European J. Biochem. 10 (1969) 268–273

# On the Biosynthesis of Cyclopenin and Cyclopenol, Benzodiazepine Alkaloids from *Penicillium cyclopium* Westling

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(Received March 29/May 19, 1969)

By feeding of isotope labelled compounds to cultures of *Penicillium cyclopium* Westling and degradation of the cyclopenin (3-benzyl-3,10-epoxy-4-methyl-2,3,4,5-tetrahydro-1*H*-1,4-benzodiazepine-2,5-dione) and cyclopenol [3-(*m*-hydroxy-benzyl)-3,10-epoxy-4-methyl-2,3,4,5-tetrahydro-1*H*-1,4-benzodiazepine-2,5-dione] formed the biosynthesis of these alkaloids was investigated:

1. The carbon skeletons of both alkaloids originate from all the C-atoms of anthranilic acid and L-phenylalanine and from the methyl group of methionine.

2. N(1) and N(4) of the diazepine ring derive from the nitrogen atoms of anthranilic acid and L-phenylalanine respectively.

3. For cyclopenol, of several phenylpropane compounds tested, only L-phenylalanine is a direct precursor. Thus, the introduction of the *m*-hydroxy group by a mixed functional oxygenase as one of the later steps of its biosynthesis appears very likely.

The benzodiazepine alkaloids (−)-cyclopenin and (−)-cyclopenol were isolated by Bracken *et al.* [1] and Birkinshaw *et al.* [2] from the culture medium of *Penicillium cyclopium*. The structure of these alkaloids (see Fig. 1) elucidated by oxidative degradation [3], recently has been confirmed by the synthesis of (±)-cyclopenin [4].

Besides chemical and physicochemical evidences, the proposal for the chemical structure of cyclopenin and cyclopenol had been influenced by biosynthetic considerations. Both alkaloids were regarded as derivatives of the cyclic dipeptide of anthranilic acid and phenylalanine. This suggestion was coroborated by investigations on the biosynthesis of viridicatin and viridicatol by *P. viridicatum* Westling. The carbon skeleton of these quinoline alkaloids derives from anthranilic acid and phenylalanine [5], by a biosynthetic pathway including cyclopenin and cyclopenol. The rearrangement of the benzodiazepine ring system of the latter alkaloids to the quinoline nucleus of the former (Fig. 1) is catalyzed by an enzyme called cyclopenase [6].

In this paper, we wish to present the results of our investigations on the biosynthesis of cyclopenin and cyclopenol, especially considering the following problems: (a) derivation of the total carbon skeleton of the alkaloids from anthranilic acid, phenylalanine, and the methyl group of methionine and, (b) incorporation of the nitrogen atoms from both, anthranilic acid and phenylalanine, and (c) origin of the *m*-hydroxyl group in cyclopenol.

Natural products with this unusual substitution pattern occur relatively seldom (for a summary see [7]). Apparently, there are the following biosynthetic routes leading to *m*-hydroxylated aromatic compounds:

a) the *p*-dehydroxylation of *m,p*-dihydroxylated aromatic acids, as carried out by the intestinal flora of higher animals (for a summary see [8, 9]),

b) the direct formation of *m*-substituted compounds from shikimic acid without involvement of phenylalanine or phenylpyruvic acid, as shown to occur in higher plants, *e. g.* formation of *m*-tyrosine and, biogenetically derived from it, 1-methyl-6-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid in *Euphorbia myrsinites* [10],

c) a direct *meta*-hydroxylation by mixed functional oxygenases (for details see [7]).

The latter pathway, which until now never has been proved directly, could also involve *m*-carboxylated derivatives, found for instance in various *Reseda* species [11], because there are several examples known in which hydroxylations are combined with simultaneous decarboxylations (summary see [7]).

In the biosynthetic pathway leading to the two fungal products volucrisporine from *Volucrispora aurantiaca* Haskins [12] and gliotoxin from *Trichoderma viride* Pers. ex Fr. [13] direct *m*-hydroxylations probably occur, because the incorporation of phenylalanine and, though to a smaller extent,

This paper represents part 5 of the series „Zur Bildung von Chinolinalkaloiden in Pflanzen". Part 4 see [6].

also of *m*-tyrosine into both compounds was demonstrated and because tyrosine and 3,4-dihydroxyphenylalanine were inactive as precursors at least for volucrisporine.

## MATERIALS AND METHODS

### *Radioactive Chemicals and General Methods*

DL-[1-$^{14}$C]Phenylalanine, DL[2-$^{14}$C]phenylalanine, DL-[3-$^{14}$C]phenylalanine, L-[*amino*-$^{15}$N]phenylalanine, and L-[U-$^{14}$C]tyrosine were commercial products. [*Carboxy*-$^{14}$C]Anthranilic acid and DL-[*Me*-$^{14}$C]methionine were synthesized following the methods described by Munsche *et al.* [14] and Melville *et al.* [15], respectively. *m*-[2-$^{14}$C]Tyrosine was synthesized from [2-$^{14}$C]glycine *via* hippuric acid and condensation of the latter with *m*-hydroxy-benzaldehyde [16]. D-[U-$^{3}$H]Phenylalanine, [U-$^{3}$H]anthranilic acid, DL-*m*-[U-$^{3}$H]tyrosine, and *m*-carboxy-[U-$^{3}$H]phenylalanine were labelled by the method of Wilzbach and purified before use first by treatment with charcoal, then by heating sucessively with dilute HCl and NaOH on a water bath and finally, if necessary, by preparative thin layer chromatography. All radioactive chemicals used were radiochromatographically pure.

For thin layer chromatography the following systems were used. For α-amino acids: *sec.*—butanol—acetic acid—water (8:2:2, v/v) on silica gel $PF_{254}$ (E. Merck AG., Darmstadt), for preparative scale, silica gel G (E. Merck AG., Darmstadt) and MN-Cellulose powder-300 (Marcherey, Nagel & Co., Düren), for analytical scale. For anthranilic acid: toluene—acetic acid—ethanol—water (80:15:5:0.5, by vol.) on silica gel G. For cyclopenin and cyclopenol and their degradation products see [17].

Radioactivity was measured in a Tricarb liquid scintillation counter. $BaCO_3$ before the measurement was decomposed by citric acid and the radioactive $CO_2$ was trapped into an alcoholic solution of triethanolamine. Tritium content of water was determined after distillation of a small amount into acetic anhydride and measurement of the acetic acid formed. $^{15}$N enrichment was determined spectrophotometrically [18]. Thin layer chromatogramms of radioactive substances were scanned with the Dünnschicht-Scanner (Lab. Prof. Dr. Berthold, Wildbad, Schwarzwald).

### *Culture of* Penicillium cyclopium

The cyclopenin and cyclopenol producing strain of *Penicillium cyclopium* Westling (SM 72) originates from a culture of *P. cyclopium* LSHTM No. 72 [1,2]. Culture condition were as described by Luckner and Mothes [5]. In the feeding experiments sterile aqueous solutions of the labelled precursors were added under sterile conditions to the culture medium.

### *Isolation of Radioactive Cyclopenin and Cyclopenol from the Culture Medium*

After the feeding time given for each experiment in Tables 1 and 3, the nutrient solution was filtered off and made alkaline with $NH_3$. The alkaloids were extracted three times with ethylacetate and, after evaporation of the solvent *in vacuo*, separated on a quantitative scale by thin layer chromatography on silica gel $PF_{254}$ (thickness of the layer 1 mm, about 30 mg alkaloid mixture per 200 mm start line, solvent system: benzene—ethylacetate—methanol—water (7:3:0.5:0.02, by vol.). The chromatograms were developed twice. Cyclopenin ($R_F$ 0.5—0.6) and cyclopenol ($R_F$ 0.2—0.4) were detected under ultraviolet light as dark zones. The alkaloids were extracted from the silica gel with acetone—methanol (1:1) in columns containing a layer of 1 g of neutral alumina to retain small amounts of viridicatine and viridicatol. After evaporation of the solvent cyclopenin and cyclopenol were crystallized to constant isotope content from aqueous methanol and acetone—benzene—heptane. The final products were colourless prismatic needles of (—)-cyclopenin (m. p. 183—184°) and colourless prisms of (—)-cyclopenol (m. p. 215°).

### *Degradation of Labelled Cyclopenin and Cyclopenol*

Oxidative degradation with hydrogen peroxide in acetic acid was carried out by the method of Mohamed and Luckner [3]. To about 20 mg of the alkaloid dissolved in 5 ml acetic acid, 1 ml 30% $H_2O_2$ was added and the solution was heated in a boiling water bath. A stream of $CO_2$-free nitrogen was passed first through the reaction mixture and then through a solution of barium hydroxide. The barium carbonate formed was separated by centrifugation, washed with water, alcohol, and ether, and dried.

After 2 hours the reaction mixture was diluted with 25 ml water and extracted four times with ether. From the organic phase benzoic acid and *m*-hydroxy-benzoic acid, respectively, were extracted with dilute aqueous $KHCO_3$. The remaining ether solution, washed with water, and dried with $Na_2SO_4$ was evaporated to dryness. The residue, recrystallized several times from methanol—water, gave 3-*N*-methyl-1,2,3,4-tetrahydroquinazolin-(2,4)-dione, m. p. 237—238°. The aromatic acids were extracted with ether from the $KHCO_3$-solution after acidification with HCl. The dry ether extract on evaporation gave the crude acid which on repeated recrystallizations, first from ether—chloroform—heptane and then from water, yielded colourless crystals of benzoic acid, m. p. 122° and *m*-hydroxybenzoic acid, m. p. 205°, respectively. Yields of degradation products were about 20%.

Acid degradation and pyrolysis were carried out by the methods of Birkinshaw *et al.* [2] and Luckner *et al.* [18], respectively.

Table 1. *Incorporation of radioactive labelled amino acids into cyclopenin and cyclopenol*
In Method A, cultures were grown for 4 days in 200 ml Erlenmeyer flasks on 50 ml nutrient solution. From the 5th to the 7th day three times 50 μmoles of the radioactive precursor dissolved in water were added to each flask. The cultures were harvested on the 10th day after inoculation. In Method B, cultures were grown in 2 l Erlenmeyer flasks on 400 ml nutrient solution. From the 5th to the 7th day three times 400 μmoles of the radioactive precursor dissolved in water were added to each flask. The cultures were harvested on the 9th day after inoculation. To the cultures, besides the radioactive precursor, 10 times the amount of the inactive substance listed was added

| Method | Precursor | Specific activity | Inactive substances added | Alkaloids formed | | | |
|---|---|---|---|---|---|---|---|
| | | | | Cyclopenin | | Cyclopenol | |
| | | | | Specific activity | Specific incorporation | Specific activity | Specific incorporation |
| | | $10^5$ counts $\times min^{-1} \times \mu mole^{-1}$ | | $10^3$ counts $\times min^{-1} \times \mu mole^{-1}$ | % | $10^3$ counts $\times min^{-1} \times \mu mole^{-1}$ | % |
| A | [*Carboxy*-$^{14}$C]anthranilic acid | 4.85 | — | 11.9 | 2.5 | 7.5 | 1.5 |
| A | DL-[1-$^{14}$C]Phenylalanine | 1.75 | — | 49 | 28 | 32.9 | 19 |
| | | | DL-Tyrosine | 47.5 | 27 | 32.3 | 18 |
| | | | DL-*m*-Tyrosine | 53.5 | 31 | 35.7 | 20 |
| | | | DL-3,4-Dihydroxy-phenylalanine | 58.3 | 33 | 31.0 | 18 |
| A | DL-[2-$^{14}$C]Phenylalanine | 1.55 | — | 59.5 | 38 | 35.5 | 23 |
| | | | Na-cinnamate | 51.5 | 33 | 40.6 | 26 |
| A | DL-[3-$^{14}$C]Phenylalanine | 0.173 | — | 3.47 | 20 | — | — |
| B | DL-[2-$^{14}$C]Phenylalanine | 0.37 | — | 5.7 | 15 | 2.14 | 5.8 |
| B | D-[U-$^{3}$H]Phenylalanine | 5.4 | — | 29 | 5.4 | 6.54 | 1.2 |
| | | | L-Phenylalanine | not isolated | | 2.78 | 0.5 |
| B | DL-[*Me*-$^{14}$C]Methionine | 0.466 | — | 3.87 | 8.3 | 1.03 | 2.1 |
| A | L-[U-$^{14}$C]Tyrosine | 2.5 | — | 3.15 | 1.30 | 1.76 | 0.70 |
| | | | DL-Phenylalanine | 1.71 | 0.69 | 1.02 | 0.41 |
| | | | DL-*m*-Tyrosine | 2.32 | 0.93 | 1.33 | 0.53 |
| | | | DL-3,4-Dihydroxy-phenylalanine | 2.29 | 0.92 | 1.08 | 0.43 |
| A | DL-*m*-[U-$^{3}$H]Tyrosine | 2.45 | — | 3.5 | 1.40 | 1.8 | 0.74 |
| | | | DL-Phenylalanine | 0.62 | 0.25 | 0.27 | 0.11 |
| B | DL-*m*-[2-$^{14}$C]Tyrosine | 2.06 | — | 0.28 | 0.14 | 0.16 | 0.08 |
| B | *m*-Carboxy-DL-[U-$^{3}$H]-phenylalanine | 42 | — | 3.2 | 0.076 | practically inactive | |
| | | | DL-Phenylalanine | 1.6 | 0.038 | | |

## RESULTS AND DISCUSSION

The experiments with radioactive labelled precursors (Tables 1 and 2) confirm the biosynthetic pathway for cyclopenin and cyclopenol deduced from their chemical structure (Fig. 1) as well as from biosynthetic studies on viridicatin and viridicatol [5]. The total carbon skeleton of both alkaloids is formed from all the C-atoms of anthranilic acid and phenylalanine and from the methyl group of methionine. The specifity of the incorporation was proved by three kinds of degradation (Fig. 1), which enabled us to determine independently the isotope content in the two nitrogen and the carbon atoms of cyclopenin and cyclopenol deriving from C-1, C-2, C-3, and the ring of phenylalanine, from the carboxyl group and the ring of anthranilic acid, and from the methyl group of methionine.

Evidently the L-isomer of phenylalanine is the direct alkaloid precursor. Feeding experiments with D-[U-$^{3}$H]phenylalanine resulted in a specific incorporation rate of about one third of that found for the DL-form. A further decrease of D-phenylalanine incorporation was observed if L-phenylalanine was added simultaneously to the nutrient solution (Table 1). The rather good incorporation of D-phenylalanine without randomization may be due to the transformation into the L-isomer *via* phenylpyruvic acid.

In the usual feeding experiments (see legend to Table 1), the radioactive precursors were added on the 5th, 6th, and 7th day to cover the mean period of alkaloid biosynthesis. Under these conditions, the specific incorporation rates into cyclopenol were always lower than into cyclopenin. At least partly

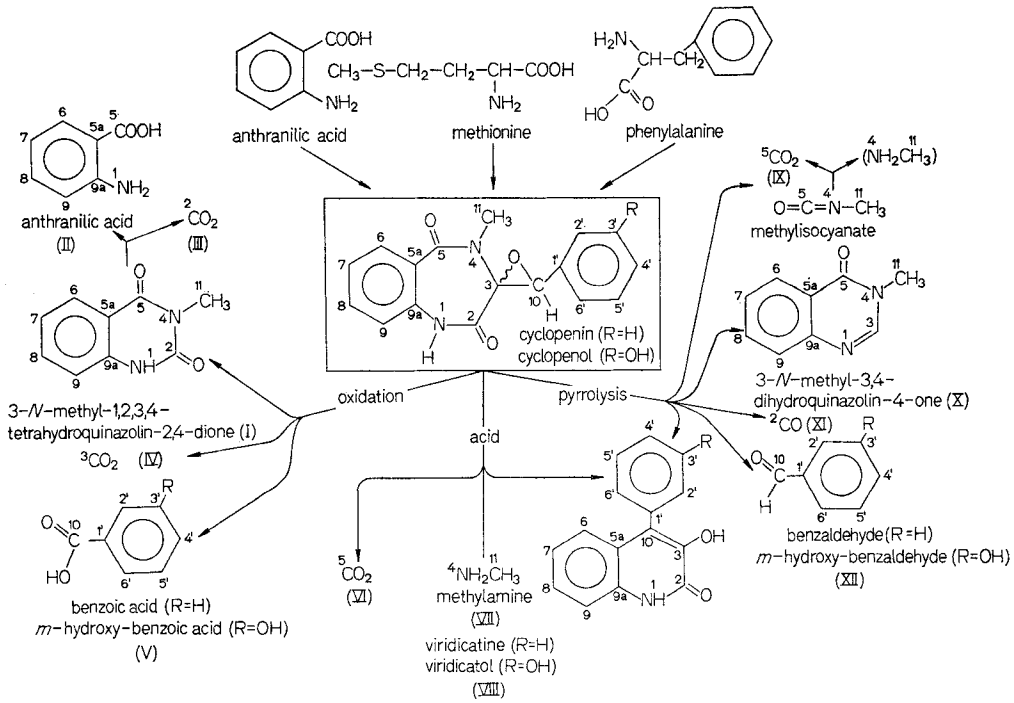


Fig. 1. *Biosynthesis and degradation of cyclopenin and cyclopenol.* The numbering of the degradation products corresponds to that of the parent substances cyn and cyl.

Table 2. *Degradation of radioactive labelled cyclopenin and cyclopenol*
Radioactivity of the degradation products I—XII (Fig. 1) is given in percent of the total activity of the alkaloid degraded, numbers in brackets represent the theoretical values

| Precursor | Alkaloid formed | Oxidation | | | | | Acid | | | Pyrolysis | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | I | II | III[a] | IV[a] | V | VI[a] | VII | VIII | VIII | IX[a] | X | XI[a] | XII |
| | | % | % | % | % | % | % | % | % | % | % | % | % | % |
| [*Carboxy*-$^{14}$C]-anthranilic acid | [5-$^{14}$C]Cyclopenin | 95 (100) | 99 (100) | — | 5 (0) | 0 (0) | 57 (100) | — | 0 (0) | — | — | — | — | — |
| | [5-$^{14}$C]Cyclopenol | — | — | — | — | — | 48 (100) | — | 0 (0) | 8 (0) | 45 (100) | 110 (100) | 4 (0) | 1 (0) |
| DL-[1-$^{14}$C]-Phenylalanine | [2-$^{14}$C]Cyclopenin | 94 (100) | 0 (0) | 90 (100) | 5 (0) | — | 3 (0) | 0 (0) | 105 (100) | — | — | — | — | — |
| | [2-$^{14}$C]Cyclopenol | — | — | — | — | — | — | — | — | 79 (100) | 2 (0) | 9 (0) | 22 (100) | — |
| DL-[2-$^{14}$C]-Phenylalanine | [3-$^{14}$C]Cyclopenin | 3 (0) | — | — | 65 (100) | — | — | — | — | — | — | — | — | — |
| | [3-$^{14}$C]Cyclopenol | — | — | — | — | — | — | — | — | 74 (100) | 0 (0) | 70 (100) | 0 (0) | 1 (0) |
| DL-[3-$^{14}$C]-Phenylalanine | [10-$^{14}$C]Cyclopenin | 2 (0) | — | — | 3 (0) | 106 (100) | — | — | — | — | — | — | — | — |
| DL-[*Me*-$^{14}$C]-Methionine | [11-$^{14}$C]Cyclopenol | — | — | — | — | — | — | 72 (100) | 0 (0) | — | — | — | — | — |

[a] In all cases more than the molar amount was formed, so that the specific activity is too low.

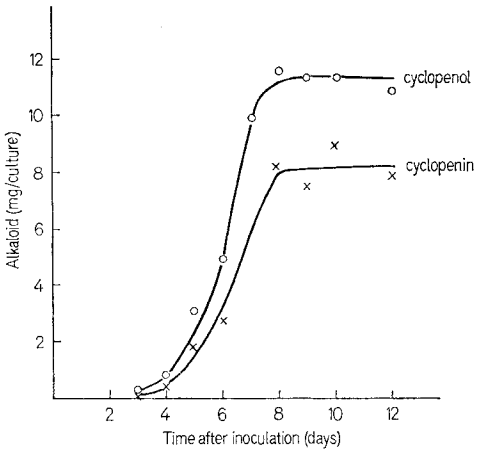


Fig. 2. *Alkaloid content in the culture medium of* Penicillium cyclopium *Westling*. Cultures were grown in 200 ml Erlenmeyer flasks on 50 ml nutrient solution. The isolation of the alkaloids followed the procedure given for the radioactive experiments. After preparative thin layer chromatography the content of cyclopenin and cyclopenol was determined by heating the alkoloid sample with 3 ml $FeCl_3$-reagent (2 ml conc. HCl + 1 ml 10% aqueous $FeCl_3$ + 97 ml water) 10 min in a boiling water bath. After cooling and addition of 3 ml ethanol the absorbance of the green solution was determined at 590 nm

phenylalanine. This assumption is supported by $^{15}N$-experiments, the results of which are presented in Table 3. A specific incorporation of the $NH_2$-groups of anthranilic acid into N-1 and for phenylalanine into N-4 of the diazepine ring was found. The N-isotope enrichment in the alkaloid formed was lower than that calculated from the $^{3}H$- and $^{14}C$-incorporation rates of the amino acids in the same experiments. For phenylalanine that can easily be explained by reversible transamination. Involvement of a phenylalanine ammonia-lyase (EC 4.3.1) is improbable because the reaction catalyzed by this enzyme is irreversible [19] and is further excluded by the results of a competition experiment with cinnamic acid (Table 1). The considerably smaller loss of $^{15}N$ in the experiment with anthranilic acid may not be significant. In these experiments the feeding time had to be shortened up to 10 hours to keep unspecific incorporation low. Earlier attempts (feeding time 72 hours) failed because a total randomization of $^{15}N$ from both amino acids had occurred.

With regard to the origin of the *m*-hydroxyl group in cyclopenol, results shown in Tables 1—3 prove phenylalanine to be a specific precursor of this hydroxylated derivative, too. The incorporation rate

Table 3. *Origin of the nitrogen atoms in cyclopenol*
Cyclopenin was not isolated in these experiments because of the small amount formed during the short feeding time necessary. The $^{15}N$ enrichment is given after subtraction of the natural $^{15}N$ content. Cultures were grown in 2 l Erlenmeyer flasks on 400 ml nutrient solution. At the 5th day after inoculation 250 ml of the nutrient solution were removed and 500 µmole of the labelled precursor were added. After 10 hours the cultures were harvested

| Precursor | Specific activity | $^{15}N$ enrichment | Alkaloid isolated and its acid degradation products (see Fig. 1) | Specific activity | $^{15}N$ enrichment | | |
|---|---|---|---|---|---|---|---|
| | | | | | Incorporation rate | Calc. | Found |
| | $10^5$ counts × $min^{-1}$ × µmole$^{-1}$ | % | | $10^3$ counts × $min^{-1}$ × µmole$^{-1}$ | % | | |
| [U-$^3$H,$^{15}$N]Anthranilic acid | 1.33 | 51 | Cyclopenol | 2.37 | 1.78 | 0.45 [b] | 0.37 |
| | | | Viridicatol (VII) | | | 0.74 [c] | 0.74 |
| | | | Methylamine (VI) | | | 0.0 [c] | 0.0 |
| DL-[2-$^{14}$C]Phenylalanine and L-[$^{15}$N]phenylalanine | 1.20 [a] | 53 | Cyclopenol | 4.4 | 3.65 | 0.96 [b] | 0.22 |
| | | | Viridicatol (VII) | | | 0.0 [c] | 0.0 |
| | | | Methylamine (VI) | | | 0.44 [c] | 0.35 |

[a] Calculated for L-phenylalanine.
[b] Values are calculated from the incorporation rate of the radioactivity of the precursor.
[c] Values are calculated from the isotope enrichment found in the cyclopenol formed.

this effect is due to the ratio between unlabelled alkaloid present at the beginning of the feeding experiments and alkaloid formed after addition of the radioactive precursor, which is about 1:2.8 for cyclopenol and 1:4.5 for cyclopenin (Fig. 2). Whether there are still other factors unequally influencing the incorporation rates is under current investigation.

As mentioned in the introduction the biosynthesis of cyclopenin and cyclopenol presumably proceeds *via* the cyclic dipeptide of anthranilic acid and

of this amino acid is not influenced by simultaneous addition of inactive tyrosine, *m*-tyrosine and dihydroxy phenylalanine (Table 1). Moreover, radioactivity from tyrosine, *m*-tyrosine, and *m*-carboxyphenylalanine was incorporated only unspecifically into cyclopenin and cyclopenol as well, showing a lower incorporation rate into cyclopenol as was found also for phenylalanine. The results with *m*-tyrosine are in contrast to earlier ones in our laboratory (published preliminarily [20]). By degradation of cyclopenin formed after feeding of

[U-$^{14}$C] tyrosine it was shown that total randomization of radioactivity had occurred.

Thus, for the biosynthesis of cyclopenol the dihydroxylation-dehydroxylation mechanism at least on the level of the phenylpropane compounds, the direct hydroxylation of phenylalanine, and the formation of cyclopenol from shikimic acid without involvement of phenylalanine as the main pathway were ruled out. Therefore a direct *m*-hydroxylation catalyzed by a mixed functional oxygenase as one of the later steps of cyclopenol formation appears very likely [21].

We are grateful to Dr. D. Munsche (Halle) for a gift of DL-[*Me*-$^{14}$C]methionine and to Dr. P. O. Larsen (Copenhagen) for a gift of *m*-carboxy-phenylalanine.

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