

Microbial Transformation of A23187, a Divalent Cation Ionophore Antibiotic

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A23187 is an ionophore antibiotic that forms dimeric complexes with divalent cations such as Mn^{2+} and Ca^{2+} . Over 200 randomly selected soil microorganisms were incubated with A23187. None of these cultures was capable of transforming this compound. In contrast, many microorganisms were able to modify the methyl ester of A23187. The transformation products produced by one culture, *Streptomyces chartreusis*, were isolated and identified as 16-hydroxy-*N*-demethyl A23187 methyl ester, 16-hydroxy-A23187 methyl ester, and *N*-demethyl A23187 methyl ester. These ester derivatives lack most of the ionophore properties of the acids and cannot readily form dimeric complexes with divalent cations. However, they could be hydrolyzed by a mild treatment with ethanolic KOH to free acids that possess good ionophore activity. The use of the ester substrate in conjunction with the hydrolysis procedure is, at the present time, the only known method for microbiologically producing A23187 derivatives.

A23187 is one of the few known naturally occurring compounds capable of transporting divalent cations across biological membranes (11). Ion transport by A23187 is mediated by a dimeric form of the molecule that complexes the cation (3, 4, 13) (Fig. 1). The relative stabilities of the complexes formed with cations are $Mn^{2+} \gg Ca^{2+} \approx Mg^{2+} \gg Sr^{2+} > Ba^{2+}$ (10).

A23187 elicits a wide range of pharmacological responses, e.g., platelet aggregation (15), insulin release (14), histamine release (6), increased cardiac contractility (8), arrest of sperm motility (1), release of slow-reacting substance (2), etc. Some of these responses have potential clinical utility, particularly if the toxicity of the compound can be reduced and the spectrum of effects can be narrowed. Structure modification of A23187 could lead to new compounds with a narrower range of cation specificity or to compounds with a completely different spectrum of ion complexation. Such derivatives may have more specific pharmacological effects or fewer toxic liabilities or both. To explore these possibilities a screening program was initiated to find microorganisms that modify A23187. Over 200 randomly selected cultures were tested, and none was found that modified the ionophore. Subsequent studies showed that many microorganisms were able to modify the methyl ester of A23187. This report describes three new derivatives of A23187 that were produced by using the methyl ester substrate.

MATERIALS AND METHODS

Microorganism. The microorganism that produced the transformation products was identified as a strain of *Streptomyces chartreusis* (L. Doolin, R. Kastner, and F. Mertz, unpublished data). This strain was

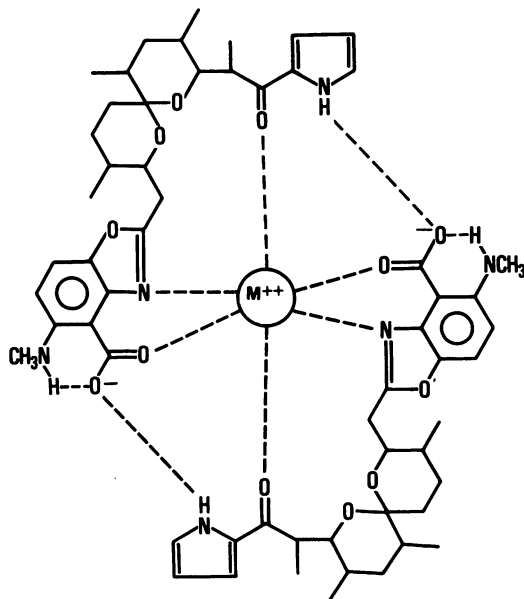


FIG. 1. Schematic representation of the dimeric complex formed by A23187 in the presence of divalent cations (3, 4, 13).

designated A47521 and deposited in the Northern Regional Research Center Collection as NRRL 11407. Stock cultures were maintained on Bennett agar slant medium. The slants were incubated for 72 to 96 h at 25 to 30°C and then stored at 4°C.

Screening program. A total of 209 randomly selected microorganisms were isolated from soil and tested for their ability to modify A23187. These microorganisms represented approximately equal numbers of bacteria, actinomycetes, and fungi. The methods used for cultivation were similar to those described in an earlier biotransformation study (7). The only differences were that bacterial cultures were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and substrate was added as a dry solid rather than in an ethanol solution. Three days after substrate addition, each culture was extracted twice with ethyl acetate. The extracts were combined, dried over Na_2SO_4 , and concentrated to dryness in vacuo. The dried extracts were dissolved in ethyl acetate to obtain a concentration of ≈ 10 mg/ml, and 10- μl samples were applied to Silica Gel G F₂₅₄ thin-layer plates (EM Laboratories, Darmstadt, Germany). The plates were developed with a solvent system consisting of chloroform-methanol-cyclohexane (1:1.5:2.8) to resolve metabolites derived from the unesterified form of A23187. Tetrahydrofuran-cyclohexane-methanol (1:1:0.1) was used to separate metabolites of A23187 methyl ester. A23187 and its derivatives were detected on the plates by their intense fluorescence when irradiated with long-wave ultraviolet light.

To confirm that the fluorescent compounds observed on thin-layer plates were transformation products of A23187 (or its methyl ester), control flasks were prepared. Control and experimental flasks were prepared by similar procedures except that the control flasks were autoclaved (two sterilization cycles at 121°C for 30 min) immediately before substrate addition. Control flasks were also prepared by not adding substrate to viable cultures. Control and experimental flasks were extracted and analyzed by similar procedures.

Production, isolation, and purification of transformation products. Larger quantities of the transformation products produced by *S. chartreusis* were obtained by cultivating the microorganism in the following medium: Cerelose, 15 g; soybean meal, 15 g; corn steep solids, 10 g; Stadex 11, 20 g; CaCO_3 , 2 g; CZA mineral mixture, 2 ml; tap water, 1 liter. The pH was adjusted to 6.6 with 5 N NaOH. CZA mineral solution was prepared by dissolving 10 g of KCl, 10 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g of FeSO_4 , and 2 ml of 12 N HCl in 100 ml of distilled water. Two liters of medium were dispensed in 200-ml aliquots into 1-liter Erlenmeyer flasks. The flasks were closed with cotton plugs and autoclaved at 121°C for 40 min. Each flask was inoculated with a loop transfer from an agar slant culture. After 96 h of cultivation, a suspension of A23187 methyl ester and polyvinylpyrrolidone was added to each flask to achieve a 0.5-mg/ml final concentration of A23187 methyl ester. The polyvinylpyrrolidone-substrate suspension was prepared by dissolving 1 part (by weight) A23187 methyl ester and 10 parts (by weight) polyvinylpyrrolidone in chloroform. The chloro-

form was then removed in vacuo. The resultant dry powder was slurried in sterile water and then added to the cultures.

Transformation products were recovered from the culture 96 h after substrate addition by extraction with ethyl acetate. The contents of each flask were pooled and extracted three times. The extracts were combined, dried with Na_2SO_4 , and evaporated to dryness in vacuo. The dry residue (1.3 g) was chromatographed on a reverse-phase high-performance liquid chromatography column (Quantum Industries, Fairfield, N.J.). The column was stainless steel (1 by 12 in., ca. 2.5 by 30.5 cm) packed with 10- to 20- μm particle size LP-1 silica treated with octadecyltrichlorosilane. The column was operated at 300 to 750 lb/in^2 with a Lapp pump (Interpace Corp., Leroy, N.Y.) to achieve a solvent flow rate of 20 ml/min. Effluent from the column was monitored with a Variscan model 635LC Detector (Varian Assoc., Palo Alto, Calif.) at 390 nm.

Two transformation products, 16-hydroxy A23187 methyl ester and *N*-demethyl-16-hydroxy A23187 methyl ester, were eluted with methanol-water (65:35). Methanol-water (90:10) was used to elute *N*-demethyl A23187 methyl ester. Final purification of all products was achieved by preparative thin-layer chromatography by using Silica Gel G F₂₅₄ plates developed with a solvent mixture consisting of tetrahydrofuran-cyclohexane-methanol (1:1:0.1).

Preparation and hydrolysis of methyl esters. The methyl ester of A23187 was prepared by reaction with diazomethane (5). Methyl ester transformation products of A23187 were hydrolyzed to free acids with ethanolic KOH. The reaction was initiated by adding 0.3 ml of a 10% KOH solution to 5.0 ml of ethanol containing 5 mg of transformation product. The mixture was reacted at 60°C for 3 h. Water (12.0 ml) was then added, and the pH of the solution was lowered to 4.5 with 0.1 N HCl. The ethanol was removed in vacuo, and the remaining aqueous suspension was extracted three times with ethyl acetate to recover about 4.5 mg of acidic product. Thin-layer chromatography and high-performance liquid chromatographic analyses indicated that >95% of the methyl ester was hydrolyzed and little or no side products were produced.

Spectral analyses. Electron impact high-resolution mass spectra were obtained with a Varian-MAT model 731 (Varian Assoc., Palo Alto, Calif.) mass spectrometer. Samples were introduced directly into the ion source.

Proton magnetic resonance spectra (360 Hz) were obtained with a Bruker model WH-360 Nuclear Magnetic Resonance Spectrometer (Bruker Instruments, Inc., Billerica, Mass.). The spectra were obtained on samples dissolved in CDCl_3 with tetramethylsilane as an internal standard.

A23187. The transformation substrate A23187 was supplied by Robert L. Hamill of the Lilly Research Laboratories.

RESULTS

Randomly selected soil microorganisms were tested for their ability to transform A23187. None of these cultures was capable of modifying the ionophore. Subsequent testing with 24 ad-

ditional microorganisms indicated that one-third of these cultures were able to transform the methyl ester of A23187. The transformation products produced by *S. chartreusis* NRRL 11407 were selected for isolation and structure determination. This selection was made because strain NRRL 11407 appeared to produce somewhat larger quantities of products than the other cultures.

Antibiotic A23187 is produced de novo by another strain of *S. chartreusis* designated NRRL 3882. *S. chartreusis* NRRL 3882 differs from *S. chartreusis* NRRL 11407 (the microorganism that modifies A23187 methyl ester) by several biochemical characteristics (e.g., pigment production, temperature optima for growth, and aerial mycelia production on some media; L. Doolin and F. Mertz, unpublished data). In addition, culture NRRL 11407 does not produce antibiotic A23187 or its methyl ester, and culture NRRL 3882 cannot produce transformation products of A23187 methyl ester.

Three transformation products of A23187 methyl ester were obtained from ethyl acetate extracts of *S. chartreusis* NRRL 11407 cultures (Fig. 2). Each product was obtained in 40- to 60-mg quantities which corresponded to 4 to 6% (by weight) yield from the substrate. Mass spectral analyses and proton magnetic resonance estab-

lished the structures as 16-hydroxy-*N*-demethyl A23187 methyl ester, 16-hydroxy A23187 methyl ester, and *N*-demethyl A23187 methyl ester (Table 1). Small amounts of *N*-demethyl A23187 were isolated from control flasks containing the substrate. This product was present as a minor component in the A23187 methyl ester preparation. About five times more *N*-demethyl A23187 methyl ester was obtained from viable cultures than could be isolated from the controls. Thus, most of the *N*-demethyl derivatives can be attributed to enzymatic *N*-demethylation by *S. chartreusis*.

Attempts were made to produce the A23187 transformation products using *S. chartreusis* and the acid form of the substrate. Chromatographic evaluation of the culture extracts did not reveal the presence of any transformation products. The acid form of A23187 and its transformation products also were not found in *S. chartreusis* cultures incubated with A23187 methyl ester.

Structure determinations. The mass spectrum molecular ion of *N*-demethyl A23187 methyl ester was 14 mass units (i.e., CH₂) less than the substrate. The fragmentation pattern was consistent with the loss of a CH₂ fragment from the benzoxazole portion of the molecule. The proton magnetic resonance spectrum of this

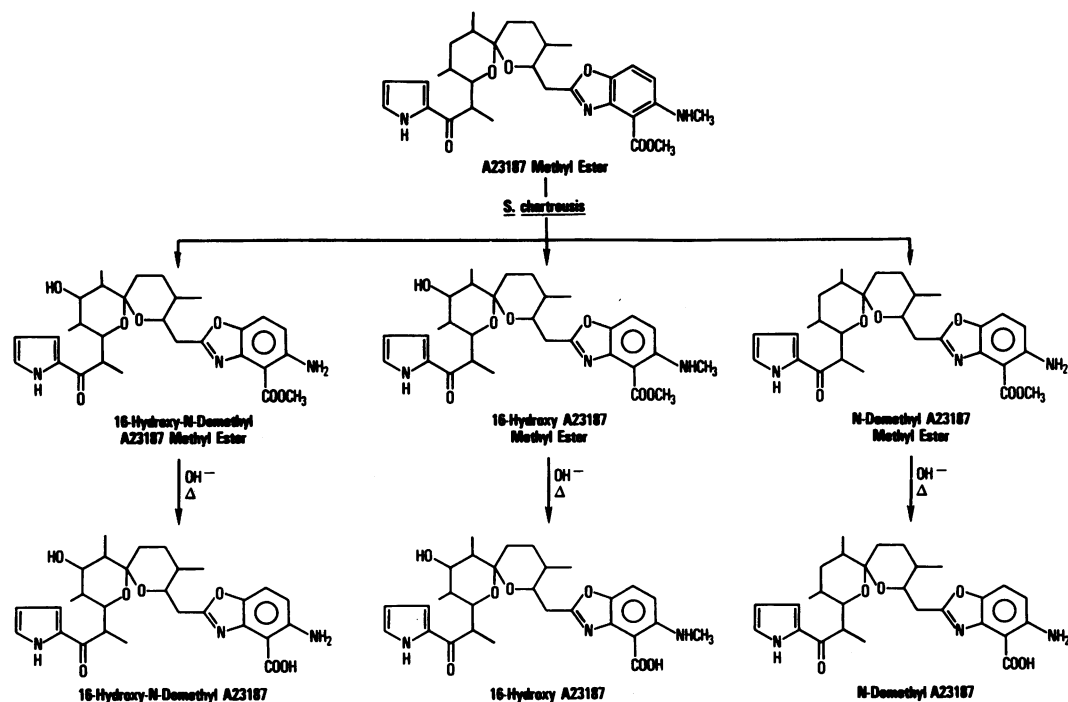


FIG. 2. Transformation products produced by *S. chartreusis* from A23187 methyl ester and hydrolysis of the ester products to their corresponding acids.

TABLE 1. *Chromatographic mobilities, high-resolution mass spectral molecular weights, and empirical formula of A23187 methyl ester and its transformation products*

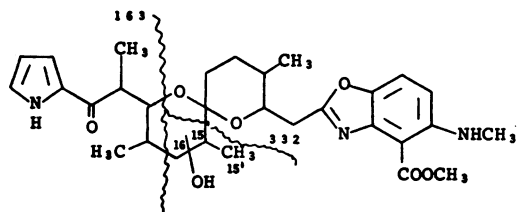
Compound	R_f^a	Mol wt		Empirical formula ^b
		Found	Calculated	
A23187 methyl ester	0.51	537.2855	537.2839	$C_{30}H_{38}N_3O_6$
16-Hydroxy- <i>N</i> -demethyl A23187 methyl ester	0.27	539.2645	539.2632	$C_{29}H_{37}N_3O_7$
16-Hydroxy A23187 methyl ester	0.37	553.2790	553.2788	$C_{30}H_{38}N_3O_7$
<i>N</i> -Demethyl A23187 methyl ester	0.44	523.2685	523.2683	$C_{29}H_{37}N_3O_6$

^a Silica Gel G F₂₅₄ thin-layer plates, tetrahydrofuran-cyclohexane-methanol (1:1:0.1).

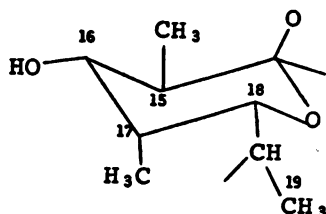
^b Calculated from the high-resolution mass spectrum.

product was essentially identical to that of the substrate (A23187 methyl ester) except for the absence of the *N*-methyl resonance near $\delta 3$.

The mass spectrum of the product assigned the 16-hydroxy A23187 methyl ester structure had a parent ion m/e 553, i.e., 1 oxygen atom greater than the substrate. The spectrum also contained fragment ions $C_{10}H_{13}NO$ (m/e 163) and $C_{17}H_{20}N_2O_5$ (m/e 332) which arise as follows:



Thus, the additional oxygen atom was associated with carbon 15, 15', or 16. Although m/e 163 is of relatively low intensity, its assignment as shown is supported by the presence of ions of the same composition in the spectra of A23187 and its methyl ester. The proton magnetic resonance spectrum of the compound was essentially identical to that of the substrate except for the presence of a doublet of doublets at $\delta 3.71$. Through a series of decoupling experiments, this resonance was shown to be due to a proton at C(16). This proton was coupled to H(17) by a coupling constant of 4.3 Hz. The remaining coupling, by elimination due to H(15), was large ($J = 10.4$ Hz), indicating that the structure and conformation of this portion of the molecule can be represented as follows:



The mass spectrum of 16-hydroxy-*N*-demethyl A23187 methyl ester contained the molecular ion $C_{29}H_{37}N_3O_7$ (m/e 539), i.e., A23187 methyl ester plus oxygen less CH_2 . The spectrum also contained ions $C_{10}H_{13}NO$ (m/e 163) and $C_{10}H_{18}N_2O_5$ (m/e 318), again indicating that the additional oxygen is associated with carbon 15, 15', or 16. The m/e 318 fragment is the *N*-demethyl analog of the same fragment that occurs in the mass spectrum of A23187 methyl ester. The proton magnetic resonance spectrum lacks an *N*-methyl resonance near $\delta 3$. Except for some small differences in the resonances of the benzoxazole portion of the molecule, the spectrum is identical to the spectrum of 16-hydroxy A23187 methyl ester.

Hydrolysis of methyl ester products. The methyl ester derivative of A23187 complexes with divalent cations "orders of magnitude" more weakly than A23187 itself (4, 9). Thus, to obtain products with better ionophore activity, the acid form of the products was generated by hydrolyzing the esters with ethanolic KOH. The acids were recovered by extraction with ethyl acetate and subjected to high-resolution mass spectral analyses. The results (Table 2) indicate that the desired acid products were obtained from the hydrolysis reactions. Initial evaluations of the acidic products indicated that they possessed ionophore specificity similar to A23187, but they were one-fifth to one-tenth as active as A23187 in activating oxygen uptake by liver mitochondria and in inhibiting Ca^{2+} -dependent mitochondrial adenosine triphosphatase (D. T. Wong, unpublished data). Other biological properties of the A23187 transformation products are currently under investigation.

DISCUSSION

Initial attempts to accomplish microbial transformations with A23187 were unsuccessful. Subsequent investigations revealed that many cultures were able to transform the methyl ester of A23187 which only weakly forms dimeric com-

TABLE 2. High-resolution mass spectral data for A23187 and its transformation products

Compound	Mol wt		Empirical formula ^a
	Found	Calculated	
A23187	523.2698	523.2682	C ₂₉ H ₃₇ N ₃ O ₆
16-Hydroxy-N-demethyl A23187	525.2466	525.2475	C ₂₈ H ₃₅ N ₃ O ₇
16-Hydroxy A23187	539.2629	539.2632	C ₂₉ H ₃₇ N ₃ O ₇
N-Demethyl A23187	509.2529	509.2526	C ₂₈ H ₃₅ N ₃ O ₆

^a Calculated from the high-resolution mass spectrum.

plexes with cations. Three transformation products produced by *S. chartreusis* were converted to acids with better ionophore activities by a facile chemical hydrolysis. At the present time, the use of the ester substrate followed by hydrolysis after the transformation step is the only way to modify A23187 microbiologically.

Selection of the methyl ester of A23187 as a substrate for modification was based on the hypothesis that the inability of microorganisms to modify the acid form was somehow associated with its ability to readily complex divalent cations. The fermentation media used to cultivate the microorganisms contained an abundance of divalent cations. Thus, when A23187 was added to the culture, it would be expected to form dimeric complexes with these ions. The molecular weight of the dimeric complex is approximately 1,100. Molecular sieving studies with bacterial cell walls suggest that the wall pores have an exclusion threshold that corresponds to a molecular weight of 1,100 and an Einstein Stokes hydrodynamic radius of 1.1 nm (12). The approximate radius of the dimeric A23187 complex is 1.0 nm (3). Thus, the dimeric complex may not be able to enter the cell because it is too large to penetrate the wall. In addition, when A23187 is in its dimeric form, many of the functional groups potentially susceptible to enzymatic modification are on the interior of the complex where they may not be accessible to enzymes. The accessibility of the functional groups of monomeric A23187 also may be determined by the solution conformation of the molecule. A23187 can exist in "open and closed" conformations (10). Thus, the methyl ester of A23187 could favor a conformation that is more readily modified enzymatically or more readily penetrates the cell or both. The contribution of these various factors to the apparent recalcitrance of A23187 might be clarified by additional experiments with cell-free enzymes derived from *S. chartreusis*.

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