METABOLIC PRODUCTS OF MICROORGANISMS. 209*

KIRROTHRICIN, A NEW MEMBER OF THE KIRROMYCIN-GROUP

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(Received for publication July 14, 1981)

Kirrothricin, a new narrow-spectrum antibiotic produced by *Streptomyces cinnamomeus* strain Tü 89, was isolated in respect to its activity against *Clostridium pasteurianum*. The molecular formula is $C_{44}H_{64}N_2O_{10}$. Kirrothricin is related to kirromycin, aurodox and other members of this group by its chemical structure, biological activity and the mode of action.

Kirromycin (=mocimycin)[1,2], a representative of a new chemical class of antibiotics, was described first in 1972. Some other antibiotics, aurodox (=X5108)[3,4], efrotomycin[5], heneicomycin (=A21A)[6], dihydromocimycin[7] and probably azdimycin[8] were found to be closely related. In the course of our screening for antibiotics active especially against *Clostridium pasteurianum*, the strain Tü 89 was striking. It was identified as *Streptomyces cinnamomeus*, which is identical with *Streptoverticillum cinnamomeus* comb. nov. (A-725)[9]. Beside aureothin[10] and 2-ethyl-5-(3-indolyl)oxazole[11] the strain produces a bright yellow compound called kirrothricin[21]. The latter proved to be a new member of the kirromycin group. In this paper we describe the fermentation, isolation, physicochemical characterization and the biological properties of kirrothricin. The chemical structure is reported in another publication[12].

Materials and Methods

Test Organisms and Media

Synthetic Medium for *Bacillus subtilis*[13]: K_2HPO_4 7 g, KH_2PO_4 3 g, Na-citrate 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1 g, glucose 2 g, agar 15 g, H_2O added 1000 ml, pH 7.2.

Synthetic Medium for *Bacillus brevis*: K_2HPO_4 13.6 g, $(NH_4)_2SO_4$ 2 g, $CaCl_2$ 10 mg, $MgSO_4 \cdot 7H_2O$ 200 mg, $FeSO_4 \cdot 7H_2O$ 0.5 mg, L-glutamic acid 5 mg, a solution of vitamins 10 ml, H_2O added 1000 ml; pH 7.2.

Solution of Vitamins[14]: Thiamine-HCl 3.4 mg, riboflavine 0.4 mg, panthotenate 5.8 mg, pyridoxal phosphate 1.9 mg, nicotinamide 2.4 mg, folic acid 4.4 mg, H_2O added 100 ml.

Complex Medium for Bacteria: Nutrient broth 8 g, NaCl 5 g, H_2O added 1000 ml.

Complex Medium for *Clostridium pasteurianum*: Meat extract 3 g, yeast extract 3 g, malt extract 3 g, peptone 20 g, glucose 5 g, ascorbic acid 0.2 g, agar 15 g, H_2O added 1000 ml; pH 7.0.

* 208. KRONE, B.; A. HINRICHS & A. ZEECK: Haloquinone, a new antibiotic active against halobacteria. II. Chemical structure and derivatives. J. Antibiotics 34: 1538~1543, 1981

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Fermentation

Streptomyces cinnamomeus (strain Tü 89) was grown on a medium composed of 4 g yeast extract, 1 g malt extract, 4 g glucose and 2 g agar, H_2O added 100 ml. The agar slants and plates were incubated at 27°C and kept at room temperature. Fermentation medium: Skim milk powder 40 g, distillers solubles 10 g, H_2O added 1000 ml; pH 7.2.

100 ml medium in Erlenmeyer flasks (500 ml, one baffle) were inoculated from agar slants of *Streptomyces cinnamomeus* and incubated 24 hours on a rotary shaker at 27°C. One liter was used for inoculation of 9 liters medium in a fermenter jar (MF-14, New Brunswick Scientific Co.) and incubated 24 hours at 250 rpm under aeration (3 liters air/hour). The cell suspension of two 10 liters fermenter jars were used as inoculum for 200 liters medium in a fermenter jar (model b-200, AG für biologische Verfahrenstechnik). After incubation for 20 hours at 1,500 rpm under aeration (3,500 liters air/hour) the fermentation broth was harvested at pH 8.0. The temperature during fermentation was held at 27°C. The mycelium was separated by filtration and discarded.

Isolation and Purification

Kirrothricin was adsorbed on Amberlite XAD-2 resin from the culture filtrate. After washing the resin with water - methanol (4: 1 and 1: 1) the antibiotic was eluted with pure acetone. The solvent was evaporated under reduced pressure and the oily residue distributed between water and $CHCl_3$. From the dried and concentrated organic layer, kirrothricin was precipitated by petroleum ether. The final purification of the remaining yellow powder was done by column chromatography (column 25×11.5 cm) on silica gel (560 g, smaller than 0.08 mm, Macharey-Nagel), which was impregnated with phosphate buffer (pH 7). Crude antibiotic (3.1 g) was dissolved in the eluant $CHCl_3$ - methanol - 33% NH_3 (80: 20: 1) and placed on the top of the column, which was cooled to −20°C. All additional eluant was kept at the same low temperature during the whole separation. The chromatography gave two yellow main zones. The zone with the greater Rf value contained aureothin and 2-ethyl-5-(3-indolyl)oxazole. The other zone contained the pure antibiotic. The separation was finished within 2.5 hours and yielded 1.6 g kirrothricin.

Kirrothricin was identified by the following methods: 1) Bioassay against *Bacillus brevis* or *Clostridium pasteurianum*, 2) on TLC plates (Aluminium sheets, silica gel 60 F_{254} pre-coated, Merck) impregnated with phosphate buffer (pH 7) by detecting the spots under UV light (254 nm) or staining with $FeCl_3$ solution (red color).

Results and Discussion

Isolation

Kirrothricin has been produced in the early log-phase (Fig. 1) and was found in the culture filtrate. To stop the fermentation when the culture broth reached a pH value of $8.0 \sim 8.1$, gave the best yields of kirrothricin (Fig. 1). The antibiotic was isolated from the culture filtrate by adsorption on XAD-2 resin and elution with acetone. The yield was $100 \sim 120$ mg per liter culture broth. Further purification was achieved by solvent distribution and chromatography on silica gel. The Rf values in comparison with other antibiotics of the kirromycin-group are shown in Table 1. All current systems containing NH_3 show kirrothricin to be the fastest one. To obtain sharp zones for kirrothricin during chromatography it is necessary to use phosphate impregnated silica gel (pH 7) and NH_3 in the solvent system. Using the first system mentioned in Table 1 kirrothricin gave two zones on the TLC plates, a slower yellow and a faster bright yellow one. By two dimensional TLC each spot splits into two spots which have the same appearance as after the first run. This observation describes a typical behaviour of kirrothricin.

Characterization

Kirrothricin is a yellow amorphous powder (dec. above 100°C), insoluble in water or *n*-pentane,

Fig. 1. Kirrothricin production (bioassay against *Bacillus subtilis*).

(1) Sediment, (2) pH, (3) activity in the culture filtrate.

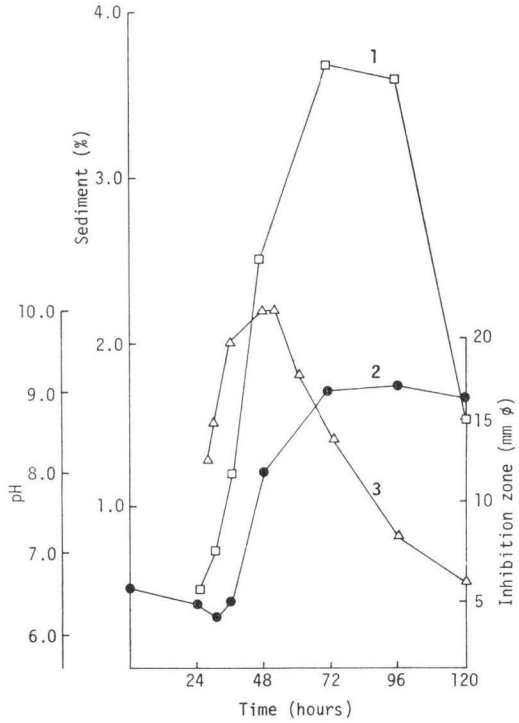


Table 1. Rf values of kirrothricin (**1**), kirromycin (**2a**), aurodox (**2b**) and efrotomycin (**2d**) on silica gel TLC plates.

Solvent system	1	2a	2b	2d
Chloroform - methanol - 33% NH_3 (80: 20: 1)	0.34	0.10	0.19	0.23
Chloroform - methanol - 25% NH_3 (80: 20: 1)	0.37	0.11	0.22	0.28
Benzene - ethanol - 25% NH_3 (65: 40: 9)	0.20	0.08	0.12	0.15
Chloroform - methanol (90: 10)	0.36	0.19	0.32	0.38

soluble in methanol, $CHCl_3$ or acetone. The antibiotic is a weak acid. Treatment with 0.02 M NaOH gave a sodium salt, which is soluble in water but not in organic solvents. Kirrothricin is unstable in acidic or basic solutions, especially under the influence of daylight and air oxygen. In solid phase it is more stable in the crude form than in the pure form after chromatography. Reaction with sodium metaperiodate leads to degradation[12], staining with $FeCl_3$ gives a red color.

The molecular formula $C_{44}H_{64}N_2O_{10}$ (M. W. 781.0) is based on the elemental analysis in connection with determinations of the molecular weight.

Anal. Calcd.: C 67.67, H 8.26, N 3.59, O 20.49%
Found: C 67.34, H 8.38, N 3.68, O 20.63%

Osmometric determination in methanol leads to a molecular weight of 782, potentiometric titration in ethanol - water (4: 1) with 0.02 M NaOH gave 771. Kirrothricin contains one *O*-methyl and one *N*-methyl group.

The optical rotation of the antibiotic is $[\alpha]_D^{20}$ −75.8° (*c* 0.01, methanol). The CD spectrum in me-

Table 2. UV absorptions of kirrothricin in different solvents.

	λ_{max} in nm (ε)
MeOH[a]	333 (sh, 21500), 324 (22100), 312 (sh, 20000), 283 (18600), 270 (sh, 16600), 231 (51000)
MeOH - NaOH	333 (29300), 320 (sh, 26900), 282 (16200), 270 (sh, 15200), 231 (46200)
MeOH - HCl[b]	360 (15500), 321 (20000), 306 (21000), 291 (20000), 282 (sh, 19300), 270 (sh, 16600), 231 (50300)
Cyclohexane - chloroform (7.3: 1)	366 (17700), 325 (19000), 312 (19300), 296 (18000), 285 (sh, 17200), 233 (44700)

[a] The spectrum depends on proportion of acid in the used methanol.
[b] The absorption is changing in course of time.

thanol shows the following COTTON effects: $\lambda_{max}([\theta]^{23})=357$ (−6000), 270 (0), 242 (−41600), 220 nm (+29200). The UV absorption (Fig. 2, Table 2) depends on the pH value of the solvent. In acidic solution the absorption at 360 nm disappears quickly whereas those at 321, 306 and 291 increase in course

Fig. 2. UV spectra of kirrothricin (Zeiss DMR 21).

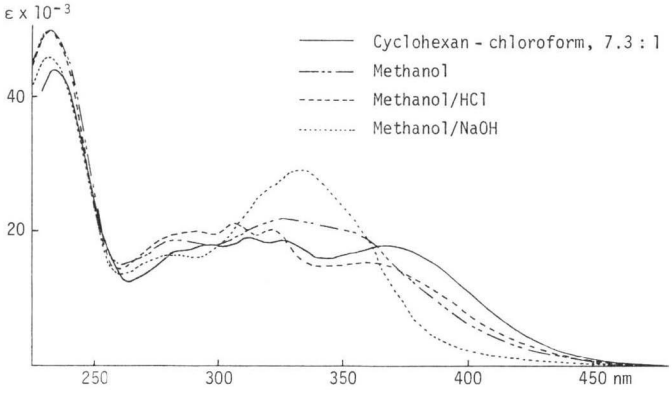


Fig. 3. IR spectrum of kirrothricin in KBr (Perkin-Elmer, Model 297).

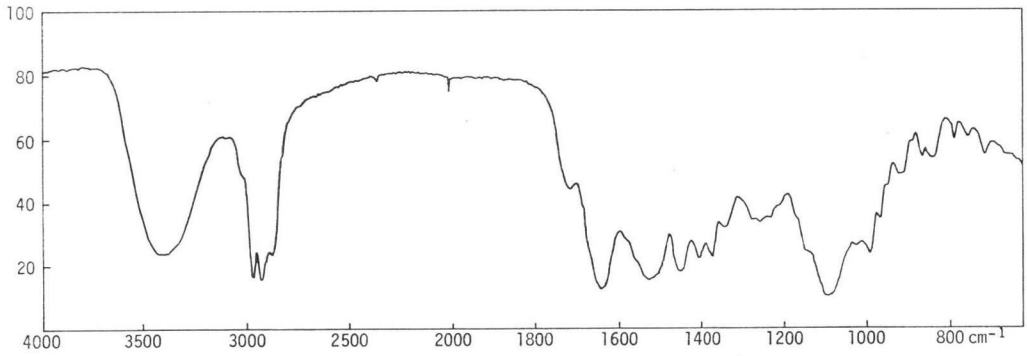
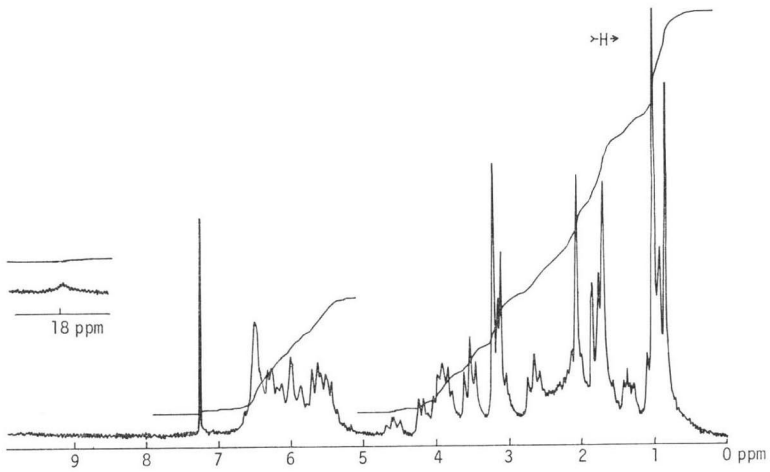


Fig. 4. PMR spectrum of kirrothricin in $CDCl_3$ (Varian FT-80, δ in ppm, TMS as external standard).



of time. Cyclohexane - $CHCl_3$ (7.3: 1) was used to get reproducible UV spectra of undissociated kirrothricin (Table 2). Compared to all other members of the kirromycin group, the absorption at longest wave-length shows a red shift of about 25~30 nm. The IR spectrum (Fig. 3) has characteristic absorption bands in the region of carbonyls and C-C double bonds at 1715, 1667 and 1650 cm^{-1}. The complex PMR spectrum of kirrothricin (Fig. 4) shows a typical OH resonance at δ 18.00. Further characteristic resonances are the triplets at δ 3.60 and δ 2.70 for 6-H_2 and 5-H_2. Signals of O-CH_3 and N-CH_3 are to be seen at δ 3.25 and 3.15.

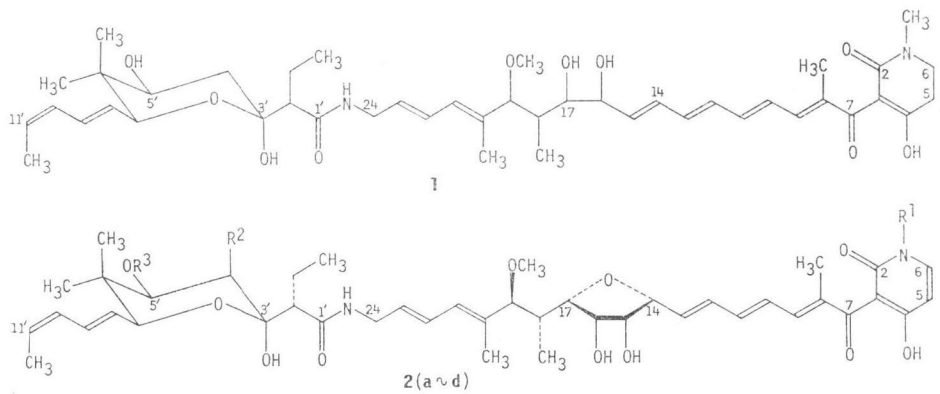
Chemical Structure

From the chemical and spectral data it is realized that kirrothricin is a new antibiotic. Its chemical structure has been worked out[12,22] and is shown in Fig. 5 in relation to the known antibiotics of the kirromycin group. All compounds have the same number of skeleton atoms suggesting that they are built up by a similar biosynthetic pathway[15].

One very important difference between kirrothricin (**1**) and the other antibiotics (**2a**~**2d**) is to be seen in the region from C-14 to C-17. In kirromycin (**2a**) these atoms are part of a tetrahydrofuran ring which carries vicinal hydroxyl groups in position 15 and 16. The formation of a ring in this region does not take place during the kirrothricin biosynthesis. An additional double bond is formed by dehydration resulting in a tetraene moiety.

The pyridone ring of the known antibiotics **2a**~**2d** is hydrogenated in **1** and 5,6-dihydro-**2a** (dihydromocimycin[7]) which are similar in this respect. This hydrogenation of the *N*-heterocycle leads to a β-triketo system which shows significant tautomerism[16]. Resonances of protons involved in the tautomeric equilibrium split up and give smaller additional signals near the main resonance. This equilibrium, combined with intermolecular interactions in concentrated solutions, causes very poor PMR spectra of kirrothricin. However, it has proven difficult to get definite kirrothricin derivatives where the tauto-

Fig. 5. Structure of kirrothricin (**1**) and related antibiotics.



	R^1	R^2	R^3	
2a	H	OH	H	Kirromycin[1,2]
2b	CH_3	OH	H	Aurodox[3,4]
2c	CH_3	H	H	Heneicomycin[6]
2d	CH_3	OH	Disaccharide $C_{15}H_{27}O_8$	Efrotomycin[5]

meric system is blocked. The antibiotic is very unstable both under acidic or basic conditions.

Just as heneicomycin (**2c**) which is a 4′-deoxy-derivative of aurodox (**2b**), kirrothricin is not oxygenated at position 4′. In conclusion kirrothricin (**1**) differs in substantial respects from kirromycin and its analogs and is a good compound for studying structure activity relations within this group. As shown below, the antimicrobial activity of **1** is similar to aurodox (**2b**), but **1** is not effective as a feed additive for animals. The vicinal hydroxyl groups in the middle of the long chain of **1** are more distant from the pyridone ring as in **2a**~**2d**. Furthermore it is expected that the conformation of the long chain may be different between **1** and **2a**~**2d**.

Biological Properties

Serial dilution tests have been evaluated. The inhibitory effect of kirrothricin on Gram-positive and Gram-negative bacteria in comparison with related antibiotics is listed in Table 3. The differences in the MIC values are very small, except of *Arthrobacter crystallopoietes, Micrococcus roseus, Chromobacterium violaceum* and *Corynebacterium* ATCC 23830 which were more susceptible to kirrothricin than to the other three substances. All four antibiotics show no activity against *Bacillus subtilis, Escherichia coli* and fungi but show very good activity against *Bacillus brevis* and *Clostridium pasteurianum*. This is the most outstanding feature of the whole kirromycin group[17].

The effect of kirrothricin in the agar diffusion test on complex media is shown in Fig. 6. *Clostridium pasteurianum* is the most susceptible microorganism. Kirrothricin is bacteriostatically active and acts on cells in the log-phase. Cells in the stationary phase remain insensitive to kirrothricin (Fig. 7).

Kirromycin was found to be the first antibiotic acting on the bacterial protein synthesis by an attack on the elongation factor Tu (EF-Tu)[17,18]. Kirrothricin is effective in the same way. It inhibits the poly (U)-directed poly(phenylalanine) synthesis to an extent of 50% with a concentration of 5×10^{-6} M and up to 96% for a concentration of 5×10^{-5} M (Fig. 8). The similarity of these results with those of kirromycin[17,18] suggests the same site of action.

Table 3. Antibacterial activity (MIC in μg/ml) of kirrothricin (**1**), kirromycin (**2a**), aurodox (**2b**) and efrotomycin (**2d**) in a serial dilution test (0.5 ml inoculum with $E_{578}=0.5$ for 4.5 ml medium).

Organism	1	2a	2b	2d
Achromobacter geminiani	>50	>50	>50	>50
Arthrobacter crystallopoietes	1	10	10	5
Arthrobacter simplex	10	50	50	50
Bacillus brevis	0.05	0.05	0.01	0.01
Bacillus subtilis	>100	>100	>100	>100
Chromobacterium violaceum	0.1	50	50	50
Clostridium pasteurianum	>0.1	>0.1	>0.1	>0.1
Corynebacterium ATCC 23830	1	10	10	5
Escherichia coli K12	>100	>100	>100	>100
Micrococcus luteus	1	5	1	5
Micrococcus roseus	0.1	1	1	1
Mycococcus spec.	50	50	1	5
Pseudomonas fluorescens	50	50	50	50
Pseudomonas saccharophila	10	10	10	10
Staphylococcus aureus	>100	>100	>100	>100
Streptomyces violaceoruber	0.5	0.5	0.5	0.5
Streptomyces viridochromogenes	0.5	0.5	0.1	0.1

Fig. 6. Effect of inhibition by kirrothricin on *Streptomyces viridochromogenes* (1), *Streptomyces violaceoruber* (2), *Bacillus brevis* (3) and *Clostridium pasteurianum* (4) in agar diffusion test (complex medium).

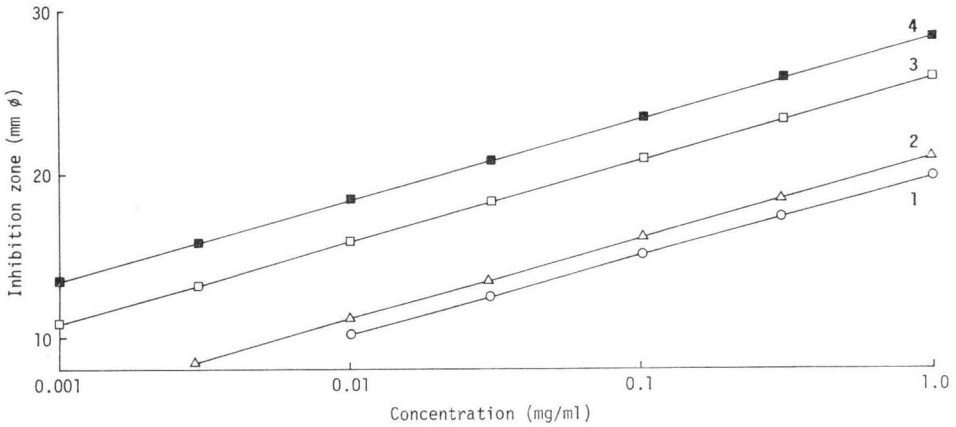
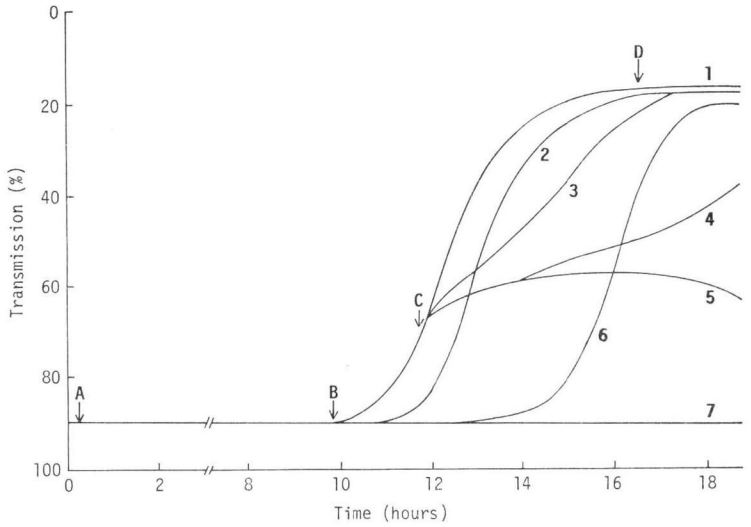
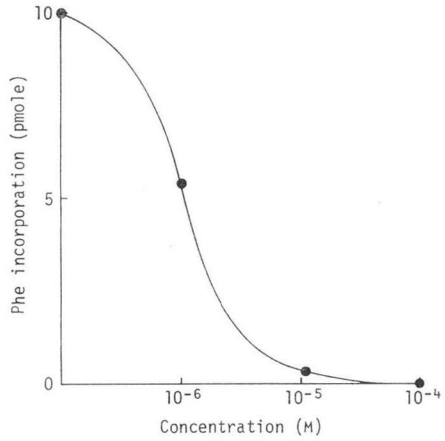


Fig. 7. Effect of inhibition by kirrothricin in complex medium (test organism: *Bacillus brevis*, 10^5 cells/ml). (1) Control and 10 μg/ml at D, (2) 0.14 μg/ml at A or B, (3) 1.0 μg/ml at C, (4) 2.5 μg/ml at C, (5) 5.0 μg/ml at C, (6) 1.4 μg/ml at A or B, (7) 2.5 μg/ml at A or B.



The role of the pyridone ring within the kirromycin group is unknown. At physiological pH the chelated enolic hydroxyl group is dissociated and the charge is distributed within an anionic region. The β-keto system will be able to complex with various cations, possibly with the important magnesium ion. In this respect kirrothricin seems to be similar to tetramic acid antibiotics like magnesidin[19] or streptolydigin[20]. So far stable salts of **1** are not observed *in vitro*. The proposed chelating effect is perhaps not responsible for the high specificity of the action of kirromycin and related antibiotics but it may assist the linkage of these antibiotics to the EF-Tu-GTP complex or influence the transport of the molecules.

Fig. 8. Effect of kirrothricin on the poly(U)-directed poly(phenylalanine) synthesis of *E. coli*.



Acknowledgments

We thank Dr. SCHEER, Bayer A.-G., Wuppertal for testing kirrothricin as feed additive and Dr. H. WOLF, University Tübingen, for his help by the investigation of the mode of action.

This work was supported by the Deutsche Forschungsgemeinschaft.

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