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Identification of astaxanthin diglucoside diesters from snow alga *Chlamydomonas nivalis* by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Abstract

A method is described for the identification of astaxanthin glucoside esters from snow alga *Chlamydomonas nivalis* by means of liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization (LC–MS/APCI). The method is based on the use of preparative HPLC and subsequent identification of astaxanthin diglucoside diesters by microbore LC–MS/APCI. The combination of these two techniques was used to identify more than 100 molecular species. The astaxanthin diglucoside diester, i.e. (all-*E*)-[di-(6-*O*-oleoyl-β-D-glucopyranosyloxy)]-astaxanthin, was also synthesized to unambiguously confirm its structure.

Keywords: *Chlamydomonas nivalis*; Snow alga; Astaxanthin diglucoside diesters; Liquid chromatography–mass spectrometry–atmospheric pressure chemical ionization

1. Introduction

Carotenoids, typically bright yellow, orange, red, or purple polyisoprenoid compounds with absorption maxima in the range of 400–500 nm are found predominantly in photosynthesizing organisms such as green plants, algae and some bacteria (Britton et al., 2004). The number of currently known carotenoids exceeds 700 (Dembitsky, 2005) and it keeps on rising – more than 1000 papers on these compounds have been published in 2006 alone. One of their main functions is to interact with chlorophyll during photosynthesis to absorb light and transfer energy; they are also able to quench singlet oxygen and free radicals and thereby protect plants from photo-oxidative damage. Carotenoids can be divided into two main groups – hydrocarbon carotenes and oxygenated xanthophylls.

Because of the presence of conjugated polyene chains in their molecule, they are usually unstable in the presence of light, heat, or oxygen. Isolation, identification, and quantitation of carotenoids are difficult and many compounds interfere in their isolation. The use of gas chromatography (GC) and GC–mass spectrometry (GC–MS) is not suitable because carotenoids are heat labile. The only useful method appears to be high-performance liquid chromatography (HPLC) with UV/visible (UV/Vis) detection or, much more conveniently, with mass spectrometry detection (LC–MS). Reversed-phase HPLC (RP-HPLC) is a preferred method, which is frequently used with C18 stationary phase, usually with gradient elution. This procedure ensures sufficiently fast elution and the chromatographic peaks are sharper. UV/vis absorbance detectors lack the selectivity and the possibility of identifying individual

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molecular species is limited because individual carotenoids are structurally highly similar. In contrast to other ionization techniques, xanthophylls and carotenes form both molecular ions and protonated molecules during positive-ion APCI (atmospheric pressure chemical ionization).

Several LC–MS methods have been reported for carotenoid analysis, including particle beam, continuous-flow fast atom bombardment, electrospray and APCI techniques (van Breemen, 1996, 1997; Careri et al., 1999; Lacker et al., 1999). The latter provide high sensitivity and highly linear detector response, suggesting that this LC–MS technique should become the standard method for carotenoid analysis (van Breemen, 1997). The first LC–MS/APCI analysis of carotenoids was published by van Breemen et al. (1996). While LC–MS/APCI analysis of free carotenoids has been comprehensively reported (van Breemen, 1996, 1997; van Breemen et al., 1996; Breithaupt and Schwack, 2000), the characterization of carotenoid esters in plant tissues has only emerged in the last few years.

Carotenoids and carotenoid esters were extracted from red pepper pods (*Capsicum annuum* L.) without saponification. Among the 42 compounds detected, 4 non-esterified, 11 mono- and 17 diesters were characterized based on their retention times, UV/vis spectra and their fragmentation patterns in collision-induced dissociation experiments in atmospheric pressure chemical ionization mass spectrometry (MS/APCI) (Schweiggert et al., 2005).

LC–MS/APCI was used to identify carotenoid esters in mandarin essential oil (Giuffrida et al., 2006). After first being analyzed by HPLC, 4 free carotenoids, 15 astaxanthin monoesters, 12 astaxanthin diesters, and 3 astacin monoesters in *Haematococcus pluvialis* were identified by LC–MS/APCI (Breithaupt, 2004).

Takaichi et al. (2003) isolated fractions with astaxanthin monoesters and diesters from Antarctic krill *Euphausia superba*. Astaxanthin esters were separated by RP-HPLC depending on the number of carbons and double bonds of esterified fatty acid(s) and identified by field desorption mass spectrometry.

A new carotenoid glycoside, astaxanthin-glucoside, was isolated from the astaxanthin-producing marine bacterium *Agrobacterium aurantiacum*, and its structure was determined by Yokoyama et al. (1995a). Another new carotenoid glucoside, astaxanthin-diglucoside plus the known astaxanthin glucoside were isolated from two transformed *E. coli* strains (Yokoyama et al., 1998).

The snow alga *Chlamydomonas nivalis* (Bauer) Wille (Chlorophyta, Chlamydomonadales), forms striking red patches in high mountain and polar regions all over the world (Kol, 1968). Since the snow environment is extremely harsh, with low temperature, high irradiance and frequent freeze–thaw cycles, for most of its life cycle, *Chlamydomonas nivalis* occurs as immotile spherical thick-walled resting stage, which can be considered as major adaptation to the extreme habitat. Flagellates are rarely observed. The red coloration of the cells is caused by the accumulation of the astaxanthin, which plays a central role in protection against high levels of UV and photosynthetically-active radiation (Bidigare et al., 1993; Gorton et al., 2001; Gorton and Vogelman, 2003). The content of astaxanthin in dark red cysts was about 20 times that of chlorophyll *a* in samples collected in Austrian Alps (Remias et al., 2005).

In this paper, based on our previous communications on constituent compounds from the algae and lichens (Dembitsky et al., 1993; Rezanka and Podojil, 1984), we examined the chemical composition of the snow alga *C. nivalis* collected from high-altitude environments with the aim of separating astaxanthin diglycoside diesters and identifying their structure by means of the LC–MS/APCI. Because reference substances were not available commercially, astaxanthin diglucoside diester {di-[(6-*O*-oleoyl-β-D-glucopyranosyl)-oxy]-astaxanthin – 18:1-G-A-G-18:1} was synthesized independently to facilitate peak assignment in complex mixtures. Major and also minor astaxanthin diglycoside diesters were characterized in positive ion mode by their specific fragmentation patterns, revealing a more complex profile than described so far. This is the first report of analysis of native carotenoid glycoside esters by LC–MS/APCI.

2. Results and discussion

The MeOH extract from the snow alga *C. nivalis* was subjected to preparative normal phase HPLC to obtain eight fractions. Table 1 gives the data obtained after RP-HPLC from fraction 4, i.e. di-(6-*O*-acyl-glucopyranosyl-oxy)-astaxanthins (FA-G-A-G-FA, where FA is fatty acid, G is glucose, and A is astaxanthin), which had the highest weight proportion. The yield referred to the wet weight of cells was 185 mg (0.93%). A total of 69 peaks were separated by LC–MS/APCI on narrow-bore columns connected in series. The chromatogram is given in Fig. 1. MS/APCI identified 105 molecular species. All astaxanthin diglycoside diesters exhibited very similar, if not identical, UV spectra (200–550 nm). Substitution by any acyl chain or glucosyl moieties did not modify the chromophore (Yamano et al., 2002; Yokoyama et al., 1995a, 1998; Giuffrida et al., 2006). All the above findings lead to the conclusion that the UV spectra of all compounds are very similar and do not provide a clue for identification of the appropriate structures.

The components of astaxanthin diglycoside diesters were also analyzed by GC after hydrolysis. Fraction 4 contained astaxanthin as the aglycone moiety. Glucose was the only sugar which was detected in fraction 4. The fatty acid composition, which was identical with the results obtained from APCI analysis, was also determined by GC–MS, see Table 2.

Optimum APCI conditions were established by using a synthetic sample, i.e. (18:1-G-A-G-18:1). The different fragmenter voltage settings used throughout this study and different cone voltage were shown to have a significant

Table 1
Quantitative distribution of astaxanthin glucoside esters from snow alga *Chlamydomonas nivalis* by means of LC–MS/APCI

Peak no.	RT (s)	Mol. spec.		% [a]	Ratio[b]	M+H	M−Acyl$_1$	M−Acyl$_2$	M−Acyl$_1$-18	M−Acyl$_2$-18	M−Acyl$_1$-G	M−Acyl$_2$-G	M−Acyl$_1$-G-18	M−Acyl$_2$-G-18
1	605	18:5	18:5	1.36		1433	1159	1159	1141	1141	997	997	979	979
2	620	18:5	16:4	1.87		1407	1133	1159	1115	1141	971	997	953	979
3	637	16:4	16:4	2.56		1381	1133	1133	1115	1115	971	971	953	953
4	690	22:6	18:5	0.88		1487	1159	1213	1141	1195	997	1051	979	1033
5	699	22:6	16:4	1.21		1461	1133	1213	1115	1195	971	1051	953	1033
6	707	18:5	18:4	1.17		1435	1161	1159	1143	1141	999	997	981	979
7	766	18:5	16:3	4.04	60	1409	1135	1159	1117	1141	973	997	955	979
		18:4	16:4		40	1409	1133	1161	1115	1143	971	999	953	981
8	786	16:4	16:3	3.35		1383	1135	1133	1117	1115	973	971	955	953
9	804	22:6	22:6	0.57		1541	1213	1213	1195	1195	1051	1051	1033	1033
10	822	22:6	18:4	0.75		1489	1161	1213	1143	1195	999	1051	981	1033
11	881	22:6	16:3	1.59		1463	1135	1213	1117	1195	973	1051	955	1033
12	909	20:4	18:5	0.99		1463	1159	1189	1141	1171	997	1027	979	1009
13	919	20:4	16:4	1.36		1437	1133	1189	1115	1171	971	1027	953	1009
14	946	18:5	18:3	2.06	50	1437	1163	1159	1145	1141	1001	997	983	979
		18:4	18:4		50	1437	1161	1161	1143	1143	999	999	981	981
15	972	18:5	16:2	4.40	20	1411	1137	1159	1119	1141	975	997	957	979
		18:4	16:3		45	1411	1135	1161	1117	1143	973	999	955	981
		18:3	16:4		35	1411	1133	1163	1115	1145	971	1001	953	983
16	994	16:4	16:2	5.56	80	1385	1137	1133	1119	1115	975	971	957	953
		16:3	16:3		20	1385	1135	1135	1117	1117	973	973	955	955
17	1099	22:6	20:4	0.64		1517	1189	1213	1171	1195	1027	1051	1009	1033
18	1124	22:6	18:3	0.68		1491	1163	1213	1145	1195	1001	1051	983	1033
19	1202	22:6	16:2	0.55		1465	1137	1213	1119	1195	975	1051	957	1033
20	1220	20:4	18:4	0.85		1465	1161	1189	1143	1171	999	1027	981	1009
21	1252	20:4	16:3	1.78		1439	1135	1189	1117	1171	973	1027	955	1009
22	1283	18:5	18:2	1.68	45	1439	1165	1159	1147	1141	1003	997	985	979
		18:4	18:3		55	1439	1163	1161	1145	1143	1001	999	983	981
23	1300	18:5	16:1	4.72	20	1413	1139	1159	1121	1141	977	997	959	979
		18:4	16:2		15	1413	1137	1161	1119	1143	975	999	957	981
		18:3	16:3		40	1413	1135	1163	1117	1145	973	1001	955	983
		18:2	16:4		15	1413	1133	1165	1115	1147	971	1003	953	985
24	1328	18:5	14:0	0.27		1387	1113	1159	1095	1141	951	997	933	979
25	1350	16:4	16:1	2.96	50	1387	1139	1133	1121	1115	977	971	959	953
		16:3	16:2		50	1387	1137	1135	1119	1117	975	973	957	955
26	1475	16:4	14:0	0.38		1361	1113	1133	1095	1115	951	971	933	953
27	1495	22:6	18:2	0.50		1493	1165	1213	1147	1195	1003	1051	985	1033
28	1503	22:6	16:1	0.66		1467	1139	1213	1121	1195	977	1051	959	1033
29	1515	22:6	14:0	0.17		1441	1113	1213	1095	1195	951	1051	933	1033
30	1527	20:4	20:4	0.72		1493	1189	1189	1171	1171	1027	1027	1009	1009
31	1562	20:4	18:3	0.77		1467	1163	1189	1145	1171	1001	1027	983	1009
32	1581	20:4	16:2	0.62		1441	1137	1189	1119	1171	975	1027	957	1009
33	1610	18:5	18:1	3.44	55	1441	1167	1159	1149	1141	1005	997	987	979
		18:4	18:2		20	1441	1165	1161	1147	1143	1003	999	985	981
		18:3	18:3		25	1441	1163	1163	1145	1145	1001	1001	983	983
34	1634	18:5	16:0	6.47	15	1415	1141	1159	1123	1141	979	997	961	979
		18:4	16:1		15	1415	1139	1161	1121	1143	977	999	959	981
		18:3	16:2		10	1415	1137	1163	1119	1145	975	1001	957	983
		18:2	16:3		20	1415	1135	1165	1117	1147	973	1003	955	985
		18:1	16:4		40	1415	1133	1167	1115	1149	971	1005	953	987
35	1666	18:4	14:0	0.23		1389	1113	1161	1095	1143	951	999	933	981
36	1689	16:4	16:0	3.53	35	1389	1141	1133	1123	1115	979	971	961	953
		16:3	16:1		50	1389	1139	1135	1121	1117	977	973	959	955
		16:2	16:2		15	1389	1137	1137	1119	1119	975	975	957	957
37	1725	16:3	14:0	0.50		1363	1113	1135	1095	1117	951	973	933	955
38	1816	22:6	18:1	1.27		1495	1167	1213	1149	1195	1005	1051	987	1033
39	1831	22:6	16:0	0.54		1469	1141	1213	1123	1195	979	1051	961	1033
40	1855	20:4	18:2	0.56		1469	1165	1189	1147	1171	1003	1027	985	1009
41	1882	20:4	16:1	0.75		1443	1139	1189	1121	1171	977	1027	959	1009
42	1905	20:4	14:0	0.20		1417	1113	1189	1095	1171	951	1027	933	1009
43	1924	18:5	18:0	2.59	10	1443	1169	1159	1151	1141	1007	997	989	979

(*continued on next page*)

Table 1 (*continued*)

Peak no.	RT (s)	Mol. spec.		% [a]	Ratio [b]	M+H	M−Acyl$_1$	M−Acyl$_2$	M−Acyl$_1$-18	M−Acyl$_2$-18	M−Acyl$_1$-G	M−Acyl$_2$-G	M−Acyl$_1$-G-18	M−Acyl$_2$-G-18
		18:4	18:1		65	1443	1167	1161	1149	1143	1005	999	987	981
		18:3	18:2		25	1443	1165	1163	1147	1145	1003	1001	985	983
44	1953	18:4	16:0	5.97	10	1417	1141	1161	1123	1143	979	999	961	981
		18:3	16:1		15	1417	1139	1163	1121	1145	977	1001	959	983
		18:2	16:2		10	1417	1137	1165	1119	1147	975	1003	957	985
		18:1	16:3		60	1417	1135	1167	1117	1149	973	1005	955	987
		18:0	16:4		5	1417	1133	1169	1115	1151	971	1007	953	989
45	1984	16:3	16:0	2.15	70	1391	1141	1135	1123	1117	979	973	961	955
		16:2	16:1		30	1391	1139	1137	1121	1119	977	975	959	957
46	2000	18:3	14:0	0.21		1391	1113	1163	1095	1145	951	1001	933	983
47	2032	16:2	14:0	0.17		1365	1113	1137	1095	1119	951	975	933	957
48	2144	22:6	18:0	0.19		1497	1169	1213	1151	1195	1007	1051	989	1033
49	2166	20:4	18:1	1.43		1471	1167	1189	1149	1171	1005	1027	987	1009
50	2185	20:4	16:0	0.61		1445	1141	1189	1123	1171	979	1027	961	1009
51	2214	18:4	18:0	2.22	10	1445	1169	1161	1151	1143	1007	999	989	981
		18:3	18:1		70	1445	1167	1163	1149	1145	1005	1001	987	983
		18:2	18:2		20	1445	1165	1165	1147	1147	1003	1003	985	985
52	2249	18:3	16:0	3.01	20	1419	1141	1163	1123	1145	979	1001	961	983
		18:2	16:1		20	1419	1139	1165	1121	1147	977	1003	959	985
		18:1	16:2		40	1419	1137	1167	1119	1149	975	1005	957	987
		18:0	16:3		20	1419	1135	1169	1117	1151	973	1007	955	989
53	2275	18:2	14:0	0.15		1393	1113	1165	1095	1147	951	1003	933	985
54	2290	16:2	16:0	1.30	40	1393	1141	1137	1123	1119	979	975	961	957
		16:1	16:1		60	1393	1139	1139	1121	1121	977	977	959	959
55	2355	16:1	14:0	0.21		1367	1113	1139	1095	1121	951	977	933	959
56	2377	14:0	14:0	0.05		1341	1113	1113	1095	1095	951	951	933	933
57	2548	20:4	18:0	0.22		1473	1169	1189	1151	1171	1007	1027	989	1009
58	2592	18:3	18:0	1.34	20	1447	1169	1163	1151	1145	1007	1001	989	983
		18:2	18:1		80	1447	1167	1165	1149	1147	1005	1003	987	985
59	2640	18:2	16:0	2.15	20	1421	1141	1165	1123	1147	979	1003	961	985
		18:1	16:1		70	1421	1139	1167	1121	1149	977	1005	959	987
		18:0	16:2		10	1421	1137	1169	1119	1151	975	1007	957	989
60	2669	18:1	14:0	0.40		1395	1113	1167	1095	1149	951	1005	933	987
61	2699	16:1	16:0	0.63		1395	1141	1139	1123	1121	979	977	961	959
62	2724	16:0	14:0	0.16		1369	1113	1141	1095	1123	951	979	933	961
63	2883	18:2	18:0	3.00	5	1449	1169	1165	1151	1147	1007	1003	989	985
		18:1	18:1		95	1449	1167	1167	1149	1149	1005	1005	987	987
64	2933	18:1	16:0	1.44	95	1423	1141	1167	1123	1149	979	1005	961	987
		18:0	16:1		5	1423	1139	1169	1121	1151	977	1007	959	989
65	3001	18:0	14:0	0.05		1397	1113	1169	1095	1151	951	1007	933	989
66	3021	16:0	16:0	0.51		1397	1141	1141	1123	1123	979	979	961	961
67	3162	18:1	18:0	0.44		1451	1169	1167	1151	1149	1007	1005	989	987
68	3233	18:0	16:0	0.18		1425	1141	1169	1123	1151	979	1007	961	989
69	3497	18:0	18:0	0.06		1453	1169	1169	1151	1151	1007	1007	989	989

[a] Abundance.

[b] The ratio of isomers, determined solely from the detector response to each one of the compounds and from the mass spectra of the respective astaxanthin glucoside esters.

impact on the fragmentation patterns. The fragmentation of the astaxanthin diglycoside diester required two different voltage values. If the cone voltage was set at 30 V in the positive mode, all compounds exhibited strong $[M+H]^+$ peaks (data not shown) but no fragmentation was observed, whereas a higher cone voltage (70 V) gave rise to fragmentation and individual daughter ions can be used for identification of molecular species. LC–MS analysis at *m/z* 400–1700 of the extract using an APCI showed $[M+H]^+$ (pseudomolecular ions) for the peaks characteristic for astaxanthin diglycoside diesters and allowed the attribution of their molecular weights. A similar situation has already been observed with astaxanthin esters (Breithaupt, 2004). The following discussion of the APCI mass spectra of the astaxanthin diglycoside diesters studied here includes speculative assignments of fragmentation pathways for each compound analyzed.

Some fragmentation of the peak at 2883 s (No. 63, 18:1-G-A-G-18:1) was evident with a fragmenter voltage of 70 V (Fig. 2 and Table 1). The parent ion at *m/z*1449 $[M+H]^+$ dissociates to six ions *m/z* 1167 $[M+H-FA_1]^+$, identical in this case with $[M+H-FA_2]^+$ *m/z* 1149 $[M+H-FA_1-H_2O]^+ = [M+H-FA_2-H_2O]^+$, *m/z* 1005 $[M+H-Glc-FA_1]^+ = [M+H-Glc-FA_2]^+$, *m/z* 987 $[M+H-Glc-FA_1-H_2O]^+ = [M+H-Glc-FA_2-H_2O]^+$, and ions common to all astaxanthin diglycoside diesters

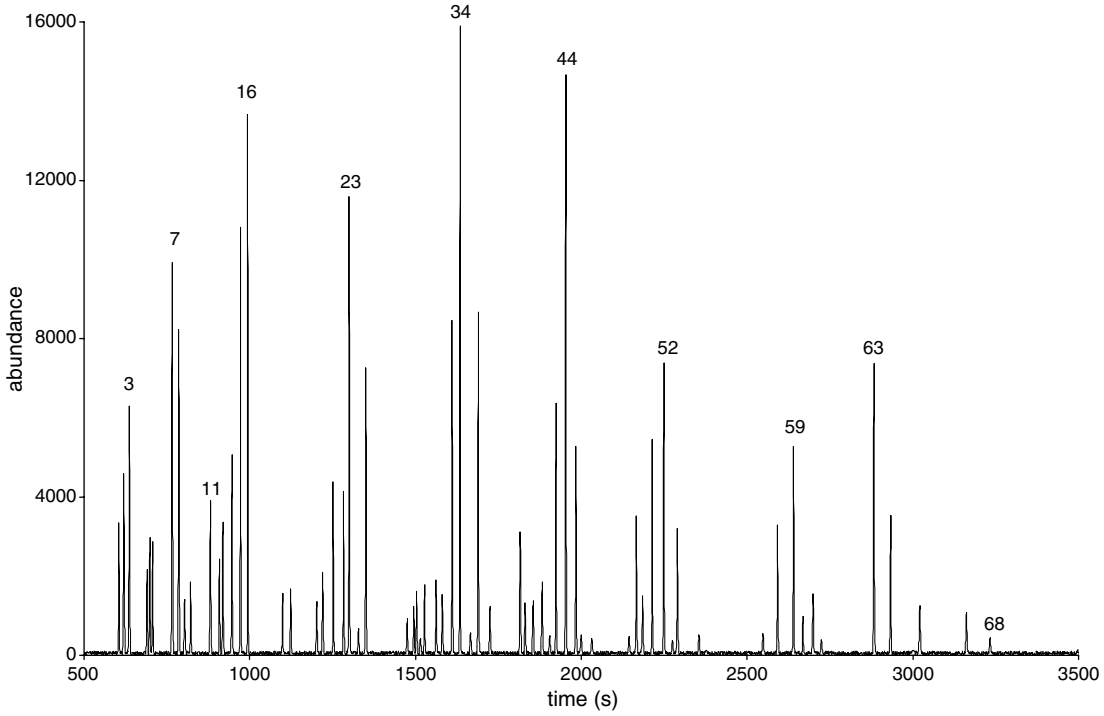


Fig. 1. Separation of astaxanthin glucoside esters from snow alga *Chlamydomonas nivalis* by means of LC–MS/APCI. For peak assignment, see Table 1.

Table 2
Fatty acid composition observed by GC–MS

Fatty acid	%
14:0	1.1
16:0	5.2
16:1	6.6
16:2	5.3
16:3	16.9
16:4	12.7
18:0	1.3
18:1	13.4
18:2	4.7
18:3	6.8
18:4	7.6
18:5	9.0
20:4	6.3
22:6	5.5

m/z 885 $[M+H-FA_1-FA_2]^+$; *m/z* 867 $[M+H-FA_1-FA_2-H_2O]^+$; *m/z* 849 $[M+H-FA_1-FA_2-2\times H_2O]^+$; *m/z* 723 $[M+H-FA_1-FA_2-Glc]^+$; *m/z* 705 $[M+H-FA_1-FA_2-Glc-H_2O]^+$; *m/z* 561 $[M+H-FA_1-FA_2-2\times Glc]^+$; *m/z* 543 $[M+H-FA_1-FA_2-2\times Glc-H_2O]^+$; *m/z* 525 $[M+H-FA_1-FA_2-2\times Glc\text{-}2\times H_2O]^+$. Fig. 2 shows a tentative pathway for the mass spectral fragmentation of 18:1-G-A-G-18:1. The other 104 compounds had very similar mass spectra, viz. Table 1. We illustrate this on the peak with retention time 1953 s (peak no. 44), which is a mixture of five molecular species: 18:4–16:0, 18:3–16:1, 18:2–16:2, 18:1–16:3, and 18:0–16:4. Its semiquantitation was done by using a previously described method (Rezanka and Sigler, 2006, 2007), which makes use of the abundance of $[M+H-FA_1]^+$ and/or $[M+H-FA_2]^+$ ions for determining peaks inseparable by HPLC. The results are documented in Table 1. Fig. 3 illustrates an excision of mass spectra of peak no. 44.

The number of papers, which described the positive soft ionization of astaxanthin derivatives is very low. It should be noted that we did not find any papers describing the MS of astaxanthin diglycoside diesters. Different xanthophyll glycoside esters have been identified in several studies, but the published data describe only the identification of pseudomolecular ions (Takaichi et al., 2001, 2003), usually in a high-resolution mode (Burgess et al., 1999; Yokoyama et al., 1995b). Several papers dealt with the analysis of acyls. The first of these studies (Breithaupt and Bamedi, 2002) describes the pseudomolecular ion $[M+H]^+$, $[M+H-H_2O]^+$ and other ions which we also identified and described (see above), i.e. $[M+H-FA_1]^+$, $[M+H-FA_1-H_2O]^+$, $[M+H-FA_1-FA_2]^+$, $[M+H-FA_1-FA_2-H_2O]^+$. The splitting was complicated by the two epoxidic oxygen atoms present in the major xanthophyll–violaxanthin – described by the authors.

Another study identified astaxanthin esters, including both monoesters and diesters, in aquatic organisms (Breithaupt, 2004). In contrast to our approach, the authors used APCI in the negative mode. Among the metabolites in phytoplankton described by Frassanito et al. (2005) only adonirubin among the many xanthophylls present was esterified by two different polyene fatty acids (18:4 and 20:5). However, the method of identification has not been described. Two other studies (Schweiggert et al., 2005; Miao et al., 2006) devoted to carotenoid esters in red

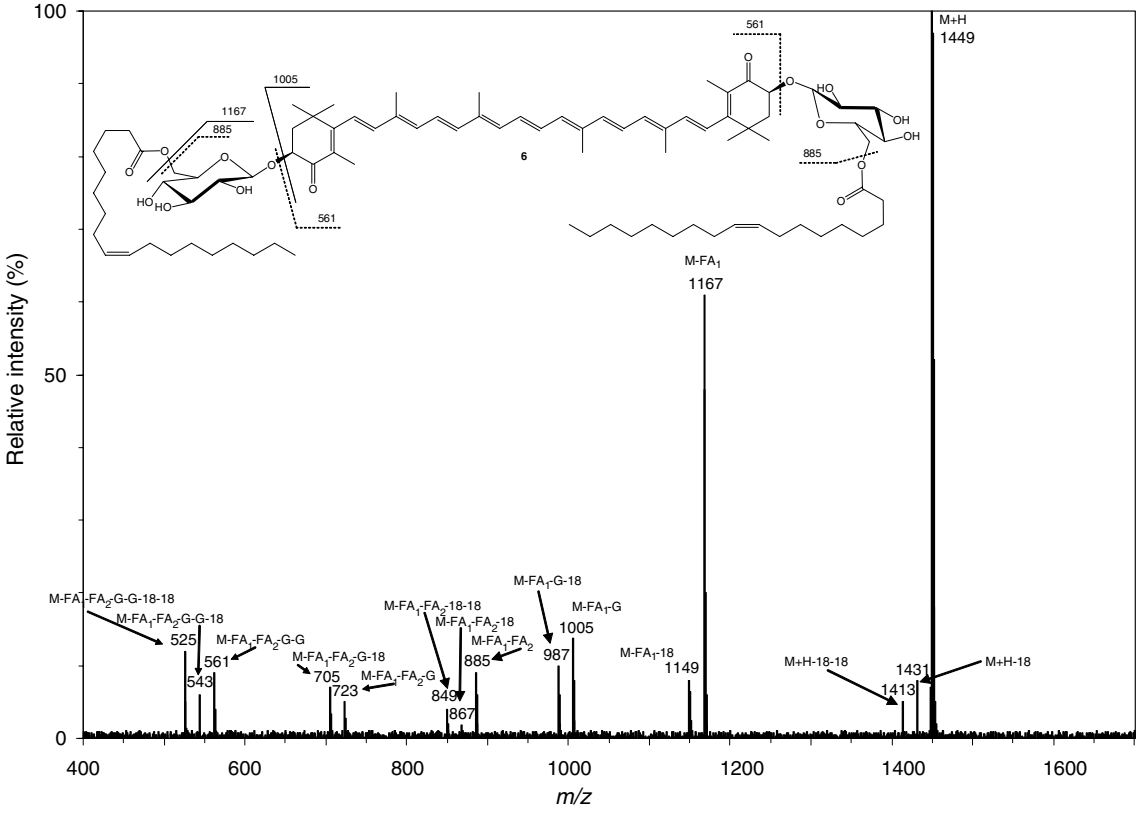


Fig. 2. APCI spectrum of astaxanthin glucoside ester in peak 63 and major fragmentation pathway of protonated astaxanthin glucoside esters di-[(6-*O*-oleoyl-β-D-glucopyranosyl)oxy]-astaxanthin, i.e. 18:1-G-A-G-18:1 in Fig. 1.

pepper pods and in mandarin essential oil also made use of MS^n APCI and identified the appropriate ions, i.e. $[M+H]^+$ and $[M+H-FA_1]^+$.

In order to fully confirm the structure, peak no. 63 (i.e. peak with retention time 2883 s) was separated by preparative HPLC. The reasons for separating this peak were manifold. Firstly, according to mass spectral analysis this peak contains only two molecular species, the symmetrical molecular species being the major component (about 95% of the total content). Secondly, this peak can be relatively easily separated from other neighboring peaks; thirdly, its abundance is among the highest, and fourthly oleic acid and/or its acylchloride is commercially available for the partial synthesis of di-(6-*O*-oleoyl-β-D-glucopyranosyloxy)-astaxanthin (Scheme 1).

The first paper concerning the synthesis of glycosides of xanthophyll was published by Pfander and Hodler (1974). These authors described the synthesis of zeaxanthin β,β-D-diglucoside using a reaction of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide and zeaxanthin under catalysis with silver carbonate. Another study (Yamano et al., 2000) described the preparation of zeaxanthin- and cryptoxanthin-β-D-glucopyranosides from appropriate aglycones, with tetra-*O*-benzoyl-α-D-glucopyranosyl bromide as a glycosyl donor and silver triflate as an activator. However, the most suitable method for the preparation of astaxanthin diglucoside esters appeared to be the sequence of reactions described in the paper by Yamano et al. (2002). Although it does not provide high yields, it involves simple reactions, low number of reaction steps and easy availability of commercial reagents.

The structure of astaxanthin-β-D-diglucoside was determined based on the identity of the data obtained by measuring the spectra of our compound (**4**) with the data published previously (Yokoyama et al., 1995a, 1998). The ^{1}H and ^{13}C NMR data, see Table 3.

The UV–vis absorption spectrum of **6** in MeOH showed a broad absorption maximum at around 489 nm and small maxima at 300 and 263 nm, indicating the presence of a conjugated system, which was comparable to that of astaxanthin-β-D-glucoside (Yokoyama et al., 1995a). The molecular formula of **6** was determined to be $C_{88}H_{136}O_{16}$ by HRFABMS. The ^{1}H and ^{13}C NMR data of **6** in CD_3OD (Table 4) indicated again the presence of astaxanthin as the carotenoid moiety, two hexose moieties, and two monounsaturated fatty acids. The ^{1}H signals of the hexose moieties showed two anomeric protons at δ_H 4.50 (*d*, $J = 7.8$ Hz). Further, we compared the ^{13}C NMR data and found that the two hexoses were β-D-glucopyranose based on the coupling constants of the anomeric protons, i.e. $^3J_{H1'-H2'} = 7.8$ Hz and $^1J_{H1'-C1'} = 169.8$ Hz (Podlasek

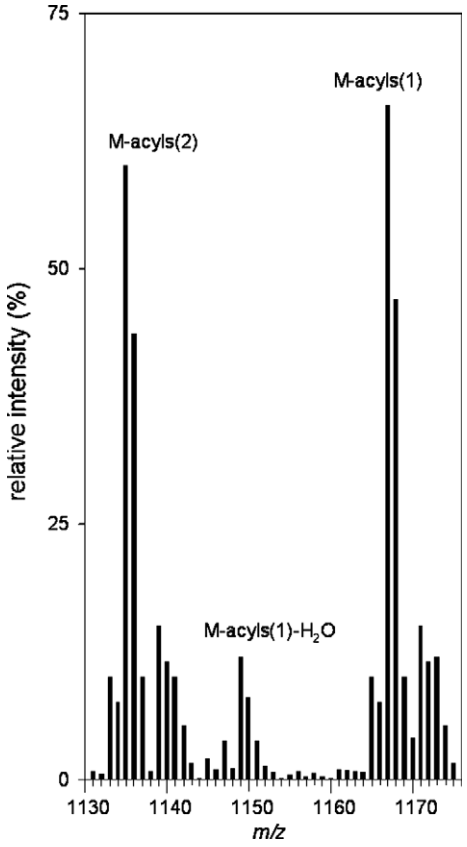


Fig. 3. Part of APCI spectrum of astaxanthin glucoside ester in peak 44 containing five molecular species: 18:4-G-A-G-16:0, 18:3-G-A-G-16:1, 18:2-G-A-G-16:2, 18:1-G-A-G-16:3, and 18:0-G-A-G-16:4.

et al., 1995). The HMBC correlations prove that the glucose moieties were attached to the hydroxyl groups at C-3 of the astaxanthin moiety and also that oleoyl moieties are bound by an ester bond to the 6-hydroxy group of glucose. The assignment of signals in the olefinic region of the spectra of **6** revealed an (*E*)-configuration for all of the double bonds, which meant that the aglycone of this compound could be identified as (*all-E*)-astaxanthin. For the determination of the absolute configuration of the carbohydrate, it was cleaved from the carotenoid by mild methanolysis and subsequently perbenzylated. The CD spectrum in the region from 200 to 250 nm of modified β-glucose in **6** belongs to the D-series because it was in accordance with published data for β-D-glucose (Kaluarachchia and Bush, 1989). Comparison of UV, MS and ^{1}H and ^{13}C NMR data of both, i.e. synthetic and natural compounds showed that the compounds are completely identical, having the structure of *di*-(6-*O*-oleoyl-β-*D*-glucopyranosyloxy)-astaxanthin.

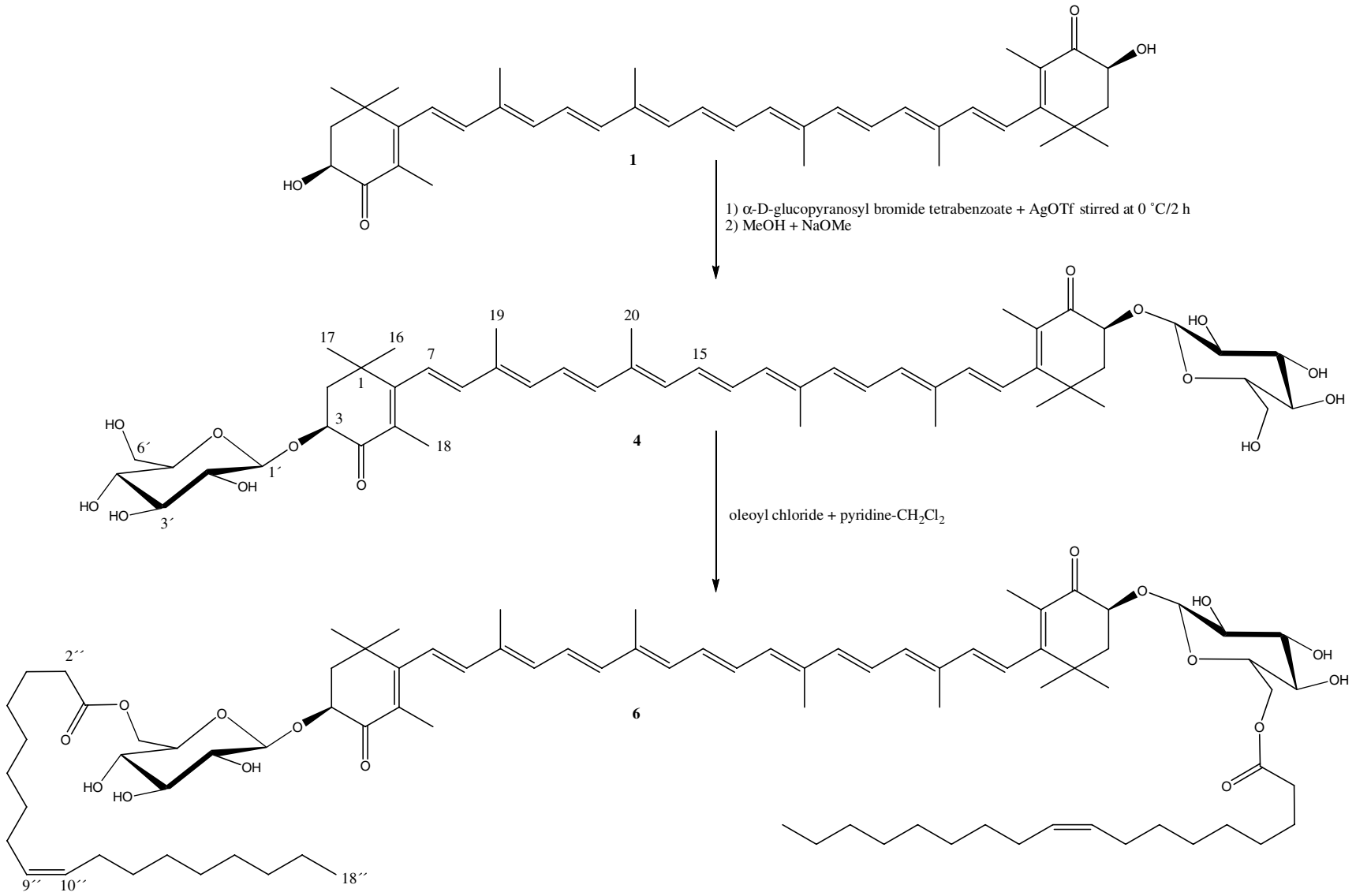
The survival of snow algae in high altitude regions is dependent on the synthesis of specific lipid components with multiple physiological functions. Secondary carotenoids function as passive photoprotectants minimizing the amount of light available for absorption by cells (Gorton et al., 2001; Gorton and Vogelman, 2003). Esterification of the relatively polar hydroxyl containing astaxanthin molecules with fatty acids allows this chromophore to be localized within cytoplasmic globules to maximize its photoprotective efficiency. Composition of red cysts similar to that found by us was described by Bidigare et al. (1993). According to their data, the fatty acid pattern of the astaxanthin fraction differs from the total fatty acid pattern of the fat extract of the alga; the astaxanthin diester fraction contained as dominant acids with one or more double bonds, i.e. more than 60% of all fatty acids were unsaturated. Although, in contrast to our findings, these acids were found also in astaxanthin diesters, astaxanthin with unsaturated fatty acids can be safely assumed to be main components of the cysts (see below).

As described in the green alga *Haematococcus pluvialis*, which forms similar amounts of secondary carotenoids as *C. nivalis*, but grows at mesophilic temperatures, astaxanthin derivatives also have antioxidant activity (Kobayashi and Okada, 2000; Boussiba, 2000).

In *C. nivalis* they may act as multipurpose protective compounds, screening the chloroplast and nucleus from potentially damaging UV and VIS radiation, quenching singlet oxygen and protecting against superoxide. Astaxanthin derivatives may also provide cryoprotection because the large volume filled by astaxanthin ester rich lipid droplets reduces the amount of cellular water and may thus reduce damage from ice crystals (Hoham, 1992). The polyenoic fatty acid moieties aid in preserving fluidity of membranes even at very low temperatures. Thus, carotenoids with their bipolar, linear, and rigid structures may be responsible for general membrane stabilization (Ourisson et al., 1987; Yokoyama et al., 1996).

In addition to all these functions, compounds with characteristic "hydrophobic–hydrophilic–hydrophobic" structures such as thermozeaxanthins and thermocryptoxanthins are assumed to stabilize membranes even at high temperature, a function considered essential, e.g., for the growth of thermophilic bacteria. Studies on thermophilic bacteria producing carotenoid glucoside esters, e.g. zeaxanthin mono- and diglucoside esters (*Thermus thermophilus*) (Yokoyama et al., 1995a, 1996) showed that membrane-associated carotenoids in most cases are monocyclic or dicyclic, with one or two C-6 acylated glucosyl units involved with characteristic acyl groups (Yamano et al., 2002).

We have developed a new LC–MS/APCI methodology that uses a single RP-HPLC gradient separation and different MS experimental set-up (positive ion mode, APCI ionization). It is suitable both for the screening of pigments and for the detection of other interesting natural products from raw extracts of microalgae such as snow algae, and will aid in identifying xanthophyll derivatives in natural sources. Analysis of carotenoids in *C. nivalis* showed the presence of astaxanthin diglucoside diesters. The C-3 hydroxyl group of astaxanthin is glucosylated to yield carotenoid glucoside; then the C-6 hydroxyl group of the glucosyl moiety is esterified with the specific fatty acids. After analyzing the LC–MS/APCI data, we identified astaxanthin derivatives



Scheme 1. Scheme of the synthesis of di-[(6-*O*-oleoyl-β-D-glucopyranosyl)oxy]-astaxanthin.

Table 3
^1H and ^{13}C NMR data of astaxanthin diglucoside (**4**) (in CD_3OD)

No.	^1H	^{13}C
1	–	37.9 *s*
2	1.95 (2H, *t*, *J* = 14.0, 14.0 Hz); 2.18 (2H, *dd*, *J* = 14.0, 5.5 Hz)	45.6 *t*
3	4.56 (2H, *dd*, *J* = 14.0, 5.5 Hz)	78.1 *d*
4	–	200.5 *s*
5	–	128.8 *s*
6	–	163.4 *s*
7	6.19 (2H, *d*, *J* = 15.8 Hz)	123.3 *d*
8	6.42 (2H, *d*, *J* = 15.8 Hz)	143.1 *d*
9	–	135.1 *s*
10	6.31 (2H, *d*, *J* = 10.9 Hz)	135.8 *d*
11	6.64 (2H, *m*)	124.6 *d*
12	6.47 (2H, *d*, *J* = 14.8 Hz)	137.8 *d*
13	–	136.5 *d*
14	6.28 (2H, *d*, *J* = 11.1 Hz)	134.0 *d*
15	6.63 (2H, *m*)	131.7 *d*
16	1.35 (6H, *s*)	26.7 *q*
17	1.21 (6H, *s*)	30.8 *q*
18	1.90 (6H, *s*)	14.3 *q*
19	1.98 (6H, *s*)	12.6 *q*
20	2.00 (6H, *s*)	12.7 *q*
1′	4.50 (2H, *d*, *J* = 7.8 Hz)	105.3 *d*
2′	3.61 (2H, *dd*, *J* = 7.8, 9.4 Hz)	73.5 *d*
3′	3.54 (2H, *t*, *J* = 9.4)	76.8 *d*
4′	3.59 (2H, *t*, *J* = 9.4)	70.0 *d*
5′	3.38 (2H, *m*)	75.9 *d*
6′	3.75 (2H, *dd*, *J* = 12.0, 5.2 Hz); 3.85 (2H, *dd*, *J* = 12.0, 3.4 Hz)	62.6 *t*

Table 4
^1H and ^{13}C NMR data of diacyl diglucoside astaxanthin (**6**) (in CD_3OD)

No.	^1H	^{13}C
1	–	37.9 *s*
2	1.95 (2H, *t*, *J* = 14.0, 14.0 Hz); 2.18 (2H, *dd*, *J* = 14.0, 5.5 Hz)	45.6 *t*
3	4.56 (2H, *dd*, *J* = 14.0, 5.5 Hz)	78.1 *d*
4	–	200.5 *s*
5	–	128.8 *s*
6	–	163.4 *s*
7	6.19 (2H, *d*, *J* = 15.8 Hz)	123.3 *d*
8	6.42 (2H, *d*, *J* = 15.8 Hz)	143.1 *d*
9	–	135.1 *s*
10	6.31 (2H, *d*, *J* = 10.9 Hz)	135.8 *d*
11	6.64 (2H, *m*)	124.6 *d*
12	6.47 (2H, *d*, *J* = 14.8 Hz)	137.8 *d*
13	–	136.5 *d*
14	6.28 (2H, *d*, *J* = 11.1 Hz)	134.0 *d*
15	6.63 (2H, *m*)	131.7 *d*
16	1.35 (6H, *s*)	26.7 *q*
17	1.21 (6H, *s*)	30.8 *q*
18	1.90 (6H, *s*)	14.3 *q*
19	1.98 (6H, *s*)	12.6 *q*
20	2.00 (6H, *s*)	12.7 *q*
1′	4.50 (2H, *d*, *J* = 7.8 Hz)	104.3 *d*
2′	3.63 (2H, *dd*, *J* = 7.8, 9.4 Hz)	74.2 *d*
3′	3.58 (2H, *t*, *J* = 9.4)	75.7 *d*
4′	3.56 (2H, *t*, *J* = 9.4)	69.6 *d*
5′	3.46 (2H, *m*)	75.1 *d*
6′	4.25 (2H, *dd*, *J* = 12.0, 5.1 Hz); 4.40 (2H, *dd*, *J* = 12.0, 2.9 Hz)	61.2 *t*
1″	–	173.5 *s*
2″	2.28 (4H, *t*, *J* = 7.1 Hz)	34.5 *t*
3″	1.60 (4H, *m*)	25.2 *t*
4″-7″	1.23-1.48 (16H, *m*)	29.0-29.8 *t*
8″, 11″	1.95-2.03 (8H, *m*)	27.3 *t*
9″, 10″	5.31 (4H, *m*)	130.1 *d*
12″-15″	1.23-1.48 (16H, *m*)	29.0-29.8 *t*
16″	1.23-1.48 (4H, *m*)	32.1 *t*
17″	1.23-1.48 (4H, *m*)	22.8 *t*
18″	0.87 (6H, *t*, *J* = 7.0 Hz)	14.2 *q*

in *C. nivalis* according to molecular mass and characteristic fragmentation patterns. Positive ion APCI mass spectra produced structurally significant fragment ions that reflect the main structure information of astaxanthin derivatives and the rule of fission. This approach is convenient for analyzing the structure and composition of astaxanthin diglucoside diesters in the snow alga.

3. Experimental

3.1. Plant material

Red snow was aseptically collected on July 28th, 2006 from surface layers of a snow field in the Pirin Mountains (south western Bulgaria). The sampling site was situated in the alpine zone near the lake Tevno ezero at an altitude of 2546 m a.s.l. (41°41′93″N, 23°29′15″E). The snow cover at the site is seasonal. Snow sample was transported into laboratory in a thermos bottle, identified under microscope and the algal biomass was kept frozen until further analysis. The red bloom was formed entirely by spherical cysts of *Chlamydomonas nivalis* (Bauer) Wille, no flagellates were observed in the sample.

3.2. Standards and isolation

Standard of astaxanthin diglucoside diester (18:1-G-A-G-18:1) was prepared as described below. All solvents were double distilled and degassed before use. All chemicals were obtained from Sigma–Aldrich, Prague.

Carotenoids were extracted with MeOH several times from the wet cell pellets (ca. 20 g) using an ultrasonicator for several seconds. The extract was partitioned with CH_2Cl_2 and H_2O, and then the CH_2Cl_2 layer was evaporated. Major carotenoid classes were purified by preparative HPLC. The yield of the astaxanthin diglycoside diesters was 185 mg. All procedures were performed under dim light and argon.

The astaxanthin diglycoside diesters (10 mg) were saponified in 10% KOH–MeOH at room temperature overnight. A fatty acid fraction obtained from saponification was partitioned between alkali solution (pH 9) and Et_2O to remove basic and neutral components. The aqueous phase, containing fatty acids, was acidified to pH 2 and extracted with hexane. The fatty acid fraction was methylated using CH_2N_2. GC–MS of a FAME mixture was done on a Finnigan 1020 B in EI mode. Splitless injection was at

100 °C, and a fused silica capillary column (Supelcowax 10; 60 m × 0.25 mm ID, 0.25 mm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range *m/z* 70–500. The structures of fatty acids were confirmed by comparison of retention times and fragmentation patterns to those of the standard fatty acid methyl esters (Supelco, Prague).

For establishment of the configuration of the glycosides, the astaxanthin diglycoside diesters (10 mg) were subjected to a mild methanolysis in 0.5 ml of 0.1 M methanolic HCl at room temperature overnight. The solvent was removed under Ar gas. To dried sample was added 0.5 ml of freshly prepared benzoic anhydride (5% benzoic anhydride and 10% (dimethylamino)pyridine in pyridine). The reaction was carried out overnight at room temperature, and the solvent removed again under Ar. The sample was dissolved in EtOAc and subjected to preparative TLC (Si 60, hexane–EtOAc, 1:2). The perbenzylated glycoside was eluted from the TLC plates, desorbed with EtOAc, filtered, and evaporated to dryness.

3.2.1. Preparation of di-[(6-O-oleoyl-β-D-glucopyranosyl)oxy]-astaxanthin

To a stirred suspension of astaxanthin (**1**) (238.4 mg, 400 μmol, mw 596), β-D-glucopyranosyl bromide tetrabenzoate **2** (791 mg, 1.2 mmol, mw 659), and powdered molecular sieve 4 Å (2 g) in dry CH_2Cl_2 (10 ml) was added silver trifluormethanesulfonate (AgOTf) (411.2 mg, 1.6 mmol, mw 257) at 0 °C. After being stirred at 0 °C for 2 h, the reaction mixture gave a residue, which was purified by HPLC (CH_2Cl_2–MeOH, 99:1) to yield the octabenzoate **3** (217.2 mg, 124 μmol, 31%, mw 1752) as a pale yellow solid. HRFABMS (*m/z*) 1753.7101 $[M+H]^+$, calcd. for $[C_{108}H_{104}O_{22}+H]^+$ 1753.7097.

To a solution of the mixture of octabenzoate **3** (200 mg, 114.2 μmol) in MeOH (10 ml) was added NaOMe (1 M in MeOH; 150 μl, 150 μmol) and the mixture was stirred at room temperature for 40 min. To this mixture was added Dowex 50W-X8 (50-100 mesh; H^+) (800 mg) and stirring continued at room temperature for a further 30 min. After Dowex was filtered off, the filtrate was evaporated to give a residue which was purified by HPLC (CH_2Cl_2–MeOH, 9:1) to yield the diglucoside **4** (97.6 mg, 106 μmol, 93%) as a yellow foam. HRFABMS (*m/z*) 921.5004 $[M+H]^+$, calcd. for $[C_{52}H_{72}O_{14}+H]^+$ 921.5000, negative FABMS *m/z* 919 $[M-H]^-$, *m/z* 757 $[M-H-162]^-$ and *m/z* 595 $[M-H-162-162]^-$; UV λ_{max} (MeOH) nm ($\log\varepsilon$) 487 (4.91), 299 (4.11), 252 (4.25). NMR data see Table 3.

A solution of the oleoyl chloride **5** (60.2 mg, 200 μmol, mw 301) in CH_2Cl_2 (1 ml) was added to a solution of the diglucoside **4** (80 mg, 87 μmol) and pyridine–CH_2Cl_2 (3 ml, 1:1). After being stirred at room temperature for 30 min, the reaction mixture was diluted with ethyl acetate, the organic layer was washed with 5% HCl, saturated $NaHCO_3$, brine and dried. Evaporation of the solution provided a residue, which was purified by HPLC (CH_2Cl_2–MeOH, 95:5) to afford the di-oleate **6** (29 mg, 23%). HRFABMS (*m/z*) 1449.9911 $[M+H]^+$, calcd. for $[C_{88}H_{136}O_{16}+H]^+$ 1449.9906. UV λ_{max} (MeOH) nm ($\log\varepsilon$) 489 (4.76), 300 (4.07), 254 (4.16). NMR data see Table 4. The yield of sequence **1** → **3** → **4** → **6** was 6.6%.

3.3. Preparative HPLC

The liquid chromatograph was the semipreparative Gradient LC System G-1 (Shimadzu, Kyoto, Japan) with two LC-6A pumps (0.5 ml/min), an SCL-6A system controller, an SPD ultraviolet detector an SIL-1A sample injector and a C-R3A data processor, with preparative column Supelcosil LC-Si HPLC column 5 μm particle size, length × ID 25 cm × 21.2 mm and/or discovery C18 HPLC column (5 μm particle size, length × ID 25 cm × 21.2 mm). UV detection at 487 nm.

3.4. LC–MS/APCI

HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, USA) and two Hichrom columns HIRPB-250AM 250 × 2.1 mm ID, 5 μm particle size, in series. This setup provided us with a high-efficiency column – approximately ~26,000 plates/250 mm. A quadrupole mass spectrometer system Navigator (Finnigan MAT, San Jose, CA, USA) was used for analysis. The instrument was fitted with an atmospheric pressure chemical ionization source (vaporizer temperature 400 °C, capillary heater temperature 250 °C, corona current 6 μA, sheath gas – high-purity nitrogen, pressure 0.4 MPa, and auxiliary gas (also nitrogen) flow rate 17 ml/min. Positively charged ions with *m/z* 100–1700 were scanned with a scan time of 0.5 s. The whole HPLC flow (0.4 ml/min) was introduced into the APCI source without any splitting. Astaxanthin diglucoside diesters were separated using a gradient solvent program with acetonitrile (ACN), dichloromethane (DCM) and methanol (MeOH) as follows: initial ACN/MeOH/DCM (50:25:25, vol/vol/vol); linear from 500 s to 3500 s ACN/MeOH/DCM 25:50:25, vol/vol/vol); held until 3600 s; the composition was returned to the initial conditions over 300 s. A peak threshold of 0.05% intensity was applied to the mass spectra. Data acquisition and analyses were performed using PC with MassLab 2.0 for Windows XP applications/operating software.

The compounds were dissolved in CD_3OD and the ^1H and ^{13}C NMR spectra were measured using a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^1H) and 125.7 MHz (^{13}C). Circular dichroism (CD) measurement was carried out under dry N_2 on a Jasco-500A spectropolarimeter at 24 °C.

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.

Acknowledgements

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