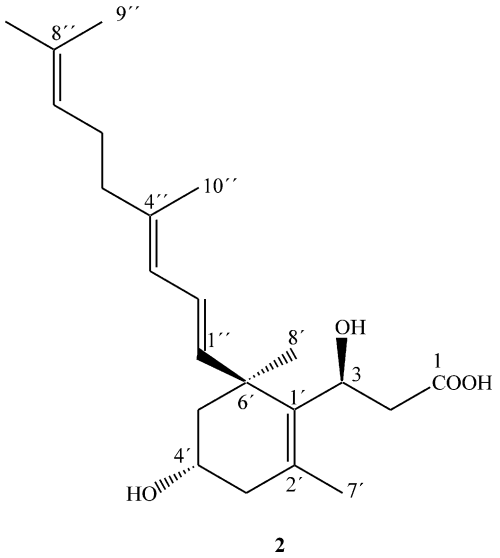

Fig. 2. Structure of woodwardine **2**.

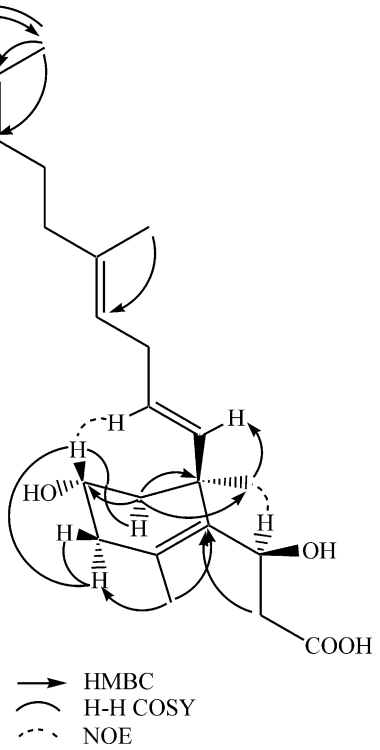

Fig. 3. HMBC, COSY and NOE correlations of compound **2**.

The ^{1}H–^{1}H COSY spectrum, enhancing the long range couplings, allowed connection of an isoprenoid chain. The ethylenic proton at 5.20 ppm (H-7″), which was vicinal to the methylene protons at 1.95 and 2.10 ppm, gave cross peaks with signals of methyl protons at 1.73 (H-9″) and 1.57 ppm (H-11″). The ethylenic proton at 5.78 ppm, H-3″, gave correlation spots with methyl protons at 1.63 ppm (H-10″) and methylene protons at 1.95 and 2.10 ppm. This proton H-3″ is coupled (J = 10.8 Hz) with the proton at 6.32 ppm (H-2″), which in turn gave an E ethylenic coupling (J = 15.3 Hz) with the proton at 5.62 ppm (H-1″). This latter proton (H-1″) gave cross peaks, on the ^{1}H–^{1}H-COSY, with H-8′ (1.07 ppm) and also with the methylene that gave the signals at 1.76 and 1.36 ppm (H-5′); these results lead to the linkage of C-5′, C-6′, and C-8′ to the remaining quartenary carbon atom (138.5 ppm, C-1′).

Finally, strong NOE were observed between: H-1″ and H-3, H-3″ and H-5″, H-5″ and H-7″, H-2″ and H-10″, and H-7″ and H-9″, indicating that they are in a *cis* relative disposition. Irradiation of H-1″ enhanced the signals of H-8′ and H = 5′ ax. This data shows the flexibility of the non-aromatic ring and of the isoprenoid chain. The geometry of the trisubstituted double bond (C-3″) was established as *E* owing to the presence of an NOE between H-3″ and H-5″ (δ 1.95) observed in the NOESY spectrum and the chemical shift of methyl carbon C-10″ (δ 16.4) of **2** (Okubo et al., 1980; Barlow and Pattenden, 1976) (Fig. 4). In the HMBC spectrum, the H-1″ proton provided a good starting point for the assignment of the proton and carbon resonances of the geranyl function. Proton H-1″ exhibited correlations with the C-5′, C-8′, C-6″ and C-3″ carbons. This permitted assignment of the C-6′ position of the geranyl group.

The relative configuration at the chiral center at C-4′ was determined by the coupling constants of the H-4′ resonance at δ_H 3.54 (*ddt*, J = 12.0, 9.2, 6.0 Hz) with the two pairs of methylene protons, which inferred an equatorial orientation of the hydroxyl group at C-4′. The absolute stereochemistry of C-4 was confirmed by Mosher ester derivatization (Ohtani et al., 1991a,b). Both the (*S*)- and (*R*)-MTPA esters of **2** were prepared (Fig. 5) and subjected to ^{1}H NMR analysis (Table 2). In the ^{1}H NMR spectrum, the H-3′$_{ax}$, H-3′$_{eq}$, and Me-2′ signals of **2a** shifted upfield due to the phenyl group, whereas in **2b**, the H-5′$_{ax}$ signal experienced shielding. Therefore, the absolute configuration of **2** at C-4′ was determined as *R*. The reaction of aglycone (**2**) having a secondary hydroxyl group on C-3 proceeded slowly and the yield of corresponding ester was very poor, therefore we used the method based on reaction of free acid with (*S*)- and (*R*)-PGME (Yabuuchi and Kusumi, 2000) for determination of absolute stereochemistry on C-3. The principle of the PGME method is following: a chiral β,β-disubstituted propionic acid is condensed with (*R*)- and (*S*)-PGME. In the diastereomeric pair of PGME amides, the protons of the (*S*)-isomer will have larger upfield chemical shifts than those of the (*R*)-isomer

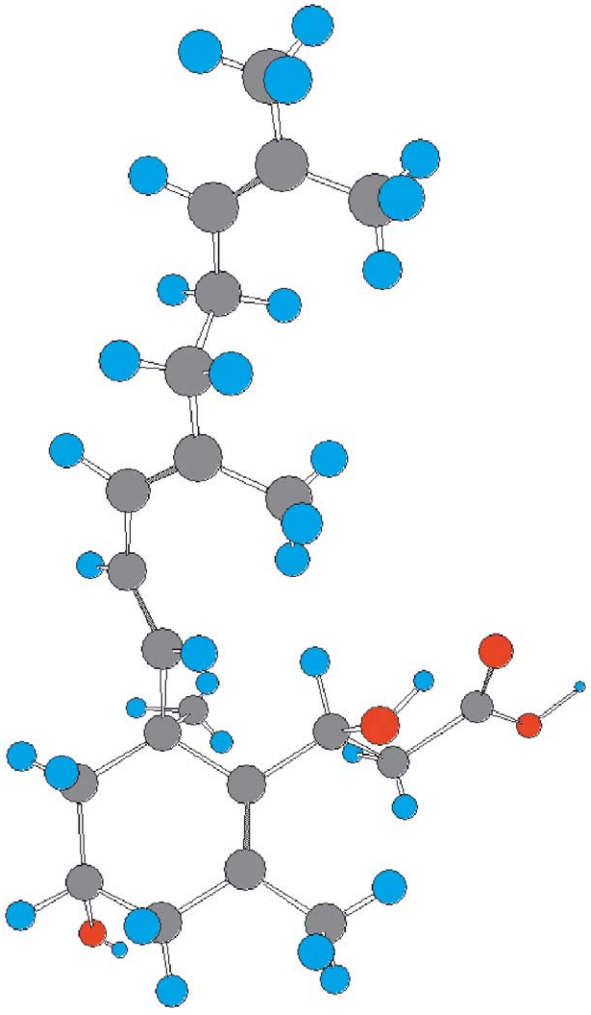

Fig. 4. 3D-MM2 models of compound **2**.

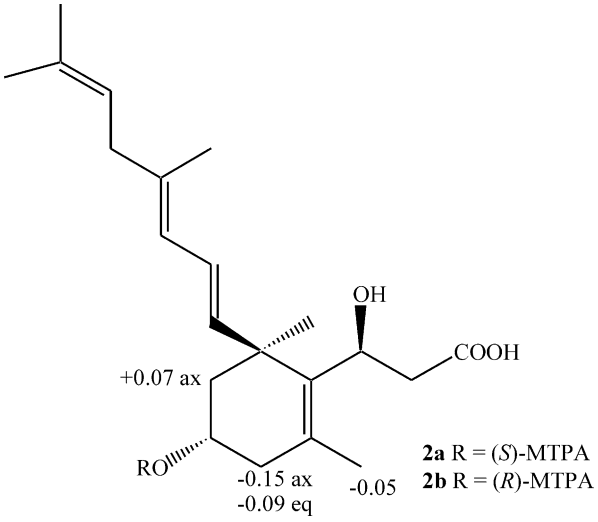

Fig. 5. $\Delta\delta = (\delta_S - \delta_R)$ values (ppm) obtained for the MTPA esters.

Table 1
^{13}C-NMR of woodwardinoside (**1**)

C no.		C no.	
1	177.0	4^1	80.2
2	42.2	5^1	76.8
3	60.7	6^1	62.1
1′	138.5	1^2	104.8
2′	132.9	2^2	75.2
3′	42.2	3^2	86.1
4′	73.8	4^2	84.8
5′	44.3	5^2	76.4
6′	24.2	6^2	62.0
7′	17.9	1^3	105.2
8′	23.5	2^3	75.0
1″	132.6	3^3	76.7
2″	127.0	4^3	70.0
3″	122.3	5^3	76.8
4″	140.7	6^3	62.3
5″	40.2	1^4	104.9
6″	25.1	2^4	75.1
7″	122.8	3^4	78.2
8″	133.9	4^4	70.8
9″	26.3	5^4	67.3
10″	16.4	1^5	111.0
11″	17.6	2^5	84.5
1^1	104.0	3^5	78.6
2^1	86.4	4^5	85.4
3^1	77.3	5^5	62.4

Table 2
^{1}H- and ^{13}C-NMR data of woodwardine (**2**)

No.	^{1}H	^{13}C
1	–	177.0
2	3.27 (1H, *dd*, *J* = 10.8, 5.0); 3.50 (1H, *dd J* = 10.8, 6.0)	42.2
3	3.64 (1H, *dd*, *J* = 6.0, 5.0)	60.7
1′	–	138.5
2′	–	132.9
3′	2.31 eq (1H, *dd*, *J* = 17.2, 6.0); 1.94 ax (1H, *dd*, *J* = 17.2, 9.2)	44.5
4′	3.54 (1H, *ddt*, *J* = 12.0, 9.2, 6.0)	67.0
5′	1.76 eq (1H, *dd*, *J* = 12.0, 6.0); 1.36 ax (1H, *t*, *J* = 12.0)	48.9
6′	–	24.2
7′	1.71 (3H, *s*)	17.9
8′	1.07 (3H, *s*)	23.5
1″	5.62 (1H, *d*, *J* = 15.3)	132.6
2″	6.32 (1H, *dd*, *J* = 15.3, 10.8)	127.0
3″	5.78 (1H, *dq*, *J* = 10.8, 1.3)	122.3
4″	–	140.7
5″	1.95 (2H, *m*)	40.2
6″	2.10 (2H, *m*)	25.1
7″	5.20 (1H, *tq*, *J* = 6.8, 1.2)	122.8
8″	–	133.9
9″	1.73 (3H, *s*)	26.3
10″	1.63 (3H, *d*, *J* = 1.3)	16.4
11″	1.57 (3H, *d*, *J* = 1.2)	17.6

owing to the diamagnetic anisotropic effect of the benzene ring. The stereochemistry of the side chain alcohol was resolved by the ^{1}H NMR analysis of **2c** and **2d** (Fig. 6). The signal of Me on C-2′ of **2d** appeared more downfield than that of **2c**, and the H-8′, H-1″ and H-2″ protons of **2d** were shielded by a phenyl group and shifted more upfield than that of **2c**. These observations indicated that the configuration at C-3 was *S*. On the basis of the above data, the absolute stereochemistry of woodwardine was assigned as **2**, i.e. (1′*E*,1″*E*,3*S*, 3″*E*,4′*R*,6′*S*)-3-[6′-(4″,8″-dimethyl-nona-1″,3″,7″-trienyl)-4′-hydroxy-2′,6′-dimethyl-cyclohex-1′-enyl]-3-hydroxy-propionic acid.

Formation of the compounds **2c**, **d**, which have MTPA ester on β-hydroxyl group, was also observed. The residue was subjected to silica gel preparative TLC with eluent *n*-hexane–acetone (3:1) to yield **2a** and **2c** or **2b** and **2d**, successively. After preparative TLC the yield of compounds **2c**, **d** was negligible and consequently the configuration of β-hydroxyl group was determined with the help of PGME esters. The low yield of esters formation on β-hydroxyl group can be explained due to the fact that it is not an isolated secondary hydroxyl group, but an β-hydroxylacid where different hydroxyl groups have different properties.

The saccharide composition of **1** was identified by GLC (after acidic hydrolysis), and the sugars were identified as glucose, arabinose and xylose. In the ^{1}H NMR spectrum of **1**, five anomeric proton signals at δ_H 4.46, 4.61, 5.01, 5.28, and 6.28 were observed, corresponding to signals at δ_C 104.0, 104.8, 104.9, 105.2, and 111.0, respectively, and indicating that **1** possesses five sugar units. Four anomeric protons (δ_H 4.46, 4.61, 5.01 and 5.28) showed β-glycosidic linkages according to the coupling constants of their anomeric protons (J = 7.5–7.8 Hz). A characteristic furanosyl broad singlet signal (δ_H 6.28, *brs*) was observed. Among the five anomeric carbons of the carbohydrate units, the chemical shift at δ 111.0 demonstrated that one of those was in the α-furanose form (Gorin and Mazurek, 1975). The configuration of all of the other sugars in the pyranose form in **1** was fully defined from the chemical shift and the coupling constant of each of the remaining anomeric protons. From the ^{1}H–^{1}H COSY and TOCSY spectra,

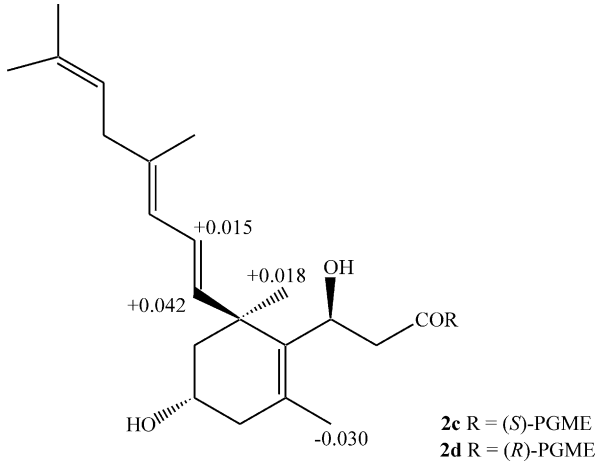

Fig. 6. $\Delta\delta = (\delta_S - \delta_R)$ values (ppm) obtained for the PGME amides.

all proton signals belonging to each sugar moiety in **1** were identified, starting with the anomeric protons. All sugar connectivities were established using NOESY and HMBC experiments. In the NOESY spectrum, cross-peak signals were observed between Glc-H-1^1 and H-4′, Glc-H-1^2 and Glc-H-4^1, Glc-H-1^3 and Glc-H-4^2, Xyl-H-1^4 and Glc-H-2^1, and Ara*f*-H-1^5 and Glc-H-3^2. The HMBC experiment showed long-range correlations between H-1^1 and C-4′, H-1^2 and C-4^1, H-1^3 and C-4^3, H-1^4 and C-2^1, and H-1^5 and C-3^2 (Fig. 1). Thus, the structure of **1** was assigned as (4′*R*)-4′-*O*-[β-D-glucopyranosyl-(1-4)-β-D-arabinofuranosyl-(1-3)-β-D-glucopyranosyl-(1-4)]-[β-D-xylopyranosyl-(1-2)]-β-D-glucopyranosyl-1-4-woodwardine, i.e. woodwardinoside (**1**).

(1′*E*,1″*E*,3*S*,3″*E*,4′*R*,6′*S*)-3-[6-(4,8-Dimethyl-nona-1,3,7-trienyl)-4-hydroxy-2,6-dimethyl-cyclohex-1-enyl]-3-hydroxy-propionic acid (woodwardine, **2**), could be synthesized (see Fig. 7). The mechanism of sesterterpene biosynthesis was recently reviewed (Dewick, 2002). Dimethylallyl diphosphate is the starting biogenic isoprene unit, along with isopentyl diphosphate. Polyprenyl diphosphate synthases (prenyl-transferases) is responsible for the alkylation of dimethylallyl diphosphate and isopentyl diphosphate resides, reactions which provide the polyprenyl diphosphate precursors for the various terpenoid families. In enzymatic cyclization, presence of cation cyclases is possible. At present, the oxidation in the biosynthesis of woodwardine **2** is not yet known; it probably includes some further biosynthetic steps.

3. Experimental

3.1. General experimental procedures

UV spectra were measured in heptane within the range of 200–350 nm by a Cary 118 (Varian) apparatus. Optical rotary dispersion (ORD) measurements were carried out under dry N_2 by a Jasco-500A spectropolarimeter at 24 °C. A Perkin-Elmer Model 1310 (Perkin-Elmer, Norwalk, CT, USA) IR spectrophotometer was used for scanning IR spectroscopy of acids and glycosides as neat films. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^{1}H), 125.7 MHz (^{13}C) in mixture of deuterated pyridine and CD_3OD (v/v 1:1). High- and also low-resolution MS were recorded using a VG 7070E-HF spectrometer (70 eV). HRFABMS (positive and/or negative ion mode) were obtained with a PEG-400 matrix. RP-HPLC was carried out using Shimadzu gradient LC system (Shimadzu, Kyoto, Japan). Gas chromatography analysis was made on a Hewlett Packard HP 5980 gas chromatograph (Hewlett Packard sro, Czech Republic).

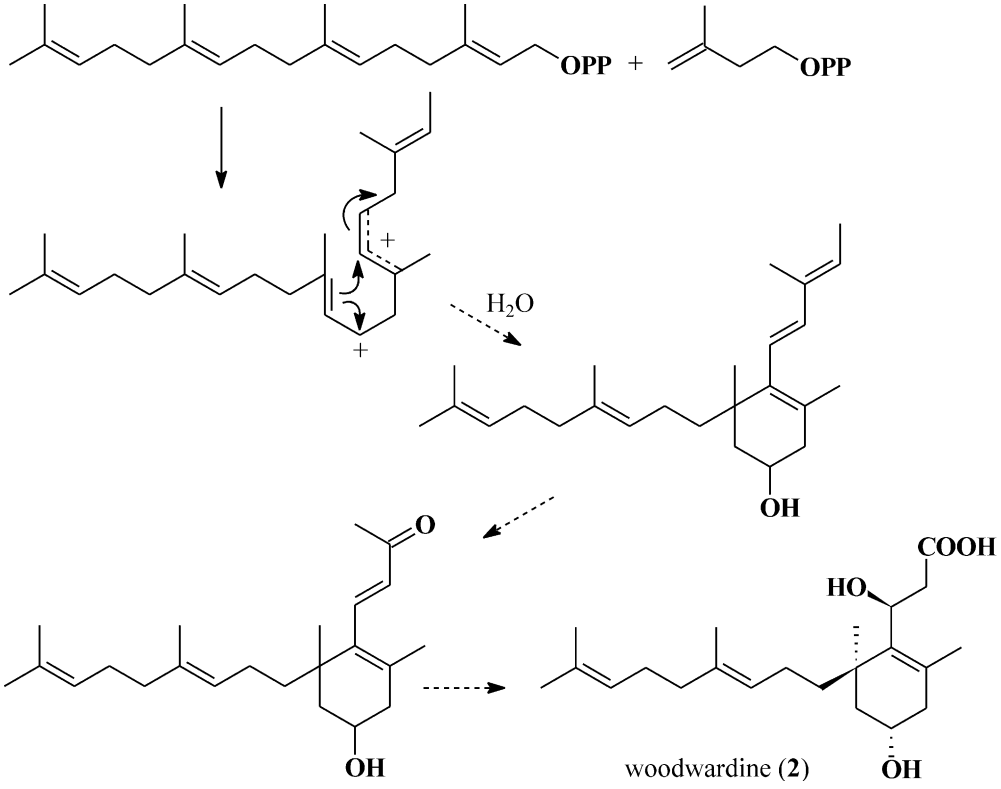

Fig. 7. Proposed biosynthetic pathways of woodwardine (**2**) in fern *Woodwardia virginica*.

3.2. Plant material, extraction and isolation

The specimens of *W. virginica* (L.) Sm. (Blechnaceae), Virginia chain fern, were collected in Montgomery County, Maryland (USA) in October 2002. Fresh fern leaves were extracted with ethanol (on the spot, immediately after collecting) and after that with ethanol–water (70:30). Both extracts were combined and evaporated to small volume. Ethanol–water extracts were separated on a Sephadex LH-20 column eluting with MeOH–H_2O (9:1), yielding three fractions. Fraction B was further fractionated by RP-HPLC on a C18-Bondapack column (30 cm×7.8 mm, flow rate 2.0 ml/min) with ACN–H_2O (1:2) to yield compound **1**.

3.2.1. Acidic hydrolysis of the glycosides

The glycoside (**1**) was refluxed in 2 N HCl (0.5 ml) for 2 h. The aglycone was extracted three times with EtOAc (10 ml). After separating the organic layer, the aqueous phase was neutralized with $NaHCO_3$, lyophilized and the residue was chromatographed on a column of silica gel (10 g), using CH_2Cl_2–MeOH–H_2O (90:10:1) to provide an acid for ^{1}H NMR analysis. The identification and the D or L configuration of sugars was determined using gas chromatography (a glass-capillary column Supelco SPB-1, the [acetylated (+)-2-butyl] derivatives were eluted as a peaks with retention times, which were identical with standards of the appropriate tetraacetyl (+)-2-butyl-saccharides) according to the method stated in Gerwig et al. (1978), with some modifications as previously described (Řezanka and Guschina, 2000).

3.2.2. (S)-MTPA and (R)-MTPA esters

To a CH_2Cl_2 solution (100 μl) of aglycone (0.3 mg), DMAP (1.0 mg), and Et_3N (2 μl), (*R*)-(−)-MTPACl (2.0 mg) was added at room temperature, and was stirred for 3 h. After evaporation of solvent, the residue was purified by silica gel TLC (hexane–AcOEt, 2:1) to provide the (*S*)-MTPA ester as colorless oil.

3.2.3. (R)-MTPA ester of aglycone

The aglycone (0.3 mg) was treated with (*S*)-(+)-MTPACl (2.0 mg) by the same procedure as described above to provide the (*R*)-MTPA ester as colorless oil (Ohtani et al. 1991a,b).

3.2.4. (S)- and (R)-PGME amides of a carboxylic acid

To a stirred solution of a 0.1 mmol carboxylic acid and 0.15 mmol (*S*)-PGME [or (*R*)-PGME] in dry 2 ml DMF were successively added 0.15 mmol BOP, 0.15 mmol HOBT, and *N*-methylmorpholine (200 μl) at 0 C. After the mixture was stirred at room temperature for 3 h, ethyl acetate was added, and the resulting solution was successively washed with 5% HCl, a saturated $NaHCO_3$ solution, and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to give a residue which was chromatographed on silica gel with hexane–ethyl acetate (3:1, v/v) as a developing solvent to afford the amides in 70–80% yield (Yabuuchi and Kusumi, 2000).

(4′*R*)-4′-*O*-[β-D-Glucopyranosyl-(1→4)-β-D-arabinofuranosyl-(1→3)-β-D-glucopyranosyl-(1→4)]-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranosyl-1-4-woodwardine, i.e. woodwardinoside (**1**), colourless powder, $[\alpha]_D^{23}$ −42.0°; UV λ_{max} (EtOH) (log ϵ) 252 nm (2.24); IR (film) ν_{max} 3450 (OH), 1680 (COOH); HRFABMS *m*/*z* 1113.4969 $(M+H)^+$, calculated for $[C_{50}H_{80}O_{27}+H]^+$ 1113.4965; negative LRFABMS *m*/*z* 1111 $[M-H]^-$, 979 $[M-H-132]^-$, 949 $[M-H-162]^-$, 847 $[M-H-2\times132]^-$, 817 $[M-H-162-132]^-$, 685 $[M-H-162-2\times132]^-$, 361 $[M-H-3\times162-2\times132]^-$; ^{1}H-NMR spectrum: 3.71 (1H, *m*, H-4′), 4.46 (1H, *d*, *J*=7.6 Hz, H-1^1), 4.61 (1H, *d*, *J*=7.5 Hz, H-1^2), 5.01 (1H, *d*, *J*=7.8 Hz, H-1^4), 5.28 (1H, *d*, *J*=7.6 Hz, H-1^3), 6.28 (1H, *brs*, H-1^5); ^{13}C-NMR spectrum, see Table 1.

(1′*E*,1″*E*,3*S*,3″*E*,4′*R*,6′*S*)-3-[6-(4,8-Dimethyl-nona-1,3,7-trienyl)-4-hydroxy-2,6-dimethyl-cyclohex-1-enyl]-3-hydroxy-propionic acid = woodwardine (2), white crystals, m.p. 125–127 °C; $[\alpha]_D^{23}$ −71.0° (*c* 0.18, MeOH); UV λ_{max} (EtOH) (log ϵ) 253 nm (2.37); IR (film) ν_{max} 3450 (OH), 2950, 2910, 1981 (COOH); HREIMS *m*/*z* 348.2305 $C_{21}H_{32}O_4$ $[M]^+$, calculated for $[C_{22}H_{34}O_4]^+$ 348.2301; ^{1}H- and ^{13}C-NMR spectra, see Table 2.

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