Detection of Phenanthroviridin Aglycone in a UV-Mutant of *Streptomyces murayamaensis*

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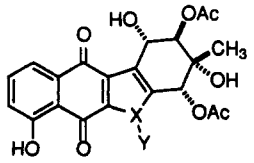
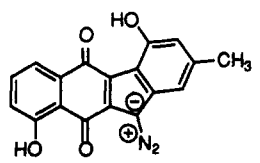
The kinamycin antibiotics, isolated from *Streptomyces murayamaensis*, were originally characterized by Omura and co-workers[1-3] as benzo[*b*]carbazoles (cf. kinamycin D, **1**) on the basis of chemical, spectroscopic, and X-ray crystallographic data.[4] Since then a number of other members of this group have been reported.[5-7] One of these, named prekinamycin by us,[5] was recently synthesized and the synthetic material did not match the natural product.[8] We have since revised the structures of *both* prekinamycin and kinamycin D as diazo-substituted benzo[*b*]fluorenes **2** and **3**, respectively.[9] We now report the detection of phenanthroviridin aglycone **4**,[10] a member of the recently discovered benzo[*b*]phenanthridine class of natural products, as a minor metabolite of *S. murayamaensis*.



1, X = N, Y = CN
3, X = C⁻, Y = $^{+}N_2$



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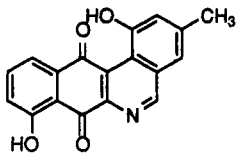

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Results and Discussion

A number of UV mutants of *S. murayamaensis* blocked in the biosynthesis of **3** have been prepared.[11] One of these was found to produce small amounts of kinafluorenone (**5**) which we have recently identified in another blocked mutant strain of *S. murayamaensis*,[11] and a number of new colored metabolites. Scale-up of the fermentation and extraction with ethyl acetate, followed by concentration and trituration with hexanes, removed nonpolar oils and yielded 2.4 g of crude material. This material was then chromatographed sequentially on Silicar CC-4, phosphate-buffered flash grade silica gel,[11] and Sephadex LH-20 to give material enriched in a red metabolite and devoid of a group of yellow metabolites. Diode array HPLC analysis of this material revealed a number of minor components (Figure 1A), some of which had been masked by one of the yellows at earlier stages. While the structures of the more major new metabolites have so far proved elusive, one of the newly-revealed minor metabolites, eluting at 19.6 min, exactly matched the retention time and UV/vis absorption spectrum of authentic **4** (Figure 1B). Coinjection with the authentic material[12,13] gave a symmetrical enhancement of the peak with an unchanged UV/vis spectrum. Further confirmation of the identity of the peak assigned to **4** was provided by thermospray (TSP) liquid chromatography mass spectrometry (LCMS): both an authentic sample of **4** and the new metabolite had identical retention times and gave an M^- ion at *m/z* 305.

Previous investigations in our laboratory have established that the kinamycins are of polyketide origin, apparently derived from a benzo[*a*]anthraquinone **6** via a decaketide **7**.[14,15] A few years ago we had predicted the existence of naturally-ocurring benzo[*b*]phenanthridines,[16] and four such compounds have subsequently been isolated from two different *Streptomyces*, including **4**, and the jadomycins.[17,18] Metabolism of **6** to a *seco*-structure, quinone **8**, could readily lead to the benzo[*b*]phenanthridines via nitrogen addition and decarboxylation,[13] while reduction of **8** to the hydroquinone **9** followed by biological Friedel–Crafts cyclization would yield the fluorene system of the kinamycins (Scheme 1).

Experimental Section

Bacterial Strains and Culture Media. The wild-type strain, and procedures for mutagenesis and screening of cultures are those specified in ref 11.

Photodiode-Array HPLC. Samples of interest were analyzed by photodiode array HPLC, using a Waters 600E gradient HPLC equipped with a Waters 990+ photodiode-array detector. The metabolites were separated on a Waters NovaPak C_{18} radial compression column (8 × 100 mm, 4-μm particle size) using a gradient of 5–95% acetonitrile in water over a period of 20 min at 1.5 mL/min. Both solvents contained 0.1% HOAc. Absorption spectra of the separated metabolites were obtained over a wavelength range of 200–650 nm with a 2-nm interval, 2 s/scan. A reference library of spectra of the kinamycins and all other characterized metabolites of *S. murayamaensis* was constructed under the same chromatographic conditions.

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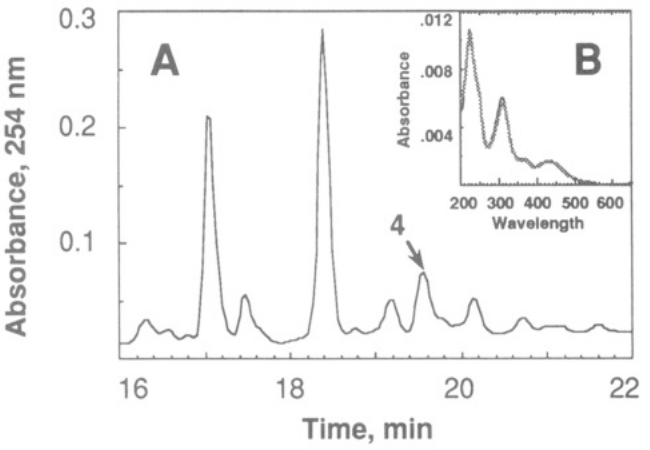

Figure 1. HPLC analysis of LH-20 Fraction from *S. murayamaensis* MC2. Sample was chromatographed on a Waters NovaPak C_{18} column (8 × 100 mm, 4-μm packing) using a linear gradient of 5–95% acetonitrile in 0.1% HOAc over 20 min at 1.5 mL/min. Absorption spectra (200–650 nm, 2-nm interval) were read every 2 s using a Waters 990+ photodiode array detector. A: chromatogram of LH-20 fraction. B: UV-vis absorption spectrum of component eluting at 19.56 min, overlaid with spectrum of authentic 4.

Thermospray Mass Spectrometry–Liquid Chromatography. Authentic phenanthroviridin aglycone and the partially purified extract from *S. murayamaensis* mutant strain MC2 were chromatographed on a Waters NovaPak C_{18} column (3.9 × 150 mm) using a gradient of 10–95% acetonitrile in water, 0.1% HOAc, over a period of 25 min at 1.2 mL/min. Mass spectrometric detection was performed with a Finnigan TSP-46 single quadrapole MS instrument, vaporizer temperature 80 °C, jet temperature 230 °C; 1-kV discharge ionization, repellor voltage off, mass range 150–800, 2 s/scan.

Production and Detection of Phenanthroviridin Aglycone (4). Kinako soy/glycerol seed cultures of strain MC2, inoculated with frozen culture on glycerol–asparagine plugs (−80 °C) were used to inoculate the production cultures in 7–10% farina. After 7 days under standard conditions,[19] the mycelium and broth were separated by centrifugation. The mycelium was suspended in deionized water, sonicated, and combined with acetone. The resulting suspension was filtered through a Celite pad and extracted further with acetone to remove as much color as possible. The acetone was removed by rotary evaporation, and the resulting precipitate and aqueous layer were extracted with ethyl acetate. The ethyl acetate extracts from 4.3 L of culture were combined, yielding 3.3 g of crude extract. After sonication and trituration with hexane to remove oils, the remaining 2.4 g

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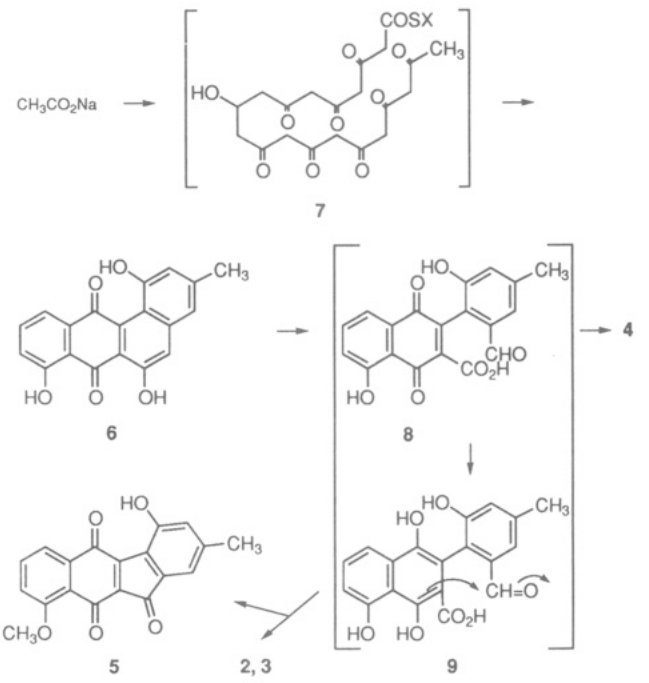
Scheme 1



residue was dried under high vacuum, dissolved in a small amount of CH_2Cl_2 (DCM), and adsorbed onto a small amount of Silicar CC-4. This was applied to a column of Silicar CC-4 (5.5 × 26 cm) in the same solvent. Colored materials were eluted with increasing proportions of EtOAc in DCM. Fractions containing a red metabolite as the major component (170 mg) were further purified on a column of phosphate-buffered flash grade silica gel[11] (pH 7.0; 2.5 × 26 cm), yielding 24 mg of material in the fraction most enriched with this metabolite. A portion (5 mg) was further resolved on Sephadex LH-20 (1.5 × 3 cm) in toluene–methanol (9:1), yielding a fraction (1 mg) that contained **4**, as indicated in the text.

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