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HUMILIXANTHIN, A NEW BETAXANTHIN FROM *RIVINA HUMILIS*

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Key Word Index—*Rivina humilis*; Phytolaccaceae; betalain; betaxanthin; humilixanthin; 5-hydroxynorvaline.

Abstract—A new betalain has been isolated from fruits of *Rivina humilis* and identified as the betaxanthin humilixanthin, the 5-hydroxynorvaline-immonium conjugate of betalamic acid. Its structure was elucidated and characterized by ^{1}H NMR spectroscopy, FAB mass and GC/MS spectrometry, UV/Vis absorption spectroscopy, high-performance liquid chromatography, thin-layer chromatography and electrophoresis. The structure of the amino acid moiety 5-hydroxynorvaline (2-amino-5-hydroxyvaleric acid) was unambiguously confirmed by comparison with synthetic reference material. Humilixanthin was also detected in fruits of *Phytolacca acinosa* and *P. bogotensis*, in petals of *Delosperma luteum*, *Lampranthus aurantiacus*, *L. peersii*, *Portulaca grandiflora*, and in the yellow-coloured root of *Beta vulgaris*.

INTRODUCTION

There is a lack of knowledge about the various structures of the yellow water-soluble, Caryophyllales-characteristic betaxanthins. So far only 9 out of at least 25 of these pigments, detectable as major components by thin-layer chromatography (TLC) and high-voltage electrophoresis, have been identified from Caryophyllales-derived betalain extracts [1–3]. Some additional 7 betaxanthins (muscaaurins) have been isolated from the fly agaric (*Amanita muscaria*) [4]. In addition, high-performance liquid chromatography (HPLC) analyses [Strack, D. unpublished] indicate that the number of betaxanthin structures is considerably greater than known from the traditional separation techniques.

Several interesting unknown major betaxanthins have been observed in extracts from the red- and yellow-coloured fruits of the neo/subtropical shrub *Rivina humilis*. One of these unknown pigments has now been isolated and its structure conclusively elucidated. This paper presents the proof that this new compound is the 5-hydroxynorvaline-immonium conjugate of betalamic acid, for which we propose the trivial name humilixanthin. Furthermore, humilixanthin has also been detected in some other members of the Caryophyllales.

RESULTS AND DISCUSSION

During the course of a betalain screening (TLC, thin-layer electrophoresis, HPLC) of members of the Phytolaccaceae we observed in extracts from yellow- and orange red-coloured fruits of *Rivina humilis* several major bright-yellow pigments which could not be identified by direct comparison with reference material (extracts with known components from the institute collection in Cologne). Only dopaxanthin and betalamic acid were readily detected by co-HPLC (R_t 20 and 36.5 min) to be two of the major components while vulgaxanthin I (R_t 9.2 min), miraxanthin I (R_t 9.5 min), and indicaxanthin (R_t 17.5 min) were among the minor ones. The orange red-coloured fruits showed in addition betanin (R_t 21.4 min), with R_t of 22.9 min most likely the betanin-sulphate ester rivinianin [5], and iso-betanin (R_t 23.5 min). One of the unknown betaxanthins (10–40% of total betaxanthins depending on the variety used, i.e. 0.3–1.2% of fruit flesh dry weight; R_t 24.5 min in HPLC) was isolated by three successive column chromatography steps. (i) on polyamide with an aqueous citric acid solution, (ii) on reversed-phase (C_{18}) silica (prep. HPLC) with a water–acetonitrile gradient, containing 0.4% formic acid, and (iii) on Sephadex LH-20 with water.

The UV/Vis spectrum of humilixanthin (in water λ_{max} nm: 258, 463 shoulder, 483) was typical for betaxanthins [6]. It showed an electrophoretic mobility similar to indicaxanthin (*ca* 9.5 cm) and an ^2R_f [two evelopments; $1-(1-R_f)^2$] value (TLC in SS1) of 0.70 (indicaxanthin, 0.58).

The ^{1}H NMR spectra of humilixanthin in CD_3OD, containing a trace of DCl, indicated that it exists in this solution as a mixture of four isomers (Table 1; Schemes 1 and 2). This is evident from the lower field signals in the one-dimensional (1D) ^{1}H NMR spectrum and from the cross peaks in the 2D ^{1}H COSY spectrum. That we are dealing with isomeric forms of the same basic structure was confirmed by the observation of only one peak in the negative ion FAB mass spectrum corresponding to a deprotonated molecular ion at *m/z* 325.

The detection of vicinal and allylic proton–proton coupling constants, determined from the 1D ^{1}H NMR spectrum and from cross peaks in the 2D COSY spectrum, provided evidence for the various fragments in the

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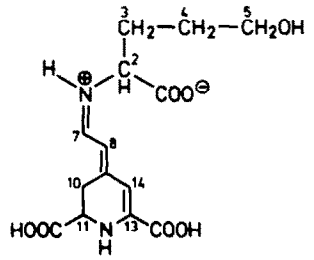
Table 1. ^{1}H NMR spectral data of humilixanthin in CD_3OD

Shifts (ppm)†	Major*		Minor	
H-2	4.90	—	4.85–4.97	—
H-3	2.52–2.34	—	2.61–2.34	—
H-4	2.27–2.08	—	2.27–2.08	—
H-5	4.10–3.75	—	4.10–3.75	—
H-7	8.610	8.684	8.927	8.94
H-8‡	6.235	5.999	5.918	5.88
H-10A	3.521	—	3.50–3.60	—
H-10B	3.162	—	3.10–3.20	—
H-11	4.527	4.537	4.471	4.441
H-14	6.419	6.332	6.761	6.794
Couplings (Hz)				
(7–8)	12.1	12.4	12.3	≃12
(10A–11)	5.5	5.2	6.1	≃6.7
(10B–11)	7.1	7.1	6.1	≃6.7
(10A–10B)	17.2			

* The precentages of the isomers, taken from the height of the signal for H-5, were 60 ('Major'), 22, 15 and 3 ('Minor').

† The shifts given to two decimal places after the point were taken from the cross peaks in the 2D ^{1}H COSY spectrum.

‡ Cross peaks in the 2D ^{1}H COSY spectrum are indicative of allylic coupling from H-8 to H-10A and H-10B.



Scheme 1. Structure of humilixanthin and numbering scheme for Table 1.

molecule (C-2–C-5 and C-7–C-11). A comparison of the chemical shifts and coupling constants with literature data [7] indicated these were characteristic of betaxanthin derivatives. The presence of the fragment -CH-CH_2-CH_2-CH_2- in the amino-acid moiety followed from the ^{1}H 2D COSY spectrum and integrals in the 1D spectrum. The shift of the methine proton was characteristic of the α-proton of the amino acid, while the low field signal of the terminal CH_2 group allowed only a limited number of possibilities for the nature of the attached group(s). Thus only ring closure with attachment to the charged nitrogen or the presence of an hydroxyl group were possible. (An amino group to give an ornithine-type chain would not account for the shifts.) The former would give rise to a known compound, indicaxanthin, which could be excluded by comparison with the properties of authentic material. Thus only the presence of an hydroxyl group is possible and this is confirmed by the FAB mass spectrum which is compatible with a M_r of 326.

The presence of several isomers in acidic solution is not unexpected as betanin, isobetanin and indicaxanthin also showed this phenomenon [7]. However, only the isomers **1** and **2** (Scheme 2) arising from isomerisation of one of the formal double bonds, were invoked to explain the low field NMR spectra. Isomerisation of the second formal double bond would create a further pair of isomers, **3** and **4**. Isomerisation caused by rotation about the central formal single bond appears unlikely as this would lead to a sterically crowded species.

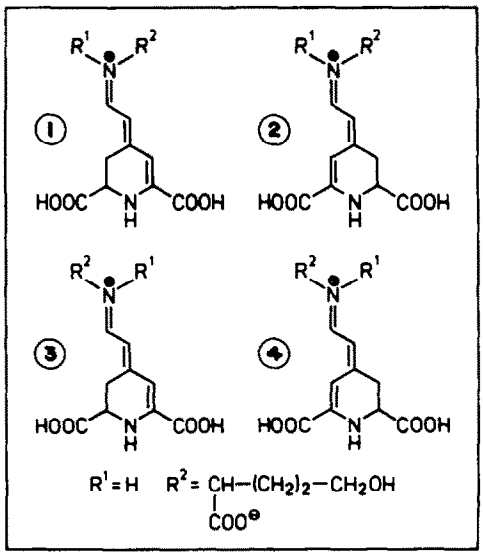
In conclusion, from the data described above, the amino acid moiety of humilixanthin was shown to be 5-hydroxynorvaline, i.e. 2-amino-5-hydroxyvaleric acid, and has been found sporadically elsewhere [8, 9]. This was unambiguously proven by direct chromatographic comparison of the degradation product from treatment of humilixanthin with 1 M HCl at 100° with chemically synthesized 5-hydroxynorvaline, which gave an identical R_f of 0.55 (TLC in SS2) and R_t of 26.6 min (HPLC, amino acid analysis).

In a survey (TLC, HPLC) of some 70 members of the Caryophyllales (plants not listed), humilixanthin was found to be also present in fruits of *Phytolacca acinosa* Roxb. and *P. bogotensis* H. B. K., in petals of *Delosperma luteum* L. Bolus, *Lampranthus aurantiacus* (DC) Schwantes in Fedde, *L. peersii* N. E. Brown, and *Portulaca grandiflora* Hook., and in the yellow-coloured root of *Beta vulgaris* L.

EXPERIMENTAL

Plant material. Rivina humilis L. and the other plants mentioned above were grown in the field or in the greenhouse of the botanic garden of the University of Cologne.

Isolation of humilixanthin. (i) Deseeded fruits (*ca* 30 g) of *Rivina humilis* (yellow-coloured) were treated with an Ultra-Turrax homogenizer (4 min) in 200 ml of 80% aq. MeOH. The homogenate was allowed to stand for 2 hr with continuous stirring. (ii) The filtrate was concd under vacuum to a few ml and



Scheme 2. Possible isomeric structures of humilixanthin detected by NMR spectroscopy (see Table 1).

transferred onto a H_2O-equilibrated polyamide column (CC-6 Perlon, 64 cm × 3 cm; Macherey-Nagel, Düren, F.R.G.) and fractionated using H_2O (250 ml) and citric acid solution (1 l. of 0.2% aq. Na citrate–0.2% aq. citric acid, 1:1, v/v pH 4.5) [6]; flow-rate of 20 ml/min. (iii) The 2nd 500 ml of the 'citrate eluate' was concd under vacuum to *ca* 3 ml. 500 μl aliquots were chromatographed by means of prep. HPLC on a prepacked Multosorb-C_{18} column (10 μm, 250 mm × 2 cm; *CS*-Chromatographie Service, Eschweiler, F.R.G.); detection at 480 nm (1.024 absorbance unit full scale); elution system: linear gradient within 80 min from 10 to 60% solvent B (0.4% HCO_2H in 50% aq. MeCN) in solvent A (0.4% HCO_2H in H_2O), then from 81 to 85 min to 100% B; flow-rate at 5 ml/min; from 6 HPLC runs the eluates (at 54–60 min) containing humilixanthin were pooled and concd under vacuum to *ca* 4 ml. (iv) The 'humilixanthin fraction' was finally purified in the cold (4°) on a Sephadex LH-20 column (64 cm × 3 cm; Pharmacia, Freiburg, F.R.G.) using *ca* 400 ml H_2O; flow-rate at 1.4 ml/min. Elution of humilixanthin was visually controlled and the eluate was taken to complete dryness.

Isolation of 5-hydroxynorvaline from humilixanthin. Humilixanthin, dissolved in 5 ml 1 M HCl, was heated for 2.5 hr at 100°, then dried under vacuum and redissolved in 1 ml H_2O [6]. This was passed through a Dowex column (4 cm × 1 cm; 50 W, H^+, 200–400 mesh; Sigma, München, F.R.G.), washed with 50 ml H_2O followed by 20 ml 2 M NH_4OH. The latter was dried under vacuum and the residue was redissolved in 1 ml 50% aq. MeOH containing 1 mM HCl.

Chemical synthesis of 5-hydroxynorvaline was achieved by reduction of glutamic acid ethyl half-ester with 3 equiv. of sodium in liquid NH_3 at −78° in the presence of 3.5 equiv. *t*-BuOH. When the blue colour of the dissolving metal persisted, 3 equiv. NH_4Cl were added and the ammonia was allowed to evaporate. A conc. aq. solution of the residue was desalted on Dowex 50 (H^+ form; 1 M NH_4OH as the eluant). The product appeared to be largely homogenous (TLC, HPLC). An aliquot was derivatized with BSTFA in dry MeCN [10] and gave a MS (Finnigan 4510 GC/MS spectrometer) for the trisilylated amino acid in agreement with literature data [11]. MS (70 eV) (%): 349 (0.11) $[M]^+$, 334 (0.09), 306 (0.08), 232 (16) $[M-(CH_3)_3Si\text{-}O\text{-}C=O]^+$, 160 (3), 147 (7), 142 (41), 128 (9), 115 (4), 100 (9), 75 (18), 73 (100), 70 (21), 59 (8), 45 (119), 43 (7).

TLC. Analysis of betaxanthins: developed twice in SS1, *iso*-PrOH–H_2O–HOAc (75:20:5; equilibration for 24 hr) on diethylaminoethyl cellulose (MN 300 DEAE, Macherey-Nagel). Analysis of amino acids: SS2, *iso*-PrOH–EtOH–H_2O–HOAc (6:7:6:1) on microcrystalline cellulose ('Avicel', Macherey-Nagel) (described for betalains in ref. [12]); detection by ninhydrin spray and subsequent heating for 10 min at 110°.

Thin-layer electrophoresis was carried out as described elsewhere [13] on 'Avicel' with KPi buffer (pH 6.65)–MeOH, 1:1; 2 hr at 40 V/cm. Plates were made with 'Avicel' suspended in KPi buffer (pH 6.65).

HPLC. The liquid chromatograph (LKB) and the data processor (Shimadzu) have been described [14]. Analysis of betalains: the column (250 mm × 4 mm) was prepacked with MN-Nucleosil 120–5 C_{18} (Macherey-Nagel). Elution system: at a flow rate of 1.5 ml/min linear gradient elution within 45 min from solvent A (1.5% H_3PO_4 in H_2O) to 45% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O). Detection was at 480 nm (see also ref. [15]). Analysis of amino acids: the column (125 mm × 4 mm) was prepacked with Shandon ODS-Hypersil 5μm (Bischoff Analysentechnik und -geräte, Leonberg, F.R.G.). Elution system: at a flow rate of 1 ml/min linear gradient elution within 60 min from solvent A (10 mM NaH_2PO_4, pH 7.2, in H_2O) to 70% solvent B (H_2O–MeOH–MeCN, 1:1:1) in solvent A. Detection of 2-mercaptoethanol-*o*-phthalaldehyde amino acid derivatives was at 340 nm [16]. The reagent for amino acid derivatization was prepared by dissolving 44 mg *o*-phthalaldehyde (Serva, Heidelberg, F. R. G.) in 1 ml MeOH, 9 ml borate buffer (pH 9.5, 400 mM), and 200 μl 2-mercaptoethanol [17]. This was mixed (1:1) for 1.5 min with amino acid solution immediately before injection.

UV/Vis spectroscopy was performed with an UVICON-810 spetrophotometer (Kontron, München, F. R. G.).

NMR and MS. ^1H NMR (400 MHz) spectra were recorded at ambient temp. on a Bruker WM-400 NMR spectrometer locked to the deuterium resonance of the solvent, CD_3OD containing a trace of DCl. A 2D COSY ^1H spectrum was recorded with a 90°-t_1-90°-FID(t_2) pulse sequence. The spectral width was F2 2451.0 Hz and F1 ± 1225.5 Hz with 1 K data points in t_2 and 512 data points in t_1. 128 pulses were taken for each t_1-increment with a relaxation delay of 0.5 sec between pulse sequences to give a total accumulation time of 15 hr. The data were multiplied by sine-bell functions and one level of zero-filling was used for both t_1 and t_2. All 1D and 2D spectra were recorded using the standard Bruker software package. Chemical shifts are recorded in ppm relative to TMS and coupling constants in Hz.

A negative fast atom bombardment (FAB) mass spectrum was recorded on a Kratos MS-50 mass spectrometer equipped with a Kratos FAB source. Glycerol was used as matrix.

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