13. 2,3- and 4,5-Secodopa, the Biosynthetic Intermediates Generated from L-Dopa by an Enzyme System Extracted from the Fly Agaric, *Amanita muscaria* L., and Their Spontaneous Conversion to Muscaflavin and Betalamic Acid, Respectively, and Betalains

by **Fernand Terradas**[1]) and **Hugo Wyler***

Institut de chimie organique, Université de Lausanne, 2, rue de la Barre, CH–1005 Lausanne

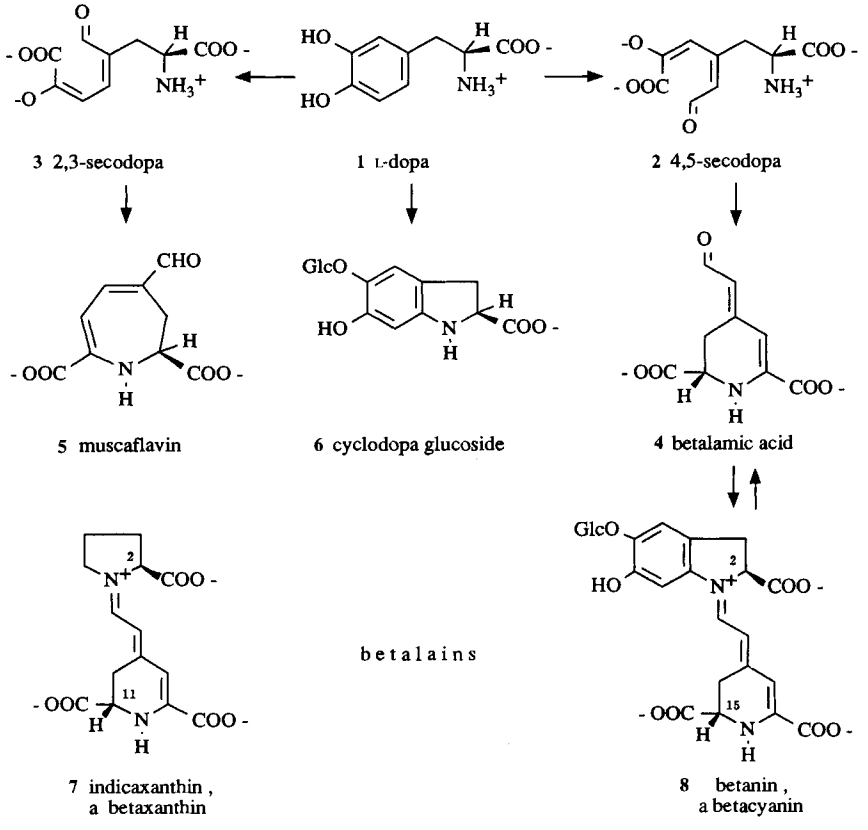
(5.X.90)

An enzyme is extracted from the red peel of *Amanita muscaria* which cleaves the C(2)–C(3) and the C(4)–C(5) bond of the aromatic ring of L-dopa (**1**) to form a mixture of 4,5-secodopa (= salt of 6-amino-2-hydroxy-4-(2′-oxoethylidene)hept-2-enedioic acid; **2**) and 2,3-secodopa (= salt of 7-amino-5-formyl-2-hydroxyocta-2,4-dienedioic acid; **3**), two hitherto hypothetical biosynthetic intermediates (see *Scheme*). Though isolation of these products has not been possible, structural evidence is inferred from reaction products, kinetics, and spectroscopical characteristics in comparison with known compounds. Secodopas **2** and **3** are characterized in dilute solution by HPLC and UV/VIS spectroscopy (anions: λ_{max} 424 and 414 nm, resp., $\varepsilon_{420} = 25\,500$; on acidification, shift to 380 and 372 nm, resp.). They cyclize without enzyme catalysis, optimally at pH 4.5–5; **3** produces muscaflavin (**5**) and **2** betalamic acid (**4**). The products are identified by direct comparison with authentic samples in HPLC, by ^{1}H-NMR of **5**, and by condensation of **4** with L-proline to form the well known betalain indicaxanthin (**7**). The enzymatic conversion of L-dopa (**1**) *via* **2** to betalamic acid (**4**; (*S*)) and its condensation with L-proline leads to pure natural indicaxanthin (**7**; (2*S*,11*S*)); correspondingly, the enzymatic conversion of D-dopa to (*R*)-betalamic acid and its condensation with L-proline produces isoindicaxanthin ((2*S*,11*R*)) which is unknown in nature. Particularly relevant is the fact that the same enzyme cleaves pyrocatechol to produce a solution of the enolate form of the known 2-hydroxy-6-oxohexa-2,4-dienoate (secopyrocatechol; **9**; see *Fig. 5*). Dissociation constants of the corresponding enolic functions in the cleavage products are determined by spectrometric titration and compared to those of known systems.

Introduction. – In the early sixties, it was realized that the vacuolary coloring system of plants is not entirely supported by anthocyanins as presumed previously. A group of families of the order *Centrospermae* uses a special palette of colorations covered by the red-violet betacyanins and the yellow betaxanthins [1a]. The originality of this choice lies in the fact that betacyanins and betaxanthins, coined betalains as a class [1b–f], are built by the same principle, the condensation of a merocyanin, betalamic acid (**4**), with amino acids to form cyanin dyes (*Scheme*). Typical representatives are the red-violet betanin (**8**) [2] of beetroot containing the unusual indoline amino acid cyclodopa glucoside (**6**) [3], and the yellow indicaxanthin (**7**) of cactus flowers or fruits containing proline [4]. Betalamic acid (**4**) has been found also free [5a] in association with betaxanthins [5b]. Surprising in this context is the fact that betaxanthins, considered taxonomically characteristic for the mentioned plant families, are produced as well in the isolated case of some fungi of the *Agaricales*, in particular in the toadstool fly agaric, *Amanita muscaria* [6].

[1]) Part of the Ph. D. thesis of *F.T.*, Université de Lausanne, 1989.

Scheme. *Biosynthetic Pathways from* L-*Dopa*. The metabolites are shown in the form prevailing in neutral medium.



A specially attractive chapter has been the biosynthetic aspect of the betalains. It was suggested [7] that betalamic acid (**4**) arises from L-dopa (= 3-hydroxy-L-tyrosine; **1**) by cleavage of the C(4)–C(5) bond of the aromatic ring to form an intermediate which by cyclization leads to **4** (*Scheme*), and that the formation of cyclodopa started as well from **1**. The proposed pathway received experimental support from incorporation studies in red beet and cactus fruits [8].

The presumed cleavage product **2** of dopa which we propose to name 4,5-secodopa[2]) has not yet been noticed in the plants. A precedent of this type of enzymatic ring cleavage is the action of enzymes from strains of *Pseudomonas* on simple catechols as shown by *Dagley* and *Hayaishi* [9] [10]; the cleavage was found to take place at the bond adjacent to the diol group, called the 'extradiol' position, and the enzymes have been classified as metapyrocatechases. Corresponding enzymes in betalain-producing plants have not yet been seized.

It appeared promising to extend the biosynthetic investigation on the formation of betalains to the fly agaric *Amanita muscaria*, especially in view of the occurrence there of

[2]) The prefix 'seco' has been applied in steroid nomenclature in a different sense, as defined by IUPAC rule S-7.4. The use of 'secodopa' as a trivial name has some precedent in the field of alkaloids, however.

an isomer of betalamic acid (**4**), muscaflavin [6a] (**5**); this compound is assumed to be the cyclisation product of 2,3-secodopa (**3**), another possible 'extradiol'-cleavage product of L-dopa (**1**). Muscaflavin may serve as additional argument in support of the biosynthetic hypothesis and has been looked for in *Centrospermae* plants without success [5b]. We noticed that red or yellow tissue parts from the cap of the fly agaric incubated with **1** at pH 7 developed yellow solutions absorbing near 420 nm; these solutions contained effectively, among other products, **4** and **5** offering the unique occasion to undertake this investigation which led us to the discovery of the intermediate secodopas.

Results. – *Enzyme Preparation and Activity.* The red epidermis peeled off the cap of *Amanita muscaria* is ground under liquid N_2, and the enzymes are extracted from the powder at 4° and pH 8. Some inactive protein part is removed by precipitation with 30% acetone/H_2O; the active enzyme is precipitated in a second fraction increasing the acetone part to 60%. Further purification steps have not been achieved; because of its instability, the enzyme preparation has been used freshly. The enzymatic activity in incubation tests with L-dopa (**1**) at pH 8 is measured from the initial linear increase of absorbance at 420 nm of the developing yellow solution. The enzyme remains well conserved in the frozen untreated tissue at −30°. But an extracted preparation left at 25° looses within 1 h *ca.* 20% of its activity. Loss of activity may be limited at 4° to 1% per h, and preparative assays are, therefore, performed under this condition.

The enzyme is remarkably active towards some other catecholic substrates. Comparing the initial rates of enzymatic action to that on L-dopa taken as 1.0, the relative rates of other substrates are for D-dopa 0.62, α-methyldopa 1.12, dopamine 1.69, *N*-acetyldopamine 0.13, pyrocatechol 0.19, and 3,4-(dihydroxyphenyl)acetate 0.04; the enzyme is inactive among others towards 3-(3,4-dihydroxyphenyl)propionate.

Products of Enzymatic Action on L-*Dopa.* Four products **2**–**5** are formed in the yellow solution of the enzymatic assay with **1** which are separable by HPLC on reversed-phase *ODS Hypersyl* with a gradient of phosphate buffer pH 6 and MeOH (detection at 420

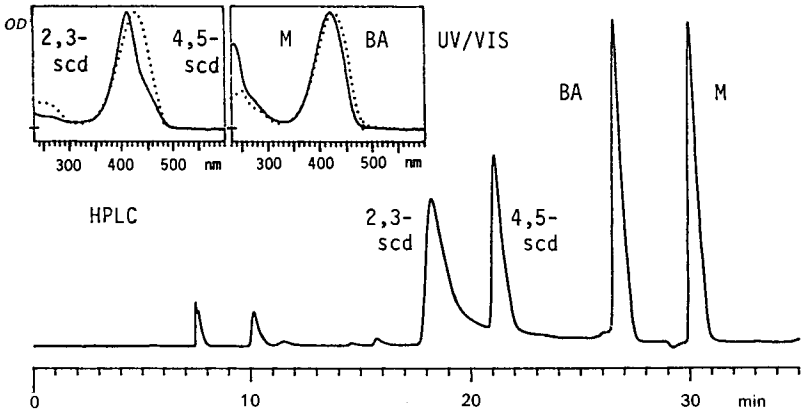


Fig. 1. *HPLC and UV/VIS spectra of the products* **2**–**5** *formed during incubation of* L-*dopa* (**1**) *with the enzyme extract from the peel of* Amanita muscaria. HPLC: *ODS Hypersyl* (5 μ); 0.05M Na_2HPO_4 buffer pH 6 containing 0.03M $(Bu_4N)H_2PO_4$ with a 0–40% MeOH gradient within 25 min, then isocratic for 10 min; detection at 420 nm. UV/VIS spectra are taken at the product peaks; 2,3-secodopa (**3**; 2,3-scd, —) and 4,5-secodopa (**2**; 4,5-scd, ·····), muscaflavin (**5**; M, —), and betalamic acid (**4**; BA, ·····).

nm; *Fig. 1*). The products ratio varies with time and pH as will be outlined later. The peaks at t_R 27 and 30.5 min are attributed to betalamic acid (**4**) and muscaflavin (**5**), respectively, in a direct comparison with authentic samples (retention times, co-HPLC). These are provided by known methods: **5** by chromatography of a pigment extract from *Amanita muscaria* following *Döpp* and *Musso* [12], and **4** by hydrolysis of betanin (**8**) with ammonia [5a] [11] and chromatography. The enzymatic products **4** and **5** have UV absorptions at 430 and 422 nm (*Fig. 1*) corresponding to those of the authentic samples.

In repeating the procedure of *Döpp* and *Musso* [12] to obtain **5** from the pigment extract (*Fig. 2*), we arrive at an essential addendum. The separation of the pigment mixture by ion-exchange chromatography on *DEAE-Sephadex A-25* with a NaCl gradient leads well to the pattern of peaks described, but owing to HPLC, it becomes evident that the zone assigned to **5** is actually composed of two overlapping components; these are separable, at least partially, by fractionation; the front fractions contain prevalently **4** and only the tail fractions pure **5**.

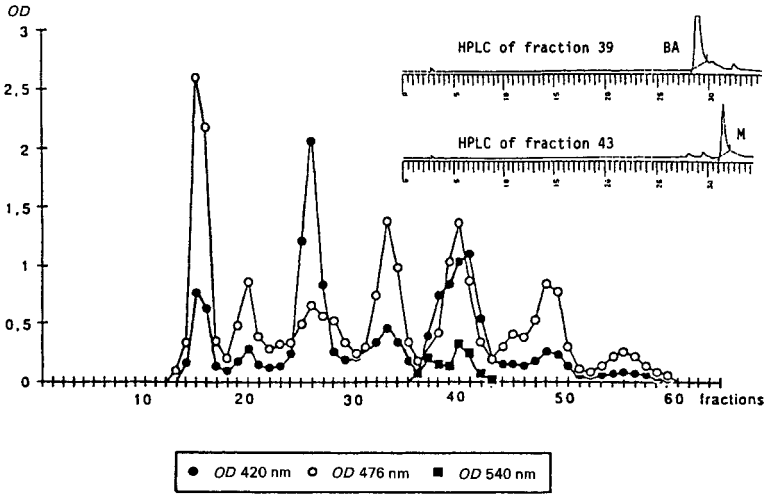


Fig. 2. *Isolation of betalamic acid* (**4**) *and muscaflavin* (**5**) *from a methanolic extract of the red peel of* Amanita muscaria. Elution profiles of the chromatography on *DEAE-Sephadex A-25* (analogue to [12]) established from spectrophotometric readings at 420, 480, and 540 nm, and identification of the components in the front and tail fractions of the muscaflavin-containing zone by HPLC (conditions: see *Fig. 1*).

The relationship of the products **2** and **3** of the enzymatic assays to the identified ones (**4** and **5**) is established in an experiment. For this purpose, the enzymatically generated mixture of the cleavage products (**2** and **3**) is separated by HPLC; when the fractions containing the pure components are left for several h at pH 5.5, **3** is converted to muscaflavin (**5**) and **2** to betalamic acid (**4**), as established by HPLC. Hence there is a strong indication that **2** and **3** are the hypothetical intermediates 4,5- and 2,3-secodopa, and further kinetical, chemical, and spectroscopical arguments will consolidate this conclusion.

Recognition of the Secodopas as Intermediary Products from Kinetics. The evolution of products in enzymatic assays with L-dopa (**1**), incubated at 4° at different pH's, is followed by HPLC analysis of daily samples evaluating spectroscopically the separated

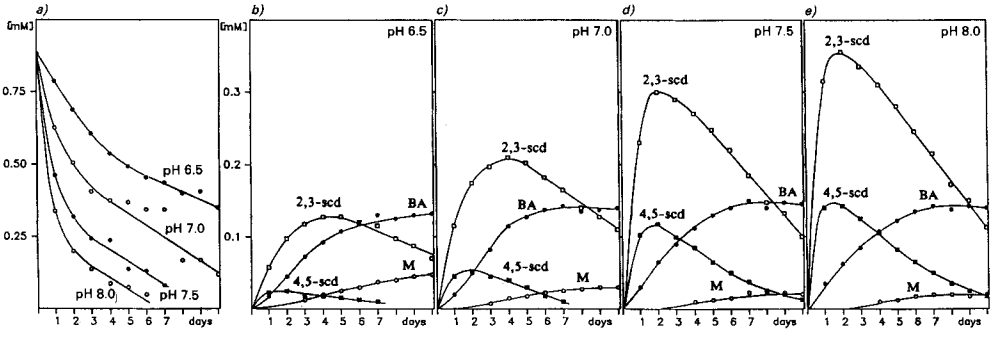


Fig. 3. *Product developments after incubation of* L-*dopa* (**1**) *with the enzyme extract from* Amanita muscaria *at different pH's.* The molar concentrations in the graphs are based on integrated absorbance peak areas of HPLC (*Table 4* in *Exper. Part*) and calculated using known molar absorptivities for **1** ($\varepsilon_{280} = 2800$), **4** and **5** ($\varepsilon_{420} = 30000$), and an extrapolated value for the unknown **2** and **3** ($\varepsilon_{420} = 25500$, see *Exper. Part*). *a*) Consumption of L-dopa (**1**) at different pH's; *b–e*) formation of products **2–5** at different pH's; 2,3-secodopa (**3**; 2,3-scd, □–□–□), 4,5-secodopa (**2**; 4,5-scd, ■–■–■), muscaflavin (**5**; M, ○–○–○), and betalamic acid (**4**; BA, ○–○–○).

peaks of **1** and the four new compounds **2–5.** *Fig. 3* shows the general picture of two parallel series of consecutive transformations dopa → secodopas **2** and **3** → products **4** and **5**, as will be explained in the following. The decrease of **1** appears to be exponential during the first days and then linear, and the fraction of enzymatically converted **1** is increased at higher pH (*Fig. 3a*). Graphically determined initial decay rates of **1** (*Table 1*) increase linearly with pH in the range 6.5 to 8 (decay should be zero near pH 6).

Table 1. *Initial Rates of Enzymatic Dopa Decay and Secodopa Formation.* Graphically evaluated from *Fig. 3* in mM/d.

pH	L-Dopa (**1**)	4,5-Secodopa (**2**)	2,3-Secodopa (**3**)	Total secodopa
6.5	– 0.1	0.04 (40%)	0.06 (60%)	0.1
7.0	– 0.27	0.07 (35%)	0.13 (65%)	0.20
7.5	– 0.47	0.17 (37%)	0.28 (63%)	0.45
8.0	– 0.74	0.24 (32.5%)	0.50 (67.5%)	0.74

The curves of formation of the two primary products **2** and **3** (*Fig. 3b–e*), suspected to be 4,5- and 2,3-secodopa, are of complex character, initial rapid increase similar to that of a first-order reaction, turning into a decrease which remains linear over several days. The total extent of this development is enhanced with pH (*Table 1*). The graphically evaluated initial rates of formation of the secodopas sum up approximately to that of L-dopa consumption confirming that the two events must be concomitant. The ratio of these rates of formation indicates fractions of *ca.* 35% of 4,5-secodopa (**2**) and *ca.* 65% of 2,3-secodopa (**3**). The rates of linear decay are proportional to the maximum amounts of the intermediates formed. The formation of the final products, **4** and **5**, delayed by an initial lag time, progresses at clearly different rates. Muscaflavin (**5**) remains very much below expectation and is even decreased with raising pH in contrast to the observed complementarity of the preceeding transformation **1** → **3**; an important part of **3** has disappeared to unknown products. The development of betalamic acid (**4**) is rather

important in proportion and nearly identical over the examined pH range, irrespective of the amount of preceeding **2**.

A supplementary information is obtained by following the transformation of the intermediates to the final products independently. Starting material is a mixture of secodopas **2** and **3**, which may be generated efficiently by a short-time enzymatic assay at pH 8. Such samples are incubated at pH's between 3 and 6.5. Within the initial 12 h, the decay of **2** and **3** exhibits each the characteristics of a first-order reaction. Similar samples which are deproteinated by addition of acetone after the enzymatic generation of **2** and **3** develop in the same way as in presence of the proteins, confirming that this conversion does not need enzyme catalysis. The formation of the final products **4** and **5**, respectively, exhibits as well characteristics of a first-order reaction but is by far not complementary to the decay of the corresponding secodopa. An illustrative view of the development within the first 2 h is given in a bar chart (*Fig. 4*) where the amounts of the disappeared

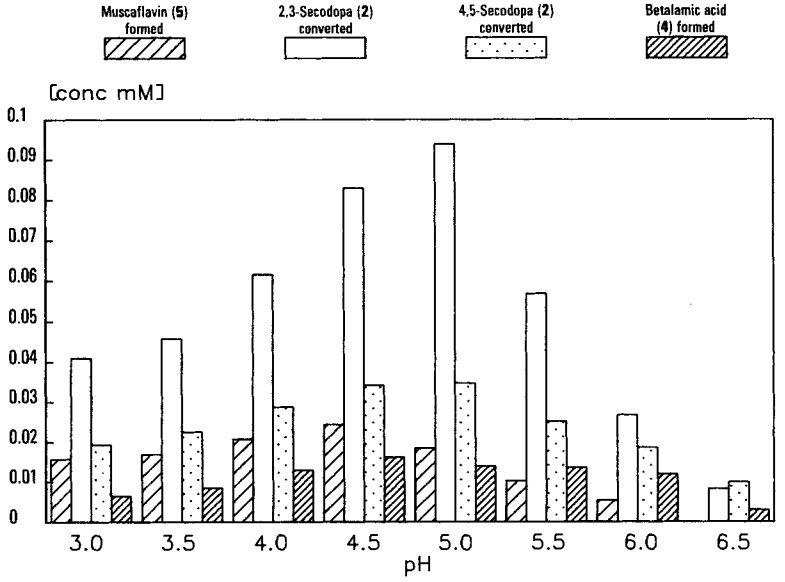


Fig. 4. *Conversion of 2,3- and 4,5-secodopa* (**3** and **2**, resp.) *to muscaflavin* (**5**) *and betalamic acid* (**4**). The concentrations of **2**–**5** are established by HPLC 2 h after incubation at the different pH's.

secodopas are compared to those of products formed. The overall profile of the disappeared amounts of **2** and **3** in function of pH is a bell-shape curve with a maximum between 4.5 and 5. The amounts of final products formed remain below those of their disappeared precursors. The optimum of product formation appears all the same to take place in the same pH range near 4.5. Remarkable is the proportion product/disappeared precursor which is generally higher for **4** than for **5**; apart from that, formation of **5** is relatively favored under acidic condition and negligible above pH 6, and that of **4** is relatively enhanced towards the neutral range.

Formation of Betaxanthins and Enantiomeric Purity of Enzymatically Generated Betalamic Acid (**4**). The addition of L-proline to the enzymatic assay leads to a characteristic

change of the kinetic picture. Only a limited amount of betalamic acid (**4**) shows up and vanishes, and in its place, a new final product absorbing at 480 nm is formed in important yield; no change is observed in the development of muscaflavin (**5**). The identity of the new product as indicaxanthin (**7**) is established by HPLC at pH 2.8[3]) in a direct comparison with an authentic sample synthesized from betanin (**8**) [11]. In fact, the synthetic sample has been prepared from a 67:33 mixture of **8** (15*S*) and its (15*R*)-isomer isobetanin, thus giving rise to two very close peaks in the HPLC, the smaller one appearing first (35%; isoindicaxanthin) followed by the bigger one (65%; **7**); the peak of the enzymatically generated product matches perfectly to the latter one.

The stereochemical implication of this result is confirmed in a complementary experiment. If an enzymatic assay with L-dopa (**1**) in presence of L-proline produces pure indicaxanthin ((2*S*,11*S*); **7**), the corresponding assay with D-dopa should lead to isoindicaxanthin ((2*S*,11*R*)). This is indeed the case, since an enzymatic assay with D-dopa in presence of L-proline produces, according to HPLC, a single compound (besides **5**) which is identical to the synthetic isoindicaxanthin (see above). Confirmation is given by an enzymatic assay with DL-dopa and L-proline which produces a 42:58 mixture (HPLC) of isoindicaxanthin and **7** (the unequal distribution might reflect the different reactivity of the enzyme towards the two enantiomers). The mixture obtained from DL-dopa and L-proline has served as HPLC standard; when it is mixed with an equal amount of mixture from the enzymatic assay with D-dopa and L-proline, the area of the isoindicaxanthin peak is enhanced, as expected from a perfect addition of the corresponding product areas. Similarly, the peak of **7** is enhanced upon addition of the product solution resulting from the assay with L-dopa (**1**) and L-proline. This experiment provides a double proof: it confirms, on one hand, the identity of betalamic acid (**4**) and, on the other hand, establishes the optical purity of enzymatically generated **4** from optically pure substrate.

An artifact is produced when *Tris* buffer is used in the enzymatic assays instead of phosphate buffer; a new product shows up (HPLC: t_R 10 min), which exhibits a UV/VIS spectrum characteristic of a betaxanthin (λ_{max} 476 nm); isolated, it liberates betalamic acid (**4**) on alcaline hydrolysis. It may be concluded that the new product is *Tris*-betalain[4]) a cyanine dye resulting from the condensation of the buffer amine with **4**. In presence of L-proline at even low concentrations, the production of *Tris*-betalain is largely suppressed, and that of indicaxanthin (**7**) becomes prevalent[5]).

Isolation of Enzymatically Generated Muscaflavin (**5**). A combined procedure is used to prepare **5** in sufficient amount for ^{1}H-NMR characterization: A mixture **2**/**3**, enzymatically generated at pH 8, is acidified to pH 5.5 which accelerates cyclization. By addition of L-proline, the less stable **4** is converted entirely to **7**, a salt which is not extracted by organic solvents. Thus, **5** can now be extracted at acidic pH and is further purified on *Sephadex LH-20*. The ^{1}H-NMR spectrum (CD_3OD) of the brownish-yellow product

---

[3]) Gradient and pH for efficient separation of betaxanthins are different from those in *Figs. 1* and *2*; see *Exper. Part* for details.

[4]) For nomenclature, see [8f].

[5]) The presence of still another product is observed with increasing concentrations of L-proline, at the expense of muscaflavin (**5**) formation. To this product (λ_{max} 438 nm), we provisionally assign the structure of a cyanine dye or merocyanin, condensation product of 2,3-secodopa (**3**) with L-proline.

corresponds to the one reported by *Musso et al.* [13] for synthetic **5** (salt in H_2O), except for some difference in the chemical shifts.

Spectrometric Titrations. The yellow, relatively stable neutral solutions of **2**/**3** display intense absorption maxima in the visible, 4,5-secodopa (**2**) at 424 nm and 2,3-secodopa (**3**) at 414 nm. On acidification, the maxima shift by 42–44 nm to 380 and 372 nm, respectively; the spectra of these protonated forms, however, fade away rather rapidly, and are available only by quick recording techniques. UV/VIS spectra recorded within the pH range of titration (*Fig. 5a, b*) provide data composed of those of the protonated

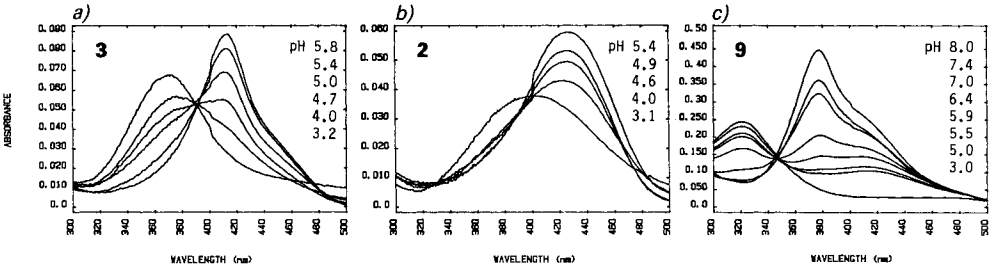
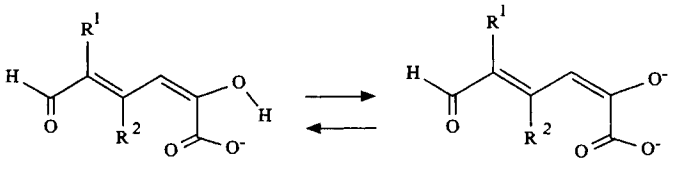


Fig. 5. *UV/VIS spectra of enol and enolate forms of* a) *2,3-secodopa* (**3**), b) *4,5-secodopa* (**2**), *and* c) *secopyrocatechol* (**9**) *in the pH range of titration.* The enol-enolate equilibrium represents the acid-base pair concerned in the titration. Deprotonation involves a red shift of the absorbance maximum.



3 $R^1 = CH_2CH(NH_3^+)COO^-$, $R^2 = H$
2 $R^1 = H$, $R^2 = CH_2CH(NH_3^+)COO^-$
9 $R^1 = R^2 = H$ (secopyrocatechol)

Table 2. *UV Absorptions and* pK_a*-Values of Glutaconaldehyde Derivatives*

	λ_{max} [nm] of AH form	λ_{max} [nm] of A^- form	pK_a
2,3-Secodopa (**3**)	372 (pH 3.2)	414 (pH 5.8)	5.0 ± 0.1
4,5-Secodopa (**2**)	380 (pH 3.1)	424 (pH 5.4)	4.8 ± 0.1
Glutaconaldehyde (pent-2-enedial)	305 [14]	362	5.75 [16]
Secopyrocatechol (**9**; see *Fig. 5*)	322 (pH 3)	378 (pH 8)	6.7 ± 0.1

and anionic forms with a characteristic isobestic point; pK_a values are evaluated from these series by means of a graphical linearization method[6]) (*Table 2*).

Most relevant is a comparison of these spectra with that of secopyrocatechol[2]) (= 2-hydroxy-6-oxohexa-2,4-dienoate; see *Fig. 5*, **9**), the known [9] cleavage product from pyrocatechol (= benzene-1,2-diol). A solution of **9** is easily generated by incubation of the enzyme extract from *Amanita muscaria* with pyrocatechol. It can be shown that the

[6]) Since uncertainty persists concerning the 'acidic end' of the spectra series, a linearization method is chosen that refers to the 'alcaline end' of titration. The pK_a data obtained are still to be considered approximately.

cleavage of pyrocatechol is accomplished by action of the same enzyme as that for L-dopa (**1**) since in assays with mixtures of **1** and pyrocatechol (*Table 7*, see *Exper. Part*), a depression of secodopa formation is noticed[7]). The shape of the UV/VIS spectrum of **9** at various pH is strikingly similar to that of 2,3-secodopa (**3**), except for the position of the maxima, giving testimony of close structural relationship (see *Fig. 5c*). On passing from the enol to the enolate form, the absorption maximum shifts quite similarly to the red by 55 nm; such a shift is also reported for glutaconaldehyde [14]. The pK_a of the enolic system **9**, 6.7, determined similarly from a spectra series in the titration range (*Fig. 5c* and *Table 2*), indicates weaker acidity than that of the secodopas.

Discussion. – The present work contributes to clarify the essential steps in the biosynthetic conversion of L-dopa (**1**) to the betalains (*Scheme*); *i.e.* the enzymatic formation of the secodopas **2** and **3** from **1**, their spontaneous cyclization to the known products muscaflavin (**5**) and betalamic acid (**4**), and the formation of betalains from **4** under neutral conditions.

The temporary existence of the secodopas **2** and **3** is demonstrated for the first time. We gathered substantial evidence for their identity despite the only limited amounts available in the dilute solutions of the enzymatic assay. Usual methods for the determination of structure are not feasable due to the instability of **2** and **3**. The strongest argument for their structure is the comparison with the known cleavage product **9** from pyrocatechol, produced by the same enzyme from *Amanita*; but in contrast to **9** which is stable in weakly alkaline medium and can be isolated, the tendency of **2** and **3** to cyclize makes their isolation problematic. On the other hand, the disappearance of **2** and **3** in acid medium yielding unidentified products is not unexpected in view of the known reactivity of similar glutaconaldehyde systems [15]. Plausible reasons why these primary intermediates have not been noticed before may be either their presence in minute amounts in the plant or their loss during the extraction procedure. The question of their occurrence in nature can now be examined profiting of HPLC characterization.

Noteworthy is the fact that the enzyme from *Amanita muscaria* acts as a metapyrocatechase on various substrates, whereas the activity of known metapyrocatechases of bacterial origin remains restricted to simple catechols. Considering the assortment of tested substances, the presence of an amino group appears to be essential for a good substrate. The enzyme of *Amanita* is obviously less stable than those of bacterial or animal origin [10b]. Whether the cleavage by the enzymes from *Amanita* at both 'extradiol' sites of L-dopa (**1**) is due to the action of one non-regiospecific enzyme or a mixture of two isoenzymes is an open question; the fact that in *Centrospermae* plants, exclusively 4,5-bond cleavage of **1** takes place advocates rather in favor of the existence of isoenzymes.

Information on the acidity of hydroxydienones such as those contained in the cleavage products of **1** and pyrocatechol has been lacking. This is certainly due to technical problems in spectra recording (now obsolete) caused by the instability of the protonated forms of such dienol systems. The only dissociation constant of this kind is that of glutaconaldehyde (pK_a 5.75) which was determined with admirable experimental effort [16].

---

[7]) We wish to acknowledge a helpful discussion with *Bruce Ganem*.

Illuminating is a comparison of the acidities of the secodopas **2** and **3** to those of known similar chromophors. Secopyrocatechol (**9**; pK_a 6.7) is a weaker acid than the unsubstituted glutaconaldehyde which can be readily interpreted as a consequence of chelation by the carboxylate group. Chelation should also be operative in the case of **2** and **3**, but in contrast, enhanced acidities (pK_a 4.8 and 5, resp.) are noted. The enolate form of **2** and **3** is thus stabilized by an effect which outweighs the contrarious effect due to chelation of the enol. This could be either a consequence of substitution of the chromophoric system or due to mutual interaction with the ammonium group in the alanine side chain (H-bonding) of **2** and **3**, an effect similar to that encountered in amino acids (see *e.g.* [17]).

The acid catalysis required for the cyclization of the secodopas **2** and **3** corroborates the needs of imine formation; a 'bell-shaped' reaction-rate profile is generally observed in the formation of imines for which the optimum is near pH 4 [18]. In principle, the spontaneous cyclization of **2** and **3** could have led to two products following the addition of the amino group to one or the other of the two carbonyl C-atoms of the conjugated system. Significant is the exclusive preference for the carbonyl C-atom neighboring the carboxylate group which appears to be related to the stability of the corresponding cyclized products.

The cyclization, obviously initiated by the nucleophilic addition of the free amino group to a carbonyl C-atom, or to a vinylogous position, depends on both the acid-base equilibria of the ammonium and of the enol function. Probably there is a relation between the pH range for optimal cyclization rates of **2** and **3** and the pK's of the enolic system.

Whereas many examples of epimeric betacyanins (as *e.g.* betanin (**8**) and isobetanin) are known, betaxanthins appear as unique species in nature. This is the first time that isoindicaxanthin is characterized analytically; in HPLC, it preceeds indicaxanthin (**7**) contrasting thus to the case of the known isomeric betacyanins [19] where the betacyanin always elutes earlier than the isobetacyanin.

The fact that, enzymatically, from the natural L-dopa, pure (*S*)-betalamic acid (**4**) is produced makes it unlikely that naturally produced betalamic acid should not be of pure (*S*)-configuration as well; hence, only betalains of (*S*,*S*)-configuration would be natural. The origin of the many isobetacyanins isolated from plants [1b] [3a] needs, therefore, to be reexamined. It is known that epimerization of betacyanins to isobetacyanins and *vice versa* takes place easily under alcaline conditions [11] [20]. A possible epimerization in the plant may not be necessarily due to enzymatic action; some general-base catalysis in the aged cell or the conditions of isolation could be responible for the artificial formation of isobetacyanins.

We gratefully acknowledge financial support from the *Swiss National Science Foundation*.

Experimental Part

1. *General.* L-Dopa (**1**) was a gift from *F. Hoffmann-La Roche AG*. Betanin (**8**) was isolated from red beet extract by repeated precipitation as Pb-salt following the procedure of [21]. The other products were purchased from *Fluka AG*. Chromatography: *DEAE-Sephadex A-25* and *Sephadex LH-20* from *Pharmacia*, *ODS Hypersyl* from *Shandon*, and *Spherisorb ODS 2* from *Phase Sep*. The centrifugations were realized in a *MSE Multex* centrifuge at 4500 rpm during 10 min at 4°. UV spectra: *Hewlett-Packard-8450A* diode array spectrophotometer.

^{1}H-NMR spectrum: *Bruker-AC-250* instrument. Buffers: The required molar quantity of salt in H_2O was adjusted to the desired pH's by addition of conc. NaOH soln. or acid (H_3PO_4 or HCl), and the volumes were completed with H_2O; 0.05M NaH_2PO_4 pH 2.8; 0.25M sodium lactate pH 3 and 3.5; 0.25M NaOAc pH 4 to 5; 0.1–0.25M *Tris*-HCl (= [tris(hydroxymethyl)methyl]amine) pH 7.5 and 8; *Tris*-HCl/DTT, 0.1M [tris(hydroxymethyl)methyl]-amine + 0.01M dithiotreitol pH 8; MES, 0.25M 2-(morpholino)ethanesulfonate pH 5.5 to 6.5; 0.25M Na_2HPO_4 pH 6.5 and 7; 0.05M $Na_2HPO_4/(Bu_4N)_2HPO_4$ 0.05M Na_2HPO_4 + 0.03M $(Bu_4N)_2HPO_4$; 0.25M $(NH_4)_2HPO_4$ pH 8; $(NH_4)_2HPO_4$/DTT, 0.1M $(NH_4)_2HPO_4$ + 0.01M dithiotreitol pH 8; $(NH_4)_2HPO_4$/DTT/glyc, 0.1M $(NH_4)_2HPO_4$ pH 8 + 0.01M dithiotreitol + 50% glycerol.

2. *HPLC Chromatography and Quantitative Evaluation of Products.* We used a *Spectra-Physics-SP8700* solvent-delivery system and for detection a *Hewlett-Packard-1040A* diode array spectrophotometer with data processing by a *HP-85B* computer. The columns filled by our own means (25 × 0.5 cm) were protected by a prefiltration cartridge (2 × 0.5 cm); the solvent flow was generally fixed to 0.9 ml/min. L-Dopa (**1**), betalamic acid (**4**), muscaflavin (**5**), 4,5- and 2,3-secodopa (**2** and **3**, resp.) were separated on *ODS Hypersyl* (5 μ) using $Na_2HPO_4/(Bu_4N)_2HPO_4$ pH 6, with a 0–40% MeOH gradient within 25 min, and then isocratic for 10 min. Betalains (betacyanins and betaxanthins) were separated on *Spherisorb ODS 2* (5 μ) with 0.05M NaH_2PO_4 pH 2.8 [19a] isocratic for 10 min, then with a 0–35% MeOH gradient within 25 min; for optimal separation of indicaxanthin (**7**)/isoindicaxanthin these conditions were slightly modified (isocratic for 5 min, then 0–20% MeOH gradient within 30 min). The absorbance at the selected wavelength was recorded through a 0.6-cm flow cell, and separated peaks were integrated yielding '*mAUs*' ('milli absorbance units second'), the product of absorbance axis and time axis. Dividing '*mAUs*' by 1000, multiplying by 0.9/60 (the solvent flow in ml/s), and dividing by the injected sample volume (inj. vol. in ml) gives the absorbance (A) of the product contained in the peak dissolved in 1 ml of soln. The molar concentration is then obtained from the *Beer-Lambert* equation: [mmol/ml] = '*mAUs*' $\cdot 10^{-3} \cdot [(0.9/60) \cdot 0.6] \cdot (1/\varepsilon) \cdot$ (1/vol. inj.).

3. *Enzymatic Assays.* 3.1. *Enzyme Preparation.* The red skin peeled from caps of the fly agaric *Amanita muscaria* was dropped into liq. N_2 and conserved frozen at −30°. Before extraction, the slices were ground under liq. N_2 in a mortar. The powder obtained was suspended for a few min in $(NH_4)_2HPO_4$/DTT pH 8 at 4° (18 ml per 4 g of peel), and the soln. was centrifuged. The pellet was resuspended in some $(NH_4)_2HPO_4$/DTT pH 8 (8 ml) and again centrifuged. To the combined supernatant (22 ml), acetone (9 ml; −30°) was added (*i.e.* 30% of total vol.), and the precipitate was centrifuged; with addition of more acetone (24 ml; completing to 60% of total vol.), another precipitate, containing the metapyrocatechase activity, was formed and centrifuged. The pellet was dissolved in $(NH_4)_2HPO_4$/DTT pH 8 (6 ml), and acetone (9 ml; 60% of total volume) was added to precipitate the proteins which were centrifuged. This pellet was dissolved in buffer or H_2O at 4° for enzymatic assays.

3.2. *Substrate Specificity of the Metapyrocatechase.* In a temperature-controlled UV cell, substrate (0.3 ml, 0.01M), $(NH_4)_2HPO_4$ pH 8 (0.6 ml, 0.25M), H_2O (2 ml), and enzyme preparation (0.1 ml of an extract from 2 g of tissue dissolved in 5 ml of $(NH_4)_2HPO_4$/DTT/glyc pH 8) were mixed at 25° (conc. of substrate in the assay 0.001M). The development of the yellow solns. was followed by measuring the absorbance at λ_{max} for a period of 20 min during which the increase remained linear. The absorbance after 10 min and, in parentheses, λ_{max} [nm] of the

Table 3. *Tests of Residual Activity of Enzymic Solutions after Storage at Different Temperatures.* Absorbance (A) at 420 nm after 30 min incubation with **1** at 25° and pH 8.

Time [min]	Temp. 4°		Temp. 25°		Temp. 37°	
	A	A_{rel}	A	A_{rel}	A	A_{rel}
0	0.87	1.00	0.87	1.00	0.87	1.00
10	0.89	1.02	0.90	1.03	0.70	0.80
20	0.92	1.06	0.85	0.98	0.52	0.60
40	0.89	1.02	0.74	0.85	0.33	0.38
60	0.88	1.01	0.76	0.87	0.21	0.24
90	0.94	1.08	0.74	0.85	0.14	0.16
120	0.90	1.03	0.67	0.77	0.06	0.07
180	0.86	0.99	0.59	0.67	0.05	0.06
240	0.84	0.97	0.58	0.67	0	0
360	0.84	0.97	0.47	0.54	0	0
1320	0.71	0.82	0	0	0	0

products and the absorbance relative to that obtained in the test with L-dopa are: L-dopa 0.119 (416, rel. 1.0); D-dopa 0.074 (416, rel. 0.62); α-methyl-L-dopa 0.133 (434, rel. 1.12); dopamine 0.201 (426, rel. 1.69); *N*-acetyl-dopamine 0.015 (384, rel. 0.13); pyrocatechol 0.023 (377, rel. 0.19); (3,4-dihydroxyphenyl)acetic acid 0.005 (382, rel. 0.04). The following substrates did not react: 3-(3′,4′-dihydroxyphenyl)propionic acid, caffeic acid, 3,4-dihydroxybenzaldehyde, and 3,4-dihydroxybenzoic acid.

3.3. *Thermostability of the Enzymic System.* Samples (1 ml) of enzyme soln. (prepared from 3.1 g of tissue and diluted in 4 ml of $(NH_4)_2HPO_4$/DTT pH 8) were left at 4, 25, and 37°. The enzymatic activity of these solns. was tested at regular intervals by assays (0.1 ml) incubated at 25° with L-dopa (**1**; 0.1 ml, 0.01M), H_2O (0.6 ml), and $(NH_4)_2HPO_4$ pH 8.0 (0.2 ml, 0.25M). After 30 min, MeOH (1.5 ml) was added to precipitate the proteins which were removed by centrifugation before measuring the absorbance of the supernatant at 420 nm (*Table 3*).

4. *Identification of the Products Enzymatically Generated from* L-*Dopa.* 4.1. *Betalamic Acid* (**4**) *from Betanin* (**8**). Conc. NH_3 was added under N_2 to a suspension of 4–5 mg of **8** in H_2O to achieve complete dissolution at pH 12.5. The soln. remained 45 min at 25° under N_2, then the pH was adjusted to 11.5 and the mixture chromatographed on *DEAE-Sephadex A-25* (25 × 2 cm, Cl^- form; 0.3 – 1M NaCl gradient) [12]. The purity of the fractions of the yellow zone collected was checked by HPLC: **4** at t_R 27 min; λ_{max} 430 nm.

4.2. *Isolation of Muscaflavin* (**5**) *Extracted from the Cap of the Fly Agaric.* Powdered red peel of *Amanita muscaria* (3.5 g) was extracted at 4° with MeOH (12 ml). The mixture was sonicated for 30 s and centrifuged. The pellet was once more extracted and centrifuged. The extracts were evaporated and the residue, dissolved in 0.3M NaCl (4 ml), was chromatographed on *DEAE-Sephadex A-25* (25 × 2 cm, Cl^- form; 0.3 – 1M NaCl gradient, flow rate 44 ml/h) [12]. The absorbance of the eluted fractions (7.3 ml) was measured at 420, 480, and 540 nm (*Fig. 2*). The zone containing **5** eluted together with a red pigment (*Fr. 38–44*, in *Fig. 2*). *Fr. 39*, examined by HPLC (0.1 ml), contained practically pure **4** (t_R 27 min; λ_{max} 430 nm) and *Fr. 43* practically pure **5** (t_R 30.5 min; λ_{max} 422 nm). The latter was concentrated under vacuum at 30° to 1 ml and stored at −30° to serve as HPLC standard.

4.3. *Identification of Enzymatically Produced Betalamic Acid* (**4**) *and Muscaflavin* (**5**). L-Dopa (**1**. 0.1 ml, 0.01M) was added to Na_2HPO_4 pH 6.5 (0.2 ml, 0.25M), H_2O (0.6 ml), and enzyme preparation (0.1 ml of an extract from 4 g of tissue and diluted in 3 ml of $(NH_4)_2HPO_4$/DTT pH 8) and incubated 4 days at 4°. HPLC (detection at 420 nm) of the yellow soln. t_R 18.5, 21.5, 27, and 30.5 min (*Fig. 1*). The peak at t_R 27 min was identical with that of **4** prepared from **8** (coinjection: 1 peak of added surfaces). The peak at t_R 30.5 min was identical with that of **5** isolated from *Amanita* [6a] (coinjection 1 peak of added surfaces).

4.4. *Correlation of 4,5-Secodopa* (**2**) *and 2,3-Secodopa* (**3**) *with* **4** *and* **5**, *Respectively.* The mixture of yellow compounds generated according to *4.3* was separated by HPLC. The zones at t_R 18.5 (2.9 ml; **3**) and 21.5 min (1.6 ml; **2**) were concentrated under vacuum at 30° and adjusted to the same volumes (0.15 ml). NaOAc buffer pH 5.5 (0.075 ml, 1M) was added to each sample. After 14 h at 4°, the products were examined by HPLC: the soln. obtained from **3** showed 1 product (t_R 30.5 min, λ_{max} 422 nm), identical and coincidant with **5**, and the soln. obtained from **2** contained also 1 product (t_R 27 min, λ_{max} 430 nm), identical with **4**.

5. *Enzymatic Cleavage Products from* L-*Dopa* (**1**). 5.1. *Enzymatic Incubation of* **1** *at Various pH's.* A mixture of 0.01M aq **1** (0.2 ml) buffer (0.4 ml, 0.25M), H_2O (1.2 ml), and enzyme preparation (0.2 ml of an extract from 8 g of tissue and diluted in 10 ml of $(NH_4)_2HPO_4$/DDT/glyc pH 8) was incubated at 4° (buffers: MES pH 6.5; Na_2HPO_4 pH 7; $(NH_4)_2HPO_4$ pH 7.5 and 8); final concentrations were 0.001M **1** and 0.05M buffer. Daily samples (0.08 ml) were taken and stored in the freezer (−30°); of the thawed samples, 0.035 ml were analyzed by HPLC at 280 (for **1**) and 420 nm (for **2**–**5**): see *Table 4*.

Table 4. *Product Development in Enzymatic Assays with* **1** *at Different pH's* (see *Fig. 3*).
The values in the table are *'mAUs'* (integrated areas of HPLC peaks); from these, molar concentrations can be calculated by the conversion factors.

Time [day]	**1** $0.255 \cdot 10^{-6}$ a)	**2** $0.028 \cdot 10^{-6}$ a)	**3** $0.028 \cdot 10^{-6}$ a)	**4** $0.0238 \cdot 10^{-6}$ a)	**5** $0.0238 \cdot 10^{-6}$ a)
pH 6.5					
0	3453	0	0	0	0
1	3091	777	2048	740	0
2	2688	846	3525	1917	0
3	2383	657	4161	2997	552
4	2116	660	4522	3893	802

Table 4 (cont.)

Time [day]	**1** $0.255 \cdot 10^{-6}$ a)	**2** $0.028 \cdot 10^{-6}$ a)	**3** $0.028 \cdot 10^{-6}$ a)	**4** $0.0238 \cdot 10^{-6}$ a)	**5** $0.0238 \cdot 10^{-6}$ a)
5	1946	543	4529	4493	1051
6	1790	435	4296	4918	1292
7	1718	366	4106	5442	1591
8	1571	0	3465	5236	1720
9	1581	0	3144	5487	1943
10	1370	0	2465	5544	1974
pH 7					
0	3453	0	0	0	0
1	2440	1592	4074	866	0
2	1979	1843	6283	2486	0
3	1583	1579	7081	3484	346
4	1470	1399	7491	4717	617
5	1453	1077	7266	5352	776
6	1355	806	6474	5770	935
7	1347	618	5994	5940	1078
8	673	355	4970	5665	1144
9	670	0	4518	5810	1276
10	468	0	3954	5833	1299
pH 7.5					
0	3453	0	0	0	0
1	1818	3704	8209	1278	0
2	1250	4211	10726	2686	0
3	985	3541	10339	3796	0
4	935	3016	9670	4736	465
5	542	2329	8820	5434	563
6	523	1765	7829	5887	665
7	311	1327	6576	6272	909
8	153	913	5312	5884	914
9	0	674	4755	6198	900
10	0	439	3564	6071	891
pH 8					
0	3453	0	0	0	0
1	1332	4987	11291	1471	0
2	773	5093	12719	2672	0
3	548	4456	11961	3562	0
4	334	3733	11103	4470	423
5	283	2977	9966	5153	497
6	193	2293	8766	5686	745
7	0	1775	7693	6012	804
8	0	1230	6145	5793	827
9	0	881	5382	6023	767
10	0	592	4048	5918	800

a) Conversion factor.

5.2. *Evaluation of an ε-Value for the Secodopas* **2** *and* **3**. The sum of the molar concentration of **2** and **3**, obtained according to *5.1*, was calculated as the difference of enzymatically converted **1** minus **4** and **5** (see *Table 4*), based on their known molar absorptivities (ε (**1**) = 2800, ε (**4** or **5**) = 30000). The ε value of secodopa are calculated for each anal. set; they decrease linearly with the time of the analysis (probably a consequence of products depletion), and the slope is nearly identical in all four series of data sets; extrapolation to zero time converges in all the series to a value ε 25500 ± 500.

5.3. *Transformation of Secodopas* **2** *and* **3** *at Different pH's.* A mixture of **2**/**3** was generated by incubation of enzyme soln. (5 ml; see *5.1*), **1** (98.5 mg, 0.05 mmol), $(NH_4)_2HPO_4$ pH 8 (5ml, 2M), and H_2O (90 ml) for 3 days at 4°. Aliquots (0.3 ml) of the resulting yellow soln. were transferred at 4° to 0.25M buffers (1.2 ml) of different pH's; sodium lactate pH's 3, 3.5; NaOAc pH's 4, 4.5, 5; MES pH's 5.5, 6, 6.5. The development of the product mixtures was followed for 12 h by HPLC of samples (0.06 ml) taken at certain time intervals (see *Table 5*).

Table 5. *Changes of Product Compositions after Incubation of an Enzymatically Generated Mixture of Secodopas* **2** *and* **3** *at Different pH's.* Substance amounts (*'mAUs'*), determined after 2 h, are converted to molar concentrations by the conversion factors (see *Fig. 4*).

Time [h]	**2** $0.0163 \cdot 10^{-6}$ [a])	**3** $0.0163 \cdot 10^{-6}$ [a])	**4** $0.0139 \cdot 10^{-6}$ [a])	**5** $0.0139 \cdot 10^{-6}$ [a])	Dopaxanthin $0.001 \cdot 10^{-6}$ [a])
pH 3					
0	4157	8716	1796	0	366
2	2969	6203	2266	1138	348
5	2341	5010	2267	1532	320
12	1490	2573	2109	2649	364
pH 3.5					
0	4157	8716	1796	0	366
2	2776	5910	2404	1220	339
5	1462	4270	2049	1674	342
12	1149	1907	2388	2885	371
pH 4					
0	4157	8716	1796	0	366
2	2398	4943	2738	1497	347
5	1294	1880	2781	2542	363
12	667	941	3106	3628	424
pH 4.5					
0	4157	8716	1796	0	366
2	2065	3635	2966	1755	330
5	898	666	3311	2937	359
12	398	329	3835	4014	330
pH 5					
0	4157	8716	1796	0	366
2	2033	2960	2808	1335	360
5	800	1024	3274	2282	392
12	traces	traces	3775	3236	459
pH 5.5					
0	4157	8716	1796	0	366
2	2613	5225	2778	743	354
5	2049	4717	2948	1195	971
12	1147	3165	4039	2037	915
pH 6					
0	4157	8716	1796	0	366
2	3013	7074	2664	388	374
5	2399	6273	3066	687	682
12	1522	4079	3421	1146	1178
pH 6.5					
0	4157	8716	1796	0	366
2	4180	8211	1327	0	252
5	2914	6646	2412	444	519
12	2235	7303	3223	559	743

[a]) Conversion factor.

6. *Formation of Betalains.* 6.1. *Enzymatic Assay with* L-*Proline in* Tris *and Hydrogen Phosphate Buffer.* Two assays were incubated at 4° with **1** (0.1 ml, 0.01M), L-proline (0.05 ml, 0.05M), H_2O (0.55 ml), enzyme preparation (0.1 ml of an extract from 4.3 g of *Amanita* dissolved in 1.5 ml of H_2O), and buffer (0.2 ml, 0.25M; *i.e. Tris*-HCl pH 7.5 and Na_2HPO_4 pH 7.5, resp.). The formation of *Tris*-betalain and indicaxanthin (**7**) was followed by HPLC at 480 nm (conditions for betalains) of aliquots (0.035 ml) injected after different incubation times (*Table 6*).

Table 6. *Amounts* (expressed as *'mAUs'*) *of Betaxanthins Formed in Enzymatic Assays with* L-*Dopa* (**1**) *and* L-*Proline*

	Time [day]					
	1	4	7	11	17	24
Tris-HCl buffer						
Tris-betalin	592	2617	2672	1871	1308	779
Indicaxanthin (**7**)	510	4947	10125	16468	20676	24151
Na_2HPO_4 buffer						
Tris-betalain	0	0	0	0	0	0
Indicaxanthin (**7**)	536	5749	11129	16196	20058	23122

6.2. *Preparation of Indicaxanthin* (**7**) *from Betanin* (**8**). To a soln. of **8**/isobetanin (3.6 mg, 0.067 mmol) in 17% NH_3/H_2O (2 ml), L-proline (7.7 mg, 0.067 mmol) was added. The mixture was left 1 h at 25°, then acidified with HCl to pH 1, and submitted to HPLC: t_R 25.7 (4985 *'mAUs'*, 65%; **7**) and 25.2 min (2688 *'mAUs'*, 35%; isoindicaxanthin). HPLC of starting material (conditions for betalains): t_R 19.8 (8789 *'mAUs'* 67%; **8**) and 21 min (4355 *'mAUs'*, 33%; isobetanin).

6.3. *Identification of Enzymatically Produced* **7**. L-Dopa (**1**; 0.1 ml, 0.01M), *Tris*-HCl pH 7.5 (0.2 ml, 0.25M), L-proline (0.5 ml, 0.02M), and enzyme preparation (0.1 ml of an extract from 2.3 g of *Amanita* and dissolved in 1 ml H_2O) were incubated 7 days at 4°. HPLC (conditions for betalains): only one peak t_R 25.5 (**7**); coinjection of enzymatically prepared **7** and synthetic **7**/isoindicaxanthin (see *6.2*) gave 2 peaks at t_R 25.1 and 25.6 min, the latter being greatly enhanced.

6.4. *Enzymatic Syntheses of Indicaxanthin* (**7**) *and Isoindicaxanthin.* Dopa (0.1 ml, 0.01M), *Tris*-HCl buffer pH 7.5 (0.05 ml, 1M), H_2O (0.7 ml), L-proline (0.05 ml, 0.2M), enzyme preparation (0.1 ml of an extract from 4.9 g of *Amanita* and dissolved in 1 ml of *Tris*-HCl/DTT pH 8) was incubated 3 days at 4°. Three such enzymatic assays were prepared identically with L-, D-, and DL-dopa as substrates, indexed as 'L', 'D', 'DL', resp. Identical aliquots of 'L', 'D', and 'DL' (0.035 ml each) and 1:1 mixtures (0.035 ml + 0.035 ml) 'L'/'DL' and 'D'/'DL' were analyzed by HPLC under modified conditions (*Spherisorb ODS 2* (5 μ) with 0.05M NaH_2PO_4 pH 2.8 [19a] isocratic for 5 min, then 0–20% MeOH gradient within 30 min) for optimal separation of a mixture of **7** (t_R 24.8 ± 0.1 min) and isoindicaxanthin (t_R 23.9 ± 0.1 min); the peak of *Tris*-betalain appears at t_R 9.6 min. Results (peak integrations in *'mAUs'*): 'D': 2661 (100%, isoindicaxanthin); 'L': 2648 (100%, **7**); 'DL': 1194 (42%, isoindicaxanthin); 1635 (58%, **7**); 'D'/'DL': 4359 (67.3%, isoindicaxanthin), 2978 (32.3%), **7**); 'L'/'DL': 1418 (21.4%, isoindicaxanthin), 5214, (78.6%, **7**).

7. *Preparative Enzymatic Formation of Muscaflavin* (= *4-Formyl-2,3-dihydro-1*H-*azepin-2,7-dicarboxylate;* **5**)[8]. A mixture of **1** (99 mg, 0.5 mmol), $(NH_4)_2HPO_4$ (2.5 ml, 2M, pH 8), and 2.5 ml of enzyme extract (from 6 g of tissue of *Amanita muscaria*, adjusted to 50 ml with H_2O) was incubated during 6 days at 4°. The soln. was acidified to pH 5.5 by adding AcOH and left 24 h at 4°. The pH was again increased to 7.5, and 287 mg of L-proline (2.5 mmol) were added. After 3 days at 4°, the soln. was acidified with conc. HCl soln. to pH 1.5 and the aq. phase extracted 4 times with 100 ml of AcOEt. The combined org. phase was dried ($MgSO_4$) and evaporated. The residue (32 mg) was chromatographed on *Sephadex LH-20* (23 × 2.2 cm, MeOH). The fractions containing pure **5** (HPLC) yielded a brownish residue (2 mg) on evaporation. ^{1}H-NMR (CD_3OD, 250 MHz): 9.38 (*s*, 1 H, CHO); 7.16 (*d*, *J* = 8.5, 0.85 H, H–C(5)); 7.16 (*s*, 0.1–0.15 H, H–C(5), H/D exchange of H–C(6) with solvent); 6.20 (*d*, *J* = 8.5, 0.85 H, H–C(6), H/D exchange with solvent); 4.28 (*dd*, *J* = 6.5, 1.5, 1 H, H–C(2)); 3.33 (*dd*, *J* = 15, 1.5, 1 H, H–C(3)); 2.93 (*dd*, *J* = 15, 6.5, 1 H, H–C(3)).

8. *Spectrometric Titration of Enzymatically Generated Solutions of the Secodopas and of the Cleavage Product from Pyrocatechol.* 8.1. *Enzymatic Assay with* **1** *in Presence of Pyrocatechol.* To *Tris*-HCl pH 8 (0.2 ml, 0.25M),

[8]) In collaboration with *Philippe Hünenberger.*

1 (0.1 ml, 0.01M), and enzyme preparation (0.1 ml of an extract from 1 g of *Amanita muscaria* peel and dissolved in 1 ml of 0.1M *Tris*-HCl pH 8), different volumes of pyrocatechol were added (0.1, 0.3, and 0.6 ml, 0.01M); one assay was left without pyrocatechol as a control experiment. The assays were adjusted with H_2O to the same volume (1 ml). After 3 h incubation at 4°, samples (0.5 ml) were analyzed by HPLC (*Table 7*).

Table 7. *Enzymatic Assay with* **1** *in Presence of Pyrocatechol.*
The product compositions (in *'mAUs'*) are determined by HPLC after 3 h of incubations; detection at 380 nm for **9** and 420 nm for **2** and **3**.

Starting material		Products		
L-Dopa (**1**) [M]	Pyrocatechol [M]	Secopyrocatechol (**9**)	4,5-Secodopa (**2**)	2,3-Secodopa (**3**)
0.001	0	—	2209	3571
0.001	0.001	traces	2003	3320
0.001	0.003	857	1772	2987
0.001	0.006	1720	1294	2087

8.2. *UV Spectroscopy of the Secodopas* **2** *and* **3**. By incubation of an enzyme preparation (0.1 ml of an extract from 3 g of *Amanita* dissolved in 0.6 ml of 0.1M of *Tris*-HCl/DTT pH 8), H_2O (1.8 ml), and **1** (0.1 ml, 0.01M) for 3.5 h at 4°, **2**/**3** was generated (conserved frozen for 12 days without notable change) and separated by HPLC in 4 runs of 0.1 ml each. The fractions containing **3** (10 ml) or **2** (7.2 ml) were pooled and concentrated under vacuum at 25° to 3.1 and 3.4 ml, resp. For the UV/VIS spectra, 0.2 ml of these solns. and 0.6 ml of different 0.25M buffers were mixed. The stability of **2** and **3** at different pH's was checked by recording spectra every 30 s during 5 min. The pH of the solns. was measured after UV recordings. Buffers: sodium lactate pH 3; NaOAc pH's 4, 4.5, and 5; MES pH's 5.5, 6, and 6.5; Na_2HPO_4 pH 7; *Tris*-HCl pH's 7.5 and 8 (*Fig. 5*).

8.3. *UV Spectroscopy of Secopyrocatechol* (**9**). Pyrocatechol (0.1 ml, 0.01M), H_2O (1.7 ml), and enzyme preparation (0.2 ml of an extract from 3 g of *Amanita* peel dissolved in 0.6 ml of 0.1M *Tris*-HCl/DTT pH 8) were incubated at 4° for 3.5 h. The mixture was deproteinated by addition of 3 ml of acetone followed by centrifugation. The yellow supernatant containing **9** was concentrated under vacuum at 25° to *ca.* 0.3 ml and adjusted with H_2O to 1.1 ml. For the spectrometric titration, 0.9 ml of 0.25M buffer and 0.1 ml soln. of **9** were mixed as described in *8.2* (*Table 8*). The linearization method for pK_a calculations was based on the formula $(OD–OD(B))\cdot 10^{pH} = (-1/K_a)\cdot OD + (1/K_a)\cdot OD(BH)$ [22]. The value $OD(B)$ is the absorption at the 'alcaline end' of titration; the value $OD(BH)$ is an uncertain constant ('acid end' of titration) but does not interfer in the calculation of the constant K_a. The value pK_a is given from $1/K_a$, value which corresponds to the slope of the line of points (calculated by the method of least squares) defined by the coordinates $(OD–OD(B))\cdot 10^{pH}$, and OD.

Table 8. *Spectrometric Titrations of Secocatechols* (based on the curves in *Fig. 5*).
Absorptions (OD) at the wavelength of the maximas corresponding to BH and B at various pH.

4,5-Secodopa (**2**)			2,3-Secodopa (**3**)			Secopyrocatechol (**9**)		
pH	OD (380)	OD (424)	pH	OD (372)	OD (414)	pH	OD (322)	OD (378)
3.1[a])	0.034	0.034	3.2	0.068	0.028	3.0[a])	0.244	0.050
4.0	0.027	0.043	4.05	0.056	0.040	5.0	0.23	0.096
4.65	0.026	0.049	4.7	0.049	0.054	5.5	0.211	0.108
4.95	0.025	0.053	5.0	0.042	0.067	5.9	0.201	0.145
5.4	0.025	0.059	5.45	0.032	0.081	6.4	0.167	0.205
5.8[a])	0.022	0.060	5.8[a])	0.027	0.089	7.0	0.108	0.324
6.8	0.022	0.064	6.8	0.016	0.100	7.4[a])	0.070	0.361
						8.0	0.070	0.446
	pK_a 4.82			pK_a 4.98			pK_a 6.72	

[a]) Values neglected for the calculation of pK_a.

REFERENCES

[1] a) H. Wyler, A. S. Dreiding, *Experientia* **1961,** *17,* 23; b) T. J. Mabry, A. S. Dreiding, 'The Betalains, a Class of Red Pigments in the Centrospermae', in 'Recent Advances in Phytochemistry', Eds. T. J. Mabry, R. E. Alston, and V. C. Runeckles, Appleton-Century-Crofts, New York, 1968, Vol. 1, p. 145–160; c) T. J. Mabry, 'Betalains, Red-Violet and Yellow Alkaloids of the Centrospermae', in 'Chemistry of the Alkaloids', Ed. S. W. Pelletier, Van Nostrand Reinhold, 1970, p. 367–384; d) T. J. Mabry, 'Betalains', in 'Encyclopedia of Plant Physiology', Eds. E. A. Bell and B. V. Charlwood, Springer, Berlin–Heidelberg, 1980, new series, Vol. 8, p. 513–533; e) M. Piattelli, 'The Betalains: Structure, Biosynthesis, and Chemical Taxonomy', Eds. P. K. Stumpf and E. E. Conn, in 'The Biochemistry of Plants', Ed. E. E. Conn, Academic Press, New York–London–Toronto–Sydney–San Francisco, 1981, Vol. 7, p. 557–575; f) W. Steglich, D. Strack, 'Betalains', in 'The Alkaloids', Ed. A. Brossi, Academic Press, New York, in press.

[2] M. E. Wilcox, H. Wyler, T. J. Mabry, A. S. Dreiding, *Helv. Chim. Acta* **1965,** *48,* 252.

[3] H. Wyler, U. Meuer, J. Bauer, L. Stravs-Mombelli, *Helv. Chim. Acta* **1984,** *67,* 1347.

[4] M. Piattelli, L. Minale, G. Prota, *Tetrahedron* **1964,** *20,* 2325.

[5] a) L. Kimler, R. A. Larson, L. Messenger, J. B. Moore, T. J. Mabry *J. Chem. Soc., Chem. Commun.* **1971,** 1329; b) H. Reznick, *Z. Pflanzenphysiol.* **1978,** *87,* 95.

[6] a) R. von Ardenne, H. Döpp, H. Musso, W. Steglich, *Z. Naturforsch., C* **1974,** *29,* 637; b) H. Musso, Tetrahedron Report No. 70, *Tetrahedron* **1979,** *35,* 2843; c) H. Döpp, S. Mauer, A. N. Sasaki, H. Musso, *Liebigs Ann. Chem.* **1982,** 254; d) M. Gill, W. Steglich, *Progr. Chem. Nat. Prod.* **1987,** *51,* 75, Ed. W. Herz, H. Grisebach, G. W. Kirby, and Ch. Tamm, Springer, Wien–New York.

[7] a) H. Wyler, T. J. Mabry, A. S. Dreiding, *Helv. Chim. Acta* **1963,** *46,* 361; b) R. Thomas, 'Biosynthetic Pathways Involving Ring Cleavage', in 'Biosynthesis of Antibiotic Substances', Eds. Z. Vanek and Z. Hostalek, Academic Press, New York–London, 1965, Chapt. 13, p. 161–162.

[8] a) H. E. Miller, H. Rösler, A. Wohlpart, H. Wyler, M. E. Wilcox, H. Frohofer, T. J. Mabry, A. S. Dreiding, *Helv. Chim. Acta* **1968,** *51,* 1470; b) L. Minale, M. Piattelli, R. A. Nicolaus, *Phytochemistry* **1965,** *4,* 593; c) G. Impellizeri, M. Piattelli, *ibid.* **1972,** *11,* 2499; d) N. Fischer, A. S. Dreiding, *Helv. Chim. Acta* **1972,** *55,* 649; e) H.-W. Liebisch, B. Matschiner, H.-R. Schütte, *Z. Pflanzenphysiol.* **1969,** *61,* 269; f) C. Chang, L. Kimler, T. J. Mabry, *Phytochemistry* **1974,** *13,* 2771; g) H. Wyler, U. Meuer, *Helv. Chim. Acta* **1979,** *62,* 1330.

[9] a) S. Dagley, W. Ch. Evans, D. W. Ribbons, *Nature (London)* **1960,** *188,* 560; b) S. Dagley, D. A. Stopher, *Biochem. J.* **1959,** *73,* 16P.

[10] a) O. Hayaishi, in 'Oxygenases', Ed. O. Hayaishi, Academic Press, New York, 1962; Y. Kojima, N. Itada, O. Hayaishi, *J. Biol. Chem.* **1961,** *236,* 2223; b) M. Nozaki, 'Oxygenases and Dioxygenases', in 'Topics in Current Chemistry', Ed. F. L. Boschke, Springer, Berlin–Heidelberg–New York, 1979, Vol. 78, p. 145.

[11] H. Wyler, M. E. Wilcox, A. S. Dreiding, *Helv. Chim. Acta* **1965,** *48,* 361.

[12] H. Döpp, H. Musso, *Z. Naturforsch., C* **1974,** *29,* 640; *Chem. Ber.* **1973,** *106,* 3473.

[13] H. Barth, G. Burger, H. Döpp, M. Kobayashi, H. Musso, *Liebigs Ann. Chem.* **1981,** 2164; p. 2171.

[14] J. Becher, M. Chr. Christensen, *Tetrahedron* **1979,** *35,* 1523.

[15] J. Becher, *Synthesis* **1980,** 589.

[16] G. Schwarzenbach, K. Lutz, *Helv. Chim. Acta* **1940,** *23,* 1147.

[17] D. L. Hughes, J. J. Bergan, E. J. J. Grabowsky, *J. Org. Chem.* **1986,** *51,* 2579.

[18] For a discussion, see 'Mechanism of Imine Formation', in 'Bioorganic Mechanisms', Eds. T. C. Bruice and S. J. Benkovic, W. A. Benjamin, New York–Amsterdam, 1966, Vol. II, p. 227–255.

[19] a) T. Adachi, M. Nakatsukasa, *Z. Pflanzenphysiol.* **1983,** *109,* 155; b) D. Strack, H. Reznik, *ibid.* **1979,** *94,* 163; c) S. J. Schwartz, J. H. von Elbe, *J. Agric. Food Chem.* **1980,** *28,* 540; d) K. R. Vincent, R. G. Scholz, *ibid.* **1978,** *26,* 812.

[20] M. E. Wilcox, H. Wyler, A. S. Dreiding, *Helv. Chim. Acta* **1965,** *48,* 1134.

[21] G. W. Pucher, L. C. Curtis, H. B. Vickery, *J. Biol. Chem.* **1938,** *123,* 61.

[22] J. Polster, H. Lachmann, 'Spectrometric Titration Curves', Verlag Chemie, Weinheim, 1989, p. 43.