FORMATION, ISOLATION, AND
IDENTIFICATION OF PRODUCTS
FROM THE INACTIVATION OF
VIRGINIAMYCIN M₁ BY
Actinoplanes utahensis

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The study of the inactivation of antibiotics by microorganisms is important in gaining an understanding of the development of resistance to antibiotics.[1] This point is crucial for the ongoing development of chemotherapeutic treatments for infectious diseases.

The virginiamycin family of antibiotics (alternatively designated as streptogramins, synergimycins, or pristinamycins) have been known for well over thirty years and have several chemotherapeutic applications.[2,3] They are a unique class of antibiotics consisting of two groups of compounds: type A as exemplified by virginiamycin M₁ (VM, **1a**; also known as pristinamycin II_A); and type B as exemplified by virginiamycin S₁ (**2**). Although the two types have quite different structures, they act synergistically to provide greatly enhanced levels of

antibacterial activity. Both types function as protein synthesis inhibitors. They bind to non-overlapping sites of the large ribosomal subunit and inhibit the peptidyltransferase reaction, thus blocking elongation of peptide chains.[4]

Despite the general use of the virginiamycins for the last few decades, the development of resistance towards VM has been very limited and in most cases has involved an altered permeability of the resistant cell to the antibiotic. Very few studies of the inactivation of VM have been reported. Previous studies have demonstrated the acetylation of the C-14 hydroxyl group of VM (**1**) by a resistant strain of *Staphylococcus aureus* to give the inactive ester derivative **1b**.[5,6] We now wish to report results of our more recent investigation of inactivation which has uncovered a bacterial species which transforms VM into two additional inactivated derivatives.

Results

A preliminary screening for resistance to VM was performed with the following ten bacterial strains: *Actinomyces albocyaneus*, *Actinoplanes armeniacus*, *Actinoplanes missouriensis*, *Actinoplanes philippinensis*, *Actinoplanes utahensis*, *Streptomyces alborubidus*, *Streptomyces albus*, *Streptomyces cyanogriseus*, *Streptosporangium roseum*, and *Streptoverticillium biverticillatum*. Only *A. albocyaneus* and *A. utahensis* yielded sufficient amounts of degradation products suitable for the present studies. Of these two strains, *A. utahensis* was chosen for detailed investigation.

The extract from a 48-hour exposure of *ca.* 25 mg of VM to *A. utahensis* was shown by TLC to contain several components. Very prominent was a spot having Rf 0.85. Extraction of the silica gel from this spot on a preparative layer plate gave *ca.* 5 mg of
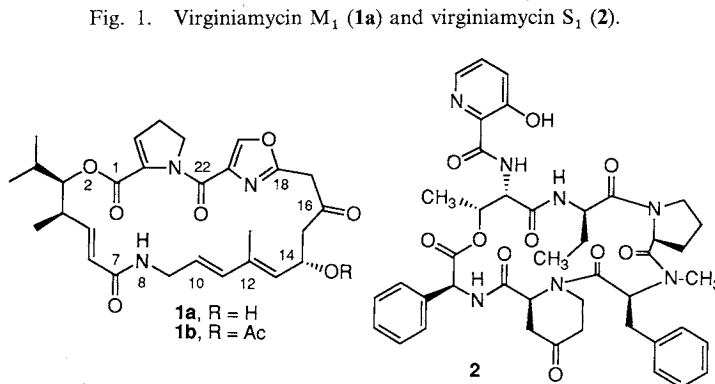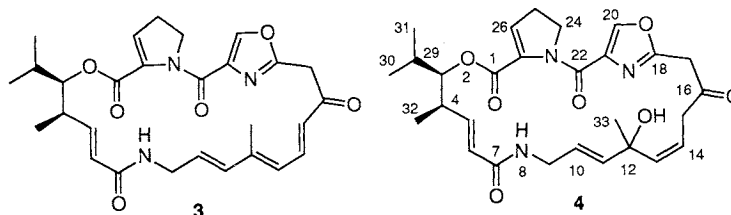
Fig. 1.   Virginiamycin M₁ (**1a**) and virginiamycin S₁ (**2**).

1a, R = H
1b, R = Ac

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Fig. 2. Isolated inactivation products from **1a**.

residue which contained two components. Further purification by preparative TLC and HPLC gave 2 mg of each of the two components. These components did not exhibit antibiotic activity *in vivo* (*i.e.* undetectable growth inhibition) as tested against *Micrococcus luteus*, *Bacillus subtilis*, and *Staphylococcus aureus*. These components were also inactive in binding to ribosomes *in vitro*. In order to demonstrate that these components were not artifacts and did not arise spontaneously upon exposure of VM to the culture medium, VM was treated under the same culture conditions but in the absence of *A. utahensis*. Following the same extraction protocol as above, the two components were not observed.

The slower component was readily characterized by ^{1}H NMR as the trienone derivative **3**. This compound, which is a formal dehydration product of VM, has been characterized previously. It has been obtained from a fermentation broth of *Streptomyces olivaceus* and by direct modification of VM.[7] For the purpose of direct comparison, a sample of **3** was obtained by the previously reported treatment of VM with methanesulfonyl chloride in pyridine at 25°C.[7] The resulting sample of **3** was shown by ^{1}H NMR, TLC, and HPLC to be identical to the sample obtained above from the incubation of VM with *A. utahensis*.
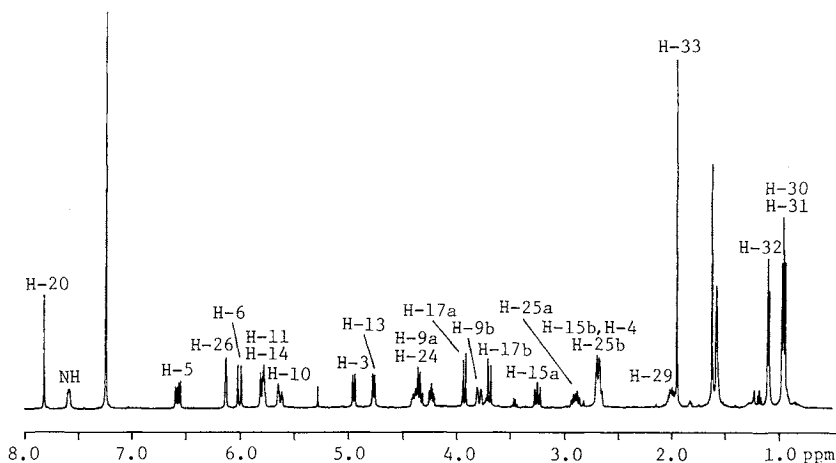
The faster component proved to be more interesting. The 500 MHz ^{1}H NMR (CDCl$_3$) spectrum (obtained on a Varian VXR-500 spectrometer at ambient temperature) (Fig. 3) provided the following data: δ 7.82 (s, H-20), 7.60 ~ 7.65 (br, NH), 6.58 (dd, $J=16.5, 8.0$ Hz, H-5), 6.14 (t, $J=2.5$ Hz, H-26), 6.01 (dd, $J=15.5, 1.0$ Hz, H-6), 5.77 ~ 5.82 (m, H-11, H-14), 5.62 (dt, $J=15.5, 4$ Hz, H-10), 4.95 (dd,

$J=10, 2$ Hz, H-3), 4.77 (d, $J=9.5$ Hz, H-13), 4.20 ~ 4.42 (m, H-9a, H-24a, H-24b), 3.93 (d, $J=13$ Hz, H-17a), 3.79 (br d, $J=19$ Hz, H-9b), 3.69 (d, $J=13$ Hz, H-17b), 3.25 (dd, $J=13.5, 11$ Hz, H-15a), 2.86 ~ 2.94 (m, H-25a), 2.66 ~ 2.72 (m, H-15b, H-4, H-25b), 2.00 (m, H-29), 1.95 (s, H-33), 1.10 (d, $J=7$ Hz, H-32), 0.97 (d, $J=7$ Hz, H-30), and 0.95 (d, $J=7$ Hz, H-31). This spectrum is similar to that of VM itself with the exception of the C-12 to C-14 region. Absent from the structure is the conjugated diene bearing a total of three alkenyl hydrogens and a methyl group. This diene unit of VM has apparently been replaced by two isolated alkene units, each bearing two alkenyl hydrogens. Also absent is the C-14 carbinol C–H. A structure that is consistent with these data is the rearranged allylic alcohol **4**. Further evidence for this structure is provided by a 2D homonuclear COSY correlation (not shown here) and by ^{13}C NMR and mass spectrometry data.[†] Especially relevant is the coupling of the alkenyl H-14 at δ 5.78 with both the alkenyl H-13 at δ 4.77 and the methylene H-15 at δ 3.25. The H-13 ~ H-14 coupling constant of 9.5 Hz is indicative of a *cis*-disubstituted alkene, whereas the H-10 ~ H-11 coupling constant of 15.5 Hz indicates retention of this *trans*-disubstituted double bond configuration from VM. Assignment of the configuration of the new chiral center at C-12 is not possible with the present data.

Discussion

The development of resistance toward antibiotics is due to any of the three following mechanisms: (1) reduced permeability (due to alteration of the synthesis or the function of permeases); (2) alteration

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Fig. 3. ^{1}H NMR (500 MHz) spectrum of compound 4.

of the target (due to mutation of a gene coding for a component of an antibiotic binding site); and (3) inactivation of the antibiotic by structural modification. The mechanisms of inactivation may in general be divided into two broad categories; (1) coupling with other groups (e.g. alkylation, acylation, adenylation, and phosphorylation) and (2) hydrolysis (e.g. β-lactam cleavage).[8,9]

Little was previously known concerning the inactivation of type A virginiamycins.[8~12] The C-14 acetoxy derivative (1b) was identified upon the action of Staphylococcus species on VM.[5] Due to the presence of the ester and amide functionalities as obvious sites of possible hydrolysis, inactivation by cleavage of VM may have been expected. In the present studies, we have instead observed the formation of the two compounds 3 and 4 by the action of A. utahensis.

From a chemical point of view, the formation of the trienone 3 by dehydration of either VM (1a) or the rearranged alcohol 4 is conceivable. Based upon preliminary data not reported above, chromatographic monitoring of the progress of VM conversion indicates rapid formation of the rearranged alcohol within the first few hours followed by much slower formation of the trienone during the following two-day period. We cannot exclude at the present time a simple chemical, non-enzymatic conversion of the rearranged alcohol to the trienone. The non-conjugated system 4 is expected to be much less stable than the fully conjugated trienone 3. The formation of the alcohol 4 from the trienone 3 is therefore less likely than the reverse.

In conclusion, the compounds 3 and 4 appear to be initial products of inactivation of VM by A. utahensis. Their formation provides information concerning a possible mechanism for the development of resistance to VM. Whether other mechanisms exist and whether subsequent cleavage of the macrocyclic ring of 3 and 4 occurs will be subjects of further studies in our laboratories.

Experimental

Actinoplanes utahensis (ATCC 14539) was grown in pH 7.5 medium, containing 4 g of yeast extract (Gibco), 10 g of malt extract (Gibco), and 4 g of glucose per liter. Cultures were shaken at 24°C in the presence of 100 ~ 200 μl/ml VM. At various times, aliquots of the cultures were centrifuged at 5,000 × g for 15 minutes. The supernatant phases were extracted with two vol of ethyl acetate, the organic phases were evaporated, and the residues were dissolved in ethanol. Antibiotic degradation products in the ethanol solutions were fractionated by TLC (0.25 mm Silica gel 60 plates with fluorescent indicator from Merck, Darmstadt, Germany) using 95 : 5 (v/v) chloroform - methanol as the developing solvent. The plates were analyzed under UV light (254 and 366 nm) and scanned with a densitometer (Chromoscan 3, Joyce-Loebl, Gateshead, U.K.). The TLC spots were eluted with ethanol, and biological activity was evaluated by measuring the growth inhibition of Micrococcus luteus either on solid medium (diameter of the halo of inhibition on liter agar) or in liquid medium (minimal inhibitory concentration, MIC, in liter broth, 16 hours, 37°C, shaken cultures). Similar results were obtained with Bacillus subtilis and Staphylococcus aureus.

The extract from a 48-hour exposure of *ca.* 25 mg of VM to *A. utahensis* was shown by TLC (10:1 chloroform - methanol) to contain several components. Very prominent was a spot having Rf 0.85. The silica gel from this spot was scraped off a preparative layer plate and was extracted with ethanol. Evaporation of the solvent gave *ca.* 5 mg of residue. Further purification was effected by preparative TLC using 92:4:4 (v/v) chloroform - methanol - acetic acid to give each of the components with a purity of 75% contaminated with 25% of the other component. Complete separation was effected using HPLC (ISCO Model 2350, UV detection at 254 nm, 220 × 4.6-mm 5 μm silica gel column, 98:2 (v/v) methylene chloride - methanol, 2 ml/minute flow rate) to give 2 mg of each of the two components having retention times of 15 and 21.5 minutes, respectively. No antibiotic activity and no ribosome binding activity were seen for these VM degradation products. The components were examined spectroscopically to give the data reported in this note.

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