CGP 4832, A NEW SEMISYNTHETIC RIFAMYCIN DERIVATIVE HIGHLY ACTIVE AGAINST SOME GRAM-NEGATIVE BACTERIA

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CGP 4832 (**5**) is a new derivative of rifamycin S, showing a very high degree of activity against certain Gram-negative bacteria, with MICs as much as 400 times lower than those of rifampicin. CGP 4832 and rifampicin inhibit DNA-dependent transcription *in vitro* to a similar extent, which excludes any difference in their effect on the target enzyme. The most plausible explanation for the potent activity of CGP 4832 is that it penetrates into bacterial cells by way of a specific mechanism. This hypothesis is corroborated by the high rate of mutations leading to bacterial strains resistant against CGP 4832.

In our search for rifamycin derivatives with good activity against Gram-negative bacteria, we discovered a new derivative, CGP 4832 (**5**, Fig. 1), with MICs for certain Gram-negative species such as *Escherichia coli* and *Salmonella* up to 400 times lower than those of rifampicin. The present paper describes the antibacterial properties of this derivative as well as some experiments aimed at determining its mechanism of action.

Materials and Methods

Chemical Methods

Organic solutions were dried with anhydrous sodium sulfate. Evaporation was carried out under water-jet vacuum. TLC was performed on Merck $60F_{254}$ Silica gel plates using the following solvent systems: DC1 (ethyl acetate), DC2 (methylene chloride - acetone, 9.5 : 0.5), DC3 (ethyl acetate - methanol, 1 : 1). Preparative chromatography was carried out on Merck 60 Silica gel. ^{1}H NMR spectral† analysis was carried out in $CDCl_3$ at 360 MHz; signals are given in δ, ppm (TMS: δ 0.0 or $CHCl_3$ δ^3 7.28).

3-Morpholinorifamycin S-21,23-acetonide (**2**)††

After addition of a few drops of concentrated sulfuric acid, a solution of 60 g 3-morpholinorifamycin S (**1**) in 600 ml anhydrous acetone and 60 ml 2,2-dimethoxypropane was left to stand for 3 hours at room temp. It was then neutralized with aqueous sodium bicarbonate solution and mixed with a concentrated NaCl solution, and the reaction product was extracted with methylene chloride. After drying and evaporation of the methylene chloride solution, 3-morpholinorifamycin S-21,23-acetonide (**2**) crystallized from ether as dark-green prisms with a metallic sheen. Recrystallization from methanol - water yielded 60 g of reddish-violet, silk-lustred crystals without mp.

Rf (DC1) 0.56, (DC2) 0.35; ^{1}H NMR δ 0.82 (s), 1.20 (s, acetonide-CH_3), 2.00 (s, 25-OOCCH_3); field desorption mass spectrum (FD-MS) m/z 820 (M^+, $C_{44}H_{56}N_2O_{13}$).

† Only signals characteristic of the respective compounds are given. All further signals correspond to the typical shifts of rifamycins[8].

†† Rifamycin skeleton numbered according to OPPOLZER *et al.*[12].

25-*O*-Deacetyl-3-morpholinorifamycin S-21,23-acetonide (3)

50 g of 3-morpholinorifamycin S-21,23-acetonide (**2**) was treated for 16 hours with 600 ml of 5% methanolic sodium hydroxide solution. Ice was then added, the solution acidified with citric acid, and the reaction product extracted with methylene chloride. After drying and evaporation of the methylene chloride solution, 30 g of 25-*O*-deacetyl-3-morpholinorifamycin S-21,23-acetonide (**3**) crystallized from ether as fine, grayish-violet-colored crystals with an mp of 215°C.

Rf (DC1) 0.54, (DC2) 0.20; ^{1}H NMR δ 0.90 (s), 1.1 (s, acetonide-CH_3), $OCOCH_3$ signal at ~2.0 missing; FD-MS *m/z* 778 (M^+, $C_{42}H_{54}N_2O_{12}$).

25-*O*-Deacetyl-25-*O*-malonic Acid-3-morpholinorifamycin S-21,23-acetonide (4)

A solution of 30 g (**3**) and 13 g malonic acid in 180 ml absolute THF was cooled to 0°C and stirred. Upon addition of 26 g dicyclohexylcarbodiimide (DCCI), the reaction began immediately. After 5 minutes, the precipitated dicyclohexylurea was removed by filtration and the filtrate extracted with ethyl acetate, washed with sodium bicarbonate, citric acid and sodium chloride solution, dried and evaporated. Upon chromatography (1 kg silica gel; eluent: ethyl acetate - methanol, 9 : 1), the residue yielded 25 g (**4**) as the main extract, which crystallized from ether as dark-bluish-green crystals (mp 170°C).

Rf (DC1) 0.11; ^{1}H NMR δ 3.36 (s), malonic acid methylene group; FD-MS *m/z* 864 (M^+, $C_{45}H_{56}N_2O_{15}$).

CGP 4832 (5)

8.7 g morpholinorifamycin-acetonide-malonic acid (**4**) was reacted with 3.38 g hydroxybenzotriazole (HBT) in 100 ml absolute THF at 0°C for 2 hours while stirring. 3.23 g 3-hydroxymethyl-1-methylpiperidine and 5.15 g DCCI were then added and the reaction mixture stirred for a further 2 hours at 0°C and thereafter for 10 hours at room temp. After removal of the precipitated dicyclohexylurea by filtration, the filtrate was concentrated to 50 ml and 20 ml of 20% sulfuric acid added. After 1 hour the solution was neutralized with sodium bicarbonate, the reaction product extracted with ethyl acetate, and the extract dried and evaporated. Chromatographic purification of the black evaporation residue on 800 g silica gel first with methylene chloride - methanol (9 : 1) yielded a small amount of an urea derivative of **4**, then the quinone of the compound sought (**5**) was obtained as the main component of an eluate with methylene chloride - methanol (2 : 1).

Rf (DC3) 0.83; ^{1}H NMR (100 MHz) (ref TMS δ 0.0) δ 2.25 (s, NCH_3+aromatic CH_3), 3.38 (s, malonic ester-CH_2); FD-MS *m/z* 935 (M^+, $C_{49}H_{65}N_3O_{15}$); UV λ_{max} (alcohol) nm (ε) 217 (28,600), 271 (27,100), 321 (14,400), 540 (2,300).

Materials

[^{3}H]CTP was purchased from Radiochemical Centre, Amersham, the nucleotides from SERVA (Heidelberg, Germany).

Bacterial Strains

E. coli F 464 and its mutants F 612, F 539, F 583 and F 515 were a generous gift from K. JANN. *E. coli* strain W 3110 was obtained from W. ARBER. All the other microorganisms were strains from our own collection.

Measurement of RNA Synthesis

Highly purified RNA polymerase holoenzyme from *E. coli* ETH 2018 was isolated according to the methods of BURGESS[1] or BERG *et al.*[2] and MANGEL[3]. The activity of the enzyme was tested as described earlier[4]. Permeabilization of bacterial cells with ether was carried out as described earlier[5,6]. DNA-dependent transcription in permeabilized cells was performed as follows: 0.25 ml assay mixture contained Tris-HCl (pH 8.0) 40 mM, spermidine-HCl (pH 8.0) 8 mM, mercaptoethanol 8 mM, $MgCl_2$ 8 mM, NH_4Cl (pH 8.0) 128 mM, K-phosphate (pH 8.0) 0.5 mM, sucrose 0.5 M, ATP, GTP and UTP 1.2 mM, [^{3}H]CTP (10 mCi/mmol) 0.4 mM and cell protein *ca.* 40 μg (*ca.* 4×10^7 permeabilized bacterial cells). The reaction was started by addition of the four nucleotides. After 10 minutes incubation at 37°C, the reaction was stopped by addition of 3 ml cold 12% trichloroacetic acid. Samples were kept in ice for

60 minutes before filtration through glass fiber filters (Whatman GF/C). The filters were washed 12 times with 5 ml of cold 5% trichloroacetic acid, then dried for 30 minutes at 100°C, and radioactivity was determined by scintillation counting. The blanks were obtained by stopping the reaction with trichloroacetic acid immediately after addition of the nucleotides.

Measurement of the Resistance Mutation Rate

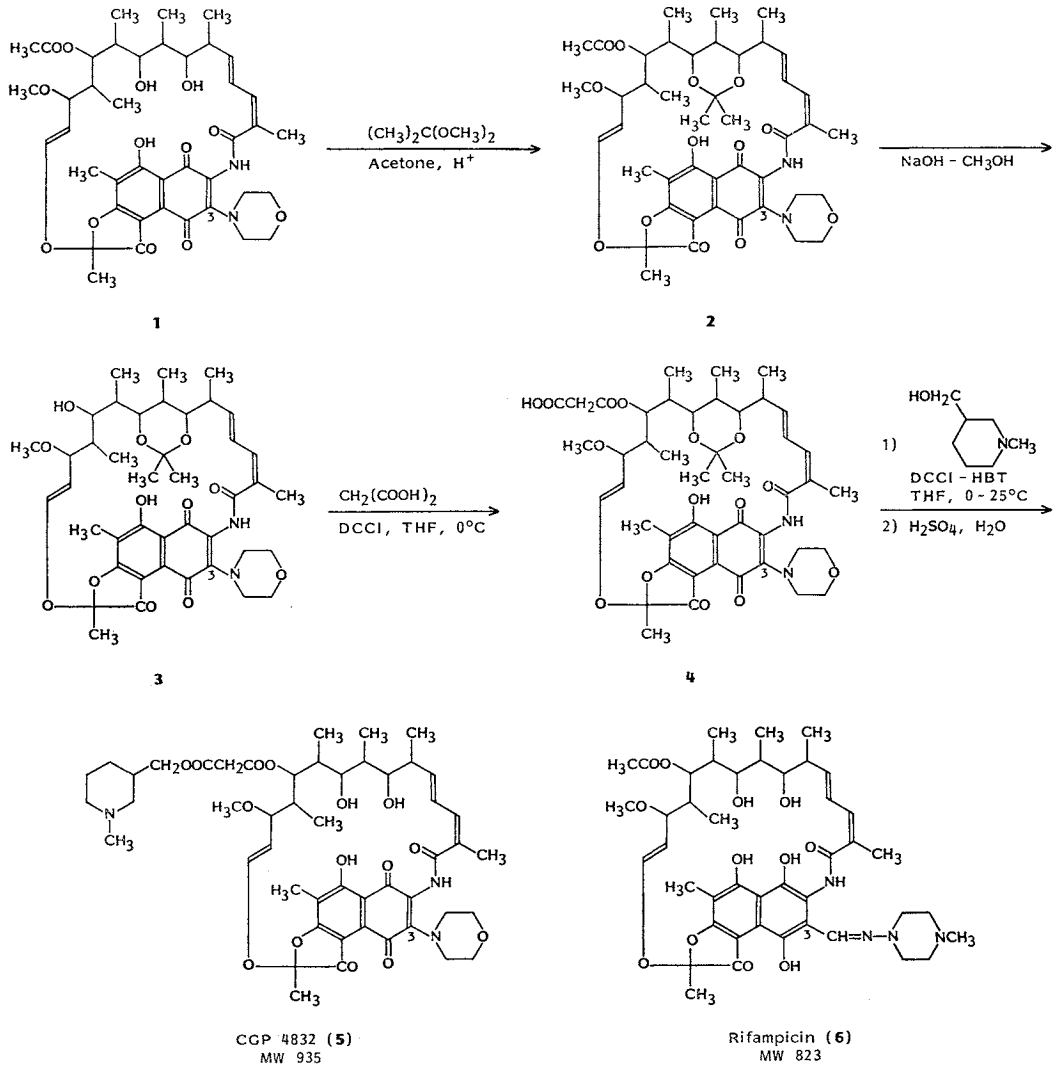
The rate of mutation towards resistance against CGP 4832 was measured using the fluctuation test described by Luria & Delbrück[7].

Results

Chemical Preparation of CGP 4832

To produce CGP 4832 (see Fig. 1), rifamycin S was first converted by reaction with morpholine to give 3-morpholinorifamycin S (**1**)[8]. In this compound the OH groups at C-21 and C-23 are pro-

Fig. 1. Formula scheme.



tected by acetonide formation (**2**) and *O*-acetyl at C-25 is saponified with NaOH to OH (**3**). Then in an unexpectedly rapid and straightforward reaction of **3** with malonic acid and DCCI, the semi-ester (**4**) was produced. That was converted by the established procedure with 3-hydroxymethyl-1-methylpiperidine, HBT and DCCI, followed by acetonide cleavage to the malonic ester derivative (**5**). In accordance with its preparation from racemic 3-hydroxymethyl-1-methyl piperidine, CGP 4832 is a diastereomeric mixture.

Antimicrobial Activity

As shown in Table 1, CGP 4832 is comparable to rifampicin in its activity against Gram-positive bacteria. Against certain Gram-negative species, however, it is 80~400 times more potent than rifampicin. In a more detailed study, 105 clinical isolates of *E. coli* and 30 of *Salmonella* were analyzed. Figs. 2 and 3 show that the MIC of rifampicin for practically all isolates of both bacterial species was around 16 μg/ml, whereas two categories of strains can be distinguished in respect of their sensitivity to CGP 4832: Two thirds of the strains are only 2~4 times more sensitive to CGP 4832 than to rifampicin, whereas the remainder are 1~3 orders of magnitude more sensitive.

Some strains of *Klebsiella* and *Enterobacter* highly sensitive to CGP 4832 have also been isolated (see Table 1). However, marked sensitivity appears to be much less common among these genera than with *E. coli* and *Salmonella.*

Effect of CGP 4832 on RNA Synthesis

To ascertain whether the greater sensitivity of the bacterial strains described above could be

Table 1. The antimicrobial spectrum of CGP 4832 and rifampicin.

Microorganism	MIC (μg/ml)	
	CGP 4832	Rifampicin
Staphylococcus aureus 10 B	0.01	0.01
S. aureus Wood 46	0.001	0.001
Streptococcus pyogenes, Aronson	0.01	0.01
S. faecalis D 3	4	1
Escherichia coli 205	8	8
E. coli 205 rifampicin-resistant	>128	>128
E. coli ETH 2018	0.01	4
E. coli K-12 W 3110	0.02	8
E. coli B 25	0.03	8
E. coli B 45	0.06	8
E. coli B 114	0.06	16
Salmonella typhimurium 277	8	16
Salmonella AX 5	0.5	16
Klebsiella pneumoniae C 14	32	32
K. pneumoniae 327	16	8
K. pneumoniae C 16	1	16
Serratia marcescens	16	16
Enterobacter cloacae P 99	16	16
E. cloacae ATCC 13047	1	16
Proteus mirabilis 774	16	4
P. vulgaris S 10	16	16
Morganella morganii 2359	8	4
Pseudomonas aeruginosa ATCC 12055	4	4

Assayed on brain heart infusion agar.

Fig. 2. MICs of CGP 4832 and rifampicin for 105 *Escherichia coli* strains (clinical isolates).
Number of strains per MIC. ● Rifampicin, ▲ CGP 4832.

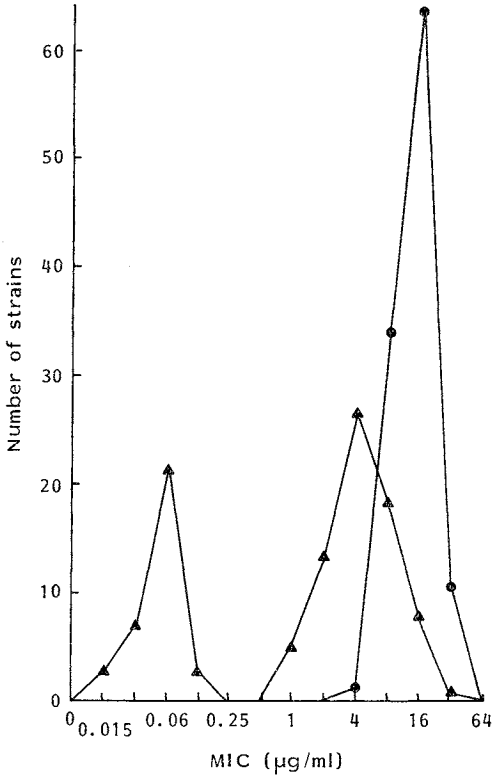


Fig. 3. MICs of CGP 4832 and rifampicin for 30 *Salmonella* strains (clinical isolates).
Number of strains per MIC. ● Rifampicin, ▲ CGP 4832.

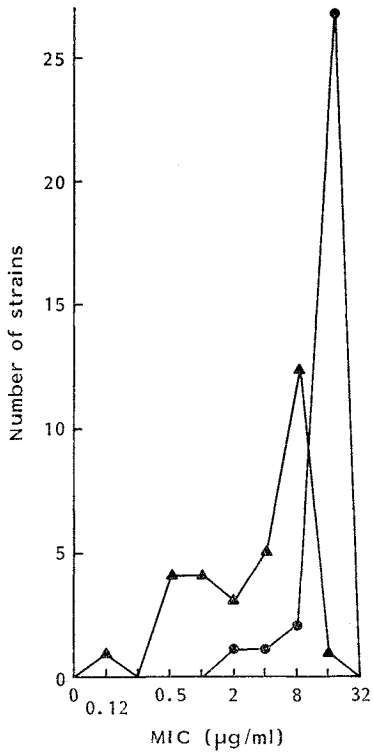


Table 2. Comparison between the antibacterial activity and the inhibition of various bacterial RNA polymerases by CGP 4832 and rifampicin.

Bacterial strains used for measuring MICs and RNA polymerase	CGP 4832		Rifampicin	
	MIC (μg/ml)	ED_{50} enzyme[a] (μg/ml)	MIC (μg/ml)	ED_{50} enzyme[a] (μg/ml)
Escherichia coli ETH 2018	0.01	0.09	4	0.02
E. coli ETH 2018, highly purified enzyme	—	0.06	—	0.02
E. coli 205	8	0.06	8	0.02
E. coli B 25	0.03	0.34	8	0.10
E. coli B 114	0.06	1.16	16	0.24
E. coli K-12 W 3110	0.02	0.05	8	0.02
Salmonella typhimurium 277	8	0.07	16	0.03
Salmonella sp. AX 5	0.5	0.14	16	0.06
Klebsiella pneumoniae C 14	32	0.08	32	0.03
K. pneumoniae C 16	1	0.6	16	0.15
Proteus vulgaris S 10	16	0.14	16	0.14

[a] Concentration of CGP 4832 or rifampicin needed to induce 50% inhibition of RNA polymerase. The substances were in the hydroquinone form. As quinones they are 2~5 times less active. The enzyme from *E. coli* ETH 2018 was highly purified; in all other cases the enzyme activity was measured by using ether permeabilized cells.

attributable to stronger inhibition of the target enzyme, DNA-dependent RNA polymerase[9,10] we analyzed a series of different bacterial strains. As shown in Table 2, there is no correlation between MIC and enzyme inhibition. A comparison with rifampicin demonstrated that, in general, CGP 4832 is 2~5 times less potent in its action on the RNA polymerase. The low MICs for certain Gram-negative strains consequently cannot be due to better enzyme inhibition.

Antibacterial Activity of CGP 4832 against *E. coli* Mutants with Defects in their Cell Envelope

An obvious explanation for the superior activity of CGP 4832 against certain bacteria might be that it penetrates more easily and more rapidly through the cell wall than rifampicin. We therefore determined the antibacterial activity of the two rifamycin derivatives against a range of *E. coli* mutants with known defects in their lipopolysaccharide structure.

The parent strain, *E. coli* F 464, contains the complete core oligosaccharide, while in the mutants F 612 to F 515 the oligosaccharides are smaller and less complete. *E. coli* F 515 contains 2-keto-3-deoxyoctanate as the only sugar moiety[11]. The data given in Table 3 clearly show that none of the lipopolysaccharide mutants tested is as sensitive to CGP 4832 as some *E. coli* wild-type strains are. There is a considerable increase in sensitivity to CGP 4832 from the wild-type F 464 to the deep-rough mutant F 515, but this is accompanied with a completely parallel increase in sensitivity to rifampicin. Defects in lipopolysaccharide structure therefore cannot be responsible for the high activity of CGP 4832 against some *E. coli* strains.

Rate of Resistance to CGP 4832

Mutants resistant to CGP 4832 were selected under two different conditions. Using 100 μg/ml of drug, resistant mutants were obtained at a rate very similar to that found with rifampicin (Table 4). However, after exposure of the bacteria to only 1 μg/ml CGP 4832 the rate of mutation was 50~100 times higher. For most mutants produced in this way, the MICs of rifampicin and CGP 4832 were similar. Thus, high sensitivity of certain *E. coli* strains against CGP 4832 can be converted in a one-step mutation to normal sensitivity, as to rifampicin.

Table 3. Antibacterial activity of CGP 4832 and rifampicin against *Escherichia coli* mutants with defects in their lipopolysaccharide structure.

For a detailed description of the mutants see ref 11.

E. coli strain	MIC (μg/ml)	
	CGP 4832	Rifampicin
F 464	32	16
F 612	32	32
F 539	2	0.5
F 583	1	0.5
F 515	0.5	0.25

Table 4. Rate of resistance of *Escherichia coli* strain B 45 to CGP 4832 and rifampicin.

Type of mutation	Selecting agent and condition	Rate of mutation ($\times 10^{-8}$)
CGP $4832^S \rightarrow$ CGP 4832^R $rif^{S\,a}$	1 μg/ml CGP 4832	50
CGP $4832^S \rightarrow rif^{R\,b}$	100 μg/ml CGP 4832	0.44
$rif^S \rightarrow rif^{R\,b}$	100 μg/ml rifampicin	1.1

[a] Mutants no longer highly sensitive to CGP 4832, but still inhibited by rifampicin and CGP 4832 at the usual concentrations of 8~16 μg/ml.

[b] Mutants resistant to at least 100 μg/ml rifampicin or CGP 4832.

rif: Rifampicin, ^{S}: sensitive, ^{R}: resistance.

Discussion

The new rifamycin derivative CGP 4832 is up to 400 times more active than rifampicin against certain Gram-negative bacteria. This greater potency is not due to stronger inhibition of the target enzyme, RNA polymerase. There are no indications of inactivation of rifampicin, or activation of CGP 4832, by the sensitive microorganisms. It is therefore reasonable to assume that the penetration of CGP 4832 into the bacterial cell is somehow facilitated. Lipopolysaccharides do not seem to play a decisive role, since mutants having defects in the lipopolysaccharide structure are affected in a similar way by CGP 4832 and rifampicin. The high mutation rate to CGP 4832-resistance could point to a single protein responsible for the transport of CGP 4832.

References

1) BURGESS, R.: A new method for the large-scale purification of *E. coli* RNA polymerase. J. Biol. Chem. 244: 6160~6167, 1969
2) BERG, D.; K. BARRETT & M. CHAMBERLIN: Purification of two forms of *E. coli* RNA polymerase and of sigma component. *In* Methods in Enzymology. Vol. 21. *Eds.*, L. GROSSMAN & K. MOLDAVE, pp. 506~519, Academic Press, New York, 1971
3) MANGEL, W. F.: Initial steps in the large-scale purification of *E. coli* RNA polymerase. Arch. Biochem. Biophys. 163: 172~177, 1974
4) WEHRLI, W.; F. KNÜSEL, J. NÜESCH & M. STAEHELIN: Interaction of rifamycin with bacterial RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 61: 667~673, 1968
5) FELIX, H. R. & W. WEHRLI: RNA synthesis in *E. coli* cells made permeable to nucleoside triphosphates by various methods. Experientia 34: 1667, 1978
6) VOSBERG, H. P. & H. HOFFMANN-BERLING: DNA synthesis in nucleotide-permeable *E. coli* cells. I. Preparation and properties of ether-treated cells. J. Mol. Biol. 58: 739~753, 1971
7) GLOVER, S. W.: Luria & Delbrück fluctuation test. *In* Experiments in Microbial Genetics. *Eds.*, R. C. CLOWES & W. HAYES, pp. 22~26, Blackwell, Scientific Publication, Oxford, 1968
8) KUMP, W. & H. BICKEL: Zur Kenntnis von Rifamycin-S. Helv. Chim. Acta 56: 2348~2377, 1973
9) WEHRLI, W.: Ansamycins, chemistry, biosynthesis and biological activity. Top. Curr. Chem. 72: 21~49, 1977
10) WEHRLI, W.: Rifampicin, mechanism of action and resistance. Rev. Infect. Dis. 5 (Suppl. 3): S407~S411, 1983
11) SCHMIDT, G.; B. JANN & K. JANN: Immunochemistry of R lipopolysaccharides of *E. coli*. Eur. J. Biochem. 16: 382~392, 1970
12) OPPOLZER, W.; V. PRELOG & P. SENSI: Konstitution des Rifamycins B und verwandter Rifamycine. Experientia 20: 336~339, 1964