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Cytotoxicity of triterpenoid saponins

Part 2: Relationships between the structures of glycosides of polygalacic acid and their activities against pathogenic *Candida* species

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Glycosides of polygalacic acid (2β,3β,16α,23-tetrahydroxy-olean-12-ene-28-oic acid) isolated from the aerial parts of *Solidago virgaurea* L. subsp. *virgaurea, Heteropappus altaicus* (Willd.) Novopokr. and *Heteropappus biennis* (Ldb.) Tamamsch. or produced by degradation of these genuine saponins were tested against humanpathogenic strains of *Candida albicans, C. glabrata, C. krusei* and *C. tropicalis* using a micro-dilution assay. The antifungal action can be influenced the variation of the etherglycosidically bonded carbohydrate units at C-3 as well as of the acylglycosidically bonded oligosaccharide at C-28 of the aglycone.

1. Introduction

Candida albicans and other species of this genus are important opportunistic fungal pathogens of man, and cause infections ranging in severity from superficial to systemic.

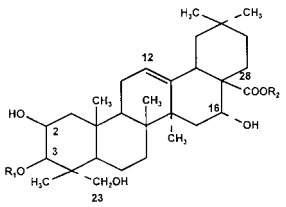
In our course of screening for new antifungal agents of medicinal plant origin we found that deacylated triterpenoid saponins, isolated after mild alkaline hydrolysis of the mixture of genuine ester saponins of *Solidago virgaurea* L., showed higher activity against several humanpathogenic *Candida* species than the mixture of ester saponins [1]. After that, the structures of the mentioned deacylated saponins were elucidated as bisdesmosidic glycosides of polygalacic acid and named as virgaureasaponins 1 [2], 2 [3] and 3 [4]. In a further study [5], it was demonstrated that the antifungal activities of bisdemosides of polygalacic acid were substantially higher than that of the correlated prosapogenins (monodesmosides) using an agar diffusion as well as a liquid shaking culture assay.

The present study extends these former results for further carbohydrate modified glycosides of polygalacic acid, e.g. the major compounds of the mixture of genuine fucosylester saponins as well as some carbohydrate chain degradation products, obtained by enzymatic hydrolysis, and for some *Candida albicans* strains with different sensitivity or resistance to the clinical used azole derivative fluconazole.

2. Investigations, results and discussion

In the first step of our investigations, two groups of glycosides of polygalacic acid (see Table 1) were tested against four *Candida* species in a concentration range of 50 μg/ml to 400 μg/ml in a micro-dilution assay (see Table 2). The first group of glycosides contained compounds with a 3-O-glucosyl unit: virgaureasaponins 1 (**1**), B (**2**), C (**3**), heteropappussaponin 5 (**4**) and prosapogenin 1 (**5**). The second group contained glycosides with a 3-O-laminaribosyl unit: virgaureasaponins 2 (**6**), D (**7**), E (**8**), heteropappussaponin 7 (**9**) and prosapogenin 2 (**10**).

Table 1: Carbohydrate moieties of tested glycosides of polygalacic acid (R_1, R_2 = H)



Compd.	R_1	R_2
1	←1glc	←1fuc2←1rha4←1xyl3←1rha
2	←1glc	←1fuc(4-hba-hba-hba)2←1rha4←1xyl3←1rha
3	←1glc	←1fuc(4-hba-hba)2←1rha4←1xyl3←1rha
4	←1glc	←1ara2←1rha(3←1api)4←1xyl
5	←1glc	Na
6	←1glc3←1glc	←1fuc2←1rha4←1xyl3←1rha
7	←1glc3←1glc	←1fuc(4-hba-hba-hba)2←1rha4←1xyl3←1rha
8	←1glc3←1glc	←1fuc(4-hba-hba)2←1rha4←1xyl3←1rha
9	←1glc3←1glc	←1ara2←1rha(3←1api)4←1xyl
10	←1glc3←1glc	Na
11	←1glc	←1fuc2←1rha4←1xyl
12	H	←1fuc2←1rha4←1xyl3←1rha

glc: β-D-glucopyranose, fuc: β-D-fucopyranose, α-L-rhamnopyranose, xyl: β-D-xylopyranose, api: β-D-apiofuranose, hba: ß-hydroxybutyric acid

Estersaponins with an acylated hydroxyl at C-4 of the fucose unit showed weaker antifungal activities as the related deacylated compounds, except **2** for *Candida albicans* and *Candida tropicalis*: **2** ≤ **1**; **3** < **1**; **7** < **6**; **8** < **6**.
The kind of sugar units of acylglycosidically bonded tetrasaccharides seems to be important, too: **1** > **4**; **6** > **9**.
One etherglycosidically bound glucose unit at C-3 of the aglycone is more effective as a disaccharide, consisting of two glucose units: **1** > **6**; **2** > **7**; **3** > **8**; **4** > **9**.
The present results support the previous findings that bisdesmosides of polygalacic acid are more active than their corresponding monodesmosides [5] and are not in agreement with results for saponins of other aglycones [6].
The most active compound **1** was characterized in previous investigations [5].
Further investigations were carried out to get informations about structural parts of this compounds which are necessary for the antifungal action. The activity of **1** was compared with that of the 4-O-fucosyl ester saponin **2** and two partial deglycosylated products, compounds **11** and **12**. Compound **11** was yielded after cleavage of the terminal rhamnose unit and **12** after loss of the terminal glucose unit of **1**, respectively (see Table 1). Five strains of *Candida albicans* with different sensitivities against fluconazole were used for this investigation (see Table 3). With the exception of one strain, **1** showed the highest antifungal activity. This experiment confirmed that the acylglycosidic bound tetrasaccharide sequence of **1** is necessary: **1** > **11**. But one glucose unit at C-3 of the aglycone is important, too: **1** > **12**. The acylation of the hydroxyl group of the fucose unit lead mainly to a lower activity, as described above.
In a previous work [7] we showed some carbohydrate structure related cytotoxic effects against two murine cancer cell lines and human erythocytes. These cytotoxic activities were influenced by both, the O-glycosylation pattern at carbon atom 3 and 28 of the aglycone. The bisdesmosides of polygalacic acid showed significant higher toxic activities than monodesmosides. The etherglycosidic linked glucose unit at C-3 of the aglycone was also found to be essential, but no difference in activity to the disaccharide laminaribose (glucose-1→3-glucose-1) at the same position was observed. The acylglycosidic carbohydrate sequence 1-fucose-2←1-rhamnose-4←1-xylose-3←1-rhamnose of the mentioned bisdesmosides showed the highest cytotoxic effect, but it was not influenced by the acylation of the hydroxyl group at C-4 of the fucose unit.
It is of interest to note, that the ester saponin **2** exhibits a remarkably higher antifungal cytotoxicity (see Table 3) than **1** in *Candida albicans* cells, which are characterised by a relative resistance against fluconazole (IC_{50}: 50 μg/ml). The molecular IC_{50} values against this strain are 0.16 μmol/l for fluconazole (mw. 306) and 0.07 μmol/l for **2** (mw. 1495.6). These results suggest that the resistance to fluconazol leads to a higher sensitivity to the estersaponin **2**. Further investigations are necessary to understand the mechanism of action of this saponin. The primary mode of action of saponins are believed to be through interaction with sterols, which results in damage to the plasma membrane by impairing its barrier function and causing leakage of the intracellular constituents. In our previous investigation [8], scanning electron microscopy showed that treatment with **1** affected the structure and the integrity of the outer surface of *Candida albicans* cells. However, further studies are necessary to acquire knowledge about the carbohydrate specific mechanisms of fungitoxicity due to the treatment with saponins.

Table 2: Inhibition (%) of the growth of 4 *Candida* species

Concentration (μg/ml)	1	2	3	4	5	6	7	8	9	10
C. albicans Y01-09										
50	0	0	0	0	0	0	0	0	0	0
100	100	100	0	0	0	0	0	0	0	0
200	100	100	100	40	0	0	0	0	0	0
400	100	100	100	50	0	100	0	0	0	10
C. tropicalis A24										
50	0	0	0	0	0	0	0	0	0	0
100	100	100	0	0	0	0	0	0	0	0
200	100	100	100	50	0	0	0	0	0	0
400	100	100	100	40	10	100	0	80	30	10
C. krusei M44781/92										
50	90	0	0	0	0	0	0	0	0	0
100	100	50	0	40	0	0	0	0	0	0
200	100	40	20	90	0	90	0	0	0	0
400	100	70	40	90	0	90	0	0	70	40
C. glabrata 14231/94										
50	0	0	0	0	0	0	0	0	0	0
100	100	0	0	0	0	0	0	0	0	0
200	100	20	0	60	0	0	0	0	0	0
400	100	10	0	90	0	90	0	0	20	10

Table 3: Inhibition (%) of the growth of 5 *Candida albicans* strains

Concentration (μg/ml)	1	11	12	2
C. albicans 4083/91 (IC_{90} Fluconazol: 0.2–0.4)				
75	0	0	0	0
100	100	0	0	100
150	100	40	0	100
200	100	80	100	100
C. albicans Y01-09 (IC_{90} Fluconazol: 0.4–0.8)				
75	10	0	0	0
100	100	100	0	90
150	100	100	0	100
200	100	100	100	100
C. albicans 1474/95 (IC_{90} Fluconazol: 1.56–3.12)				
75	70	0	0	0
100	100	100	0	0
150	100	100	0	10
200	100	100	100	100
C. albicans 1399II/95 (IC_{90} Fluconazol: 6.2)				
75	0	0	0	0
100	60	40	0	0
150	100	60	0	0
200	100	100	100	0
C. albicans Stamm C (IC_{90} Fluconazol: 50)				
75	0	0	0	30
100	0	0	0	100
150	30	0	0	100
200	100	60	30	100

3. Experimental

3.1. Isolation and preparation of triterpenoid glycosides

The virgaureasaponins 1 (**1**), B (**2**), C (**3**), 2 (**6**), D (**7**) and E (**8**) and the related prosapogenins were characterised in [9], the heteropappussaponins 5 (**4**) and 7 (**9**) in [10], the triterpenoid glycoside **11** and the acylglycose **12** in [11].

3.2. Yeasts

The *Candida albicans* strain Y01-09 was purchased from Pfizer/Mack, Illertissen, Germany. The other strains were obtained from clinical specimens processed in the Mycology Laboratory of the Robert Koch Institute in Berlin.
Inocula were prepared from yeasts grown on neutral Sabouraud glucose agar for 24 h at 37 °C by suspending and diluting them in 0.4% NaCl.

3.3. Microdilution assay

Microtiterplates were filled with suspensions containing a liquid nutrient consisting of a sodium phosphate buffered casitone/glucose medium supplemented with ions of potassium, iron, magnesium, trace elements and vitamins (see [12]) as well as of 10^4 cells/ml, 2 µl DMSO/ml with or without (control) treatment of different amounts of test substances.
After incubation in a water-saturated chamber for 18 h at 37 °C, the resulting turbidities were measured at 630 nm (see [12]).
The inhibitions (%) of the yeast growth were calculated by consideration the optical densities of the medium as well as the control.

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