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KINAMYCIN BIOSYNTHESIS. SYNTHESIS, DETECTION, AND INCORPORATION OF KINOBSCURINONE, A BENZO[*b*]FLUORENONE

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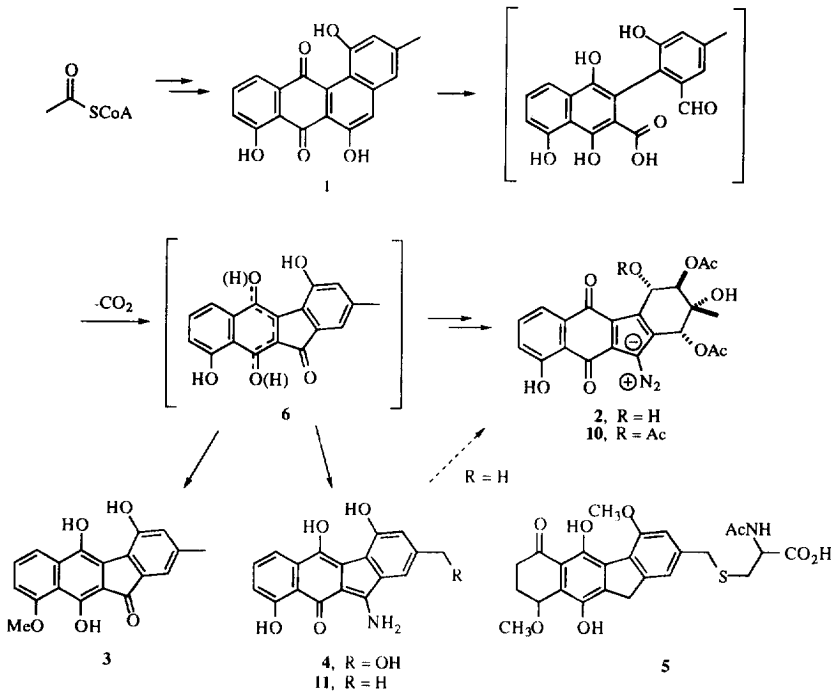
Abstract: The kinamycin antibiotics, derived from a benz[*a*]anthraquinone precursor, were recently shown to be 5-diazobenzo[*b*]fluorene quinones. A 5-ketobenzo[*b*]fluorene quinone has been synthesized and shown to be present in *Streptomyces murayamaensis* fermentations, and a deuterium-labeled sample of this metabolite, kinobscurinone, has been incorporated biosynthetically into kinamycins C and D.

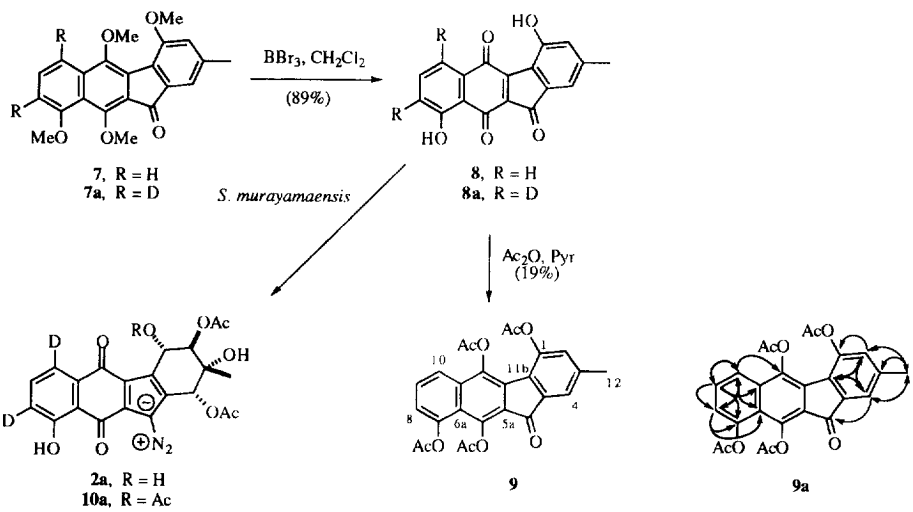
The kinamycin antibiotics, first isolated from *Streptomyces murayamaensis*,[1-5] were originally reported as *N*-cyanobenzo[*b*]carbazoles. We subsequently demonstrated their polyketide origin,[6,7] with the benz[*a*]anthraquinone dehydrorabelomycin, **1**, identified as an early biosynthetic intermediate (Scheme 1).[8] Recently we have shown that the kinamycins are actually 5-diazobenzo[*b*]fluorenes (e.g. kinamycin D, **2**).[9,10] Benzo[*b*]fluorenes had only recently been found to occur in Nature (e.g. kinafluorenone **3**, also produced by *S. murayamaensis*,[11] the aminofluorene **4**, produced by *S. viridochromogenes*,[12] and cysfluoretin, **5**, produced by another *Streptomyces* sp.[13]). We now report that the kinamycins are derived from **1** via a benzo[*b*]fluorenone intermediate **6**.

We had previously synthesized the tri-*O*-methylkinafluorenone, **7**,[14,15] and this now served as our starting material. Treatment with BBr_3 in CH_2Cl_2 (Scheme 2) afforded one major, highly colored (purple-red) product, **8**,[16] which proved to be an "NMR-silent" species, a behavior previously observed by Seto, *et al.* for the stealthins (i.e **4**).[12] As with **4**, variation of NMR solvent and temperature failed to yield ^1H- or ^{13}C NMR spectra. Acetylation of **8** with acetic anhydride in pyridine[11] yielded the tetra-acetate **9**,[17] at the hydroquinone oxidation level. An HMBC experiment[18] provided the long-range $^1H - ^{13}C$ correlations (**9a**), and these established the substitution pattern. Additionally, treatment of **9** with methanolic HCl, without exclusion of oxygen, regenerated **8**. A similar *in situ* reduction of the iminoquinone discorhabdin D upon treatment with acetic anhydride/pyridine has previously been observed.[19] The quinone oxidation level of **8** is based on the following chemical and spectroscopic data. Reduction with dithionite yielded a new, fluorescent product with a UV/vis spectrum (λ_{max} 220, 274, 322 (sh), and 498 nm) similar to that of **3** (λ_{max} 210, 228 (sh), 274, 325 (sh), 384, and 494 nm), and treatment of **8** with hydroxylamine[20] yielded a single product (data not shown) which had a UV/vis spectrum (λ_{max} 212, 254, 282, 470, and 545 nm) similar to that of **8** (λ_{max} 224, 252, 272, 477, and 578 nm). The high resolution mass spectrum of this latter compound (calcd for $C_{18}H_{11}NO_5$ 321.0637, found 321.0636) was consistent with the quinone oxidation level. This same compound was also prepared by treatment of **7** with hydroxylamine followed by BBr_3 deprotection of the resulting stereoisomeric mixture of oximes.

Scheme 1



Scheme 2



Compound **7a**, specifically labelled with deuterium at H-8 and H-10, was next prepared by treatment of **7** (38.2 mg) with trifluoroacetic acid-d_1.[8] Essentially complete deuteration (≥ 96% exchange, quantitative recovery) at these positions was established by the lack of ^{1}H NMR resonances at δ7.77 and 6.89, and from the EI mass spectrum (m/z 366, 100%, M+2; 365, 8%, M+1).[21] After BBr_3 deprotection, the resulting material, **8a** (31.2 mg), was fed as a solution in DMSO (3.2 mL) to actively growing cultures of *S. murayamaensis* (four 400–mL fermentations in 2-L Erlenmeyer flasks containing glycerol-asparagine medium[22]) in four pulses at 3-hour intervals from 12 to 21 h after inoculation with a seed culture. Work-up afforded 63.4 mg of a ~1:1 mixture of kinamycins C, **10a**, and D, **2a**. Both compounds have nearly identical ^{1}H chemical shifts for the aromatic resonances[2,7] (H-8 Δ δ = 0.10 ppm, H-10 Δ δ = 0.09 ppm) and the differences would be less than the ^{2}H NMR line widths: we had previously observed that the resonances for H-8 (δ7.23) and H-10 (δ7.71) of **2** could not be resolved in ^{2}H NMR spectra.[7] The mixture of **2a** and **10a** was analyzed directly by ^{2}H NMR.[23] A broad ^{2}H resonance centered at δ7.5 was observed (chemical shift and deuterium quantitation reference: natural abundance CH^2HCl_2 at δ5.32), which corresponded to deuterium at H-8 and H-10 of **2a** and **10a**. The deuterium enrichment was calculated to be 0.18% per site, based on HPLC analysis[11] of the fermentation that showed 65 mg of **2a** and **10a** had been produced, and this corresponded to a 0.22% incorporation of **8a**.

Concurrent with the feeding experiment, a separate fermentation of *S. murayamaensis* was monitored for kinamycin production and for the presence of **8**. Samples were periodically removed, acidified, mixed with an equal volume of EtOAc, and sonicated to disrupt the mycelium. The organic layer was filtered and concentrated, and the residue was dissolved in CH_2Cl_2:MeOH (10:1) and analyzed by HPLC. Kinamycins were first detected between 12 and 14 hours and reached a maximum between 24 and 36 hours after inoculation of the production broth. A peak with the retention time and UV/vis spectrum of **8** was first observed in the 12 hour sample and was present through 24 hours, but was missing by 36 hours. Co-injection with authentic **8** enhanced the peak but did not alter the UV/vis spectrum.

We have demonstrated the production of **8** by *S. murayamaensis* and its incorporation into kinamycins. Since *in vivo* redox chemistry could readily interconvert quinone and hydroquinone, we are presently unable to say whether **8** or the corresponding hydroquinone lies directly on the kinamycin biosynthetic pathway. This question will be addressed in future cell-free work. Regardless, it is clear that a benzo[*b*]fluorenone is an intermediate in kinamycin biosynthesis (Scheme 1). The occurrence of the aminofluorene **4** in another *Streptomyces* indicates that **11** may be the next intermediate in the pathway.

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16. IR (KBr) 2960, 1717, 1614 cm^{-1}; UV_{max} 580, 488, 272, 252, 224 nm; EIMS *m/z* (rel intensity) 308.0 (100%), 307.0 (66%); HRMS (CI, positive mode) *m/z* calcd for $C_{18}H_{13}O_5$ [$(M + 3H)^+$ from *in situ* reduction of the quinone] 309.0763, found 309.0791.
17. Pale yellow needles: mp 222.6-224.1 °C; IR 1773.7, 1711.8 cm^{-1}; UV_{max} 420, 290, 214 nm; ^1H NMR ($CDCl_3$) δ 7.82 (dd, 1H, J = 8.5, 1.1 Hz), 7.59 (dd, 1H, J = 8.5, 7.7 Hz), 7.48 (q, 1H, J = 0.7 Hz), 7.18 (dd, 1H, J = 7.7, 1.1 Hz), 7.01 (q, 1H, J = 0.7 Hz), 2.55 (s, 3H), 2.54 (s, 3H), 2.46 (s, 3H), 2.42 (s, 3H), 2.41 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 188.45, 169.24, 168.66, 168.31, 167.78, 148.36, 145.75, 142.83, 142.44, 138.55, 137.97, 134.84, 131.71, 130.62, 130.00, 128.63, 123.27, 122.95, 122.69, 120.92, 21.07, 20.88; EIMS *m/z* (rel intensity) 476.0 (M^+, 3%), 434.0 (10%), 392.0 (24%), 350.0 (29%), 308.0 (100%); HREIMS *m/z* calcd for $C_{26}H_{20}O_9$ 476.1107 found 476.1106.
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21. Mp 108.2-110.4 °C; ^1H NMR ($CDCl_3$) δ 2.39 (s, 3H), 3.88 (s, 3H), 3.98 (s, 3H), 4.01 (s, 3H), 4.03 (s, 3H), 6.92 (s, 1H), 7.22 (s, 1H), 7.47 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.42, 56.15, 56.35, 62.44, 63.12, 107.90 (t, J = 22.9 Hz), 115.84 (t, J = 23.4 Hz), 117.15, 118.95, 121.48, 122.79, 126.76, 127.75, 129.68, 137.19, 138.80, 141.30, 146.40, 155.04, 155.23, 159.34, 190.43; EIMS *m/z* (rel intensity) 366.2 ($[M+2]^+$, 100%), 365.2 ($[M+1]^+$, 8%); HREIMS calcd for $C_{22}H_{18}{}^2H_2O_5$ 366.1436, found 366.1437.
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23. Kinamycins C and D (47.6 mg) in CH_2Cl_2 (0.345 g): ^2H NMR conditions: sweep width 1433 Hz; 4K data points zero-filled to 16K; pulse width 90°; 38662 scans. Integration of aromatic deuterium compared with that of the natural abundance solvent line indicated 8.5 times natural abundance.

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