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Ala(0)-actagardine, a New Lantibiotic from Cultures of *Actinoplanes liguriae* ATCC 31048

LÁSZLÓ VÉRTESY*, WERNER ARETZ, ALAIN BONNEFOY†, EBERHARD EHLERS, MICHAEL KURZ, ASTRID MARKUS, MATTHIAS SCHIELL, MARTIN VOGEL, JOACHIM WINK and HERBERT KOGLER

Hoechst Marion Roussel Deutschland GmbH,
D-65926 Frankfurt, Germany
†Hoechst Marion Roussel France,
102 Route de Noisy, F-93235 Romainville, France

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The actagardine-producing strain *Actinoplanes liguriae* ATCC 31048, forms an additional lantibiotic when it is cultured on mannitol and soya meal. The new compound, Ala(0)-actagardine (**1**), has been isolated by solid-phase extraction followed by a two-step chromatographic separation. The molecular formula of **1** is $C_{84}H_{129}N_{21}O_{25}S_4$. Its chemical structure was determined by 2D-NMR analysis and was further confirmed by an amino acid analysis, Edman degradation, and partial synthesis from actagardine. **1** exhibits a slightly higher biological activity than the parent compound actagardine. The synthetic analogs Lys(0)-actagardine (**2**) and Ile(0)-actagardine (**3**) demonstrate also antibacterial activities and emphasize the importance of the *N*-terminus for further derivatization.

Antibiotics that inhibit bacterial cell wall biosynthesis are of major interest in the development of new therapeutics[1]. In particular, it is the increasing resistance of pathogenic microorganisms that makes the search for new therapeutic principles a constant challenge. However, innovative medicines must not only have novel mechanisms of action, they also need to be well tolerated. Thus, one suitable target for antibacterial chemotherapy is the biosynthesis of peptidoglycan (murein), without which the bacterium cannot survive but for which there are no corresponding enzymes in warm blooded animals — this means that peptidoglycan synthesis inhibitors generally should have a low toxicity.

Lantibiotics[2] are lanthionine-containing cyclic peptides synthesized by Gram-positive bacteria, which exert their antibiotic action particularly against foreign Gram-positive bacteria and anaerobic prokaryotes. A subgroup of lantibiotics, comprising mersacidin[3,4] and actagardine[5,6], has recently attracted particular attention on account of a specific inhibitory action[7] on peptidoglycan biosynthesis. Both mersacidin and actagardine have been shown to act by complexing the so-called lipid II, undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc. None of the antibiotics currently used in medicinal practice have a similar site of action in the bacterial metabolism, which means that the small lantibiotics mentioned above promise access to novel antibacterial agents. In fact, even though the spectrum of activity of mersacidin[8] and of actagardine also encompasses resistant pathogens, neither antibiotic has so far found therapeutic use, owing either to gaps in the antibacterial spectrum or to relatively weak activity. Furthermore, semisynthetic modifications of the *C*-terminus of actagardine have failed to bring about any decisive activity improvement[9], and the question whether an improvement in their spectra of activity can be achieved by chemical or molecular-biological variation of the natural lantibiotics has so far remained unanswered.

We have investigated cultures of the actagardine-producing strain *Actinoplanes liguriae* (ATCC 31048) in a number of nutrient media and have discovered a new naturally occurring lantibiotic, Ala(0)-actagardine (**1**), whose antimicrobial activity differs from the antibiotic spectrum

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of actagardine. In this paper we describe the isolation and structural elucidation of the new antibiotic along with some of its synthetic analogs.

Materials and Methods

Fermentation Conditions

Actinoplanes liguriae ATCC 31048, was stored frozen in liquid nitrogen as a vegetative cell suspension in 10% dimethyl sulfoxide. This suspension (1 ml) was used to inoculate a seed medium (100 ml) containing 1.5% (w/v) agar. This seed stage medium was a solution of starch 1% (w/v), glucose 1% (w/v), peptone 0.5% (w/v), corn steep liquor 0.25% (w/v), and yeast extract 0.2% (w/v) in tap water, which was adjusted to pH 6.0 and then sterilized in an autoclave for 20 minutes at 121°C. Spiked 500 ml flasks, each containing 120 ml of the above seed stage medium, were inoculated with plugs from a culture plate. The inoculated flasks were incubated on a gyratory shaking table for 10~14 days at 28°C and 140 rpm and the contents were then used to inoculate 30 liters of production medium in three 12 liter jar fermenters. The production medium, which contained soy bean flour 2% (w/v), mannitol 2% (w/v), and a few drops of a solution of polyol in ethanol (antifoaming agent), was cultured for 48 hours at 28°C with 5 l/minute aeration and an agitation at 200 rpm.

The progress of the fermentation was monitored by HPLC using a Pharmacia LKB pump model 2252, a steel column (4.0×250 mm) packed with Nucleosil 100-5 C_{18} AB (Macherey-Nagel, Düren, Germany), and a flow rate of 1.0 ml/minute. The mobile phase was an aqueous solution of 35% acetonitrile in 20 mM potassium phosphate buffer, pH 7.

Isolation of Ala(0)-actagardine (**1**)

The filtered fermentation broth (27 liter) was passed through a column of MCI Gel CHP20P (Mitsubishi Chemical Industries) (3 liter, 11.3×30 cm). The column was eluted with a linear elution gradient of 5~50% isopropanol in water. The active fractions were combined, concentrated *in vacuo*, and freeze-dried to give 4 g of crude product. 3 g of this material was purified further by size exclusion chromatography on Fractogel HW-40 F (E. Merck, Darmstadt, Germany) (4 liter, 10×50 cm) with 50% methanol in water as solvent and a flow rate of 50 ml/minute. The active fractions gave after drying 240 mg of crude **1**. Final purification was by preparative HPLC (Pharmacia LKB pump 2248). The pre-purified product was loaded onto a Nucleosil $12C_{18}$AB column (2.5×25 cm) and the column was eluted with a gradient of 5~30% acetonitrile in 0.05% trifluoroacetic acid. The homogeneous active fractions were concentrated and freeze-dried to give pure **1** (185 mg) in the form of a white powder.

Synthesis of Ala(0)-actagardine (**1**) Starting from Acta gardine

Actagardine (94.5 mg, 0.05 mmol) was dissolved in anhydrous dimethylformamide (10 ml) and treated with Boc-Ala-O-*N*-hydroxysuccinimide (14.4 mg) and triethylamine (100 μl). The reaction mixture was left to stand at ambient temperature and progress of the reaction was followed by analytical HPLC. After 72 hours the reaction was quenched by dilution with water (100 ml) and the resulting mixture was purified on a LiChrosorb Select B RP_8 column (2.5×25 cm, E. Merck). The column was eluted with a gradient of 20~50% acetonitrile in water. The fractions containing pure Boc-Ala(0)-actagardine were combined, concentrated *in vacuo*, and freeze-dried. Yield 57 mg, 0.023 mmol.

The derivative (30 mg, 0.015 mmol) was dissolved in 5 ml of 60% trifluoroacetic acid. After 90 minutes cleavage of the protecting group was complete. The reaction mixture was then diluted to 100 ml and purified by preparative HPLC (LiChrospher 5 C_{18} column, 1×25 cm, E. Merck), with elution over 30 minutes with a gradient of 25~50% acetonitrile in 0.1% trifluoroacetic acid. The homogeneous fractions were freeze-dried to give 25 mg (0.013 mmol) of pure **1**. ESI-MS: *m/z* 1962 $(M+H)^+$, corresponding to a molecular formula of $C_{84}H_{129}N_{21}O_{25}S_4$.

Synthsis of Lys(0)-actagardine (**2**)

Actagardine (94.5 mg, 0.05 mmol) was dissolved in 10 ml of anhydrous dimethylformamide in a 100 ml flask. To this was added di-Boc-lysine-O-*N*-hydroxysuccinimide (22 mg, 0.05 mmol) and triethylamine (100 μl), and the reaction mixture was stirred at ambient temperature. After 14 days the reaction was complete. The mixture was then diluted to 100 ml with water and purified by preparative HPLC using a 2.5×25 cm column packed with LiChrosorb Select B (E. Merck). The column was eluted over 30 minutes with a gradient of 20~50% acetonitrile. The product-containing fractions, identified by analytical HPLC, were combined and the resulting material was freeze-dried to give 33 mg (0.015 mmol) of di-Boc-Lys(0)-actagardine.

To remove the protecting groups, the derivative (25 mg, 0.011 mmol) was dissolved in 5 ml of 60% trifluoroacetic acid. After 90 minutes cleavage of the protecting groups was complete. This reaction mixture was again diluted to 100 ml and purified by preparative HPLC (LiChrospher 5

C_{18} column, 1×25 cm, E. Merck), with elution over 30 minutes with a gradient of 25~50% acetonitrile in 0.1% trifluoroacetic acid. The homogeneous fractions were freeze-dried to give 14 mg (0.007 mmol) of pure Lys(0)-actagardine. ESI-MS: *m/z* 2019 $(M+H)^+$, corresponding to $C_{87}H_{136}N_{22}O_{25}S_4$.

Synthesis of Ile(0)-actagardine (**3**)

Actagardine (189 mg, 0.1 mmol) was dissolved in 10 ml of anhydrous dimethylformamide and treated with Boc-Ile-O-*N*-hydroxysuccinimide (33 mg, 0.1 mmol) and triethylamine (100 µl). The reaction mixture was left to stand at ambient temperature and progress of the reaction was followed by analytical HPLC. After 96 hours the reaction was quenched by dilution with water (100 ml) and the resulting mixture was purified on a LiChrosorb Select B RP_8 column (2.5×25 cm, E. Merck). The column was eluted with a gradient of 20~50% acetonitrile in water. The fractions containing pure Boc-Ile(0)-actagardine were combined, concentrated *in vacuo*, and freeze-dried. Yield 210 mg, corresponding to 0.1 mmol.

The derivative (100 mg, 0.048 mmol) was dissolved in 5 ml of 60% trifluoroacetic acid. After 90 minutes cleavage of the protecting group was complete. This reaction mixture was then diluted to 100 ml and purified by preparative HPLC (LiChrospher 5 C_{18} column, 2.5×25 cm, E. Merck), with elution over 30 minutes with a gradient of 25~50% acetonitrile in 0.1% trifluoroacetic acid. The homogeneous fractions were freeze-dried to give 35 mg (0.017 mmol) of pure Ile(0)-actagardine (**3**). ESI-MS: *m/z* 2004 $(M+H)^+$, corresponding to $C_{87}H_{135}N_{21}O_{25}S_4$.

NMR Spectroscopy

NMR spectra were recorded at 37°C on Bruker DRX 500 or DRX 600 spectrometers (operating at a basic frequency of 500.13 or 600.13 MHz for protons) using solutions of the compounds in DMSO-d_6 (3~14 mg in 600 µl: see Table 2).

The data were processed on INDY workstations (Silicon Graphics) with XWINNMR software[10]. For all compounds a set of homonuclear (DQF-COSY[11] and NOESY[12]) and heteronuclear (HSQC[13] and HMBC[14]) experiments were performed. Homonuclear 2D experiments were recorded with a spectral width of 11 ppm, 1024 increments in t_1 and 4096 complex data points in t_2. A mixing time of 100 ms was used for NOESY spectra.

HSQC spectra were recorded with 1024 increments of 2048 complex data points in t_2 and a spectral width of 8 ppm in the proton and 140 ppm in the carbon dimension. HMBC spectra were acquired with a sweep width of 11 ppm in the proton and 180 ppm in the carbon dimension using 1024 increments with 4096 complex data points and a mixing time of 62.5 ms for the development of long range correlations. In addition, an optimally folded spectrum (to a spectral width of 80 ppm along f_1) was recorded to improve the resolution of the partially overlapping carbonyl resonances.

Results and Discussion

Fermentation and Isolation

The strain *Actinoplanes liguriae* ATCC 31048 forms actagardine in a nutrient medium containing 3% glycerol, 0.2% casein peptone, 0.1% K_2HPO_4, 0.1% NaCl, and 0.05% $MgSO_4 \cdot 7H_2O$, as described by Parenti et al.[15]. Fermentation of the strain in this medium as described in the experimental section, followed by isolation of the antibiotic, gave pure actagardine which was unambiguously identified on the basis of its mass spectra and 2D NMR spectra[5].

Fermentations of *Actinoplanes liguriae* ATCC 31048 in the poorly digestible soya meal/mannitol culture medium gave a product **1**, which had an HPLC retention time (12.9 minute) longer than that of actagardine (11.5 minute). This new antimicrobially active substance was isolated by solid-phase extraction, molecular sieve chromatography, and preparative HPLC.

Structural Elucidation of Ala(0)-actagardine

The physico-chemical data of **1** are summarized in Table 1. The molecular mass of the isolated compound was determined by ESI-MS (*m/z* 1960.8 for $(M+H)^+$). This differs from actagardine by 71 Da, which corresponds to an additional alanine residue. This assumption was corroborated by quantitative amino acid analysis: Ser (1), Me-Lan (2), Glu (1), Lan (>1), Gly (2), Ala (2), Val (2), Ile (2) and Leu (1) which revealed an increase only in the amount of alanine. The discrepancy between the amino acid analysis and the molecular weight could be attributed to a third Me-Lan moiety which, oxidized to the sulfoxide as is known for actagardine, has not been resolved from lantinonine in our chromatographic system.

The NMR spectroscopic analysis of **1** in DMSO was started by assigning the individual spin systems from the DQF-COSY spectra. All expected amide resonances were resolved in the one-dimensional proton-NMR spectrum at 500 MHz (14 mg/600 µl, at 45°C). Partially overlapping resonances at 8.26 ppm (attributed later to Ser^2 and Glu^{11})

Table 1. Physicochemical properties of Ala(0)-actagardine (**1**).

	Ala(0)-actagardine
Appearance:	colorless powder
$[\alpha]_D^{25}$ (H_2O):	-30.5° (*c* 0.5)
UV λ_{max} (MeOH) nm (log ε):	280 (3.71)
ESI-MS (*m/z*):	1960.8 (M + H^+)
IR ν_{max} (KBr) cm^{-1}:	3400, 3000, 2900, 1680, 1525
Molecular formula:	$C_{84}H_{129}N_{21}O_{25}S_4$
Molecular weight:	1961.21
Solubility	
soluble in:	MeOH, DMSO, dimethylformamide, water
insoluble in:	hexane

Table 2. Sequential NMR correlations of Ala(0)-actagardine.

		HMBC-correlations					NOE-correlations	
Position	δ [^{13}C]	α_i	α_{i+1}	β_i	NH_{i+1}	δ_{NH}[^1H]	NH→ α_{i-1}	NH → β_{i-1}
0	169.51		x	x	x	8.01		
1	169.27		x		x	8.56	x	
2	170.48	x	x	x	x	8.26	x	x
3	168.90	x	x		x	8.60	x	
4		(x)*				8.15	(x)	
5	171.20	x			x	7.45	x	x
6	170.14	x	x			8.49	(x)	
7		(x)				8.32	x	
8	171.45				x	7.64	x	x
9	171.59	x				7.60	x	
10						8.38	x	x
11						8.26	(x)	
12	170.78	x	x			7.33	x	
13	170.03	x	x			8.14	x	
14	168.33	x	x			7.86	x	
15	170.63	x	x			7.78	x	
16	170.96	x				7.59	x	
17	170.66	x				7.58		
18	171.52	x		x	x	8.18	x	
19						8.34	x	

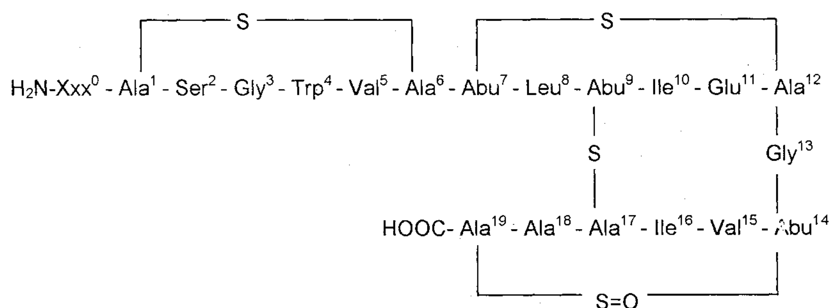
*ambiguous correlations in brackets ( )

and 8.14 ppm (attributed to Trp[4] and Gly[13]) could be distinguished from their different line-shape or multiplicity. The NH-C_αH correlations in the COSY spectrum allowed for the unambiguous assignment of all amide protons (see Table 2). However at 4.55 and 4.56 ppm three α-protons are indistinguishable. This overlap could be resolved using a TOCSY spectrum for correlations from the amide proton to the well resolved β-resonances. The three amino acids thus could be identified as valine, (β-Thio-) α-aminobutyric acid and (β-Thio-) alanine.

The assignment of ^{13}C-resonances was carried out *via* HSQC- and HMBC-spectra. The HSQC spectrum allowed for the assignment of all CH pairs without overlap (see Table 2). Ambiguities resulting from overlapping proton resonances could be resolved using the expected carbon chemical shifts for individual amino acids.

Sequential assignment of the peptide started with the HMBC spectra, utilizing ^1H-^{13}C long range couplings from

Fig. 1. Chemical structure of Ala(0)-actagardine (**1**), Lys(0)-actagardine (**2**) and Ile(0)-actagardine (**3**).



Ala(0)-actagardine (**1**) : Xxx^0 = Ala
Lys(0)-actagardine (**2**) : Xxx^0 = Lys
Ile(0)-actagardine (**3**) : Xxx^0 = Ile

the carbonyl carbon (i-position) to the amide-resonances (i+1 position) and the α-resonances (i or i+1 position) (see Fig. 1).

Sequential NOEs were also evaluated (NH_i to $C_{\alpha}H_{i-1}$) to give a complete set of information on the peptide sequence (see Table 2). The interpretation of homonuclear correlations was impeded by the overlap of three α-resonances and the overlapping amide-resonances. The overlap of Ser^2-NH and Glu^{11}-NH (8.26 ppm) could be unraveled because the sequential assignment of Ser^2 has been established by ^{1}H-^{13}C long range correlations. Similarly the overlapping resonances of Gly^{13}-NH (assigned from heteronuclear long-range couplings) and Trp^4-NH (8.14 ppm) could be resolved and NOE correlations to Gly^3-α and -α' (4.02 and 3.20 ppm) as well as to Trp^4-α were attributed to Trp^4-NH.

In addition to the two overlapping amide-resonances three α-resonances (assigned to Val^5, Abu^7 and Ala^{12} at 4.56 and 4.55 ppm) increase the ambiguities in the interpretation of the NOESY spectrum. Only one of these ambiguities could be resolved because NOE effects could be observed from the NH proton of Leu^8 (7.71 ppm) also to the side-chain protons of the preceding amino acid (to Abu^7 α and β, 4.56 ppm and 3.60 ppm).

Combining the sequence information of HMBC and NOESY spectra (see Table 2) two fragments were assigned, one stretching from Ala^0 to Ile^{10}. The other spanning the residues Ala^{12} to Ala^{19}. Because the first fragment clearly has been identified as the *N*-terminus of the peptide, the remaining glutamic acid could be placed either in position 11 or at the *C*-terminus. Though the amide resonance of glutamic acid (8.26 ppm) is overlapping with Ser^2-NH, the NOE-correlation to Ile^{10}-α can be attributed to Glu^{11}-NH, because sequential assignment for Ser^2 is already achieved by ^{1}H-^{13}C long range correlations.

This peptide sequence was consistent with the revised structure of actagardine[5)], the additional alanyl residue being located at the *N*-terminus.

The position of the sulfide bridges could be deduced from either ^{1}H-^{13}C long range correlations, NOE-correlations or the interpretation of carbon chemical shifts. The sulfoxide bridging causes a lowfield shift of the adjacent β-resonances by nearly 10 ppm, as compared with the sulfide bridges in lanthionine or methyl-lanthionine. This led to the identification of the sulfoxide-bridge between Abu^{14} and Ala^{19} (β-carbon resonances at 55.64 ppm and 51.53 ppm as compared with 43~47 ppm for the β-resonances of Abu (7 and 9) and 32~36 ppm for the β-resonances of Ala (1, 6, 12 and 17)). This bridge is further supported by the strong NOE correlation between Abu^{14}-γ (1.05 ppm) and both β-resonances of Ala^{19} (at 3.07 and 2.97 ppm). The sulfide bridge between Ala^1 and Ala^6 was supported by the NOE from Ala^6-NH (8.66 ppm) to Ala^1-β (at 3.35 ppm). Overlapping signals of Abu^7- and Abu^9-β and γ resonances (3.60 ppm and 1.19 ppm) as well as Ala^{12}- and Ala^{17}-β protons (2.88/2.56 ppm and 2.87 and 2.62 ppm) prohibit the identification of these sulfide bridges from NOE effects or $^3J_{CH}$ couplings. However, from the β-carbon of Ala^{17} (at 35.70 ppm) long range correlations to the H-α resonance within the same resonance (4.49 ppm) as well as a weak correlation to 4.77 ppm (Abu^9-α) could be observed. The corresponding correlation of Ala^{12}-β (at 35.26 ppm) to a proton signal at 4.59 ppm can be assigned to either Ala^{12}- or Abu^7-α.

Table 3-1. Chemical shifts of actagardine, Ala(0)-actagardine (**1**), Lys(0)-Actagardine (**2**) and Ile(0)-Actagardine (**3**).

	Actagardin	Ala^0 at 318 K		Ile^0 at 310 K		Lys^0 at 310 K	
Amino-acid	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
Xxx^0	-	**Ala**		**Ile**		**Lys**	
CO		169.51					
NH			8.01		8.00		8.07
α		48.35	2.89	56.82	3.75	51.97	3.85
β / β′		17.35	1.38	36.17	1.87	30.50	1.74
γ / γ′				23.39	1.53 / 1.17	20.88	1.40
δ				11.04	0.86	26.40	1.52
ε			β-Me :	14.56	0.98	38.48	2.73
NH							7.64
Ala^1							
CO		169.27					
NH	8.18		8.56		8.62		8.68
α	4.29	50.80	4.73	50.73	4.76	50.09	4.78
β / β′	3.35 / 2.64	34.21	3.40 / 2.60	34.13	3.41 / 2.59	33.78	3.46 / 2.54
Ser^2							
CO		170.48					
NH	8.84		8.26		8.26		8.38
α	4.47	55.04	4.35	54.95	4.37	54.78	4.37
β / β′	3.71 / 3.60	61.23	3.65	61.22	3.66	61.22	3.64
OH	5.12		5.10		5.14		5.16
Gly^3							
CO		168.9					
NH	8.53		8.60		8.63		8.70
α / α′	4.02 / 3.20	43.44	3.97 / 3.26	43.35	4.00 / 3.27	43.47	3.98 / 3.23
Trp^4							
CO							
NH	7.77		8.15		8.19		8.31
α	4.53	54.28	4.48	54.10	4.49	54.18	4.47
β / β′	3.23 / 3.01	27.62	3.31 / 2.98	27.55	3.33 / 2.96	27.55	3.36 / 2.95
4		110.41	-				
5	7.24	123.28	7.14	123.24	7.16	123.11	7.13
6		127.08	-				
7	7.34	111.28	7.33	111.16	7.34	111.22	7.34
8	7.07	120.83	7.06	120.73	7.07	120.78	7.07
9	6.99	118.22	6.98	118.12	6.99	118.14	6.99
10	7.66	118.10	7.55	118.02	7.57	117.96	7.55
11		136.00					
NH	10.79		10.74		10.77		10.77

Table 3-2. Chemical shifts of actagardine, Ala(0)-actagardine (**1**), Lys(0)-Actagardine (**2**) and Ile(0)-Actagardine (**3**).

	Actagardin	Ala^{0} at 318 K		Ile^{0} at 310 K		Lys^{0} at 310 K	
Amino-acid	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
Val^{5}							
CO		171.20					
NH	7.42		7.45		7.48		7.47
α	4.36	56.73	4.56	56.66	4.58	56.21	4.68
β	1.96	31.50	2.05	31.44	2.06	31.93	2.06
γ	0.88	17.76	0.91	17.64	0.90	17.47	0.93
γ′	0.86	19.02	0.86	18.95	0.87	18.97	0.87
Ala^{6}							
CO		170.14					
NH	8.66		8.49		8.52		8.62
α	4.94	53.47	4.70	53.33	4.71	53.31	4.70
β / β′	3.20 / 2.81	32.79	2.96 / 2.58	32.58	2.99 / 2.56	31.97	2.96 / 2.53
Abu^{7}							
CO							
NH	8.48		8.32		8.42		8.41
α	4.59	57.83	4.56	57.79	4.58	57.80	4.61
β	3.60	43.95	3.60	44.02	3.61	44.15	3.62
γ	1.19	19.96	1.18	19.85	1.18	19.89	1.18
Leu^{8}							
CO		171.45					
NH	7.71		7.64		7.62		7.62
α	4.60	51.01	4.63	50.83	4.67	50.70	4.74
β / β′	1.44	41.66	1.42 / 1.48	41.70	1.42 / 1.48	41.98	1.43 / 1.46
γ	1.48	24.03	1.48	23.94	1.49	24.55	1.52
δ	0.85	22.38	0.84	22.40	0.85	22.35	0.85
δ′	0.85	22.62	0.86	22.52	0.87	22.57	0.86
Abu^{9}							
CO		171.59					
NH	7.67		7.60		7.65		7.75
α	4.77	55.77	4.75	55.66	4.78	55.73	4.75
β	3.61	46.38	3.57	46.56	3.57	46.85	3.56
γ	1.19	20.10	1.21	20.02	1.22	20.09	1.23
Ile^{10}							
CO							
NH	8.45		8.38		8.42		8.47
α	3.77	60.00	3.76	60.04	3.75	60.16	3.73
β	1.62	35.71	1.61	35.58	1.60	35.55	1.60
γ / γ′	1.07 / 1.00	24.75	1.06 / 1.64	24.67	1.07 / 1.66	24.74	1.08 / 1.67
γ-Me	0.87	14.70	0.88	14.56	0.89	14.53	0.88
δ	0.87	11.59	0.86	11.51	0.87	11.63	0.88

Table 3-3. Chemical shifts of actagardine, Ala(0)-actagardine (**1**), Lys(0)-Actagardine (**2**) and Ile(0)-Actagardine (**3**).

	Actagardin	Ala^{0} at 318 K		Ile^{0} at 310 K		Lys^{0} at 310 K	
Amino-acid	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
Glu^{11}							
CO							
NH	8.36		8.26		8.29		8.22
α	3.68	55.86	3.69	55.95	3.69	55.91	3.67
β / β′	2.26 / 2.16	24.49	2.18	24.50	2.23 / 2.16	24.49	2.22 / 2.14
γ	2.32	30.98	2.32	30.86	2.33	30.84	2.34
δCO		173.75					
Ala^{12}							
CO		170.78					
NH	7.33		7.33		7.37		7.40
α	4.58	55.47	4.55	55.63	4.58	55.74	4.58
β / β′	2.88 / 2.56	35.26	2.87 / 2.56	35.29	2.88 / 2.56	35.57	2.88 / 2.55
Gly^{13}							
CO		170.03					
NH	8.22		8.14		8.17		?
α / α′	4.17 / 3.55	44.18	4.17 / 3.54	44.06	4.22 / 3.53	44.01	4.23 / 3.48
Abu^{14}							
CO		168.33					
NH	7.90		7.86		7.89		7.87
α	4.36	55.00	4.38	54.92	4.38	54.85	4.36
β	3.35	55.64	3.34	55.63	3.36	55.88	3.33
γ	1.05	6.94	1.06	6.83	1.07	6.88	1.07
Val^{15}							
CO		170.63					
NH	7.87		7.78		7.80		7.75
α	4.07	60.03	4.10	59.93	4.12	59.84	4.10
β	2.07	30.02	2.04	29.89	2.05	29.96	2.03
γ	0.88	19.15	0.88	19.09	0.88	19.08	0.88
γ′	0.88	18.56	0.87	18.45	0.89	18.46	0.88
Ile^{16}							
CO		170.96					
NH	7.57		7.59		7.59		7.65
α	3.90	59.64	3.87	59.50	3.89	59.63	3.85
β	1.86	35.69	1.88	35.67	1.86	35.60	1.86
γ / γ′	1.52 /1.15	24.55	1.51 / 1.12	24.50	1.52 / 1.14	24.55	1.51 / 1.16
γ-Me	0.89	15.53	0.89	15.43	0.90	15.42	0.90
δ	0.82	10.62	0.83	10.52	0.86	10.47	0.86

Table 3-4. Chemical shifts of actagardine, Ala(0)-actagardine (**1**), Lys(0)-Actagardine (**2**) and Ile(0)-Actagardine (**3**).

	Actagardin	Ala^0 at 318 K		Ile^0 at 310 K		Lys^0 at 310 K	
Amino-acid	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
Ala^{17}							
CO		170.66					
NH	7.62		7.58		7.62		7.61
α	4.49	53.13	4.49	53.18	4.51	53.37	4.50
β / β′	2.87 /2.62	35.70	2.88 / 2.59	35.65	2.90 / 2.59	35.74	2.89 / 2.57
Ala^{18}							
CO		171.52					
NH	8.18		8.18		8.22		8.29
α	4.04	48.89	4.04	48.70	4.06	48.77	4.03
β	1.24	15.34	1.23	15.17	1.24	15.21	1.23
Ala^{19}							
CO							
NH	8.34		8.34		8.35		8.34
α	4.40	47.16	4.45	47.12	4.44	47.13	4.46
β / β′	3.07 / 2.95	51.53	3.07 / 2.93	51.43	3.08 / 2.95	51.37	3.10 / 2.92

Peptide sequencing by Edman degradation, though giving only partial information, was in agreement with the proposed structure. This revealed a 7-unit fragment, Ala-X-Ser-Gly-Trp-Val-X, consistent with the structure of **1**, where X represents an unidentified amino acid or the absence of any cleavage.

The structures of the synthetic actagardine analogs (synthetic **1**, **2**, and **3**) were confirmed by mass spectrometry (ESI-MS, revealing molecular ions of *m/z* 2019 for **2** and 2004 for **3**) and the ^1H- and ^{13}C-NMR resonances were assigned (Table 3) from sets of COSY, NOESY, and HSQC spectra. NOEs were used to establish the sequential assignment.

Synthesis of Some *N*-acyl Actagardine Derivatives

From the first preliminary antimicrobial activity determinations it was already clear that Ala(0)-actagardine had better antibacterial activity than actagardine itself. To obtain additional confirmation of the structure and of its good activity some derivatives were partially synthesized from actagardine. Thus, on treatment in anhydrous dimethylformamide in the presence of triethylamine, actagardine underwent coupling at the *N*-terminus with the Boc-protected[16] O-*N*-hydroxy-succinimide-activated[17] amino acid ester (Boc=*tert*-butoxycarbonyl). The Boc-protected reaction products were purified by reverse phase HPLC, which was followed by cleavage of the protecting group with 60% trifluoroacetic acid. Further chromatographic separation gave respectively pure Ala(0)-actagardine (**1**), $(M+H)^+$ 1960.9, corresponding to $C_{84}H_{129}N_{21}O_{25}S_4$, Lys(0)-actagardine (**2**), $(M+H)^+$ 2019, corresponding to $C_{87}H_{136}N_{22}O_{25}S_4$, and Ile(0)-actagardine (**3**), $(M+H)^+$ 2004, corresponding to $C_{87}H_{135}N_{21}O_{25}S_4$.

The identity of the products was established, over and above the determination of their molecular weights, from their NMR data. In HPLC the synthetic Ala(0)-actagardine co-elutes with the naturally isolated **1**.

Biological Activities

The antibacterial activity of Ala(0)-actagardine was

Table 4. Comparative antibacterial data on mersacidin, **1**, and actagardine.

	MIC [μg/ml]		
	Mersacidin	**1**	**Actagardine**
Staph. aureus SG 511	1.56	6.25	12.5
Staph. aureus 285	1.56	6.25	12.5
Staph. aureus 503	0.39	3.13	6.25
Staph. aureus FH 1982	12.5	12.5	12.5
Staph. aureus 701 E	6.25	12.5	12.5
Staph. aureus 707 E	12.5	12.5	25
Staph. aureus 9 Tüb.	3.13	6.25	6.25
Staph. aureus 8236	1.56	6.25	6.25
Staph. epidermidis ZH2c	6.25	6.25	25
Staph. epidermidis 6098W	6.25	12.5	25
Staph. epidermidis 763	6.25	6.25	25
Staph. epidermidis 5747IIW	3.13	6.25	3.13
Staph. epidermidis 291	6.25	12.5	25
Staph. epidermidis 799	6.25	6.25	25
Enteroc. faecium Md8B	25	6.25	3.13
Enteroc. faecium VR1	25	50	50
Enteroc. faecium VR2	50	50	50
Streptoc. pyogenes VR3	25	25	50
Streptoc. pyogenes 308A	3.13	6.25	6.25
Streptoc. pyogenes 77A	0.78	0.195	0.195
Escherichia coli DB10	> 40	> 40	> 40
Pseudomonas aeruginosa 1771	> 40	> 40	> 40
Bacteroides fragilis 312	> 128.0	> 128.0	> 128.0
Bacteroides ovatus 103	> 128.0	> 128.0	> 128.0
Sphaerophorus varius 5262	> 128.0	> 128.0	> 128.0
Peptostreptoc. anaerob. 932	> 128.0	128.0	64.0
Propionibacterium acnes 6919	4.0	1.0	1.0
Propionibacterium acnes 6922	2.0	1.0	0.5
Clostridium tetani 9406	16.0	8.0	8.0
Clostridium perfringens 194	0.5	0.5	0.5

Abbreviation: *Staph.*; *Staphylococcus*, *Enteroc.*; *Enterococcus*, *Streptoc.*; *Streptococcus*

determined in serial dilution tests by comparison with the activities of the parent compound actagardine and the related lantibiotic mersacidin. Table 4 shows the minimal inhibitory concentrations against a broad spectrum of bacteria. Against many Gram-positives such as staphylococci and streptococci, and against some anaerobic microorganisms, **1** shows similar activity to the comparison lantibiotics. The activity of **1** exceeds that of actagardine, but is not as high as that of mersacidin. The compounds are inactive against Gram-negative microorganisms, but they also have a weak activity against Gram-positive enterococci. Since the antibacterial spectrum of the new compound Ala(0)-actagardine differs somewhat from that of the known lantibiotics, the lysine (**2**) and isoleucine (**3**) analogs of **1** were synthesized and their activity against Gram-positive bacteria was compared with that of mersacidin (Table 5). Against enterococci the new derivatives were in some cases found to be more active than mersacidin, thus demonstrating the possibility of fine-tuning the antibacterial action spectrum of this antibiotic by derivatization at the actagardine *N*-terminus.

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Table 5. Inhibitory action of actagardine derivatives on Gram-positive bacteria.

	1	2	3	Mersacidin
Staphylococcus aureus 011HT3	20	10	2.5	0.6
S. aureus 011HT3 + 50 % serum	40	5	5	5
S. aureus 011HT18	>40	>40	>40	20
S. epidermidis 012GO20	>40	>40	>40	40
S. aureus 011HT1	1.2	0.6	1.2	0.15
S. aureus 011DU5	40	10	10	2.5
S. aureus 011CB20	>40	40	>40	20
S. aureus 0121O64	>40	>40	>40	40
S. epidermidis 012GO42	>40	>40	>40	40
Staph. coagulase neg. 012HT5	>40	>40	>40	20
Streptoc. pyogenes O2A1SJ1	0.04	0.04	0.04	0.6
Streptoc. pyogenes 02A1UC1	0.04	0.04	0.04	1.2
Streptoc. pyogenes 02A1FI6	0.04	0.04	0.04	0.3
Streptoc. gr. G 02G0CB2	20	5	10	1.2
Streptoc. pneumoniae 030BI2	2.5	2.5	1.2	5
Streptoc. milleri 02milGR12	40	40	>40	5
Streptoc. mitis 02mitGR16	20	10	10	10
Enteroc. faecalis 02D2HM9	>40	40	>40	>40
Enteroc. faecalis 02D2UC5	20	40	40	>40
Enteroc. faecalis 02D2DU18	5	5	5	>40
Enteroc. faecalis 02D2HT10	>40	>40	>40	>40

Abbreviation: see Table 4

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