MIXED FUNCTIONAL OXYGENATIONS DURING THE BIOSYNTHESIS OF CYCLOPENIN AND CYCLOPENOL, BENZODIAZEPINE ALKALOIDS OF *PENICILLIUM CYCLOPIUM* WESTLING.

INCORPORATION OF MOLECULAR OXYGEN AND NIH-SHIFT

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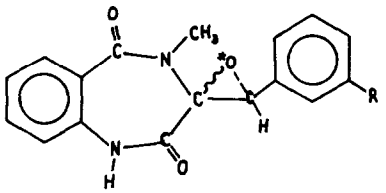
1. Introduction

(−)-Cyclopenin (cyn) and (−)-cyclopenol (cyl) (fig. 1) are benzodiazepine alkaloids produced by certain strains of *Penicillium cyclopium* Westling [1]. From the chemical and biochemical point of view both alkaloids are derivatives of the cyclic dipeptide of anthranilic acid and L-phenylalanine [2, 3]. The epoxide ring in cyn and cyl and the *m*-hydroxy group in cyl suggest the participation of mixed functional oxygenases (E.C. 1.14), catalysing the incorporation of molecular oxygen in the biosynthesis of both compounds.

Epoxides are the primary products of the mixed functional oxygenation of unsaturated substrates (for a summary see [4]). During aromatic hydroxylations the epoxide intermediates are unstable and rearrange to the hydroxy compounds via carbonium ions which usually stabilize by a 1,2-anionotropy, the NIH-shift [5]. This process results in a translocation of the hydrogen (or other substituents) replaced by the hydroxy group to the *o*-position. The hydroxy group of cyl offers an opportunity to study the NIH-shift induced by a *m*-hydroxylation.

In this paper we present results of experiments on the incorporation of molecular oxygen into the epoxide ring of cyn and cyl and into the *m*-hydroxy group of cyl, and also on the NIH-shift connected with the *m*-hydroxylation during cyl biosynthesis and with the *p*-hydroxylation of phenylalanine.



R = H = cyclopenin
R = *OH = cyclopenol

Fig. 1. (Oxygens deriving from molecular oxygen are indicated by an asterisk.)

Abbreviations: cyn = cyclopenin, cyl = cyclopenol.

2. Materials and methods

D,L-Phenylalanine-^{14}COOH and $^{18}O_2$ were commercial products. *o*-, *m*-, and *p*-T-D,L-phenylalanine were prepared by reductive tritiation of *o*-bromo-, *m*-chloro-, and *p*-chloro-D,L-phenylalanine respectively

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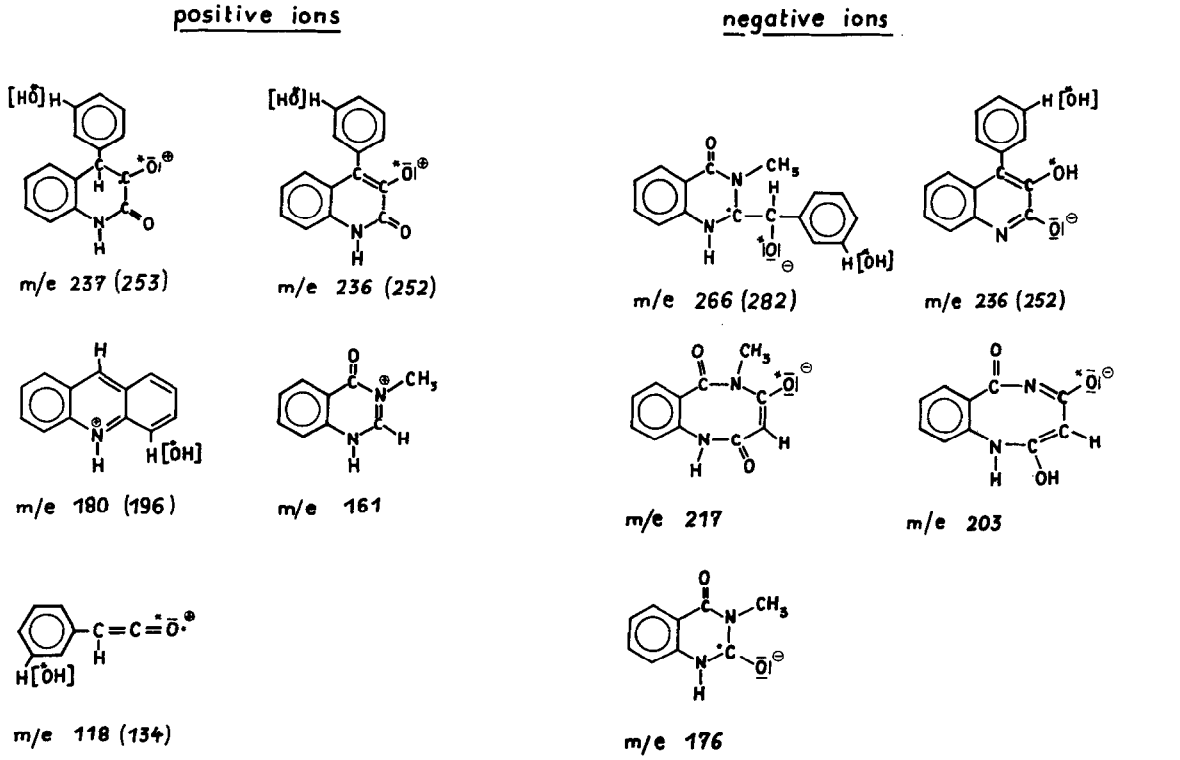

Fig. 2. Most probable structures of the positive and negative fragment ions of table 1 (oxygens deriving from the epoxide and the *m*-hydroxy group are indicated by an asterisk).

on a Pd-charcoal catalyst *. Oxidation of the tritiated phenylalanines to benzoic acid and determination of the T-distribution according to ref. [6] proved about 98% of the radioactivity to be localized in the indicated positions.

For culture of *Penicillium cyclopium*, isolation of the alkaloids formed and measurement of the radioactivity see ref. [3].

The mass spectra were recorded on the Dresdener Molekülmassenspektrographen of the Forschungsinstitut M. v. Ardenne, Dresden [7] **. For a detailed discussion of the spectra see refs. [8, 9].

* We gratefully acknowledge gifts of *m*- and *p*-chloro-D,L-phenylalanine from Chas. Pfizer & Co., Inc., Groton (USA). The reductive tritiation done by Dr. Uhlenhut (Leipzig) is very much appreciated.

** We thank Dr. R.Tümmler for recording the mass spectra.

2.1. *Isolation, iodination and decarboxylation of* L-*tyrosine*

Fresh mycelium of *P. cyclopium* (about 1.5 g dry weight) was thoroughly washed and homogenized in 20% acetic acid. The homogenate was centrifuged and the clear supernatant evaporated to dryness in vacuo. The residue was extracted with alcohol-HCl (10:1). After filtration 1 mmole of inactive L-tyrosine was dissolved in the extract and the amino acid precipitated with pyridine. It was recrystallized to constant specific activity from 50% acetic acid.

The L-tyrosine obtained was iodinated with KI/I_2 in an aqueous solution of methylamine to give 3,5-diiodo-L-tyrosine, which was recrystallized from 60% acetic acid, m.p. 195–7°.

For the thermic decarboxylation of tyrosine in diphenylamine see ref. [10].

Table 1

Specific incorporation of molecular oxygen ($^{18}O_2$) into cyclopenin and cyclopenol. Mass spectrographic evaluation of the isotope distribution.

	Positive spectra							Negative spectra					
m/e **	Cyclopenin peak intensity *		^{18}O-enr.	Cyclopenol peak intensity *		^{18}O-enr.	*m/e* **	Cyclopenin peak intensity *		^{18}O-enr.	Cyclopenol peak intensity *		^{18}O-enr.
	air	$^{18}O_2$	% ***	air	$^{18}O_2$	% ***		air	$^{18}O_2$	% ***	air	$^{18}O_2$	% ***
294 (310)	0.910	1.005	(O_e)	0.193	0.365	(O_e, O_h)	294 (310)	0.095	0.045	(O_e)	0.728	0.358	(O_e, O_h)
296 (312)	0.026	0.185	13	0.000	0.049	12	296 (312)	0.002	0.007	12	0.041	0.007	14
237 (253)	0.235	0.324	(O_e)	0.477	0.354	(O_e, O_h)	266 (282)	0.120	0.126	(O_e)	0.423	0.205	(O_e, O_h)
239 (255)	0.000	0.029	8	0.011	0.054	11	268 (284)	0.000	0.011	8	0.011	0.025	9
236 (252)	0.177	0.227	(O_e)	0.321	0.235	(O_e, O_h)	236 (252)	0.221	0.191	(O_e)	0.731	0.305	(O_e, O_h)
238 (254)	0.015	0.072	19	0.045	0.053	11	238 (254)	0.000	0.020	9	0.029	0.046	10
180 (196)	0.175	0.248	(–)	0.209	0.082	(O_h)	217	0.970	0.953	(O_e)	1.755	1.480	(O_e)
182 (198)	0.000	0.002	1	0.006	0.010	9	219	0.035	0.164	12	0.111	0.149	4
161	0.673	0.880	(–)	0.248	0.235	(–)	203	0.106	0.095	(O_e)	0.493	0.205	(O_e)
163	0.012	0.019	1	0.000	0.000	0	205	0.000	0.012	11	0.013	0.015	5
118 (134)	0.325	0.519	(O_e)	0.127	0.105	(O_e, O_h)	176	0.205	0.243	(–)	1.023	0.560	(–)
120 (136)	0.110	0.228	10	0.000	0.015	13	178	0.000	0.000	0	0.019	0.006	–1

P.cyclopium was cultured on 400 ml nutrient solution in a 2 l Erlenmeyer flask with a central tube. In experiments with $^{18}O_2$ after 6 days the air in the culture vessel was removed by oxygen-free nitrogen. An ampoule containing 1 l O_2 (23% ^{18}O-enrichment) was fitted to the top of the flask. The labelled oxygen was consumed by the mould within 2–3 days. It was substituted continuously by oxygen-free nitrogen.

* The peak intensities are corrected with respect to the natural ^{13}C and ^{15}N content of the ions.

** If two figures are given for the *m/e* values the first refers to the cyn and the second to the cyl spectra. The peaks with *m/e* 294 (310) represent the molecular ions. For the most probable structures of the fragment ions see fig. 2.

*** (O_e) and (O_h) indicate the presence of the oxygen derived from the epoxide ring and from the *m*-hydroxy group respectively, (–) indicates the absence of both (cf. the formula in fig. 2).

Table 2
Specific incorporation of D,L-phenylalanine-T-^{14}C into cyn, cyl and tyrosine by *P. cyclopium*. Hydroxylation induced NIH-shift.

	o-T-D,L-phenylalanine-^{14}COOH			*m*-T-D,L-phenylalanine-^{14}COOH			*p*-T-D,L-phenylalanine-^{14}COOH		
	T	^{14}C	T/^{14}C	T	^{14}C	T/^{14}C	T	^{14}C	T/^{14}C
	I/min nmole			I/min nmole			I/min nmole		
D,L-phenylalanine	5780	395	14.6	5260	453	11.6	3660	438	8.4
Cyclopenin	1000	70.4	14.2	541	47.8	11.3	422	60.1	7.0
Cyclopenol	241	17.6	13.7	111	14.5	7.6	118	16.9	7.0
L-tyrosine *	4.95	0.49	10.2	0.80	0.12	6.8	2.00	0.40	5.0
3,5-diiodo-L-tyrosine	5.30	0.46	11.5	0.03	0.12	–	0.08	0.34	–
Tyramine	5.25	0.01	–	0.20 **	0.02	–	0.20 **	0.01	–
CO_2 ***	–	0.27		–	0.06		–	0.21	

Cultures of *P. cyclopium* were grown on 50 ml nutrient solution in 200 ml Erlenmeyer flasks. On the 6th and 7th day after inoculation 50 μmole D,L-phenylalanine-T-^{14}C was added to each flask. The cultures were harvested on the 8th day.

* The low specific radioactivity of tyrosine compared with cyn and cyl is due to the dilution with inactive tyrosine during the isolation procedure.

** The considerable loss of tritium during the thermic decarboxylation is probably due to a diphenylamine catalyzed exchange of the hydrogen in *o*-position to the hydroxy group. As to be expected no exchange is observed in that tyrosine derived from *o*-T-phenylalanine.

*** In all three cases the CO_2 contained only about 50% of the calculated specific radioactivity. Because the tyramine formed was almost free of ^{14}C evidently only approximately half of the CO_2 derives from the carboxy group of tyrosine.

3. Results and discussion

Feeding of $^{18}O_2$ to cultures of *P. cyclopium* and subsequent mass spectrographic evaluation of the ^{18}O-content of cyn and cyl formed (table 1) prove the epoxide oxygen present in both alkaloids and the *m*-hydroxy group of cyl to derive from molecular oxygen. These groups therefore are introduced by mixed functional oxygenases. The isotope enrichment of both alkaloids was about 13%. In cyl it is almost evenly distributed between the two labelled oxygen atoms. Therefore the ^{18}O-enrichment per ogygen labelled in cyn is twice that in cyl, a consequence of the fact that at the beginning of the feeding experiment more cyl was present than cyn [3].

To show the specificity of the oxygen incorporation, the data of the molecular ions are compared with those of a number of fragment ions in table 1. The most probable structures of the selected fragment ions, without consideration of resonance forms, are reproduced in fig. 2.

To characterize the *m*-hydroxylating enzyme system participating into cyl biosynthesis, experiments with specifically ring-tritiated D,L-phenylalanine-^{14}COOH were performed (table 2). Only with *m*-T-D,L-phenylalanine-^{14}COOH a change of the T/^{14}C ratio in cyl due to a specific loss of tritium could be observed, though more than half of the T-label is retained. This indicates a partial NIH-shift, which in two independent experiments resulted in the same T-retention of about 35%, assuming that only one of the two *m*-positions is hydroxylated.

In addition to the alkaloids L-tyrosine was isolated in these experiments. The results of its degradation (iodination and thermic decarboxylation) (table 2) demonstrate that it had been formed directly from phenylalanine by a *p*-hydroxylation connected with a NIH-shift. In this case the T-retention is at least 60%, based on the T/^{14}C ratio in the *p*-T-D,L-phenylalanine-^{14}COOH. However, the usual value of the T-retention (>90%) is obtained, taking into account the presently unexplainable diminution of the

T/^{14}C ratio also found in those tyrosine preparations deriving from *o*- and *m*-T-phenylalanine-^{14}COOH respectively.

The above results are the basis for a more detailed comparison of the *m*- and *p*-hydroxylating enzyme systems of *P. cyclopium*. Experiments with cell-free preparations are under investigation.

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