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NEOBETANIN: ISOLATION AND IDENTIFICATION FROM *BETA VULGARIS*

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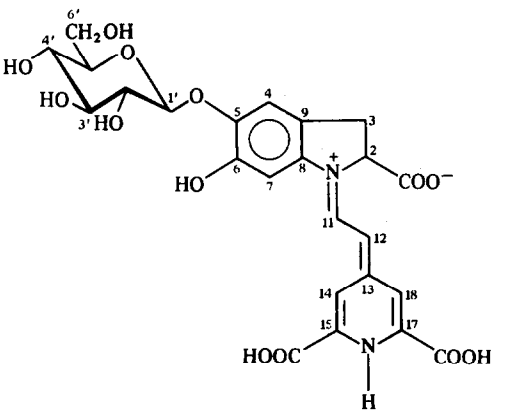
Key Word Index—*Beta vulgaris*; Chenopodiaceae; red beet; betalain; neobetanin.

Abstract—A new natural plant constituent has been isolated from the root of red beet (*Beta vulgaris* subsp. *vulgaris* var. *conditiva*) and identified as the orange-coloured water-soluble pigment 5-*O*-β-D-glucopyranosylneobetanidin (neobetanin). Its structure was elucidated and characterized by ^{1}H and ^{13}C NMR spectroscopy, FAB mass spectrometry, absorption spectroscopy, enzymatic determination of the glucose moiety, high-performance liquid chromatography and thin-layer electrophoresis. ^{1}H nuclear Overhauser enhancement spectra unambiguously identified the position of attachment of the sugar residue. Neobetanin was also found to occur as a minor constituent in petals of *Opuntia ficus-indica*, *Portulaca grandiflora*, *Zygocactus truncatus* and in fruits of *Phytolacca bogotensis* and *Rhipsalis warmingiana*.

INTRODUCTION

The red-violet betacyanins, yellow betaxanthins and the yellow betalamic acid [1, 2] are highly water-soluble pigments which are considered to be of taxonomic significance in families of the Caryophyllales (= Centrospermae) [3, 4]. Unexpectedly betalains were also shown to occur in mushrooms [3] which was interpreted as an example of biochemical convergence [5].

The basic structure of betacyanins was deduced from structural elucidation of the chemically formed 14,15-dehydrobetacyanins (neobetacyanins). Thus the aglycone of the prototype of all betalains, the 'red beet pigment' betanin (5-*O*-β-D-glucopyranosylbetanidin) was identified after conversion of betanidin into neobetanidin [1]. To the best of our knowledge, naturally occurring neobetalains have not yet been found in plants and we describe here for the first time isolation and identification of neobetanin (5-*O*-β-D-glucopyranosylneobetanidin, **1**) from a plant source.



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RESULTS AND DISCUSSION

Electrophoretic analyses of 80% aqueous methanol extracts of fresh samples of the root of red beet (*Beta vulgaris* L. subsp. *vulgaris* var. *conditiva* Alef.) resulted in a pattern of major red- and yellow-coloured zones: betanin, isobetanin and amaranthin [6; Reznik, unpublished], and vulgaxanthin I, vulgaxanthin II, indicaxanthin and betalamic acid [7; Reznik, unpublished]. The latter yellow pigments were also found in roots of *Beta vulgaris* L. subsp. *vulgaris* var. *alba* DC. [8]. One minor orange-coloured pigment (**1**) which we repeatedly observed in extracts from various organs of betalain-bearing plants showed low electrophoretic mobility relative to those of betanin (E_b = neobetanin : betanin) and indicaxanthin (E_i = neobetanin : indicaxanthin) [3] (SI: $E_b = 0.36$, $E_i = 0.17$; SII: $E_b = 0.59$, $E_i = 0.30$). This compound awaited structural elucidation. HPLC analyses [9] revealed that **1** constituted about 5% of the total betalain content in red beet roots. Compound **1** showed a retention related to that of betanin (R_b = R_f of neobetanin: R_f of betanin) of R_b 1.18.

In this study **1** was isolated from red beet roots and its structure was elucidated on the basis of the data presented below. From the spectral data ($\lambda_{max}^{H_2O}$ nm: 267, 306, 470; $\lambda_{max}^{H_2O}$ nm: 298, 365) **1** could not be conclusively assigned to either the betacyanins nor to the betaxanthins. Also we were unable to produce the betalain-characteristic C-15 diastereoisomer by treatment with a citric acid solution [10] which indicated the absence of the chiral centre at C-15. There was no detectable isobetalain formed (thin-layer electrophoresis and HPLC). Thus it could be assumed that **1** is a neobetalain. In agreement with this assumption **1** gave a yellow colour (λ_{max} nm: 401) in alkaline solution (pH *ca* 10 in aq. NaOH), shifting to pink (λ_{max} nm: 500) when acidified (aq. HCl) to pH *ca* 1 [4].

Integration of the ^{1}H spectrum and ^{1}H homodecoupling identified the various spin systems of **1**. The com-

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parison with previous ^1H data [1], together with the number of signals in the ^1H broad band decoupled ^{13}C spectrum and their multiplicity in the SFORD ^{13}C spectrum, unambiguously identified **1** as a β-glucopyranosyl derivative of neobetanidin. A positive ion fast atom bombardment mass spectrum showed a molecular ion $[M+H]^+$ at *m/z* 549, confirming the molecular formula of $C_{24}H_{24}N_2O_{13}$, and a fragmentation pattern showing the loss of one hexose moiety. In addition, glucose was identified and quantified by enzymatic determination (UV-method) through conversion of hydrolytically liberated glucose to gluconate-6-phosphate by the aid of hexokinase and glucose-6-phosphate and gave a ratio of neobetanidin : glucose of 1 : 1. This substantiated the NMR data. The attachment of the sugar moiety at C-5 of the aglucone was deduced unambiguously from the ^1H nuclear Overhauser enhancement difference spectra. These same spectra confirmed the assignment of signals in the ^1H NMR spectrum, while those of the ^{13}C NMR spectrum were assigned from a comparison of literature data for phenylalanine, 4-vinylpyridine and the appropriate indole alkaloids.

In a survey (thin-layer electrophoresis) of 177 members of Caryophyllales (plants not listed), **1** was found to be present as a minor constituent in petals of *Opuntia ficus-indica* Mill. (Cactaceae), orange-flowered *Portulaca grandiflora* Hook. (Portulacaceae) and *Zygocactus truncatus* K.Sch. (Cactaceae) and in fruits of *Phytolacca bogotensis* H.B.+K. (Phytolaccaceae) and *Rhipsalis warmingiana* K.Sch. (Cactaceae). This finding documents the occurrence of a new class of orange-coloured pigments in the Caryophyllales.

In summary the data prove that the structure of the orange betalain, isolated from the root of red beet, is neobetanin (5-*O*-β-D-glucopyranosylneobetanidin, **1**). It would be of interest to investigate the *in vivo* reaction, leading to this structure, possibly catalyzed by a desaturase acting either on betalamic acid, prior to its incorporation into the betalain structure, or on the intact betalain.

EXPERIMENTAL

Plant material. Red beet (*Beta vulgaris* subsp. *vulgaris* var. *conditiva* Alef.) was grown in the botanic garden of the Institute of Botany at the University of Cologne. Other plants came from a greenhouse of the same institute.

Extraction and isolation. Root material (1000 g) of fresh 2-month-old red beet plants was cut into small pieces and extracted with 80% aq. MeOH with an Ultra-Turrax homogenizer (2–3 min). After filtration a small aliquot of the filtrate was taken for analytical experiments and the remaining extract was concd under vacuum to a vol. of *ca* 100 ml. Portions of 25 ml were fractionated on a polyamide column (CC-6 AC, 3.5 × 70 cm; Macherey & Nagel, Düren, West Germany) using H_2O (500 ml), MeOH (650 ml) and 10% HOAc in MeOH (500 ml). This eluted almost all of the red beet pigments with the exception of **1**. The column was then washed with MeOH (500 ml) prior to the elution of **1** with *ca* 0.04% NH_4OH in MeOH (1–2 l.). From four chromatographic runs the fractions containing **1** were pooled and evaporated to dryness under vacuum. The resulting residue was redissolved in a few ml of H_2O and chromatographed twice on a Sephadex LH-20 column (2.5 × 80 cm; Pharmacia, Uppsala, Sweden) using H_2O as a solvent. The orange-coloured eluant (**1**) was collected in four fractions which were subsequently freeze-dried. Yield was about 20 mg.

Thin-layer electrophoresis was carried out as described elsewhere [3, 11, 12]. System I (SI) on microcrystalline cellulose (Avicel, Macherey & Nagel, Düren, West Germany): KPi buffer (pH 6.65)–MeOH, 1:1, system II (SII) on methylcellulose-cellulose MN 300 (4:1): KPi buffer (pH 6.65). Thin-layer plates were made with cellulose suspended in KPi buffer (pH 6.65).

Hydrolysis and identification of glucose. A sample of **1** was dissolved in 1 N aq. HCl and this soln was kept at 100° for about 60 min. The hydrolysate was concd to a small vol. and a few ml MeOH was added. This was repeated × 3 and the soln was taken to complete dryness. The residue was redissolved in buffer solution, adjusted to the appropriate pH, and enzymatic glucose determination was performed using the test kit for spectrophotometric analysis (formation of NADPH) from Boehringer, Mannheim, West Germany [13].

NMR. ^1H and ^{13}C NMR spectra were recorded, at ambient temperature in DMSO-d_6, on a Bruker WM-400 (400 MHz) and a Bruker AM-300 (75 MHz) NMR spectrometer, respectively. Chemical shifts are reported in ppm relative to TMS. The following abbreviations are used to indicate the signal multiplicities in the ^1H NMR spectrum and in the SFORD ^{13}C NMR spectrum: *s* = singlet, *d* = doublet, *t* = triplet, *m* = multiplet and *br* = broad.

MS. Positive ion FAB MS were recorded on a Kratos MS 50 S mass spectrometer equipped with a Kratos FAB source. Glycerol, acidified with oxalic acid, was used as matrix.

5-O-β-D-Glucopyranosylneobetanidin (*neobetanin*, **1**). ^1H NMR (DMSO-d_6): δ8.433 (1H, *d*, H-11, $J_{11,12}$ = 13.3), 7.982 (2H, *br s*, H-14, H-18), 7.321 (1H, *s*, H-7), 7.006 (1H, *s*, H-4), 5.595 (1H, *d*, H-12), 4.811 (1H, *dd*, H-2, $J_{2,3A}$ = 10, $J_{2,3B}$ = 2.5), 4.546 (1H, *d*, H-1′, $J_{1',2'}$ = 6.8), 3.716 (1H, *d*, H-6′A, $J_{6'A,6'B}$ = 12), 3.491 (1H, *dd*, H-3A, $J_{3A,3B}$ = 16), 3.479 (1H, *dd*, H-6′B, $J_{6'B,5'}$ = 6), 3.31–3.23 (3H, *m*, H-2′ H-3′, H-5′), 3.148 (1H, *dd*, H-4′, $J_{3',4'}$ = 9, $J_{4',5'}$ = 9), 3.022 (1H, *dd*, H-3B). ^{13}C NMR (DMSO-d_6): δ181.29 (*s*, C-2 CO), 171.90 (*s*, C-15 CO, C-17 CO), 161.58, 160.56 (2 × *s*, C-15, C-17), 147.37, 141.29, 139.81, 137.41 (4 × *s*, C-5, C-6, C-8, C-13), 119.43 (*s*, C-9), 117.71 (*d*, C-11), 115.32 (*d*, C-14, C-18), 114.91 (*d*, C-4), 103.69 (*d*, C-1′), 98.72 (*d*, C-12), 98.41 (*d*, C-7), 77.23, 75.73 (2 × *d*, C-5′, C-3′), 73.32 (*d*, C-2′), 69.80 (*d*, C-4′), 62.11 (*d*, C-2), 60.77 (*t*, C-6′), 32.81 (*t*, C-3). FAB MS *m/z*: 549 $[M+H]^+$, 387 $[M-C_6H_{11}O_5+2H]^+$. ^1H nuclear Overhauser enhancement difference spectra in DMSO-d_6 showed negative enhancements as follows. Irradiation at 7.321 (H-7) gave enhancements of the signals at 8.433 (H-11) and at 7.983 (H-14, H-18). Irradiation at 7.006 (H-4) gave an enhancement at 4.546 (H-1′), while irradiation at 4.546 (H-1′) gave enhancements at 7.006 (H-4) and at 3.31 to 3.23 (H-3′ and H-5′).

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REFERENCES

1. Mabry, T. J., Wyler, H., Sassu, G., Mercier, M., Parikh, I. and Dreiding, A. S. (1962) *Helv. Chim. Acta* **45**, 640.
2. Mabry, T. J., Dreiding, A. S. (1968) in *Recent Advances in Phytochemistry* (Mabry, T. J., Alston, R. E. and Runeckles, V. C., eds) Vol. 1, p. 145. Appleton-Century-Crofts, New York.
3. Reznik, H. (1980) in *Pigments in Plants* (Czygan, F.-C., ed.) 2nd edn, p. 370. Gustav Fischer, Stuttgart.
4. Mabry, T. J. (1980) in *Secondary Plant Products* (Bell, E. A. and Charlwood, B. V., eds) *Encyclopedia of Plant Physiology New Series*, Vol. 8, p. 513. Springer, Berlin.
5. Piattelli, M. (1976) in *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., ed.) p. 560. Academic Press, London.
6. Piattelli, M. and Minale, L. (1964) *Phytochemistry* **3**, 547.

7. Piattelli, M., Minale, L. and Prota, G. (1965) *Phytochemistry* **4**, 121.
8. Strack, D. and Reznik, H. (1979) *Z. Pflanzenphysiol.* **94**, 163.
9. Strack, D., Engel, U. and Reznik, H. (1981) *Z. Pflanzenphysiol.* **101**, 215.
10. Piattelli, M. and Minale, L. (1964) *Phytochemistry* **3**, 547.
11. Reznik, H. (1957) *Planta* **49**, 406.
12. Reznik, H. (1975) *Ber. Dtsch. Bot. Ges.* **88**, 179.
13. Boehringer Mannheim GmbH (1980) *Methods of Enzymatic Food Analysis.*