PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF ACTAGARDINE† AND SOME ACID HYDROLYSIS PRODUCTS

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(Received for publication February 22, 1985)

Actagardine (originally designated as metabolite B or gardimycin) is a polypeptide antibiotic produced by fermentation of *Actinoplanes* strains ATCC 31048 and 31049. During the course of repeated fermentations two new compounds, coded D and E, were isolated. Some physico-chemical and biological characteristics of actagardine and of both compounds are presented. Compound D is derived from chemical transformation of actagardine.

In previous papers[1,2] it was reported that fermentation of *Actinoplanes liguriae* ATCC 31048 and *Actinoplanes garbadinensis* ATCC 31049 produced actagardine and two related metabolites A and C[3]. During the course of studies to improve the procedure of recovery and purification, the formation of two new compounds coded D and E was noticed.

In this paper additional characteristics of actagardine not described previously are reported together with some physico-chemical and biological properties of compounds D and E.

Chemistry

The fermentation broth[3] was shown by HPLC to contain actagardine and metabolites A and C (traces) but not compounds D and E (Fig. 1). The broth was filtered and extracted at pH 3 with butanol. Upon concentration of the extracts and precipitation with ether a crude material containing actagardine (9%), metabolites A (7%) and C (1%), and compounds D (3%) and E (1%) was obtained. Actagardine and compounds D and E were separated by a variety of preparative column chromatographies. Some physico-chemical properties of the three compounds are shown in Table 1.

Actagardine Free Acid

The amino acid mixture, obtained by both acid and base hydrolysis, was analyzed by GC and GC-MS after derivatization as *N*-trifluoroacetylmethyl (*N*-TFA) esters. The composition previously reported[2] was confirmed. In addition, it was established that the sulfur-containing amino acids, previously characterized as lanthionine and β-methyllanthionine[4], are present as one and three units respectively, and that alanine, valine, leucine and isoleucine are in the L-configuration.

Actagardine shows two acidic and one basic function. The pK_{MCS} values were determined in methylcellosolve (MCS) - water, 4:1 solution, and their proposed attributions, based on the amino acids present and on the titration in the presence of formaldehyde are shown in Table 1. These three acidic and/or basic functions ionize in non-aqueous solvents, *i.e.* in DMSO - acetic acid, 5:95 the

† Actagardine is the recommended International Nonproprietary Name (WHO) of the antibiotic previously designated as gardimycin.

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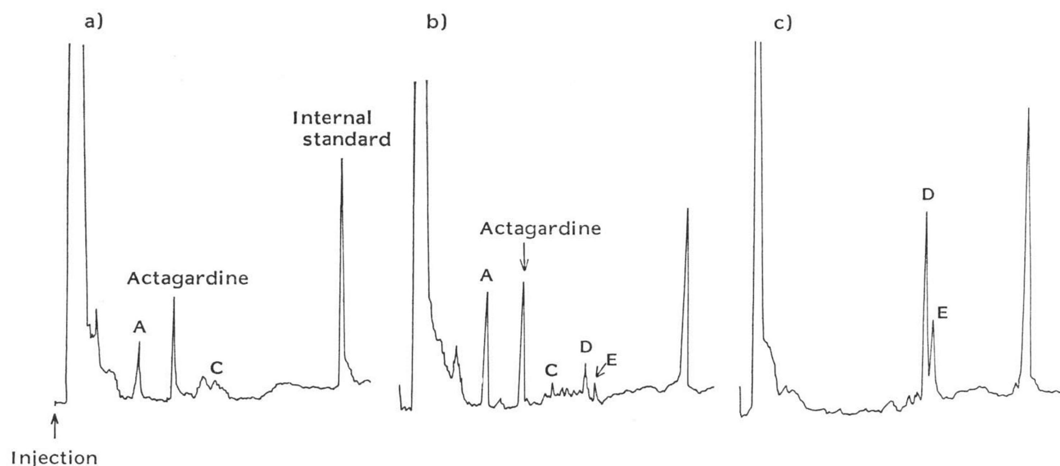
Fig. 1. HPLC examination of: a) harvested broth, b) crude powder and c) enriched material containing compounds D and E.

Apparatus: Two Waters mod 6000 A pumps; 710 A wisp; injector Rheodyne mod 7120 (loop 50 μl); detector Perkin-Elmer LC 15 (254 nm). Flow rate 2 ml/minute; column size 4.6×250 mm; stationary phase: LiChrosorb RP-8, 10 μm; mobile phase: linear gradient from 0.025 M aq NaH_2PO_4 - CH_3CN, 7:3 to 0.025 M aq NaH_2PO_4 - CH_3CN, 2:8 in 30 minutes; column temp: ambient; injection 50 μl.

Sample preparation: a) The filtered broth (2 ml) was diluted with 1 ml of a mixture of 0.025 M NaH_2PO_4 (pH 4) - CH_3CN, 2:8 and 1 ml of a solution of 1 μg/ml of anthracene (internal standard) in CH_3CN was added; b) the powders were dissolved (150 μg/ml) in the same solvent mixture.



compound can be titrated with perchloric acid with one titration slope attributed to a basic function while in pyridine the compound can be titrated with tetrabutylammonium hydroxide with two different titration slopes attributed to two acidic functions with different strengths. It is worthwhile mentioning that the compound in aqueous solution exists as acidic zwitterion, while in non-aqueous solution it does not. The eqW obtainable from the described titration is 1,950. Since actagardine free acid strongly retains crystallization water and solvents MW ~ 1,890 is calculated.

The IR spectrum (Nujol) shows bands at 3300 and 3060 (ν_{NH}), 1660 (amide I), 1525 (amide II) and 1235 cm^{-1} (amide III).

The ^1H NMR spectrum* shows ~65 aliphatic protons in the region δ 0.8 ~ 2.5 ppm; ~55 protons on carbons bonded to oxygen or nitrogen in the region δ 2.5 ~ 5.5 ppm; 5 protons of aromatic type at δ 7.10, 7.20, 7.35, 7.50 and 7.75 ppm; 20 protons of amidic and phenolic type in the region δ 6.8 ~ 10.8 ppm.

The ^{13}C NMR spectrum** allows the following attributions in the various spectral regions: 1) δ 0 ~ 60 ppm: about 51 sp^3 carbons attached to C, CO, and NH; 2) δ 105 ~ 135 ppm: 8 aromatic carbons; 3) δ 160 ~ 170 ppm: about 18 carbons (NHCO and COOH).

FAB-MS shows peaks in the range m/z 1,889 ~ 1,895.

Compound D

The amino acid analysis under the conditions reported for actagardine showed that the only

* Recorded at 270 MHz in DMSO-d_6 solution before and after addition of D_2O on a Bruker WH 270 Cryospectrometer (TMS δ=0.00 ppm as internal reference).

** Recorded on the same Bruker spectrometer at 67.88 MHz in DMSO-d_6-^{12}C solution.

Table 1. Physico-chemical properties of compounds D and E in comparison with actagardine.

	Actagardine	Compound D	Compound E
MP (°C, dec)	>240	>265	>240
Anal[a] (%) C	52.03	53.80	50.04
H	7.14	7.46	6.89
N	14.32	12.82	11.54
S	6.40	5.13	4.69
Weight loss (%)	6.6	2.1	9.6
Inorganic residue (%)	0.3	3.7	1.7
TLC[b]	0.25	0.52	0.61
HPLC retention time (minutes)[c]	9.16	14.20	14.87
IR[b] ν_{max} (cm^{-1})	3300, 3060 (ν NH); 1660 (amide I); 1525 (amide II); 1235 (amide III)	3300, 3060 (ν NH); 1730 ($\nu_{C=O}$ ester); 1650 (amide I); 1530 (amide II); 1235 (amide III)	3300, 3060 (ν NH); 1730 ($\nu_{C=O}$ ester); 1655 (amide I); 1530 (amide II); 1245 (amide III)
UV[b] λ_{max}^{MeOH} (nm)	278, 287	272 (sh), 280, 289	270 (sh), 280, 288
pK_{MCS}	≤4 (COO^- of an α-amino acid in peptide linkage),[d] 4.2 (COO^- of a β-*S*-α-amino acid), 6.3 (NH_3 of a β-*S*-α-amino acid)	No titrable functions (HCl, NaOH)	No titrable functions (HCl, NaOH)
Amino acid composition	Ser (1), Glu (1), Gly (2), Ala (1), Val (2), Ile (2), Leu (1), Try (1), lanthionine (1), β-methyllanthionine (3)	Ser (1), Glu (1), Gly (2), Ala (1), Val (2), Ile (2), Leu (1), Try (1), lanthionine (1), β-methyllanthionine (2)	Not determined

[a] C, H, N were determined on samples previously dried at 140°C in inert atmosphere. The weight loss was measured by thermogravimetric analysis. The inorganic residue was determined after heating the samples at 900°C in oxygen atmosphere. The values are corrected for inorganic residue and for weight loss (S).
[b] See introduction to Experimental Section.
[c] See Fig. 1. Internal standard, anthracene (retention time, 21.56 minutes).
[d] EqW 1,950±20.

difference is the absence of one β-methyllanthionine unit. It addition, the IR spectrum shows a relatively strong band at 1730 cm^{-1} attributable to an ester group (not present in actagardine) and the acid-base titration with either HCl or NaOH does not reveal any titratable function. The UV spectrum shows that tryptophan is unmodified. No satisfactory FAB-MS was obtained.

The information described above suggests that compound D is derived from actagardine. In fact, compound D was isolated from a solution of actagardine in butanol, acetone and 2 N HCl kept for three days at room temperature. Taking into account this transformation and the comparison of the physico-chemical data of actagardine and of compound D, the latter appears to be the butyl ester of the actagardine moiety resulting from the loss of one β-methyllanthionine unit. It can be hypothesized, based also on the values of the ionizable functions, that β-methyllanthionine may be a terminal unit of the polypeptide skeleton linked to a carboxyl of an amino acid (likely a glutamic residue) through an amide bond that generates the butyl ester after mild hydrolysis.

Table 2. *In vitro* and *in vivo* antibacterial activities.[a]

Organisms	MIC (μg/ml)			ED_{50} (mg/kg/day), sc[b]	
	Actagardine	Compound D	Compound E	Actagardine	Compound D
Staphylococcus aureus ATCC 6538	100	25	>50		
S. aureus TOUR	25	6.25	>50	56.6	24.5
S. aureus TOUR[c]	50	12.5	>50		
S. aureus TOUR[d]	25	6.25	>50		
Streptococcus pyogenes C 203	1.6	0.8	50	0.47	0.33
S. pneumoniae UC 41	25	6.25	>50		
Gram-negative bacteria	>100	>50	>50		

[a] See ref. 5).
[b] Administration in suspension in 0.5% aq Methocel solution.
[c] Inoculum 10^6 cells/ml.
[d] In the presence of 30% bovine serum.

Compound E

Some physico-chemical data are given in Table 1. No further investigation was carried out.

Biological Results

The *in vitro* and *in vivo* biological data, obtained by methods previously described[5)], are reported in Table 2. Compound D is twice as active as actagardine against *Streptococcus pyogenes* C 203 and four times more active than actagardine against *S. pneumoniae* UC 41, *Staphylococcus aureus* ATCC 6538 and *S. aureus* TOUR in the *in vitro* tests.

It is also more effective than the parent compound against experimental infections in mice with either *S. pyogenes* C 203 or *S. aureus* TOUR, after sc administration. Nevertheless, it resulted ineffective at doses up to 150 mg/kg when given orally to mice infected with *S. pyogenes* C 203. Compound D (LD_{50} 1,250 mg/kg, mice, ip) is about 2.5 times more toxic than actagardine (3,310 mg/kg). Compound E shows practically no *in vitro* activity up to 50 μg/ml against the selected organisms.

Conclusions

Compound D, one of the two compounds isolated from acid butanolic extracts of fermentation broth, is an artifact. Although the structure of actagardine itself is unknown, comparison of some physico-chemical data and of the analysis of the acid hydrolysates shows that compound D is an esterified fragment of actagardine and it still possesses good biological activity.

Experimental

Evaporation was carried out with a rotary evaporator at 45°C under vacuum. Column chromatographies were done on silica gel 0.06~0.2 mm (Merck). TLC was done on silica gel Merck F_{254} plates, eluting mixture BuOH - AcOH - H_2O, 4:1:1; the spots were detected by UV light at 254 nm. IR spectra were determined with a Perkin-Elmer 421 spectrophotometer, as Nujol mulls. UV spectra were recorded with a Unicam SP 800 A apparatus, in MeOH solutions. Gas-chromatograms of the amino acid *N*-TFA methyl esters were recorded with a Perkin-Elmer 3920/B gas-chromatograph equipped with flame ionization detector. GC-MS was done by a Varian Mat 112 instrument equipped with a Varian 101 MS computer.

Production and Isolation of Actagardine Complex

The antibiotic activity was assayed in the filtered broth, in powders and in the chromatographic fractions by the agar diffusion method (using *Micrococcus luteus* ATCC 9341 as test organism) and by HPLC (Fig. 1).

Actinoplanes garbadinensis ATCC 31049 was fermented[1] in a 200-liter fermentor for 96 hours (microbiological titer of 250 μg/ml); HPLC analysis (Fig. 1). The harvested broth was filtered with a rotating filter precoated with Clarcel Flow-MA filter-aid washing the panel with H_2O (20 liters). The solution (180 liters, pH 8.0) was brought to pH 3 with 3 N HCl while cooling at 5°C. A suspension was obtained that was extracted with BuOH (2×50 liters). The extracts were combined and concentrated to a final volume of 2 liters. On cooling and adding 5 liters of ether a solid separated that was filtered and washed with ether (114 g).

Separation of the Products

Crude material (114 g) was dissolved in 1 liter of a mixture BuOH - AcOH - H_2O, 1: 1: 1. The solution was diluted with 5 liters of BuOH and adsorbed on a column containing 3 kg of silica gel prepacked with a mixture BuOH - AcOH - H_2O, 15: 1: 1 at a rate of 500 ml/hour. The column was diluted at the same rate with 3 liters each of the following mixtures of BuOH - AcOH - H_2O, 18: 1: 1, 15: 1: 1, 14: 1: 1, 13: 1: 1, 12: 1: 1, 11: 1: 1, 10: 1: 1, collecting 500 ml fractions. Fractions containing compounds D and E (16~27) were combined and concentrated to a small volume. By adding ether a precipitate formed that was filtered off, washed with ether and dried (14 g; HPLC: compound D 22%; compound E 7%; actagardine and metabolite A traces). Fractions 36 to 49 were combined and treated as described above obtaining 18 g of a powder containing actagardine (45%) and metabolite A (5%).

a) The crude material (14 g) was dissolved in 200 ml of MeOH and 40 g of silica gel was added. The solvent was evaporated, the residue was slurried with 300 ml of Me_2CO. The column was developed using 2 liters each of the following Me_2CO - H_2O - MeOH mixtures, 96: 2: 2, 98: 1: 1, 93: 3.5: 3.5, 92: 4: 4, at a rate of 600 ml/hour, collecting 200 ml fractions. Fractions 21 to 36 were combined, BuOH (640 ml) was added and the mixture was concd to a small volume. By cooling and adding ether a precipitate formed that was filtered obtaining 0.6 g of compound E (HPLC titer 80%) which was crystallized from 50 ml of *iso*-PrOH (yield 0.25 g).

Fractions 57 to 69 were combined and worked up in the same way obtaining 2.4 g of compound D (HPLC titer 90%) which was dissolved in 100 ml of a mixture CH_3CN - H_2O, 9: 1 and loaded on a column prepacked with 240 g of silica gel developing with the same solvent mixture. Fractions of 100 ml were collected and those containing the desired product were combined. BuOH was added and the CH_3CN was evaporated under vacuum at room temp. The remaining solution was washed three times with H_2O, then concentrated to a small volume. On cooling, a solid separated which was collected, washed with ether and dried under vacuum at 50°C overnight obtaining 1.8 g of pure compound D.

b) The crude material (18 g) was purified as described above a) obtaining 3.2 g of actagardine monosodium salt that was dissolved in 1 liter of a mixture H_2O - MeOH, 9: 1. The resulting solution was extracted with 1.5 liters of BuOH. The organic phase was separated and, after adding 100 ml of AcOH, it was washed with distilled water (2×1 liter), then concentrated to a small volume. By adding ether a solid separated that was collected and washed with ether, Me_2CO, then with ether, obtaining 2.1 g of actagardine free acid.

Preparation of Compound D from Actagardine

A solution of 4 g of actagardine sodium salt in 1 liter of the mixture BuOH - Me_2CO - 2 N HCl, 1.5: 2: 2 was stirred at room temp for three days; the solution was concd at 60°C to a final volume of 200 ml, then extracted with 200 ml of 0.1 M phosphate buffer, pH 8.5. The organic layer was repeatedly washed with 200 ml of H_2O, then concentrated to a small volume. On the addition of ether, a crude solid separated which was collected and purified on a silica gel column by the same procedure described above yielding 1.8 g of the product. A highly pure sample for analysis (0.38 g) was obtained by repeating the chromatographic purification.

Determination of Amino Acid Composition

N-TFA Methyl Esters[6]: 20 mg of either actagardine or compound D was suspended in 2 ml of 6 N HCl or in 3 ml of a 30% $Ba(OH)_2$ solution (for tryptophan determination). The reaction mixtures were heated at 110°C for 14 hours. The dried hydrolysates were treated with MeOH - HCl (80°C, 3 hours) than with trifluoroacetic anhydride in methylene chloride (room temp, 2 hours).

GLC and GC-MS Analysis: Injector temp 270°C; detector temp 270°C; carrier gas (N_2) flow rate 30 ml/minute; injected volume 1 µl. Glass columns were packed with: A, 3% OV 17 on Chromosorb W HP 80~100 mesh (2 m×2 mm I.D.), temperature programmed from 150 to 250°C at 4°C/minute; B, Vetrabond (Supelco No. 2-18171), temperature programmed from 70 to 230°C at 4°C/minute.

Resolution of the Enantiomers: Capillary column Chirasil Val (25 m) (Applied Science Labs., Pennsylvania, USA); temperature programmed from 60 to 230°C at 4°C/minute.

Acknowledgments

The authors thank A. De Paoli and P. Ferrari for NMR and IR spectra, G. C. Allievi for UV spectra and acid-base titrations, and Profs. G. G. Gallo and G. C. Lancini for helpful discussions.

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