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5-*n*-Alkylresorcinols from the green microalga *Apatococcus constipatus*

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Abstract

Mixtures of six 5-*n*-alkylresorcinol (ARs) homologues were isolated from acetone extracts of four isolates of the unicellular green microalga *Apatococcus constipatus*. The pattern of homologues in different algal isolates was diverse. The predominant compounds were 1,3-dihydroxy-5-*n*-heneicosylbenzene (AR $C_{21:0}$) and 1,3-dihydroxy-5-*n*-tricosylbenzene (AR $C_{23:0}$) or 1,3-dihydroxy-nonadecylbenzene (AR $C_{19:0}$), depending on the strain. ARs were identified by chromatographic and spectroscopic means.

Keywords: *Apatococcus constipatus*; *Chaetophraceae*; *Leptosiroideae*; Green microalga; Alkylresorcinols; Resorcinolic lipids

1. Introduction

The green microalgae belonging to the genus *Apatococcus* were found mainly in the cover crusts on Norway spruce (*Picea abies*) needles (Sochting, 1997) as well as on some Spanish monuments (Flores et al., 1997) or different kinds of insulators in Tanzania (Gubanski et al., 2000). Although *Apatococcus* algae are cosmopolitan, little is known about their lipidic constituents.

The group of the natural polyketides 5-*n*-alkylresorcinols (ARs) is known to occur in 11 families of higher plants (Kozubek and Tyman, 1999). Their occurrence in lower plants such mosses and algae, is however poorly documented. So far, algal resorcinolic lipids were identified first of all as unsaturated (Gregson et al., 1977), (di)acetylated (Stodola et al., 1973) or ether derivatives (Metzger, 1994). In this paper, we report that the green microalga *A. constipatus* could be taken into account as a new natural source of alkylresorcinols.

2. Results and discussion

From four *A. constipatus* isolates (A, B, G and H), a set of six ARs homologues was isolated. Acetone extracts were separated on TLC silica gel plates and bands of resorcinolic lipids were visualised by staining with diazonic salt Fast Blue $B \times BF_4$. These bands exhibited characteristic reddish-violet colour and R_f values identical to those of authentic 1,3-dihydroxy-5-alkylbenzenes (Kozubek and Tyman, 1995). The structure of the isolated compounds was elucidated using a combination of chromatographic and spectroscopic methods. The UV spectra with two close peaks at λ_{max} 278 and 282 nm, showed no significant differences with those of standards. The IR spectra exhibited the presence of characteristic peaks of ARs' residues, such as aryl, hydroxyl and alkyl groups and were in good agreement with data from the IR-spectra reference library (Pouchert, 1981). The EIMS spectra showed the occurrence of peaks at m/z 123 and 124, characteristic of alkylresorcinols. Indeed, the peak at m/z 123 is due to the dihydroxytropylium ion formed by direct β-fission, while the base peak at m/z 124 is due to McLafferty rearrangement involving a hydrogen atom of the side chain. The 123/124-abundance ion ratio of about 1 to 5 is in agreement with a *meta* position for the hydroxyl groups in the aromatic ring (Vincieri et al., 1981; Tyman, 1991, 1996). We have not found any unsaturated homologues. Final identification, relative composition and total amount of ARs in studied materials were obtained by GC–MS analysis. The acetone extracts, partially purified on TLC Si 60 plates, were converted into

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ditrimethylsilylether derivatives and subjected to GC–MS analysis. In the four algal isolates the C_{15} and C_{25} homologues were newly identified, together with the already identified C_{17} to C_{23} homologues. The retention times and mass spectra of the algal ARs ditrimethylsilylether derivatives in the GC/EIMS were coincident with those of authentic standards. Thus, unequivocal similarity of analysed compounds to alkylresorcinols substituted with an alkyl chain at the 5 position of the aromatic ring, was deduced. The relative composition and total amounts of ARs, estimated from the base peak ion at *m/z* 268, common to all 5-alkylresorcinol molecules, are presented in Table 1. Significant differences in the relative total amount and relative composition of AR homologues in the four strains can be noticed. In particular, strain H, which showed the lowest AR total amount exhibits a relative composition different from those of strains A and B, while those of algae A and B are almost identical. Furthermore, the composition of the dominant homologues in strains G and H was rather similar. Thus individual *A. constipatus* isolates can differ from each other regarding ARs content and composition. Observed divergences are due to these strains represent different biotypes. Thereby, it results from the natural variability directly determined by complex genetic factors.

Our results, along with prior reports (Stodola et al., 1973; Gregson et al., 1977; Metzger, 1994), indicate that algae could be equivalent sources of ARs, both as native or as complex derivatives. It appears that these kinds of phenolic lipids might be, in fact, common among algae. Moreover, our findings could be useful for the *Apatococcus* genus' chemotaxonomy. The studied alga was proved for the first time to be a natural source of these compounds according to available on-line databases retrieval.

3. Experimental

3.1. General

IR spectrum was measured as KBr film. UV spectrum was measured with EtOH solution. TLC was conducted with precoated silica gel Si 60 F_{254} plates (0.25 mm thickness, Merck 5745) and RP HPTLC plates (Merck 5914).

IR: ν_{max}^{KBr} cm^{-1} 3500-3200, 2919, 2850, 1711, 1632, 1599, 1500, 1472, 1378, 1315, 1198, 1143, 1158, 958, 808.

3.2. Plant material

The algal crusts were collected from silicone-rubber and porcelain insulator in Tanzania, in 1999. The isolation of individual algal strains and their identification was carried out at the Department of Agricultural Microbiology, the Agricultural University of Wroclaw, Poland. Four isolates were separated and termed as A, B, G and H, respectively. All these isolates have been clasified as the green microalga *A. constipatus* Printz (the *Chaetophraceae* family and *Leptosiroideae* subfamily). Agar-solidified Slamer's medium was used for alga cultivation (Slamer et al., 1971). Algal cultures were grown on Petri's dishes at 24°C for 30 days. The light period lasted 12 h per day. The light intensity was *ca*. 700 lux. Afterwards, algal cells were suspended in 0.1 M $MgSO_4$ and lyophilised.

3.3. Extraction and isolation

Dried algal samples (62 mg of A, 100 mg of B, 109 mg of G and 68 mg of H, respectively) were extracted once with Me_2CO at room temp. for 24 h. The volume of Me_2CO sufficient to soak the materials completely was applied. After filtration, Me_2CO was removed by vacuum evaporation. The resulting extracts (1.50, 1.25, 1.80 and 1.35 mg) were separated by two-dimensional chromatography on prep TLC Si 60 plates (20×20 cm) using first $CHCl_3$:EtOAc (85:15, v/v) and then *n*-hex:Et_2O:HCO_2H (70:30:1, by vol.) as eluents. Gel areas containing compounds of interest were scrapped off the plates and then extracted with $CHCl_3$:EtOAc (85:15, v/v) for 30 min. After filtration and removal of the solvent the residues were dissolved in $CHCl_3$ and used for further analyses.

3.4. Sample preparation for ARs analyses by GC–EIMS

Acetone extracts were separated on Si 60 gel plates (8×10 cm) using $CHCl_3$:EtOAc (85:15, v/v). The ARs mixture, re-extracted with EtOAc containing 1% AcOH

Table 1
Contents of 5-*n*-alkylresorcinols and relative compositions in four strains of *A. Patococcus constipatus*.

Isolate	Content (ng/mg of dry weight)	ARs' relative composition (%)					
		$C_{15:0}$	$C_{17:0}$	$C_{19:0}$	$C_{21:0}$	$C_{23:0}$	$C_{25:0}$
A	5.7	1.0	1.5	29.6	51.2	13.4	3.2
B	2.3	1.3	2.9	28.3	51.3	13.4	2.9
G	4.4	1.0	7.1	11.9	34.5	35.9	8.6
H	0.2	8.1	1.4	12.2	33.7	35.8	8.8

after removal of the solvent, was dissolved in EtOAc (100 μl). Seventy microlitres of the sample was transferred into a glass capillary-tube (ϕ *ca.* 2 mm, 5 cm). After removal of the solvent *N*-methyl-*N*-trimethylsilyltrifluoroacetimide (MSTFA, 5 μl) was added and the tube was sealed and allowed to stand for 30 min at 70°C.

3.5. Qualitative and quantitative determination of ARs by GC–EIMS

One microlitre of the derivatized sample was injected into GC equipment (HP 5890 Series II Gas Chromatograph) connected to mass spectrometer (JEOL SX-100 Mass Spectrometer at 70 eV): gas (He) flow rate of 1 ml min^{-1}, DB-1 column (G&L Science, ϕ 0.25 mm×15 m, 0.25 μm film thickness). Oven temp. was programmed as follows: 80°C for 1 min, then 30°C min^{-1} up to 230°C, 10°C min^{-1} to 320°C and 320°C for 2 min. The sample injection temperature was 250°C. Due to the better separation of the tested homologues, application of ditrimethylsilyl ARs derivatization was recommended. Identification of each AR homologue was deduced from the molecular ion and common base peak ion at *m*/*z* 268 which is characteristic of ditrimethylsilyl AR derivatives. The retention times and molecular ions were 9.4 min (M^+ 464, AR $C_{15:0}$), 10.6 min (M^+ 492, AR $C_{17:0}$), 11.7 min (M^+ 520, AR $C_{19:0}$), 12.9 min (M^+ 548, AR $C_{21:0}$), 14.0 min (M^+ 576, AR $C_{23:0}$) and 15.0 min (M^+ 604, AR $C_{25:0}$), respectively. The relative compositions and total amounts of the homologue were estimated from the area of the peaks in the ion chromatogram. Authentic AR $C_{15:0}$, used for preparation of the standard calibration curve, showed a linear relationship between 1 and 10 ng.

3.6. Reference compounds and other chemicals

Pure standards of 1,3-dihydroxy-5-*n*-alkylbenzene homologues with side chains from $C_{13:0}$ to $C_{27:0}$ were isolated from mature wheat grains according to the method described by Kozubek (1985). Synthetic 1,3-dihydroxy-5-*n*-pentadecylbenzene was obtained from Sigma-Aldrich (Poznan, Poland). Solvents and remaining chemicals of the highest available purity were from POCh (Gliwice, Poland).

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References

Flores, M., Lorenzo, J., Gomez-Alcaron, G., 1997. Algae and bacteria on historic monuments at Alcala de Henares, Spain. Int. Biodeterior. Biodegrad. 40, 241–246.

Gregson, R.P., Kazlauskas, R., Murphy, P.T., Wells, R.J., 1977. New metabolites from the brown alga *Cystophora torulosa*. Aust. J. Chem. 30, 2527–2532.

Gubanski, S. M., Fernando, M. A. R. M., Pietr, S. J., Matula, J., Kyaruzi, A., 2000. Effects of biological contamination on insulator performance. Submitted for the 5th International Conference on Properties and Applications of Dielectric Materials ICPADM-94, X'ian, China, June 2000.

Kozubek, A., 1985. Isolation of 5-*n*-alkyl, 5-*n*-alkenyl- and 5-*n*-alkdienyl-resorcinol homologs from rye grains. Acta Aliment. Pol 9, 185–189.

Kozubek, A., Tyman, J.H.P., 1995. Cereal grain resorcinolic lipids: mono and dienoic homologues are present in rye grains. Chem. Phys. Lipids 78, 29–35.

Kozubek, A., Tyman, J.H.P., 1999. Resorcinolic lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. Chem. Rev. 99, 1–26.

Metzger, P., 1994. Phenolic ether lipids with an *n*-alkenylresorcinol moiety from a Bolivian strain of *Botryococcus braunii* (Arace). Phytochemistry 36, 195–212.

Pouchert, C.J., 1981. The Aldrich Library of Infrared Spectra. Aldrich Chemical Co, 3rd Edition. Milwaukee, WI.

Slamer, R.Y., Kunisawa, R., Handel, M.R., Cohen-Bazire, G., 1971. Purification and properties of unicellular blue-green algae (order *Chrooccoccales*). Bacteriol. Rev. 35, 171–205.

Sochting, U., 1997. Epiphyllic cover on spruce needles in Denmark. Ann. Bot. Fenn. 34, 157–164.

Stodola, F.H., Weisleder, D., Vesonder, R.F., 1973. A new dialkylresorcinol from *Stemphylium majusculum*. Phytochemistry 12, 1797–1798.

Tyman, J.H.P., 1991. The chemistry of non-isoprenoid phenolic lipids. In: Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry, Vol 9. Elsevier, Amsterdam, pp. 313–381.

Tyman, J.H.P., 1996. Synthetic and Natural Phenols, Elsevier, Amsterdam.

Vincieri, F.F., Vinzenzini, M.T., Vanni, P., 1981. Extraction of active compounds from sarcotesta of *Ginkgo biloba* seed: inhibition of some dehydrogenase activity. Riv. Ital. E.P.P.O.S 63, 79–82.