Kinamycin Biosynthesis. Synthesis, Isolation, and Incorporation of Stealthin C, an Aminobenzo[*b*]fluorene

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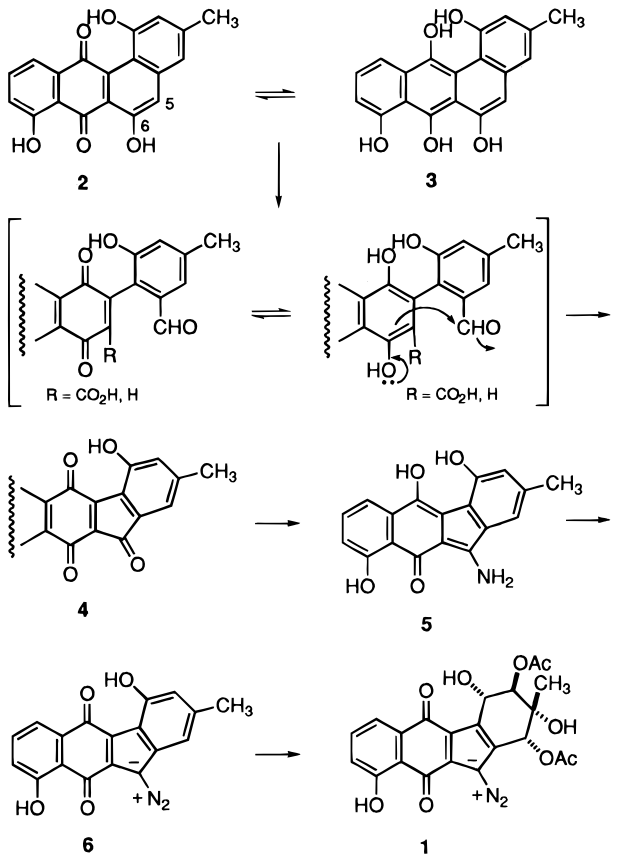
A new intermediate in the biosynthesis of the benzo[*b*]fluorene antibiotic, kinamycin D, has been identified. 11-Amino-4,5,9-trihydroxy-2-methyl-10H-benzo[*b*]fluoren-10-one was synthesized and shown to be present in extracts of *Streptomyces murayamaensis* fermentations. A deuterated sample was prepared and shown to be specifically incorporated into kinamycin D. This new intermediate, now named stealthin C, is also the probable hydroxylation substrate for the biosynthesis of stealthin A by *S. viridochromogenes*.

Since the first members were reported in 1970,[1] nearly 20 kinamycins have been identified from a variety of actinomycetes.[2−7] Their structures were recently corrected and shown to be 5-diazobenzo[*b*]fluorenes (e.g., kinamycin D, **1**, produced by *Streptomyces murayamaensis*),[8,9] thus greatly expanding the number of known, naturally occurring diazo compounds, as well as greatly expanding the number of benzo[*b*]fluorenes, a recently discovered class of metabolites.[6,10−13] The kinamycins were previously shown to be polyketide in origin,[14,15] with the benzo[*a*]anthraquinone dehydrorabelomycin, **2**, as an early intermediate (Scheme 1).[16] Oxidative cleavage of **2**—or its hydroquinone **3**—is apparently followed by a biological Friedel−Crafts closure and oxidation.[17] The benzo[*b*]fluorenone, kinobscurinone, **4**, was recently shown to be a more advanced intermediate in kinamycin biosynthesis.[7] We now report the synthesis of an aminobenzo[*b*]fluorene, which we have named stealthin C, **5**, its isolation from an *S. murayamaensis* culture, and its incorporation into kinamycin D.

Scheme 1



Results and Discussion

On the bais of the structures of kinobscurinone,[7] prekinamycin, **6**,[4,8,20,21] and the stealthins A and B reported by Seto,[10] a new stealthin, **5**, seemed a logical intermediate between **4** and **6**. None of the pathways to naturally occurring diazo compounds have as yet been elucidated, but the nitroso analog of **5** appeared to be a reasonable candidate for an intermediate closer to **6**.

To test these ideas, the nitrosobenzo[*b*]fluorene **7** and **5** were synthesized from 4,5,9,10-tetra-*O*-methylkinob-

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.

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(15) Sato, Y.; Gould, S. J. *J. Am. Chem. Soc.* **1986**, *108*, 4625−4631.
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(17) One reviewer has suggested closure may occur on a carboxylate equivalent. Since phenanthroviridin aglycon[18] and the jadomycins[19] are derived from an aldehyde, we prefer closure at this oxidation state. The correct sequence remains to be determined experimentally.
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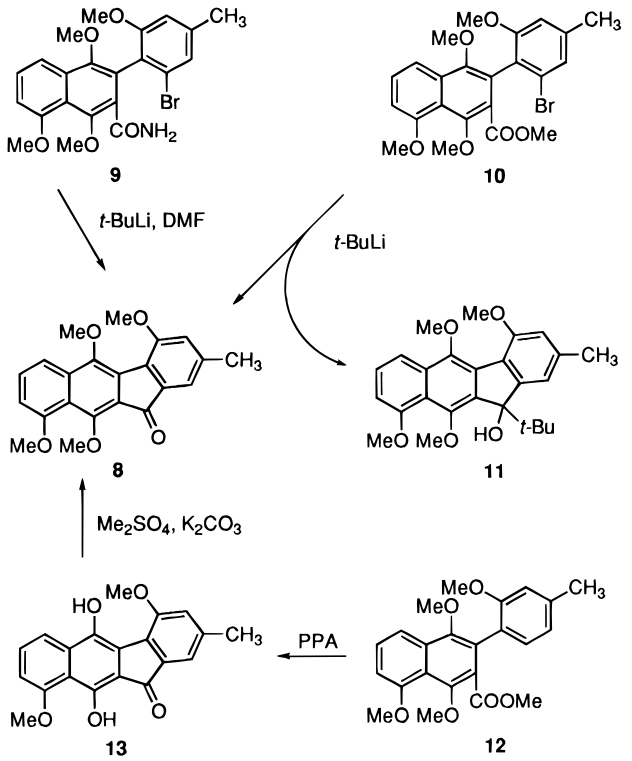
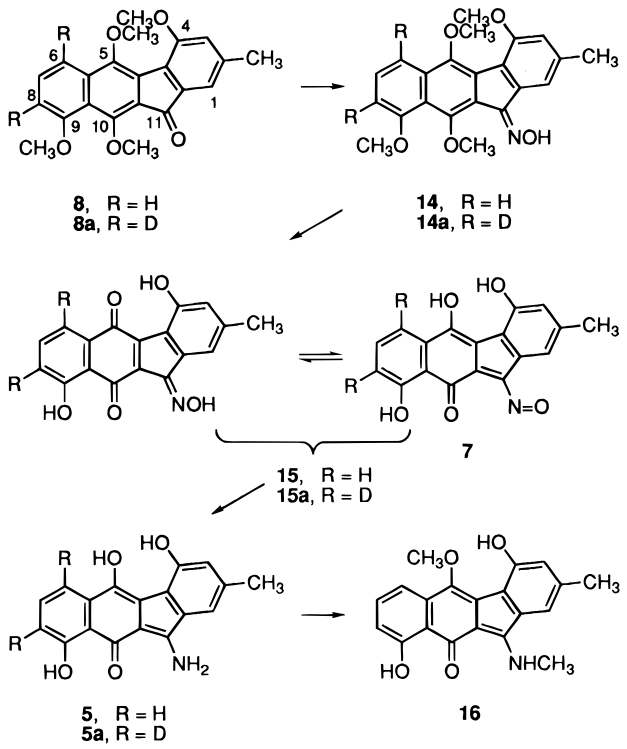
Scheme 2



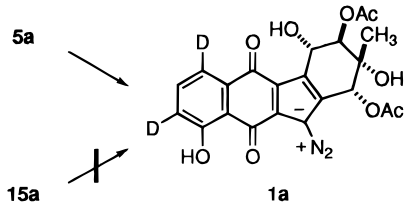
Scheme 3



scurinone, **8**.[22] This compound was originally obtained as a minor product (3% yield) from the base-promoted cyclization of bromoamide **9** (Scheme 2).[23] It was subsequently obtained in 40% yield from bromo ester **10**, but this also afforded 35% of the tertiary alcohol **11**. The best preparation (72% yield) has proven to be cyclization of ester **12**[24] with polyphosphoric acid to give **13**,[21] followed by remethylation of the hydroquinone. Treatment of **8** with hydroxylamine yielded the oxime **14**, which was demethylated[24] with BBr_3 in CH_2Cl_2 to give **15** (Scheme 3). As with **4** and the stealthins A and B, **15** was an "NMR silent" compound, so we could not determine whether the oxime or nitroso form predominates. Nonetheless, it gave a single peak by HPLC, a distinct UV−vis absorption spectrum (photodiode array detection), and HRMS consistent with the structure. The same compound was obtained by the reverse sequence: demethylation to **4** followed by hydroxylamine treatment. Compound **15** was then reduced with dithionite to afford **5**, also an "NMR silent" compound. We have recently determined that the NMR silence of **4**, **5**, and **15** is due to the presence of mixed oxidation states that include a free radical under ambient conditions.[25] The HPLC retention times and UV−vis spectra (photodiode array detection) of both **15** and **5** were entered into a database library.

Concurrent with the completion of the syntheses, a large-scale fermentation of *S. murayamaensis* mutant strain MC2 was worked up by Sephadex LH-20 chromatography to replenish our stock of kinafluorenone.[6] This approach, milder than used previously, now allowed the isolation of additional metabolites not previously recognized. Each was examined by photodiode array-detected HPLC. A number of these exhibited a stealthin-like chromophore.[26] One gave a perfect match of retention time and UV−vis spectrum with synthetic **5**. As expected, it was NMR silent, and its HREI mass spectrum matched the formula $C_{18}H_{13}NO_4$. Using the protocol previously used for stealthin A,[10] a portion of this material was treated with methyl iodide in acetone. This yielded four products that were separable by HPLC. One of these was characterized as the dimethyl derivative **16**. The ^1H and ^{13}C NMR values for **16** were consistent with the analogous derivative of stealthin A.

Compound **8** was deuterated to give **8a**,[7] and this was converted to **15a** and then to **5a**. Each was dissolved in DMSO and fed to cultures of *S. murayamaensis*. Workup afforded kinamycin D in each case. ^2H NMR analysis of the sample obtained from the culture to which **15a** had been added showed that the kinamycin D was completely devoid of deuterium enrichment. However, the sample (**1a**) obtained from the **5a** feeding showed a deuterium resonance at δ 7.5, clearly indicating enrichment at H-8 and H-10. Calculations referenced to the natural abundance deuterium in the NMR solvent (CH_2Cl_2) indicated a 0.44% enrichment per site (~20 × natural abundance) and a 0.67% incorporation of the material that had been fed.



These results demonstrate that stealthin C, **5**, is produced by *S. murayamaensis* and is an intermediate

(22) We are now using the numbering system for benzo[*b*]fluorenes, rather that what we had used for kinafluorenone (ref 6).

(23) Gore, M. P.; Gould, S. J.; Weller, D. D. *J. Org. Chem.* **1991**, *56*, 2289−2291.

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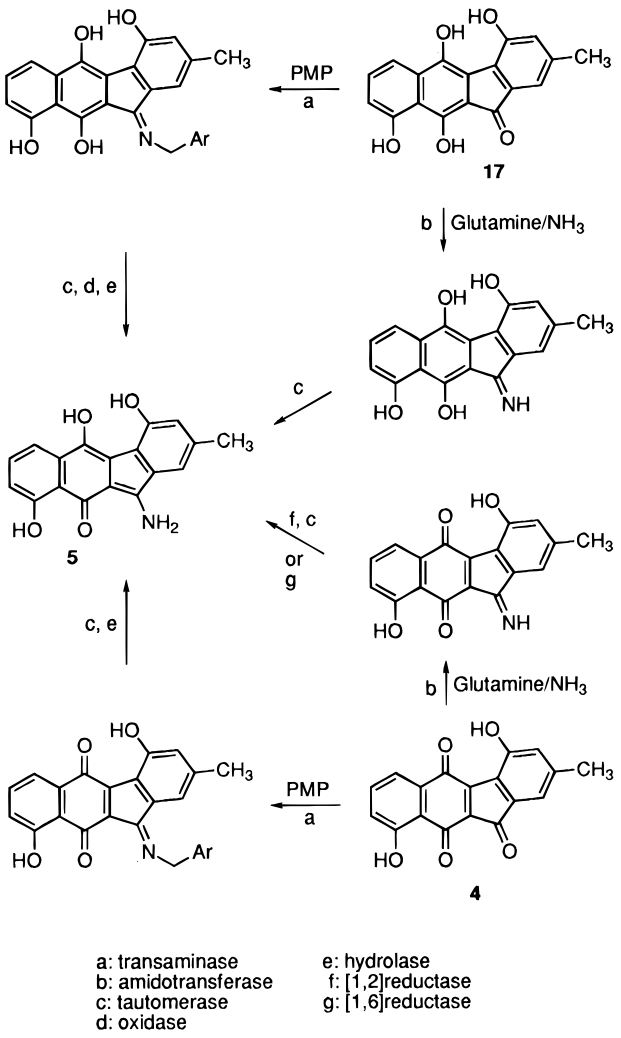
(25) Gould, S. J.; Melville, C. R. Unpublished results.

(26) UV spectra of stealthins A and B were kindly provided by Professor H. Seto.

Scheme 4



in kinamycin biosynthesis. It is very likely that this is the substrate in *S. viridochromogenes*[10] for hydroxylation leading to stealthin A.

Quinones, hydroquinones, and (presumably) semiquinones are found among the naturally occurring benzo[*b*]fluorenes, indicating that the redox potential between these forms is not large. It is, therefore, not self-evident whether the amination substrate to generate **5** is the quinone kinobscurinone or its hydroquinone **17**. As shown in Scheme 4, a glutamine- or ammonia-dependent amidotransferase would appear to be more efficient with the hydroquinone as substrate, whereas a pyridoxamine-dependent aminotransferase would appear to be more efficient with the quinone substrate. This intriguing issue will require cell-free studies for resolution.

The formation of N−N bonds (e.g., hydrazines, azoxys, diazos) in nature is not well-understood.[27−30] Prior to revising the structures of the kinamycins, only a few primary diazo and diazonium natural products were known, and none had been studied biosynthetically. Each of the two nitrogens of the azoxy compounds elaiomycin[28,29] and valanimycin[30,31] come from different amine sources, and it has been shown that valanimycin biosynthesis involves a hydoxylamine intermediate. The hydrazine derived from agaratine is derived from *p*-aminobenzoic acid and glutamine in an apparent two-step process.[32] The results reported here reveal that the diazo group of the kinamycins is also formed by stepwise addition of the two individual nitrogens and that this does not involve a nitroso benzo[*b*]fluorene intermediate.

Experimental Section

Growth of Bacterial Strains. *S. murayamaensis* strains MC1[6] and MC2[18] were cultured as described previously.[33]

Diode Array-Detected HPLC. During fermentation and extraction, the metabolites were analyzed by gradient reversed-phase HPLC with diode array detection, as described previously.[6]

Purification of Stealthin C, 5. An 80-h culture of *S. murayamaensis* strain MC2 was acidified to pH 3 and centrifuged to separate the cells from the broth. The cells were stirred overnight with acetone (800 mL), filtered with Celite, and extracted twice more with acetone (300 mL each time). The combined acetone extracts were concentrated, the residue partitioned between EtOAc and brine, and the EtOAc extract dried and concentrated to a purple oil (7 g). Much of the oil was removed by trituration with petroleum ether, affording a purple solid (452 mg). This material was fractionated in four portions on a Sephadex LH-20 column (3.0 × 31 cm), using acetone−MeOH (1:1) as eluant. Five fractions were collected in order: yellow-brown (203 mg), orange (189 mg), pink (27 mg), magenta (7 mg), and a mixed purple and green fraction (36 mg). The orange fraction contained kinafluorenone,[6] the pink fraction contained a single component (**5**), and the other fractions were mixtures. Stealthin C, **5**, gave no detectable NMR signals but was characterized as follows: IR (KBr) 3440, 3317 (sh), 3209 (sh), 1637, 1617, 1247 cm^{-1}; UV_{max} (HPLC conditions: 70% MeCN in H_2O, 0.1% HOAc) 214, 276, 340, 506, 540 nm; HREIMS *m/z* 307.0844 ($C_{18}H_{13}NO_4$, 0.1 Δmmu).

***O5,N*-Dimethylstealthin C, 16.** A portion of the pink fraction (23 mg) was suspended in acetone (5 mL) and sonicated to disperse it. Finely powdered K_2CO_3 (138 mg) was added, and the mixture was degassed with Ar. Methyl iodide (0.6 mL) was added dropwise and the mixture stirred for 2 d. After neutralization with 1 M HCl, EtOAc (10 mL) and MeOH (2 mL) were added. The solvents were removed by rotary evaporation, and the mixture was extracted with EtOAc until all pink color was removed from the aqueous layer. The EtOAc extract was dried and concentrated to dryness, yielding a purple solid (20.5 mg). Thin-layer chromatography on silica gel in CH_2Cl_2 revealed four products. Separation of the products was accomplished by HPLC (20 × 0.8 cm, 4 μm, C18 NovaPak, Waters Assoc., UV detection) with isocratic elution (1.2 mL/min, 85% MeOH, 15% sodium acetate buffer, 0.05 M, pH 6.5). Four fractions were collected from each injection (330 μg). Each of the four fractions possessed a similar chromophore to that of the starting material with slightly shifted UV_{max}. Only the latest eluting fraction, **16** (4.4 mg), was characterized: IR (KBr) 3440, 3317 (sh), 3209 (sh), 1637, 1617 cm^{-1}; ^1H NMR (DMSO-d_6) δ 14.50 (s, 1H, exch), 10.37 (br, 1H, exch), 9.68 (s, 1H, exch), 7.45 (t, 1H, *J* = 8 Hz), 7.43 (s, 1H), 7.16 (d, 1H, *J* = 8 Hz), 6.91 (s, 1H), 6.70 (d, 1H, *J* = 8 Hz), 3.91 (s, 3H), 3.58 (d, 3H, *J* = 6 Hz), 2.35 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 176.7, 163.4 , 162.7, 152.3, 152.0, 141.0, 138.9, 134.0, 133.8. 133.0, 123.9, 121.6, 120.6, 117.6, 113.5, 112.8, 105.2, 64.1, 32.9, 21.0; HREIMS *m/z* 335.1159 ($C_{20}H_{17}NO_4$, 0.2 Δmmu).

Synthesis of 8 from Base-Promoted Cyclization of Bromo Ester 10. A solution of bromo ester **10** (100 mg, 0.21

(27) LaRue, T. A. *Lloydia* **1977**, *40*, 307−321.
(28) Parry, R. J.; Mueller, J. V. *J. Am. Chem. Soc.* **1984**, *106*, 5764−5765.
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mmol) in dry THF (10 mL) was cooled to −80 °C. Under Ar protection, *t*-BuLi (0.14 mL, 1.7 M in pentane, 1.1 equiv) was added, and the resulting mixture was stirred for 1.5 h. The reaction was then quenched with dilute HCl and NH_4Cl and warmed to rt. EtOAc workup gave a brown residue that was then subjected to flash silica gel column chromatography (3% EtOAc in CH_2Cl_2). This first gave a mixture of the tertiary alcohol **11** and unreacted **10** as a yellow oil. This was followed by pure **8**[24] as a yellow solid (30 mg, 39%). The tertiary alcohol **11** was separated from the starting material by recrystallization from EtOAc and hexane as light brown crystals (30 mg, 34%). The recovered starting material was collected as a yellow solid (20 mg, 20%).

Tertiary alcohol **11**: mp 162−165 °C; IR 3555, 3072, 2935, 2837, 1611, 1577, 1465, 1372, 1288 cm^{-1}: ^1H NMR ($CDCl_3$) δ 7.89 (d, 1H, J = 8 Hz), 7.39 (t, 1H, J = 8 Hz), 7.03 (s, 1H), 6.90 (d, 1H, J = 8 Hz), 4.01 (s, 3H), 3.99 (s, 3H), 3.86 (s, 3H), 3.84 (s, 3H), 3.78 (s, 1H), 2.43 (s, 3H), 0.90 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 155.9, 154.5, 150.1, 148.9, 145.7, 138.9, 136.3, 133.6, 129.0, 126.0, 123.3, 119.4, 118.3, 116.1, 112.9, 106.9, 89.5, 63.7, 62.6, 56.3, 55.9, 41.0, 26.0, 21.8; EIMS m/z 421, 365; HREIMS m/z 421.2015 ($(M - H)^+$, $C_{26}H_{29}O_5$, 0.1 Δmmu); 365.1389 ($M^+ - C_4H_9$, $C_{22}H_{21}O_5$, 0.0 Δmmu).

Synthesis of 8 by Methylation of 13. A mixture of **13**[21] (76.4 mg, mmol), potassium carbonate (1.5 g), sodium dithionite (0.15 g), and dimethyl sulfate (1.0 mL) in acetone (50 mL) was heated at reflux under Ar for 15 h. The acetone was evaporated, and the mixture was stirred with 30 mL of 1 N NaOH for 2 h at room temperature. Methylene chloride workup gave a yellow residue that was subjected to column chromatography (silica gel, 3% EtOAc in CH_2Cl_2) to give **8** as a light yellow solid (80 mg, 96%).

[6,8-$^2H_2$]-4,5,9,10-Tetramethoxy-2-methyl-11*H*-benzo[*b*]-fluoren-11-one, 8a. **8** (0.20 g, 0.55 mmol) in 5 mL of deuterated trifluoroacetic acid (99% D) was stirred at 35−40 °C under Ar for 3 d. Solvent was evaporated in vacuo, and the residue was chromatographed on a short flash silica gel column (2.5 × 10 cm), eluting with 3% EtOAc in CH_2Cl_2. This gave **8a** as a light orange solid (0.185 g, 92%): mp 168−171 °C; ^1H NMR ($CDCl_3$) δ 7.47 (s, 1H), 7.23 (s, 1H), 6.92 (s, 1H), 4.03 (s, 3H), 4.02 (s, 3H), 3.98 (s, 3H), 3.89 (s, 3H) and 2.40 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 190.4, 159.3, 155.2, 155.0, 146.4, 141.3, 138.8, 137.2, 129.7, 127.7, 126.8, 122.8, 121.5, 118.9, 117.2, 116.1 (t), 108.2 (t), 63.1, 62.4, 56.3, 56.1 and 21.4; EIMS m/z 366 (M^+, 100), 365 (18.7), 364 (2.6); HREIMS 366.1437 ($C_{21}H_{18}D_2O_5$, −0.1 Δmmu).

4,5,9,10-Tetramethoxy-2-methyl-11*H*-benzo[*b*]fluoren-11-one Oxime, 14. To a solution of **8** (7.9 mg, 0.022 mmol) in EtOH (2.20 mL) and pyridine (2.20 mL) was added $NH_2OH·HCl$ (8.1 mg, 0.12 mmol). After the mixture was stirred at rt 3 d under Ar, evaporation gave an orange gum that was suspended in H_2O and extracted with EtOAc. This was dried, filtered, and concentrated to give 8.3 mg (99%) of a stereoisomeric mixture of oximes as a yellow solid. The major isomer was isolated by flash silica gel chromatography (40% EtOAc in hexanes): mp 231.1−233.6 °C; IR (KBr) 1602, 1578, 1369, 1340, 1270, 1059, 978 cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.40 (s, 1H), 8.14 (s, 1H), 7.72 (d, 1H, J = 8 Hz), 7.45 (dd, 1H, J = 8, 8 Hz), 7.05 (s, 1H), 7.01 (d, 1H, J = 8 Hz), 3.95 (s, 3H), 3.93 (s, 3H), 3.78 (s, 3H), 3.75 (s, 3H), 2.50 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 157.2, 154.7, 150.9, 149.3, 145.9, 140.5, 133.8, 133.1, 128.7, 127.5, 126.7, 124.2, 122.2, 120.5, 115.5, 115.3, 107.5, 62.5, 60.3, 56.3, 55.9, 21.5; EIMS m/z (rel intensity) 378 (100), 364 (52); HREIMS m/z 379.1419 ($C_{22}H_{21}NO_5$, −0.1 Δmmu). This material reverted to the mixture on standing in DMSO.

[6,8-$^2H_2$]-4,5,9,10-Tetramethoxy-2-methyl-11*H*-benzo[*b*]-fluoren-11-one Oxime, 14a. The deuterated tetra-*O*-kinobscurinone **8a** (180 mg, 0.49 mmol) was dissolved in EtOH (10 mL) and pyridine (10 mL). $NH_2OH·HCl$ (0.20 g, 2.9 mmol) was added, and the resulting solution was stirred at room temperature for 3 d under Ar. Removal of solvent gave a light brown residue that was partitioned between H_2O and CH_2Cl_2. The aqueous phase was extracted further (3 × 50 mL), and the organic phase was then washed with H_2O, dried, and concentrated to give a light brown residue that was purified by flash silica gel column chromatography (2.5 × 20 cm). Elution with 10% EtOAc in CH_2Cl_2 gave a light brown residue (6.5 mg) containing mainly the *Z*-isomer of the desired product. Further elution with 50% EtOAc in CH_2Cl_2 gave pure *E*-isomer (180 mg, 96%): mp 237−243 °C; IR (KBr) 2926, 1602, 1576, 1458, 1355, 1324, 1062, 980 cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.42 (s, 1H), 8.15 (s, 1H), 7.46 (s, 1H), 7.06 (s, 1H), 3.97 (s, 3H), 3.94 (s, 3H), 3.79 (s, 3H), 3.77 (s, 3H) and 2.41 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 157.1, 154.5, 150.9, 149.3, 145.8, 140.5, 133.8, 133.0, 127.2, 126.6, 124.2, 122.7, 122.2, 120.4, 115.5, 62.5, 60.3, 56.2, 55.9, 21.5; HRFABMS m/z 382.1621 ($(M + H)^+$, $C_{21}H_{19}D_2NO_5$, −0.3 Δmmu).

Kinobscurinone Oxime, 15. A THF solution of **14** (15.4 mg, 0.04 mmol) was evaporated *in vacuo* and the resulting solid suspended in CH_2Cl_2 (2.00 mL) and cooled to −78 °C. Boron tribromide (1 M in CH_2Cl_2, 0.82 mL) was added dropwise under Ar over 0.2 h and the resulting black solution allowed to slowly warm to rt. After 14 h, the flask was packed in ice, MeOH (200 mL) was added, and stirring was continued for 1 d. The solid precipitate was collected by filtration to give 9.1 mg (69%) of **15** as a purple solid: mp >300 °C; IR (KBr) 3233, 1628, 1586, 1457, 1379, 1270, 1041 cm^{-1}; UV_{max} 210, 254, 284, 380, 468, 555 nm; ^1H and ^{13}C NMR unobserved; EIMS m/z (rel intensity) 321 (M^+, 78), 306 (100), 278 (29); HREIMS m/z 321.0636 ($C_{18}H_{11}NO_5$, −0.1 Δmmu).

[6,8-$^2H_2$]Kinobscurinone Oxime, 15a. The deuterated oxime **14a** (180 mg, 0.47 mmol) was dissolved in dry CH_2Cl_2 (40 mL) and cooled to −78 °C. Boron tribromide (1.0 M in CH_2Cl_2, 7.5 mL, 7.5 mmol) was added dropwise under Ar over 5 min. The resulting deep red mixture was stirred for another 3 h at −78 °C and then allowed to warm to room temperature very slowly. After 15 h, MeOH (0.3 mL) and H_2O (0.1 mL) were added while the flask was cooled in an ice−water bath. The resulting mixture was then stirred at rt for another 6 h. H_2O was then added, and the mixture was extracted repeatedly with CH_2Cl_2 containing 10% methanol. The combined organic layer was then washed with H_2O. Evaporation of the solvent gave a purple residue (115 mg, 75%). HPLC (monitored at 254 nm) showed a major peak (t_R 19.3 min) with a UV/vis spectrum matching that of **15**. Because of its low stability, this compound was used directly in the next step.

[6,8-$^2H_2$]Stealthin C, 5a. Compound **15a** (60 mg, 0.19 mmol) was dissolved in MeOH (20 mL). An aqueous solution of sodium dithionite (0.16 g in 10 mL of H_2O) was added, and a deep red color developed immediately. After being stirred for 5 min, the mixture was extracted repeatedly with 5% MeOH in CH_2Cl_2. The combined organic phase was washed with H_2O and then concentrated. The residue was purified on a Sephadex LH-20 column (1.5 × 15 cm) eluting with 10% methanol in acetone. This gave **5a** as a red residue (46 mg, 80%). This compound slowly decomposes when it is dry but is more stable when in solution (MeOH or DMSO), especially when kept cold and protected from light. EIMS indicated 45% d_0, 38% d_1, and 17% d_2; HRFABMS m/z 309.0968 ($C_7H_{11}D_2NO_4$, −0.2 Δmmu).

Incorporation of Stealthin C into Kinamycin D. A glucose−soybean flour seed culture of *S. murayamaensis* was maintained at 26−7 °C and 280−290 rpm for 48 h. This was used to inoculate the glycerol−asparagine production medium (5% inoculum). Five production cultures (400 mL broth in 2 L flasks) were inoculated: three for feeding, one for a blank control, and one for a DMSO control. These were kept at 26−7 °C and 280−290 rpm. Synthetic **5a** (22.4 mg in 2.4 mL of DMSO) was added to three flasks (0.2 mL per addition per flask) at 12, 15, 18, and 21 h after inoculation. DMSO was also added in the same amount to one of the control flasks. After 48 h, the cultures were harvested. The combined broths to which **5a** had been fed were adjusted to pH 2.8 with 1 N HCl and filtered through Celite, and the mycelium was sequentially washed with EtOAc (200 mL), acetone (300 mL), and EtOAc (300 mL). The combined organic phase was concentrated to ~100 mL and extracted with EtOAc. This was then combined with an EtOAc extract of the original filtrate and then dried, filtered, and concentrated to give 1.2 g of crude material. Chromatography on flash silica gel (2.5 × 20 cm) and elution with 10% EtOAc in $CHCl_3$ gave 22 mg of crude kinamycin D, which was rechromatographed to give 18.5 mg

of pure kinamycin D as an orange solid: ^{2}H NMR (CH_2Cl_2) δ 7.5 (broad, H-8 and H-10), 5.3 (solvent).

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Supporting Information Available: Characterization data (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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