PERGAMON

Phytochemistry 60 (2002) 639–646

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Glycosides of polyenoic branched fatty acids from myxomycetes

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Received 23 November 2001; received in revised form 18 April 2002

Abstract

The determination of chemical structures of five novel compounds, i.e. one multibranched polyunsaturated fatty acid ((2*E*,4*E*,7*S*,8*E*,10*E*,12*E*,14*S*)-7,9,13,17-tetramethyl-7,14-dihydroxy-2,4,8,10,12,16-octadecahexaenoic acid) and its four glycosides from seven different myxomycetes is described. The absolute configuration of both hydroxyl groups was determined. The glycosides containing glucose, mannose and rhamnose. These compounds were identified by means of ^{1}H and ^{13}C NMR, MS, UV and IR spectra. Three of them were identified in *Arcyria cinerea* (Bull.) Pers., two in *A. denudata* (L.) Wetts., and *A. nutans* (Bull.) Grev., *Fuligo septica* (L.) Wigg., *Lycogala epidendrum* (L.) Fries, *Physarum polycephalum* Schwein., and *Trichia varia* Pers. contained one of the identified glycosides each.

Keywords: Myxomycetes; Glycosides; Polyunsaturated branched fatty acids

1. Introduction

Myxomycetes, commonly called slime molds, are some of nature's most extraordinary organisms in that they exhibit characteristics of both fungi and animals. In the trophic (feeding) stage, the slime mold moves about as a mass of protoplasm (the plasmodium) feeding on bacteria, spores and other organic matter, much like an amoeba. Myxomycetes in the feeding stage are most often found under the bark of decaying logs or between layers of leaf litter. Plasmodia may be white or bright red, orange or yellow. When the food supply is exhausted or other unfavorable conditions occur, the plasmodium changes, producing structures, called fruiting bodies, whose reproductive spores when released, germinate and begin the life cycle anew (Stephenson and Stempen, 2000).

Generally, myxomycetes produce unsaturated compounds, e.g. in *Fuligo septica*, the polyene pigment fuligorubin A was isolated (Casser et al., 1987). Further polyenes from myxomycetes are the ceratiopyrones from *Ceratiomyxa fructiculosa* (Velten et al., 1995) and compounds mentioned in a review article on the chemistry of slime moulds (Steglich, 1989). Other coloring substances with polyenoic unsaturated chain such as chrysophysarin A from *Physarum polycephalum* (Eisenbarth and Steffan, 2000), physarochrome A (Steffan et al., 1987), polycephalins B and C (Nowak and Steffan, 1998), or physarorubinic acid (Nowak and Steffan, 1997) were also described.

In our continuing search (Rezanka, 1993; Rezanka and Guschina, 2000) for unusual bioactive compounds from organisms growing in extreme environmental conditions, we recently collected several myxomycetes (see Table 1). One acid and four glycosides were isolated from seven species of slimemoulds.

2. Results and discussion

The myxomycetes (Table 1) were extracted and the extract was separated on Sephadex LH-20. The fractions were further purified by RP-HPLC to give one acid (**1**) and four glycosides (**2**–**5**)—see Table 1, which were identified by IR, UV, MS and ^{1}H and ^{13}C NMR spectral data.

The molecular formula of **1** (Fig. 1) was established as $C_{22}H_{32}O_4$ from the HRFAB–MS and NMR spectral analyses. The UV spectrum showed two maxima, one at 268 nm, i.e. conjugated triene and another at 242 nm

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0031-9422/02/$ - see front matter
PII: S0031-9422(02)00159-0

($\Delta^{2,3}$ and $\Delta^{3,4}$ conjugated acid) (Scott, 1964). The IR spectrum featured bands at 3400 (free OH) and 1710 cm^{-1} (unsaturated conjugated acid) (Crews et al., 1998).

NMR data of **1** are shown in Tables 2 and 3. In the ^1H NMR spectrum of **1**, nine olefinic protons were observed in the range between 5.11 and 7.23 ppm. A combination of ^1H–^1H DQFCOSY and ^1H–HMBC experiments revealed the partial structure of the unsaturated branch of **1**. In the ^1H–^1H DQFCOSY spectrum, the protons at 5.90 and 6.25 ppm (labeled H-2 and H-4, respectively) revealed couplings to the proton at 7.23 (H-3). In the HMBC spectrum the proton at 5.90 ppm (H-2) showed cross peaks with C-1. The ^1H–^1H DQFCOSY spectrum also revealed the couplings of protons at 6.73 (H-10) and 6.40 ppm (H-12) with the proton at 6.94 ppm (H-11). HMBC experiment results revealed the coupling of H-12 with C-13, C-14 and C-21, and both H-8 and H-10 showed couplings with C-9.

The geometry of double bonds $\Delta^{2,3}$, $\Delta^{4,5}$ and $\Delta^{10,11}$ was confirmed to be all *E* by the coupling constants of $J_{2,3}=15.6$, $J_{4,5}=15.6$ and $J_{10,11}=15.0$ Hz. Also the chemical shift (δ 14.8, 12.6 respectively) of CH_3-20 and CH_3-21 suggested 8*E* and 12*E* geometries. Methyl signals at 1.82 (3H, *s*), 1.79 (3H, *s*) and 1.64 (3H, *d*) were typical for the ^1H NMR spectrum of *E*-oriented carotenoids (Ravi et al., 1981).

The substitution at C-14 was confirmed by the COSY correlation between H-14 and the C-15 methylene protons at δ 2.19 and δ 2.25 and by the HMBC correlation between C-14 and H-21.

The absolute configuration at C-14 was determined to be *S* by Mosher's method (Ohtani et al., 1991a,b). The ^1H NMR spectra of the (*S*)- and (*R*)-MTPA esters, **1*S*** and **1*R***, were recorded and based on the Δ (δ **1*S***-δ **1*R***) values; the *S* configuration was assigned for the C-14 alcohol, see Fig. 1. The reaction with MTPACl did not esterify the tertiary hydroxyl group on C-7. We therefore used oxidative splitting and chromatography of appropriate methyl esters on a chiral capillary column, and determined the absolute configuration of compound **1**. As shown in Table 4, many non-chiral and only one chiral compound were isolated from the reaction mixture, i.e. (2*S*)-2-methyl-2-hydroxybutanedioic acid (citramalic acid). Baseline separations were obtained on the chiral capillary column (conditions are described in Experimental) with an appropriate phase. First, the standards of both enantiomers of citramalic acid were chromatographed as methyl esters. Second, the oxidation products were chromatographed, also as methyl esters and the retention times of the corresponding peaks were compared (Table 4).

Thus the full structure of **1** was (2*E*,4*E*,7*S*,8*E*,10*E*,12*E*,14*S*) - 7,9,13,17 - tetramethyl - 7,14 - dihydroxy - 2,4,8,10,12,16-octadecahexaenoic acid.

Interpretation of the ^1H and ^{13}C NMR spectra of compound **2** (Tables 2 and 3) indicated the presence of five methyls, three methylenes, 15 methines and five quaternary carbons. The ^{13}C NMR spectrum also indicated oxygenated functionalities: a conjugated acid and a hexose sugar moiety giving a molecular formula of $C_{28}H_{42}O_9$, which was confirmed by the high-resolution FAB–MS *m*/*z* 523.2911 $(M+H)^+$. The protonated carbons were all assigned using an HSQC NMR experiment. An unsaturation number of 8, in conjunction with the presence of the carboxyl group and six double bonds, suggested the presence of one ring.

The splitting pattern and the respective *J* values of the protons in the olefinic region of the ^1H spectrum indicated proton H-11 (δ 6.97 ppm) to be coupled to both protons H-10 (δ 6.73 ppm) and H-12 (δ 6.44 ppm) in a *E* geometry typical for carotenoids. A substructure consisting of C-8 to C-18 was constructed, which was further supported by ^1H–^1H COSY correlations H-12–H-21, H-14–H-15, H-18–H-22. The HMBC NMR spectrum showed connectivity from the H-20 to C-8 and C-9, indicating that methyl 20 was attached at C-9. Similarly, H-21 showed HMBC correlations to C-14 and C-12, which suggested that the methyl (C-21) was attached at C-13. Further HMBC connectivities were found from

Table 1
Distribution of the polyenoic compounds identified in the seven species of slime moulds collected at Central Bohemia

Slime mould species	Description and weight (lyophilized in g)	The fatty acid (**1**) and its glycosides (**2**–**5**)[b] (mg /X g)				
		1	**2**	**3**	**4**	**5**
Arcyria cinerea (Bull.) Pers.	Stalked sporangia, ochraceous-light brown, 12.VIII. 2001;4.7	4.8	–[a]	14.2	–	–
A. denudata (L.) Wetts.	Stalked sporangia, brick red brown, 7.IX. 2001; 5.8	5.1	–	3.4	–	–
A. nutans (Bull.) Grev.	Stalked sporangia, bright-pale yellow, 2.IX. 2001; 3.7	1.9	–	9.6	–	–
Fuligo septica (L.) Wigg.	Gregarious aethalia pale pink, 6.VII. 2001; 14.4	–	–	–	7.8	–
Lycogala epidendrum (L.) Fries	Aethalia, pinkish gray, 25.X. 2001; 11.3	–	4.2	–	–	–
Physarum polycephalum Schwein.	Sessile sporangia, greenish yellow, 23.IX. 2001; 12.8	–	–	–	–	3.3
Trichia varia Pers.	Sessile sporangia, yellow-green-brown, 22.IX. 2001; 13.2	–	–	8.5	–	–

[a] Less than 0.1 mg/X g of lyophilized weight.
[b] Names of the compounds are included in the text.

C-9 to H-10 and H-11; C-12 to H-10 and H-14; C-16 to H-14, H-15, H-18, and H-22; and C-17 to H-15, H-18, and H-22, thus confirming the C-8–C-18 substructure. NOE difference experiments for H-11–H-20 and H-21, H-10–H-12, H-16–H-18 firmly established the all *E* configuration of the octadecahexaene system. The ^{1}H-^{1}H COSY spectrum also showed that five of the deshielded methines (H-1′–H-5′) and the deshielded methylene H-6′ were in the same spin system, which suggested the presence of a hexose sugar moiety. A literature search showed that the ^{13}C chemical shifts most closely resembled those of a glucose sugar in the pyranose form (Breitmaier and Voelter, 1989).

All four glycosides were hydrolyzed under acidic conditions. Unfortunately, after hydrolysis, the solution was very intensively coloured. The luminous yellow-orange color with $\lambda_{max} = 455$ nm rapidly darkened to brown and it was impossible to extract the free acid from reaction solution. We assumed, on the basis of Woodward-Fieser rules, that during hydrolysis, the two molecules of water are eliminated and conjugated octaenoic acid was formed, which is rapidly destroyed.

To determine the absolute configuration of carbohydrates in the glycosides, the free sugars were prepared from glycosides by enzymatic hydrolysis. Compound **2** was hydrolyzed by β-D-glucosidase (EC 3.2.1.21 from almonds), compound **3** by α-D-glucosidase (EC 3.2.1.20 from yeast), **4** by α-D-mannosidase (EC 3.2.1.24 from jack beans) and **5** by hesperidinase (hesperidin-α-1,6-rhamnosidase (EC 3.2.1.40) from *Aspergillus niger*, which contains both α-L-rhamnosidase and β-D-glucosidase. Acetylated-2-butyl derivatives were obtained after

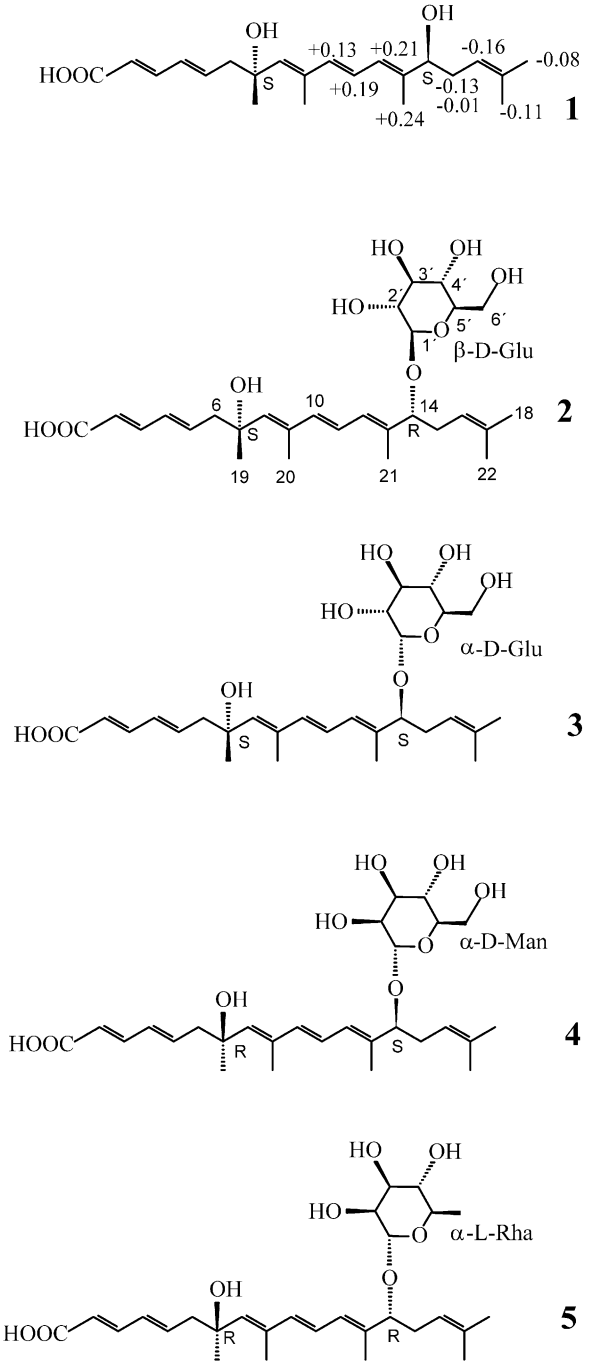

Fig. 1. The structures of hydroxy-polyenoic branched fatty acid (**1**) and glycosides (**2**–**5**) from myxomycetes.

Table 2
^{13}C NMR data of **1**–**5**, solvent see Experimental

Carbon No.	Compound				
	1	**2**	**3**	**4**	**5**
1	169.5	169.4	170.0	169.3	169.1
2	122.2	122.3	122.0	122.5	122.1
3	146.6	146.4	146.5	146.3	146.8
4	132.5	132.1	132.4	132.6	132.4
5	131.7	131.6	131.8	131.4	131.9
6	42.8	43.0	42.7	42.9	43.1
7	71.8	72.0	71.9	71.7	72.1
8	136.3	129.4	131.0	132.7	133.5
9	141.0	141.1	141.4	141.2	140.9
10	132.9	132.4	132.6	132.7	132.5
11	130.3	130.1	130.2	130.4	130.2
12	126.7	126.5	126.4	126.6	126.4
13	143.7	140.1	141.1	142.3	138.4
14	77.0	84.4	81.4	80.2	86.1
15	24.7	20.3	20.3	19.4	21.2
16	120.8	120.8	120.7	120.8	120.8
17	134.0	133.9	134.2	133.7	134.2
18	25.6	25.8	25.4	25.9	25.4
19	27.9	27.8	27.6	27.7	27.4
20	14.8	14.3	14.4	14.9	14.5
21	12.6	12.2	12.3	12.8	12.5
22	18.0	17.9	18.2	18.1	18.4
1′		101.7	97.1	98.2	97.4
2′		74.8	71.7	72.9	72.7
3′		77.9	75.3	72.6	72.5
4′		72.5	71.4	68.9	73.2
5′		76.8	73.9	75.3	69.7
6′		68.1	62.2	63.1	18.6

Table 3
^{1}H NMR data of **1**–**5**, solvent see Experimental

H No.	**1**	**2**	**3**	**4**	**5**
2	5.90 (1H, *d*, *J* = 15.6)	5.91 (1H, *d*, *J* = 15.4)	5.92 (1H, *d*, *J* = 15.5)	5.93 (1H, *d*, *J* = 15.7)	5.91 (1H, *d*, *J* = 15.3)
3	7.23 (1H, *dd*, *J* = 15.6, 10.7)	7.24 (1H, *dd*, *J* = 15.4, 10.9)	7.28 (1H, *dd*, *J* = 15.5, 10.8)	7.30 (1H, *dd*, *J* = 15.7, 11.0)	7.27 (1H, *dd*, *J* = 15.3, 10.4)
4	6.25 (1H, *dd*, *J* = 15.6, 10.7)	6.24 (1H, *dd*, *J* = 15.4, 10.9)	6.30 (1H, *dd*, *J* = 15.5, 10.8)	6.26 (1H, *dd*, *J* = 15.9, 11.0)	6.22 (1H, *dd*, *J* = 15.7, 10.4)
5	6.15 (1H, *dt*, *J* = 15.6, 8.1)	6.17 (1H, *dt*, *J* = 15.4, 8.2)	6.13 (1H, *dt*, *J* = 15.5, 8.0)	6.16 (1H, *dt*, *J* = 15.9, 7.9)	6.14 (1H, *dt*, *J* = 15.7, 8.2)
6	2.39 (2H, *m*)	2.35 (2H, *m*)	2.40 (2H, *m*)	2.36 (2H, *m*)	2.38 (2H, *m*)
8	5.65 (1H, *s*)	5.65 (1H, *s*)	5.65 (1H, *s*)	5.65 (1H, *s*)	5.65 (1H, *s*)
10	6.73 (1H, *d*, *J* = 15.0)	6.70 (1H, *d*, *J* = 15.8)	6.79 (1H, *d*, *J* = 15.5)	6.77 (1H, *d*, *J* = 15.3)	6.76 (1H, *d*, *J* = 15.4)
11	6.94 (1H, *dd*, *J* = 15.0, 11.0)	6.97 (1H, *dd*, *J* = 15.8, 11.0)	6.96 (1H, *dd*, *J* = 15.5, 11.4)	6.93 (1H, *dd*, *J* = 15.3, 11.2)	6.95 (1H, *dd*, *J* = 15.4, 10.9)
12	6.40 (1H, *d*, *J* = 11.0)	6.44 (1H, *d*, *J* = 11.0)	6.41 (1H, *d*, *J* = 11.4)	6.47 (1H, *d*, *J* = 11.2)	6.39 (1H, *d*, *J* = 10.9)
14	3.95 (1H, *dd*, *J* = 7.9, 5.2)	4.13 (1H, *dd*, *J* = 7.9, 5.2)	4.27 (1H, *dd*, *J* = 7.9, 5.2)	4.31 (1H, *dd*, *J* = 7.9, 5.2)	4.11 (1H, *dd*, *J* = 7.9, 5.2)
15	2.19 (1H, *m*); 2.25 (1H, *m*)	2.23 (1H, *m*); 2.29 (1H, *m*)	2.28 (1H, *m*); 2.37 (1H, *m*)	2.27 (1H, *m*); 2.32 (1H, *m*)	2.24 (1H, *m*); 2.30 (1H, *m*)
16	5.11 (1H, *t*, *J* = 6.7)	5.11 (1H, br*t*, *J* = 7.0)	5.12 (1H, br*t*, *J* = 7.3)	5.13 (1H, br*t*, *J* = 7.0)	5.14 (1H, br*t*, *J* = 6.9)
18	1.52 (3H, *d*, *J* = 1.2)	1.51 (3H, *d*, *J* = 1.4)	1.54 (3H, *d*, *J* = 1.3)	1.53 (3H, *d*, *J* = 1.3)	1.50 (3H, *d*, *J* = 1.5)
19	1.29 (3H, *s*)	1.33 (3H, *s*)	1.31 (3H, *s*)	1.32 (3H, *s*)	1.34 (3H, *s*)
20	1.82 (3H, *s*)	1.84 (3H, *s*)	1.80 (3H, *s*)	1.82 (3H, *s*)	1.85 (3H, *s*)
21	1.79 (3H, *s*)	1.78 (3H, *s*)	1.81 (3H, *s*)	1.84 (3H, *s*)	1.80 (3H, *s*)
22	1.64 (3H, *d*, *J* = 1.2)	1.65 (3H, *d*, *J* = 1.4)	1.62 (3H, *d*, *J* = 1.3)	1.60 (3H, *d*, *J* = 1.3)	1.63 (3H, *d*, *J* = 2.6)
1′		4.82 (1H, *d*, *J* = 7.1)	5.01 (1H, *d*, *J* = 2.7)	4.32 (1H, *d*, *J* = 1.2)	4.98 (1H, *d*, *J* = 2.6)
2′		3.54 (1H, *dd*, 8.9, 7.1)	3.59 (1H, *dd*, *J* = 2.7, 9.4)	3.69 (1H, *dd*, *J* = 1.2, 2.5)	3.92 (1H, *dd*, *J* = 2.6, 2.5)
3′		3.60 (1H, *t*, *J* = 8.9)	3.71 (1H, *t*, *J* = 9.4)	3.73 (1H, *dd*, *J* = 2.5,9.0)	3.71 (1H, *dd*, *J* = 2.5, 9.4)
4′		3.42 (1H, *t*, *J* = 8.9)	3.44 (1H, *t*, *J* = 9.4)	3.50 (1H, *t*, *J* = 9.0)	4.28 (1H, *t*, *J* = 9.4)
5′		3.47 (1H, *m*)	3.75 (1H, *m*)	3.32 (1H, *ddd*, *J* = 9.0, 9.0, 5.7)	4.11 (1H, *dq*, *J* = 9.4, 6.5)
6′		3.75 (1H, *dd*, 12.0, 5.2)	3.89 (1H, *dd*, 11.8, 1.9)	3.67 (1H, *dd*, *J* = 11.4, 9.0)	1.35 (3H, *d*, *J* = 6.5)
		3.95 (1H, *dd*, 12.0, 2.2)	3.78 (1H, *dd*, 11.8, 5.4)	3.48 (1H, *dd*, *J* = 11.4, 5.7)	

derivatization and they were analyzed by gas chromatography using a glass-capillary column (Supelco SPB-1) (Gerwig et al., 1978). The acetylated (+)-2-butyl derivatives (see Experimental) were eluted as the peaks with retention times identical with those of tetraacetyl (+)-2-butyl-D-glucose, tetraacetyl (+)-2-butyl-D-mannose and tetraacetyl (+)-2-butyl-L-rhamnose, respectively, see experimental. These results revealed that **2** and **3** contained D-glucoses, **4** D-mannose and **5** L-rhamnose (Fig. 1).

The signals of five oxymethine protons in trans diaxial conformations ($J = \sim 8$ Hz) and one oxymethylene group indicated the presence of a β-glucopyranosyl group. Subsequently, **2** was hydrolyzed by β-D-glucosidase, furnishing an aglycone and D-glucose.

In the ^{1}H NMR spectrum of **2**, the unusual 14-*O*-glycosylation was indicated by downfield shifts of H-14 (+0.18 ppm) and H-15 (+0.04 ppm) with respect to acid **1** as parent compound. Similarly, in the ^{13}C NMR spectra of **2** (Table 2) 14-*O*-glycosylation was confirmed by the diagnostic downfield shift of C-14 (+7.4 ppm) and by upfield shift of the related C-15 (−4.4 ppm) and C-13 (−3.6 ppm) carbons with respect to acid **1**.

The absolute configuration of **2** was established by ^{13}C NMR spectroscopy. Comparison of the ^{13}C NMR chemical shifts of glycoside **2** with those of **1** (Tables 2 and 3) revealed that a larger glycosidation shift at C-15 (|4.4| ppm) than that at C-13 (|3.6| ppm) was observed in pyridine-(D_5) as solvent. Application of the glycosidation shift rule (Seo et al., 1978; Tori et al., 1977; Kasai et al., 1977) to these shifts indicated the configuration at C-14 of the glycoside **2** to be *R* and thus the glycoside **2** was confirmed to be (14*R*)-14-*O*-β-D-glucopyranoside of *S,R*-**1** acid.

It is well known that protons of a hexose in a chair conformation will have different coupling constants depending on their orientation in space. Axial-axial proton couplings will be the largest (7–10 Hz), whereas equatorial-axial or equatorial-equatorial couplings will be smaller, i.e. 2–3 Hz (Bork and Thogersen, 1982).

Table 4
The presence of degradation products (determined by chiral capillary GC) after oxidation of compounds **1**–**5**

Methyl ester	RT of products after degradation (min^{-1})					
	Standards	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]
Acetic	2.15	2.17	2.17	2.16	2.18	2.17
Pyruvic	6.58	6.60	6.59	6.60	6.61	6.60
Oxalic	8.12	8.11	8.12	8.12	8.11	8.12
Malonic	11.76	11.77	11.75	11.76	11.77	11.76
2*R*-Citramalic	18.27	–	–	–	18.30	18.31
2*S*-Citramalic	18.96	19.01	18.97	18.99	–	–

[a] The acetone was also presented.

The ^{1}H NMR spectrum of **3** was typical for a glycoside with sugar region showing one anomeric proton doublet, with coupling constant characteristic of α-anomers ($J_{1,2} = 2.7$ Hz). The rest of the glycosidic protons were resolved well enough to allow for most of the coupling constants to be measured. Assignment of the different resonances to each of the residues was achieved through COSY and TOCSY experiments. The large coupling constants found for protons H-2′, H-3′ and H-4′ of the spin system of the residue were consistent with glucose moiety (see Table 3).

In the spectrum of **3**, the glycosidation shift at C-15 (|4.4|) and at C-13 (|2.6| ppm) is the same as in **2** but with α-glucose; the configuration of **3** thus must be *S*, and the glycoside is then (14*S*)-14-*O*-α-D-glucopyranoside of *S,S*-**1** acid.

In compound **4** the large vicinal coupling constants, $^3J_{\text{H-3′-H-4′}} = 9.0$ Hz and $^3J_{\text{H-4′-H-5′}} = 9.0$ Hz, and the NOE between H-3′ and H-5′ indicated that H-3′, H-4′ and H-5′ protons were located in an axial position. The observation of NOE between H-4′ and H-2′ proton suggested that H-2′ was oriented in an equatorial position. From these data and from enzymatic hydrolysis, we deduced that the sugar is a D-mannopyranoside. β-Configuration was determined by an NOE correlation from H-1′ to H-3′ and H-5′, and by a small coupling constant $^1J_{\text{C-1′-H-1′}} = 157.5$ Hz ($<<166$ Hz) (Kasai et al., 1977).

Finally, the connectivity was accomplished from the ^{1}H–^{13}C correlations; the long range couplings from anomeric proton H-1′ (δ 4.32) to oxygenated methine carbon C-14 (δ 80.2). In the spectrum of **4**, the glycosidation shifts are at C-15 (|5.3|) and at C-13 (|1.4| ppm). Based on the results of the NMR studies described above, the structure of **4** ((14*S*)-14-*O*-α-D-mannopyranoside of *R,S*-**1** acid) was determined as shown in Fig. 1.

The observed value of $J_{\text{H-1′-H2′}} = 2.5$ Hz from compound **5**, which is close to the literature (Breitmaier and Voelter, 1989) reported value of 2.8 Hz. NOEs confirmed that the hexose sugar was α-rhamnopyranose. HMBC connectivities from H-14 to C-1′ as well as C-14 and H-1 indicated the position of attachment of the α-rhamnopyranose moiety. From enzymatic hydrolysis we also deduced that the sugar must be L-α-rhamnose. The glycosidation shifts of **5** are opposite to those in **2**, **3** and **4** and are at C-15 (|3.5|); at C-13 (|5.3| ppm) and the structure of the glycoside therefore must be (14*R*)-14-*O*-α-L-rhamnopyranoside of *R,R*-**1** acid.

3. Experimental

3.1. General experimental procedures.

UV spectra were measured in heptane within the range of 200–350 nm by a Cary 118 (Varian) apparatus.

A Perkin-Elmer Model 1310 (Perkin-Elmer, Norwalk, CT, USA) IR spectrophotometer was used for scanning IR spectroscopy of acids and glycosides as neat films. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^{1}H), 125.7 MHz (^{13}C) in mixture of deuterated pyridine and CD_3OD (v/v 1:1). High- and also low-resolution MS were recorded using a VG 7070E - HF spectrometer (70 eV). HRFABMS (positive and/or negative ion mode) were obtained with a PEG-400 matrix. RP-HPLC was carried out using Shimadzu gradient LC system (Shimadzu, Kyoto, Japan). Gas chromatography analysis was made on a Hewlett Packard HP 5980 gas chromatograph (Hewlett Packard, Czech Republic).

The following compounds: acetic acid, acetone, malonic, oxalic, pyruvic, (*R*)-(−)-citramalic acid (i.e. 2-hydroxy-2-methylbutanedioic acid or 2-methylmalic acid) and (*S*)-(+)-citramalic acid, α-D-glucosidase (EC 3.2.1.20) from bakers yeast, β-D-glucosidase, (EC 3.2.1.21) from almonds, α-D-mannosidase (EC 3.2.1.24) from jack beans, hesperidin-α-1,6-rhamnosidase (EC 3.2.1.40) from *Aspergillus niger* (contains both α-L-rhamnosidase and β-D-glucosidase) were purchased from Sigma-Aldrich (Prague, Czech Republic).

3.2. Plant material

The specimens of slime-moulds were collected in wet weather about 30 km south of Prague (Central Bohemia), for more details, see Table 1. They were identified taxonomically and are kept in our department.

3.3. Extraction and isolation

Samples of slime-moulds (see Table 1) were separately extracted by 90% butanol. Chromatography of every extract on a Sephadex LH-20 column (100×5 cm) with elution with MeOH gave organic fractions (8 ml) checked by two-dimensional TLC [silicagel plates, *n*-BuOH–AcOH–H_2O (12:3:5) and $CHCl_3$–MeOH–H_2O (40:9:1)] and combined in to two main fractions. Fraction A was further fractionated by RP-HPLC on a C18-Bondapak column (30 cm×7.8 mm, flow rate 2.0 ml/min) with MeOH–H_2O (4:1) to yield only compound **1**. Fraction B was separated by RP–HPLC with MeOH–H_2O (1:2) to yield the compounds **2**–**5**, see Table 1.

3.4. Acid hydrolysis

The glycoside (~1 mg) was refluxed in 2 N HCl (0.5 ml) for 2 h. The hydrolysate was extracted three times with EtOAc (10 ml). After separating the organic layer, the aqueous phase was neutralized with $NaHCO_3$ and lyophilized.

3.5. Enzymatic hydrolysis

A solution of glycoside (~1 mg) in acetate buffer (pH 4.4, 10 ml) was treated with α-glucosidase, ß-glucosidase, α-mannosidase or hesperidinase for 48 h at 37 °C. The reaction solution was evaporated to dryness, and the residue was chromatographed on a column of silica gel (10 g) using CH_2Cl_2–MeOH–H_2O (90:10:1) to provide acids for ^{1}H NMR analysis.

3.6. Oxidative cleavage

A stream of 4% ozone was passed through a solution (Kroft et al., 1981) of the given compound (~1 mg) in dichloromethane (2 ml) at –78 °C for 5 min. The solution was flushed with nitrogen and concentrated. The residue was dissolved in 90% HCOOH (0.7 ml) and 30% hydrogen peroxide (0.3 ml) was added. After gentle heating the mixture was heated under reflux for 70 min. The mixture was concentrated and the residue was dissolved in methanol (0.5 ml) and treated with etheral diazomethane.

3.7. Chiral chromatography

FS capillary column HYDRODEX ß-3P ID 0.25 mm, length 25 m, with the stationary phase [heptakis-(2,6-di-*O*-metyl-3-*O*-pentyl)-β-cyclodextrine] from Macherey-Nagel GmbH & Co. KG, Düren, Germany was used. Oven temperature: 50–150 °C at 2 °C/min, then to 240 °C at 5 °C/min, carrier gas helium, 20 cm/s, detector FID, 300 °C, injection of 1 μl mixture in methylene chloride (for standards: containing 0.5 mg/ml of each sample), split (100:1), 300 °C.

3.8. Identification of component sugars of glycosides 2–5

The identification and the D or L configuration of sugars (i.e. D-mannose, L-rhamnose and D-glucose) was determined using GC–MS (Gerwig et al., 1978), with some modifications, as previously described (Rezanka and Guschina, 2000) with SPB-1 (Supelco) column (30 m×0.25 mm I.D.). Briefly, the nitrogen was bubbled through a solution of the lyophilized sample (0.5 mg) after methanolysis of **2**–**5** in (+)-2-butanol (300 μ1) and acetyl chloride (50 μ1), and the ampoule was then sealed. After butanolysis at 80 °C for 8 h, the solution was neutralized with Ag_2CO_3. After centrifugation at 2000 rpm for 10 min the supernatant solution was concentrated under reduced pressure at 45 °C. The residue was treated with acetic anhydride (100 μ1) in pyridine (200 μ1). Heating at 70 °C for 30 min brought about acetylation.

The column temperature was programmed to rise 5 °C/min from 170 to 260 °C. The flow rate of the helium carrier gas through the column was 1.5 ml/min.

The temperature of the injector was 270 °C. The retention times (in minutes) were 17.07 and 19.20 for D-Glu, 14.43 and 16.57 for D-Man and 8.91 and 10.16 for L-Rha, respectively. Differences from the appropriate standards were smaller than ±0.03 min.

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

(*S*)-MTPA ester of free acid (**1**) and aglycones from glucosides **2**–**5** (Ohtani et al., 1991a,b). To a CH_2Cl_2 solution (100 μl) of aglycone (0.3 mg), DMAP (1.0 mg), and Et_3N (2 μl) was added (*R*)-(−)-MTPACl (2.0 mg) at room temperature, and stirring was continued for 3 h. After evaporation of solvent, the residue was purified by silica gel TLC (hexane–AcOEt, 2:1) to provide the (*S*)-MTPA ester as a colourless oil.

(*R*)-MTPA esters of free acid (**1**) and of aglycones from glucosides **2**–**5**. Each aglycone (0.3 mg) was treated with (*S*)-(+)-MTPACl (2.0 mg) by the same procedure as described above to provide the (*R*)-MTPA ester as a colourless oil.

(2*E*,4*E*,7*S*,8*E*,10*E*,12*E*,14*S*) - 7,9,13,17 - tetramethyl - 7,14-dihydroxy-2,4,8,10,12,16-octadecahexaenoic acid **1**, white needles, m.p. 74.8 °C, $[\alpha]_D^{24}$ +13.8 ° (*c* 0.07, $CHCl_3$), UVλ_{max} (EtOH, nm) 242 (log ε 3.42) and 268 (log 3.17); IR (KBr) (cm^{-1}): 3400 (OH), 2950, 2920, 1710 (unsaturated conjugated acid); HREIMS *m*/*z*: 360.2304 (M^+, calc. for $[C_{22}H_{32}O_4]^+$ 360.2300; LREIMS *m*/*z* (%), 360 $[M]^+$ (8), 345 $[M–Me]^+$ (11), 342 $[M–H_2O]^+$ (54), 324 $[M\text{-}2xH_2O]^+$ (63), 316 $[M–CO_2]^+$ (41), 298 $[M–CO_2–H_2O]^+$ (58); ^1H NMR and ^{13}C NMR spectra, see Tables 2 and 3.

14-*O*-β-D-Glucopyranosyl-(2*E*,4*E*,7*S*,8*E*,10*E*,12*E*,14*R*) 7,9,13,17 - tetramethyl - 7,14 - dihydroxy - 2,4,8,10,12,16 - octadecahexaenoic acid (**2**), white powder, $[\alpha]_D^{23}$ −34°; HRFABMS *m*/*z* 523.2911 $(M+H)^+$, calc. for $[C_{28}H_{43}O_9]^+$ 523.2906; negative FABMS *m*/*z* 521 $(M-H)^-$, 359 (M–H-162); ^1H and ^{13}C NMR spectra, see Tables 2 and 3.

14-*O*-α-D-Glucopyranosyl-(2*E*,4*E*,7*S*,8*E*,10*E*,12*E*,14*S*) 7,9,13,17 - tetramethyl - 7,14 - dihydroxy - 2,4,8,10,12,16 - octadecahexaenoic acid (**3**), white powder, $[\alpha]_D^{23}$ +11; HRFABMS *m*/*z* 523.2910 $(M+H)^+$, calc. for $[C_{28}H_{43}O_9]^+$ 523.2906; negative FABMS *m*/*z* 521 $(M-H)^-$, 359 (M–H-162); ^1H and ^{13}C NMR spectra, see Tables 2 and 3.

14-*O*-α-D-Mannopyranosyl-(2*E*,4*E*,7*R*,8*E*,10*E*,12*E*,14*S*)-7,9,13,17-tetramethyl-7,14-dihydroxy-2,4,8,10,12,16-octadecahexaenoic acid (**4**), white powder, $[\alpha]_D^{23}$ +42°; HRFABMS *m*/*z* 523.2908 $(M+H)^+$, calc. for $[C_{28}H_{43}O_9]^+$ 523.2906; negative FABMS *m*/*z* 521 $(M-H)^-$, 359 (M–H-162); ^1H and ^{13}C NMR spectra, see Tables 2 and 3.

14-*O*-α-L-Rhamnopyranosyl-(2*E*,4*E*,7*R*,8*E*,10*E*,12*E*,14*R*)-7,9,13,17-tetramethyl-7,14-dihydroxy-2,4,8,10,12,16-octadecahexaenoic acid (**5**), white powder, $[\alpha]_D^{23}$ +37°; HRFABMS *m*/*z* 507.2963 $(M+H)^+$, calc. for $[C_{28}H_{43}O_8]^+$ 507.2958; negative FABMS *m*/*z* 505 $(M-H)^-$, 359 (M–H-146); ^1H and ^{13}C NMR spectra, see Tables 2 and 3.

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