150. Cyclodopa Glucoside (= (2*S*)-5-(β-D-Glucopyranosyloxy)-6-hydroxyindoline-2-carboxylic Acid) and its Occurrence in Red Beet (*Beta vulgaris* var. *rubra* L.)

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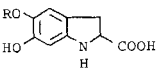
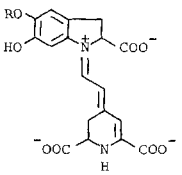
Summary

Cyclodopa glucoside (= (2*S*)-5-(β-D-glucopyranosyloxy)-6-hydroxyindoline-2-carboxylic acid; **1a**) is prepared from betanin (**2a**) *via* a base exchange reaction. Its purification and physical properties are described. The compound is *N*-formylated in dilute HCOOH solution. Cyclodopa glucoside is contained in considerable amount in red-beet juice varying from 0.07 to 1.9 mmol/kg or from 12 to 46% relative to the content of betanin. The occurrence of free cyclodopa glucoside supports its role as intermediate in betanin biosynthesis.

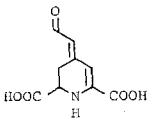
Introduction. – Cyclodopa glucoside (**1a**), has only been noticed as constituent part of betanin (**2a**) [1], the pigment of red beet (*Beta vulgaris* var. *rubra* L.) where it is bound as immonium salt to the chromophoric moiety, betalamic acid (**3**) [2]. Betanin is the best known representative of the red-violet pigments named betacyanins [3]; together with the yellow betaxanthins, they form a group of pigments named betalains occurring in a distinct group of plant families of the order *Centrospermae* [4].



1a R = Glucopyranosyl (Glc)
1b R = H



2a R = Glc
2b R = H



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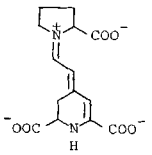

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The formation of cyclodopa glucoside (**1a**) appears to be specifically related to that of betacyanins. We proposed that both parts of betanin, **1a** and **3** derive from the same precursor, dopa. Experiments showed good incorporation into the betalamic-acid but a rather poor one into the cyclodopa-glucoside moiety [5]. Concerning cyclodopa formation, it is known that *in-vitro* oxidation of dopa leads to dopaquinone which at neutral pH spontaneously cyclizes to cyclodopa (**1b**); this, however, immediately undergoes further oxidation to dopachrome [6]. It is still open to question whether in biosynthesis intermediary formed cyclodopa is intercepted and glucosylated.

Cyclodopa (**1b**) was identified as a product of alkaline or strong-acid degradation of betanidin (**2b**) [7]. Its glucoside **1a** has not been thoroughly described but mentioned

occasionally. *Sciuto, Oriente & Piattelli* [8] have obtained it in solution by cleavage of betanin (**2a**) with SO_2 and characterized it by transformation into the crystalline hexa-*O*-acetyl methyl ester. We have isolated **1a** after cleavage of betanin (**2a**) in some biosynthetic studies [5]. In the present paper we give a detailed description of cyclodopa glucoside (**1a**) and report on its occurrence in red beet.

Results and Discussion. – *Cyclodopa Glucoside* (**1a**) *from Betanin* (**2a**). The preparation of **1a** is based on an earlier procedure in which the cyclodopa moiety of betanin (**2a**) was substituted by another amine or amino acid in presence of dilute NH_3 (see *e.g.* the synthesis of indicaxanthine (**4**), using proline [9] [10]). This base-exchange reaction has been applied since to interconvert betalains [11].

From an acidified mixture containing **2a**, **4**, and proline we have now isolated **1a**, after repeated chromatography on a *Sephadex SP C25* cation exchanger (see *Exper. Part*). Pure **1a** was eluted after the betalains **2a** and **4** and crystallized slowly from a chilled concentrate (m.p. 159.5–162.5°).

On titration of a $4.6 \cdot 10^{-2}$M solution of the trifluoroacetate of **1a**, three steps are discerned of pK_a 2.7, 5.1, and 9.7; these account, respectively, for the carboxyl/carbox-

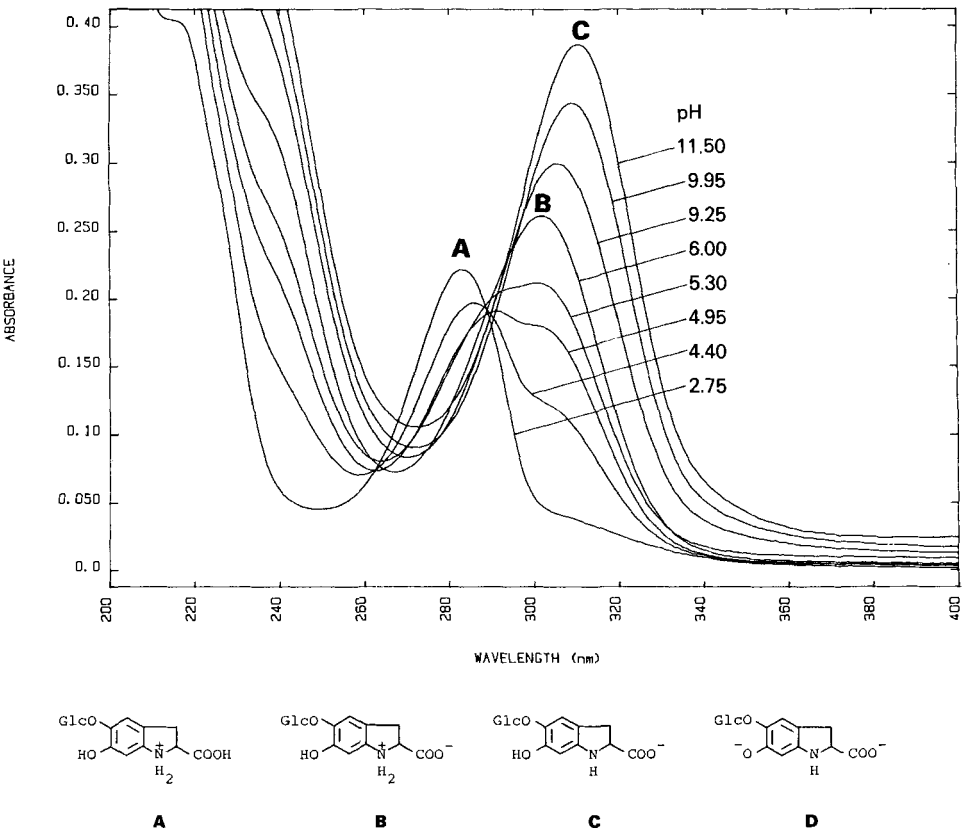


Fig. 1. *UV-spectra of cyclodopa glucoside at different pH's*

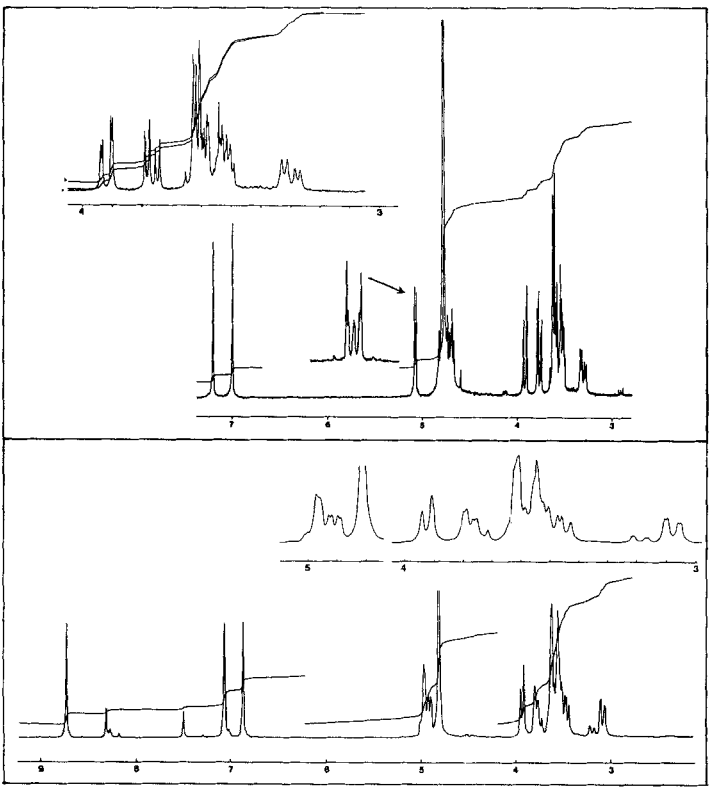


Fig. 2. *^{1}H-NMR spectra (D_2O) of cyclodopa glucoside* (**1a**) (top) *and of its* N-*formylated derivative* **5** (bottom)

ylate, indolinium/indoline, and catechol/catecholate equilibria. Correspondingly, **1a** is readily distinguished from the common proteic amino acids; having an isoelectric point of *ca.* 3.9, **1a** takes a place between the acidic and the neutral amino acids and separates clearly from these on paper electrophoresis as well as in ion-exchange chromatography.

The UV spectrum changes with pH in agreement with the protonation stages **A–D** of **1a** (see *Fig. 1*). This allowed the determination of pK_{a1} 1.58, pK_{a2} 4.75, and pK_{a3} 9.42 (*c* (**1a**) = $7.7 \cdot 10^{-5}$ mol/l).

Below pH 3.8, only a single absorption band appears at 283 nm (ε = 3400) corresponding to the indolinium stage **B**; from pH 3.8 to pH 7.4 there is a gradual change to the absorption spectrum of the indoline form **C** with infl. at 235 (ε = 5100) and λ_{max} 302 nm (ε = 3940); finally, with increasing alkalinity, the catecholate form **D** is reached absorbing at 310 nm (ε = 5000); at low pH's, the missing step leading to the fully protonated species **A** is indicated by a smooth change of the extinction at λ_{max} 217 nm, whereas the maximum at 283 remains unaffected.

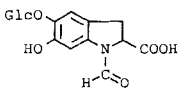
The ^{1}H-NMR signals (CF_3COOH) of the cyclodopa part of **1a** are nearly identical to those reported for the aglucone **1b** [6], except for H–C(4) in *ortho*-position to the glucosyloxy rest which is deshielded by 0.24 ppm *(Table 1)*. As expected, the δ's in CF_3COOH solution are shifted to lower field compared to those observed in D_2O solution.

Table 1. *Chemical Shifts and Coupling Constants in the NMR Spectra of* **1a** *and* **5**

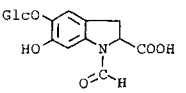
	Cyclodopa glucoside (**1a**)		*N*-Formylcyclodopa glucoside (**5**) in D_2O		*β*-D-Glucopyranose [12] in D_2O
	in CF_3COOH	in D_2O	(*E*) 61%	(*Z*) 39%	
H–C(2)	5.34 (*dd*, $^3J(2,3\alpha) = 9.5$)	4.68 (*dd*, $^3J(2,3\alpha) = 9.7$)	4.91 (*dd*, $^3J(2,3\alpha) = 11.5$)	4.99	
H_β–C(3)	3.61 (*dd*, $^3J(2,3\beta) = 6$)	3.30 (*dd*, $^3J(2,3\beta) = 6.4$)	3.08 (*dd*, $^3J(2,3\beta) = 3.9$)	3.19	
H_α–C(3)	3.89 (*dd*, $^2J(3\alpha,3\beta) = 16.5$)	3.53 (*ddd*, $^2J(3\alpha,3\beta) = 16.1$, $^4J(3\alpha,4) = 1.9$)	3.46 (*dd*, $^2J(3\alpha,3\beta) = 16.2$)	3.52	
H–C(4)	7.29	6.99 (*s*)	7.06 (*s*)	7.06 (*s*)	
H–C(7)	7.34	7.19 (*s*)	6.86 (*s*)	7.50 (*s*)	
HC=O			8.73 (*s*)	8.31 (*s*)	
H–C(1′)	5.17 (*d*, J(1′,2′) = 6)	5.07 (*m*)	4.96 (*m*)	4.99 (*m*)	4.65 (*d*)
H–C(2′)		3.57–3.66 (*m*)	3.5–3.7 (*m*)		3.27 (*dd*)
H–C(3′)		3.57–3.66 (*m*)			3.45 (*dd*)
H–C(4′)	4.17 (*m*)	3.49–3.54 (*m*)			3.35 (*dd*)
H–C(5′)		3.57–3.66 (*m*)			3.42 (*ddd*)
H_A–C(6′)		3.92 (*dd*, $^3J(6'A,5') = 2.3$, $^2J(6'A,6'B) = -12.4$)	3.93 (*dd*, $^3J(6'A,5') < 2$, $^2J(6'A,6'B) = -12.3$)		3.89 (*dd*)
H_B–C(6′)		3.76 (*dd*, $^3J(6'B,5') = 5.3$)	3.77 (*dd*, $^3J(6'B,5') = 4.2$)		3.72 (*dd*)

Comparing the ^{1}H-NMR shifts (D_2O) of the glucoside moiety of **1a** to those of β-D-glucopyranose [12], increased deshielding is observed the closer the protons are situated with respect to the phenolic ring, most affected being the anomeric proton H–C(1') as well as H–C(2') and H–C(5'). The signals of H–C(2'), H–C(3'), and H–C(4'), therefore, overlap in a complex *m*, and the anomeric proton appears in virtual coupling as an 8-line *m* (see enlarged section in *Fig. 2*, top)[1]).

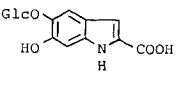
N-*Formylcyclodopa Glucoside* (**5**). For the *Sephadex* chromatography of **1a**, we used diluted HCOOH as eluant. After slow concentration and on standing of these solutions of **1a**, the single UV absorption of **1a** is superimposed by absorptions at 255 and 305 nm arising from *N*-formylcyclodopa glucoside **5**. Air exposed solutions of **1a** in HCOOH contain besides **5** another product which most probably is the glucoside **6** of 5,6-dihydroxyindol-2-carboxylic acid. *N*-Formylcyclodopa glucoside (**5**) can be easily separated on *Sephadex SP C25* from the much slower migrating **1a**, whereas **6** partially overlaps with **5**.



5 *(E)*



5 *(Z)*



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The new compounds **5** and **6** have characteristic UV spectra, and the structure of the *N*-formyl derivative **5** is confirmed by the ^{1}H-NMR spectrum *(Fig. 2, Table 1)*.

The formyl derivative **5** exhibits two maxima at 253 (11100) and 307 (10500) nm analogous to other *N*-acylcyclodopa derivatives [6], and the spectrum of the indol **6** is very similar to that of the parent substance λ_{max} 315, infl. 255 nm). Two rotamers of **5** are distinguished from splitted ^{1}H-NMR signals for CHO and H–C(7): in the prevalent (*E*)-form (61%), the CHO signal is at lower field than that of the (*Z*)-form due to the influence of the benzene ring, and inversely, H–C(7) is at lower field in the (*Z*)-form since deshielded by the CHO group. At high resolution, slight shifts of the signals of H–C(2) and 2H–C(3) are revealed: H–C(2) is a little more shielded in the (*E*)- than in the (*Z*)-form, and the 2H–C(3), inversely, are more deshielded; the reasons for these shifts are not obvious.

Formylation in dilute HCOOH solutions has not been noticed before; normally, mixtures of HCOOH and Ac_2O giving mixed anhydride are used as acylating agent. We have treated indoline in dilute HCOOH and obtained the *N*-formyl derivative which was prepared before by treatment with HCOOH in toluene with azeotropic distillation [15]. The formylation reaction may be rationalized considering the low pK_a (about 5) of the indolinium ion; at moderate acidity, the conjugate base adds as nucleophile to undissociated HCOOH leading to the kinetically stable amide.

Cyclodopa Glucoside (**1a**) *in Red Beet*. Hitherto, **1a** has not been noticed in red-beet juice since its concentration may be too low to be detected directly (*e.g.* on paper electrophoresis) in presence of the prevailing other ingredients. The occurrence of **1a** as artifact during isolation could be precluded by instantaneous acidification of the red-beet juice blocking thus enzymatic intervention. To isolate and quantify the compound, we used chromatography on *Sephadex SP C25* as well as HPLC.

Purification by ion-exchange chromatography is performed in two steps. After removal of sugars and betalains at pH 3, the comparatively small amounts of **1a** elute still superimposed with the trail of the colored

[1]) An analogous experience was encountered with other glucosides by *A. de Bruyn*, University of Gent, Belgium (personal communication).

products. Complete purification necessary for quantitative spectroscopic determination is achieved in a second chromatography at pH 3.5. Direct quantization is feasable by reverse-phase HPLC with 0.05M sodium dihydrogenphosphate (pH 4.5); under these conditions, **1a** elutes shortly after dopa and is followed by tyrosine, whereas other compounds, in particular betalains, are more retained and may be retrieved using a gradient with increasing part of MeOH; quantitative determination is obtained by UV spectroscopy.

The discovery of free cyclodopa glucoside (**1a**) in the plant shows above all that this compound is an intermediate in biosynthesis which has to be taken into account in investigations. The amount of **1a** present in beet root varies with age: In a young (45 days old) plant, 1.9 μmol/g f.w. (g fresh weight) were found, a mature, stored beet root contained only 0.07 μmol/g f.w. These data become more significant if compared to the amount of total betacyanins (*i.e.* betanin and prebetanin): the content of **1a** represents up to 46% of total betacyanins (4.1 μmol/g f.w.) in the young root and only 12% (0.58 μmol/g f.w.) in old species. The occurrence of **1a** in such amounts is of considerable interest in biosynthetic studies during the development of the plant.

We thank Prof. Dr. *J. Lauterwein* for his help in the determination and interpretation of NMR spectra. We acknowledge financial support from the *Swiss National Science Foundation.*

Experimental Part

General. UV spectra: *Unicam SP1800; Hewlett-Packard 8450 A* diode array spectrophotometer; *Cecil 202.* ^{1}H-NMR spectra: *Bruker WP 80* and *WH 360.* MS: *Finnigan.* Titration: Radiometer.

Cyclodopa Glucoside (**1a**) *from Betanin* (**2a**). Degassed 0.6N NH_3 (25 ml) was distilled in a closed evacuated system by gentle heating and condensed by cooling into a mixture of betanin[2] (**2a**; 214 mg, 72% pure; 0.28 mmol) and excess proline (349 mg, 3 mmol). After 1 h at r.t., the solvent was removed in the same way. The residue was dissolved in 3 ml of 0.07M AcOH (pH 3) and chromatographed with the same solvent on *Sephadex SP C25* (H^+-form, 31 × 3 cm) monitoring the elution by UV at 284 nm. Following the forerunnings (68 ml), an orange zone containing neobetanin was eluted (164 ml; 0.09 mmol, calc. on the base of ε(469 nm) = 18200)[3]. The column now displayed the following separation pattern (noticed from bottom to top): rose (8 cm), violet (4 cm, **2a**), dark yellow (13 cm, indicaxanthin (**4**)), orange (0.5 cm) and faint yellow (4 cm). Accordingly, the following fraction sequence was collected: red-violet (240 ml; **2a**, 0.046 mmol); yellow (435 ml; **4**, 0.135 mmol); faint yellow to colorless (716 ml); colorless (425 ml; **1a**, 0.2 mmol). Some weakly colored zones remained on the column: rose (2 cm), yellowish (9 cm), rose (11 cm), brownish (9 cm). *Cyclodopa glucoside* (**1a**): m.p. 159.5–162.5° (browning > 167°), $[\alpha]_D^{20.5°} = -110.7°$ (H_2O, pH 3; c = 4.65 mg/ml). Paper electrophoresis: *Eb* values (migration relative to that of **2a**) −0.27 at pH 2.4, +0.09 at pH 4.5. Paper chromatography (BuOH/AcOH/H_2O 4:1:5): R_f 0.09. Detection with 0.1% aq. sodium metaperiodate followed by benzidine (1.8 g in EtOH/H_2O/acetone/0.2N HCl 50:50:20:10) [14] or diazotized 4-nitro-*O*-anisidine [8] UV (H_2O, pH 3): 284 (3400). UV (H_2O, pH 11): 311 (5000). IR (KBr; band intensity *s* unless stated otherwise): 3540, 3470, 3185, 3230, 3160, 2980, 2950, 2935, 2920, 2885, 2865, 2580*m*, 2500*m*, 1643, 1628, 1600, 1505, 1460, 1450, 1415, 1395, 1380–1370, 1350, 1328, 1295, 1273, 1255, 1230, 1208, 1191, 1661, 1140, 1131, 1121*m*, 1108, 1090, 1068, 1058, 1050, 1032, 1022, 1015, 1008, 990*m*, 943*m*, 912*w*, 900*m*, 865, 850, 830, 785, 755, 713, 698–90, 665, 629, 611. ^{1}H-NMR: see *Table 1, Fig. 2.* MS: 357 (trace), 274 (0.6), 252 (0.6), 223 (2), 194 (11, $C_6H_{11}O_5^+$), 177 (1), 150 (5), 149 (4.5), 98 (9), 81 (16), 71 (100), 69 (23), 68 (23).

Determination of pK_a *by UV.* The UV (*Hewlett Packard* diode array spectrophotometer) were measured in 2 series: 1. Aliquots of **1a** in H_2O were added to aq. HCl and spectra immediately taken; the pH of the solutions was measured thereafter; 2. A solution of **1a** in H_2O (pH 2.75) was gradually alkalinized while circulating through a flow cell with the aid of a peristaltic pump. After pH readings spectra were recorded. Results are noted in *Table 2* (see also *Fig. 1*).

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[2] Isolated from red beet and partially purified by precipitation [13].

[3] Neobetanin (= 14,15-dehydrobetanin) is usually present in aged betanin samples.

Table 2. *UV Measurements of* **1a** *at Different pH*

pH	λ_{max}	*OD*	*OD* 283 nm	*OD* 302 nm	*OD* 310 nm
0	218	0.792	0.49	–	–
0.5	217	0.777	0.49		
1.0	217	0.783	0.492		
1.35	217	0.793	0.494		
2.0	217	0.834	0.505		
2.35	217	0.837	0.499		
2.75	283	0.222	0.222	0.047	0.037
3.40	283	0.223	0.223	0.056	0.045
3.95	284	0.217	0.216	0.072	0.059
4.40	286	0.197	0.192	0.125	0.104
4.95	292	0.190	0.167	0.179	0.150
5.30	301	0.212	0.166	0.212	0.179
5.70	302	0.245	0.145	0.245	0.206
6.00	302	0.261	0.140	0.261	0.221
6.40	302	0.269	0.135	0.269	0.228
6.90	302	0.274	0.134	0.274	0.233
7.85	302	0.276	0.135	0.276	0.235
8.50	303	0.281	0.133	0.280	0.249
8.80	304	0.285	0.132	0.283	0.259
9.25	305	0.299	0.128	0.291	0.287
9.50	308	0.316	0.125	0.298	0.311
9.95	309	0.344	0.122	0.308	0.343
10.35	310	0.368	0.124	0.317	0.368
11.50	311	0.387	0.134	0.330	0.386
12.05	311	0.334	0.138	0.334	0.392

Titration. The NMR sample in CF_3COOH was evaporated and redissolved in 5.0 ml of H_2O. Titration with 0.05N NaOH needed for step 1 (equiv. point pH 3.85) 1.22 ml, for step 2 (equivalence point pH 7.4) 0.63 ml and for step 3 (equiv. point pH 10.8) 0.70 ml. The pK_{a1} value 2.7 was evaluated after deduction of the approx. amount of base necessary to compensate still contained excess acid (0.56 ml); pK_{a2} was found 5.1, pK_{a3} 9.7.

Isolation of **1a** *from Red Beet.* Slices of beet root (264 g) were disintegrated in a centrifugal juice mill and the strained juice of pH 5.6 (130 ml) was led directly into HCOOH (1.5 ml) to give a final solution of pH 2.9; this was further acidified to pH 2.7. A fine, dark precipitate formed and was eliminated by centrifugation and filtration of the supernatant. The pigment content was determined spectrophotometrically (1 ml of sample diluted in 250 ml of H_2O). Total betacyanin: 1.17 µmol/ml of juice (betanin/prebetanin; λ_{max} 538 nm, $\varepsilon = 60000$); total betaxanthin: 0.97 µmol/ml of juice (vulgaxanthins; λ_{max} 478 nm, $\varepsilon = 42000$; the betaxanthin content was evaluated in deducing the contribution of betacyanin from the absorption at 478 nm which is 0.325 of that at 538 nm; the spectrum of the extract showed, however, an apparent λ_{max} 484 nm). In the spectrum of fresh juice, an important λ_{max} is seen at 266 nm which decayed in the aging solution to about 1/3 and a sh appeared at 300 nm.

Of this extract, 50 ml were separated on *Sephadex SP C25* (H^+-form, 3 × 30 cm) using 0.009M HCOOH of pH 2.7 and taking fractions of 20 ml: forerunnings (168 ml); violet (63 ml); red and yellow (20 ml); yellow, turning reddish within 10 h (64 ml; λ_{max} 421 and 530 nm); yellow (399 ml); red-violet, betanin main zone (400 ml; λ_{max} 538 nm); red and yellow (252 ml); weak colored, containing **1a** (793 ml); faintly colored (200 ml). All the fractions were tested on paper electrophoresis (pH 2.4) before being collected. The fractions between ml 168 and 315 contained sugars as detected with *Partridge*'s reagent. The fraction of **1a** was purified in a second chromatography on *Sephadex SP C25* (3 × 18 cm) conditioned at pH 3.5. Colored bands migrated in the order: orange, violet, yellow, and rose. The fractions eluted in the order: colorless (43 ml); orange (21 ml); faint yellow (43 ml; λ_{max} 254, 308, min. 236, 278 nm; *N*-formylcyclodopa glucoside (**5**), 0.0021 mmol); red-violet (43 ml; λ_{max} 238 nm); yellow (43 ml; λ_{max} 474 nm; betaxanthin-type UV/VIS); faint yellow (139 ml); colorless (15 ml); colorless (290 ml; **1a**, 0.0052 mmol).

Purification of an Aged Solution of Cyclodopa Glucoside. A HCOOH-containing solution of 1a (68 mg) which had been stored for 3 months in a refrigerator was chromatographed on *Sephadex Sp C25* (15 × 3 cm) at pH 3.5. Following the forerunnings (43 ml), 2 sharp bands appeared (monitored at 284 nm) comprising each 43 ml and containing **5** and **6**, resp.; a large empty part followed (280 ml), then a weakly rose band (86 ml) before **1a** was eluted (450 ml). *N-Formyl cyclodopa glucoside (= (2S)-1-formyl-5-(β-D-glucopyranosyloxy)-6-hydroxyindoline-2-carboxylic acid;* **5**): Paper electrophoresis: E_b values (migration relative to that of **2a**) +0.5 at pH 2.4 (0.1M HCOOH), +0.75 at pH 4.5 (0.05M pyridinium formiate). Paper chromatography (BuOH/AcOH/H_2O 4:1:5): R_f 0.20. UV (H_2O): 253 (11100), 307 (10500); min. 232 (6500), 276 (2400). UV (H_2O/2 drops of 0.1N NaOH): 255 (sh, 8800), 324 (10200); min. 286 (1800) ^{1}H-NMR: *Table 1, Fig. 2.*

5-(β-D-Glucopyranosyloxy)-6-hydroxyindol-2-carboxylic Acid (**6**). The identity of this compound was concluded from the characteristics of its UV, very similar to that of the parent aglucone. UV (MeOH, neutral): 251 (0.165), 312 (0.312); min. 240 (0.160), 271 (0.11). UV (MeOH, alkaline): 264-5 (0.15), 331 (0.32); min. 252 (0.095), 284 (0.11). UV (H_2O, neutral): 310 (0.42); infl. 246 (0.23); min. 268 (0.14). UV (H_2O, alkaline): 326 (0.40); infl. 252 (0.24); min. 280 (0.12).

Formylation of Indoline. Indoline (1.8 g) was dissolved in a mixture of H_2O (5 ml) and HCOOH (1.8 g). After 2 h at r.t., H_2O was added (20 ml) and the solution extracted with Et_2O. The Et_2O extract was washed with dil. HCl, $NaHCO_3$, and brine, dried over Na_2SO_4, and evaporated: 0.9 g of *N*-formylindoline, m.p. 55–60°; recrystallized from CH_2Cl_2/hexane, m.p. 63–64° ([15]: 62–63°).

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