166. Enzyme-Mediated Regioselective Acylations of Flavonoid Disaccharide Monoglycosides

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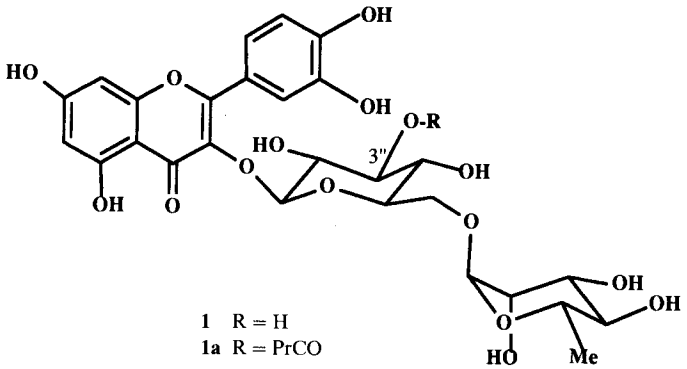
Flavonoid disaccharide monoglycosides have been acylated by the catalytic action of the protease subtilisin in anhydrous pyridine. The effects of the nature of the sugars and of the interglycosidic bonds on the regioselectivity of the reactions have been analyzed. The selectivity was excellent with rutin (**1**), hesperidin (**2**), naringin (**6**), and quercetin 3-*O*-[*O*-(β-D-glucopyranosyl)-(1→4)-α-L-rhamnoside] (**9**), giving single monoesters on their glucose moieties (see **1a**, **2a**, **6a**, and **9b**, resp.); quite interestingly, in the last compound, acylation did not occur at the free primary OH group but at the secondary OH–C(3‴). On the other hand, a mixture of mono- and diesters was obtained with the flavonoid peltatoside (**7**).

Introduction. – The proteolytic enzyme subtilisin (protease from *Bacillus licheniformis*) has been used recently in organic solvents to catalyze the regioselective acylation of polyhydroxylated compounds [1], including flavonoid monosaccaride monoglycosides [2].

To extend the applicability of this acylation procedure to structurally related flavonoids and to gain more information on the relationships between substrate structure and enzyme activity and regioselectivity, we looked at the more complex flavonoid disaccharide monoglycosides. These important compounds are widely distributed in nature (vegetable sources), where they are often present esterified with different acids at specific positions of their disaccharide moieties [3]. Among these esters, the most frequently encountered are the cinnamoyl, *p*-coumaroyl, and feruloyl derivatives, some of which may be of pharmaceutical interest. *E.g.*, the *p*-coumaroyl derivatives of quercetin and kaempferol 3-*O*-biosides are the major components of the extract from *Ginkgo biloba*, and it is believed that these esters significantly contribute to the effect on the symptoms of cerebrovascular insufficiency and poor arterial circulation displayed by the extract [4].

Direct selective nonenzymatic acylation of these disaccharides is still a distant target because of the present lack of suitable reagents. This, plus the structural complexity of these glycosides makes enzyme-catalyzed acylations a promising investigation. We now report on the subtilisin-catalyzed esterification of some representative flavonoid disaccharide monoglycosides, which not only produced new acyl derivatives, but also revealed a remarkable behaviour of this enzyme with respect ot the regioselectivity of the reaction.



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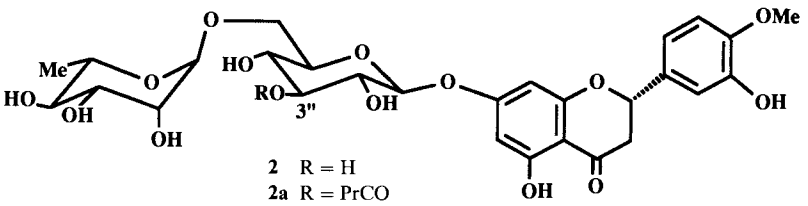
1a R = PrCO



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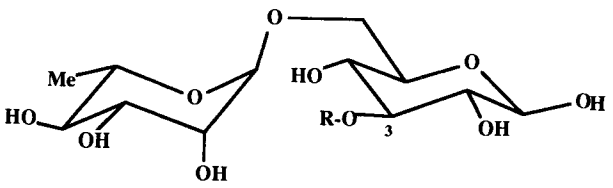

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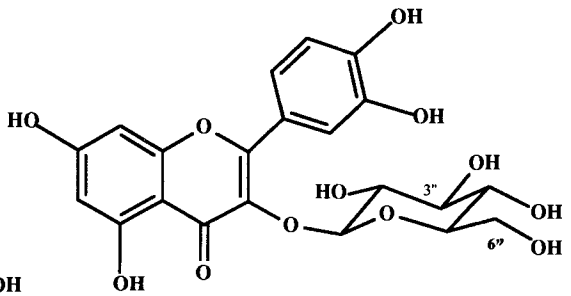

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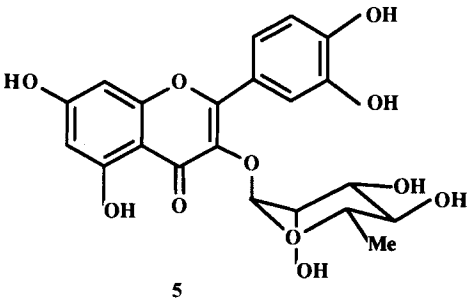

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Results and Discussion. – We started our investigation with two easily available compounds, rutin (**1**) and hesperidin (**2**). Both compounds have the same disaccharidic moiety, rutinose (= 6-*O*-(α-L-rhamnopyranosyl)-D-glucose; **3**), which is linked to OH–C(3) of the quercetin aglycone in **1** and to OH–C(7) of hesperetin in **2**. Rutinose has only secondary OH groups. When a solution of **1** (or **2**) in anhydrous pyridine was treated at 45° with an excess of trifluoroethyl butanoate in the presence of subtilisin (lyophilized from an aqueous solution at pH 7.8), a *ca.* 65% conversion was observed after 48 h[1]). Both reactions resulted almost exclusively in the formation of single products which were isolated after the usual workup and chromatographic purification, and were identified as 3″-*O*-butanoylrutine (**1a**) and 3″-*O*-butanoylhesperidin (**2a**) on the basis of their spectroscopic properties.

The negative FAB-MS of **1a** contained the quasimolecular ion at *m/z* 679, in accordance with the introduction of one butanoate in **1** and few other ions due to the aglycone moiety. More relevant information was gained from the analysis of B/E daugther-ion spectrum of $[M - H]^-$ which contained, *i.a.*, a clear fragment at *m/z* 533 derived from the unimolecular loss of an unsubstituted rhamnose moiety in the gas phase. The acylation of glucose, and thus of the 'internal' sugar, was established by the ^{13}C-NMR spectrum (*Table 1*) of **1a** which also allowed unequivocal assignment of the esterification site. On comparison with the spectrum of **1** [6], different chemical shifts for some of the glucose C-atoms of **1a** were observed: C(3″) was found downfield (+0.9 ppm), whereas C(2″) and C(4″) appeared at higher fields (−2.0 and −2.4 ppm, resp.), as expected from the attachment of the butanoyl residue at OH–C(3″) of glucose [2] [7]. Accordingly, the ^{1}H-NMR spectrum contained, *i.a.*, a downfield *t* at 4.84 ppm. (J = 10 Hz), due to the proton at the deshielded C(3″) [2], as suggested by examination of the selectively ^{1}H-decoupled ^{13}C-NMR spectrum. In addition, irradiation of H–C(1″) at 5.45 ppm (*d*, J = 7.5 Hz) allowed the identification of H–C(2″) at *ca.* 3.40 ppm (*dd*, J = 7.5 and 10 Hz; simplified to a *d*, J = 10 Hz). Moreover, this *dd* became a *d* (J = 7.5 Hz) on decoupling of the *t* at 4.84 ppm. In the same way, the structure of **2a** was established (see *Exper. Part* and *Table 1*).

Table 1. *^{13}C-NMR Data* ((D$_6$)DMSO) *of the Disaccharide Moiety of* **1**–**3**, **6**, *and* **9** *and of Their Butanoyl Derivatives*

	1[a])	**1a**	**2**[a])	**2a**	**3**[a])		**3a**		**6**[a])	**6a**	**9**[b])[2])	**9b**[2])
					α	β	α	β				
Glu, C(1″)	101.0	100.8	100.5	100.5	93.5	96.8	93.0	96.8	96.6	96.5	101.0	100.8
Glu, C(2″)	73.8	71.8	72.9	70.6[e])	72.3	74.9	70.8	73.2	75.3	75.3	73.9	71.6
Glu, C(3″)	76.1	77.0	76.2	76.9	73.6	76.5	75.7	77.8	76.4	76.1	76.6	76.9
Glu, C(4″)	70.3[c])	67.9	70.6	67.5	70.8	70.6	68.9	68.9	68.8	69.3	69.1	66.7
Glu, C(5″)	75.6	75.6	75.4	75.1	71.4	75.6	71.2	75.5	76.1	73.0	76.2	76.3
Glu, C(6″)	66.9	66.5	66.0	65.5	68.2	67.9	68.0	67.7	59.7	62.5	60.3	59.7
Rha, C(1‴)	100.5	100.6	99.3	98.9	101.6		101.7		99.7	99.7	99.0	99.3
Rha, C(2‴)	70.1[c])	70.5[d])	70.2	70.4[e])	70.9		70.9		69.6[f])	69.6[f])	71.8	71.7
Rha, C(3‴)	69.7	70.3[d])	69.5	70.2[e])	71.9		71.9		69.7[f])	69.7[f])	70.4[g])	70.4[g])
Rha, C(4‴)	71.6	71.8	72.0	72.0	72.9		72.9		71.1	71.1	81.5	81.5
Rha, C(5‴)	68.1	68.2	68.2	68.3	69.5		69.6		67.5	67.5	70.3[g])	70.2[g])
Rha, C(6‴)	17.3	17.6	17.7	17.7	17.5		17.4		17.3	17.2	17.4	17.4

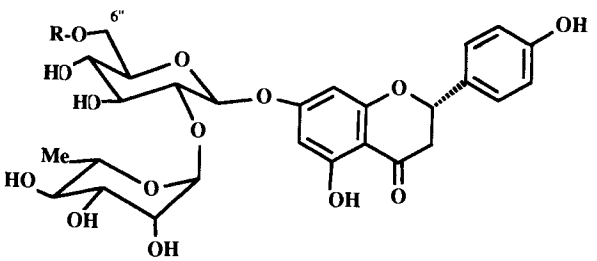
[a]) See [6].
[b]) See [10].
[c])[d])[e])[f])[g]) Assignments may be reversed.

[1]) It should be pointed out that no appreciable conversion was observed in the absence of the enzyme or in the presence of subtilisin inactivated by phenylmethanesulfonyl fluoride, which specifically reacts with an essential residue of the enzyme-active site [5]. These data rule out the artifactual origin of the process and demonstrate the need for an intact active site for the enzymatic reactions.

[2]) For convenience, in *Table 1* (*only*) doubly primed numbering of **9**, **9a**, and **9b** refers to the glucose and threefold primed numbering to the rhamnose moiety; systematic numbering is given in the *General* and *Exper. Part*.

To the best of our knowledge, flavonoid rutinosides acylated at the glucose moiety (such as **1a** and **2a**) have not been previously found in nature or obtained by synthesis. The preferred acylation of glucose with respect to the rhamnosyl unit was reminiscent of the behaviour of the previously studied isoquercitrin (**4**) and quercitrin (**5**) [2]. The selectivity for OH–C(3″) of glucose was expected since OH–C(6″) is blocked in the intersugar linkage. In addition, selectivity was independent of the presence and nature of the aglycone: indeed, rutinose (**3**) was cleanly acylated under similar conditions to the 3-*O*-butanoyl derivative **3a** as the unique product (see *Exper. Part* and *Table 1*).

At this point, we tested the behaviour of the rhamnoglucoside naringine (**6**) in which the interglycosidic linkage is between C(1‴) of rhamnose and C(2″) of glucose, leaving the



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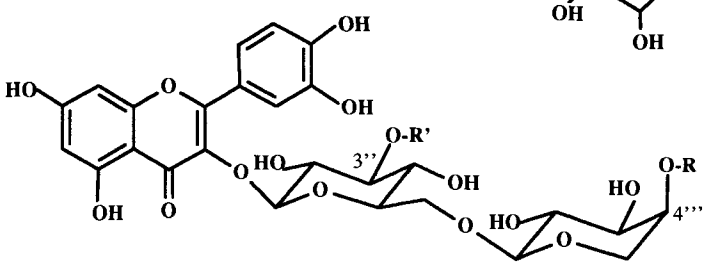
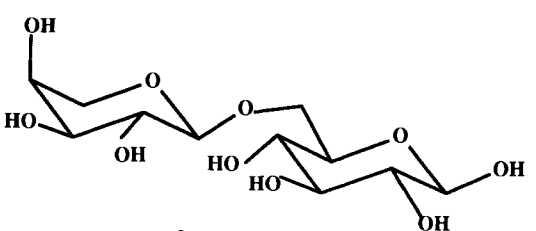
6a R = PrCO



7 R = R′ = H
7a R = PrCO, R′ = H
b R = H, R′ = PrCO
c R = R′ = PrCO



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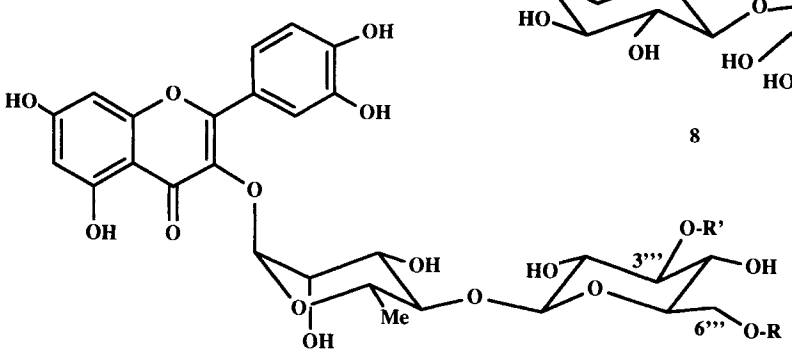

9 R = R′ = H
9a R = (*E*)-4-OH-C_6H_4-CH=CHCO (= *p*-coumaroyl); R′ = H
b R = H, R′ = PrCO

primary OH–C(6″) accessible. Usual enzymatic butanoylation of **6** occurred with 33% conversion, again to a single product, which turned out to be the expected 6″-*O*-butanoyl ester **6a** (^{13}C-NMR (*Table 1*): $\Delta\sigma$(C(6″)) = +2.8 and $\Delta\sigma$(C(5″)) = −3.1 ppm for **6a** *vs.* **6** [6]; ^{1}H-NMR: downfield shift of CH_2(6″) (*AB* of *ABX* at 4.32 and 4.03 ppm)).

In the subsequent step, we investigated the effect of replacing rhamnose with an other sugar. For this, we chose peltatoside (**7**) which contains the disaccharide vicianose (= 6-*O*-(α-L-arabinopyranosyl)-D-glucose; **8**) at OH–C(3) of quercetin. When **7** was reacted under the usual conditions, a complex mixture of at least six products was obtained, as shown by HPLC. Three of them were identified as being 4‴-*O*-butanoylpeltatoside (**7a**), 3″-*O*-butanoylpeltatoside (**7b**), and 3″,4‴-di-*O*-butanoylpeltatoside (**7c**).

FAB-MS source and gas-phase spectra established that only one butanoate residue was present in **7a** and **7b**, on the arabinosyl and glucosyl parts, respectively. On the other hand, two butanoate moieties were present in **7c**, one on each sugar moiety. As the ^{13}C-NMR spectrum of peltatoside (**7**) had not been previously attributed, we determined the acylation sites by a careful investigation of the ^{1}H-NMR spectra using extensive decoupling experiments. Peltatoside (**7**) exhibited 2 *d* at 5.36 (J = 7 Hz) and 3.94 ppm (J = 7 Hz) for the anomeric protons of the glucose and arabinose moiety. These signals were almost the same in the spectrum of **7a** which showed an additional br. *s* at 4.72 ppm. The latter could only be attributed to H–C(4‴): H–C(2‴) was excluded on the basis of multiplicity and decoupling of H–C(1‴); H–C(3‴) was excluded because a large *trans*-diaxal coupling constant value is expected for J (H–C(2‴), H–C(3‴)) [8]. In **7b**, the anomeric proton was shifted at 5.47 ppm (*d*, J = 7 Hz) and a *t* appeared at 4.86 ppm (J = 9 Hz, H–C(3″)). By applying the same methodology as for rutine (**1**), we established the attachment of butanoate at C(3″). The ^{1}H-NMR of diester **7c** had the same signals as those of **7a** and **7b**, with both H–C(3″) and H–C(4‴) present at 4.87 and 4.76 ppm. The structures of the three other products could not be identified because of the difficulties of isolating them in pure form. FAB-MS data suggested that two of them were monoesters, while the third one was a diester.

From the data reported above (and from those summarized in a previous communication [2]), it is evident that subtilisin is not able to acylate the rhamnose unit. Substitution of this sugar with arabinose causes a dramatic effect on the regioselectivity of the reaction, since the 'external' sugar can be acylated, too.

Recently, the major components of the pharmaceutical extract of *Ginkgo biloba* were isolated and identified as the 6‴-*O*-*p*-coumaroyl esters (= (*E*)-3-(4-hydroxyphenyl)prop-2-enoates) of the unusual quercetin and kaempferol glycosides kaempferol 3-*O*-[*O*-(β-D-glucopyranosyl)-(1→4)-α-L-rhamnoside] [9] and quercetin 3-*O*-[*O*-(β-D-glucopyranosyl)-(1→4)-α-L-rhamnoside] (**9**) [10]. These flavonol glycosides are unique in the sequence of their disaccharides, with rhamnose and not glucose linked to OH–C(3) of the aglycone. Therefore, these compounds appeared suitable substrates for concluding the present study, since the accumulated information led us to expect to obtain dominant acylation at OH–C(6‴) of the external glucose moiety, and, therefore, subtilisin would have afforded the same substitution pattern as in the natural esters, indicating possible use of a microbial enzyme for the preparation of potentially valuable pharmacological compounds of plant origin.

Quercetin 3-*O*-{*O*-[6‴-*O*-(*p*-coumaroyl)-β-D-glucopyranosyl]-β-(1→4)-α-L-rhamnoside} (**9a**) was isolated as suggested by *Anton* and coworkers [10]. Mild alkaline hydrolysis yielded **9**, which was fully characterized by FAB-MS, ^{13}C- and ^{1}H-NMR (see *Exper. Part* and *Table 1*). Treatment of **9** in anhydrous pyridine in the presence of subtilisin and an excess of trifluoroethyl butanoate resulted in moderate conversion (40%) after 48 h, yielding a single product which was isolated in the usual way and characterized as **9b**.

The attachment of the butanoate residue to the glucose moiety was suggested by FAB-MS data. However, to our surprise, inspection of the ^{13}C-NMR revealed that acylation had occurred at OH–C(3‴) and not at HO–C(6‴) of the glucose moiety (see *Table 1*[2])). In fact, although the C(3‴) shift was small (only +0.3 ppm downfield), the acylation of the OH group at this position was strongly supported by the expected shifts of C(2‴) and C(4‴) (–2.3 and –2.5 ppm upfield, resp.) [10]. Therefore, the structure of **9b** was identified as quercetin 3-*O*-[*O*-(3‴-*O*-butanoyl)-β-D-glucopyranosyl)-(1→4)-α-L-rhamnoside].

Conclusions. – We have shown that flavonoid disaccharide monoglycosides can be acylated successfully, on a preparative scale, by the catalytic action of the protease subtilisin in anhydrous pyridine. The regioselectivity is excellent for four of the five compounds **1**, **2**, **6**, **7**, and **9** tested. The results, summarized in *Table 2*, show an exclusive preference for the glucose moiety in disaccharides containing L-rhamnose and D-glucose (see **1**, **2**, **6**, and **9**). Both monosaccharidic moieties are esterified similarly if the disaccharide is composed of L-arabinose and D-glucose (see **7**). In each example, glucose is acylated at OH–C(6) or OH–C(3), but the acylation site can not be predicted *a priori* with confidence, as evidenced by the results obtained with compound **9**. Further studies are necessary to clarify the factors that regulate the approach of the carbohydrate nucleophile to the active site and, consequently, the selectivity of the enzyme.

Table 2. *Position of Acylation of the Disaccharide Moieties*

Compound	Disaccharide moiety	Position of acylation[a])
1, **2**	6-*O*-(α-L-rhamnopyranosyl)-D-glucose	C(3) of Glu
6	2-*O*-(α-L-rhamnopyranosyl)-D-glucose	C(6) of Glu
7	6-*O*-(α-L-arabinopyranosyl)-D-glucose	C(3) of Glu, C(4) of Ara, and others (not identified)
9	4-*O*-(β-D-glucopyranosyl)-L-rhamnose	C(3) of Glu

[a]) Glu = Glucose; Ara = arabinose.

Experimental Part

1. *General.* Subtilisin (EC 3.4.21.14, protease from *B. licheniformis*) was obtained from *Sigma*; prior to use, this enzyme was dissolved in H_2O, and the soln. adjusted to pH 7.8 and freeze-dried [1a]. Rutin (**1**), hesperidin (**2**), and naringin (**6**) were from *Aldrich*, while peltatoside (**7**) was purchased from *Extrasynthese*. Pyridine (anal. grade) was used without further purification apart from drying by shaking with 3-Å molecular sieve (*Merck*). Trifluoroethyl butanoate was prepared as described [1b]. Enzymatic transesterifications were followed by HPLC: *JASCO 880/PV* pump, *JASCO 870* UV/VS detector; *Erbasil* 10 μm C_{18}/*M* column (250 mm × 4.6 mm); 15-min linear gradient from 10 to 60% MeCN in H_2O (containing 0.1% CF_3COOH); flow rate 1 ml/min, readings at 254 nm. TLC: precoated silica gel *60* F_{254} plates from *Merck*; *A* = AcOEt/AcOH/H_2O 15:1:1, *B* = AcOEt/AcOH/H_2O/HCOOH 15:1:2:1, *C* = AcOEt/MeOH/H_2O 9:1.5:0.5, *D* = AcOEt/AcOH/H_2O/HCOOH 50:1:2:1. Optical rotation: at 589 nm (Na line) and 25°; *Perkin-Elmer-141* polarimeter. ^{1}H-NMR (200 MHz) and ^{13}C-NMR spectra (50.2 MHz): *Varian XL-200*. FAB-MS: *VG-anal-70-70-EQ-HF* instrument equipped with own source.

2. *3″-O-Butanoylrutin* (= *3-{[3-O-Butanoyl-6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy}-5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4*H*-1-benzopyran-4-one*; **1a**). Subtilisin (400 mg) was added to 15 ml of anh. pyridine containing 0.82 mmol (500 mg) of **1** and 4.1 mmol of trifluoroethyl butanoate. The suspension was shaken at 250 rpm and at 45°. After 48 h, HPLC showed that 67% of **1** had been acylated. The enzyme was removed by filtration, the solvent evaporated, and the crude residue purified by chromatography (silica gel, *A*): 290 mg (53%), of **1a**. Amorphous solid. TLC (*B*): R_f 0.68. HPLC: t_R 12.3. $[\alpha]_D = -6.7$ ($c = 0.36$, MeOH). ^{1}H-NMR

(200 MHz, (D_6)DMSO): sugar moiety: 5.45 (*d*, H–C(1″)); 4.84 (*t*, H–C(3″)); aliph. chain 2.30 (*t*); 1.56 (*m*); 0.90 (*t*). FAB-MS: 679 (100, $[M - H]^-$), 609 (2), 301 (84), 285 (22).

3. *3″-O-Butanoylhesperidin* (= *(2S)-7-{[3-O-Butanoyl-6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy}-2,3-dihydro-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyran-4-one*; **2a**). Hesperidin (500 mg, 0.82 mmol) was reacted as described above. After 48 h, HPLC showed that 65% of **2** had been acylated. Usual workup and purification by chromatography (silica gel, *A*) furnished 250 mg (45%) of **2a**. Amorphous solid. TLC (*B*): R_f 0.67. HPLC: t_R 13.0. $[\alpha]_D = -68.0$ (*c* = 0.5, MeOH). ^{1}H-NMR (200 MHz, (D_6)DMSO): sugar moiety: 5.46 (*d*, H–C(1″)); 4.90 (*t*, H–C(3″)); aliph. chain: 2.30 (*t*); 1.55 (*m*); 0.86 (*t*). FAB-MS: 679 (43, $[M - H]^-$), 609 (2), 301 (100), 285 (11).

4. *3-O-Butanoylrutinose* (= *3-O-Butanoyl-6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranose*; **3a**). Rutinose (**3**; 500 mg, 1.5 mmol) was dissolved in 10 ml of anh. pyridine containing 4.5 mmol of trifluoroethyl butanoate. Subtilisin (200 mg) was added and the suspension shaken at 250 rpm and at 45° for 48 h. Usual workup and purification by chromatography (silica gel, *C*) furnished 186 mg (32%) of **3a**. Oil. FAB-MS: 395 (100, $[M - H]^-$), 307 (98), 265 (41), 205 (80), 163 (91).

5. *6″-O-Butanoylnaringin* (= *(2S)-7-{[6-O-Butanoyl-2-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy}-2,3-dihydro-5-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one*; **6a**). To naringin (500 mg, 0.86 mmol) and trifluoroethyl butanoate (4.3 mmol) in 15 ml of anh. pyridine, subtilisin (400 mg) was added, and the suspension was shaken at 45° for 48 h (33% conversion by HPLC). Usual workup furnished 176 mg (0.27 mmol) of **6a**. Amorphous solid. TLC (*B*): R_f 0.53. HPLC: t_R 13.2. $[\alpha]_D = -95.9$ (*c* = 0.37, MeOH). ^{1}H-NMR (200 MHz, (D_6)DMSO): sugar moiety: 5.16 (*d*, H–C(1″)); 4.03 (*dd*, CH_2(6″)); aliph. chain: 2.20 (*t*); 1.45 (*m*); 0.76 (*t*). FAB-MS: 649 (97, $[M - H]^-$), 579 (1), 467 (4), 398 (2), 271 (100).

6. *Acylation of Peltatoside* (**7**). Subtilisin (400 mg) was added to 15 ml of anh. pyridine containing 1.08 mmol (500 mg) of **7** and 3.2 mmol of trifluoroethyl butanoate. The suspension was shaken at 250 rpm and at 45°. After 48 h, HPLC showed that 78% of **7** had been acylated. The enzyme was removed by filtration, the solvent evaporated, and the crude residue first purified by chromatography (silica gel, *A*): 300 mg (57%) of a mixture of monobutanoyl derivatives of **7** and 25 mg (5%) of a mixture of dibutanoyl derivatives of **7**. Further purification by prep. HPLC (*Partisil 10 ODS-3 Whatman* column, 25 × 2.1 cm; MeCN/H_2O 1:3) allowed the isolation of 2 (over at least 4) monoesters and 1 diester, **7a–c**.

3″-O-Butanoylpeltatoside (= *3-{[6-O-(α-L-Arabinopyranosyl)-3-O-butanoyl-β-D-glucopyranosyl]oxy}-5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4H-1-benzopyran-4-one*; **7b**). ^{1}H-NMR (200 MHz, (D_6)DMSO): sugar moiety: 5.37 (*d*, H–C(1″)); 4.86 (*t*, H–C(3″)); 3.96 (*d*, H–C(1‴)); aliph. chain: 2.31 (*t*); 1.58 (*m*); 0.93 (*t*). FAB-MS: (100, $[M - H]^-$), 595 (2), 532 (6), 301 (24), 285 (6).

4‴-O-Butanoylpeltatoside (= *3-{[6-O-(4-O-Butanoyl-α-L-arabinopyranosyl)-β-D-glucopyranosyl]oxy}-5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4H-1-benzopyran-4-one*; **7a**). ^{1}H-NMR (200 MHz, (D_6)DMSO): sugar moiety: 5.37 (*d*, H–C(1″)); 4.72 (br. *s*, H–C(4‴)); 3.99 (*d*, H–C(1‴)); aliph. chain: 2.23 (*t*); 1.52 (*m*); 0.86 (*t*). FAB-MS: 665 (100, $[M - H]^-$), 595 (2), 463 (5), 301 (49), 285 (12).

3″,4‴-Di-O-butanoylpeltatoside (= *3-{[3-O-Butanoyl-6-O-(4-O-butanoyl-α-L-arabinopyranosyl)-β-D-glucopyranosyl]oxy}-5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4H-1-benzopyran-4-one*; **7c**). ^{1}H-NMR (200 MHz, (D_6)DMSO): sugar moiety: 5.48 (*d*, H–C(1″)); 4.87 (*t*, H–C(3″)); 4.76 (br. *s*, H–C(4‴)); 3.99 (*d*, H–C(1‴)); aliph. chain: 2.33, 2.24 (2*t*); 1.60, 1.52 (2*m*); 0.93, 0.87 (2*t*). FAB-MS: 774 (100, $[M - H]^-$), 664 (5), 532 (10), 301 (85), 285 (20).

7. *Quercetin 3-O-[O-(β-D-Glucopyranosyl)-(1→4)-α-L-rhamnoside]* (= *3-{[6-Deoxy-4-O-(β-D-glucopyranosyl)-α-L-mannopyranosyl]oxy}-5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4H-1-benzopyran-4-one*; **9**). Quercetin 3-*O*-{*O*-[6‴-*O*-(*p*-coumaroyl)-β-D-glucopyranosyl]-(1→4)-α-L-rhamnoside} (135 mg), isolated by silica-gel chromatography from a crude extract of *Ginkgo biloba*, was dissolved in 15 ml of MeOH containing 0.5% (*w*/*v*) of KOH. After 10 h at r.t., the ester was completely hydrolyzed. MeOH was evaporated and the residue dissolved in 15 ml of H_2O. Following acidification to pH 3 and extraction of the *p*-coumaric acid (= (*E*)-3-(4-hydroxyphenyl)prop-2-enoic acid) with Et_2O the H_2O layer was lyophilized. Anh. pyridine was added and the solid residue (inorg. salts) filtered off. Pyridine was subsequently evaporated furnishing 98 mg of **9**. Amorphous solid. ^{1}H-NMR (200 MHz, (D_6)DMSO): sugar moiety: 5.58 (*d*, H–C(1″)); 4.29 (*d*, H–C(1‴)); 0.91 (*d*, CH_3(6″)). FAB-MS: 609 (100, $[M - H]^-$), 447 (6), 301 (91).

8. *Acylation of* **9**. To a soln. of **9** (0.16 mmol, 98 mg) in 3 ml of anh. pyridine, trifluoroethyl butanoate (0.8 mmol) and subtilisin (100 mg) were added, and the suspension was shaken at 45° for 48 h (45% conversion by

HPLC). Usual workup and purification by chromatography (silica gel, *D*) furnished 17 mg (15%) of *quercetin 3-O-[O-(3‴-O-butanoyl-β-D-glucopyranosyl)-(1→4)-α-L-rhamnoside]* (= *3-{[4-O-(3-O-butanoyl-β-D-glucopyranosyl)-6-deoxy-α-L-mannopyranosyl]oxy}-5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4H-1-benzopyran-4-one*; **9b**). TLC (*B*): R_f 0.58. FAB-MS: 679 (82, $[M - H]^-$), 467 (13), 447 (5), 300 (100), 285 (47).

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