METABOLIC PRODUCTS OF MICROORGANISMS. 239†

BACIMETHRIN ISOLATED FROM *STREPTOMYCES ALBUS* IDENTIFICATION, DERIVATIVES, SYNTHESIS AND BIOLOGICAL PROPERTIES

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Bacimethrin (**1**), known as a thiamine antagonist produced by *Bacillus megatherium*, was isolated from *Streptomyces albus* and has been further characterized by NMR spectra and acetylation. A new easy three step synthesis for **1** is described. The biological activity of **1**, and its mode of action were discussed. There are indications that bacimethrin inhibits the phosphorylation of 4-amino-5-hydroxymethyl-2-methylpyrimidine (Pyr-OH) during thiamine biosynthesis.

In the course of our screening we especially look for strains which are producing more than one secondary metabolite. In the culture broth of *Streptomyces albus* (strain Tü 2031), isolated from a soil sample collected in Mexico, we detected by chemical and biological methods azomycin[2], bicycloamide[3] and a third compound, which shows striking activity against Gram-positive and Gram-negative bacteria on synthetic medium. The activity was completely antagonized by thiamine, leading us to suspect it was bacimethrin (**1**), which had been isolated by UMEZAWA *et al.*[4] and YONEHARA *et al.*[5] from *Bacillus megatherium* in 1962. Until now no other producer of this natural thiamine antagonist has been described. In this paper we report the isolation of bacimethrin from *Streptomyces*, its acetylation, a new easy synthesis and its probable mode of action.

Bacimethrin and its Acetates

Bacimethrin (**1**) was isolated from the culture filtrate of *Streptomyces albus* by adsorption on Amberlite XAD-4 and elution with methanol - water. Further purification was attained by chromatography on silica gel in chloroform - methanol systems and recrystallization from methanol yielding at least 8~10 mg/liter culture broth. **1** extinguishes UV-light on Silica gel F_{254}, turns orange on TLC plates with vanillin - sulfuric acid after heating and turns blue in the Barrollier-test. The physicochemical data are in agreement with those given for bacimethrin (**1**)[4]. Additionally the electron impact mass spectrum (EI-MS) shows the molecular ion at *m/z* 155 ($C_6H_9N_3O_2$ by high resolution) and the ^1H and ^{13}C NMR data fit the formula (Table 1).

Acetylation of **1** in acetic anhydride with pyridine as catalyst at room temperature yielded quantitatively the monoacetate **2**[5], with sodium acetate at 100°C the diacetate **3** and the triacetate **4**. Rf

† See ref 1.

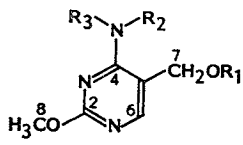
Table 1. NMR data of bacimethrin (**1**) in DMSO-d_6 (δ values in ppm).

Position	^{1}H NMR (80 MHz)	^{13}C NMR (50.3 MHz)
2	—	164.6[a] s
4	—	163.6[a] s
5	—	111.1 s
6	7.75 s	154.6 d
7	4.25 s	57.5 t
8	3.72 s	53.5 q
OH	4.92 br	—
NH_2	6.59 br	—

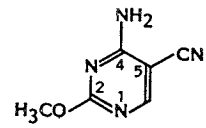
[a] The assignment is not quite certain.

Table 2. Rf values of bacimethrin (**1**) and its derivative on silica gel TLC plates.

Compound	$CHCl_3$ - MeOH (9 : 1)
1	0.45
2	0.52
3	0.60
4	0.76
5	0.57
7	0.59
9	0.45
Fluorescein	0.38



1 $R_1 = R_2 = R_3 = H$
2 $R_1 = Ac$ $R_2 = R_3 = H$
3 $R_1 = R_2 = Ac$ $R_3 = H$
4 $R_1 = R_2 = R_3 = Ac$



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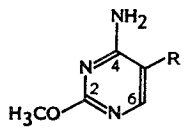
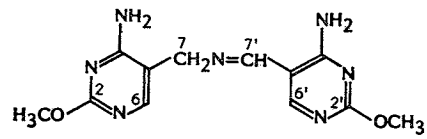


6 R = CH=NH
7 R = CHO
8 $R = CH_2NH_2$



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values are given in Table 2. The structure of the acetates could be derived from the mass spectra and the ^{1}H NMR data. The signal of the methylene group shifts to lower fields ($\Delta\delta$ 0.65~0.75) by acetylation of the hydroxy group, the acetoxy group is observable at δ 2.04~2.10, the *N*-acetyl group at δ 2.50 or δ 2.30, respectively. 6-H shows a successive paramagnetic shift going from **1** to **4**, the maximum is $\Delta\delta$ 0.86. There is no evidence from the spectra that the *N*-acetates present another tautomeric form of the possible amino-imino tautomerism than that given in **3** and **4**.

Synthesis

Bacimethrin (**1**) was first synthesized by KOPPEL *et al.*[6]. They chose a pyrimidine derivative as starting material, which needs three steps to be synthesized in acceptable yield. We start with the 4-amino-5-cyano-2-methoxypyrimidine (**5**), which in one step is easily available[7]. Hydrolysis of the cyano group under acidic or alkaline conditions failed, because of the instability of the methoxy group. The cyano group of **5** could be reduced by lithium aluminum hydride in tetrahydrofuran to the imine

6, which was hydrolyzed during the workup procedure with water to the aldehyde **7**. The unexpected low yield (50%) of this step could be explained by a condensation of **7** with the amine **8**, which was formed as a minor product during the reduction. The yield of the azomethine **9** shows that nearly 25% of the aldehyde **7** has been lost. Efforts to enhance the yield of **7** by changes of the temperature, the reaction time and/or the equivalents of the reducing agent failed. Bacimethrin (**1**) was then made by reduction of the aldehyde **7** with sodium borohydride in methanol (90%). The synthesis can be carried out in a larger scale, the reaction time is short and the desired products can be separated easily.

Biological Properties

In the disc-diffusion assay bacimethrin inhibits the growth of *Bacillus subtilis* and *Escherichia coli* only on a chemical defined medium. The inhibitory diameter caused by 1 μg was nearly 30 mm. While the monoacetate **2** is nearly as active as bacimethrin, increasing acetylation caused a drastic reduction of the inhibition.

In order to investigate the effect of bacimethrin on growing *E. coli* K-12 cells, photometric experiments were conducted in a chemical defined medium (Fig. 1). The inhibitory effect of the antibiotic is intensified by increasing concentrations up to the value of 1.3 μg/ml (MIC value). The minimal growth concentration (MGC) to thiamine for *E. coli* mutants was also determined in these experiments. The MGC value for *E. coli* K-12 *thiA* amounted to 4 pg/ml, for *E. coli* K-12 *thiC* to 200 pg/ml.

In cross tests it could be established that thiamine, pyridoxine and 4-amino-5-aminomethyl-2-methylpyrimidine (Pyr-NH_2) antagonized the effect of bacimethrin on *E. coli*. For the first two compounds a concentration-independent antagonism was observed, while for Pyr-NH_2 a concentration dependent antagonism was evident. This was verified in photometric experiments (Tables 3 and 4).

In this test, thiamine is about 10^6 times more effective as an antagonist than pyridoxine. It is also noteworthy that a multiple of the MIC can be antagonized by the MGC of thiamine. An

Fig. 1. Growth of *Escherichia coli* at variable bacimethrin concentrations.

Curve 1; without bacimethrin, curve 2; addition of 0.17 μg/ml bacimethrin, curve 3; addition of 1.3 μg/ml bacimethrin.

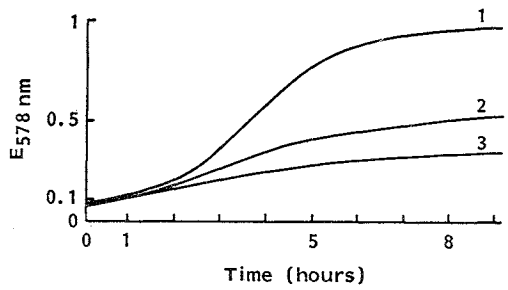


Table 3. Concentration dependency of bacimethrin - thiamine and bacimethrin - pyridoxine antagonism in photometer experiments on *Escherichia coli* K-12.

Culture No.	**1** (μg/ml)	Thiamine (pg/ml)	$E_{578\,nm}$ (after 14 hours)	**1**: thiamine
1	0.0	0.0	0.6	—
2	40	0.0	0.1	
3	40	3.3	0.52	1.2×10^7
4	40	6.6	0.6	6.1×10^6
5	40	1.6	0.6	2.5×10^7

Culture No.	**1** (μg/ml)	pyridoxione (μg/ml)	$E_{578\,nm}$ (after 14 hours)	**1**: pyridoxine
6	40	1.6	0.3	25

Table 4. Concentration dependency of bacimethrin - Pyr-NH_2 antagonism in photometer experiments on *Escherichia coli* K-12.

Culture No.	1 (μg)	Pyr-NH_2 (μg)	$E_{578\ nm}$ (after 14 hours)	1 : Pyr-NH_2
1	0.0	0.0	0.92	—
2	40	0.0	0.12	∞
3	40	0.2	0.72	200
4	240	0.2	0.20	1,200

Table 5. Incorporation of ^3H-labeled Pyr-NH_2 in thiamine biosynthesis of *Escherichia coli* K-12 (values in cpm).

Culture 1; without bacimethrin, culture 2; addition of 8.3 μg/ml bacimethrin, culture 3; addition of 16.6 μg/ml bacimethrin.

Compounds	4 hours			6 hours			7 hours		
	Culture 1	Culture 2	Culture 3	Culture 1	Culture 2	Culture 3	Culture 1	Culture 2	Culture 3
Thiamine phosphates	—	—	—	410	50	30	490	320	402
Thiamine	56	—	—	45	145	230	—	191	292
Pyr-NH_2	340	413	388	130	495	230	120	480	515
Pyr-OH	89	96	82	—	190	140	—	192	320
Pyr-OH/Pyr-NH_2	0.26	0.23	0.21		0.38	0.61		0.40	0.62

—: No activity detectable.

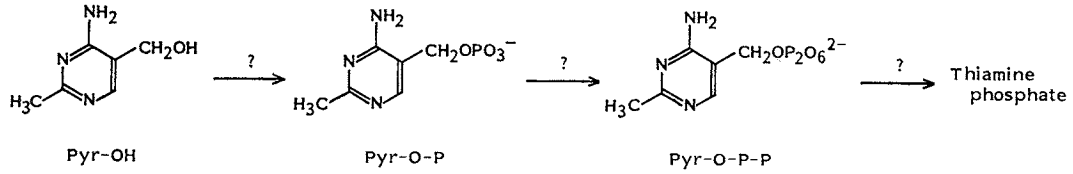
antogonism is still observable even if the addition of thiamine or Pyr-NH_2 occurs 2.5 hours after the addition of the antibiotic. Thus the antagonism is independent of the time of addition.

Thiamine auxotrophic mutants of *E. coli* are not inhibited by bacimethrin. This indicates that the thiamine biosynthesis may be the only possible site of action for this antibiotic, as was also demonstrated by incorporation tests with ^3H-labeled Pyr-NH_2 on *E. coli* K-12. The concentrate of the culture supernatant, obtained according to the methods described in the Experimental section, was analyzed by TLC. The TLC's were evaluated by scraping off the appropriate Rf value regions and measuring their activity by liquid scintillation counting after growing for various hours. At the time-point t=0 only Pyr-NH_2 was detectable. At time interval t=4 the greater part of the radioactivity had not yet been metabolized. At t=6 and t=7 Pyr-NH_2 and the biosynthesis products (4-amino-5-hydroxymethyl-2-methylpyrimidine (Pyr-OH), thiamine and thiamine phosphates) were detectable, the synthesis still proceeding (Table 5). Pyr-O-P and Pyr-O-P-P could not be detected in any of the cultures. As a comparison between the cultures the quotient of Pyr-OH/Pyr-NH_2 was determined; this quotient is a gauge of the ratio of the rate of the two reactions (Pyr-NH_2→Pyr-OH and Pyr-OH→Pyr-O-P). The latter reaction appears to be slowed by bacimethrin, more so by higher concentration than by lower.

Discussion

A first indication of the site of action of bacimethrin lies in the antagonism of thiamine and pyridoxine[8]. A thiamine concentration, which is the minimum required to permit the growth of thiamine auxotrophic *E. coli* mutants is effective enough to completely antagonize ten-fold the MIC level of bacimethrin. The thiamine antagonism is not dependent on the proportion of bacimethrin concentration to thiamine concentration. Thiamine enables growth even when it is introduced to the cells after they have been inhibited for 2.5 hours. In this case, also, the low concentration of 2 pg/ml is sufficient to antagonize 40 μg/ml of bacimethrin. Pyridoxine exhibits a much weaker, time-dependent

Fig. 2. Thiamine biosynthesis in *Escherichia coli*[9,10].



antagonism than thiamine. The primary site of action of bacimethrin is therefore most probably thiamine biosynthesis. As even the most minimal thiamine concentration is able to cancel the effects of bacimethrin completely, the thiamine metabolism is most likely the only significant site of action for bacimethrin.

As the pyridoxine antagonism is time-dependent, it is conceivable that an inhibition of the antibiotic uptake could occur. In the case of thiamine antagonism, an influence on transport as chief cause can be ruled out, because the thiamine is effective even when added to the culture after the bacimethrin. If bacimethrin would compete with thiamine on the coenzyme level, a competitive (concentration-dependent) antagonism would be expected. It would not be understandable that even the minimal amount of thiamine required for growth could totally cancel the effects of bacimethrin. According to these results bacimethrin effects the biosynthesis of thiamine.

The pyrimidine moiety of thiamine (Pyr-OH) and its preliminary compound (Pyr-NH_2) are also effective as non time-dependent antagonists. The antagonism is competitive probably because bacimethrin prevents the incorporation of Pyr-OH in thiamine. If it would hinder the synthesis of Pyr-OH, then the minimal need of Pyr-OH of *E. coli* would suffice to cancel the action of bacimethrin and the antagonism would not be competitive. The thiazole moiety of thiamine has no influence on the bacimethrin action. The biosynthesis of thiamine[9,10] is shown in Fig. 2.

The site of action thought to be one of the three steps which are labeld with question marks. It seems most plausible that bacimethrin competes with the molecule on the enzyme which carries the same electrical charge as it does. This is Pyr-OH. However, it cannot be ruled out that either the bacimethrin is phosphorylated or that a rise in the Pyr-OH level also leads to a rise in the Pyr-O-P or Pyr-O-P-P concentrations. Incorporation of ^3H-labeled Pyr-NH_2, which was metabolized to Pyr-OH, led to an increase in the Pyr-OH level in the bacimethrin-influenced cells, however, no Pyr-O-P or Pyr-O-P-P could be detected. In addition, the bacimethrin appeared to be unaltered. These results support the assumption that bacimethrin inhibits the phosphorylation of Pyr-OH.

Experimental

General

UV spectra were recorded using a Zeiss DMR 21 spectrometer, IR spectra in pressed KBr disks using a Perkin-Elmer model 298 spectrometer. The NMR spectra were determined with a Varian FT-80 or XL-200, respectively. Chemical shifts (δ in ppm) are reported relative to internal tetramethylsilane. The EI-MS (70 eV) were obtained on an Varian MAT 731 using direct probe insert, high resolutions with perfluorokerosine as a standard. TLC was performed on silica gel (Merck, Kieselgel 60 F_{254}, 5×6 cm, 0.25 mm), column chromatography on Silica gel 60 (>0.08 mm, Macherey & Nagel) or Sephadex LH-20 (Pharmacia), preparative TLC on silica gel P UV 254 (Macherey & Nagel).

Bacterial Strains

The standard strains for the activity determination of bacimethrin and the investigation of the biological properties of this compound were obtained from the stock culture collection in our laboratories, from ATCC or from CGSC. The antibiotic producing microorganism (Tü 2031) was a new soil isolate (soil sample from Mexico), classified according to HÜTTER[11] and BERGEYS[12] as *Streptomyces albus*.

Biological Assay

Preparation of Bacterial Cultures: The medium was inoculated with an overnight culture in a ratio of 1 : 100 and incubated at 37°C in Erlenmeyer flasks with side intrusion on a vibrator at 120 rpm. For maintenance slant tubes were inoculated with 0.1 ml of an overnight culture and incubated at 37°C for 24 hours.

Minimal Medium for Photometric Tests (DM-medium)[13]: KH_2PO_4 (3.0 g), K_2HPO_4 (7.0 g), NaCl (0.1 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), $(NH_4)_2SO_4$ (1.0 g), D-glucose (4.0 g), sterilized separately, deionized water (1.0 liter).

Growth Curve Registration on Photometer: The medium was inoculated with an overnight culture which had grown in DM-medium such that the $E_{578\ nm}$ (measured with an Eppendorf-photometer) fell between 0.02 and 0.1. 6 ml of this solution were pipetted into each cuvette. The vitamins and bacimethrin were added in MeOH solution so that the final concentration of MeOH in the culture was less than 1.6%. The increase in the extinction value was measured.

Incorporation Tests with ^{3}H-labeled Pyr-NH_2 on *E. coli* K-12: Logarithmically growing *E. coli* K-12 cells were separated from DM-medium by centrifuging, washed with DM-medium and resuspended in DM-medium ($E_{578\ nm}$=0.1). Of this suspension, 3 ml respectively, were shaken with bacimethrin for 30 minutes at 37°C in a water bath. The ^{3}H-labeled Pyr-NH_2 (30.00 cpm≃0.3 μg) was then added. After 2, 4, 6 and 7 hours, respectively, 600 μl samples were removed. The samples were centrifuged using an Eppendorf-centrifuge for 5 minutes and the supernatant was concentrated to *ca.* 50 μl using a speed Vac Concentrator. The concentrate was analyzed using TLC.

Chemical Assay

Siliconisation of Culture Flasks: The untreated culture flasks introduced varying amounts of thiamine into the cultures, which sufficed to influence the effect of bacimethrin. The flasks were therefore rinsed out with *ca.* 1 N sodium hydroxide and then washed with deionised water until neutral. After drying the flasks were washed with silicone; the coating was hardened by drying the flasks in a drying cabinet at 100°C for an hour.

Synthesis of ^{3}H-labeled Pyr-NH_2: ^{3}H-labeled Pyr-NH_2 was synthesized similar to the unlabeled Pyr-NH_2[14]. Dioxan, which possesses no transferable protons, was used as solvent instead of ethanol. 1 mg Pyr-CN was reduced with 10 ml tritium. After 12 hours 10 ml cyclohexen were added to bind the tritium not used in the reaction. After 2 days the liquid was distilled off using a water jet vacuum pump. The yield of Pyr-NH_2 was 5% of the theoretical yield (50 μg Pyr-NH_2, 0.2 mCi). The yield was determined in plate diffusion tests with *E. coli* K-12 *thiC*.

Characterization of Synthetic Products: A chemical characterization was conducted on silica gel using TLC plates. Pyr-OH had an Rf value of 0.8 in an eluent solution of $CHCl_3$ - MeOH (1 : 1); all other intermediate products of the thiamine biosynthesis remained at the application line. This system was used to remove Pyr-OH from the mixture of labeled substances (Pyr-NH_2, Pyr-O-P, Pyr-O-P-P, thiamine, thiamine-P and thiamine-P-P) before proceeding further. The chromatogram which had been developed in $CHCl_3$ - MeOH was developed again in a mixture of acetic acid - BuOH - H_2O (20 : 60 : 20). In this eluent mixture thiamine had an Rf value of 0.2, Pyr-NH_2 an Rf value of 0.4 and both of the thiamine phosphates remained at the application line. On cellulose TLC plates with acetone - acetic acid (1 : 1) as the eluting solution Pyr-OH had an Rf value of 0.8, Pyr-O-P of 0.45 and Pyr-O-P-P of 0.23[15]. The Rf value for Pyr-OH was reproducible, but for those of Pyr-O-P and Pyr-O-P-P no reference substances were available.

Fermentation Studies

Streptomyces albus was cultured at 27°C for 72 hours in a medium consisting of soybean meal 2% and mannitol 2% (100 ml medium in 500-ml Erlenmeyer flask with one intrusion). The pH value was adjusted to 7.5 before autoclaving. Cultures grown for 48 hours were used as inoculum for the 20-liter fermentor (Giovanola Frères SA, Monthez, Switzerland). Bacimethrin was produced in this tank under the following conditions: 10% inoculum was transferred to a 20-liter fermentor containing 18 liters medium (s.a.) and run at 27°C, 800 rpm and 8 liters air/minute. These conditions

were transferred to the 200-liter fermentor (Giovanola Frères SA, Monthez, Switzerland). A 20-liter fermentor, 24 hours old, was used as inoculum for the 200-liter fermentor. The culture was harvested after 48 hours cultivation.

Isolation of 4-Amino-5-hydroxymethyl-2-methoxypyrimidine (Bacimethrin, **1**)

The culture filtrate (pH 7.6, 200 liters) was added to a column of Amberlite XAD-4, the biological active substances were eluted with MeOH - H_2O (1 : 1), the eluate was evaporated to dryness. The crude residue (73 g) was extracted twice with 500 ml MeOH. The concentrated extract was chromatographed on a silica gel column (9×20 cm) with $CHCl_3$ - MeOH (8 : 2). The fractions containing bacimethrin (TLC control) were collected and evaporated to dryness. The residue (3.5 g) was suspended in 40 ml MeOH, the undesolved substance consists of nearly pure **1** and was recrystallized from a small quantity of MeOH as colorless plates: MP 175°C; Rf see Table 2; IR (KBr) cm^{-1} 1658, 1612; UV λ_{max}^{MeOH} nm (ε) 273 (6,200), 229 (6,600); $\lambda_{max}^{MeOH-HCl}$ nm (ε) 263 (7,700), 231 (6,400); ^1H and ^{13}C NMR see Table 1; EI-MS *m/z* (abundance) 155 (100%, M^+, high resolution calcd for $C_6H_9N_3O_2$ and found: 155.0692), 138 (13%), 125 (71%).

Anal Calcd for $C_6H_9N_3O_2$: C 46.43, H 5.85, N 27.08.
Found: C 46.34, H 5.77, N 27.11.

5-Acetoxymethyl-4-amino-2-methoxypyrimidine (Bacimethrin-monoacetate, **2**)

10 mg of bacimethrin (**1**), dissolved in 1 ml of pyridine, were treated with 5 drops of acetic anhydride at room temp. After 10 minutes toluene was added and the mixture evaporated to dryness under reduced pressure. The white residue was purified by preparative TLC (20×20 cm, $CHCl_3$ - MeOH (85 : 15)). The main zone was eluted with acetone yielding 11 mg (86%) **2** as a white solid: MP 131°C; Rf see Table 2; IR (KBr) cm^{-1} 3425, 3140, 1723, 1646, 1602; UV λ_{max} (MeOH and MeOH - NaOH) nm (ε) 273 (6,800), 231 (8,500); $\lambda_{max}^{MeOH-HCl}$ nm (ε) 263 (8,200), 232 (8,000); ^1H NMR (80 MHz, $CDCl_3$) δ 2.07 (s, acetyl), 3.87 (s, 2-OCH_3), 4.96 (s, 7-H_2), 5.60 (br, NH_2), 7.96 (s, 6-H); EI-MS *m/z* (abundance) 197 (92%, M^+, high resolution calcd for $C_8H_{11}N_3O_3$ and found: 197.0800), 167 (15%), 155 (23%, M−CH_2CO), 154 (21%), 138 (100%, M−CH_3COO), 137 (37%), 110 (18%), 95 (27%), 43 (46%).

Anal Calcd for $C_8H_{11}N_3O_3$: C 48.73, H 5.62, N 21.31.
Found: C 49.04, H 5.73, N 21.43.

4-Acetamino-5-acetoxymethyl-2-methoxypyrimidine (Bacimethrine-diacetate, **3**)

30 mg bacimethrine (**1**) were stirred with 50 mg sodium acetate in 3 ml acetic anhydride 4 hours at 100°C. After cooling off, the reaction mixture was poured on water and the diacetate **3** extracted with $CHCl_3$. Preparative TLC (20×20 cm; $CHCl_3$ - MeOH (9 : 1)) and chromatography on Sephadex LH-20 (column: 100×2.5 cm, MeOH) yielded 25 mg (55%) **3** as a white solid: MP 142°C; Rf see Table 2; IR (KBr) cm^{-1} 1725, 1700, 1627, 1580; UV λ_{max}^{MeOH} nm (ε) 278 (8,000), 231 (7,800); $\lambda_{max}^{MeOH-HCl}$ nm (ε) 279 (10,450), 235 (7,300); $\lambda_{max}^{MeOH-NaOH}$ nm (ε) 274 (6,500), 229 (6,800); ^1H NMR (80 MHz, $CDCl_3$) δ 2.10 (s, *O*-acetyl), 2.52 (s, *N*-acetyl), 3.97 (s, 2-OCH_3), 5.00 (s, 7-H_2), 8.30 (s, 6-H), 8.59 (br, NH); EI-MS *m/z* (abundance) 239 (8%, M^+, high resolution calcd for $C_{10}H_{13}N_3O_4$ and found: 239.0906), 197 (25%), 196 (85%, M−CH_3CO), 155 (19%), 154 (100%, M−CH_3CO−CH_2CO), 138 (51%), 60 (82%), 43 (99%).

4-*N*,*N*-Diacetamino-5-acetoxymethyl-2-methoxypyrimidine (Bacimethrin-triacetate, **4**)

4 was prepared in the same manner as the diacetate **3**, but the reaction time was extended to 5 hours. Proceeded from 30 mg of bacimethrin (**1**), 25 mg (46%) **4** were yielded as an oily, water sensible substance: Rf see Table 2; IR (KBr) cm^{-1} 1740, 1728, 1600; UV λ_{max} (MeOH and MeOH - HCl) nm (ε) 273 (7,100), 220 (13,000); $\lambda_{max}^{MeOH-NaOH}$ nm (ε) 274 (9,500), 229 (10,500), not reversible; ^1H NMR (80 MHz, $CDCl_3$) δ 2.04 (s, *O*-acetyl), 2.30 (s, 2×*N*-acetyl), 4.02 (s, 2-OCH_3), 4.90 (s, 7-H_2), 8.61 (s, 6-H); EI-MS *m/z* (abundance) 281 (1%, M^+), 239 (15%, M−CH_2CO), 196 (40%, M−CH_2CO−CH_3CO), 154 (70%, M−2CH_2CO−CH_3CO), 138 (14%), 42 (100%).

Anal Calcd for $C_{12}H_{15}N_3O_5$: C 51.24, H 5.38, N 14.94.
Found: C 51.18, H 5.43, N 14.88.

4-Amino-5-cyano-2-methoxypyrimidine (**5**)

By the method of TAYLOR *et al.*[7] we obtained **5** as colorless amorphous solid (40%): MP 210~213°C; Rf see Table 2; IR (KBr) cm^{-1} 2230, 1675, 1606, 1587; UV λ_{max}^{MeOH} nm (ε) 286 (6,500), 241 (10,800); $\lambda_{max}^{MeOH-HCl}$ nm (ε) 274.5 (5,800), 241 (10,800); ^1H NMR (80 MHz, DMSO-d_6) δ 3.80 (s, 2-OCH_3), 7.25 (br, NH), 7.75 (br, NH), 8.40 (s, 6-H); EI-MS *m/z* (abundance) 150 (71%, M^+, high resolution calcd for $C_6H_6N_4O$ and found: 150.0542), 120 (100%), 93 (29%), 91 (53%), 44 (100%).

4-Amino-5-formyl-2-methoxypyrimidine (**7**)

A solution of 0.63 g of $LiAlH_4$ in 120 ml anhydrous THF was added within 30 minutes to a chilled suspension of 3 g of **5** in 200 ml THF. After stirring for 30 minutes at 0°C and 1 hour at room temp the mixture was decomposed with H_2O, neutralized with 2 M HCl under reduced pressure. The residue was dissolved in 15 ml 2 M HCl and filtered, **7** was precipitated by adding 6 M NaOH until pH 6. 1.5 g (50%) white solid: MP 185~187°C; Rf see Table 2; IR (KBr) cm^{-1} 3393, 3135, 1687, 1645, 1598; UV λ_{max}^{MeOH} nm (ε) 295 (8,300), 254 (7,800); $\lambda_{max}^{MeOH-HCl}$ nm (ε) 265 (sh), 250 (8,000); ^1H NMR (80 MHz, DMSO-d_6) δ 3.85 (s, 2-OCH_3), 8.00~8.20 (br, NH_2), 8.55 (s, 6-H), 9.67 (s, CHO); EI-MS (70 eV) *m/z* (abundance) 153 (100%, M^+, high resolution calcd for $C_6H_7N_3O_2$ and found 153.0538), 123 (74%), 95 (44%), 68 (80%).

4-Amino-5-*N*-(4-amino-2-methoxy-5-pyrimidylmethylidene)aminomethyl-2-methoxypyrimidine (**9**)

After the precipitation of **7** the remaining filtrate was concentrated and allowed to crystallize overnight. 1.35 g (46%) **9** were obtained as colorless solid: MP 216~218°C; Rf see Table 2; IR (KBr) cm^{-1} 1640, 1610; UV λ_{max}^{MeOH} nm (ε) 288 (14,500); $\lambda_{max}^{MeOH-HCl}$ nm (ε) 263 (17,200); ^1H NMR (80 MHz, DMSO-d_6) δ 3.76 (s, 2-OCH_3), 3.84 (s, 2'-OCH_3), 4.42 (br s, 7-H_2), 6.86 (br, NH_2), 7.81 (s, 6-H), 7.90 (br, NH), 8.20 (s, 7'-H), 8.37 (s, 6'-H), 8.85 (br, NH); EI-MS *m/z* (abundance) 289 (11%, M^+, high resolution calcd for $C_{12}H_{15}N_7O_2$ and found: 289.1287), 138 (100%, $M-C_6H_6N_4O$), 109 (30%), 95 (83%), 69 (40%), 68 (54%), 67 (39%), 66 (34%), 54 (100%), 53 (70%), 52 (100%), 43 (50%).

Bacimethrin (**1**)

In a few portions 0.5 g sodium borohydride were added to suspension of 1 g **7** in 40 ml MeOH and stirred 30 minutes at room temp. The solvent was evaporated and the product purified by preparative TLC (20×20 cm, $CHCl_3$ - MeOH (85:15)) yielding 0.9 g (89%) **1**, which in all respects was identical with natural bacimethrin.

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