Pergamon

PII: S0031–9422(98)00047–8

Phytochemistry, Vol. 49, No. 2, pp. 585–588, 1998
© 1998 Elsevier Science Ltd. All rights reserved
Printed in Great Britain
0031–9422/98/$–see front matter

INTRAMOLECULAR STABILIZATION OF ACYLATED BETACYANINS

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

WILLIBALD SCHLIEMANN* and DIETER STRACK

Institut für Pflanzenbiochemie (IPB), Abteilung Sekundärstoffwechsel, Weinberg 3, D-06120 Halle (Saale), Germany

(*Received* 17 *October* 1997)

Key Word Index—Betacyanin; betanin; amaranthin; lampranthin II; celosianin II; racemization; stability; intramolecular stacking.

Abstract—Racemization and stability of the betacyanins, betanin (betanidin 5-*O*-glucoside) and amaranthin (betanidin 5-*O*-glucuronosylglucoside), under acidic conditions were compared with those of the corresponding feruloyl derivatives, lampranthin II and celosianin II. Both acylbetacyanins showed a reduced racemization velocity and celosianin II in addition an enhanced stability, possibly caused by intramolecular association between the betanidin and the feruloyl moieties. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Anthocyanins acylated with aromatic acids, mainly hydroxycinnamic acids, exhibit a marked bathochromic shift in light absorption (copigmentation effect) and an enhanced colour stability [1–6]. Acylation of betacyanins, e.g. esterification of the sugar moieties of betanidin 5-*O*-glucoside (betanin, **1**), betanidin 6-*O*-glucoside (gomphrenin I) and betanidin 6-*O*-sophoroside with hydroxycinnamic acids also leads to bathochromic shifts of light absorption [7, 8]. NMR spectroscopic analyses showed ^{1}H chemical shift differences between gomphrenin I, betanidin 6-*O*-sophoroside and its respective acylated derivatives [7, 8] as well as between **1** and lampranthin II (6′-*O*-*E*-feruloylbetanin, **2**) [7], indicating molecular association of the aromatic acids and the chromophore, betanidin.

We report here on experiments showing a reduced racemization and an enhanced stability of acylbetacyanins as compared to the corresponding non-acylated betacyanins, possibly caused by intramolecular stacking of the acyl residues.

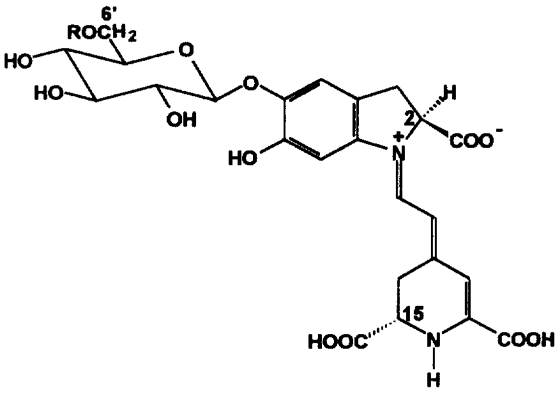
RESULTS AND DISCUSSION

The present HPLC-PDA studies revealed bathochromic shifts of light absorption of **2** (λ_{max} 541 nm) and **4** (λ_{max} 545 nm) compared to **1** and **3** (both λ_{max} 537 nm). Since **2** and **4** are major betacyanin components in callus cultures of *Portulaca grandiflora* Hook. (data not shown) and suspension cultures of *Chenopodium rubrum* L. [9], respectively, both compounds were readily isolated and purified to compare their stabilities with those of the non-acylated precursors, **1** and **3**, under conditions which enhance racemization. In early studies on betacyanin identification, betanin has been isomerized to a betanin–isobetanin mixture (6:4) by treatment with 5% aqueous citrate for 36 h at room temperature [10]. To increase the racemization velocity, 1 N HCl was added to the betacyanins **1**–**4** and the progress of the reaction was analyzed by HPLC for 8 h at room temperature (Figs 1 and 2). Whereas **1** and **3** racemize in a linear time-dependent manner (2.2% and 1.5% h^{-1}, respectively), both the feruloylated derivatives, **2** and **4**, exhibited a markedly reduced racemization (0.4% h^{-1}), possibly caused through steric hindrance by the feruloyl moieties approaching the reaction site. Compound **4** showed in addition a clearly enhanced resistance against hydrolytic attack.

The degradation products of **1** in acidified solutions have been suggested to be betalamic acid and *cyclo*-Dopa 5-*O*-glucoside [11]. By monitoring the racemization and degradation of **1** with HPLC and detection wavelengths of 540, 405 and 283 nm, the accumulation of a very polar compound (R_t 1.8 min) with the characteristic UV spectrum of *cyclo*-Dopa 5-*O*-glucoside (λ_{max} 283 nm) [12] was observed. Betalamic acid (R_t 19.5 min, λ_{max} 405 nm) was only detected in small amounts due to its high instability at low pH

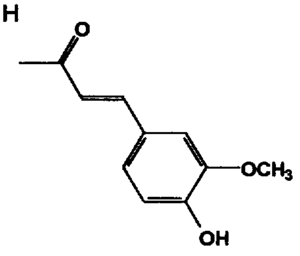
---

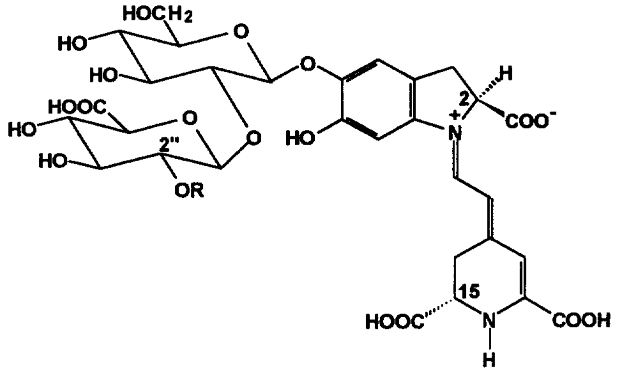
* Author to whom correspondence should be addressed.



1 R= H

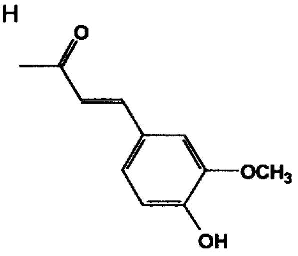
2 R=





3 R= H

4 R=



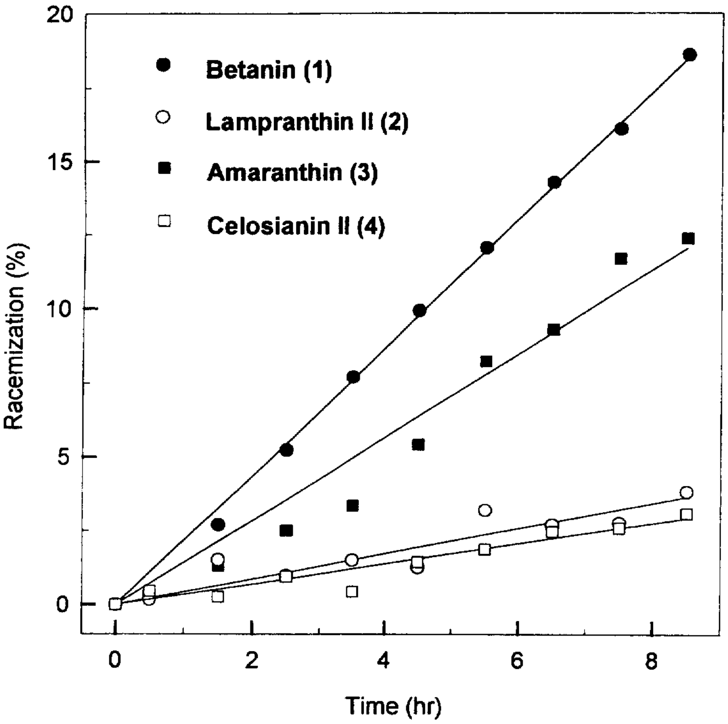


Fig. 1. Time course of the racemization of **1**–**4** (conversion of 15*S* into 15*R*) in 0.5 N HCl at room temperature.

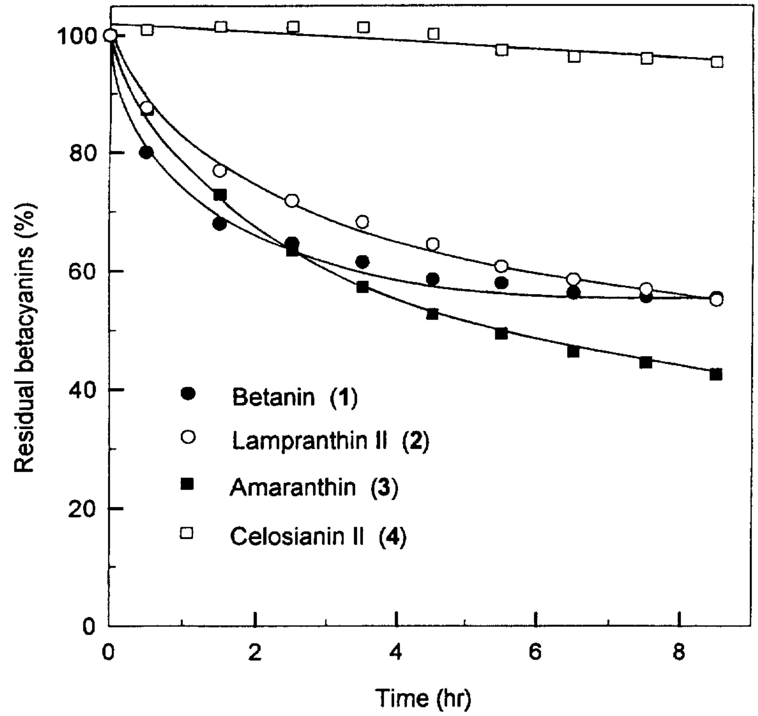


Fig. 2. Time course of the degradation of **1**–**4** in 0.5 N HCl at room temperature.

(data not shown). Hydrolysis of the glucosidic bond and the ester linkage were not observed.

Racemization of **1** proceeds in the partial transformation of the (*S*)-betalamic acid moiety to the (*R*)-form with the retention of configuration of the (2*S*)-*cyclo*-Dopa 5-*O*-glucoside part. Since the racemization is accompanied by degradation due to partial hydrolysis of the aldimine bond, it was not possible to decide whether the racemization proceeds at the intact betacyanins or, less likely, partly at the liberated betalamic acid which can form the detected (2*S*/15*R*)-isomer in a reverse reaction.

Since racemization starts at C-15 of the betacyanins and the hydrolytic attack at the aldimine bond, both reactions appear to proceed independently. This is obvious comparing the reaction kinetics of **2** in Figs 1 and 2. Indeed, besides the structure of the aromatic acyl groups, an important factor in the stacking process is the linkage to the glycosyl moiety which has to allow sufficient conformational flexibility for the intramolecular association on the betanidin skeleton. The binding of the feruloyl residue at the glucuronosyl moiety of a disaccharide in **4** obviously leads to a higher flexibility of the acylglycoside moiety which effectively protects the aldimine bond against hydrolytic attack.

To substantiate the assumptions above, different conformers of **4** have been sampled using a systematic search for the torsional angles related to single bonds between the four major subunits (betanidin, glucose, glucuronic acid and ferulic acid). Subsequent energy minimization at semiempirical level [13] of **4** showed that the energetically most favourable structures probably have a stretched conformation, although the high flexibility of the molecule also permits U-shaped structures (3–5 kcal mol^{-1} higher energy compared to stretched types) (data not shown) which have to be postulated for intramolecular stacking. Due to the size of **4** and the high number of conformers, other, more accurate quantum chemical methods, were not applicable. The flexibility of the molecule makes a definite assignment of the structures by comparison of relative energies for different conformations problematic.

In summary, the acylation of the betacyanins which leads to bathochromic shifts of light absorption of the betanidin moiety (copigmentation effect), results in a reduced racemization velocity of **2** and **4** and in addition to an enhanced stability of **4** in comparison with the corresponding non-acylated betacyanins, the latter most likely caused by intramolecular stacking.

EXPERIMENTAL

Purification of betacyanins

Freshly harvested cell culture material and red beetroots were frozen in liquid N_2, homogenized in a mortar and extracted with 80% aq. MeOH for 30 min. Purification was performed by semi-prep. HPLC on an analytical column with a fraction collector (0.5 ml fractions). Betanin (**1**) was isolated from beetroot (*Beta vulgaris* L. subsp. *vulgaris* var. *conditiva* Alef.), lampranthin II (6′-*O*-*E*-feruloylbetanin) (**2**) from a callus culture of *Portulaca grandiflora* Hook., amaranthin (**3**) and celosianin II (2″-*O*-*E*-feruloylamaranthin) (**4**) from suspension-cultured cells of *Chenopodium rubrum* L.

Racemization and degradation procedure

The initial isomer ratio of the betacyanins (5.5 nmol in 110 μl H_2O) was analyzed by HPLC (10 μl injection which corresponds to 0.5 nmol betacyanin). To start the racemization, 100 μl 1 N HCl was added to the injection vial (final pH of 0.4) 30 min after starting the HPLC run. The progress of racemization was monitored every hour by automatical injection of 20 μl, corresponding again to 0.5 nmol betacyanin. From the peak areas of the betacyanins and their increasing amounts of the isoforms, the racemization and degradation were calculated. The data are the mean from two measurements.

HPLC

The liquid chromatograph and data processor (Millenium software) from Waters™ Millipore (Eschborn, Germany) were used. The chromatograph was equipped with a column prepacked with Nucleosil C_{18} (250 × 4 mm i.d., 5 μm, Macherey–Nagel, Düren, Germany) and a linear gradient within 40 min from solvent A (1% aq. HCO_2H) to 30% solvent B (80% aq. MeCN) in A + B at a flow rate of 1 ml min^{-1} was applied. Injections (10 or 20 μl for analysis, 100 μl for semi-prep. HPLC) were carried out by an automatic sampler. Compounds were detected at their λ_{max} (ca ~540 nm) by a photodiode array detector (215–600 nm).

Acknowledgements—The authors thank Barbara Kolbe (IPB) for experimental assistance, Dr H. Böhm (Deutsches Institut für Ernährungsforschung, Bergholz-Rehbrücke, Germany) for providing callus cultures of *Portulaca grandiflora* Hook. and Dr M. Meyer (Institut für Molekulare Biotechnologie, Jena, Germany) for biocomputing. This work was supported by the Deutsche Forschungsgemeinschaft (Bonn) and the Fonds der Chemischen Industrie (Frankfurt/M.).

REFERENCES

1. Brouillard, R. and Dangles, O., in *The Flavonoids: Advances in research since* 1986, ed. J. B. Harborne, Chapman and Hall, London, 1993, p. 565.
2. Strack, D. and Wray, V., in *The Flavonoids: Advances in research since* 1986, ed. J. B. Harborne, Chapman and Hall, London, 1993, p. 1.
3. Goto, T., Tamura, H., Kawai, T., Hoshino, T., Harada, N. and Kondo, T., *Annals of the New York Academy of Sciences*, 1986, **471**, 155.
4. Goto, T. and Kondo, T., *Angewandte Chemie*, 1991, **103**, *Int. Ed.*, **30**, 17.
5. Yoshida, K., Kondo, T. and Goto, T., *Tetrahedron Letters*, 1991, **32**, 5579.
6. Glässgen, W. E., Wray, V., Strack, D., Metzger, J. W. and Seitz, H. U., *Phytochemistry*, 1992, **31**, 1593.
7. Heuer, S., Wray, V., Metzger, J. W. and Strack, D., *Phytochemistry*, 1992, **31**, 1801.
8. Heuer, S., Richter, S., Metzger, J. W., Wray, V., Nimtz, M. and Strack, D., *Phytochemistry*, 1994, **37**, 761.
9. Strack, D., Bokern, M., Marxen, N. and Wray, V., *Phytochemistry*, 1988, **27**, 3529.
10. Piattelli, M. and Minale, L., *Phytochemistry*, 1964, **3**, 547.
11. Huang, A. S. and von Elbe, J. H., *Journal of Food Science*, 1985, **50**, 1115.
12. Wyler, H., Meuer, U., Bauer, J. and Stravs-Mombelli, L., *Helvetica Chimica Acta*, 1984, **67**, 1348.
13. Stewart, J. J. P., *Journal of Computer-Aided Molecular Design*, 1990, **4**, 1.