SYNTHESIS AND ANTIBACTERIAL ACTIVITY OF SOME DERIVATIVES OF THE ANTIBIOTIC THERMORUBIN

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A series of derivatives has been prepared from the antibiotic thermorubin, some of which show a substantial modification of the original structure. The antibacterial activities are reported.

Thermorubin (**1**) is an antibiotic produced by fermenting *Thermoactinomyces antibioticus* which was shown to possess a high activity against Gram-positive bacteria and a good activity against selected Gram-negative bacteria[1] (see also Tables 1 and 2).

The protection previously shown[1] (ED_{100} 3 mg/kg) in mice infected intraperitoneally with *Streptococcus pyogenes* SKF 13400 has to be considered only indicative because the antibiotic was administered intraperitoneally. However, when tested under the same experimental conditions, it was inactive up to 200 mg/kg both by oral and subcutaneous administration, whereas it showed a certain degree of protection (approximately ED_{50} 50 mg/kg) when administered iv (M. BERTI, personal communication). The poor solubility in water and a substantial inactivation by serum proteins probably accounts for this lack of activity.

The mechanism of action of thermorubin was studied first by PIRALI *et al.*[2] who showed that it is bacteriostatic and acts on the protein synthesis by inhibiting the formation of the complex f Met-tRNA-ribosome - mRNA before the formation of the first peptidic bond. Recent studies[3,4] on the nature of the complex thermorubin-ribosome showed that the inhibition occurs only in the presence of the initiation factors.

Following the determination of the structure[5] a program for the chemical modification of thermorubin was established in an attempt to obtain derivatives with *in vivo* activity.

A series of compounds have been synthesized that are shown in Scheme 1. Due to the different chemical reactivity of the various functions present in thermorubin (**1**) treatment with conventional reagents gave both the expected derivatives and some compounds where the original structure was greatly modified. The preparation of compounds **5** to **9** and **12**, and the assignment of the structures were previously reported[6]. The synthesis and some physico-chemical characteristics of compounds **2** to **4** and **10**, **11** are described in the present paper together with some biological properties of all the compounds.

Chemistry

The general reaction pattern is reported in Scheme 1. The carboxymethyl group in position 3 undergoes the normal reactions of esters. By treating thermorubin (**1**) with aqueous sodium hydroxide followed by acidification and extraction the corresponding acid **2** was obtained which, by action of

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Scheme 1.

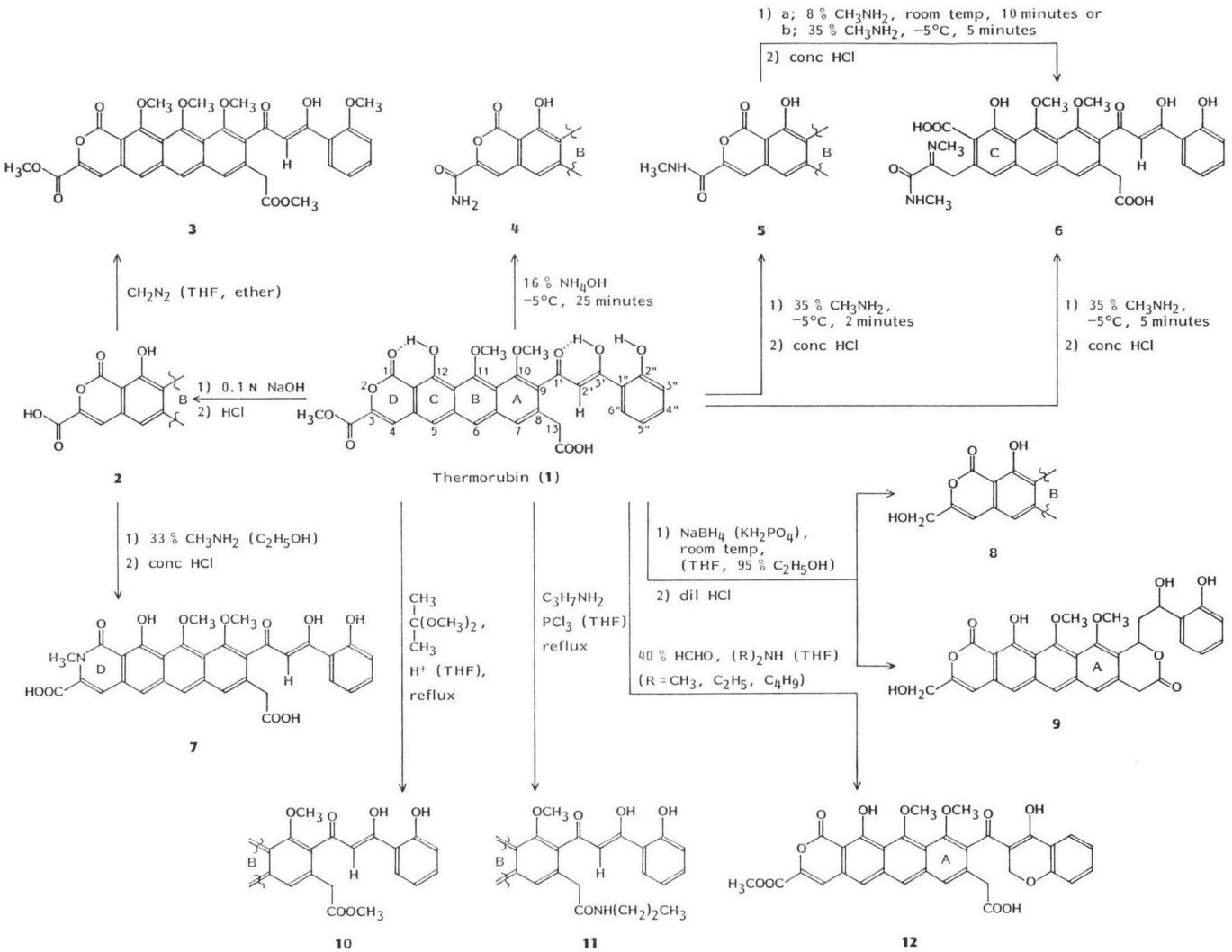
1) a; 8 % CH3NH2, room temp, 10 minutes or
b; 35 % CH3NH2, −5°C, 5 minutes
2) conc HCl
3
4
5
6
CH2N2 (THF, ether)
16 % NH4OH
−5°C, 25 minutes
1) 35 % CH3NH2,
−5°C, 2 minutes
2) conc HCl
1) 35 % CH3NH2,
−5°C, 5 minutes
2) conc HCl
1) 0.1 N NaOH
2) HCl
2
Thermorubin (1)
1) 33 % CH3NH2 (C2H5OH)
2) conc HCl
1) NaBH4 (KH2PO4),
room temp,
(THF, 95 % C2H5OH)
2) dil HCl
8
9
7
CH3
C(OCH3)2,
CH3
H+ (THF),
reflux
C3H7NH2
PCl3 (THF)
reflux
40 % HCHO, (R)2NH (THF)
(R = CH3, C2H5, C4H9)
10
11
12

Table 1. *In vitro* antibacterial activity (MIC, μg/ml) of thermorubin (**1**) and derivatives.

Organism	Compound											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Staphylococcus aureus ATCC 6538	0.006	50	>100	0.05	0.05	1.6	50	0.025	1.6	0.1	12.5	0.012
S. aureus TOUR	0.05	>100	>100	0.4	0.1	6.2	>100	0.1	12.5	1.6	50	0.1
S. aureus TOUR[a]	25	>100	>100	100	25	>100	>100	25	>100	>100	>100	>50
Streptococcus pyogenes C 203 SKF 13400	0.025	>100	>100	0.1	0.1	12.5	100	0.05	50	1.6	100	0.4
S. pneumoniae UC 41	0.05	50	>100	0.05	0.05	25	>100	0.05	25	0.1	100	0.8
Proteus vulgaris X19H ATCC 881	3.12	>100	>100	12.5	1.6	>100	>100	12.5	>100	>100	>100	50
Escherichia coli SKF 12140	6.25	>100	>100	25	6.2	>100	>100	12.5	>100	>100	>100	>50
Pseudomonas aeruginosa ATCC 10145	25	>100	>100	25	12.5	>100	>100	12.5	>100	>100	>100	>50

[a] Determined in the presence of 30% bovine serum.

Table 2. *In vitro* antibacterial activity (MIC, μg/ml) of thermorubin (**1**) and some derivatives against selected organisms.

Organism	Compound		
	1	**5**	**8**
Salmonella typhi ATCC 10749	12.5	25	25
S. paratyphi ATCC 9150	12.5	25	25
S. choleraesuis ATCC 10708	12.5	50	50
S. september L 369*	12.5	25	25
S. enteritidis L 371*	6.25	12.5	12.5
Shigella flexneri ser. 3 ATCC 11836	1.6	6.25	6.25
S. dysenteriae ATCC 9583	6.25	12.5	12.5
S. sonnei L 246*	6.25	12.5	25
Clostridium difficile ATCC 9689	0.8	6.25	1.6
C. difficile ATCC 17857	3.12	6.25	1.6
C. difficile ATCC 17858	6.25	25	6.25
Propionibacterium acnes ATCC 6919	0.4	3.12	3.12
P. acnes ATCC 6922	0.4	3.12	3.12
P. acnes ATCC 25746	0.2	3.12	3.12
Bacteroides fragilis ATCC 23745	0.4	3.12	3.12
B. fragilis ATCC 25285	0.1	0.8	3.12
B. fragilis s. sp. thet. ATCC 8492	0.1	0.4	3.12
B. fragilis s. sp. thet. ATCC 12290	0.8	3.12	3.12
B. fragilis VPI 9032	0.1	0.8	1.6

* Clinical isolates.

diazomethane, led to the already known[5] trimethylthermorubin (**3**).

Amides **4** and **5** were obtained by reacting **1** with aqueous solutions of ammonia and methylamine respectively. These reactions were carried out at -5°C and carefully monitored in order to prevent further reactions of the newly formed amides. In particular, **5** reacts with another molecule of methylamine leading to **6** in which the unsaturated lacton ring is open and a *N*-methylketiminic group is formed at position 3[6].

By allowing acid **2** to react with ethanolic methylamine compound **7** is obtained in which an unsaturated lactam ring is present instead of the lactonic one[6].

The sodium borohydride reduction of **1**, in the presence of potassium dihydrogenphosphate necessary for the complete reaction of the starting material, gives the alcohol **8**. During the neutralization of the large excess of the reducing reagent compound **9** is also formed[6]. In this molecule, besides the reduction of the ester group, the 1,3-diketone is reduced too, while a lactonization between the carboxyl group of the acetic side chain and the newly formed hydroxyl group in position 1′ occurred.

Derivatives of the acetic side chain in position 8 of thermorubin are difficult to obtain; no amides or esters could be obtained by means of the commonly used coupling reagents such as dicyclohexylcarbodiimide and carbonyldiimidazole. By allowing **1** to react in THF with a large excess of 2,2-dimethoxypropane[7] under acidic catalysis the methyl ester **10** is isolated in good yields. Treatment of **1** with a 6: 1 complex of *n*-propylamine and PCl_3[8] in THF gives small amounts of the amide **11**. Finally, **1** reacts with formaldehyde and several secondary amines under the Mannich reaction conditions[9] giving in every case the same product **12** in which a methylenic group links the phenolic hydroxyl in position 2″ at the 2′ position.

In the Experimental section the procedures for the preparation of compounds **2** to **4** and **10**, **11**

are reported together with some NMR data and MS values (obtained by Direct Inlet System LC-MS[6]) which are relevant for the determination of the structures. The synthesis of compounds **5** to **9** and **12** has been described previously[6].

Biological Properties and Structure-activity Relationships

The *in vitro* antibacterial activities (MIC) of the compounds are reported in Table 1. Compounds **4**, **5** and **8** in which only the carbomethoxy group of **1** in position 3 is modified maintain the spectrum of activity of the parent compound although MIC values are generally higher.

Compounds **2**, **3** and **7** are inactive; this result was expected for **3** in which all the hydroxylated functions of the molecule are blocked as methyl ethers or esters, but was rather surprising for **2** and **7**. The activity of **2** on a cell-free extract of *Escherichia coli*[2] is on the other hand only slightly lower than that of **1** determined in the same experiment (data not shown). This result suggests that the free carboxyl group in position 3 has a negative effect on the transport of the compound across the bacterial cell-wall and may explain also the inactivity of **7**.

Compounds **10** and **11**, in which the carboxyl of the acetic side chain is derivatized, are inactive against the Gram-negative strains; **10** retains a moderate activity against the Gram-positive strains while **11** is definitely less active.

A pattern of activity similar to that of **10** is shown by compounds **6** and **9** in which major structural modifications have been introduced. Compound **12**, in which only the disappearance of the phenolic hydroxyl in position 2″ is remarkable, is less active than **1** on streptococci and Gram-negative strains.

The *in vitro* activity of compounds **5** and **8** was also determined in comparison with thermorubin (**1**) against strains of *Shigella* and *Salmonella* and on Gram-positive and Gram-negative anaerobes. All the compounds had a comparable activity against *Shigella* and *Salmonella* strains. Thermorubin was more active than its derivatives on anaerobic bacteria, in particular on *Propionibacterium acnes* and *Bacteroides fragilis*; the activities of the other two compounds were generally comparable, but compound **5** was more active than compound **8** against 3 strains of *B. fragilis* (Table 2).

The MIC of thermorubin and its derivatives **5** and **8** against staphylococci and *E. coli* in the absence and presence of bovine and horse serum and bovine albumin are shown in Table 3. The presence of 30% serum strongly reduced the antibacterial activity of the compounds against all the strains tested; there was a 1,024 to 32,000 fold increase in MIC of thermorubin and a 64 to 2,048 fold increase in MIC of its derivatives compared with the serum-free broth.

MIC of all the compounds were no more than 32 fold higher in the presence of 30% bovine albumin, with the exception of MIC of thermorubin which increased 128 times against *Staphylococcus aureus* ATCC 6538. An increase in serum from 30 to 50% seems to have a more pronounced effect.

Against *E. coli* the decrease in activity of compounds **5** to **8** was difficult to compare with that of thermorubin because the derivatives precipitated at the high concentration in the presence of serum and albumin.

In Vivo Evaluation

In vivo activities of thermorubin (**1**) and compounds **4**, **5** and **8** were compared by using a mouse model intraperitoneal infection with *S. aureus* TOUR. Compounds **4** and **8** were inactive up to 200 mg/kg both by oral and subcutaneous administration. Compound **5** showed a slight improvement

Table 3. Influence of 30% or 50% (in brackets) bovine (BS) and horse (HS) serum or bovine albumin (BA) on the *in vitro* activity of thermorubin (**1**) and selected derivatives against various bacterial strains.

Organism	MIC (μg/ml)											
	Thermorubin (**1**)				Compound **5**				Compound **8**			
	—	BS	HS	BA	—	BS	HS	BA	—	BS	HS	BA
S. aureus	0.0015	12.5	50	0.2	0.05	25	100	0.8	0.012	12.5	nd	nd
ATCC 6538	—	(50)	(100)	(0.4)	—	(100)	(400)	(0.8)	—	nd	nd	nd
S. aureus	0.05	50	nd	nd	0.1	50	nd	nd	0.1	25	6.25	0.8
TOUR	—	nd	nd	nd	—	nd	nd	nd	—	(12.5)	(12.5)	(1.6)
S. aureus L 780*	0.012	25	50	0.2	0.1	50	100	0.8	0.1	50	100	1.6
S. aureus L 793*	0.05	100	100	1.6	0.1	25	100	0.8	0.1	25	100	0.8
S. aureus L 1096*	0.006	50	50	0.2	0.05	25	100	0.8	0.1	50	100	1.6
S. epidermidis L 1142*	0.012	12.5	50	0.2	0.1	25	100	0.8	0.1	50	100	1.6
E. coli	6.25	400	400	>400	12.5	>100	>100	400	25	>200	>200	>200
SKF 12140	—	(>400)	(>400)	(>400)	—	(>100)	(>100)	(400)	—	(>200)	(>200)	(>200)

* Clinical isolates.

nd: Not determined.

with respect to **1** by sc route (ED_{50} 140 mg/kg) whereas it was still inactive (ED_{50} >200 mg/kg) by oral administration. Compound **5** had LD_{50}'s of 133 mg/kg iv, ≃750 mg/kg ip and >2,000 mg/kg po in mice.

Experimental

All compounds were analyzed for C, H and N. The analytical results were within ±0.4% of the theoretical values.

Melting points were determined in open glass capillaries, using a Büchi apparatus and are uncorrected.

The reactions were monitored by TLC on Kieselgel 60 F_{254} plates (Merck) developed with a mixture of $CHCl_3$ - MeOH, 9: 1 and the spots detected by UV light at both 254 and 360 nm.

IR spectra were recorded as nujol mull with a Perkin-Elmer model 580 spectrophotometer.

^1H NMR spectra were recorded at 270 MHz with a Brucker WH-270 spectrometer in DMSO-d_6 solution with TMS as internal standard (δ 0.00 ppm).

EI mass spectrum of compound **10** was recorded on a Hitachi RMU-6L instrument with a Direct Insertion System (DIS) heated to 180°C, electron energy 70 eV, ion source temperature 250°C; the other compounds were analyzed by DIS LC/MS on a HP 5985 B instrument in both positive and negative ionization (only positive ions are reported in this paper). The instrument was equipped with a RP-MP RP-8 column (10 cm) eluted with CH_3CN - H_2O, 75: 25, CH_3CN - THF - H_2O, 70: 10: 20, or CH_3CN - DMSO - H_2O, 70: 10: 20, according to the compound solubilities. The eluents were also used as reactant gases for the chemical ionization.

Compound **2**

A soln of **1** (2.0 g, 3.33 mmol) in 0.1 N NaOH (100 ml) was stirred for 2 hours at room temp, after which TLC indicated complete reaction. Ice was added then the reaction mixture was acidified with conc HCl and extracted with EtOAc (2×200 ml). The combined extracts were washed with H_2O to neutrality and then dried (Na_2SO_4). Compound **2** crystallized as orange crystals upon concentration (0.87 g, 45%); mp 210°C (dec); Rf 0.0; IR ν_{max} cm^{-1} 3300~2200, 1725, 1688, 1650, 1635, 1610, 1580, 1542; NMR δ 12.7 (1H, br s, CH_2COOH), 16.1 (1H, br s, COOH); LC/MS *m/z* 587 ($(M+H)^+$), 451, 425, 163. *Anal* ($C_{31}H_{22}O_{11}$) C, H.

Compound **3**

A 1.5% soln of CH_2N_2 in dry ether (20 ml) was added dropwise to a soln of **2** (0.87 g, 1.48 mmol) in dry THF (50 ml). After five minutes the reaction was completed (TLC). AcOH (1 ml) was carefully added, followed by H_2O (300 ml). The aqueous phase, made acidic with a few drops of HCl, was extracted with EtOAc (2×100 ml), the combined extracts were washed with H_2O then dried over Na_2SO_4.

After concentration to a small vol crude **3** ppt by addition of petroleum ether (0.87 g). Pure **3** was obtained by column chromatography (silica gel Merck 0.06~0.2 mm loaded with 4.8% aqueous potassium dihydrogen phosphate, eluted with $CHCl_3$ - MeOH, 99: 1) as a yellow ppt from $CHCl_3$ - petroleum ether (0.1 g, 11%); mp 118~120°C (ref[5] 121~123°C).

Compound **4**

A soln of **1** (3.0 g, 5 mmol) in 16% aqueous ammonia (70 ml) was stirred for 25 minutes at −5°C, after which TLC revealed complete disappearance of the starting material. The reaction mixture was poured into ice and worked up as described for compound **2**, obtaining 1.6 g (55%) of **4** as red crystals from EtOAc; mp >270°C; Rf 0.40; IR ν_{max} cm^{-1} 3480, 3360, 3320, 3300~2500, 1745, 1675, 1650, 1610, 1585, 1550; NMR δ 7.87 (1H, s, $CONH_2$), 8.07 (1H, s, $CONH_2$), 12.4 (1H, br s, CH_2COOH); LC/MS *m/z* 600 ($(M+H+14)^+$), 586 ($(M+H)^+$), 450, 424, 163. *Anal* ($C_{31}H_{23}NO_{11}$) C, H, N.

Compound **10**

2,2-Dimethoxypropane (100 ml) and a few drops of 96% H_2SO_4 was added to a soln of **1** (2.0 g,

3.33 mmol) in THF (70 ml). The reaction mixture was refluxed for 16 hours after which it was cooled to room temp and filtered. To the filtrate Na_2CO_3 (1 g) was added and the suspension stirred until acidity was no longer detected. After filtration the soln was concd to dryness and taken up in EtOAc. Compound **10** was obtained as orange crystals from EtOAc (0.7 g, 34%); mp 204~206°C; Rf 0.98; IR ν_{max} cm^{-1} 3200~2500, 1740, 1725, 1660, 1630, 1610, 1580, 1550, 1490; NMR δ 4.02 (3H, s, CH_2COOCH_3); EI-MS *m/z* 628 ($(M+14)^+$), 614 M^+, 479, 452, 162. *Anal* ($C_{33}H_{26}O_{12}$) C, H.

Compound **11**

To a soln of *n*-propylamine (1.67 ml, 20 mmol) in dry THF (20 ml) cooled at 0°C, PCl_3 (0.29 ml, 3.33 mmol) was added with stirring. The resulting soln was allowed to come to room temp after which **1** (3.0 g, 5 mmol), dissolved in 60 ml of dry THF, was added. The reaction mixture was warmed to 50°C and stirred for 2 hours. After this time TLC showed the disappearance of **1** and a predominant yellow spot (Rf 0.95) together with some spots of the decomposition compounds.

After cooling the reaction mixture was filtered from the undissolved material, diluted with H_2O, acidified with a few drops of concd HCl and then extracted with EtOAc (2×200 ml). Crude **12** (1.5 g) was obtained as a brown ppt from EtOAc - petroleum ether. Column chromatography (silica gel 0.2~0.06 mm Merck, eluted with CH_2Cl_2 - MeOH, 98: 2) gave 0.6 g of **11** which was purified by preparative TLC (silica gel 60 F_{254} Merck eluted with $CHCl_3$ - MeOH, 95: 5). Yield 0.2 g (6%); mp >270°C; Rf 0.95; IR ν_{max} cm^{-1} 3300, 3200~2500, 1730, 1660, 1620, 1610, 1580, 1550, 1490; NMR δ 0.72 (3H, t, J=7.0 Hz, $NHCH_2CH_2CH_3$), 1.30 (2H, tq, J=6.5 Hz, J=7.0 Hz, $NHCH_2CH_2CH_3$), 2.93 (2H, dt, J=6.5 Hz, J=6.0 Hz, $NHCH_2CH_2CH_3$), 3.97 (3H, s, $COOCH_3$), 7.98 (1H, t, J=6.0 Hz, $NHCH_2CH_2CH_3$); LC/MS *m/z* 642 ($(M+H)^+$), 506, 480, 163. *Anal* ($C_{35}H_{31}NO_{11}$) C, H, N.

Determination of Antibacterial Activity

Minimal inhibitory concentration (MIC) was determined using the two-fold dilution method in microtiter system. The media used were: Brain heart infusion broth (Difco) supplemented with 2% bovine serum for streptococci; Antibiotic medium 3 (Difco) for staphylococci and Gram-negative bacteria. Anaerobic bacteria were tested on Schaedler agar supplemented with 1% vitamine K_1 and 1% hemin.

The final inoculum was approximately 10^3 and 10^2 colony-forming units (cfu) per ml for Gram-positive and Gram-negative bacteria, respectively. Anaerobs were inoculated by multipoint inoculator (inoculum 10^4 cfu per spot) and incubated in anaerobic atmosphere (N_2 - CO_2 - H_2, 80: 10: 10). MIC was read as the lowest concentration which showed no visible growth after incubation at 37°C for 18~24 hours or 48 hours (anaerobic bacteria). Experimental infection; groups of five mice were infected intraperitoneally with *S. aureus* TOUR. Inocula were adjusted so that untreated animals died of septicemia within 48 hours. Animals were treated once daily for three days starting immediately after infection. On the 10th day the value for the ED_{50} in mg/kg/day was calculated by the method of SPEARMAN and KÄRBER[10], on the basis of the percentage of surviving animals at each dose.

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