ACTA CHEMICA SCANDINAVICA 18 (1964) 1591–1595

The Role of Propionic Acid in *Penicillium baarnense*. Formation of Homoorsellinic Acid by Utilization of Propionic Acid

KLAUS MOSBACH

Institute of Biochemistry, University of Lund, Lund, Sweden

From the medium of *Penicillium baarnense* a new metabolite has been isolated and shown to be 2-ethyl-4,6-dihydroxy-benzoic acid (homoorsellinic acid). This compound, the synthesis of which is described in this paper, could only be detected in cultures grown on Czapek–Dox medium when supplied with propionic acid. By the administration of $CH_3CH_2{}^{14}COONa$, radioactive homoorsellinic acid was obtained. Results from the chemical degradation suggest a biosynthetic route of formation in which one propionyl-CoA unit is condensed with three malonyl-CoA units. The added propionate-1-^{14}C gave rise to radioactive orsellinic acid as well, the labeling pattern of which was shown to correspond to that obtained from acetate-1-^{14}C.

Several compounds are known in which propionic acid participates as a structural unit. In the case of fatty acids, Bressler and Wakil [1] found, that propionyl-CoA can substitute for acetyl-CoA in condensing with malonyl-CoA to form long-chain fatty acids. Horning *et al.*[2] have reported that a purified fatty acid-synthesizing system from rat adipose tissue catalyzes the synthesis of C_{15} acids from propionyl-CoA and malonyl-CoA. The macrolide aglycones of some antibiotics are derived entirely from C_3 units (erythromycin [3–5]) or from $C_3 + C_2$ units (methymycin [6]). ε-Pyrromycinone,[7] the aglycone in a series of antibiotics, is biosynthesized from nine acetate (or malonate) units and one propionate unit. The above mentioned antibiotics are all produced by *Actinomycetes*. No report has yet appeared on a participation of propionic acid in the biosynthesis of secondary natural products produced by fungi.

Orsellinic acid from *Penicillium baarnense* [8] as well as from the lichen depside gyrophoric acid in *Umbilicaria pustulata* [9] is known to be formed from one acetyl-CoA and three malonyl-CoA. In this paper it is shown that propionyl-CoA can substitute for acetyl-CoA as the "starter" molecule in the formation of the higher homolog of orsellinic acid. Upon addition of sodium propionate (1 mmole/100 ml of Czapek-Dox solution) to a surface culture of *Penicillium*

baarnense a new metabolite could be detected by paper chromatography. This compound appeared in the medium along with orsellinic acid in a ratio of about 1:5. Its chromatographic behaviour as well as its color reactions showed a close similarity to orsellinic acid, thus suggesting the above mentioned substitution of propionate for acetate. In order to prove the identity of this metabolite, 2-ethyl-4,6-dihydroxy-benzoic acid or homoorsellinic acid was synthesized.

This synthesis started off with a condensation of acetoacetic ester with the ethylester of β-ethyl acrylic acid. In analogy to the described synthesis of orsellinic acid,[10–12] the intermediate steps were as follows: ethylester of dihydro-homoorsellinic acid → ethylester of dibromo-homoorsellinic acid → ethylester of homoorsellinic acid → homoorsellinic acid. The hydrolysis of the ester of homoorsellinic acid in 10 % sodium hydroxide at room temperature for some weeks did not proceed without simultaneous decarboxylation. Instead hydrolysis in concentrated sulfuric acid at room temperature for a few hours proved more favorable.

The synthetic homoorsellinic acid was then compared with the new metabolite formed on addition of propionic acid to the medium and showed identical chromatographic behaviour in different solvent systems as well as identical color reactions. To a culture of *Penicillium baarnense* was then administered 0.250 mC of $CH_3CH_2{}^{14}COONa$. After 8 days of incubation the formed radioactive homoorsellinic acid was isolated and after addition of 570 mg of carrier homoorsellinic acid recrystallized to constant radioactivity.

For further identification a part of the radioactive acid was decarboxylated to homoorcinol and submitted to paper chromatography in different solvent systems. Scanning of the papers in a strip counter showed that the R_F values of the radioactive peaks obtained were identical with those of homoorcinol located by spraying with calcium hypochlorite.

In the course of degradation the total radioactivity was determined. Decarboxylation yielded the radioactivity of the carboxyl group. A modified Kuhn—Roth oxidation followed by Schmidt degradation of the propionic acid and total combustion of ethylamine gave the radioactivity of the side chain and its adjacent carbon atom in homoorsellinic acid.

EXPERIMENTAL

Culture conditions. Penicillium baarnense v. Beyma was grown on Czapek—Dox medium as surface culture in a 500 ml Erlenmeyer flask containing 150 ml of medium. After one weeks growth, 0.250 mC of $CH_3CH_2{}^{14}COONa$ with a specific activity of 7 mC/mmole was added to the medium.

Isolation. After 8 days of incubation the mycelium was filtered off and the medium extracted with ether after acidification. To the extract was added a total of 570 mg of synthetic homoorsellinic acid and the formed radioactive homoorsellinic acid isolated by the washing out technique. Repeated recrystallisations from a mixture of water and acetic acid gave 150 mg of homoorsellinic acid of constant radioactivity.

The values and color reactions of homoorsellinic acid and its decarboxylation product homoorcinol were as follows:

	Solvent systems			Color reactions	
	B	D	E	$FeCl_3$	$Ca(ClO)_2$
Homoorsellinic acid	0.63	0.05	0.42	red violet	red
Orsellinic acid	0.47	0.02	0.28	»	»
Homoorcinol	0.57	0.08	0.86	—	»
Orcinol	0.38	0.05	0.90	—	»

The solvent systems used as well as R_F values given for orsellinic acid and orcinol are those reported by L. Reio.[15]

Synthesis of homoorsellinic acid (2-ethyl-4,6-dihydroxy-benzoic acid). *Ethylester of dihydro-homoorsellinic acid.* 3 g of sodium was dissolved in 43 ml abs. ethanol. To the sodium ethylate were added 18.2 g of acetoacetic ester and 18 g of the ethylester of β-ethyl acrylic acid. After refluxing for several hours on a water bath, the formed sodium salt of dihydro-homoorsellinic acid was filtered off, acidified with diluted H_2SO_4 and the free ester extracted with ether. After evaporation of the ether and extensive drying in a desiccator, a whitish solid residue was obtained. The ester was recrystallized from petroleum ether. Yield 10 g, m.p. 68—71°. (Found: C 62.1; H 7.5; O 30.4. Calc. for $C_{11}H_{16}O_4$: C 62.3; H 7.6; O 30.2).

Ethylester of dibromo-homoorsellinic acid. 8 g of the ethylester of dihydro-homoorsellinic acid was dissolved in 32 ml of glacial acetic acid and brominated with 18 g of bromine in 32 ml of glacial acetic acid. The formed crystalline precipitate was filtered off and recrystallized from ethanol. Yield 11.3 g, m.p. 80°. (Found C 36.0; H 3.4; O 17.4. Calc. for $C_{11}H_{12}O_4Br_2$: C 35.9; H 3.3; O 17.4).

Ethylester of homoorsellinic acid. 8 g of the ethylester of dibromo-homoorsellinic acid was dissolved in 130 ml 2 N NaOH and hydrogenated after addition of 10.7 g of catalyst (palladium on calcium carbonate). After removal of the catalyst, the filtrate was acidified with dil. HCl and the precipitate isolated and recrystallized from ethanol. Yield 3.8 g, m.p. 85°. (Found: C 62.8; H 6.8; O 30.6. Calc. for $C_{11}H_{14}O_{14}$: C 63.0; H 6.7; O 30.3).

Homoorsellinic acid. 2.5 g of the ethylester of homoorsellinic acid was dissolved in 12.5 g of conc. H_2SO_4 and kept at room temperature for 3 h. After hydrolysis the mixture was poured onto ice and extracted with ether. The ether phase was treated with 0.5 M $NaHCO_3$. After acidification of the bicarbonate solution the latter was acidified and extracted with ether. Evaporation of the ether gave homoorsellinic acid which was recrystallized from a mixture of water and acetic acid (2.5:1 parts by volyme). Yield 0.8 g, m.p. 168—169°. (Found: C 59.4; H 5.6; O 35.3. Calc. for $C_9H_{10}O_4$; C 59.3; H 5.5; O 35.2).

Homoorcinol. 50 mg of homoorsellinic acid was suspended in 5 ml of glycerol. The decarboxylation was performed in a slow current of N_2 at 155° for 15 min. To the glycerol then was added 50 ml of water and the mixture extracted with ether. After evaporation of the ether a white solid remained which sublimed at 760 mm Hg and 155°. Yield 40 mg, m.p. 98°. (Found: O 23.3. Calc. for $C_8H_{10}O_2$: O 23.2).

Degradation. 5 mg of the radioactive homoorsellinic acid were submitted to wet combustion by the van Slyke—Folch method and the resulting carbon dioxide trapped as barium carbonate. 45 mg of homoorsellinic acid was decarboxylated to homoorcinol by heating in glycerol at 155° and the evolved carbon dioxide isolated as barium carbonate. 100 mg of the same acid was submitted to a modified Kuhn—Roth oxidation.[13] (5 N chromic acid:conc. H_2SO_4:H_2O = 4:1:25 parts by volume). In order to prevent further oxidation of the propionic acid formed, the volatile organic acids were continuously steam distilled during the course of reaction. The evolved carbon dioxide was flushed through the closed system with N_2 and collected as barium carbonate for radioactive analysis. For further purification of the propionic acid, the concentrated distillate was applied to a partition column of Celite using phosphate buffer pH 6.5 as stationary phase.[14] The propionic acid was eluted with a mixture of 5 % butanol in chloroform as mobile phase.

The propionic acid was degraded by a Schmidt reaction and the carboxyl group isolated as barium carbonate. The ethylamine produced in the Schmidt reaction was oxidized and the carbon dioxide trapped as barium carbonate.

Radioactive analysis. In the degradation series, measurements were performed in a liquid scintillation counter with the barium carbonate samples suspended in a gel of Aerosil in a toluene solution of 2,5-diphenyloxazole.

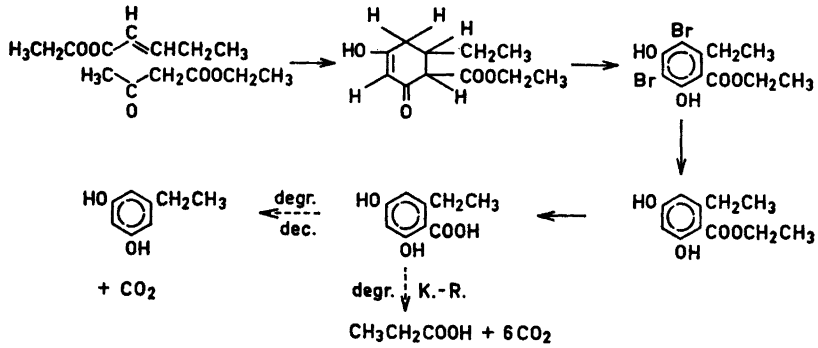

Fig. 1. Scheme of synthesis and degradation of homoorsellinic acid.

RESULTS AND DISCUSSION

As can be seen from the figures given in Table 1, 84 % of the total radioactivity found in homoorsellinic acid is located in carbon atom 2, which originates from the carboxyl group in propionic acid. The remaining radioactivity

Table 1. Propionate-1-^{14}C derived homoorsellinic acid.

Compound	cpm/mmole × 10^{-3}
Homoorsellinic acid	232.9
Propionic acid	195.6
carboxyl group (p.a.)	190.4
ethylamine (p.a.)	4.1
Carboxyl group	9.8
Kuhn—Roth carbon dioxide	11.7

of 16 % is probably distributed equally between carbon atoms 4,6, and the carboxyl group. As will be reported elsewhere, the administered carboxyl-labeled propionic acid in the described experiment above gave rise to radioactive orsellinic acid as well. The labeling pattern of the latter acid corresponds to that obtained from acetate-1-^{14}C. It follows that there is a pool of carboxyl-labeled malonyl-CoA derived from propionate-1-^{14}C present, which also is utilized for the biosynthesis of homoorsellinic acid. The somewhat too high radioactivity obtained from Kuhn—Roth carbon dioxide is probably due to a partial degradation of propionic acid occurring during oxidation. Thus these results indicate a biogenetic mechanism leading to the formation of homoorsellinic acid in which one propionyl-CoA unit is condensed with three malonyl-CoA units.

As has been mentioned above, homoorsellinic acid can only be detected in Czapek—Dox medium of *Penicillium baarnense* which has been supplied with propionic acid whereas orsellinic acid is normally found. From the figures obtained, no distinction can be made between an induced formation of this homoacid by a new enzyme or a biosynthesis involving the same enzyme complex responsible for orsellinic acid formation. However, in view of the work of Horning *et al.* on fatty acid biosynthesis using different acyl-CoA derivatives, it seems more likely that in the biosynthesis of this group of secondary natural products condensation of a *non*-specific acyl-CoA "starter" with malonyl-CoA units can take place on the same active site of the enzyme complex.

Further studies with other potential starter units such as short branched-chain fatty acids, malonamic acid, odd numbered fatty acids etc. could give rise to a variety of new compounds and provide a better understanding for the conditions of "controlled biosynthesis".

REFERENCES

1. Bressler, R. and Wakil, S. J. *J. Biol. Chem.* **237** (1962) 1441.
2. Horning, M. G., Martin, D. B., Karmen, A. and Vagelos, P. R. *J. Biol. Chem.* **236** (1961) 669.
3. Vanek, Z., Majer, J., Babicky, A., Liebster, G. and Veres, K. *Proc. 2nd Intern. Conf. Peaceful Uses Atomic Energy* **25** (1958) 143.
4. Grisebach, H., Achenbach, H. and Hofheinz, W. *Z. Naturforsch.* **15b** (1960) 560.
5. Corcoran, J. W., Kaneda, T. and Butte, J. C. *J. Biol. Chem.* **235** (1960) PC 29.
6. Birch, A. J., Pride, E., Rickards, R. W., Thompson, P. J., Dutcher, J. D., Perlman, D. and Djerassi, C. *Chem. Ind. (London)* **1960** 1245.
7. Ollis, W. D., Sutherland, J. O., Codner, R. C., Gordon, J. J. and Miller, G. A. *Proc. Chem. Soc.* **1960** 347.
8. Mosbach, K. *Naturwiss.* **48** (1961) 525.
9. Mosbach, K. *Acta Chem. Scand.* **18** (1964) 329.
10. Mosbach, K. *Acta Chem. Scand.* **14** (1960) 457.
11. von Schilling, R. and Vorländer, D. *Ann.* **308** (1899) 195.
12. Sonn, A. *Ber.* **61** (1928) 926.
13. Karrer, P. *Helv. Chim. Acta* **39** (1956) 1263.
14. Sakami, W. *Handbook of Isotope Tracer Methods*, Chap. **21** (1955) 107.
15. Reio, L. *J. Chromatog.* **1** (1958) 338.

Received June 1, 1964.