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The Mechanism of Action of Bacimethrin, a Naturally Occurring Thiamin Antimetabolite

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Abstract—The mechanism of bacimethrin (**2**) toxicity has been determined. This compound is converted to 2′-methoxy-thiamin pyrophosphate (**10**) by the thiamin biosynthetic enzymes. Of the seven thiamin pyrophosphate utilizing enzymes in *Escherichia coli*, 2′-methoxy-thiamin pyrophosphate inhibits α-ketoglutarate dehydrogenase, transketolase, and deoxy-D-xylulose-5-phosphate synthase. Bacimethrin does not cause repression of the genes coding for the thiamin biosynthetic enzymes. © 2001 Published by Elsevier Science Ltd.

Bacimethrin (**2**), a natural product isolated from *Bacillus megaterium*[1–3] and from *Streptomyces albus*,[4] is toxic to bacteria and yeast growing in minimal media and has a minimum inhibitory concentration (MIC) in the low micromolar range. The toxicity can be reversed by adding thiamin to the culture medium. This observation, and the structural similarity between bacimethrin and the pyrimidine precursor (**1**) to thiamin phosphate (**5**), led to the suggestion that bacimethrin may be an inhibitor of thiamin biosynthesis. An alternative hypothesis for the mechanism of bacimethrin toxicity suggests that it is converted to 2′-methoxy-thiamin pyrophosphate (**10**) which could then either inhibit some or all of the thiamin pyrophosphate-dependent enzymes in the cell or repress transcription of the thiamin pyrophosphate biosynthetic genes. Since we have previously overexpressed and characterized all of the enzymes needed for the conversion of **1** to thiamin pyrophosphate (**6**), these hypotheses can now be examined rigorously.[5]

Bacimethrin is a Substrate for the Thiamin Biosynthetic Enzymes

The enzymatic route for the conversion of hydroxymethylpyrimidine (HMP, **1**) to thiamin pyrophosphate **6** by the thiamin biosynthesis pathway is shown in Figure 1.[5] Two phosphorylations of HMP (**1**) give HMP-pyrophosphate (**4**).[6] This is then coupled with thiazole phosphate (**11**) to give thiamin phosphate (**5**).[7,8] A final phosphorylation completes the biosynthesis of **6**.[9]

To determine if bacimethrin (**2**) is a substrate for the thiamin biosynthetic enzymes, it was synthesized[4,10] and treated with a mixture consisting of partially purified HMP-P kinase, thiamin phosphate synthase, thiamin phosphate kinase, thiazole phosphate (**11**),[11] and ATP. 2′-Methoxy-thiamin pyrophosphate (**10**) in the reaction mixture was assayed by first oxidizing it to the highly fluorescent thiochrome[8] analogue with potassium ferricyanide followed by HPLC analysis. A reference sample of 2′-methoxy-thiamin pyrophosphate (**10**) was prepared by the pyrophosphorylation of 2′-methoxy-thiamin using yeast thiamin pyrophosphokinase[12] and was used to calibrate the assay. Thiamin pyrophosphate (**6**) was synthesized from **1** and **11** under identical conditions and assayed in a similar manner. The results of these experiments (Fig. 2) demonstrate that the last three thiamin pyrophosphate biosynthetic enzymes can catalyze the conversion of bacimethrin (**2**) to 2′-methoxy-thiamin pyrophosphate (**10**) at a rate that is 6 times faster than the rate of conversion of the natural substrate HMP (**1**) to thiamin pyrophosphate (**6**). The observed enzymatic conversion of bacimethrin (**2**) to 2′-methoxy-thiamin pyrophosphate (**10**) suggests that the latter compound is responsible for the antibiotic activity

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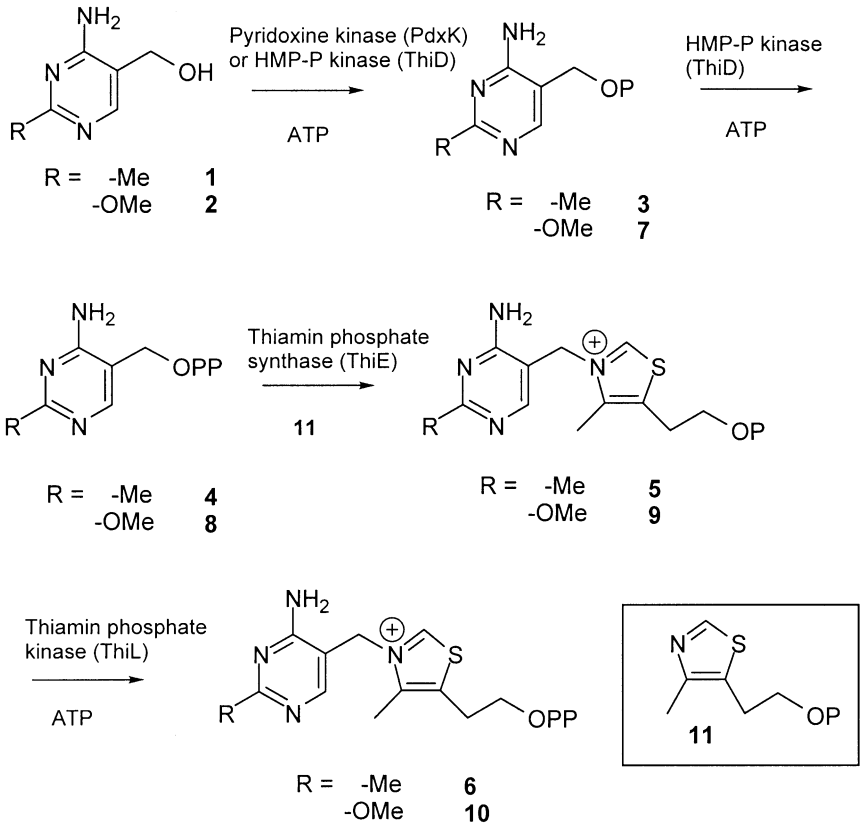

Figure 1. The thiamin biosynthetic pathway in *Escherichia coli*.

of bacimethrin because toxicity from any of the intermediates would be alleviated by further metabolism. This analysis is also consistent with the recent characterization of a bacimethrin-resistant mutant in *Salmonella typhimurium*. The mutation is in the HMP-P kinase gene and blocks the conversion of **2** to **7**, the first step in the conversion of bacimethrin to 2′-methoxy-thiamin pyrophosphate.[13]

To confirm that 2′-methoxy-thiamin pyrophosphate (**10**) is the toxic metabolite of bacimethrin, we tested the antibiotic activity of 2′-methoxy-thiamin. This compound, which is presumably converted to 2′-methoxy-thiamin pyrophosphate (**10**) in vivo, inhibited the growth of *E. coli* at concentrations that were 15 times lower than bacimethrin, and the inhibition was reversed by the addition of thiamin.

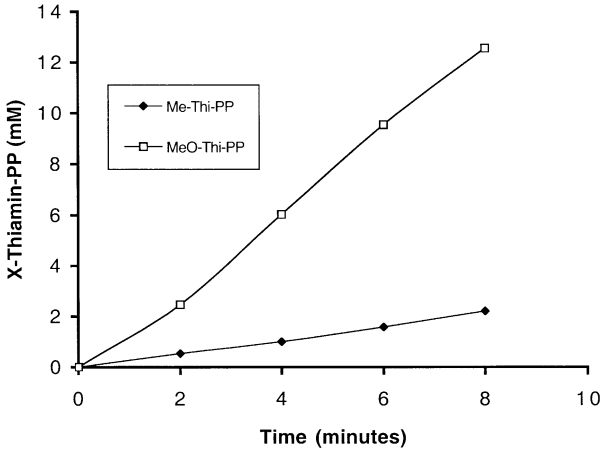

Figure 2. The enzymatic synthesis of 2′-methoxy-thiamin pyrophosphate (**10**) and thiamin pyrophosphate (**6**) from an equimolar mixture of HMP (**1**) and bacimethrin (**2**, 50 μM).

Methoxy-thiamin Pyrophosphate Inhibits a Subset of the Thiamin Dependent Enzymes in *E. coli*

The thiamin pyrophosphate requiring enzymes in *E. coli* are shown in Table 1. Also shown in the table are the nutritional requirements resulting from mutations in each of the corresponding genes. SHCHC synthase, an enzyme involved in menaquinone biosynthesis, can be excluded as an inhibition target of bacimethrin (**2**) because menaquinone is not required during aerobic growth.[14] Nutrient supplements that restore growth to transketolase mutants[15] fully counteract the toxicity of bacimethrin, suggesting that transketolase is a major target. In contrast, nutrient supplements that restore growth to acetolactate synthase mutants[16] have no effect on the toxicity of bacimethrin, demonstrating that this enzyme is not a major target for inhibition. The toxicity of bacimethrin is greatly reduced when *E. coli* is grown under anaerobic conditions. This suggests that α-ketoglutarate dehydrogenase, pyruvate dehydrogenase or pyruvate oxidase are possible targets for inhibition.[17] The toxicity of bacimethrin is fully counteracted by the addition of lysine and methionine, but not acetate, to the culture medium. This demonstrates that α-ketoglutarate dehydrogenase,[18] and not pyruvate dehydrogenase

Table 1. Inhibition of the thiamin-dependent enzymes in *E. coli* by 2′-methoxy-thiamin pyrophosphate[a]

Enzyme target	Nutritional requirements of mutant	Cell growth in the absence of bacimethrin	Cell growth in the presence of bacimethrin (300 μM) + nutrient supplements[c]
Deoxy-D-xylose-5-P synthase	3-Methyl-2-butenol	+ + +	+ + +
SHCHC synthase	Not essential	+ + +	Not tested
Transketolase	Tyr, Phe, Trp, pyridoxine and aromatic vitamins[b]	+ + +	+ + +
Acetolactate synthase	Val and Ile	+ + +	—
α-Ketoglutarate dehydrogenase	Anaerobisis or	+ + +	+ +
	Lys and Met	+ + +	+ + +
Pyruvate dehydrogenase	Anaerobisis or	+ + +	+ +
	Acetate	+ + +	—
Pyruvate oxidase	Anaerobisis or	+ + +	+ +
	Acetate	+ + +	—

[a]All cultures were grown in DM minimal medium with glucose as the carbon source.
[b]Aromatic vitamins: *p*-hydroxybenzoate, *p*-aminobenzoate and 2,3-dihydroxybenzoate.
[c]Nutritional requirements used at concentrations indicated in the reference which describe the mutant in question.

or pyruvate oxidase,[18–21] is the target. Finally, 3-methyl-2-butenol also counteracts the toxicity of bacimethrin. This compound can complement regulatory mutants containing reduced levels of deoxy-D-xylulose-5-phosphate (DXP) synthase.[22] This enzyme is required for the biosynthesis of ubiquinone, menaquinone, pyridoxal phosphate, and thiamin. The observation that downregulated mutants in this gene do not require either pyridoxal phosphate or thiamin suggests that growth limiting amounts of DXP synthase have a greater effect on ubiquinone and menaquinone biosynthesis than on pyridoxal phosphate or thiamin biosynthesis because 3-methyl-2-butenol can be converted to isopentenyl pyrophosphate but not to DXP. Thus, of the seven thiamin pyrophosphate requiring enzymes in *E. coli*, α-ketoglutarate dehydrogenase, transketolase, and DXP synthase are the major targets for inhibition by 2′-methoxy-thiamin pyrophosphate. However, we do not yet understand how nutrient supplements that compensate for the inhibition of one thiamin pyrophosphate requiring enzyme can also compensate for inhibition of the other two thiamin pyrophosphate requiring enzymes.

There are three thiamin regulated gene clusters in *E. coli* (*thiCEFSGH*, *thiMD*, and *tbpAthiPQ*). To determine if bacimethrin causes the repression of these genes, we used the recently developed *E. coli* gene chip[23] to compare the transcriptional profiles of *E. coli* grown to mid log phase and treated with bacimethrin (13 μg/mL, 10×MIC) for 30 min, treated with thiamin (0.34 μg/mL) for 30 min and a control sample to which no additional small molecules were added. The relative expression of *thiC*/*thiM*/*tbpA* for the thiamin treated sample compared to the control was 0.05:0.07:0.01 demonstrating the expected strong repression of all three gene clusters by thiamin. In contrast, the relative expression of *thiC*/*thiM*/*tbpA* for the bacimethrin treated sample compared to the control was 0.75:0.35:1.53. Thus, while bacimethrin does have an effect on the transcription of the thiamin biosynthetic genes, this effect is small relative to the effect of thiamin. The transcriptional profile analysis also revealed that the transcript levels of at least 30 genes were induced (>4-fold) and at least 15 other gene transcripts were repressed after exposure to bacimethrin. Based on the reported function of these genes, no specific cellular response to bacimethrin toxicity could be deduced. Consequently, further work is necessary to determine if bacimethrin or 2′-methoxy-thiamin pyrophosphate directly alters gene transcript levels as part of its mechanism of cellular toxicity.

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