β-Carboline Alkaloids from *Ribes nigrum* L.

St. Blech and H. Budzikiewicz

Institut für Organische Chemie der Universität, Greinstraße 4, D-50939 Köln, Bundesrepublik Deutschland

D. Dill

Merz + Co. GmbH & Co., Eckenheimer Landstraße 100–104, D-60318 Frankfurt, Bundesrepublik Deutschland

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From *Ribes nigrum* L. two β-carboline alkaloids (deshydroxymethyl flazin (**1**) and flazin methyl ether (**2**)) could be isolated in minute amounts. The structure elucidation is described. The two compounds are present in the freshly pressed juice, but they are also formed during storage of the juice by condensation of tryptophane with ascorbic acid or its degradation products. This could be shown by adding ^{14}C-labelled ascorbic acid to the juice. After four weeks' incubation radioactive **1** and **2** could be isolated.

Introduction

In plant extracts as well as in preparations from plant products as, *e.g.*, soy sauce occasionally small amounts of furyl-β-carboline alkaloids have been detected. They can be considered as arising from condensation products of tryptophane with ascorbic acid or its degradation products such as furfural. As products of this type are formed *in vitro* when tryptophane and ascorbic acid are kept for some time in an acidic solution it has been an open question whether the furyl-β-carboline alkaloids are actually formed during storage of the plant products or are present in the plant cells and hence genuine natural products. In the following experiments with the juice of black currants (*Ribes nigrum* L.) will be described which shed some light on this question.

Results and Discussion

From the concentrated juice of black currants (*Ribes nigrum* L.) two fluorescent compounds could be isolated in minute amounts (*ca.* 3 mg each from 100 kg fresh juice, *i.e. ca.* 30 ppb) by repeated chromatographic steps (see Experimental), *viz.* 1-(2-furyl)-pyrido[3,4-b]indol-3-carboxylic acid (**1**) and 1-(5-methoxymethyl-2-furyl)-pyrido[3,4-b]indol-3-carboxylic acid (**2**). Their elemental compositions were determined by mass spectrometry as $C_{16}H_{10}N_2O_3$ and $C_{18}H_{14}N_2O_4$. The fragmentation patterns are straight forward: **1** ($M^{+\cdot}$ *m/z* 278) loses CO_2 followed by ·CHO (*m/z* 205) and HCN/H_2CN· (*m/z* 178/177). **2** ($M^{+\cdot}$ *m/z* 322) also loses CO_2 (*m/z* 278), but the further degradation is governed by the presence of the CH_2OCH_3 group: Both, *m/z* 322 and 278 eliminate ·OCH_3 (*m/z* 291 and 247, resp.). The ion *m/z* 278 loses CH_3OCH_2CO· in analogy to the loss of ·CHO above (*m/z* 205), *m/z* 247 loses ·CHO (*m/z* 218). All these processes were confirmed by exact mass measurements.

The ^1H and ^{13}C data of **1** and **2** are compiled in Tables I and II. It can be seen that the signals for

Table I. ^1H NMR data of **1** and **2.**

	1		**2**	
H(4)	8.84 (s)		8.85 (s)	
H(5)	8.40 (d)	7.7 Hz	8.41 (d)	7.8 Hz
H(6)	7.32 (dd)	7.7/8.3 Hz	7.34 (dd)	7.8/8.2 Hz
H(7)	7.62 (dd)	7.7/8.3 Hz	7.63 (dd)	7.8/8.2 Hz
H(8)	7.81 (d)	8.3 Hz	7.82 (d)	8.2 Hz
H(3′)	7.48 (d)	3.4 Hz	7.45 (d)	3.4 Hz
H(4′)	6.82 (dd)	1.7/3.4 Hz	6.77 (dd)	3.4 Hz
H(5′)	8.01 (d)	1.7 Hz	–	
NH	11.85 (s)		11.66 (s)	
COOH	–		–	
CH_2	–		4.64 (s)	
CH_3	–		3.34 (s)	

Reprint requests to Prof. Dr. H. Budzikiewicz.
Telefax: +49-221-470-5102.

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Table II. ^{13}C NMR data of **1** and **2.**

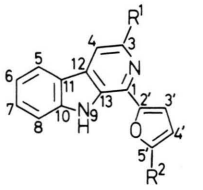
	1	**2**
C(1)	137.1	137.1
C(3)	132.2	132.2
C(4)	115.9	115.9
C(5)	121.9	122.0
C(6)	120.4	120.5
C(7)	128.8	128.9
C(8)	113.0	112.9
C(10)	141.6	141.5
C(11)	120.8	120.9
C(12)	129.8	129.9
C(13)	132.0	132.0
C(2′)	152.3	151.7
C(3′)	110.2	111.1
C(4′)	112.4	111.9
C(5′)	144.2	153.2
CO	166.5	166.5
CH_2	–	65.5
CH_3	–	57.2

the β-carbolin system are very close to each other for the two compounds. The assignments were confirmed by H,H- and H,C-COSY experiments.

Both ^1H spectra show the typical 4-spin system of an unsymmetrically disubstituted benzene ring. Furtheron, a sharp singulet of the H-4 and a broad one for the indole-NH can be observed. While **1** exhibits the typical 3-spin system of an α-substituted furan ring (confirmed by COSY) **2** shows that of an α,α′-disubstituted furan ring and in addition the signals for an aromatically bound CH_2OCH_3 group. The substitution patterns in the benzene and the furan rings were confirmed by the observed multiplicities of the various signals and by H,H-COSY.

H,C-COSY experiments allow a correlation of the directly connected H and C atoms. Due to the large number of quaternary carbon atoms additional structural correlations were possible only with the aid of long-range correlation experiments which allow to establish connectivities over 2 or 3 bonds. The 4 protons of the benzene ring show all theoretically possible ^3J correlation peaks and so the ^{13}C signals of the 2 remaining carbon atoms (C-10 and C-11) could be identified. In addition, H-5 couples with the β-carbon of the indole system (C-12). The pyrrol ring carbons (C-10–C-13) show ^2J and ^3J coupling, resp., with the NH proton. The proton of the pyridine ring couples *i.a.* with C-11, C-12 and C-13 which establishes its location at C-4. In addition, the vicinity of H-4 and H-5 can be shown by NOE experiments. Coupling of H-4 with C-3 (^2J) and the COOH carbon (^3J) determines the position of the latter.

The α-substitution of the furan ring of **1** and the α,α′-disubstitution for **2** are confirmed by ^2J and ^3J coupling. In addition, in the case of **2** correlation signals are observed between the CH_2 group and C-5′ as well as with the OCH_3 group. The substitution of C-1 of the β-carboline system by the furan ring was confirmed by an additional NOE experiment: Saturation of the NH resonance leads to an increase both of the H-8 signal and that of H-3′ of the furan ring.



1: R^1 = COOH, R^2 = H
2: R^1 = COOH, R^2 = CH_2OCH_3
3: R^1 = COOH, R^2 = CH_2OH
4: R^1 = H, R^2 = CH_2OH
5: R^1 = R^2 = H

2 is the methyl ether of flazin (**3**) (Nakatsuka *et al.,* 1986) while **1** is identical with a product obtained *i.a.* when tryptophane and ascorbic acid were kept for two months in a phosphate buffer solution (Kanamori *et al.,* 1980, Kanamori *et al.,* 1981) (incidentally, the assignments for the ^{13}C signals given by the authors based on reference data only could be corrected by the correlation experiments described above, see Table II). The furanyl β-carboline perlolyrin (Gessner *et al.,* 1988) **4** (decarboxylated flazin; the name perlolidin (Nakatsuka *et al.,* 1986) is an erroneous quotation from Jeffreys, 1970) was isolated from *Lolium perenne* L. (Jeffreys, 1970), *L. chuangxiong* (quoted in Nakatsuka *et al.,* 1986), *Codonopsis lanceolata, Polygala tenuifolia* Lk., *Lycium chinense* Mil. and *Panax ginseng* C.A. Mey. (Han *et al.,* 1986). Perlolyrin and flazin have also been found in sake (Japanese rice wine) (Nakatsuka *et al.,* 1986), rice vinegar (Nakatsuka *et al.,* 1986; Kihara, 1987, 1991) and old soy sauce (Nakatsuka *et al.,* 1986; Kihara, 1991), the age of which could be correlated with the concentration of the two compounds (Kihara,

1990) (a quality control is thus possible by HPLC analysis (Kihara, 1989)).

1 and its decarboxylation product **5** were detected amongst the reaction products of tryptophane and ascorbic acid and were shown to be mutagenic for various bacterial strains (Kanamori *et al.*, 1980, 1981). It was suggested that these compounds might be formed during storage and cooking of food preparations which contain free tryptophane and ascorbic acid. This raises the question whether perlolyrin from *Lolium* etc. as well as **1** and **2** from *Ribes nigrum* L. are genuine plant products or are formed in the aqueous extracts. Jeffreys (1970) first approached this problem by using a special extraction procedure of air-dried *Lolium* (benzene with triethylamine) considering that this extract should be free of amino acids, proteins and sugars and prevent oxidation reactions. He concluded that perlolyrin (**4**) was actually present in the grass. Following his reasoning we prepared fresh juice from deep-frozen black currants and extracted it with ether. An ether extract should not contain highly polar compounds as sugars, ascorbic acid or tryptophane (the presence of amino acids could be excluded by a negative ninhydrine test). Both **1** and **2** were present in the ether extract. Especially the formation of the aliphatic methoxyl group of **2** cannot be due to a methylation by solvents during the isolation. Flazin (**3**) could not be detected in the ether extract. To test whether the formation of **1** and **2** continued in the juice during storage ^{14}C-labelled L-ascorbic acid was added to freshly prepared juice which was then kept at 37 °C for four weeks. Subsequent work-up demonstrated the presence of radioactive **1** and **2.** Hence it can be concluded that **1** and **2** are formed by (most probably) non-enzymatic condensation reactions of tryptophane with transformation products of L-ascorbic acid in the fruit (or at the latest after destruction of the cell walls during pressing) and that this process continues afterwards (*cf.* the results with soy sauce mentioned above).

To test the reaction of L-tryptophane with L-ascorbic acid we repeated the experiment of Kanamori (1980) both at pH 7.0 (0.1 M phosphate buffer) and (to be closer to the conditions in the currants juice) at pH 2.5 (0.1 M citrate buffer) (37 °C, 4 weeks). In both cases only **1** was obtained and neither **2** nor **3** could be detected (note that Kanamori also obtained **1** and its decarboxylation product **5**). The formation of **1** in this experiment is readily understandable: In acidic media ascorbic acid is degraded *i.a.* to furfural (Tokuyama *et al.*, 1971) which in turn reacts with tryptophane to give furyl-tetrahydro-β-carboline (Kanamori *et al.*, 1984) which can be dehydrogenated easily to the fully aromatic system. Also a reaction with furan-2-carboxylic acid is conceivable giving dihydro-β-carboline. Furan-2-carboxylic acid is formed *i.a.* by acid treatment of dehydroascorbic acid (v. Euler and Hasselquist, 1956) present in the reaction product mixture of ascorbic acid with amino acids (Kanamori *et al.*, 1980).

O-Methylflazin (**2**) as well as flazin (**3**) and its decarboxylation product perlolyrin (**4**) have been found only in plant extracts and preparations from those *(v. supra)*. **3** and **4** can be considered as the condensation products of tryptophane (**4** with subsequent decarboxylation) with 5-hydroxymethyl-furan-2-carboxylic acid, a dehydration/cyclization product of dihydroascorbic acid or of 2-desoxy-ascorbic acid (shown to be present in the reaction product mixture of ascorbic acid with amino acids (Kanamori *et al.*, 1980)). The methylation step leading to **2** must, however, be due to an O-methyltransferase-catalyzed reaction leading to 6-O-methylascorbic acid or to one of its accordingly methylated transformation products.

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Experimental

Spectroscopy

The ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AM 300 spectrometer operated at 300 MHz and 75.5 MHz. Chemical shifts are given on the δ (ppm) scale with TMS as internal standard. The ^{1}H,^{1}H-COSY experiments were effected with 32 scans/experiment and calculated with a sine function shifted by π/2 (Aue *et al.*, 1976). For the inverse ^{1}H,^{13}C correlations for **1** and **2** HMQC experiments were performed with GARP decoupling (Bax *et al.*, 1983). The frequency range was

3086 and 2703 Hz, resp. for F_2 (2 k data points) and 4902 and 6039 Hz, resp. for F_1, 256 measurements with 16 scans each in t_1; calculations in both directions as above. For the inverse $^1H,^{13}C$ long-range correlations undecoupled HMBC experiments were used (Bax and Summers, 1986). The frequency range was 3086 and 2994 Hz, resp. for F_2 (2 k data points) and 5285 and 7163 Hz, resp. in F_2. The delay for developing the long-range couplings was varied between 50 and 80 ms, 512 measurements with 16 scans each in t_1, after zero-filling for 1024 data points calculation was performed using a sine function shifted by $\pi/2$ in both directions.

Mass spectra were obtained with a Finnigan MAT INCOS 50 XL, the exact mass measurements (deviations from calculated values < 1 mmu with a Finnigan MAT 8230 instrument. Column chromatography was carried out on silica gel 60 (70–230 mesh, ASTM, Merck, Darmstadt), analytical and preparative HPLC on a Waters model 6000 A with a U6K injector using Nucleosil 100-C_{18} (5 μm, Macherey & Nagel, Düren) with a flow rate of 0.8 and 10 ml/min, TLC on micropolyamid F 1700 (50 μm, Schleicher & Schüll, Dassel).

Isolation of **1** *and* **2**

24 kg of 6.6-fold concentrated juice of *Ribes nigrum* L. (Haarmann & Reimer, Holzminden) were exhaustively extracted with diethyl ether in a Soxhlet-type apparatus. The extract was dried over $MgSO_4$, filtered, and the ether removed with a rotatory evaporator. The oily residue (180 g) was chromatographed on silica gel columns (4×150 cm) using hexane/acetone (40:60) and the first yellow eluate was collected. After evaporation of the solvent the residue (12 g) was separated by RP-HPLC (column: 250×10 mm) with methanol/0.1 N acetic acid (60:40). The fraction eluating after 14 min was collected, freed from the solvent (0.9 g) and separated by analytical RP-HPLC (column 4×250 mm) with acetonitrile/0.1 N acetic acid (40:60) to get the crude fractions of **1** (15 mg, R_f = 19.4 min) and **2** (14 mg, R_f = 21.8 min). Both of them were further purified by TLC on micropolyamide with methanol/2-butanone (60:40). The bands with R_f values 0.60 (**1**) and 0.68 (**2**) were scraped off and extracted with dimethyl formamide. The solvent was removed and the residues were rechromatographed by analytical RP-HPLC. Yield 3.8 mg of **1** and 3.4 mg of **2.**

Work-up of fresh fruit

2.5 kg deep-frozen black currants (Mainfrucht Obstverwertung GmbH, Gorchsheim) were warmed to room temperature and pressed, seeds and cell material was removed by repeated sifting and centrifugation (yield 1.8 l). One half of the juice was extracted immediately with ether, worked up and chromatographed as described above (yield 1.2 g). The presence of **1** and **2** was shown by co-injection with authentic material.

^{14}C Experiments

To the other half of the juice of the fresh fruits 1.85 MBq (0.18 GBq/mmol) [L-1-^{14}C]ascorbic acid (DuPont, NEN Division, Bad Homburg) were added. After incubation for two months at 37 °C the juice was extracted with ether (see above) and the ether removed at 40 °C. The oily residue was dissolved in 1 ml acetonitrile/0.1 N acetic acid (2:3) and separated in 2.5 μl portions by isocratic HPLC (column: 250×4 mm) with the same solvent. Fractions were collected every 30 sec. After 10 runs fractions with identical retention times were combined, 12 ml scintillation cocktail Quicksafe A (Zinser, Frankfurt) was added and the radioactivity was determined with a scintillation counter (Beckmann-Counter LS 5000 TD). The fractions with R_f = 25 and 28 min were found to exhibit increased radioactivity. These retention times correspond to those of **1** and **2** under identical chromatographic conditions.

Reaction of tryptophane with ascorbic acid

As described by Kanamori *et al.* (1980, 1981) 117.4 g (0.67 mol) L-ascorbic acid and 17.0 g (0.08 mol) L-tryptophane in 0.1 M phosphate buffer (pH 7.2) or 0.1 M citrate buffer (pH 2.5) were kept for two months at 37 °C. The reaction mixture was extracted with ether for 48 h, the extract was dried with $MgSO_4$ and freed from the solvent *i.v.* The main product (**1**) was isolated by RP-HPLC and identified by EI-MS, ^1H and ^{13}C NMR (*cf.* above). No other β-carboline derivatives could be detected by spectroscopic means.

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