Vol. 95, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1980 Pages 1017-1022

ISOLATION OF 5-(β-HYDROXYETHYL)-4-METHYLTHIAZOLE-2-CARBOXYLIC ACID, A METABOLITE RELATED TO THIAMINE BIOSYNTHESIS IN *Escherichia coli*.

Bernard Estramareix and Michel Thérisod, Laboratoire de Chimie Organique Multifonctionnelle, Université de Paris-Sud, 91405 Orsay Cédex, France.

Received June 4,1980

Cells of *Escherichia coli* derepressed for thiamine biosynthesis excrete a new growth factor for a thiazole-less *E. coli* mutant. This factor has been identified as 5-(β-hydroxyethyl)-4-methylthiazole-2-carboxylic acid. This compound, though being closely connected to the biosynthesis of the thiazole moiety of thiamine, is likely not a precursor.

Recent published evidences strongly suggest that the five carbons chain in the thiazole moiety 1 (HET) of thiamine originates from some highly functionalized precursor, probably closely connected to a sugar : in 1977, we found (1) that several strains of *E. coli* excrete small amount of glycol 2, and showed that it shares a common precursor with HET; pyruvate on the one hand and glyceraldehyde phosphate on the other, have been found as respective precursors of fragments C-4, C-8 and C-5, C-6, C-7 of the five carbons chain of HET (2); the pentulose derivative might originate from hexose along classical pathways in yeast (3). In *E. coli* and *Salmonella typhimurium*, carbon atom C-2 (4,5) and nitrogen (6) come from tyrosine and sulfur from cysteine (7,8). To date, we do not know in which order these precursors do assemble. Any scheme should be consistent with the presence in incubation media of some strains of *E. coli* of a new growth factor for a thiazole-less mutant, acid 3, the isolation and metabolic origin of which is the subject of this paper.

Material and methods.

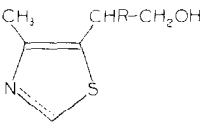
Organisms.- The following *Escherichia coli* mutants were used : a pyramine[1] auxotroph 70-17; a shikimate auxotroph 83-1; a HET[1] auxotroph 26-43, which was a generous gift from Dr Nose, Prefectural School of Medicine, Kyoto.

Derepression of thiamine biosynthesis.- Strain 70-17 was cultivated in the presence of a suboptimal 25 nM concentration of pyramine. Strain 83-1 was cultivated in the presence of adenosine (9). The washed cells were resuspended in a synthetic minimal medium (9) with glucose (0.4 %).

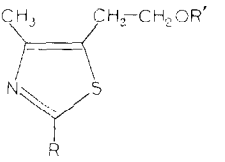
Incubations.- The cells were incubated for 1.5 h at 37° in stagnant flasks and then centrifuged. Addition to the incubation medium of suitable factors (tyrosine for strain 83-1, pyramine for strain 70-17) gave high yield of

1. Abbreviations used : Pyramine, 2-methyl-4-amino-5-hydroxymethylpyrimidine; HET, 5-(β-hydroxyethyl)-4-methylthiazole.

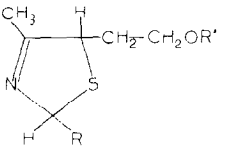
0006-291X/80/151017-06$01.00/0



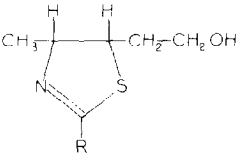
1 R=H
2 R=OH



3 R=COOH, R'=H
4 R=Li, R'=Li
5 R=$CO_2^-NH_3^+C_6H_{11}$, R'=H
6 R=CO_2CH_3, R'=H
7 R=CH_2OH, R'=H
8 R=COOH, R'=PO_3H_2



9 R=CO_2CMe_3, R'=CH_3CO
10 R=CO_2CH_3, R'=H
11 R=COOH, R'=H
12 R=COOH, R'=PO_3H_2
15 R=H, R'=H



13 R=$COOC_2H_5$
14 R=COOH

thiamine pyrophosphate in the cells. Evaporation of the supernatant to dryness *in vacuo* gave a residue which was extracted with methanol (0.1-0.5 ml).

Chromatography and bioautography.- Aliquots (5-30µl) of the methanolic solution were spotted on silicagel or cellulose plates (0.2 mm) which were developped in eluents A ($CHCl_3$-methanol, 60-40, for silicagel only), B (2-propanol-NH_4OH-water, 70-10-10), C (2-propanol- ethylacetate-acetate buffer pH 5, 1-1-1), D (n-butanol-acetic acid-water, 2-1-1). Bioautographies with cells of strain 26-43 were performed as described (10). Growth zones could be located as red spots after incubation for 12-16 h at 37°.

Tracer studies.- Parallel experiments with labelled precursors were run with normal and derepressed cells. Extracts of supernatants were streaked as 15 cm bands on silicagel plates. After development, the active zones located by bio-and-radio-autography (Kodirex film, 6 days contact) were eluted and examined by TLC in another system.

In one experiment with $|^{35}S|$sulfate, thiazole 3 (0.78 mmole) was added as carrier. Chromatography of the methanolic extract on a Dowex-50 (cyclo-hexylamine) column gave the salt 5 which was recrystallyzed from acetone-ethanol. Diazomethane esterification gave ester 6 which was purified by two silicagel column chromatography ($CHCl_3$-methanol, 96-4 and then ether). Ester 6 was reduced by $LiAlH_4$ to the diol 7, which was also purified by silicagel column chromatography ($CHCl_3$-methanol, 9-1). The concentrations of 6 and 7 in the chromatography fractions were estimated from the known values, respectively ε_{256} 8500 for 6 and ε_{250} 4900 for 7, and their molar radioactivities determined

Incubations with homogenates.- Derepressed, washed cells of strain 83-1 (30mg dry weight) were resuspended either in a 0.05 M pH 7 potassium phosphate buffer

or a 0.05 M pH 7.5 tris-HCl buffer. Sonication (12 x 15s) followed by centrifugation (5min., 5000g, 0°C) gave a supernatant which was used in the assays. Mixtures of sodium salt of thiazolic or thiazolinic acid (10^{-4}M), $MgCl_2$ (20mM), cell homogenate (0.5ml) in buffer (total volume 1.5ml) were incubated for 2 h at 37°C. Control runs without homogenate were made. Phosphate hydrolysis after incubation in tris-buffer was achieved by adjusting the pH to 8.6 with KOH and keeping for 3 h at 37°C in the presence of an alkaline phosphatase from calf intestine (2.5mg of the purified enzyme obtained from Calbiochem).

Chemical syntheses.- These will be described in detail elsewhere. Some properties are given below :

5-(β-hydroxyethyl)-4-methylthiazole-2-carboxylic acid, 3.
Prepared by carboxylation of the Li derivative 4, m.p. 101° (dec.) (alcohol-acetone).

Cyclohexylammonium salt, 5. (61% yield from 1).
m.p. 173° (ethanol-acetone, 1-2), with the expected composition;
$\lambda_{max}^{pH\ 0}$ 297nm, ε 8500; $\lambda_{max}^{pH\ 7}$ 288nm, ε 7900.

5-(β-hydroxyethyl)-2-methoxycarbonyl-4-methylthiazole, 6.
By CH_2N_2 esterification of 3 (50%); m.p. 92° (toluene);
λ_{max}^{MeOH} 297nm, ε 8400; with the expected ^{1}H NMR spectrum.

Thiazole phosphate 8.- Prepared from 6 (200 mg) by treatment with β-cyanoethylphosphate followed by hydrolysis in the presence of LiOH at room temperature. It was isolated as the bis-cyclohexylammonium salt, purified by dissolving in water ethanol mixture (1-5), precipitating with acetone (2 volumes). Yield 750 mg. $\lambda_{max}^{pH\ 7}$ 288nm. It contained one P atom and two moles of cyclohexylamine per mole.

5-(β-hydroxyethyl)-2-methoxycarbonyl-4-methyl-3-thiazoline, 10.
The ter-butoxycarbonyl analogue 9 was prepared by the general procedure of Asinger (11). Alkaline methanolysis gave 10 as a pale yellow syrup. Oxidation with $FeCl_3$ in methanol at 60° for 1 h gave the crystalline thiazolic ester 6 in 70% isolated yield.

5-(β-hydroxyethyl)-4-methyl-3-thiazoline-2-carboxylic acid, 11.-
Prepared by alkaline hydrolysis of 10. R_F 0.3 (solvent C). The cyclohexylammonium salt was a syrup.

Thiazoline phosphate, 12.- Prepared by phosphorylation of 10 with $POCl_3$ in pyridine at -10°, as a syrup. R_F 0.1 (solvent B). Quantitatively hydrolysed back to 11 by alkaline phosphatase.

2-Ethoxycarbonyl-5-(β-hydroxyethyl)-4-methyl-2-thiazoline, 13.-
4-amino-3-benzylthio-1-pentanol (picrate, m.p. 160-63°C) was hydrogenolyzed to 4-amino-3-mercapto-1-pentanol by Na in liquid NH_3 and this was condensed with diethyliminooxalate chlorhydrate (12) to give compound 13.

5-(β-hydroxyethyl)-4-methyl-2-thiazoline-2-carboxylic acid, 14.-
By alkaline hydrolysis of 13. Cyclohexylammonium salt, m.p. 117-8° (dec.);
$\lambda_{max}^{pH\ 7}$ 270nm, ε 1580; with the expected composition.

Results

TLC (solvent A) and bioautography indicated the presence of several growth factors for the mutant 26-43, in the incubation medium of derepressed cells of mutant 70-17, the known thiazoles 1 and 2 and a new compound Y, R_F 0.15-0.2, giving a more intense spot than 2 and distinct from the phosphates of 1 and 2, R_F 0. Derepressed cells of strain 83-1 could also synthesize Y, but only in the presence of exogenous tyrosine. In its absence, incubation in the presence of HET gave thiamine but neither 2 nor Y. Non derepressed cells of both strains

Table. Incorporation of radioactive precursors into compound Y by cells of strain 83-1.

Labelled precursor	Concentration M	molar radioactivity mCi/mmole	dry weight of incubated cells mg	total radioactivity in Y DPM
DL[1-^{14}C]tyrosine	2.5×10^{-5}	57	15	25 600
DL[2-^{14}C]tyrosine	2.5×10^{-5}	39	8	18 000
DL[3-^{14}C]tyrosine	2.5×10^{-5}	45	8	0
$Na^{35}SO_4$	1.25×10^{-4}	80	22	206 000

did not synthesize Y in appreciable amount. Y appeared homogeneous by TLC (solvents A, B, C) and was stable when heated for 1 hour at 100° in aqueous solution at pH 7, but decomposed fully at pH 2 to a derivative migrating as HET. On paper electrophoresis, Y migrated as an anion at pH higher than 4.

Tracer experiments (table) indicated that Y incorporated C-1 and C-2 of tyrosine and sulfur of sulfate. Such properties are consistent with structure 3 This so far unknown acid was synthesized and characterized as the cyclohexylammonium salt 5. Acid 3 can be detected by bioautography at the 25 ng level. Its behaviour on TLC was the same as that of Y in four solvent systems.

Assuming no dilution of label, the recovered activities indicated the excretion of c.a. 0.05 nmole of Y per mg of cells (dry weight).

Finally, ^{35}S labelled compound Y obtained by incubation in the presence of [^{35}S]sulfate (80 mCi/mmole) and purified by three TLC was added to the salt 5 (0.24 mmole). Radioactive crystals were thus obtained, the molar radioactivity remaining constant through several recrystallizations (630 dpm/μmole). Alternatively, carrier 3 (0.78 mmole) was added directly to the incubation medium; diazomethane esterification gave ester 6 (4000-4300 dpm/μmole). $LiAlH_4$ reduction gave the diol 7 with no decrease of molar radioactivity (3970-4050 dpm/ mole). Assuming no dilution of label, the yield of compound Y excreted was 0.24mmole/mg dry cells. This is much higher as above because of the more efficient isolation and in good agreement with visual estimation of the size of bioautography spots.

Although solid acid 3 decarboxylates at 100°, it seems indefinitely stable in water solution at pH 7 and 37°C.

The rate of decarboxylation of the phosphate 8 by homogenates of 1 mg cells 83-1 was less than 10^{-2}nmole/h while the rate of HET biosynthesis by 1mg of intact cells was 1nmole/h.

Among possible precursors of thiazoles 1 and 3, the new 2-thiazoline 14 was prepared by synthesis, but was inactive in bioautography, even at the

500ng level. On the other hand, the new syrupy thiazolinic acid 11, a diastereoisomeric mixture, is a fairly instable compound and there was evidence for the presence of 3 (1-5%) and 1 even in a freshly prepared aqueous solution of this 3-thiazoline.

Treatment with $FeCl_3$ in ethanol or ferricyanide in water at pH 7 (1 h at 60°C or 16 h at 20°C) gave the thiazole 1 (40%). Decarboxylation appears concerted with oxidation for the thiazolic acid 3 being inert in this system is not an intermediate, and furthermore, the 3-thiazoline 11 is not decarboxylated to the known (13) fairly stable compound 15 in the absence of oxidant.

In bioautography, the 3-thiazoline 11 is active at the 1ng level but this may reflect chemical oxidation on the plate. In the presence of cells extracts and oxidants such as NAD^+ and $NADP^+$, its conversion to thiazole does not exceed the small one found in blank runs.

Discussion

The above related experiments show that two strains of *E. coli*, apparently normal for the biosynthesis of the thiazole moiety of thiamine excrete an hitherto unknown compound, namely 5-(β-hydroxyethyl)-4-methylthiazole-2-carboxylic acid. As suggested by its structure, its biosynthesis is closely linked to that of HET, since the derepression of thiamine biosynthesis enhances the excretion of the acid whose amount is then nearly half that of the synthesized thiamine; furthermore, tyrosine is an indispensable factor for excretion of the acid, just as for biosynthesis of HET; two carbons of tyrosine incorporate into the acid : the C-2 which incorporates also into HET and the C-1 which is the precursor of the carboxylic carbon.

The thiazolic acid is certainly not a metabolic derivative of HET since exogenous tyrosine is indispensable for its excretion by a tyrosine-less mutant even in the presence of HET. Conversely, can the thiazolic acid be a precursor of HET ? The thiazole requiring organism 26-43 which allowed to detect this new factor on bioautography is presumably able to derive some thiamine from it. But in liquid medium, no growth of this organism is detectable in the presence of 20 ng/mg of thiazolic acid within 48 h while HET allows at 5 ng/mg a full growth within 14 h. The very high sensitivity of the triphenyltetrazolium method in bioautographies should be recalled in this connection.

Homogenates prepared from cells with high thiamine biosynthesis activity showed no appreciate ability to decarboxylate the thiazolic acid and its phosphate.

The meaning of these negative experiments is not obvious. Perhaps the thiazolic acid 3 cannot enter the cells and some cofactors are missing in the homogenate. But more probably the acid 3, like the glycol 2, is not a precursor

of HET. It would be the stable end-product of spontaneous evolution of a precursor of HET.

This common precursor is probably structurally close to the thiazolic acid. It is neither the 2-thiazoline 14, which is a too stable compound, nor the 3-thiazoline 11, which is spontaneously converted more readily to HET than to the thiazolic acid; furthermore cell extracts are not able to increase the rate of conversion of this unstable 3-thiazoline.

In the biosynthesis of HET from tyrosine, both the carboxyl and the p-hydroxybenzyl fragments have to be eliminated at some steps, the last one as p-hydroxybenzyl alcohol (14). In the biosynthesis of acid 3, elimination of the aromatic fragment has occured first. The order may be the same in the biosynthesis of HET.

Acknowledgments.- The authors wish to thank Dr Y. Nose, Kyoto Prefectural University of Medicine, who kindly supplied the strain 26-43. They are grateful to Prof. S. David for his encouragement and continuous support.

References

1. Thérisod,M., Gaudry,D. and Estramareix,B.(1978) Nouv.J.Chim.2, 119-121.
2. White,R.H. (1978) Biochemistry 17, 3833-3840.
3. White,R.L., Spenser,I.D. (1979) J.Amer.Chem.Soc. 101, 5102-5104.
4. Estramareix,B. and Thérisod,M. (1972) Biochim.Biophys.Acta 273, 275-282.
5. Bellion,E., Kirkley,D.H. and Faust,J.H. (1976) Biochim.Biophys.Acta 437, 229-237.
6. White,R.H. and Rudolph,F.B. (1978) Biochim.Biophys.Acta 542, 340-347.
7. Bellion,E. and Kirkley,D.H. (1977) Biochim.Biophys.Acta 497, 323-328.
8. Estramareix,B., Gaudry,D. and Thérisod,M. (1977) Biochimie 59,857-859.
9. Newell,P.C. and Tucker,R.G. (1966) Biochem.J.100, 512-516.
10. Newell,P.C. and Tucker,R.G. (1968) Biochem.J. 106, 279-287.
11. Thiel,M., Asinger,F. and Schmiedel,K. (1958) Liebigs Ann.Chem.611,121-131.
12. Zaitseva,E.L., Yakubovich,A., Braz,G.I. and Bazov,V.P. (1965) Chemical Abstracts 62, 562h.
13. Thiel,M., Asinger,F. and Stengler,W. (1958) Liebigs Ann.Chem. 619,161-168.
14. White,R.H. (1979) Biochim.Biophys.Acta 583, 55-62.