

5,5',6,6'-Tetrahydroxy-3,3'-biindolyl from Beetroot (*Beta vulgaris*) Peel Extract

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A compound of unusual structure was isolated from red beetroot (*Beta vulgaris*) peel extract and identified as 5,5',6,6'-tetrahydroxy-3,3'-biindolyl based on the combination of NMR and MS studies.

Introduction

Recently, plant material extracts containing phenolic compounds have been screened as potential new sources of natural antioxidants. Beet extracts, especially the extracts from the peel, have shown strong antioxidant activity in these evaluations resulting in increased attention to the natural products present in beetroot (Vinson *et al.*, 1998; Kähkönen *et al.*, 1999). As part of our ongoing investigation into the distribution and properties of known phenolics in red beetroot peel (Kähkönen *et al.*, 1999; Kujala *et al.*, 2000), a novel compound, 5,5',6,6'-tetrahydroxy-3,3'-biindolyl (**1**, Fig. 1), was isolated. It was shown to be a phenolic compound by a modified Folin-Ciocalteu phenol test (Nurmi *et al.*, 1996) and was thus of prime interest to our work despite its highly unstable nature when isolated.

Materials and Methods

Extraction and isolation

Red beetroots (*Beta vulgaris*, cultivar “Rubia”) were washed, hand-peeled, and the collected peels cut into small pieces and stored at –25° C until lyophilisation. After the lyophilised plant material was reduced to powder by mortar and pestle, 96 g of the peel was extracted in 8.0 g portions by homogenising (Ultra-Turrax T25, Janke & Kunkel, IKA-Labortechnik, Germany) each portion twice for four min with 160 ml of 80% aq. MeOH. Samples were centrifuged (10 min, 1500×g) and the combined extracts evaporated to syrup under re-

duced pressure. The syrups were stirred with MeCN (3 × 50 ml for 30 min) in a planar mixer (Promax 2020, Heidolph, Germany). The combined MeCN extracts of all syrups were taken to dryness under reduced pressure and the residue was extracted with EtOAc. The EtOAc was evaporated to dryness under reduced pressure and the water-soluble fraction of the extract was removed to water. A small portion of the sample was acetylated with the remainder subjected to semipreparative purification using a LiChrocart column {Lichrospher 100 RP-18, 250 × 10 mm I. D., 10 µm, Merck, Darmstadt, Germany; elution with MeCN (A) and HCOOH – water (B) (1:99, v/v), 0–10 min, 95% B followed by 10–25 min, 5–16% A in B (linear gradient) with detection at 305 nm}. From the combined fractions, a distinct and attractive yellow/orange isolate (as an aqueous solution) was obtained which quickly discoloured to a dark brown solution (several hours) followed by the formation of black precipitate (one to several days).

A syrup was also prepared as mentioned earlier from 4.0 g of dried peel and dissolved in water (6.0 ml) for HPLC-MS analysis.

NMR

NMR spectra were acquired on a JEOL Alpha 500 NMR spectrometer operating at 500.16 MHz for ¹H and 125.78 MHz for ¹³C. Spectra were recorded at 25° C in D₂O; both ¹H and ¹³C spectra were referenced internally to TMS (0 ppm for both). 1D proton and carbon spectra were ac-

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quired under normal single-pulse conditions. Both FG HSQC and FG HMBC experiments were acquired in magnitude mode with spectral widths and resolution appropriately optimised from the 1D spectra and both spectra utilised a $^1J_{\text{HC}}$ coupling of 145 Hz whilst HMBC correlations were optimised for long-range $^nJ_{\text{HC}}$ couplings of 8 Hz. ^1H NMR: δ/ppm 7.217 (s, couplings unresolved, H2/H2'); 7.139 (d + unresolved coupling, $J_{\text{H4,H7}} = 0.3$ Hz, H4/H4'); 7.034 (d + unresolved coupling, $J_{\text{H7,H4}} = 0.3$ Hz, H7/H7'). The labile protons were not observed. ^{13}C NMR: δ/ppm 144.37 (s, C6/C6'); 142.11 (s, C5/C5'); 133.61 (s, C7a/C7a'); 127.47 (d, C2/C2'); 123.75 (s, C3a/C3a'); 108.54 (d, C4/C4'); 103.11 (s, C3/C3'); 101.00 (d, C7/C7a'). UV, λ_{max} : 278, 304 nm.

MS-MS and HPLC-ESI-MS

Mass spectra were acquired on a VG ZabSpec instrument for EI^+ and FAB^+ (glycerol) measurements using a direct insert probe. Accurate mass measurements in EI^+ mode were performed by peak matching technique using PFK as a reference substance. $\text{ESI}^{+/-}$ (aq. MeOH by direct infusion or via HPLC inlet) measurements were acquired on a Sciex API 365 triple-quadrupole LC/MS/MS system. Masses were scanned from m/z 80 to 1100 in 0.3 amu steps. HPLC conditions have been described previously (Kujala *et al.*, 2000).

Results and Discussion

Compound **1** is novel, despite its corresponding monomer 5,6-dihydroxyindole being well known (Beer *et al.*, 1954; Benigni and Minnis, 1965; Napolitano *et al.*, 1985; Palumbo *et al.*, 1987; d'Ischia *et*

al., 1990; d'Ischia *et al.*, 1991) and the fact that indoles possess a propensity to dimerise (Napolitano *et al.*, 1985; Palumbo *et al.*, 1987; d'Ischia *et al.*, 1990; d'Ischia *et al.*, 1991; Bergman *et al.*, 1995). The 2,2'-linked isomer is actually known (Napolitano *et al.*, 1985; Bergman *et al.*, 1995) and it readily forms under catalysed oxidising conditions from the monomer (it is clearly distinct from the material isolated here based on ^{13}C NMR shifts, however); and furthermore many other related dimers incorporating mixed linkage have also been reported (Palumbo *et al.*, 1987; d'Ischia *et al.*, 1990; d'Ischia *et al.*, 1991). Mixed linkage species incorporating the 3 position in particular have also been reported (Bu'Lock and Harley-Mason, 1951; Kaneko *et al.*, 1981), and indeed the 3,3'-dimer of unsubstituted indole itself is known (Kaneko *et al.*, 1981). These compounds have been intensely studied because they and their *ortho*-quinoid oxidation products form the basis of the animal pigment eumelanine (Bergman *et al.*, 1995). Many of these references also emphasised the labile and/or intractable nature of the samples at hand.

After isolation, the sample was subjected to only a limited array of NMR techniques because of both the sample amount and the labile nature of the sample. Nevertheless a sufficient amount of data was acquired that enabled the structural elucidation of **1** – after some debate and in conjunction with the MS data. From the ^1H NMR spectrum, only three aromatic signals were attributable to the structure each of which had either the smallest of discernible couplings or unresolvable couplings to the other two signals. From the ^{13}C NMR spectrum, seven signals (three methine and four quaternary) were readily detectable; with the eighth only realisable as a signal belonging to the structure by the correlation prominent in the HMBC spectrum. Evidently this quaternary carbon (C3/C3') has an extremely long relaxation time and under the normal conditions with which ^{13}C spectra are acquired in our laboratory (45° pulse, 3.8 sec recycle time), this signal was not observable. With a sample devoid of protons, the pivotal experiment that afforded the structure of the compound was HMBC, the reproduction of which is depicted in Fig. 2. Of note is that the two protons at 7.14 (H4/H4') and 7.03 ppm (H7/H7') each have two *strong* correlations to two quaternary carbons, but they lack any commonality; and the

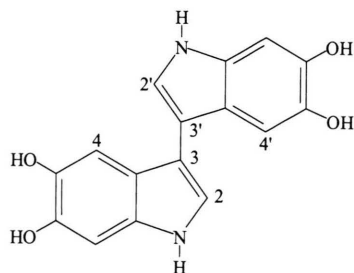


Fig. 1. The structure of 5,5',6,6'-tetrahydroxy-3,3'-biindolyl (**1**) indicating the numbering system in use. The two rings are depicted without regard to their actual orientation.

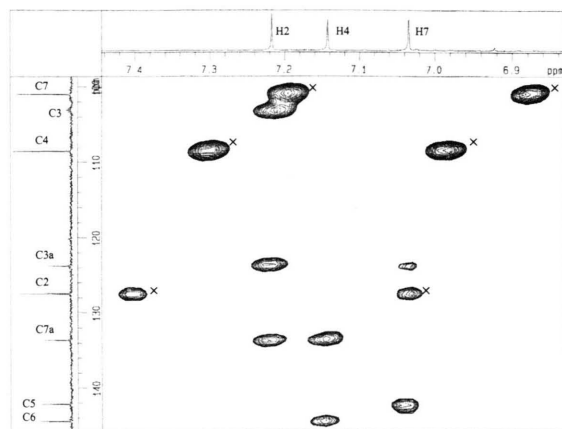


Fig. 2. The HMBC spectrum of **1** showing the decisive correlations leading to the structural elucidation of the compound. Of particular note is the very strong correlation between H2 and C3. Correlations marked with a cross are unsuppressed one bond couplings due to poor selection of $^1J_{\text{HC}}$. At lower threshold levels weaker correlations become visible, namely H7→C6, H7→C7a, and H4→C5 (not discussed in the text).

third proton at 7.22 ppm has three correlations with two of them in common with the other two protons. Thus, although the lack of any strong coupling between the protons, together with the fact that no NOE could be detected between them spoke for a system in which all three are essentially isolated from one another, they are clearly localised in a compact system. One manner by which this is attainable is to have two of the protons positioned *para* to one another in a tetrasubstituted six-membered aromatic. This provides a ready solution for each of these two protons at 7.14 and 7.03 ppm which are long-range coupled and which both correlate to two quaternary carbons, but without any commonality. The relationship of the third proton then clearly must be such that it is present in a second aromatic ring, which is fused to this first six-membered one. It is quickly apparent that a second fused six-membered ring would not be consistent with the third proton being able to correlate to the two carbons it shares in common with the other two protons (i.e. what is observed) and simultaneously preclude any long-range correlations between protons and methine carbons and any NOEs between the protons. Although the latter two points constitute negative evidence, mass spectroscopic evidence (see below) actually indicated a lack of available atoms to

form a six-membered aromatic ring in any case. Thus the monomeric portion was postulated as depicted in Fig. 1 using the atom composition available from accurate mass measurements (see below) together with the ^{13}C chemical shifts. The lack of a second methine group (by NMR) or other suitable atoms (by MS) together with the final realisation that the correct molecular weight of the compound was 296 amu led to the arrival at the final, symmetric and dimeric structure which was consistent with the observed chemical shifts, in particular the ^{13}C shifts.

The mass spectroscopic evidence did not initially alleviate the quandaries presented by the NMR (namely seven carbons), and indeed the evidence appeared to be quite ambiguous and for a considerable period of time the two techniques appeared to be inconsistent with one another. The compound is quite unstable, even within the confines of the NMR tube, and the equivalent molecular ion was not always forthcoming under FAB^+ or $\text{ESI}^{+/-}$ conditions. Depending on the particular day and set of conditions, either the equivalent parent ion or the equivalent parent ion of the apparent monomer was observable under $\text{ESI}^{+/-}$ conditions, these irregularities in detection occurred even for successive analytical sample considerably hampering the identification and isolation processes. Although by NMR there is clearly no detectable amount of the monomer present, we cannot discount the possibility that by ESI^+ the 149 ion is not a fragment ion (whole or in part) and it really does emanate from the actual presence of monomer in the MS samples and is therefore present in the beetroot extract itself. Monomer could also be converted to the dimer (**1**) at some point in the processing or possibly even separated from **1** in the purification process.

When observable, the positive- and negative-ion ESI spectra of the extract showed peaks at m/z 297 and 295, respectively, which corresponded to the protonated, $[\text{M}+\text{H}]^+$, and deprotonated, $[\text{M}-\text{H}]^-$, molecules of biindolyl. MS/MS experiments demonstrated that the molecular ion of **1** loses fragments of mass 17 and 18 (presumably, OH^\bullet and H_2O) in both negative- and positive-ion modes. The $[\text{M}+\text{H}]^+$ species produced a pair of peaks at m/z 148–149 (40–50% RA), whereas the $[\text{M}-\text{H}]^-$ ions produced a group of weaker peaks at m/z 146–148 (10–15% RA). Formation of the

ions with m/z values in the 146–149 range is consistent with the biindolyl structure, in which the cleavage of the central C-C bond is accompanied by hydrogen transfers. The negative-mode ions with m/z 148, similarly to the parent ions, decompose further by losing fragments of 17 and 18 amu.

The FAB⁺ spectra of a sample dissolved in D₂O (i.e. the NMR sample) showed a cluster of feeble peaks at m/z 298–300 (< 10% RA) and a group of prominent peaks at m/z 150–153 (25–70% RA). The decreased intensity of the molecular ions at m/z 298–300 and the presence of a cluster of peaks at m/z 131–133 (25–50% RA, presumably corresponding to the losses of D₂O and HDO from m/z 150–153) suggest that the parent biindolyl structure is very labile and tends to fragment readily even under FAB⁺ conditions.

It is therefore not surprising that mass peaks above m/z 160 were not seen in the EI⁺ spectra of the sample (introduced directly as a D₂O solution). A cluster of peaks at m/z 150–156 (27% of the total ion current, base peak at m/z 153) corresponding to the monomer indole initially suggested an apparent molecular ion (with varying

degrees of deuteration). Interestingly, losses of (H,D)₂O from ions with m/z 150–156 are less prominent under EI⁺ conditions in comparison to FAB⁺ spectra. With a sample introduced using H₂O, either the apparent monomer parent ion (149) or the fragment ion (148) was predominant. Treatment of **1** with Ac₂O was performed in order to stabilise the compound to effect observation of a M⁺ ion, but only fragment ions corresponding to mono- and diacetylated 5,6-dihydroxyindole were observed. The accurate mass measurements of the deuterated and acetylated samples under EI⁺ conditions are presented in Table I. The consistency of these two sets of results, however, formed the cornerstone for acceptance of the C₈H_xNO₂ composition of the “unit”, and therefore ultimately the composition of the molecule.

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Table I. Mass peaks from EI⁺ spectra with their compositions as determined by accurate mass measurements.

Sample	m/z	Formula	Found	Calculated	Deviation, ppm
Acetylated	149	C ₈ H ₇ NO ₂	149.0477	149.0477	0.1
“	191	C ₁₀ H ₉ NO ₃	191.0586	191.0582	1.9
”	233	C ₁₂ H ₁₁ NO ₄	233.0698	233.0688	4.3
Deuterated	150	C ₈ H ₆ DNO ₂	150.0539	150.0540	1.7
“	151	C ₈ H ₅ D ₂ NO ₂	150.0597	150.0602	3.5
”	152	C ₈ H ₄ D ₃ NO ₂	150.0659	150.0665	4.0

- Beer R. J. S., Broadhurst T. and Robertson A. (1954), The chemistry of the melanins. Part V. The autoxidation of 5: 6-dihydroxyindoles. *J. Chem. Soc.*, 1947–1953.
- Benigni J. D. and Minnis R. L. (1965), The syntheses of 5,6-dihydroxyindole and some of its derivatives. *J. Heterocyclic Chem.* **2**, 387–392.
- Bergman J., Koch E. and Pelcman B. (1995), 2,2'-Biindolyl revisited. Synthesis and reactions. *Tetrahedron* **51**, 5631–5642.
- Bu'Lock J. D. and Harley-Mason J. (1951), Melanin and its precursors. Part II. Model experiments on the reactions between quinones and indoles, and consideration of a possible structure for the melanin polymer. *J. Chem. Soc.*, 703–712.
- d'Ischia M., Napolitano A., Tsiakas K. and Prota G. (1990), New intermediates in the oxidative polymerisation of 5,6-dihydroxyindole to melanin promoted by the peroxidase/H₂O₂ system. *Tetrahedron* **46**, 5789–5796.
- d'Ischia M., Napolitano A. and Prota G. (1991), Peroxidase as an alternative to tyrosinase in the oxidative polymerization of 5,6-dihydroxyindoles to melanin(s). *Biochim. Biophys. Acta* **1073**, 423–430.
- Kaneko T., Matsuo M. and Iitaka Y. (1981), Reactions of indole with hydroxyl radicals and x-ray crystal structure of a novel indole trimer, 14-acetyldiindolo [2,3-a:2',3'-c] carbazole. *Chem. Pharm. Bull.* **29**, 3499–3506.
- Kujala T. S., Loponen J. M., Klika K. D. and Pihlaja K. (2000), Phenolics and betacyanins in red beetroot (*Beta vulgaris*) root: distribution and effect of cold storage on the content of total phenolics and three individual compounds. *J. Agric. Food Chem.* **48**, 5338–5342.
- Kähkönen M. P., Hopia A. I., Vuorela H. J., Rauha J.-P., Pihlaja K., Kujala T. S. and Heinonen M. (1999), Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* **47**, 3954–3962.
- Napolitano A., Corradini M. G. and Prota G. (1985), A reinvestigation of the structure of melanochrome. *Tetrahedron Lett.* **26**, 2805–2808.
- Nurmi K., Ossipov V., Haukioja E. and Pihlaja K. (1996), Variation of total phenolic content and individual low-molecular-weight phenolics in foliage of mountain birch trees (*Betula pubescens* ssp. *Tortuosa*). *J. Chem. Ecol.* **22**, 2023–2040.
- Palumbo P., d'Ischia M. and Prota G. (1987), Tyrosinase-promoted oxidation of 5,6-dihydroxyindole-2-carboxylic acid to melanin. Isolation and characterization of oligomer intermediates. *Tetrahedron* **43**, 4203–4206.
- Vinson J. A., Hao Y., Su X. and Zubik L. (1998), Phenol antioxidant quantity and quality in foods: vegetables. *J. Agric. Food Chem.* **46**, 3630–3634.