5-*n*-Alkylresorcinols from the Nitrogen-fixing Soil Bacterium *Azotobacter chroococcum* Az12

Robert Zarnowski[a,§,*], Yoshikatsu Suzuki[b], Ewa D. Zarnowska[c,#], Yasuaki Esumi[b], Arkadiusz Kozubek[c], and Stanislaw J. Pietr[a]

[a] Department of Agricultural Microbiology, Agricultural University, Wroclaw, Poland
[b] Polymer Chemistry Laboratory, RIKEN Institute (The Institute of Physical and Chemical Research), Wako-shi, Saitama, Japan
[c] Department of Lipids and Liposome Technology, Wroclaw University, Wroclaw, Poland
[§] Present address: Department of Medical Microbiology and Immunology, 424 Medical Science Centre, University of Wisconsin – Madison, 1300 University Ave, Madison, WI 53706, USA. Fax: +1-(608)-2656717. E-mail: robert@plantpath.wisc.edu
[#] Present address: Department of Anaesthesiology, University of Wisconsin – Madison, Madison, WI, USA

* Author for correspondence and reprint requests

Z. Naturforsch. **59 c**, 318–320 (2004); received January 16/February 27, 2004

A mixture of five saturated 5-*n*-alkylresorcinol homologues was isolated from vegetative cells of the nitrogen-fixing soil bacterium *Azotobacter chroococcum* Az12. Their structures were established by spectrometry (^{1}H NMR, EI-MS, FAB-MS, FAB-MS/MS) and chromatography (GC, TLC) means.

Key words: Nitrogen-fixing Bacteria, Resorcinolic Lipids, Phenols

Introduction

5-*n*-Alkylresorcinols (ARs) are long-chain, odd-numbered homologues of orcinol (1,3-dihydroxy-5-methylbenzene) deriving from the polyketide metabolic pathway (Kozubek and Tyman, 1999). The existence of ARs has been demonstrated in few bacterial genera including *Mycobacterium* spp. (Bu'Lock and Hudson, 1969), *Streptomyces* spp. (Tsuge *et al.*, 1992), and *Pseudomonas* spp. (Kanda *et al.*, 1975). ARs have also been shown in *Azotobacter vinelandii*, but their occurrence was attributed to metabolically altered cysts only (Batrakov *et al.*, 1977; Reusch and Sadoff, 1979; Su *et al.*, 1981). Further experiments (Kozubek *et al.*, 1996) reported the presence of ARs also in vegetative cells of *A. chroococcum*, however, their real function remains unknown. In this study, we thoroughly describe the isolation and characterization of ARs biosynthesised in vegetative cells of *A. chroococcum* Az12.

Experimental

The bacterium *A. chroococcum* Az12 was isolated from the organic layer of arable land collected near Wroclaw, Poland, and identified by the method of Thompson and Skerman (1979). The batch culture (5.0 l) was grown on Burk's nitrogen-free medium with 1.0% glucose (Dalton and Postage, 1968) for 5 d at 30° C. Afterwards, cells were collected by centrifugation (3000 × *g*, 10 min), washed with 0.1 M $MgSO_4$, freeze-dried and extracted with 20 ml of acetone for 3 × 24 h at room temperature. Supernatants were filtered and extracted three times with equal volumes of EtOAc. The extracts were concentrated *in vacuo*, redissolved in 1 ml of 10% MeOH in $CHCl_3$ and filtered in order to remove undissolved material. Next, the extracts were again concentrated, redissolved in 1 ml of pure MeOH and applied to flash column chromatography on Sephadex LH-20 (ϕ 20 × 410 mm). Elution was carried out with MeOH and 2-ml fractions were collected. After partial evaporation of the solvent, fractions were examined on TLC Si60 plates. 5% vanillin dissolved in concentrated sulfuric acid was used as a TLC spray reagent. Resorcinol-positive fractions (No. 12–16) were analysed by ^{1}H NMR (300 MHz, Bruker AC300+; Bruker BioSpin Co., Billerica, MA, USA) in $CDCl_3$ with TMS as an internal standard. Further purification of those fractions was carried out on preparative TLC Si60 plates (0.25 × 250 × 250 mm) using 15% EtOAc in $CHCl_3$ for chromatogram development. Spots on the gel containing compounds of interest were scraped off the plates and eluted with 30 ml of

0939–5075/2004/0500–0318 $ 06.00

10% MeOH in $CHCl_3$. The eluate was concentrated and chromatographed on a small silica gel column with 5% EtOAc in CH_2Cl_2. The fraction of pure ARs was collected and analyzed by low- and high-resolution FAB-MS and FAB-MS/MS (negative mode, JMS HX 110/110A tandem mass spectrometer; JEOL, Tokyo, Japan) (Suzuki *et al.*, 1999). Ions that were produced by bombarding the sample with 6 keV Xe atoms were accelerated through a potential of 10 kV, and 3-nitrobenzyl alcohol (3-NBA) was used as a matrix. For GC/EI-MS, ditrimethylsilyl-derivatives of ARs were used (Zarnowski *et al.*, 2000b). 1 μl of the derivatized sample was injected into a HP 5890 Series II gas chromatograph equipped with a DB-5MS column (ϕ 0.25 mm × 15 m, 0.25 μm film thickness; Agilent Technologies, Palo Alto, CA, USA) and connected to a HP 5973 mass selective detector. Analysis was done at 70 eV and helium was used as a carrier gas with a flow rate of 1 ml min^{-1}. Oven temperature was programmed as follows: 80 °C for 2 min, then 10 °C per min up to 200 °C, 5 °C min^{-1} to 260 °C, then 10 °C min^{-1} to 310 °C. The sample injection temperature was 280 °C. Standards of original ARs were previously isolated from rye grains (Kozubek, 1985).

Results and Discussion

The extraction of vegetative cells (3.2 g dry weight) of *A. chroococcum* Az12 as well as its post-culture medium yielded 124 and 278 mg of extracts, respectively. The AR-containing fraction (4.8 mg) was purified from the cell extract by column chromatography on Sephadex LH-20 followed by preparative TLC, whereas no ARs were detected in the post-culture medium extract. Our finding is in a good agreement with previously published studies of ARs in *A. vinelandii* (Reusch and Sadoff, 1979; Su *et al.*, 1981), however remains inconsistent with the report by Kozubek *et al.* (1996), who demonstrated the presence of small quantities of ARs also in supernatants. From a physico-chemical point of view, secretion of certain amounts of ARs into the culture liquid medium is possible due to their amphiphilic character and, in fact, has been found in few microorganisms (Zarnowski *et al.*, 2000a; Zarnowski, Hendrich and Pietr, unpublished). AR molecules exhibit an affinity to biological membranes, because their partition coefficient in an octanol/water system is more than 7.4 (Kozubek and Tyman, 1999). Apparently, the previous report on ARs found in the supernatant might be due to the contamination with incompletely spun down bacterial cells. On the other hand, the supernatant examined contained some AR-like compounds that could be detected on TLC plates. Those substances had R_f values identical with authentic rye keto- and hydroxy-AR derivatives, but their isolation was beyond the scope of this work.

Further analysis of the AR fraction obtained indicated that ARs consist of five homologues substituted exclusively with saturated carbon side-chains. The gross structure of those compounds has been elucidated using spectrometric techniques. The ^{1}H NMR spectrum recorded in $CDCl_3$ revealed signals at δ 6.20 (2H, d, J = 2.0 Hz), 6.14 (1H, t, J = 2.0 Hz), 2.46 (2H, t, J = 7.6 Hz), 1.56 (2H, broad m), 1.25 (broad s), 0.88 (3H, t, J = 6.5 Hz) ppm, which are characteristic of AR molecules (Suzuki *et al.*, 1996). In addition, the detection of common two fragment peaks at *m/z* 267 and 268 in GC/EI-MS, specific for TMS derivatives of ARs, confirmed the presence of 3,5-dihydroxy-alkylbenzene structures in the analysed fraction characteristic for ARs. Indeed, the peak at *m/z* 267 is due to the dihydroxytropylium ion formed by direct β-fission, while the base peak at *m/z* 268 is due to the McLafferty rearrangement occurring *via* transition complex formation of a hydrogen atom of the side chain. The 267/268-abundance ion ratio of 1 to 4 or of 1 to 5 is in agreement with the *meta* position of two hydroxyl groups in the aromatic ring (Vincieri *et al.*, 1981). Subsequently, AR homologues were identified by the detection of quasi-molecular ions in the negative FAB-MS and of molecular ions in GC/EI-MS spectra (Table I). The latter technique allowed five molecular ions with *m/z* from 464 through 492, 520, 548 to 576 to be distinguished, whereas FAB-MS detected only four of them. Interestingly, there was no difference in terms of long-chain AR analysis, but some discrepancies were observed for short-chain homologues, indicating thereby the vast superiority of GC/EI-MS. Those ions found correspond with a series of five AR homologues substituted with $C_{15:0}$, $C_{17:0}$, $C_{19:0}$, $C_{21:0}$ and $C_{23:0}$ side-chains and their retention times (in min) were 11.8, 13.3, 14.8, 16.5, 18.7 and 21.9, respectively. The predominant AR identified was 1,3-dihydroxy-5-*n*-heneicosylbenzene (AR $C_{21:0}$) (Fig. 1), whereas homologues substituted with C_{19}, C_{23}, C_{17} and C_{15} alkyl side-chains were detected in minor

Table I. 5-*n*-Alkylresorcinols in vegetative cells of *A. chroococcum* Az12.

Identification method	Homologue composition[a] [%]				
	$C_{15:0}$	$C_{17:0}$	$C_{19:0}$	$C_{21:0}$	$C_{23:0}$
GC/EI-MS	*t*	0.9	18.8	73.4	6.6
FAB-MS	*n.d.*	*t*	8.8	83.6	6.2
FAB-MS/MS	*n.d.*	*n.d.*	linear	linear	linear

[a] Standard error did not exceed 2%.
t = Trace (less than 0.05%).
n.d. = not detected.

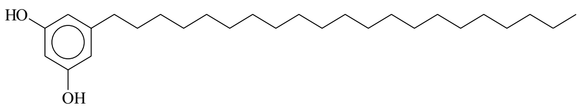

Fig. 1. 1,3-Dihydroxy-5-*n*-heneicosylbenzene, the major alkylresorcinol of *A. chroococcum*.

quantities (Table I). Afterwards, the exact molecular formulae were determined using high-resolution FAB-MS. In this case, three major homologues with $C_{19:0}$ (calcd. 375.3263, found 372.3263), $C_{21:0}$ (calcd. 403.3587, found 403.3576) and $C_{23:0}$ (calcd. 431.3869, found 431.3889) side-chains were detected. The final identification was achieved using negative FAB-MS/MS (Table I). The spectra of AR $C_{19:0}$, AR $C_{21:0}$ and AR $C_{23:0}$ revealed a sequence of odd-mass series that was regularly spaced by 14 amu after an initial loss of the methyl group. Thus, the saturated carbon side-chains of those compounds were confirmed to be linear only. No unsaturated homologues of ARs were found in *A. chroococcum* Az12.

Acknowledgements

The authors are grateful to Dr. Waldemar Rymowicz (Agricultural University, Wroclaw, Poland) for the use of biofermentors.

Batrakov S. G., Pridachina N. N., Kruglyak E. B., and Novogrudskaya E. D. (1977), Phenolic lipid from *Azotobacter chroococcum*. Khim. Prirod. Soed. (USSR) **4**, 494–499.

Bu'Lock J. D. and Hudson A. T. (1969), Beta-leprosol: the identification of a trialkylresorcinol from bacterial lipids. J. Chem. Soc. **60**, 61–63.

Dalton H. and Postage J. R. (1968), Effect of oxygen on growth of *Azotobacter chroococcum* in bath and continuous cultures. J. Gen. Microbiol. **34**, 463–469.

Kanda N., Ishizaki N., Inoue N., Oshima M., and Handa A. (1975), DB-2073, a new alkylresorcinol antibiotic. I. Taxonomy, isolation and characterization. J. Antibiot. (Tokyo) **28**, 935–942.

Kozubek A. (1985), Isolation of 5-*n*-alkyl-, 5-*n*-alkenyl- and 5-*n*-alkadienyl-resorcinol homologs from rye grains. Acta Aliment. Polon. **9**, 185–198.

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

Kozubek A., Pietr S. J., and Czerwonka A. (1996), Alkylresorcinols are abundant lipid components in different strains of *Azotobacter chroococcum* and *Pseudomonas* spp. J. Bacteriol. **178**, 4027–4030.

Reusch R. N. and Sadoff H. L. (1979), 5-*n*-Alkylresorcinols from encysting *Azotobacter vinelandii*: isolation and characterization. J. Bacteriol. **139**, 448–453.

Su C.-J., Reusch R. N., and Sadoff H. L. (1981), Isolation and characterization of several unique lipids from *Azotobacter vinelandii* cysts. J. Bacteriol. **147**, 80–90.

Suzuki Y., Esumi Y., Hyakutake H., Kono Y., and Sakurai A. (1996), Isolation of 5-(8′*Z*-heptadecenyl)-resorcinol from etiolated rice seedlings as an antifungal agent. Phytochemistry **41**, 1485–1489.

Suzuki Y., Esumi Y., and Yamaguchi I. (1999), Structures of 5-alkylresorcinol-related analogues in rye. Phytochemistry **52**, 281–289.

Thompson J. P. and Skerman V. B. D. (1979), Azotobacteriaceae: the Taxonomy and Ecology of the Aerobic Nitrogen-fixing Bacteria. Academic Press, London.

Tsuge N., Mizokami M., Imai S., Shimazu A., and Seto H. (1992), Adipostatins A and B, new inhibitors of glycerol-3-phosphate dehydrogenase. J. Antibiot. **45**, 886–891.

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

Zarnowski R., Lewicka T., and Pietr S. J. (2000a), Production and secretion of 5-*n*-alkylresorcinols by *Fusarium culmorum*. Z. Naturforsch. **55c**, 846–848.

Zarnowski R., Suzuki Y., Esumi Y., and Pietr S. J. (2000b), 5-*n*-Alkylresorcinols from the green microalga *Apatococcus constipatus*. Phytochemistry 55, 975–977.