Communications to the Editor

[Chem. Pharm. Bull. 34(8)3534—3537(1986)]

BIOSYNTHESIS OF PATULIN; IN VITRO CONVERSION OF GENTISYL ALCOHOL INTO PATULIN BY MICROSOMAL ENZYME(S) AND RETENTION OF ONE OF THE CARBINOL PROTONS IN THIS REACTION

Hiroshi Iijima, Yutaka Ebizuka and Ushio Sankawa*

Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo 113, Japan

The late stage of patulin biosynthesis was studied using cell-free preparations of Penicillium patulum NRRL 2159A. The substrate for the ring cleavage reaction was established to be gentisyl alcohol, which was converted into patulin by a microsomal enzyme(s). Incubation of [1'-^{14}C,$^{3}H_2$]-gentisyl alcohol with the microsomal preparation revealed that one of the carbinol protons of this substrate is retained in patulin. This answers the long-standing question why the side chain protons of aromatic intermediates are not incorporated into patulin in feeding experiments.

KEYWORDS —— patulin; biosynthesis; mycotoxin; Penicillium patulum; gentisyl alcohol; gentisaldehyde; microsome; monooxygenase

Patulin is one of the most commonly occurring mycotoxins produced by a number of Penicillia and Aspergilli. Its biosynthesis has attracted considerable interest among many workers.[1] Some of the enzymes involved in the patulin biosynthesis have been studied extensively,[2,3] and gentisaldehyde has been regarded as the last aromatic intermediate leading to patulin (Fig. 1). Scott et al. reported the in vitro conversion of gentisaldehyde to patulin by a soluble cell-free preparation of P. patulum in the presence of NADPH and ATP. They suggested a dioxygenase mechanism for the ring cleavage reaction.[3]

Meanwhile we conducted feeding experiments using $^{18}O_2$ and [1-^{13}C,$^{18}O_2$]-acetate as tracers and found that a monooxygenase rather than a dioxygenase is involved in the ring cleavage of the aromatic intermediate.[4] This led us to re-examine the in vitro formation of patulin with cell-free systems employing gentisaldehyde and gentisyl alcohol as alternate substrates, since most monooxygenases in eukaryotic organisms are found in the particulate fractions.[5]

P. patulum NRRL 2159A was shake-cultured for 50 h at 28° C in Czapek-Dox

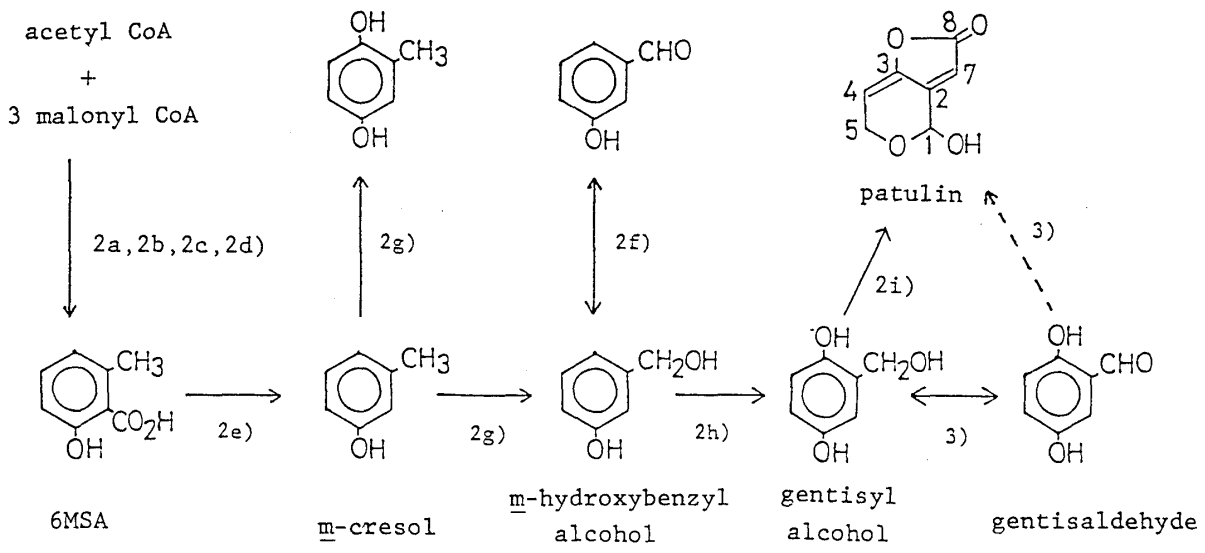

Fig.1. Biosynthetic Scheme of Patulin

Figures indicate references where corresponding enzymes are described.

medium as described.[4] Fresh mycelia were homogenized in 50 mM potassium phosphate buffer (pH 7.5), containing 4 mM $MgCl_2$ and 2 mM ascorbate using a Waring blender. After low-speed centrifugation (10,000 g for 20 min), the crude cell-free extract was separated into soluble and particulate protein fractions by centrifugation at 100,000 g for 60 min. Both fractions were further centrifuged at 100,000 g for 60 min to avoid mutual contamination.

[ring-^{3}H]-Gentisaldehyde[6] or [ring-^{3}H]-gentisyl alcohol[7](1.5 μM; 4.2 x 10^4 dpm) was incubated with enzyme preparations (soluble protein, 0.2 mg or microsomal protein, 3 mg) in the presence of dimethylpteridine (13 μM), α-ketoglutarate (300 μM) and NADPH (400 μM) at 30°C for 3 h. Non-labeled patulin (20 μg) was added to each reaction mixture and extracted with AcOEt after acidification with N-HCl. An aliquot of this extract was subjected to HPLC[8] and the radioactivity in the patulin peak was determined by a liquid scintillation counter. Patulin was formed only when gentisyl alcohol was incubated with the microsomal fraction. In order to verify the formation of patulin, the rest of the AcOEt extract was purified by preparative TLC.[9] To this, carrier patulin (100 mg) was added and the product was recrystallized from $CHCl_3$ to give a constant specific activity (conversion ratio, 0.27%). Thus the substrate for ring cleavage was determined to be gentisyl alcohol rather than gentisaldehyde. The former substrate was converted into patulin by microsomal enzyme(s).

A most interesting question remained unsolved in the patulin biosynthesis: Why in feeding experiments are the side chain protons of aromatic precursors such as m-hydroxybenzyl alcohol, gentisyl alcohol and gentisaldehyde not incorporated into the corresponding patulin hemiacetal proton at C-1 ?[4,10] To answer this question the behavior of the carbinol protons of gentisyl alcohol was investigated using [1'-^{14}C,$^3H_2$]-gentisyl alcohol as a substrate.

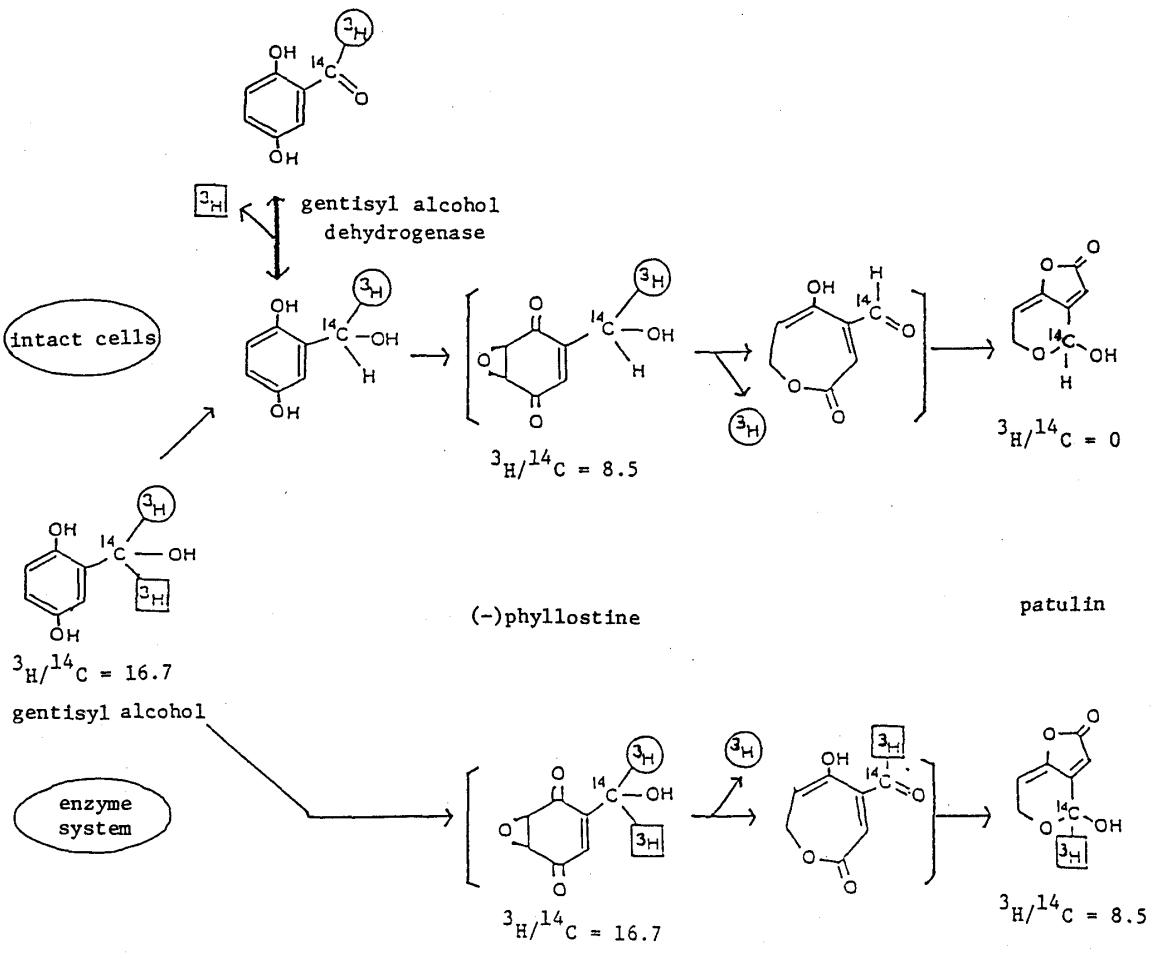

Fig. 2

[1'-^{14}C,$^3H_2$]-Gentisyl alcohol[11] (1.8 μmol, ^3H; 1.00 x 10^7dpm, ^{14}C; 6.0 x 10^5dpm, $^3H/^{14}C$=16.7) was incubated with microsomal protein (5.6 mg) at 30°C for 2 h, and worked up as described above. Then carrier patulin (100 mg) was added to the product. Even after repeated recrystallization this patulin sample retained about half of the ^3H relative to ^{14}C ($^3H/^{14}C$=8.5. conversion ratio based on ^{14}C, 0.03%). The same substrate (30 mg) incubated with washed mycelia (2.5 g, fr. wt.) at 30°C for 12 h yielded patulin devoid of ^3H (conversion ratio based on ^{14}C, 7.7%).

This clearly demonstrates that the loss of both carbinol protons of gentisyl alcohol is not a mechanistic requirement for the conversion into patulin. The loss can be attributed to the presence of gentisyl alcohol dehydrogenase, which is reported to occur in the soluble protein fraction.[3] Thus we can conclude that in intact cells, gentisyl alcohol dehydrogenase, which is not on the direct route to patulin, first exchanges one of prochiral carbinol protons by rapidly reversible oxido-reduction between gentisyl alcohol and gentisaldehyde, and then a microsomal enzyme(s) removes the other proton of opposite chirality in the course of conversion into patulin, possibly via (-)phyllostine whose intermediacy is

suggested by Gaucher et al.[12] (Fig. 2).

ACKNOWLEDGEMENT This work was supported in part by a Grant-in Aid to Y.E. from the Ministry of Education, Science and Culture, which is gratefully acknowledged.

REFERENCES AND NOTES

1) L.Zamir, "The Biosynthesis of Mycotoxins," ed. by P.S.Steyn, Academic Press, New York, 1980, p.223.

2) a) P.Dimroth, H.Walter and F.Lynen, Eur. J. Biochem., 13, 98 (1970); b) A.I.Scott, T.P.Gareth and U.Kicheis, Bioorg. Chem., 1, 380 (1971); c) A.I.Scott, L.C.Beadling N.Georgopapadakau and C.R.Subbarayan, Bioorg. Chem., 3, 238 (1974); d) P.Dimroth, E.Ringelmann and F.Lynen, Eur. J. Biochem., 68, 591 (1976); e) R.J.Light, Biochim. Biophys. Acta, 191, 430 (1969); f) P.I.Forrester and G.M.Gaucher, Biochemistry, 11, 1108 (1972); g) G.Murphy, G.Fogel, G.Krippahl and F.Lynen, Eur. J. Biochem. 49, 443 (1974); h) G.Murphy and F.Lynen, Eur. J. Biochem., 58, 467 (1975); i) This paper.

3) A.I.Scott and L.Beadling, Bioorg. Chem., 3, 281 (1974).

4) H.Iijima, H.Noguchi, Y.Ebizuka, U.Sankawa and H.Seto, Chem. Pharm. Bull., 31, 362 (1983).

5) O.Hayaishi, "Molecular Mechanism of Oxygen Activation," ed. by O.Hayaishi, Academic Press, New York, 1974, p.1.

6) Prepared by exchange reaction with $^{3}H_2O$ in the presence of triethylamine as a catalyst.

7) Prepared from [ring-^{3}H]-gentisaldehyde by $NaBH_4$ reduction.

8) Column: TSK-LS410(4.6 x 300 mm); solvent: 30 % aq. MeOH containing 1% AcOH; detection: UV_{254}; flow rate: 0.6 ml/min; Rt(patulin): 7.5 min.

9) Precoated TLC plate (E.Merck, Art.5715), Benzene/Dioxane/AcOH=95/25/4.

10) A.I.Scott, L.Zamir, G.T.Phillips and M.Yalpani, Bioorg. Chem., 2, 124 (1973).

11) [1'-^{14}C]-Gentisyl alcohol was prepared from [1'-^{14}C]-salicylic acid by Elbs oxidation, methylation with CH_2N_2 and reduction with LAH. This sample was mixed with [1'-$^{3}H_2$]-gentisyl alcohol prepared from gentisaldehyde by [^{3}H]-$NaBH_4$ reduction.

12) a) J.Sekiguchi and G.M.Gaucher, Biochemistry, 17, 1785 (1978); b) Idem, Biochemical J., 182, 445 (1979); c) Idem, Can. J. Microbiol., 25, 881 (1979).

(Received June 18, 1986)