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Antibiotics GE23077, Novel Inhibitors of Bacterial RNA Polymerase

I. Taxonomy, Isolation and Characterization

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GE 23077 factors A1, A2, B1 and B2 are novel antibiotics isolated from fermentation broths of an *Actinomadura* sp. strain. GE23077 antibiotics are cyclic peptides, which inhibit *Escherichia coli* RNA polymerase at nM concentrations. Both rifampicin-sensitive and rifampicin-resistant polymerases are inhibited, whereas *E. coli* DNA polymerase and wheat germ RNA polymerase are substantially not affected. In spite of the potent activity on the enzyme, the antibiotics generally show poor activity against whole cell bacteria. The spectrum of activity is restricted to *Moraxella catarrhalis*, including clinical isolates, with partial activity against *Neisseria gonorrhoeae* and *Mycobacterium smegmatis*.

In the course of a screening program for new antibacterial agents produced by uncommon actinomycetes, the novel cyclic heptapeptide antibiotics GE 23077 factors A1, A2, B1 and B2 have been isolated (Figure 1). This paper deals with the taxonomy and fermentation of the producer strain, the isolation of the antibiotics and their biological activity.

Materials and Methods

Taxonomy of Producing Strain

The mycelium, grown in the seed medium described below, was harvested by centrifugation and washed twice in sterile one quarter strength Oxoid Ringer's solution. Aliquots of the suspension were streaked in a cross-hatched manner onto various media recommended by Shirling and Gottlieb[1)] and several media recommended by Waksman[2)]. The ability to use a variety of carbohydrates was determined in ISP8 medium[1)] containing the carbon source at a final concentration of 2% (w/v). All media were incubated at 28°C for 21 days. Colour was assessed in natural daylight, using the Colour Atlas[3)]. Microscopic examination and cell dimensions were measured using the culture grown on one-tenth strength humic acid medium[4)] for seven days at 28°C. The enantiomeric form of diaminopimelic acid was determined according to Staneck and Roberts[5)]. Whole cell sugars were determined by gas chromatography[6)]. Polar lipids and isoprenoid quinones were extracted according to Minnikin *et al.*[7)]. Menaquinones were analyzed by HPLC[8)]. Fatty acids were analyzed as described by Kroppenstedt *et al.*[9)].

Fermentation

The strain was grown in a seed medium consisting of glucose 2%, yeast extract 0.2%, soybean meal 0.8%,

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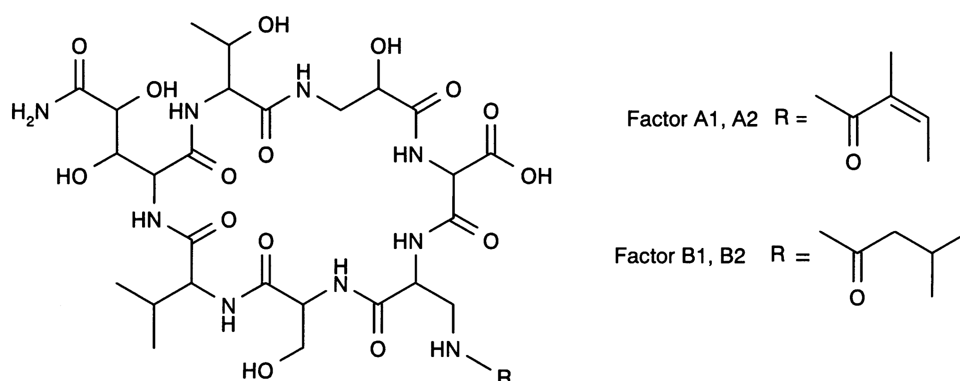
Fig. 1. Chemical structure of GE23077 factors A1, A2, B1, B2.



tryptone 0.5%, NaCl 0.1% and $CaCO_3$ 0.4%. A 72-hour culture grown in Erlenmeyer flasks at 28°C and 200 rpm was inoculated (2%) into 3 liters and, after 48 hours incubation, into 200 liters of the same medium. The 3 and 200 liters (working volumes) fermenters were run at 28°C with 0.5 v/v/minute aeration and 900 and 200 rpm stirring, respectively. Production was monitored by HPLC and with the assay of RNA polymerase inhibition. Growth was measured as packed mycelium volume (PMV%) by centrifuging 10 ml culture in graduated glass tubes.

Isolation of GE 23077 Complex

Harvested broth was filtered and the mycelium was removed by filtration with Hyflo filter matrix. The filtrate was stirred batch-wise with S112 polystyrene resin (The Dow Chemical Co., Midland, MI, USA). The resin (6 liters) was then recovered, washed with water and eluted with a mixture of acetone : water : *n*-butanol 8 : 1 : 1 (v/v). A crude preparation of GE23077 complex was obtained by column (47×7 cm) chromatography on S112 resin, previously equilibrated with 0.1 M $(NH_4)_2SO_4$, eluted at 25 ml/minute flow rate, with a 240 minutes linear gradient from 0% to 100% of phase B in phase A. Phase A was 100 mM ammonium sulphate buffer (pH 7) and phase B was water. The crude preparation was further purified by chromatography on a column containing silanised silica gel (E. Merck; Darmstadt F. R. Germany) equilibrated with 0.1 M ammonium sulphate buffer (pH 7). The column was eluted at a flow rate of 60 ml/minute using a medium pressure apparatus (Buchi Preparative LC-system B680-A, Flawil, Switzerland). GE23077 complex was eluted by increasing stepwise the percentage of methanol in 0.1 M ammonium sulphate buffer (pH 7), dried under reduced pressure and submitted to several cycles of washing with methanol. The complex was further purified by chromatography on 20 ml Supelclean LC-SAX silica based anionic exchange resin (Supelco Inc; Bellefonte, USA) equilibrated in water. After repeated washing with water, a 160 minutes linear gradient was applied from 0 to 1 mM HCl. The eluted fractions were analyzed by HPLC and those containing GE23077 were pooled and lyophilized to yield a white powder.

Purification of Factors A1, A2, B1 and B2

Factors in GE23077 complex were isolated by repeated chromatographic runs of preparative HPLC on a 250×10 mm Supelcosil LC8 column, 5 μm, (Supelco Inc; Bellefonte, USA) eluted at 4 ml/minute flow rate with a 25 minutes linear gradient from 50% to 80% of phase B, followed by 5 minutes elution with 80% of phase B. Phase A was methanol : 100 mM ammonium sulphate, pH 7 buffer, 5 : 95 (v/v), and Phase B was methanol : water 2 : 8 (v/v). UV detection was at 232 nm.

HPLC Analysis

Fermentation and purification processes were monitored by HPLC on a 250×4.6 mm Supelcosil LC8 column, 5 μm, (Supelco Inc; Bellefonte, USA) eluted at 1 ml/minute flow rate in isocratic mode for 25 minutes of 82% Phase A and 18% Phase B. Phase A and B were solutions of methanol : 40 mM ammonium formate buffer 1 : 99 (v/v) and methanol : 40 mM ammonium formate 70 : 30 (v/v), respectively. Factor A1, B1, A2 and B2 eluted with *ca.* 10.3, 11.9, 14.8 and 16.1 minutes retention times,

respectively. UV detection was at 232 nm.

Inhibition of RNA Polymerase

Inhibition of RNA polymerase was determined in a cell free transcription assay, modified from BURGESS[10]. Briefly, the [H^3]-UTP incorporation in RNA was measured in the material precipitated upon addition of trichloroacetic acid (TCA). The reaction mixture (50 μl) contained 50 mM Tris-HCl (pH 8), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), 10 μg/ml BSA, 20 μg/ml calf thymus DNA, 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.5 μCi ^{3}H-UTP and 0.5 U of *Escherichia coli* RNA polymerase enzyme (Epicentre Technology; Madison WI, USA). 5 μl of the antibiotic solution were added to 45 μl of the reaction mixture, incubated at 37°C for 15 minutes and then quenched with 150 μl of ice-cold 10% (w/v) TCA. After 30 minutes in ice, the well content was collected on glass-fiber filters (Filtermat A, Wallac, Turku, Finland) and radioactivity was determined in a β-Plate scintillation counter (Wallac). In the same assay, activity was also measured versus the rifampicin resistant *E. coli* RNA polymerase (Promega; Madison WI, USA), the wheat germ RNA polymerase (Epicentre Technology; Madison WI, USA) and the *Bacillus subtilis* RNA polymerase (kind gift from Prof. A. GALIZZI, Pavia University, Pavia Italy).

Inhibition of DNA Polymerase

Inhibition of DNA polymerase activity was tested using a procedure similar to the RNA-polymerase assay. Briefly, the reaction mixture (50 μl) contained 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.2 mM DTT, 10 μg/ml BSA, 20 μg/ml calf thymus DNA, 0.02 mM dATP, 0.02 mM dCTP, 0.02 mM dGTP, 0.3 μCi ^{3}H-TTP and 1 U of *E. coli* DNA polymerase enzyme (Promega; Madison WI, USA). 5 μl of antibiotic solution were added to 45 μl of reaction mixture, incubated at 37°C for 15 minutes and then quenched with 150 μl of ice-cold 10% (w/v) TCA. Filtration and radioactivity counting were performed as in the RNA polymerase assay.

Antimicrobial Activity

Antimicrobial activity of GE23077 complex on *E. coli*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *B. subtilis*, *Mycobacterium smegmatis* were determined by broth microdilution in cation-adjusted Difco Mueller Hinton Broth. Other organisms tested by broth microdilution included *Streptococcus pyogenes* (Difco Todd Hewitt broth), *Haemophilus influenzae* (Difco Brain Heart Infusion Broth+Difco 1% (v/v) supplement C) *Neisseria gonorrhoeae* (Difco GC base Broth+BBL 1% (v/v) IsoVitaleX), *Corynebacterium jeikeium* (DifcoTripticase Soy Broth+10% fetal calf serum), *E. coli* sp. and *E. coli* ATCC 25922 (Invitrogen Terrific Broth buffered with 50 mM Sodium Phosphate pH 7). Inocula were 10^4 CFU/ml. All strains were incubated at 35°C in air, except *H. influenzae* and *N. gonorrhoeae* which were incubated in 5% CO_2. Incubation time was 18~24 hours except for *M. catarrhalis*, *N. gonorrhoeae*, *H. influenzae* and *M. smegmatis* that were grown for 48 hours.

Results

Description and Taxonomy of Producing Strain

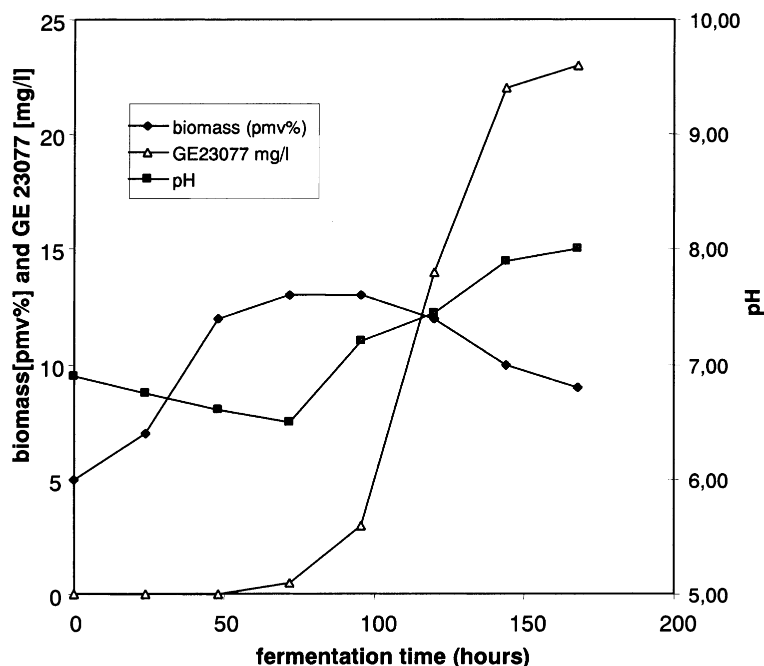
The strain producing GE23077 antibiotics grew well on many standard solid media. Colonial appearance, substrate and aerial mycelium colour and pigment production are reported in Table 1. The strain was able to utilize starch and grew well on glucose, cellobiose, cellulose and xylose. It moderately grew also on arabinose, fructose, galactose, inositol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, ribose, salicin, sucrose when used as sole carbon source. Microscopic examination of vegetative mycelium revealed the presence of extensively branched hyphae (0.5 μm in diameter). No fragmentation was observed. The aerial mycelium contained slightly twisted chains of large spores (1.2~1.4 μm in diameter). No pseudosporangia were observed. Spore dimensions exceeded those of the mycelium, giving rise to a "segmented" appearance of the spore chain. The strain contained *meso*-2,6-diaminopimelic acid as the sole peptidoglycan diamino acid in the whole cell hydrolysate. Sugar analysis showed madurose in whole cell hydrolysate. Fatty acid profile consisted of branched, saturated and unsaturated fatty acids. The 14-methylpentadecanoic acid, *iso*-16, the 10-methylheptadecanoic acid, and particularly the tuberculostearic acid (10 methyloctadecanoic acid) were the predominant constituents. Phosphatidyl inositol, phosphatidyl inositol mannosides, phosphatidyl glycerol and diphosphatidyl glycerol were the major polar lipids found. The more abundant menaquinone found was an esahydrogenated menaquinone with nine isoprene units MK-9 (H_6) followed by smaller portions of the tetrahydrogenated MK-9 (H_4) and octavehydrogenate MK-9 (H_8) variants. The strain was classified as *Actinomadura* sp. in accordance with KROPPENSTEDT *et al.*[9] and deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) with the accession number 13491.

Table 1. Cultural characteristics of the strain producing GE23077 antibiotics.

MEDIUM	GROWTH[a)]	VEGETATIVE MYCELIUM[b)]	AERIAL MYCELIUM	DIFFUSIBLE PIGMENT
Bennet's	+++	Pink patches, glutinous texture, translucent, amorphous with a diffuse edge	absent	None
Calcium malate	+++	very pale pink, matt texture with a diffuse edge	white, abundant	None
Czapek Glucose	+++	dull pink, glutinous texture, convoluted with an entire edge	absent	None
Czapek Sucrose	+++	pink, glutinous texture with an entire edge	light pink, abundant	None
Egg albumin	+++	very pale pink 6 opaque, glutinous texture, with edge entire; discolouration at growing edge	white	None
Glucose asparagine	++	light pink 6 white speckles, glutinous texture with edge entire	absent	None
Hickey and Tresner	+++	pale yellow 6 opaque glutinous texture (matt at edges), convoluted with an entire edge	white, sparse	None
ISP2	+++	pale pink speckles, glutinous texture with edge entire	absent	None
ISP3	+++	pink (2-E-9), flat, smooth	Pale pink 6 white tufts	None
ISP4	+++	Deep pink (2-I-10), dimpled with edge entire	absent	None
ISP5	++	very pale pink (2-C-1) speckles 6 opaque, glutinous texture with edges diffuse	white tufts, more concentrated at edges	None
ISP6	+++	pale yellow (deeper yellow patches) 6 opaque glutinous texture, convoluted with an entire edge	absent	None
ISP7	+++	pink 6 opaque, glutinous texture with edges diffuse	white tufts (mainly at growing edge)	None
Nutrient	+++	Yellow 6 opaque glutinous texture, convoluted with an entire edge	white tufts (mainly at growing edge)	None
Oatmeal	++	very pale pink (4-C-1), flat, smooth ,dimpled, amorphous	absent	None
Potato	+++	pink (4-D-8), glutinous texture, convoluted with an entire edge	Absent	None
Sabouraud	++	opaque glutinous texture, with an entire edge	Absent	None
Skimmed milk	+++	pink (4-F-8), glutinous texture, with an entire edge; clearing zones around growing edge	Absent	None
Potato glucose	+++	pink (2-H-8), matt texture, convoluted	very light pink	None

a) ++: moderate growth; +++: good growth
b) Codes in parenthesis indicate nearest available colour codes [3)]

Fig. 2. Time-course of *Actinomadura* sp. fermentation in a 200 liters bioreactor.



Fermentation

The production of GE23077 antibiotics started, as shown in Figure 2, 50 hours after inoculum, when *Actinomadura* sp. entered the stationary phase of growth, and reached its maximum (in the range from 20 to 25 mg/liter of GE23077 complex) between 120 and 160 hours.

Isolation and Physico-chemical Properties

GE23077 complex consisted of two couples of isomers: factors A1, A2 (molecular weight 803 amu) and factors B1, B2 (molecular weight 805 amu), as shown by the HPLC profile reported in Figure 3. During the isolation of individual peaks an equilibration between factors A1 and A2 and factors B1 and B2 was observed. In particular the purified factor A1 equilibrates to a mixture of A1 and A2, as well as A2 equilibrates to a mixture of A1 and A2. The same behavior was observed for purified factors B1 and B2. The equilibration rate was accelerated at acidic and basic conditions. Unlike in aqueous solutions, GE 23077 factors A2 and B2 were completely converted into factors A1 and B1 after 12 hours in DMSO at room temperature. Therefore, the structure elucidation by NMR was carried out in this solvent to circumvent problems of signal overlapping. The structure determination is described in a following paper[11].

Fig. 3. HPLC analysis of GE23077 complex.

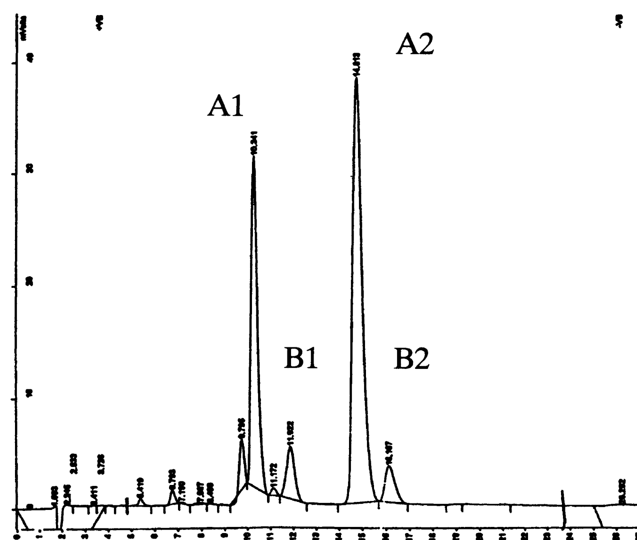
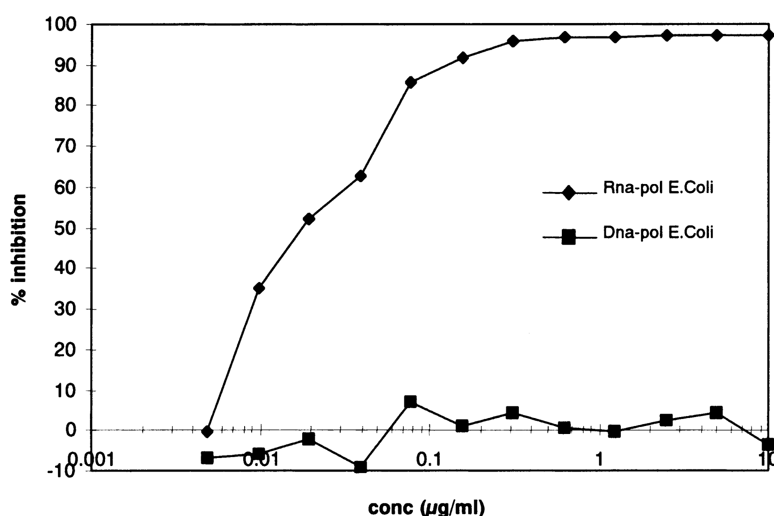


Fig. 4. Activity of GE23077 complex on *E. coli* RNA polymerase and *E. coli* DNA polymerase.



Biological Activity

GE23077 complex showed an IC_{50} on *E. coli* and *B. subtilis* RNA polymerase at 0.02 μg/ml (25 nM). An *E. coli* rifampicin resistant RNA polymerase was also inhibited at similar concentrations (IC_{50} 0.04 μg/ml). The individual GE23077 factors A1, A2, B1 and B2 inhibited *E. coli* RNA polymerase, showing IC_{50}=0.15, 0.035, 0.1 and 0.02 μg/ml, respectively. *E. coli* DNA polymerase and wheat germ RNA polymerase were not inhibited even at high antibiotic concentrations (IC_{50} >1000 μg/ml). Figure 4 shows the dose response curve of *E. coli* RNA and DNA polymerase inhibition. Details on the mode of action of GE23077 antibiotics on RNA polymerase will be presented elsewhere.

The spectrum of antibacterial activity of GE23077 complex is reported in Table 2. The majority of the microorganisms tested were not inhibited even at high antibiotic concentrations. *M. catarrhalis* was however susceptible to the complex with MIC in the 4~8 μg/ml range. Against this pathogen the individual factors A1, A2, B1 and B2 showed comparable potency. The antibiotics had also marginal activity against *N. gonorrhoeae* (MIC 256 μg/ml) and *M. smegmatis* (MIC 512 μg/ml).

Discussion

GE23077 antibiotics and rifampicin are structurally unrelated compounds. They both inhibit bacterial RNA polymerase with comparable potency in cell free experiments and are substantially inactive against bacterial DNA polymerase and wheat germ RNA polymerase. GE23077 antibiotics, however, are equally potent against both rifampicin resistant and sensitive *E. coli* RNA polymerases. GE23077 antibiotics show activity only against a restricted group of bacteria, showing significant inhibition against clinical isolates of *M. catarrhalis* and marginal activity against *N. gonorrhoeae* and *M. smegmatis*. The majority of the bacteria tested were not susceptible, including the bacteria whose RNA polymerase is sensitive. Both *B. subtilis* and *E. coli* are equally insensitive when grown either in minimal or in complex media (data not reported). Thus the antibiotics do not behave as an antimetabolite, rather they seem unable to reach intracellular concentrations resulting in growth inhibition. *E. coli acr*AB and *tol*C[12] impaired mutants are moderately susceptible, thus indicating that the *acr*AB *tol*C efflux system contributes to resistance to the GE23077 antibiotics. However, the lack of activity in whole cells may be due to additional factors and this is still matter of investigation. Interestingly, *M. catarrhalis* is susceptible. The activity found against clinical isolates of *M. catarrhalis* suggests that GE23077 may potentially acquire activity against other pathogens through chemical modifications. The cyclic heptapeptide structure of GE23077 is unique among known RNA polymerase inhibitors and generally among known microbial products. GE 23077 is constituted

Table 2. Antimicrobial activity of GE23077 complex.

	MIC (μg/ml)		
Strain	**GE23077**	**rifampicin**	**penicillin G**
76 *Moraxella catarrhalis* -ATCC8176-	128	0.03	0.03
3292 *Moraxella catarrhalis* -U503 clinical isolate-	8	0.06	0.03
3293 *Moraxella catarrhalis* -U501 clinical isolate-	4	0.06	16
3294 *Moraxella catarrhalis* -W501 clinical isolate-	8	0.06	0.13
997 *Neisseria gonorrhoeae* -ISM68/126 clinical isolate-	256	0.06	0.03
1600 *Neisseria gonorrhoeae* -clinical isolate-	512	0.03	0.03
731 *Neisseria gonorrhoeae* -SB2053 clinical isolate-	1024	0.06	0.5
970 *Haemophilus influenzae* -type b ATCC19418-	>1024	0.06	0.25
3782 *Escherichia coli* -*E.coli*_ SP-	32	0.06	16
1281 *Escherichia coli* -ATCC25922-	>1024	8	64
3793 *Escherichia coli* -VECO2004(*acr*AB)-	256	1	1
3794 *Escherichia coli* -VECO2005(*acr*R)-	>256	2	16
3795 *Escherichia coli* -VECO2066(*tol*C)-	128	2	2
3796 *Escherichia coli* -VECO2054(MC1061)-	>256	4	16
G1640 *Escherichia coli* -K12 hyperpermeable-	>1024	0.13	Nd
2508 *Corynebacterium jeikeium* -ATCC43734-	>1024	0.03	>128
819 *Staphylococcus aureus* -Smith-	>1024	0.015	0.06
49 *Streptococcus pyogenes* -C203-	>1024	0.06	0.03
102 *Bacillus subtilis* -ATCC6633-	>1024	0.015	0.008
3754 *Mycobacterium smegmatis*	512	2	128
1671 *Mycobacterium smegmatis* -ATCC35797-	>1024	16	>128

of six α amino acids and one β amino acid. A similar ring backbone was found in the weakly cytotoxic cyanobacterium product nostophycin[13], which is however structurally different from GE23077 in the ring substituents and whose activity on RNA polymerases is unknown.

In conclusion, GE23077 antibiotics constitute a new class of bacterial RNA polymerase inhibitors.

Acknowledgments

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