SYNTHESIS AND BIOLOGICAL ACTIVITY OF SOME AMIDE DERIVATIVES OF THE LANTIBIOTIC ACTAGARDINE

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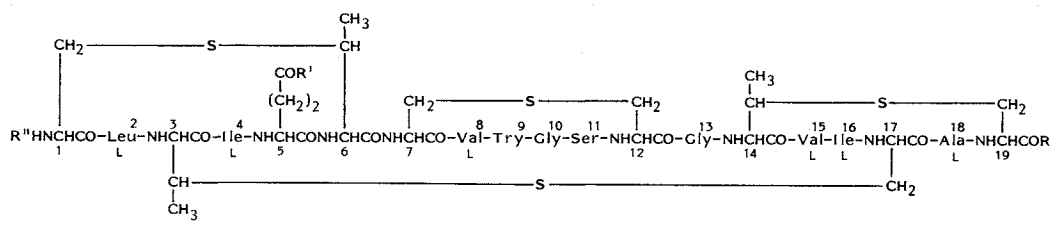
A series of basic carboxamides of actagardine (**1**), a lantibiotic possessing good antistreptococcal activity, were synthetized. Some physico-chemical characteristics, in particular charge and lipophilicity, that influence water solubility were determined. The *in vitro* and *in vivo* activity was evaluated. The monocarboxamides were generally more active than actagardine against selected Gram-positive bacteria. The 3,3-dimethylamino-1-propylamide hydrochloride (**4**) showed good water solubility, bactericidal action and favourable antibacterial activity and it appears to be a suitable drug for further investigation.

Actagardine (**1**)[1~3] is a lanthionine-containing polypeptide antibiotic, belonging to the family of lantibiotics,[4] whose structure was recently elucidated.[5] It consists of 14 aliphatic amino acids (including one lanthionine and three β-methyllanthionines) and one aromatic (tryptophan) amino acid which generate a polypeptide chain where thioether bridges form four rings. It possesses one acidic function belonging to the glutamic acid side chain (γ-COOH). The terminal amino and carboxyl groups belong to the alanine moieties of two β-methyllanthionines. The sequence is shown in Fig. 1. FAB-MS measurements[5] account for one more oxygen, whose position has not yet been established.

Actagardine, as well as its related compound designated as metabolite D,[3] possesses good *in vitro* activity against Gram-positive bacteria, particularly Streptococci, and obligate anaerobes.[3,6] It shows a good *in vivo* efficacy in experimental *Streptococcus pyogenes* septicemia in the mouse upon subcutaneous administration and low toxicity (mice, ip). Its mechanism of action consists in the specific inhibition of peptidoglycan biosynthesis in the bacterial cell wall.[7]

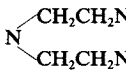
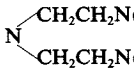
The need to improve the biological properties of actagardine prompted us to prepare some chemical derivatives. As a preliminary approach a series of basic monocarboxamides (**2**~**10**) was synthesized. Two diamides (**11** and **12**) and one *N*-acyl derivative (**13**) were also prepared.

Fig. 1. Sequence of actagardine (**1**).



Actagardine R, R'=OH R''=H

Table 1. Derivatives of actagardine

Compound	R	R′	R″	Yield (%)	Solubility in H_2O (mg/ml)	
					pH 7.3	pH 4.0
2	$NHCH_2CH_2NH_2$	H	H	10	20	80
3	$NH(CH_2)_4NH_2$	H	H	10	30	100
4	$NH(CH_2)_3N(CH_3)_2$ ·HCl	H	H	52	200	600
5		H	H	15	nd	100
6		H	H	10	nd	200
7		H	H	15	<10	60
8		H	H	43	<10	45
9		H	H	20	20	<10
10		H	H	44	<10	20
11	NHC_2H_5	NHC_2H_5	H	88	nd	nd
12	$NHCH_2COOC_2H_5$	$NHCH_2COOC_2H_5$	H	76	nd	nd
13	H	H	$CO(CH_2)_{14}CH_3$	60	nd	nd

[a] See Experimental section.

[b] C, H, N, S were determined on samples previously dried at 140℃ in inert atmosphere. The weight loss was atmosphere. The analytical values found after correction for weight loss and inorganic residue were in agreement

[c] Values determined in MCS-H_2O (4:1) by titration with either 0.1 N NaOH or 0.1 N HCl.

[d] Free base.

[e] Actagardine (acid form) is insoluble in water. The value given is log P between *n*-octanol and 0.1 M phosphate

nd: Not determined.

Chemistry

Compounds **2**～**10** (Table 1) were obtained by reaction of actagardine (**1**) with the selected amines in *N*,*N*-dimethylformamide (DMF) in the presence of diphenylphosphorylazide (DPPA) at 0～5℃. An excess of the reactant was used to minimize side reactions involving either the free amino function of actagardine or the unprotected primary or secondary amino groups of the reactant itself. A sufficient amount of triethylamine (TEA) was added to catalyze the reaction and to free the base when the hydrochloride of the reactant was used.

For the synthesis of the *N*-di(2-aminoethyl) monocarboxamide (**5**) protection of the primary amino groups of diethylenetriamine by way of the benzylidene group was necessary. The protecting functions of the dibenzylidene intermediate, which was not isolated, were then removed with diluted HCl at room temperature.

When the amidating agent was ethylamine or glycine ethyl ester diamides **11** and **12** were obtained. This is somewhat surprising and difficult to be interpreted considering that the same 1:4 molar ratio of actagardine to the amine was used. The only difference is the structure of the reacting amines and the isoelectric point of the final amides. In fact, basic monocarboxamides were obtained with amines carrying additional amino groups, while only dicarboxamides were obtained when a monoalkylamine or an amino

(R, R′ and R″ see Fig. 1)[a].

Titration (EW)		pK_{MCS} (MCS-H_2O)	log P		UV λ_{max} nm ($E^{1\%}_{1cm}$)	MW	Formula[b]
HTBA (pyridine)	$HClO_4$ (AcOH)		pH 7	pH 3			
2,400	1,154	9.7	−0.174	−0.973	280 (21.00)	1,932.4	$C_{83}H_{130}N_{22}O_{23}S_4$
nd	1,058	9.8	−0.468	−0.922	280 (19.11)	1,960.4	$C_{85}H_{134}N_{22}O_{23}S_4$
1,082[c] (nd)[d]	2,119[c] (1,142)[d]	9.2	−0.790	−1.644	279 (22.36)	2,010.9	$C_{86}H_{137}ClN_{22}O_{23}S_4$
nd	750	nd	−0.680	−1.353	279 (25.67)	1,975.1	$C_{85}H_{135}N_{23}O_{23}S_4$
nd	814	nd	−0.066	nd	279 (25.04)	2,031.6	$C_{89}H_{143}N_{23}O_{23}S_4$
2,289	1,098	8.4	−0.133	−0.733	280 (22.12)	1,958.7	$C_{85}H_{132}N_{22}O_{23}S_4$
2,084	1,036	7.5	0.214	−0.955	280 (24.08)	1,972.4	$C_{86}H_{134}N_{22}O_{23}S_4$
2,160	1,038	7.5	1.084	−0.562	280 (22.00)	2,026.5	$C_{90}H_{140}N_{22}O_{23}S_4$
2,037	905	7.2	nd	−0.145	278 (23.14)	1,972.45	$C_{86}H_{134}N_{22}O_{23}S_4$
—	2,008	—	nd	nd	280 (25.00)	1,944.4	$C_{85}H_{134}N_{22}O_{22}S_4$
—	2,190	—	nd	nd	290 (29.00)	2,060.5	$C_{89}H_{138}N_{22}O_{26}S_4$
1,012	—	—	nd	nd	280 (29.00)	2,128.7	$C_{97}H_{154}N_{20}O_{25}S_4$

measured by thermogravimetry. The inorganic residue was determined after heating the samples at 900℃ in oxygen with the calculated ones.

buffer pH 7.3.

acid ester was used.

Finally, the treatment of actagardine with an activated alkyl ester led to the acylation of the terminal amino group (compound **13**). The structure of the 3,3-dimethylamino-1-propylamide hydrochloride (**4**) was confirmed by ^1H NMR studies[5]. All the signals of the core peptide were present. In particular, a strong NOE effect between the amide proton of the propylamino side chain and the CH_α resonance of the terminal alanine demonstrated that the amide formation occurs at the Ala-19 residue.

The structures of the other amide derivatives were established by analogy of their ^1H NMR spectra with that of compound **4**.

The IR spectra of these compounds were substantially unmodified with respect to that of actagardine. They were in accordance with the polypeptide structures but were not diagnostic enough for the formation of new amide bonds because of the presence of the initial strong peptide bands (1660, 1525 and 1235 cm^{-1}). Only for compound **12** a band at 1740 cm^{-1} ($\nu_{C=O}$ ester) confirmed the presence of the ethylglycine moiety.

The UV spectra showed the same absorption pattern as in actagardine[3] indicating that the tryptophan unit was unaffected.

Acid-base titrations in methylcellosolve (MCS)-H_2O (4:1) showed that additional basic functions were present in compounds **2**~**10**, but were absent in compounds **11**~**13**. The equivalent weights (EWs), determined in non-aqueous solvents for each compound by titration with hydroxytetrabutylamine (HTBA)

Table 2. *In vitro* antibacterial activity[a].

Organism	MIC (μg/ml)												
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**1** (actagardine)
Staphylococcus aureus Tour	6.2	6.2	12.5	50	50	25	12.5	25	6.2	12.5	25	100	25
S. aureus Tour[b]	25	25	25	50	50	50	25	50	12.5	25	25	>100	50
S. aureus Tour[c]	3.1	3.1	6.2	12.5	6.2	25	12.5	12.5	6.2	6.2	12.5	>100	25
Streptococcus pyogenes C 203 SKF 13400	0.2	0.2	0.4	0.8	0.8	0.4	0.8	0.4	0.8	0.8	0.8	12.5	1.6
S. pneumoniae UC 41	12.5	12.5	6.2	12.5	25	6.2	6.2	6.2	6.2	12.5	12.5	25	25
S. mitis L 1320[d]	3.1	3.1	3.1	3.1	6.2	3.1	0.8	1.6	0.8	nd	nd	nd	12.5
S. faecalis L 1321[d]	25	25	50	100	100	25	50	25	12.5	nd	nd	nd	100
S. sanguis L 1322[d]	50	50	50	25	100	50	12.5	50	6.2	nd	nd	nd	100
S. sanguis L 1324[d]	50	25	50	50	100	50	12.5	50	6.2	nd	nd	nd	100
S. salivarius L 1323[d]	0.4	0.4	0.8	0.8	6.2	0.4	0.05	0.8	0.05	nd	nd	nd	3.1
S. bovis L 1325[d]	50	50	25	25	25	25	12.5	25	12.5	nd	nd	nd	100

[a] See Experimental section.
[b] Inoculum 10^6 cfu/ml.
[c] In the presence of 30% bovine serum.
[d] Clinical isolates.
nd: Not determined.

in pyridine and/or perchloric acid in acetic acid, were in accordance with the structures assigned.

Preliminary data of the water solubility at neutral and slightly acidic pH indicated that a certain degree of correlation exists between solubility and basicity of these derivatives, the most basic compounds **2**~**6** being the most soluble. In particular, the amide **4** was about 60 times more soluble than actagardine at pH 4 and about 4 times at the physiological pH.

Biological Activity

All the compounds showed an antibacterial activity similar to that of actagardine (**1**). With the exception of compound **13**, the carboxamides were generally more active than actagardine with MICs 2 to 8 times lower than those of the parent compound (Table 2). The inoculum size did not influence the *in vitro* activity nor did the presence of serum.

The compounds showed a high activity against *S. pyogenes*, *Streptococcus salivarius*, and *Streptococcus mitis*: compounds **8** and **10** were the most active in particular on *S. salivarius* (MIC: 0.05 μg/ml) and *S. mitis* (MIC: 0.8 μg/ml), while against *S. pyogenes* compounds **2** and **3** showed the highest activity (MIC: 0.2 μg/ml). The compounds were less active against the other Streptococci tested.

All monocarboxamides (**2**~**10**) had excellent efficacy in the murine model of *S. pyogenes* septicemia upon sc administration (Table 3) being 2~4 times more effective than actagardine. In contrast, diamides **11** and **12** were less active *in vivo* than the unmodified antibiotic, likely due to their low hydrosolubility at the physiological pH. None of these amides was effective orally up to 300 mg/kg, as was the parent compound.

Among the monocarboxamides the 3,3-dimethyl-1-propylamide hydrochloride (**4**) was selected for further evaluation. Its bactericidal activity was determined in comparison with actagardine (Fig. 2). Against growing cells of *S. pyogenes* compound **4** showed a bactericidal activity comparable to that of actagardine, but at lower concentrations. For both antibiotics 99% and >99.9% of cells were killed within 5 and 24 hours of incubation with concentrations 10 times the MIC; for the amide **4** at least 99.9% killing was obtained after 24 hours of exposure to a con-

Table 3. Activity of the amide derivatives in the murine model of *Streptococcus pyogenes* C 203 SKF 13400 septicemia in comparison with actagardine[a].

Compound	ED_{50}, sc (mg/kg/day)
2	0.14
3	0.19
4	0.23
5	0.62
6	0.29
7	0.25
8	0.24
9	0.22
10	0.19
11	1.17
12	1.06
13	>20
1 (actagardine)	0.81

[a] See Experimental section.

Fig. 2. Bactericidal activity of compound **4** and actagardine (**1**) against *Streptococcus pyogenes* C 203 SKF 13400.

○ Control, □ actagardine (1.6 μg/ml), ▼ actagardine (16 μg/ml), ▲ compound **4** (0.4 μg/ml), ● compound **4** (4 μg/ml).

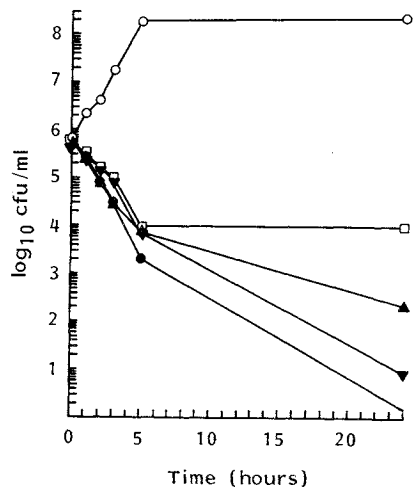


Table 4. Activity of monocarboxamide **4** in the murine model of septicemia in comparison with selected antibiotics[a].

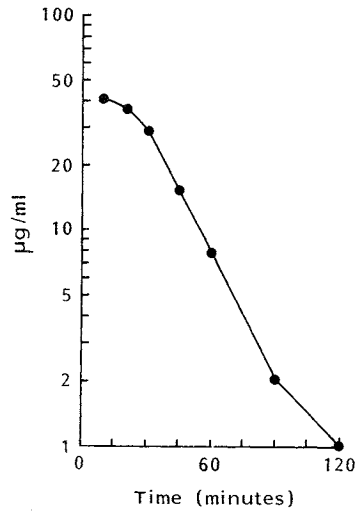
Compound	*Streptococcus pyogenes* C 203 SKF 13400		*Streptococcus pneumoniae* UC 41	
	MIC (μg/ml)	ED_{50} (mg/kg/day)	MIC (μg/ml)	ED_{50} (mg/kg/day)
4	0.4	0.23	6.25	3.5
Benzylpenicillin	0.01	0.29	0.02	20
Ampicillin	0.02	0.1	0.02	4.1
Erythromycin	0.05	0.44	0.01	26
Cephaloridine	0.01	0.03	0.02	0.93
Lincomycin	0.02	0.62	0.5	0.76

[a] See Experimental section.

centration equal to the MIC (0.4 μg/ml). The bactericidal activity did not increase with concentrations equal to 20 and 40 (actagardine) or 100 (compound **4**) times the MIC.

Table 4 compares the sc ED_{50} values of compound **4** with those of several other antibiotics in the experimental septicemia in the mouse. The MIC for the test organisms are also given. Taking into account both infections compound **4** compares well with the selected antibiotics, and had the most favorable *in vitro*/*in vivo* ratio.

Fig. 3. Plasma kinetics of compound **4** in mouse after sc administration of a single 20 mg/kg dose.



The pharmacokinetic of compound **4** in the mouse serum was studied at a dose of 20 mg/kg sc (Fig. 3). Peak level was attained by the first time point (10 minutes), then levels declined with an apparent terminal $t_{1/2}$ of .3 hours; the area under the curve was 27.4 μg·hour/ml. The very high concentrations found in the urine (about 1,000 μg/ml at 1 hour and 500 μg/ml at 4 hours) indicated that the compound is excreted through the kidney. The LD_{50} in mice was 1,225 mg/kg, ip and 700 mg/kg, iv.

Though limited to one compound, the negligible antibacterial activity of *N*-acyl derivative (**13**) (Table 2) indicated that this type of modification is very likely to be detrimental to the antimicrobial properties of actagardine.

Experimental

Evaporation of solvents was carried out with a rotary evaporator at 40°C under reduced pressure.

The reactions were monitored by TLC on Silica gel plates (F_{254}, Merck) developed with a CH_3CN-0.1 M phosphate buffer pH 7.0 mixture (75 : 25, for the piperazinyl derivatives; 60 : 40, for the other compounds). The spots were detected by both UV light at 254 nm and charring with H_2SO_4 at 120°C.

The homogenicity of final products was checked by HPLC analyses which were performed on a column Hibar (250×4 mm; Merck) prepacked with LiChrosorb RP-8 (10 μm), using a Waters chromatograph equipped with a pump Mod. M 45, a UV detector Mod. 440 at 254 nm and connected to a data system SP 4000 (Spectra Physics), and a 20-μl loop injector Rheodyne Mod. 7125. Mobile phase: CH_3CN-0.1 M

phosphate buffer pH 7.5 (40 : 60). Flow rate: 1 ml/minute.

IR spectra (Nujol) were recorded with a Perkin-Elmer Mod. 580 spectrometer.

UV spectra were recorded with a Perkin-Elmer Mod. 320 UV-VIS spectrophotometer in MeOH solution.

Acid-base titrations were carried out in both aqueous MCS - H_2O (4 : 1) and non-aqueous (pyridine or AcOH) solvents. The pK_{MCS} values of the additional basic functions were determined in MCS - H_2O (4 : 1) solution by titration of the compounds with 0.01 N NaOH. The presence of the free amino group of actagardine (pK_{MCS} 6.3) in the derivatives was confirmed by titration with 0.01 N HCl. The EWs were obtained by titration with either HTBA in pyridine or $HClO_4$ in AcOH.

Partition coefficients (log P) were determined between *n*-octanol and H_2O in distilled water (pH 7) and in 0.1 M acetate buffer (pH 3.0). The concentration of the products in each phase was determined spectrophotometrically (UV).

Basic Monocarboxamides (Compounds **2**~**10**)

General Procedure: To a stirred solution of 1 mmol of actagardine (**1**) and 4 mmol of the appropriate diamine in 100 ml of DMF, a solution of 2.5 mmol of DPPA in 20 ml of DMF was added dropwise over 30 minutes while cooling at 0~5℃. The reaction mixture was kept at 5℃ for 6 hours and at room temperature overnight. On adding 500 ml of Et_2O a solid separated which was collected, washed with 100 ml of Et_2O, and redissolved in 500 ml of a BuOH - H_2O - MeOH (45 : 45 : 10) mixture. The organic layer was separated, washed with 100 ml of H_2O, and then concentrated to a small volume. On adding Et_2O, a solid separated which was collected and washed with Et_2O. The crude material so obtained showed at least four spots by TLC. It (1 g) was dissolved in a CH_3CN - 0.01 M phosphate buffer pH 8.0, (85 : 15) mixture (60 ml) and the resulting solution was loaded on a column of silica gel (200 g; 0.2~0.06 mm, Merck), eluting with the following organic solvent - aqueous buffer mixtures: 85 : 15 (0.2 liter), 80 : 20 (0.4 liter), 75 : 25 (0.8 liter), 70 : 30 (0.8 liter), and 65 : 35 (0.8 liter); 50-ml fractions were collected which were checked by TLC. Those containing the desired pure product were pooled, one volume of BuOH was added to prevent foaming, and then most of the CH_3CN was evaporated. The remaining solution was extracted with H_2O; afterward the organic phase was concentrated to a small volume. On standing at room temperature overnight (in some cases cooling was necessary) a solid separated which was collected, washed with Et_2O, and then dried under vacuum at 50℃ overnight. The title compounds were so obtained as the free bases.

Compound **4**

To a stirred solution of 1 mmol of actagardine 3,3-dimethylamino-1-propylamide, prepared as described above, in 200 ml of H_2O, 10 ml of 0.1 N HCl was added dropwise while cooling at 5℃. The resulting solution (pH 4.95) was extracted with BuOH, and then the organic layer was separated and concentrated to a small volume. On addition of Et_2O, a solid separated which was collected, washed with Et_2O, and then dried under vacuum at 50℃ overnight, yielding the title compound, as the hydrochloride.

Compound **5**

By treatment of actagardine with dibenzylidenediethylenetriamines as described in the above general procedure, the diprotected amide was obtained, which was dissolved (1 g) in 200 ml of a 0.1 N HCl - DMF (9 : 1) mixture at room temperature with stirring. After standing overnight, 250 ml of BuOH was added. The aqueous layer was adjusted at 7.0 with a 3% (w/v) aqueous $NaHCO_3$ and the organic layer was separated; afterward it was concentrated to a small volume. On adding Et_2O, a solid separated which was collected, washed with Et_2O, and then it was purified on a silica gel column as previously described.

Compound **9**

To a stirred solution of 1 mmol of actagardine and 4 mmol of cyclopentylpiperazine dihydrochloride in 10 ml of DMF, 10 mmol of TEA was added while cooling at 0℃; afterward the usual general procedure was followed.

Dicarboxamides (Compounds **11** and **12**)

To a stirred solution of 1 g (about 0.5 mmol) of actagardine, 2.1 mmol of ethylamine or glycine ethyl

ester, as the hydrochlorides, and 0.5 ml (about 3.6 mmol) of TEA in 100 ml of DMF, a solution of 0.27 ml (1.23 mmol) of DPPA in 2.5 ml of DMF was added dropwise at 0~5℃ in 15 minutes. After 6 hours at 5℃, the reaction mixture was kept at room temperature overnight. On adding Et_2O (about 500 ml), a solid separated which was collected and redissolved in 100 ml of cold 0.01 N HCl. The solution was extracted with 100 ml of BuOH. The organic layer was washed with 100 ml of 0.1 M phosphate buffer pH 7.38, then with H_2O; afterward the solvent was evaporated and the solid residue was redissolved in 30 ml of 95% EtOH. The resulting solution was filtered. On adding Et_2O, a solid separated which was collected, washed with Et_2O, and then dried under vacuum at room temperature overnight, yielding the appropriate title compound, as the free base.

Compound **13**

To a stirred solution of 0.378 g (1 mmol) of hexadecanoic acid *p*-nitrophenyl ester in 25 ml of DMSO, 0.400 g (about 0.2 mmol) of actagardine and 0.070 ml (0.5 mmol) of TEA were added at room temperature. After 6 hours, the reaction mixture was kept at room temperature in the dark for 1 week; afterward it was adjusted at pH 3.5 with 3 N HCl, and then it was poured into 200 ml of ice cold H_2O. Extraction with BuOH and evaporation of the organic solvent yielded a crude oily residue which was triturated with Et_2O. The separated solid was collected, washed with Et_2O, and then dried under vacuum at room temperature overnight to give 0.27 g of the title compound.

Determination of Antibacterial Activity

Susceptibility Testing

MIC was determined by 2-fold serial dilution method in microtiter (Staphylococci) or in tube (Streptococci). Brain heart infusion broth (Difco) was used; it was supplemented with 2% bovine serum when Streptococci were tested. The inocula were approximately 10^3 or 10^6 cfu/ml to determine the influence of inoculum size on antibacterial activity. The influence of serum was determined on *Staphylococcus aureus* Tour by adding 30% bovine serum to the medium. MIC was defined as the lowest concentration that prevents visible growth after incubation at 37℃ for 18~24 hours.

Bactericidal Activity

The bactericidal activity of compound **4** was compared to that of actagardine. The antibiotics were added at concentrations equal or multiple of the MIC to growing cells of *S. pyogenes* C 203 SKF 13400 in Todd-Hewitt broth (Difco). The cultures were incubated at 37℃ in a water bath with shaking and viable cells were counted at intervals.

Experimental Infection

Experimental septicemia was induced in groups of 5 mice by ip injection of a suspension of the test pathogens. Inocula had been adjusted so that the untreated animals died within 48 hours. Mice were treated by sc or po route once a day for 3 days starting about 30 minutes after infection. On the 10th day the ED_{50} infected animals, expressed in mg/kg was calculated on the bases of the percentage of surviving mice at each dose, by the Spearmann and Kärber method.[8]

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References

1) PARENTI, F.; H. PAGANI & G. BERETTA: Gardimycin, a new antibiotic from *Actinoplanes*. I. Description of the producer strain and fermentation studies. J. Antibiotics 29: 501~506, 1976
2) CORONELLI, C.; G. TAMONI & G. C. LANCINI: Gardimycin, a new antibiotic from *Actinoplanes*. II. Isolation and preliminary characterization. J. Antibiotics 29: 507~510, 1976
3) MALABARBA, A.; M. LANDI, R. PALLANZA & B. CAVALLERI: Physico-chemical and biological properties of actagardine and some acid hydrolysis products. J. Antibiotics 38: 1506~1511, 1985
4) KELLNER, R.; G. JUNG, T. HÖRNER, H. ZÄHNER, N. SCHNELL, K.-D. ENTIAN & F. GÖTZ: Gallidermin: A new lanthionine-containing polypeptide antibiotic. Eur. J. Biochem. 177: 53~59, 1988

5) KETTENRING, J. K.; A. MALABARBA, K. VÉKEY & B. CAVALLERI: Sequence determination of actagardine, a novel lantibiotic, by homonuclear 2D NMR spectroscopy. J. Antibiotics 43: 1082～1088, 1990
6) ARIOLI, V.; M. BERTI & L. G. SILVESTRI: Gardimycin, a new antibiotic from *Actinoplanes*. III. Biological properties. J. Antibiotics 29: 511～515, 1976
7) SOMMA, S.; W. MERATI & F. PARENTI: Gardimycin, a new antibiotic inhibiting peptidoglycan synthesis. Antimicrob. Agents Chemother. 11: 396～401, 1977
8) FINNEY, D. J.: The Spearman-Kärber method. *In* Statistical Method in Biological Assay. *Ed.*, C. Griffin and Co., pp. 524～530, C. Griffin and Co., 1952