Pergamon

PII: S0031-9422(97)01039-X

Phytochemistry, Vol. 49, No. 1, pp. 153–156, 1998

Printed in Great Britain
0031-9422/98/$–see front matter

ENZYMATIC HYDROLYSIS OF THE CYTOTOXIC TRITERPENOID GLYCOSIDE VIRGAUREASAPONIN 1

GERD BADER,* VICTOR WRAY,† ULRICH JUST‡ and KARL HILLER

Institut für Pharmazie, Mathematisch-Naturwissenschaftliche Fakultät I, Humboldt-Universität zu Berlin, Goethestrasse 54, D-13086 Berlin, Germany; † Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Germany; ‡ Bundesanstalt für Materialforschung und -prüfung, D-12205 Berlin, Germany

(*Received in revised form* 23 *October* 1997)

Key Word Index—*Solidago virgaurea*; Asteraceae; enzymatic degradation; polygalacic acid; bisdesmosides; acylglycosides; virgaureasaponin 1; cytotoxic activity.

Abstract—The cytotoxic compound, virgaureasaponin 1, was converted using several optimized enzyme-catalysed hydrolyses to the 28-*O*-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside (**2**), and the 28-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside (**3**) and 28-*O*-β-D-xylyopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside (**4**) both lacking the glucose moiety at C-3 of the aglycone. The terminal rhamnose of the acylglycosidic bonded tetrasaccharide was cleaved by naringinase to give compound **2**. The new acylglycosides **3** and **4** were obtained with the help of a relatively crude β-glucuronidase preparation, but the cleavage of the sapogenin bonded glucose was impossible using several β-glucosidase preparations directly. These derivatives were used for the investigation of the relationship between the saponin carbohydrate structure and their cytotoxic activity.

INTRODUCTION

In previous work [1] we have elucidated the structures of the four main ester saponins from the aerial parts and roots of *Solidago virgaurea* L. subsp. *virgaurea*. Mild alkaline hydrolysis of a mixture of the genuine ester saponins of this plant gave two major deacylated products, virgaureasaponin 1 [2] and virgaureasaponin 2 [3]. Virgaureasaponin 1 possesses a number of differently bonded sugar moieties and is consequently a suitable primary target for obtaining generally applicable information for the enzymatic cleavage of triterpenoid glycosidic systems. Such information is of importance as it permits an investigation of the relationship between the carbohydrate structure of triterpenoid glycosides and their cytotoxicity [4]. In addition, significantly large quantities of virgaureasaponin 1 are isolatable which allows the production of sufficient material for structure/activity studies. This paper deals with the enzymatic degradation of virgaureasaponin 1 by the use of various commercially available glycosidase preparations and different reaction conditions (pH, temperature, enzymatic activity, saponin concentration, reaction time) to yield two new acylglycoses and one known bisdesmoside of polygalacic acid for investigation of their cytotoxicities against cancer cells.

RESULTS AND DISCUSSION

Virgaureasaponin 1 (**1**) was obtained as one of two major products after the mild alkaline hydrolysis of the mixture of genuine estersaponins of *Solidago virgaurea* L. subsp. *virgaurea* [1–3]. Treatment of **1** with naringinase led to compound **2**, that is formed by cleavage of the terminal rhamnose unit. β-glucosidase, cellulase and hesperidinase gave no TLC detectable enzymatic hydrolysis under the following conditions: enzyme concentrations of 0.1–0.8%, saponin concentrations of 0.04%, pH values of 3.8–7.0 and reaction times of ca 240 h. It appears that these enzyme preparations with β-glucosidase activity are too specific and are prevented sterically from attack on the sapogenin bonded glucose unit. It is interesting to note that these enzymes are able to cleave the terminal glucose of oligosaccharide units of other triterpenoid saponins under similar conditions [5].

Cleavage of the terminal glucose unit of **1** was possible, however, using a crude β-glucuronidase preparation that was expected to show different enzymatic activities. The product of this reaction was the acylglycose, **3**. A further acylglycose (**4**) was obtained by treatment of **3** with naringinase. A two-step reaction

* Author to whom correspondence should be addressed.

of **1** with β-glucuronidase to cleave the glucose unit followed by naringinase to cleave the terminal rhamnose, gave the same product. However the reverse order of enzyme treatment gave **4** only as a minor compound and showed additional cleavage of the xylose unit. The simultaneous treatment with both enzymes led to the aglycone polygalacic acid. Clearly the sensitivity of the various cleavages and consequently the final hydrolysis product is governed not only by the types of enzyme preparation but also by the order and combinations in which they are used.

For identification of compounds **2**, **3**, and **4**, the various enzymatic hydrolysis products were isolated from the aqueous reaction mixtures by extraction with *n*-butanol and purified by middle pressure liquid chromatography (MPLC). Yields of 22% for **2**, 8% for **3** and 7% for **4** relative to the starting material **1** were obtained from this procedure. Acid hydrolysis and TLC investigation of these showed that **2** contained glucose, fucose, rhamnose and xylose, whereas **3** and **4** contained only fucose, rhamnose and xylose. The alkaline hydrolysis of **1** and **2** gave polygalacic acid-3-*O*-glucoside (**5**). The molecular weights of the three derivatives were determined by MALDI-TOF-MS. The sodiated molecular ions $[M+Na]^+$ were readily identified and showed characteristic fragmentation patterns.

The identity of the derivatives was confirmed from a comparison of the ^1H and ^{13}C NMR data with those of **1**. No difficulties were encountered in assigning the anomeric sugar protons and the methyl protons of the 6-deoxy sugars as well of those of the aglycone moieties of **2**, **3**, and **4**. Compounds **2** and **4** differ from **1** in the absence of signals for the terminal rhamnose, i.e. an anomeric proton with *J* ca 2 Hz and an aliphatic secondary methylproton. The signals for the terminal glucose were absent in the ^1H NMR spectra of **3** and **4**. The ^{13}C NMR signals assigned to the aglycone moiety of **2**, **3**, and **4** were in accordance with those of **1**. In all cases the signals were essentially identical for all carbons apart from those of C-3 in **3** and **4** where the loss of the terminal C-3 glucose causes the expected upfield shift for this carbon. These data unambiguously demonstrate that the sapogenin part of these glycosides remains impervious to attack under the enzymatic conditions used.

The signals of the terminal rhamnose of **1** were absent in the ^{13}C NMR spectra of **2** and **4**, while **3** and **4** showed no signals for a terminal glucose. The chemical shifts of the xylose units of **2** and **4** changed (C-3 moved upfield by 6 and C-4 downfield by 1.2 ppm) in agreement with the loss of the terminal rhamnose to give a terminal xylose for these glycosides (Table 1).

The acylglycoses **3** and **4** were established to be 28-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside (**3**) and 28-*O*-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranside (**4**) of 2β,3β,16α,23-tetrahydroxyolean-12-ene-28-oic acid.

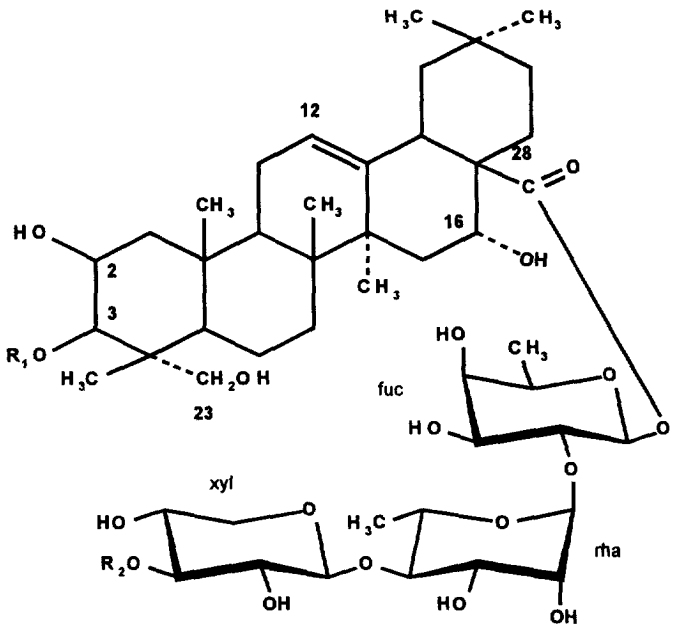
Table 1. ^{13}C NMR spectral data and atom type from DEPT data for the sugar moieties of compounds **1**–**4** (in CD_3OD)

	1	**2**	**3**	**4**	Atom type
Glc-1	105.3	105.48			CH
2	76.3	76.12			CH
3	78.15	78.24			CH
4	71.0	71.13			CH
5	77.7	77.74			CH
6	62.4	62.30			CH_2
Fuc-1	95.1	95.16	95.11	95.17	CH
2	75.4	75.41	74.75	74.69	CH
3	75.0	74.68	74.58	74.15	CH
4	73.5	73.64	73.62	73.64	CH
5	72.6	72.70	72.68	72.70	CH
6	16.45	16.51	16.53	16.50	CH_3
Rha^1-1	101.3	101.13	101.38	101.14	CH
2	72.0	71.94	72.02	71.94	CH
3	72.25	72.29	72.27	72.30	CH
4	84.55	84.36	84.69	84.34	CH
5	68.8	68.75	68.83	68.76	CH
6	18.35	18.31	18.40	18.31	CH_3
Xyl-1	107.1	107.15	107.25	107.14	CH
2	76.6	76.72	76.80	76.72	CH
3	84.3	78.24	84.40	78.24	CH
4	69.9	71.13	69.92	71.14	CH
5	67.1	67.31	67.25	67.31	CH_2
Rha^2-1	102.4		102.59		CH
2	72.25		72.38		CH
3	72.25		72.34		CH
4	74.0		74.04		CH
5	70.0		70.06		CH
6	17.85		17.82		CH_3

Rha^1: inner rhamnose, Rha^2: terminal rhamnose.

They are new compounds and have not been reported in the plant kingdom, as far as we are aware. Compound **2** has the same structure as bellidiastrosid C_2 from *Aster bellidiastrum* (L.) Scop. [6] and as bernardioside C_1 from *Bellis bernardii* Boiss. et Reuter [7].

Compounds **1**–**5** were tested for cytotoxic activities against two murine tumor cell lines (YAC-1; P-815) *in vitro*. Compound **1**, as well as the genuine acylated saponins with this basic structure, showed IC_{50} values in the range 2–4 μmol l^{-1}. Compound **2** gave significantly higher IC_{50} values in the range 16 to 33 μmol l^{-1}. For these examples, it can be concluded that the tetrasaccharide bonded at the C-28 position of polygalacic acid is more essential for exerting cytotoxic activities than a trisaccharide. The prosapogenin of **1** and **2**, polygalacic acid-3-*O*-glucoside (**5**), with a free carboxyl function is nearly devoid of activity (IC_{50} values of 188–255 μmol l^{-1}). On the other hand, glycosylation at C-3 of the sapogenin is also necessary, as the acylglycoses **3** and **4** showed weaker cytotoxic activities with IC_{50} values of 16 to 46 μmol l^{-1} [4]. Thus it appears that there is a minimum polarity requirement at ring A and between rings D/E of the triterpenoid which governs the cytotoxicity of these



	R_1	R_2
1	1Glc	1Rha
2	1Glc	H
3	H	1Rha
4	H	H

compounds. The exact nature of these polarity requirements will depend on the degree of glycosylation and/or hydroxylation of the aglycone.

EXPERIMENTAL

General procedures

^1H and ^{13}C NMR spectra were recorded at 300°K on a Bruker WM 400 NMR spectrometer (^1H NMR at 400.13 MHz, ^{13}C NMR at 100.63 MHz) locked to the major deuterium resonance of the solvent, CD_3OD. MALDI-TOF-MS were recorded on a Kratos Kompact MALDI III (Shimadzu) in the reflection mode. Samples were prepared in a matrix of 2,5-dihydroxybenzoic acid (10 mg ml^{-1} soln in EtOH–H_2O (1:1)). Sample ions were generated by laser desorption at 337 nm with a pulse width of 3 ns and then accelerated through 20 kV in the positive ion mode.

TLC, MPLC, HPLC and alkaline hydrolysis

See Ref. [5].

Preparation of virgaureasaponin 1

See Ref. [2].

Virgaureasaponin 1 (**1**). $C_{59}H_{96}O_{27}$ (calc. 1237.4), MALDI-TOF-MS: *m/z* 1260.5: $[M+Na]^+$, 593.4: $[M-Pga-Hex+Na]^+$, 689.8: $[M-Dhex-Dhex-Pent-Dhex+Na]^+$; ^1H NMR: aglycone moiety δ 0.82, 0.92, 0.99, 1.01, 1.35, 1.42 (each 3H, *s*, Me of C-26, C-29, C-30, C-24, C-25 and C-27); sugar moieties δ 1.26 (3H, *d*, $J = 6.5$ Hz, Me of Fuc), 1.37 (3H, *d*, $J = 6.2$ Hz, Me of Rha^1), 1.29 (3H, *d*, $J = 6.2$ Hz, Me of Rha^2), 4.48 (1H, *d*, $J = 7.7$ Hz, anomeric proton of Glc), 5.35 (1H, *d*, $J = 8.1$ Hz, anomeric proton of Fuc), 5.42 (1H, *d*, $J = 1.8$ Hz, anomeric proton of Rha^1), 4.53 (1H, *d*, $J = 7.5$ Hz, anomeric proton of Xyl), 5.19 (1H, *d*, $J = 1.8$ Hz, anomeric proton of Rha^2); ^{13}C NMR: aglycone moiety δ 71.3 (C-2), 84.1 (C-3), 123.6 (C-12), 144.6 (C-13), 74.5 (C-16), 66.1 (C-23), 14.9 (C-24), 17.7 (C-25), 17.9 (C-26), 27.3 (C-27), 177.3 (C-28), 33.3 (C-29), 24.9 (C-30).

Enzymatic hydrolysis of **1** *to* **2** *using naringinase.* Compound **1** (100 mg) was mixed with 250 ml 10% aq. EtOH and 1000 mg naringinase (Sigma N 1385, 0.15 U mg^{-1}) from *Penicillium decumbens*, adjusted to pH 4.8 with diluted HCl and shaken 10 h at 40°.

The mixt. was shaken 2 × with 250 ml water-saturated *n*-BuOH. The butanolic phase was evaporated *in vacuo* and submitted to silica gel MPLC with the eluent $CHCl_3$–MeOH–H_2O (65.3:28.6:6.1) to give 22 mg of **2**. *Compound* **2**: mp 218–223°; $C_{53}H_{86}O_{23}$ (calc. 1091.2), MALDI-TOF-MS: *m/z* 1114.4: $[M+Na]^+$, 447.3: $[M-Pga-Hex+Na]^+$, 689.7: $[M-Dhex-Dhex-Pent+Na]^+$; ^1H NMR: aglycone moiety δ 0.82, 0.92, 0.99, 1.00, 1.34, 1.43 (each 3H, *s*, Me of C-26, C-29, C-30, C-24, C-25 and C-27); sugar moieties δ 1.26 (3H, *d*, $J = 6.3$ Hz, Me of Fuc), 1.37 (3H, *d*, $J = 6.1$ Hz, Me of Rha^1), 4.48 (1H, *d*, $J = 7.7$ Hz, anomeric proton of Glc), 5.34 (1H, *d*, $J = 8.2$ Hz, anomeric proton of Fuc), 5.46 (1H, *d*, $J = 1.5$ Hz, anomeric proton of Rha^1), 4.53 (1H, *d*, $J = 7.3$ Hz, anomeric proton of Xyl). ^{13}C NMR: aglycone moiety δ 71.22 (C-2), 83.92 (C-3), 123.58 (C-12), 144.77 (C-13), 74.13 (C-16), 65.66 (C-23), 14.74 (C-24), 17.66 (C-25), 17.81 (C-26), 27.27 (C-27), 177.27 (C-28), 33.39 (C-29), 24.86 (C-30).

Enzymatic treatment of **1** *with β-glucosidase preparations.* β-Glucosidase (Merck, 2.5 U mg^{-1}) from *Amydalus* species, cellulase (Merck, 0.005 U mg^{-1}) from *Aspergillus niger* and hesperidinase (Sigma, 0.01 U mg^{-1}) were tested as detailed in Ref. [5].

Enzymatic hydrolysis of **1** *to* **3** *using β-glucuronidase.* Compound **1** (crude, 1000 mg) was mixed with 300 ml H_2O and 10 ml β-glucuronidase type HP-2 (Sigma G 1707, 95 U ml^{-1}) from *Helix pomatia*, adjusted to pH 4.5 with diluted HCl and shaken 22 h at 38°. The mixt. was shaken 2 × with 300 ml water-saturated *n*-BuOH. The butanolic phase was evaporated *in vacuo* and submitted to silica gel MPLC with the eluent AcOEt–MeOH–H_2O (73.7:13.8:12.5) and RP-18 MPLC with the eluent MeOH–H_2O (65:15) to give 76 mg of **3**. *Acylglycose* **3**: mp 227–229°; $C_{53}H_{86}O_{22}$ (calc. 1075.2), MALDI-TOF-MS: *m/z* 1098.4: $[M+Na]^+$, 593.4: $[M-Pga+Na]^+$; ^1H NMR: aglycone moiety δ 0.82, 0.92, 0.99, 0.99, 1.35, 1.42 (each 3H, *s*, Me of C-26, C-29, C-30, C-24, C-25 and C-27); sugar moieties δ 1.26 (3H, *d*, $J = 6.3$ Hz, Me of Fuc), 1.37 (3H, *d*, $J = 6.2$ Hz, Me of Rha^1), 1.29 (3H, *d*, $J = 6.2$ Hz, Me of Rha^2), 5.35 (1H, *d*, $J = 8.2$ Hz, anomeric proton of Fuc), 5.42 (1H, *d*, $J = 1.5$ Hz, anomeric proton of Rha^1), 4.53 (1H, *d*, $J = 7.4$ Hz, anomeric proton of Xyl), 5.18 (1H, *d*, $J = 1.5$ Hz, anomeric proton of Rha^2). ^{13}C NMR: aglycone moiety δ 72.27 (C-2), 76.51 (C-3), 123.61 (C-12), 144.74 (C-13), 74.04 (C-16), 68.35 (C-23), 14.29 (C-24), 17.71 (C-25), 17.91 (C-26), 27.22 (C-27), 177.35 (C-28), 33.38 (C-29), 24.87 (C-30).

Enzymatic hydrolysis of **1** *to* **4** *using β-glucuronidase following by naringinase.* Compound **1** (crude, 750 mg) was mixed with 750 ml H_2O and 7.5 ml β-glucuronidase type HP-2 (Sigma G 1707, 95 U ml^{-1}) from *Helix pomatia*, adjusted to pH 4.5 with diluted HCl and shaken 12 h at 38°. The mixt. was shaken 2 × with 375 ml water-saturated *n*-BuOH. The butanolic phase was evaporated *in vacuo* and the residue was dissolved in 1500 ml 10% aq. EtOH and mixed with 1500 mg of naringinase (Sigma N 1385, 0.15 U mg^{-1}) from *Penicillium decumbens*, adjusted to pH 4.5 with diluted HCl and shaken for 10 h at 40°. The mixt. was partitioned twice with 750 ml water-saturated *n*-BuOH. The combined butanolic phase was evaporated *in vacuo* and submitted to silica gel MPLC with the eluent AcOEt–MeOH–H_2O (90:11:9) to give 55 mg of **4**. *Acylglycose* **4**: mp 213–214°; $C_{47}H_{76}O_{18}$ (calc. 929.1), MALDI-TOF-MS: *m/z* 952.0: $[M+Na]^+$, 447.6: $[M-Pga+Na]^+$; ^1H NMR: aglycone moiety δ 0.82, 0.92, 0.96, 0.99, 1.34, 1.42 (each 3H, *s*, Me of C-26, C-29, C-30, C-24, C-25 and C-27); sugar moieties δ 1.26 (3H, *d*, $J = 6.4$ Hz, Me of Fuc), 1.37 (3H, *d*, $J = 6.2$ Hz, Me of Rha^1), 5.34 (1H, *d*, $J = 8.2$ Hz, anomeric proton of Fuc), 5.46 (1H, *d*, $J = 1.6$ Hz, anomeric proton of Rha^1), 4.53 (1H, *d*, $J = 7.4$ Hz, anomeric proton of Xyl). ^{13}C NMR: aglycone moiety δ 72.30 (C-2), 76.13 (C-3), 123.59 (C-12), 144.77 (C-13), 73.64 (C-16), 67.68 (C-23), 14.15 (C-24), 17.66 (C-25), 17.81 (C-26), 27.27 (C-27), 177.29 (C-28), 33.38 (C-29), 24.86 (C-30).

Acidic hydrolysis for identification of sugars and the aglycone moiety. Hydrolyses were performed in HCl steam saturated chambers directly on TLC plates at 100°. Glc, Fuc, Rha and Xyl were detected for **1** and **2**, whereas Fuc, Rha and Xyl were found for **3** and **4**. The sapogenin polygalacic acid was identified with the help of authentic samples and its characteristic degradation pattern using the same technique.

Acknowledgements—We thank I. Rahn, S. Streich (Humboldt-University) and B. Jaschok-Kentner (GBF) for technical assistance. Financial support for this work was provided by the Deutsche Forschungsgemeinschaft (Hi 505/2-1/2-2).

REFERENCES

1. Bader, G., Wray, V. and Hiller, K., *Planta Med.*, 1995, **61**, 158.
2. Hiller, K., Bader, G. and Schulten, H.-R., *Pharmazie*, 1987, **42**, 541.
3. Hiller, K., Bader, G. and Dube, G., *Pharmazie*, 1987, **42**, 744.
4. Bader, G., Plohmann, B., Hiller, K. and Franz, G., *Pharmazie*, 1996, **51**, 414.
5. Bader, G., Streich, S., Gründemann, E., Flatau, S. and Hiller, K., *Pharmazie*, 1997, **52**, 836.
6. Schöpke, Th., Al-Tawaha, Ch., Wray, V. and Nimtz, M., Meyer, A. and Hiller, K., *Phytochemistry*, 1995, **40**, 1489.
7. Schöpke, Th., Thiele, H., Hiller, K., Wray, V., Nimtz, M., *J. Nat. Prod.*, 1996, **59**, 939.