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Isolation and structure elucidation of four new triterpenoid estersaponins from fruits of *Pittosporum tobira* AIT.

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Abstract—Four new triterpenoid estersaponins, tentatively designated as $IIIA_2$ (**1**), $IIIA_3$ (**2**), $IIIB_2$ (**3**), and $IIIC_4$ (**4**) have been isolated from fruits of *Pittosporum tobira* AIT. and their structures elucidated by spectroscopic and chemical analyses. All the four compounds consist of an acylated pentacyclic triterpenoid aglycone which bears the same oligosaccharide portion. The crude saponin mixture (CIDI) was found to show significant in vitro and in vivo cytotoxicity in different human cancer cell lines. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Pittosporum tobira AIT. (Pittosporaceae) is a broadleaf evergreen shrub, native to China and Japan, and also common on the Mediterranean coasts, with small creamy white flowers, fragrant orange blossoms; the fruits are green and showy, turning to brown capsules that eventually split open, exposing orange seeds. A saponin mixture[1] obtained from its leaves was reported to possess antibiotic activity,[2] and three triterpenoid sapogenins, R_1-barrigenol, its 21-*O*-angelate and 21-*O*-angeloyl-barringtogenol C, have been isolated from the leaves.[3] More recently, two novel C_{69} carotenoids containing an α-tocopherol moiety have been found in its seeds.[4] Our investigation regarded the ethanolic extracts of the fruits of *P. tobira* AIT.; we report herein on the isolation and characterization of four acylated triterpenoid estersaponins with anticancer activity.

2. Results and discussion

A crude saponin mixture, tentatively designated as CIDI,[5] isolated from ethanolic extracts of the fruits, was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) on a 250×4 mm ID ODS Hypersil column, without any preliminary derivatization (Fig. 1).

According to Halsall,[6] triterpenoids containing carbon–carbon double bonds have a maximum absorbance close to 200 nm, largely due to the position and degree of substitution of the double bond. For the detection of the considered structures, we used a wavelength of 210 nm, because of the limitations imposed by the use of chromatographic solvents and additives, with a UV cut-off at 205 nm. On the other hand, we excluded the use of refractive index (RI) detection, as it prevents the application of gradient elution, which is necessary in the case of complex mixtures containing species with large retention differences. In this work, we successfully introduced the use of the evaporative light scattering detector (ELSD) connected in series after the UV detector, under a variety of experimental conditions, in order to circumvent the low UV detectability of the saturated components. Recently, three saponins were identified in the methanolic extracts of leaves from *Hedera helix* L. by HPLC with ELS detection.[7]

Under reversed-phase chromatographic conditions (Fig. 1) we observed for CIDI saponins three main elution zones that we divided according to decreasing polarity: 'zone I' contains the most polar components of the saponin mixture, with retention factors less than 2 ($k' \leq 2$); 'zone II' includes compounds with medium polarity ($2 < k' \leq 8$) and very low UV absorption; 'zone III' is characterized by the presence of the less polar components ($k' > 8$), which indeed showed the highest UV absorption. Zone III represented half by weight of the whole saponin mixture.

Using further RP-HPLC investigations, we were able to identify, in the less polar elution zone III, three groups of

Keywords: *Pittosporum tobira* AIT. fruits; triterpenoid acylated saponins; R_1-barrigenol; reversed-phase HPLC; evaporative light scattering detector (ELSD); anticancer drugs.

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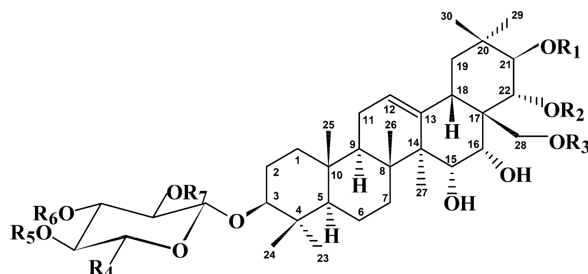
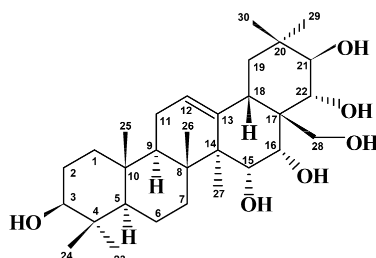


$IIIA_2$ (**1**)	R_1 = 2-acetoxy-2-methylbutanoyl	R_2 = acetyl	R_3 = H	R_4 = COOH	R_5 = α-L-ara*f*	R_6 = α-L-ara*p*	R_7 = β-D-glu*p*
$IIIA_3$ (**2**)	R_1 = angeloyl	R_2 = acetyl	R_3 = H	R_4 = COOH	R_5 = α-L-ara*f*	R_6 = α-L-ara*p*	R_7 = β-D-glu*p*
$IIIB_2$ (**3**)	R_1 = angeloyl	R_2 = H	R_3 = acetyl	R_4 = COOH	R_5 = α-L-ara*f*	R_6 = α-L-ara*p*	R_7 = β-D-glu*p*
$IIIC_4$ (**4**)	R_1 = angeloyl	R_2 = angeloyl	R_3 = H	R_4 = COOH	R_5 = α-L-ara*f*	R_6 = α-L-ara*p*	R_7 = β-D-glu*p*
Compd **5**	R_1 = H	R_2 = H	R_3 = H	R_4 = COOH	R_5 = α-L-ara*f*	R_6 = α-L-ara*p*	R_7 = β-D-glu*p*
Compd **7**	R_1 = H	R_2 = H	R_3 = H	R_4 = COOMe	R_5 = α-L-ara*f*	R_6 = α-L-ara*p*	R_7 = β-D-glu*p*
Compd **8**	R_1 = H	R_2 = H	R_3 = H	R_4 = CH_2OH	R_5 = α-L-ara*f*	R_6 = α-L-ara*p*	R_7 = β-D-glu*p*



R_1-barrigenol[8] (**6**)

saponins (named IIIA, IIIB and IIIC), among which four major triterpenoid saponins, closely related to each other, were considered and tentatively designated as $IIIA_2$ (**1**), $IIIA_3$ (**2**) from group IIIA, $IIIB_2$ (**3**) from group IIIB, and $IIIC_4$ (**4**) from group IIIC.

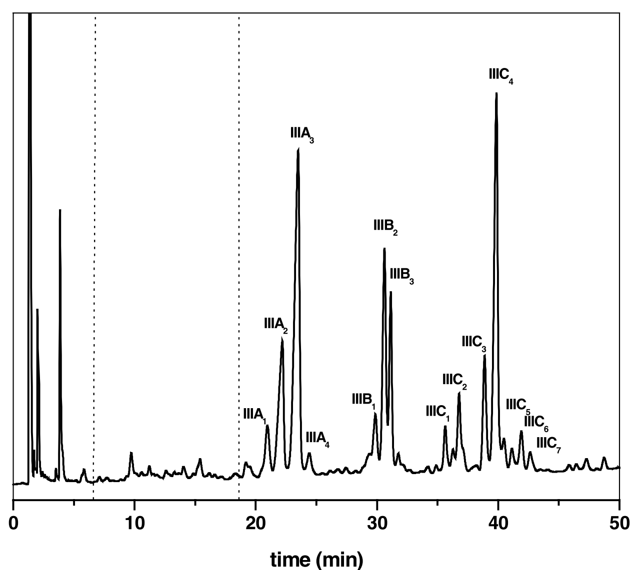


Figure 1. RP-HPLC chromatogram of CIDI. Column: 250×4 mm ID ODS Hypersil. Mobile phase, A: water/acetonitrile 90/10+0.1% acetic acid; B: water/acetonitrile 20/80+0.1% acetic acid; gradient from 25 to 30% B in 15 min (curve 2), to 40% B in 15 min (curve 5), to 45% B in 10 min (curve 6), isocratic for 5 min, to 55% B in 5 min (curve 6), isocratic for 5 min, to 25% B in 5 min (curve 6). Flow rate 1 ml/min. T=40°C. UV detection at 210 nm. Dot lines separate the three elution zones of CIDI mixture.

The four saponins were successfully isolated in an almost pure state (>95%) by repeated preparative and semi-preparative RP-HPLC experiments (Section 3), and fully characterized by comparison between their physico-chemical and spectroscopic properties.

The first ^1H and ^{13}C NMR analyses of $IIIA_2$ (**1**), $IIIA_3$ (**2**), $IIIB_2$ (**3**), and $IIIC_4$ (**4**) revealed a great number of analogies for the four compounds: a rich high-field aliphatic region containing at least seven methylene signals, a series of signals in the region between 3.00 and 5.00 ppm, and the presence of an olefinic signal between 5.40 and 5.50 ppm. Differences were discernible for some olefinic signals, for the resonance frequencies of two mutually coupled double doublets and for some methyl signals in the region between 2.00 and 2.50 ppm. A combined analysis of NMR with MS data suggested the presence of an acylated triterpenoid aglycone with an attached oligosaccharide portion, for all the four saponins.

Alkaline hydrolysis of the crude saponin mixture CIDI afforded a unique deacylated saponin (**5**), thus restraining the differences between the compounds to a diverse pattern of acylation for the oxygenated positions of the aglycone portion. A unique oligosaccharide portion for all the four saponins was then presumed to occur. On acidic hydrolysis under ordinary conditions (HCl/MeOH), **5** gave an aglycone with molecular formula $C_{30}H_{50}O_6$ (**6**), as suggested by combined NMR and FAB-MS data. Interpretation of mono- (1D) and two-dimensional (2D) NMR data allowed us to identify, in compound **6**, an oleane-12-ene skeleton, with six oxygenated positions (3β, 15α, 16α, 21β, 22α, and 28),

corresponding to an already known sapogenol, called R_1-barrigenol.[8] This structure, first isolated by Nozoe[9] from *Barringtonia racemosa* BLUME (Myrtaceae), was also found by White[10] and his co-workers in *Pittosporum undulatum* VENT.

To elucidate the nature of the oligosaccharide chain, the 1D and 2D NMR spectra of compound **5** were carefully examined. The ^{13}C NMR spectrum of **5** displayed four anomeric signals (δ 101.1, 102.7, 103.8 and 107.2), indicating the presence of four sugar units, three of which should be neutral, and one presumably acidic (a uronic acid). The presence of a carboxylic acid functionality was indicated by a carbon signal at δ 176.7 in the ^{13}C NMR spectrum and an absorption maximum at 1720 cm^{-1} in the FT-IR spectrum. Analysis of the 2D-COSY, 2D-TOCSY and 2D-HSQC spectra allowed the assignment of all the ^{1}H and ^{13}C resonances of the oligosaccharide moiety, suggesting the presence of a β-glucopyranose (β-glc*p*), an α-arabinopyranose (α-ara*p*), and an α-arabinofuranose (α-ara*f*). The presence of a β-glucopyranosuronic acid (β-glcA*p*) was also assumed.

On acidic hydrolysis with trifluoroacetic acid (TFA), **5** gave the same pure aglycone obtained with HCl/MeOH (**6**), together with a crude sugar mixture which was analyzed by HPLC, without any further purification, on a (300+150)×4 mm ID APS2 Hypersil columns system (mobile phase, water/acetonitrile 22/78+4 mmol/l ammonium formate. Flow rate 1 ml/min, T=20°C, ELSD at air temperature=85°C, air flow=6.0 l/min). The HPLC chromatogram of the sugar mixture (Fig. 2) showed the presence of the neutral sugars arabinose and glucose in the relative ratio 2:1, obtained by titration with standard solutions of the same sugars. By semi-preparative HPLC (Section 3), the two sugars were isolated and fully characterized by FT-IR and NMR analyses, and definitely identified as arabinose (2 mol equiv.) and glucose (1 mol equiv.).

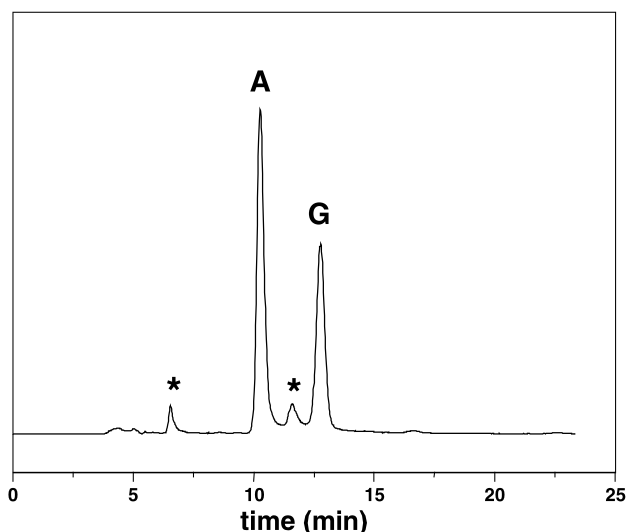


Figure 2. Crude sugar mixture obtained by acidic hydrolysis of **5**. Columns system: (300+150)×4 mm ID APS2 Hypersil. Mobile phase, water/acetonitrile 22/78. Flow rate 1 ml/min. T=20°C, ELSD detection at air temperature=85°C, air flow=6.0 l/min. A=arabinose; G=glucose; asterisks denote unknown impurities.

For the identification of the suspected uronic acid, the alkaline hydrolyzate of CIDI (compound **5**) was methylated with CH_2N_2, providing a monomethyl ester derivative **7**, which was fully characterized by comparison between its physico-chemical and spectroscopic properties. Reduction of **7** with $NaBH_4$ furnished the saponin **8**, which was in turn hydrolyzed with TFA, giving rise to a sugar chain consisting of two arabinose and two glucose units, as determined by HPLC titration under the chromatographic conditions above described for the sugar analysis. This confirmed the nature of the uronic acid as a β-glucopyranosuronic acid residue.

A further HPLC analysis of the sugar mixture obtained by acidic hydrolysis of **5** was performed by the use of a chiroptical detector (ORD) connected in series before the ELS detector (Fig. 3). In this way, we were able to assign the following absolute configurations for the sugars: L(+)-arabinose, D(+)-glucose and D(+)-glucuronic acid.

Comparison of the ^{13}C chemical shifts for anomeric carbons of glycosyl residues with those for the corresponding methyl glycosides[11] suggested the unusual presence of a three substituted β-D-glucopyranosuronic acid unit, with terminal positions for the β-D-glucopyranose, the α-L-arabinopyranose, and the α-L-arabinofuranose units. Several attempts to remove the sugars from the alkaline hydrolyzate of CIDI (compound **5**) by enzymatic hydrolysis (β-glucuronidase and hesperidinase) were almost completely unsuccessful.

In order to establish the glycosilation positions and the connectivities between the sugar chain and the aglycone moiety, a two-dimensional heteronuclear ^{1}H–^{13}C NMR long range correlation (HMBC) experiment was performed. In particular, ^3J cross-peak correlations between the anomeric protons of β-D-glc*p* (4.99 ppm), α-L-ara*p* (4.80 ppm), α-L-ara*f* (5.03 ppm) and C-2 (δ 77.8), C-3 (δ 79.3) and C-4 (δ 75.0) of the pyranose ring of the uronic acid, respectively, confirmed the presence of a three-substituted β-D-glucopyranosuronic unit. The connection of the glucopyranose at C-2, of the arabinopyranose at C-3, and of the arabinofuranose at C-4 was indeed proved by three further correlation peaks observed in the HMBC spectrum, indicative of long range couplings between protons 2, 3 and 4 of the uronic acid and the anomeric carbons of β-D-glc*p*, α-L-ara*p* and α-L-ara*f*, respectively. Moreover, a correlation between the anomeric proton of the β-D-glcA*p* unit (4.51 ppm) and C-3 (δ 91.5) of the aglycone moiety unequivocally permitted the linking position of the saccharide chain to be located on the aglycone itself.

Analogous considerations, deriving from the analysis of NMR data sets collected on the native saponins and on their hydrolyzed products, were indicative of the presence of a common skeleton for all the compounds, consisting of 3-*O*-[β-D-glucopyranosyl-(1→2)]-[α-L-arabinopyranosyl-(1→3)]-[α-L-arabinofuranosyl-(1→4)]-β-D-glucuronopyranosyl-R_1-barrigenol.

The combined analysis of the FAB-MS with ^{1}H and ^{13}C NMR data revealed the presence of three different acyl residues for the saponins $IIIA_2$, $IIIA_3$, $IIIB_2$ and $IIIC_4$. In particular, for saponin $IIIA_2$ (**1**), the functionalization, at

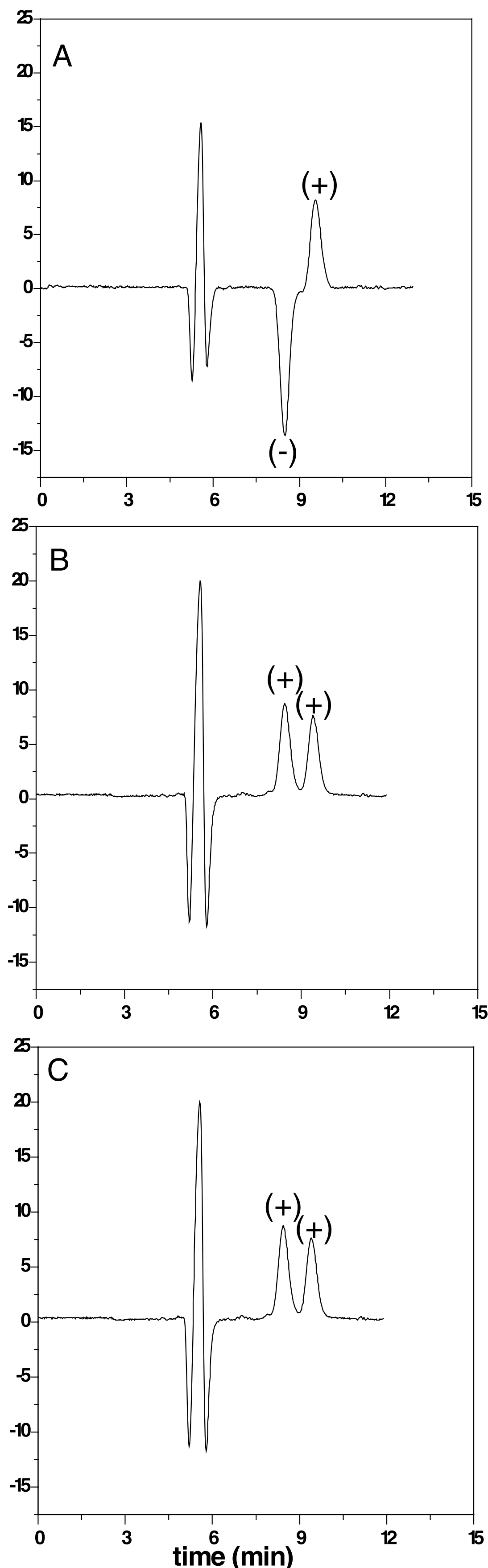


Figure 3. Chiroptical analysis of CIDI sugars. Columns system: (300+150)×4 mm ID APS2 Hypersil. Mobile phase, water/acetonitrile 22/78+4 mmol/l ammonium formate. Flow rate 1 ml/min. T=20°C. ORD detection, gain×16; response: standard. A=standard mixture of D(−)-arabinose and D(+)-glucose; B=standard mixture of L(+)-arabinose and D(+)-glucose; C=sugar mixture obtained by acidic hydrolysis of **8**.

C-21 and C-22, respectively by a 2-acetoxy-2-methylbutanoyl and by an acetyl residue, was suggested by a downfield shift for protons H-21 (5.72 ppm) and H-22 (5.48 ppm), with respect to the correspondent protons in **5** (Table 1). Moreover, in the HMBC experiment, H-21 showed a long-range correlation with the acyl carbon of the 2-acetoxy-2-methylbutanoyl residue, while H-22 showed a long-range correlation with the acyl carbon of the acetyl residue. Saponin IIIA$_3$ (**2**) revealed the presence of an angeloyl (*Z*-2-methyl-2-butenoyl) residue at C-21 and of an acetyl group at C-22. In addition, in this case, H-21 and H-22 were downshifted (5.78 and 5.50 ppm, respectively) and displayed HMBC correlations with the acyl carbon of the angeloyl (170.3 ppm) and with the acyl carbon of the acetyl residue (173.6 ppm), respectively. Saponin IIIB$_2$ (**3**) also showed substitution by both angeloyl and acetyl residues, but at C-21 and C-28, respectively. This was deduced by a downfield shift for H-21 (5.64 ppm) and H-28 (3.86 and 3.99 ppm), while the relative positions of the angeloyl and the acetyl residues were confirmed by HMBC correlations. Saponin IIIC$_3$ (**4**) showed two angeloyl functions at C-21 and C-22, as suggested by a downfield shift of H-21 and H-22 and by their relative HMBC correlations.

By using HPLC, FAB-MS and various NMR techniques, the overall structures of four major saponins isolated from fruits of *P. tobira* AIT. were unequivocally identified as (**1**) 3-*O*-[β-D-glucopyranosyl(1→2)]-[α-L-arabinopyranosyl(1→3)]-[α-L-arabinofuranosyl(1→4)]-β-D-glucuronopyranosyl-21-(2-acetoxy-2-methylbutanoyl)-22-acetyl-R$_1$-barrigenol (saponin IIIA$_2$, molecular formula $C_{61}H_{96}O_{29}$), (**2**) 3-*O*-[β-D-glucopyranosyl(1→2)]-[α-L-arabinopyranosyl(1→3)]-[α-L-arabinofuranosyl(1→4)]-β-D-glucuronopy-ranosyl-21-angeloyl-22-acetyl-R$_1$-barrigenol (saponin IIIA$_3$, molecular formula $C_{59}H_{92}O_{27}$), (**3**) 3-*O*-[β-D-glucopyranosyl(1→2)]-[α-L-arabinopyranosyl(1→3)]-[α-L-arabinofuranosyl(1→4)]-β-D-glucuronopyranosyl-21-angeloyl-28-acetyl-R$_1$-barrigenol (saponin IIIB$_2$, molecular formula $C_{59}H_{92}O_{27}$), and (**4**) 3-*O*-[β-D-glucopyranosyl(1→2)]-[α-L-arabinopyranosyl(1→3)]-[α-L-arabinofuranosyl-(1→4)]-β-D-glucuronopyranosyl-21-angeloyl-22-angeloyl-R$_1$-barrigenol (saponin IIIC$_4$, molecular formula $C_{62}H_{96}O_{27}$).

The aglycone moiety of saponin IIIA$_2$ [21-(2-acetoxy-2-methylbutanoyl)-22-acetyl-R$_1$-barrigenol] was also recently found by Burczyk[12] in *Hacquetia epipactis* (Apiaceae). The aglycone portion of saponin IIIC$_4$ (21-angeloyl-22-angeloyl-R$_1$-barrigenol) was also found by Dimbi[13] and his co-workers in *Dodonaea viscosa* (L.) Jacq. (Sapindaceae) and by Chen[14] in the fruits of *Xanthoceras sorbifolia* BUNGE (Sapindaceae).

The unusual presence of a consecutively 1,2,3,4-tetra-substituted glycosidic linkage in the glucuronic acid part of

Table 1. ^{1}H and ^{13}C NMR chemical shifts δ (ppm) of the aglycone moieties for CIDI saponins **1–4**

Carbon	Saponin IIIA$_2$ (**1**)		Saponin IIIA$_3$ (**2**)		Saponin IIIB$_2$ (**3**)		Saponin IIIC$_4$ (**4**)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	0.98–1.67	39.6	0.98–1.68	39.6	0.97–1.68	40.4	0.98–1.68	38.7
2	1.79–1.87	26.4	1.79–1.87	26.4	1.76–1.97	27.2	1.80–1.80	25.7
3	3.22	92.3	3.22	92.0	3.18	91.9	3.20	90.9
4	–	39.9	–	39.9	–	40.3	–	39.0
5	0.79 d (11.4)	56.3	0.79 d (11.4)	56.3	0.84 d (11.6)	57.1	0.80 d (11.4)	55.2
6	1.43 bd (11.4), 1.58	18.8	1.41–1.56	18.8	1.45 dd (11.6, 4.1), 1.60	19.8	1.40 bd (11.4), 1.60	18.2
7	1.70–1.75	36.7	1.70 bd (12.5), 1.75 bd (12.5)	37.4	1.80–1.80	37.6	1.70–1.75	35.9
8	–	41.6	–	41.8	–	42.3	–	41.1
9	1.57	47.6	1.57	47.7	1.60	48.5	1.60	46.7
10	–	37.6	–	37.3	–	38.1	–	36.6
11	1.90–1.97	24.3	1.90–1.96	24.1	1.94–1.94	25.0	1.95–1.95	23.5
12	5.50 bt (3.7)	126.4	5.50 bt (3.7)	126.8	5.44 bt (3.6)	127.3	5.52 bt (3.6)	126.1
13	–	142.6	–	142.7	–	143.5	–	141.9
14	–	47.2	–	47.3	–	47.8	–	47.1
15	3.82 d (4.3)	68.1	3.84	68.1	3.88 d (3.9)	68.6	3.78 d (4.8)	67.3
16	3.88 d (4.3)	72.9	3.91	73.3	3.98 d (3.9)	73.2	3.84 d (4.8)	72.8
17	–	48.5	–	48.2	–	48.3	–	47.5
18	2.52 dd (3.9, 13.5)	41.1	2.56	41.1	2.49 dd (4.2, 13.6)	42.2	2.50	40.2
19	1.22 dd (3.9, 13.5), 2.56 t (13.5)	47.1	1.21 2.62, t (13.6)	47.3	1.21 dd (4.2, 13.6), 2.64 t (13.6)	48.0	1.20–2.50	46.2
20	–	36.6	–	36.2	–	36.4	–	35.5
21	5.72 d (10.0)	80.3	5.78 d (9.9)	80.3	5.64 d (9.8)	82.7	5.86 d (10.3)	79.0
22	5.48 d (10.0)	73.5	5.50 d (9.9)	75.1	3.96 d (9.8)	72.5	5.55 d (10.3)	73.0
23	1.09 s	28.1	1.09 s	28.1	1.08 s	29.1	1.08 s	27.3
24	0.89 s	16.6	0.89 s	16.6	0.89 s	16.8	0.89 s	16.0
25	1.01 s	15.8	1.00 s	15.9	1.01 s	16.1	1.01 s	15.2
26	1.02 s	17.5	1.02 s	17.3	1.02 s	17.8	1.02 s	16.9
27	1.40 s	20.7	1.40 s	20.6	1.38 s	21.1	1.43 s	20.0
28	3.11 d (11.4), 3.31 d (11.4)	63.7	3.14 d (11.1), 3.31 d (11.1)	64.0	3.86 d (10.7), 3.99 d (10.7)	66.3	3.10 d (11.4), 3.35 d (11.4)	62.8
29	0.92 s	29.2	0.90 s	29.2	0.91 s	29.8	0.91 s	28.5
30	1.06 s	19.6	1.09 s	19.7	1.07 s	19.9	1.11 s	19.1
31	–	173.4	–	170.3	–	170.3	–	170.3
32	–	82.2	–	128.3	–	129.9	–	128.1
33	1.97–1.80	30.4	6.18 q (7.2)	139.1	6.11 q (7.1)	138.1	6.14 q (7.2)	139.0
34	0.95 t (7.5)	6.75	1.95	15.7	2.01	15.9	1.90	15.3
35	1.57 s	20.6	1.87	20.3	1.98	20.9	1.85	20.1
36	–	172.1	–	173.6	–	172.8	–	170.3
37	2.10 s	19.9	2.04 s	20.8	2.08 s	20.9	–	128.1
38	–	173.4	–	–	–	–	6.14 q (7.2)	139.0
39	2.10 s	21.1	–	–	–	–	1.90	15.3
40	–	–	–	–	–	–	1.85	20.1

Due to severe overlapping in the ^{1}H spectrum, only detectable signal multiplicities and relative *J*'s (Hz) are reported.

CIDI saponins was previously found only by Higuchi[15] in a triterpenoid saponin obtained from the leaves of *P. undulatum*, and later by Seo[16] in plant samples from *Pittosporum viridiflorum* SIMS; in the first case, the corresponding sapogenin[17] was an acylated derivative of A_1-barrigenol,[8] which differs from R_1-barrigenol for the absence of the oxygenated position at C-21. In the second case, the sugar chain differed for the coexistence of arabinopyranose and arabinofuranose with opposite absolute stereochemistry. To the best of our knowledge, however, saponins **1**, **2**, **3**, **4** are new natural products, and the described derivatives (compounds **5**, **7**, and **8**) have never been reported in the literature.

The crude ethanolic extract CIDI was found to show antiproliferative and apoptotic effect on human cell lines of colon adenocarcinoma, melanotic melanoma, breast carcinoma, pancreas adenocarcinoma, neuroblastoma and medulloblastoma.[18–21] The effects on cellular growth are concentration- and treatment time-dependent, and should be attributed to the blocking of G_0-G_1 cycle phase and to a significant apoptosis induction. In the acute toxicity test, performed in mice and rats, the LD_{50} was 25 mg/kg and 1275 mg/kg in the i.p. and oral routes, respectively. By intravenous administration, the dose of 5 mg/kg was found to be the maximal, with not lethal and side-effect levels. The anticancer activity was evaluated against the main murine tumor models (sarcoma 180, ascitic and solid Lewis lung carcinoma, ascitic and solid B16 melanotic melanoma).

With regard to topical administration, Phase I and II double-blind studies with a CIDI containing topicum (0.5%) have demonstrated a comparable activity with respect to a 5-fluorouracyl containing topical formulation (Efudix®, 5%), in the treatment of human cutaneous basaliomas. A Phase III pharmacological study with a 2% CIDI cream recently revealed a significantly higher efficacy ($p<0.05$) than that of Efudix®, with an optimal tolerance. The activities of the isolated saponins have not been tested yet.

Table 2. ^{1}H and ^{13}C NMR chemical shifts δ [ppm] of the carbohydrate moieties for CIDI saponins **1–4**

Position	Saponin IIIA$_2$ (**1**)		Saponin IIIA$_3$ (**2**)		Saponin IIIB$_2$ (**3**)		Saponin IIIC$_4$ (**4**)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
β-D-glcA*p*								
1′	4.57 d (5.2)	104.2	4.57	104.2	4.58 d (6.5)	105.4	4.60 d (4.6)	103.7
2′	3.97	78.2	3.97	78.2	3.96	79.9	3.92	78.1
3′	3.96	79.4	3.96	79.4	3.95	80.2	3.94	78.7
4′	3.84	74.8	3.84	74.8	3.96	75.0	3.84	74.7
5′	3.84	76.3	3.84	76.3	3.89	77.2	3.84	76.8
6′	–	a	–	a	–	a	–	a
β-D-glc*p*								
1′	5.02 d (7.9)	101.5	5.02 d (7.9)	101.5	5.02 d (8.1)	103.3	4.95 d (8.1)	101.3
2′	3.27 dd (7.9, 9.7)	74.8	3.27	74.8	3.24 dd (8.1, 9.2)	76.4	3.21	74.5
3′	3.48 t (9.2)	76.6	3.48 t (9.2)	76.6	3.41 t (9.2)	78.2	3.46 t (9.2)	76.3
4′	3.19 t (9.2)	71.2	3.19	71.2	3.19 (9.2)	72.5	3.20	70.8
5′	3.38 ddd (1.8, 7.5, 9.2)	77.1	3.38	77.1	3.34	78.2	3.38	76.6
6′	3.62–3.89	61.9	3.62–3.89	61.9	3.64–3.87	63.4	3.62–3.87	61.8
α-L-ara*p*								
1′	4.85	103.0	4.85 d (5.5)	103.0	4.93 d (7.9)	104.3	4.80 d (7.5)	102.7
2′	3.63	71.7	3.63	71.7	3.61	73.2	3.59	71.6
3′	3.64	73.0	3.64	73.0	3.54	74.9	3.61	72.8
4′	3.90	68.9	3.90	68.9	3.80	70.3	3.90	68.7
5′	3.62–3.92	66.5	3.62–3.92	66.5	3.53–3.89	67.6	3.64–3.86	66.3
α-L-ara*f*								
1′	5.10 bs	107.5	5.10 bs	107.5	5.15 bs	109.0	5.03 bs	107.2
2′	4.09 bd (1.7)	81.2	4.09 bs	81.2	3.99	82.4	4.05 bs	80.9
3′	3.86	77.8	3.86	77.8	3.82	79.9	3.84	77.5
4′	4.43 q (4.8)	85.2	4.43 q (4.2)	85.2	4.47	87.5	4.39 q (4.3)	84.9
5′	3.71 dd (5.7, 12.5), 3.79 dd (3.7, 12.5)	61.8	3.71 dd (5.1, 12.0), 3.79 dd (3.7, 12.0)	61.8	3.68 dd (4.9, 12.5), 3.72 dd (3.7, 12.5)	63.4	3.69–3.77	61.8

Due to severe overlapping in the ^{1}H spectrum, only detectable signal multiplicities and relative *J*′s (Hz) are reported.
[a] Detectable only in the alkaline hydrolyzate of CIDI (compound **5**).

3. Experimental

3.1. General experimental procedures

The NMR spectra were recorded using a Bruker DRX600 spectrometer at 323 K for all compounds, except for **6**, whose NMR spectra were recorded at 298 K. All the measurements were performed by dissolving the samples (15 mg) in 0.5 ml of CD_3OD/D_2O (50/50, v/v). All the 2D NMR spectra were acquired in the phase-sensitive mode with the transmitter set at the solvent resonance and time proportional phase increment (TPPI) used to achieve frequency discrimination in the ω_1 dimension. The standard pulse sequence and phase cycling were used for COSY, TOCSY, HSQC and HMBC spectra. The NMR data were processed on a Silicon Graphic Indigo2 Workstation using UXNMR software.

Optical rotations were measured on a Jasco P-1030 polarimeter. FT-IR spectra (KBr) were recorded on a Jasco FT/IR 430 spectrometer. Mass spectra were obtained by electrospray ionization (ESI) on a Waters ZMD single quadrupole instrument, equipped with a Model 75–72 Whatman nitrogen generator and a Model Harvard Pump 11 microprocessor single syringe. Melting points, which are uncorrected, were determined using a Büchi B-545 microscope.

HPLC analytical separations were performed on a Waters 2690 Separation Module, equipped with a Rheodyne Model 8125 20-μl injector. Different detectors were used, including a Model M486 programmable multi-wavelength detector, a Model PL-EMD 960 evaporative light scattering detector (Polymer Laboratories), and a Jasco OR-990 chiroptical detector. Semi- and preparative separations were carried out on a Waters Delta Prep 3000 chromatographic system, equipped with a Rheodyne Model 7010 5-ml loop injector. Chromatographic data were collected and processed using the Millennium 2010 Chromatography Manager software. Thin layer chromatography was carried out on 0.25 mm silica gel coated plates (Merck 60 F_{254}) using UV detection.

3.2. Extraction and isolation

The green fruits of *P. tobira* AIT. were collected from the Mediterranean coasts of central Italy (Lazio), over years 1992–1996, in different periods of the year. The fresh material, after washing and mechanical mincing, was kept in ethanol 95% (50/50, w/w) for at least 30 days. Chloroform was then added to the hydroalcoholic solution (50/50, v/v), and the organic phase was separated and concentrated at 40°C under reduced pressure to obtain a volume of about 1/30 of the starting solution. The crude saponin mixture was precipitated with 2-propanol (1/7, v/v) and filtered on Celite® 521; the green residue was then dissolved in ethanol/water (80/20, v/v) and filtered again on charcoal. Removal of the solvent under reduced pressure provided a white amorphous water-soluble powder, named CIDI (1.5 g of white powder per kilogram of fresh green fruits).

The crude saponin mixture CIDI was submitted to a preliminary purification by preparative RP-HPLC on an Amberchrom CG-161s, 20–50 μm (190×50 mm ID) column [mobile phase, A: water/acetonitrile 90/10+0.1% acetic acid; B: water/acetonitrile/2-propanol 20/40/40+0.1% acetic acid; at 35% B for 20 min. (isocratic), to 75% B in 15 min (curve 6), at 75% B for 30 min (isocratic), to 35% B in 5 min (curve 6). Flow rate 20 ml/min, UV detection at 220 nm]. Fractions IIIA, IIIB, and IIIC were collected (54–80% purity, checked by analytical RP-HPLC).

The saponin-containing fraction IIIA was purified by semi-preparative RP-HPLC on a Hyperprep ODS 12 μm (185×25 mm ID) column [mobile phase, A: water/acetonitrile 90/10+0.1% acetic acid; B: water/acetonitrile/2-propanol 20/40/40+0.1% acetic acid; C: water/acetonitrile 20/80+0.1% acetic acid; linear gradient from 35% B to 35% C in 15 min, to 40% C in 20 min, to 45% B in 5 min, to 55% C in 5 min, to 35% B in 5 min. Flow rate 15 ml/min, UV detection at 220 nm]. Fractions $IIIA_2$ (**1**, about 80 mg per gram of CIDI) and $IIIA_3$ (**2**, about 55 mg per gram of CIDI) were collected (95–99% purity, checked by analytical RP-HPLC).

The saponin-containing fraction IIIB was purified by semi-preparative RP-HPLC under the same chromatographic conditions used for fraction IIIA, affording saponin $IIIB_2$ (**3**, about 45 mg per gram of CIDI) (95–99% purity, checked by analytical RP-HPLC).

The saponin-containing fraction IIIC was purified by semi-preparative RP-HPLC on a Hyperprep ODS 12 μm (185×25 mm ID) column [mobile phase, A: water/acetonitrile 90/10+0.1% acetic acid; B: water/acetonitrile/2-propanol 20/40/40+0.1% acetic acid; linear gradient from 25% B to 30% B in 15 min, to 40% B in 15 min, to 45% B in 10 min, at 45% B for 5 min (isocratic), to 55% B in 5 min, at 55% B for 5 min (isocratic), to 25% B in 5 min. Flow rate 15 ml/min, UV detection at 220 nm]. Fraction $IIIC_4$ (**4**, about 60 mg per gram of CIDI) was collected (96–99% purity, checked by analytical RP-HPLC).

3.2.1. 3-*O*-[β-D-Glucopyranosyl(1→2)]-[α-L-arabinopyranosyl(1→3)]-[α-L-arabinofuranosyl(1→4)]-β-D-glucuronopyranosyl-21-(2-acetoxy-2-methylbutanoyl)-22-acetyl-R_1-barrigenol (saponin $IIIA_2$, 1). Amorphous white powder. Mp 241–243°C. $[\alpha]_D^{25}=-25.5$ (c=0.21, MeOH/water 50/50; purity: 95%). Anal. calcd for $C_{61}H_{96}O_{29}$: C, 56.65; H, 7.48. Found: C, 56.43; H, 7.45. IR ν_{max}: 3418, 2961, 1705, 1646, 1457, 1389, 1241, 1161, 1080, 1044 cm^{-1}. ^1H NMR signals as given in Tables 1 and 2. ^{13}C NMR signals as given in Tables 1 and 2. ESI-MS (pos.): m/z found 1315.5 ($[M+Na]^+$), $C_{61}H_{96}O_{29}$ requires 1292.6037 (monoisotopic).

3.2.2. 3-*O*-[β-D-Glucopyranosyl(1→2)]-[α-L-arabinopyranosyl(1→3)]-[α-L-arabinofuranosyl(1→4)]-β-D-glucuronopyranosyl-21-angeloyl-22-acetyl-R_1-barrigenol (saponin $IIIA_3$, 2). Amorphous white powder. Mp 250–252°C. $[\alpha]_D^{25}=-20.3$ (c=0.24, MeOH/water 50/50; purity: 96%). Anal. calcd for $C_{59}H_{92}O_{27}$: C, 57.46; H, 7.52. Found: C, 57.23; H, 7.49. IR ν_{max}: 3418, 2961, 1705, 1646, 1457, 1389, 1241, 1161, 1080, 1044 cm^{-1}. ^1H NMR signals as given in Tables 1 and 2. ^{13}C NMR signals as given in Tables 1 and 2. ESI-MS (pos.): m/z found 1255.4 ($[M+Na]^+$), $C_{59}H_{92}O_{27}$ requires 1232.5826 (monoisotopic).

3.2.3. 3-*O*-[β-D-Glucopyranosyl(1→2)]-[α-L-arabinopyranosyl(1→3)]-[α-L-arabinofuranosyl(1→4)]-β-D-glucuronopyranosyl-21-angeloyl-28-acetyl-R_1-barrigenol (saponin $IIIB_2$, 3). Amorphous white powder. Mp 216–218°C. $[\alpha]_D^{25}=-6.4$ (c=0.24, MeOH/water 50/50; purity: 99%). Anal. calcd for $C_{59}H_{92}O_{27}$: C, 57.46; H, 7.52. Found: C, 57.20; H, 7.50. IR ν_{max}: 3418, 2961, 1705, 1646, 1457, 1389, 1241, 1161, 1080, 1044 cm^{-1}. ^1H NMR signals as given in Tables 1 and 2. ^{13}C NMR signals as given in Tables 1 and 2. ESI-MS (pos.): m/z found 1250.0 ($[M+NH_4]^+$), $C_{59}H_{92}O_{27}$ requires 1232.5826 (monoisotopic).

3.2.4. 3-*O*-[β-D-Glucopyranosyl(1→2)]-[α-L-arabinopyranosyl(1→3)]-[α-L-arabinofuranosyl(1→4)]-β-D-glucuronopyranosyl-21-angeloyl-22-angeloyl-R_1-barrigenol (saponin $IIIC_4$, 4). Amorphous white powder. Mp 261–263°C. $[\alpha]_D^{25}=-30.0$ (c=0.25, CH_3CN/water 70/30; purity: 95%). Anal. calcd for $C_{62}H_{96}O_{27}$: C, 58.48; H, 7.60. Found: C, 58.26; H, 7.58. IR ν_{max}: 3418, 2961, 1705, 1646, 1457, 1389, 1241, 1161, 1080, 1044 cm^{-1}. ^1H NMR signals as given in Tables 1 and 2. ^{13}C NMR signals as given in Tables 1 and 2. ESI-MS (pos.): m/z found 1295.6 ($[M+Na]^+$), $C_{62}H_{96}O_{27}$ requires 1272.6139 (monoisotopic).

3.3. Alkaline hydrolysis of CIDI providing 5

The crude saponin mixture named CIDI (5.0 g) was dissolved in MeOH (170 ml) containing 4% aqueous K_2CO_3 (20 ml) and the solution was refluxed for 1.5 h. The mixture was concentrated under reduced pressure to remove the organic solvent, and the resulting aqueous solution was freeze-dried (P=5×10^{-2} mbar, T=5°C). The crude alkaline hydrolyzate (5.4 g) was purified by preparative RP-HPLC [μ-Bondapack C_{18}, 15–20 μm, 150 Å (100×40 mm ID)+Deltapack C_{18}, 15 μm, 100 Å (100×40 mm ID) columns system, mobile phase A: water/acetonitrile 90/10+0.1% acetic acid, B: water/acetonitrile 20/80+0.1% acetic acid; at 10% B for 10 min (isocratic), to 50% B in 20 min (curve 6), to 90% B in 10 min (curve 6), at 90% B for 10 min (isocratic). Flow rate 50 ml/min, UV detection at 210 nm], affording pure **5** (1.2 g, 1.1 mmol).

3.3.1. Compound 5. White amorphous powder. Mp 229–231°C. $[\alpha]_D^{20}=-14.8$ (c=0.52, MeOH/water 50/50). Anal. calcd for $C_{52}H_{84}O_{25}$: C, 56.31; H, 7.63. Found: C, 56.10; H, 7.60. IR ν_{max}: 3288, 2935, 1720, 1654, 1559, 1077 cm^{-1}. ^1H NMR aglycone moiety: δ 5.45 (bt, 1H, J=3.8 Hz, H-12), 4.01 (bd, 1H, J=3.5 Hz, H-16), 3.94 (d, 1H, J=10.0 Hz, H-21), 3.90 (1H, H-15), 3.76 (d, 1H, J=10.0 Hz, H-22), 3.42 (d, 1H, J=11.4 Hz, H-28), 3.28 (d, 1H, J=11.4 Hz, H-28), 3.22 (1H, H-3), 2.38 (t, 1H, J=13.5 Hz, H-19), 2.30 (dd, 1H, J=3.8, 13.5 Hz, H-18), 1.93 (1H, H-11), 1.86 (1H, H-11), 1.83 (2H, H-2), 1.70 (2H, H-7), 1.68 (1H, H-1), 1.58 (1H, H-6), 1.53 (1H, H-9), 1.43 (dq, 1H, J=4.1, 12.0 Hz, H-6), 1.34 (s, 3H, H-27), 1.11 (dd, 1H, J=3.9, 13.5 Hz, H-19), 1.08 (s, 3H, H-23), 1.07 (s, 3H, H-30), 1.03 (1H, H-1), 1.02 (s, 3H, H-26), 0.98 (s, 3H, H-25), 0.91 (s, 3H, H-29), 0.89 (s,

3H, H-24), 0.80 (d, 1H, J=12.0 Hz, H-5). ^{1}H NMR carbohydrate moieties: β-D-glcA*p*, δ 4.51 (d, 1H, J=6.4 Hz, H-1′), 3.91 (1H, H-2′), 3.91 (1H, H-3′), 3.72 (1H, H-4′), 3.72 (1H, H-5′); β-D-glc*p*, δ 4.99 (d, 1H, J=7.6 Hz, H-1′), 3.87 (1H, H-6′), 3.61 (1H, H-6″), 3.46 (t, 1H, J=9.2 Hz, H-3′), 3.37 (1H, H-5′), 3.24 (1H, H-2′), 3.19 (t, 1H, J=9.2 Hz, H-4′); α-L-ara*p*, δ 4.80 (d, 1H, J=7.9 Hz, H-1′), 3.90 (1H, H-4′), 3.89 (1H, H-5′), 3.62 (1H, H-5″), 3.62 (1H, H-3′), 3.57 (1H, H-2′); α-L-ara*f*, δ 5.03 (bs, 1H, H-1′), 4.39 (q, 1H, J=4.2 Hz, H-4′), 4.06 (bs, 1H, H-2′), 3.82 (1H, H-3′), 3.77 (dd, 1H, J=3.8, 12.3 Hz, H-5′), 3.69 (dd, 1H, J=5.8, 12.3 Hz, H-5″). ^{13}C NMR aglycone moiety: δ 142.8 (s, C-13), 125.3 (d, C-12), 91.5 (d, C-3), 78.8 (d, C-21), 75.2 (d, C-22), 71.7 (d, C-16), 67.3 (d, C-15), 65.5 (t, C-28), 55.4 (d, C-5), 47.6 (s, C-17), 47.1 (t, C-19), 47.1 (s, C-14), 47.0 (d, C-9), 41.1 (s, C-8), 40.7 (d, C-18), 39.7 (s, C-4), 39.0 (t, C-1), 36.6 (s, C-10), 36.0 (t, C-7), 35.4 (s, C-20), 29.4 (q, C-29), 27.3 (q, C-23), 25.8 (t, C-2), 23.4 (t, C-11), 20.1 (q, C-27), 18.3 (t, C-6), 18.1 (q, C-30), 17.0 (q, C-26), 16.0 (q, C-24), 15.3 (q, C-25). ^{13}C NMR carbohydrate moieties: β-D-glcA*p*, δ 176.7 (s, C-6′), 103.8 (s, C-1′), 79.3 (d, C-3′), 77.8 (d, C-2′), 76.9 (d, C-5′), 75.0 (d, C-4′); β-D-glc*p*, δ 101.1 (s, C-1′), 76.7 (d, C-5′), 76.3 (d, C-3′), 74.5 (d, C-2′), 70.8 (d, C-4′), 61.8 (t, C-6′); α-L-ara*p*, δ 102.7 (s, C-1′), 72.7 (d, C-3′), 71.6 (d, C-2′), 68.7 (d, C-4′), 66.3 (t, C-5′); α-L-ara*f*, δ 107.2 (s, C-1′), 84.9 (d, C-4′), 81.1 (d, C-2′), 77.6 (d, C-3′), 61.8 (t, C-5′). ESI-MS (pos.): *m/z* found 1109.2 ([M+H]$^{+}$), 1126.0 ([M+NH$_4$]$^{+}$), $C_{52}H_{84}O_{25}$ requires 1108.5302 (monoisotopic).

3.4. Acidic hydrolysis of compound 5 providing R$_1$-barrigenol (6)

Compound **5** (150 mg, 0.13 mmol) in MeOH (10 ml) was refluxed in 2 M HCl (10 ml) for 3 h, following its complete disappearance from the reaction liquid phase by RP-HPLC on a 250×4 mm ID ODS Hypersil column [mobile phase, A: water/acetonitrile 90/10+0.1% acetic acid; B: water/acetonitrile 20/80+0.1% acetic acid; at 15% B for 2 min (isocratic), to 50% B in 15 min (curve 6), to 90% B in 5 min (curve 6), to 15% B in 5 min (curve 6). Flow rate 1 ml/min, T=40°C, UV detection at 210 nm and ELS detection at air temperature=80°C, air flow=6.0 l/min]. During reflux, a white precipitate was observed. Partial removal of the solvent under reduced pressure and cooling of the residue to 4°C overnight provided a white fine solid which was collected by ultra filtration on a Low Binding Durapore® 0.45 μm ultrafree-CL unit (Millipore Corporation, Bedford, MA, USA). The precipitate was then washed with cold water and freeze-dried (P=5×10^{-2} mbar, T=5°C). The residue was crystallized from MeOH to give compound **6** (60 mg, yield 92%), corresponding to R$_1$-barrigenol.[8]

3.4.1. Compound 6. Colorless needles. Mp 310–312°C. $[\alpha]_D^{20}$=+41.0 (c=0.30, dioxane). Anal. calcd for $C_{30}H_{50}O_6$: C, 71.11; H, 9.95. Found: C, 70.83; H, 9.91. IR ν_{max}: 3350, 2962, 1457, 1388, 1034 cm^{-1}. ^{1}H NMR and ^{13}C NMR (solvent: pyridine-d_5) data are superimposable (±0.1 ppm) with those reported in the literature[22] for R$_1$-barrigenol. ESI-MS (pos.): *m/z* found 524.4 ([M+NH$_4$]$^{+}$), 1030.4 ([2M+NH$_4$]$^{+}$), 1536.3 ([3M+NH$_4$]$^{+}$), $C_{30}H_{50}O_6$ requires 506.3607 (monoisotopic).

3.5. Methylation of compound 5 providing 7

Compound **5** (100 mg, 0.090 mmol) was dissolved in MeOH (10 ml) and the solution stirred at room temperature in a dark flask. An ethereal solution of CH_2N_2 (5 ml) (obtained from Diazald®, Aldrich Chemical Co.) was added to the solution, and the reaction progress was monitored by RP-HPLC, as described for the acidic hydrolysis of compound **5**. Solvent removal and crystallization of the solid residue from Et$_2$O gave the monomethyl ester **7** (86 mg, yield 81%).

3.5.1. Compound 7. White plates. Mp 248–250°C. $[\alpha]_D^{20}$=−10.5 (c=0.50, MeOH/water 50/50). Anal. calcd for $C_{53}H_{86}O_{25}$: C, 56.67; H, 7.72. Found: C, 56.44; H, 7.69. IR ν_{max}: 3421, 2950, 1742, 1631, 1077 cm^{-1}. ^{1}H NMR aglycone moiety: δ 5.40 (bt, 1H, J=3.8 Hz, H-12), 3.93 (d, 1H, J=10.0 Hz, H-21), 3.89 (bd, 1H, J=3.5 Hz, H-16), 3.89 (1H, H-15), 3.75 (d, 1H, J=10.0 Hz, H-22), 3.45 (d, 1H, J=11.4 Hz, H-28), 3.28 (d, 1H, J=11.4 Hz, H-28), 3.14 (1H, H-3), 2.40 (t, 1H, J=13.5 Hz, H-19), 2.27 (dd, 1H, J=3.8, 13.5 Hz, H-18), 1.90 (2H, H-11), 1.75 (2H, H-2), 1.71 (2H, H-7), 1.64 (1H, H-1), 1.54 (1H, H-6), 1.54 (dq, 1H, J=4.1, 12.0 Hz, H-6), 1.52 (1H, H-9), 1.34 (s, 3H, H-27), 1.10 (dd, 1H, J=3.9, 13.5 Hz, H-19), 1.06 (s, 3H, H-23), 1.04 (s, 3H, H-25), 1.01 (s, 3H, H-30), 1.00 (s, 3H, H-26), 0.96 (1H, H-1), 0.96 (s, 3H, H-29), 0.87 (s, 3H, H-24), 0.71 (d, 1H, J=12.0 Hz, H-5). ^{1}H NMR carbohydrate moieties: β-D-glcA*p*, δ 4.56 (d, 1H, J=6.4 Hz, H-1′), 4.06 (1H, H-5′), 3.96 (1H, H-2′), 3.95 (1H, H-3′), 3.90 (1H, H-4′); β-D-glc*p*, δ 4.97 (d, 1H, J=7.6 Hz, H-1′), 3.87 (1H, H-6′), 3.61 (1H, H-6″), 3.45 (t, 1H, J=9.2 Hz, H-3′), 3.35 (1H, H-5′), 3.24 (1H, H-2′), 3.19 (t, 1H, J=9.2 Hz, H-4′); α-L-ara*p*, δ 4.80 (d, 1H, J=7.9 Hz, H-1′), 3.90 (1H, H-4′), 3.89 (1H, H-5′), 3.61 (1H, H-5′), 3.59 (1H, H-2′), 3.58 (1H, H-5″); α-L-ara*f*, δ 4.90 (bs, 1H, H-1′), 4.33 (q, 1H, J=4.2 Hz, H-4′), 4.04 (bs, 1H, H-2′), 3.85 (1H, H-3′), 3.75 (dd, 1H, J=3.7, 12.3 Hz, H-5′), 3.66 (dd, 1H, J=5.8, 12.3 Hz, H-5″). ^{13}C NMR aglycone moiety: δ 143.9 (s, C-13), 125.9 (d, C-12), 92.2 (d, C-3), 79.4 (d, C-21), 76.1 (d, C-22), 72.5 (d, C-16), 68.2 (d, C-15), 66.7 (t, C-28), 56.5 (d, C-5), 48.3 (s, C-17), 48.1 (d, C-9), 48.1 (t, C-19), 47.8 (s, C-14), 41.9 (d, C-18), 41.8 (s, C-8), 40.1 (t, C-1), 40.0 (s, C-4), 37.6 (s, C-10), 36.9 (t, C-7), 36.3 (s, C-20), 30.0 (q, C-29), 28.5 (q, C-23), 26.6 (t, C-2), 24.3 (t, C-11), 21.1 (q, C-27), 19.2 (q, C-30), 19.1 (t, C-6), 17.8 (q, C-26), 16.8 (q, C-24), 16.1 (q, C-25). ^{13}C NMR carbohydrate moieties: β-D-glcA*p*, δ 105.1 (s, C-1′), 79.7 (d, C-3′), 79.0 (d, C-2′), 75.2 (d, C-5′), 75.0 (d, C-4′); β-D-glc*p*, δ 102.5 (s, C-1′), 77.4 (d, C-3′), 77.3 (d, C-5′), 75.5 (d, C-2′), 71.9 (d, C-4′), 62.8 (t, C-6′); α-L-ara*p*, δ 103.7 (s, C-1′), 73.8 (d, C-3′), 72.5 (d, C-2′), 69.6 (d, C-4′), 67.3 (t, C-5′); α-L-ara*f*, δ 108.1 (s, C-1′), 85.6 (d, C-4′), 81.9 (d, C-2′), 77.9 (d, C-3′), 62.4 (t, C-5′). ESI-MS (pos.): *m/z* found 1140.5 ([M+NH$_4$]$^{+}$), $C_{53}H_{86}O_{25}$ requires 1122.5458 (monoisotopic).

3.6. Reduction of monomethyl ester 7 with NaBH$_4$ providing 8

Compound **7** (160 mg, 0.14 mmol) was dissolved in dry cooled MeOH (4 ml) and stirred for 15 min. A solution of NaBH$_4$ (130 mg) in dry MeOH (1 ml) was added dropwise, and the reaction mixture was stirred at room temperature for

1 h. Then, it was neutralized with acetic acid and evaporated under reduced pressure. The residue obtained was purified by semi-preparative RP-HPLC [μ-Bondapack C_{18}, 20 μm, 150 Å (150×16 mm ID) column, mobile phase A: water/acetonitrile 90/10+0.1% acetic acid, B: water/acetonitrile 20/80+0.1% acetic acid; at 15% B for 3 min (isocratic), to 90% B in 5 min (curve 6), to 15% B in 10 min (curve 6). Flow rate 7 ml/min, UV detection at 210 nm], to give compound **8** (130 mg, yield 85%).

3.6.1. Compound 8. White plates. Mp 252–254°C. $[\alpha]_D^{20}=-14.0$ (c=0.31, MeOH). Anal. calcd for $C_{52}H_{86}O_{24}$: C, 57.03; H, 7.91. Found: C, 56.80; H, 7.50. IR ν_{max}: 3408, 2930, 1640, 1389, 1077 cm^{-1}. ^{1}H NMR aglycone moiety: δ 5.41 (bt, 1H, J=3.8 Hz, H-12), 4.09 (bd, 1H, J=3.8 Hz, H-16), 3.96 (d, 1H, J=10.0 Hz, H-21), 3.92 (1H, H-15), 3.78 (d, 1H, J=10.0 Hz, H-22), 3.48 (d, 1H, J=11.4 Hz, H-28), 3.29 (d, 1H, J=11.4 Hz, H-28), 3.22 (1H, H-3), 2.48 (t, 1H, J=13.5 Hz, H-19), 2.29 (dd, 1H, J=3.8, 13.5 Hz, H-18), 1.93 (1H, H-11), 1.91 (1H, H-11), 1.78 (2H, H-7), 1.77 (2H, H-2), 1.69 (1H, H-1), 1.62 (1H, H-9), 1.58 (1H, H-6), 1.45 (dq, 1H, J=4.6, 12.0 Hz, H-6), 1.38 (s, 3H, H-27), 1.11 (s, 3H, H-23), 1.09 (dd, 1H, J=3.9, 13.5 Hz, H-19), 1.06 (s, 3H, H-26), 1.04 (1H, H-1), 1.03 (s, 3H, H-25), 0.97 (s, 3H, H-29), 0.95 (s, 3H, H-30), 0.89 (s, 3H, H-24), 0.83 (d, 1H, J=11.8 Hz, H-5). ^{1}H NMR carbohydrate moieties: β-D-glc*p*, δ 4.51 (d, 1H, J=7.4 Hz, H-1′), 3.93 (1H, H-3′), 3.90 (1H, H-2′), 3.89 (1H, H-6′), 3.78 (1H, H-4′), 3.76 (1H, H-6″), 3.39 (1H, H-5′); β-D-glc*p*, δ 5.04 (1H, J=7.7 Hz, H-1′), 3.86 (1H, H-6′), 3.63 (1H, H-6″), 3.41 (t, 1H, J=9.2 Hz, H-3′), 3.34 (1H, H-5′), 3.24 (1H, H-2′), 3.18 (t, 1H, J=9.2 Hz, H-4′); α-L-ara*p*, δ 4.95, (d, 1H, J=7.7 Hz, H-1′), 3.90 (1H, H-5′), 3.81 (1H, H-4′), 3.63 (1H, H-2′), 3.59 (1H, H-5″), 3.55 (1H, H-3′); α-L-ara*f*, δ 5.18 (bs, 1H, H-1′), 4.47 (q, 1H, J=4.1 Hz, H-4′), 4.03 (bs, 1H, H-2′), 3.85 (1H, H-3′), 3.74 (dd, 1H, J=3.7, 11.8 Hz, H-5′), 3.69 (dd, 1H, J=4.8, 12.3 Hz, H-5″). ^{13}C NMR aglycone moiety: δ 143.8?(s, C-13), 126.1 (d, C-12), 91.8 (d, C-3), 79.6 (d, C-21), 77.1 (d, C-22), 72.9 (d, C-16), 68.4 (d, C-15), 67.8 (t, C-28), 56.9 (d, C-5), 48.6 (s, C-17), 48.4 (t, C-19), 48.1 (d, C-9), 47.9 (s, C-14), 42.2 (s, C-8), 42.2 (d, C-18), 40.3 (s, C-4), 40.1 (t, C-1), 37.8 (s, C-10), 37.1 (t, C-7), 36.4 (s, C-20), 30.2 (q, C-29), 28.6 (q, C-23), 27.1 (t, C-2), 24.4 (t, C-11), 20.8 (q, C-27), 19.2 (t, C-6), 18.8 (q, C-30), 17.8 (q, C-26), 16.8 (q, C-24), 16.1 (q, C-25). ^{13}C NMR carbohydrate moieties: β-D-glcA*p*, δ 105.7 (s, C-1′), 80.5 (d, C-3′), 80.2 (d, C-2′), 77.4 (d, C-5′), 73.4 (d, C-4′), 61.9 (t, C-6′); β-D-glc*p*, δ 103.0 (s, C-1′), 78.3 (d, C-5′), 78.1 (d, C-3′), 76.3 (d, C-2′), 72.6 (d, C-4′), 63.4 (t, C-6′); α-L-ara*p*, δ 104.1 (s, C-1′), 74.7 (d, C-3′), 73.1 (d, C-2′), 70.4 (d, C-4′), 67.4 (t, C-5′); α-L-ara*f*, δ 108.9 (s, C-1′), 87.2 (d, C-4′), 82.1 (d, C-2′), 79.3 (d, C-3′), 63.2 (t, C-5′). ESI-MS (pos.): *m/z* found 1112.6 ($[M+NH_4]^+$), $C_{52}H_{86}O_{24}$ requires 1094.5509 (monoisotopic).

3.7. Isolation of CIDI sugars

Compound **5** (100 mg, 0.090 mmol) was hydrolyzed with 2 M trifluoroacetic acid (TFA; 18 ml) by treating it in a sealed tube at 100°C for 1 h, following its complete disappearance from the reaction liquid phase by RP-HPLC, under the chromatographic conditions described for the acidic hydrolysis of compound **5**. The mixture, containing a white precipitate, was evaporated under reduced pressure to remove traces of TFA, and the resulting aqueous solution was filtered to separate the white solid, identified as **6** (36 mg) with an authentic sample. The clear filtrate obtained was freeze-dried (P=5×10^{-2} mbar, T=5°C) to give a crude sugar mixture (70 mg) which was directly chromatographed, without any further purification, on a 250×10 mm ID APS LiChrosorb 10 μm column (mobile phase, water/acetonitrile 25/75. Flow rate 3 ml/min, T=20°C, RI detection at room temperature). The sugars collected were identified as arabinose (10 mg) and glucose (5 mg) by comparison of their physico-chemical and spectroscopic properties with those of authentic sugar samples. The identification of the glucuronic acid was made by chemical degradation (Section 2).

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References

1. Hostettmann, K.; Marston, A. *Saponins*. Cambridge University: Cambridge, 1995.
2. Suegirev, D. P. *Tr. Gos. Nikitsk, But. Sada* **1959**, *30*, 36.
3. Yoshioka, I.; Hino, K.; Matsuda, A.; Kitagawa, I. *Chem. Pharm. Bull.* **1972**, *20*, 1499.
4. Fujiwara, Y.; Maoka, T. *Tetrahedron Lett.* **2001**, *42*, 2693.
5. D'Arrigo, C. Chemioterapico antineoplastico di origine vegetale ad elevata selettività e tossicità fortemente ridotta, e procedimento per la sua preparazione. Italian Patent 01245700, October 14, 1994. Antineoplastic chemoterapeutic of plant origin, having high selectivity and greatly reduced toxicity, and process for the preparation thereof. Japanese Patent 2048431, April 25, 1996. European Patent 0541780, September 18, 1996. US Patent 5558866, September 24, 1996.
6. Halsall, T. G. *Chem. Ind. (London)* **1951**, 867.
7. Crespin, F.; Calmes, M.; Elias, R.; Maillard, C.; Balansard, G. *Chromatographia* **1994**, *38*, 183.
8. Itô, S.; Ogino, T.; Sugiyama, H.; Kodama, M. *Tetrahedron Lett.* **1967**, *24*, 2287.
9. Nozoe, T. *J. Chem. Soc. Jpn* **1934**, *55*, 746.
10. Cole, A. R. H.; Downing, D. T.; Watkins, J. C.; White, D. E. *Chem. Ind.* **1955**, 254.
11. Kitagawa, I.; Yoshikawa, M.; Imakura, Y.; Yosioka, I. *Chem. Pharm. Bull.* **1974**, *22*, 1339.
12. Burczyk, J.; Reznicek, G.; Baumgarten, S.; Hugh-Bloch, M.;

Jurenitsch, J.; Schröder, H.; Werz, U.; Haslinger, E. *Phytochemistry* **1995**, *39*, 195.
13. Dimbi, M. Z.; Kapundu, M.; Darimont, E.; Warin, R.; Delude, C.; Hulus, R. *Bull. Soc. Chim. Belg.* **1985**, *94*, 141.
14. Chen, Y.; Takeda, T.; Ogihara, Y.; Iitaka, Y. *Chem. Pharm. Bull.* **1984**, *32*, 3378.
15. Higuchi, R.; Fujioka, T.; Iwamoto, M.; Komori, T.; Kawasaki, T.; Lassak, E. V. *Phytochemistry* **1983**, *22*, 2565.
16. Seo, Y.; Berger, J. M.; Hoch, J.; Neddermann, K. M.; Bursuker, I.; Mamber, S. W.; Kingston, D. G. I. *J. Nat. Prod.* **2002**, *65*, 65.
17. Higuchi, R.; Komori, T.; Kawasaki, T.; Lassak, E. V. *Phytochemistry* **1983**, *22*, 1237.
18. Barone, D.; Peroglio, F.; Yu, P.; D'Arrigo, C.; Ghezzi, A. Presented at the XXIX National Congress of the Italian Pharmacological Society, Florence, June 20–23, 1999.
19. D'Arrigo, C.; Fagnano, N.; Guarnieri, D. Presented at the First International Oncological Meeting of the Val Gardena, Ortisei, September 30–October 2, 1999.
20. D'Arrigo, C. Oral communication presented at the 10° Congresso Internazionale di Medicina Ortomolecolare, Milan, February 18–21, 2000.
21. Meco, D.; Riccardi, A.; De Felice, M.; Fagnano, N.; D'Arrigo, C.; Rutella, S.; Riccardi, R. Presented at the 92nd Annual Meeting of the American Association for Cancer Research, New Orleans, March 24–28, 2001.
22. Konoshima, T.; Lee, K. H. *J. Nat. Prod.* **1986**, *49*, 650.