Kinafluorenone, a Benzo[*b*]fluorenone Isolated from the Kinamycin Producer *Streptomyces murayamaensis*

Martha C. Cone, Chris R. Melville, Makarand P. Gore, and Steven J. Gould*

Department of Chemistry, Oregon State University, Corvallis, Oregon 97331-4003

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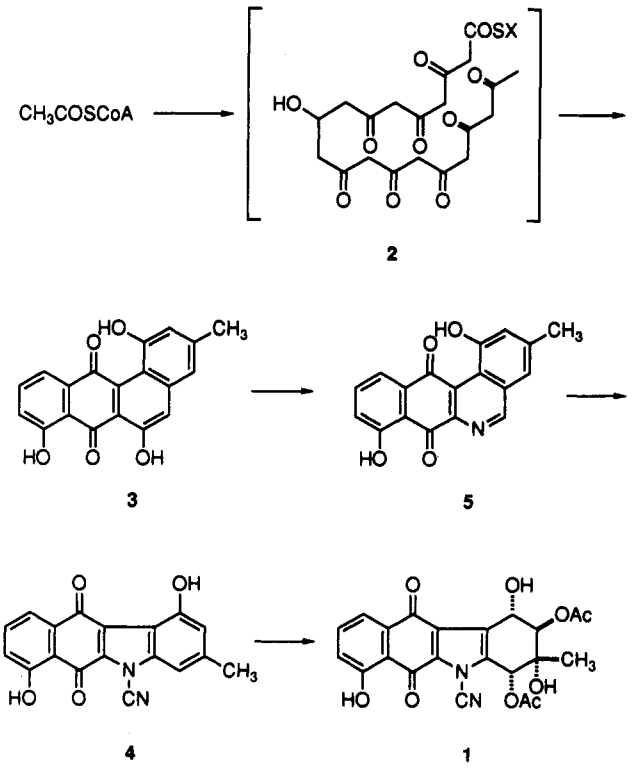
A strain of *Streptomyces murayamaensis* blocked in the biosynthesis of the kinamycin antibiotics was obtained by nitrosoguanidine mutagenesis. The major colored metabolite of the mutant strain was characterized by NMR spectroscopy and X-ray crystallography as a benzo[*b*]fluorenone, the first of this class obtained from a natural source. A rationale is presented for kinafluorenone biogenesis and for its relationship to kinamycin biosynthesis.

For nearly 20 years, *Streptomyces murayamaensis* was the only known source of the kinamycin antibiotics.[1-3] Recently, new kinamycins have been isolated from two other actinomycetes.[4,5] These antibiotics contain a novel benz[*b*]carbazole skeleton with an attached cyanamide moiety.

We have previously established that the kinamycins, as represented by kinamycin D, **1**, are derived from acetate, apparently through a decaketide **2**, which is transformed to the benzo[*b*]carbazole skeleton via the benz[*a*]-anthraquinone **3**, dehydrorabelomycin (Scheme I).[6] An unprecedented oxidation/nitrogen insertion/ring contraction process via a putative benzo[*b*]phenanthridine derivative was invoked to explain the formation of prekinamycin, **4**.[7] Although at the time the benzo[*b*]-phenanthridine ring system was unknown among natural products, three members of this class have subsequently been isolated from other *Streptomyces*.[8,9] We have recently identified one of these, phenanthroviridin aglycon, **5**, in extracts of mutant strain *S. murayamaensis* MC2.[10]

Except for **5**, all reported metabolites of *S. murayamaensis* have been obtained from the wild-type strain, often by maniuplation of the culture medium to increase production of minor components.[11] Chemical synthesis of putative intermediates, combined with biosynthetic feeding studies, have yielded useful information about the properties of the metabolites we are seeking to isolate. However, so far, only **3** has been shown to be incorporated into **1**.[6,12] The insolubility of the synthetic putative intermediates, impermeability of the cells, or both, presumably have prevented their incorporation into **1**.

Scheme I



The development of blocked mutant strains has been used to study the biosynthesis of many microbial products, among them polyketide antibiotics.[13] We report herein the isolation of a new metabolite, kinafluorenone, the first biosynthetically-derived benzo[*b*]fluorenone, from a mutant strain of *S. murayamaensis* blocked in the biosynthesis of the kinamycins. A possible mechanism for the biogenesis of this product and a rationale for its relationship to kinamycin biosynthesis are also presented.

Results and Discussion

Growth Characteristics and Metabolite Profile of the Blocked Mutant. Strain MC1, isolated from amongst the survivors of mutagenesis of the wild-type spore suspension with nitrosoguanidine, has no detectable antibiotic activity. Screening the strain in five different production media yielded no evidence of kinamycin production. Colonies of strain MC1 are bluish-purple in

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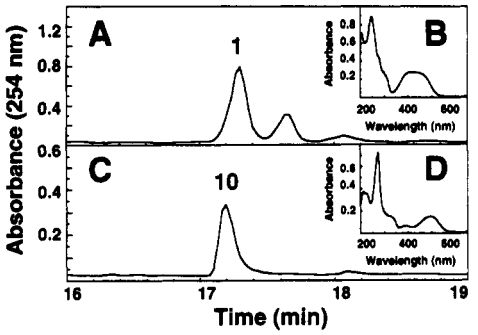


Figure 1. Portions of HPLC chromatogram of ethyl acetate extracts of oatmeal-broth cultures of *S. murayamaensis* wild-type strain (A) and blocked mutant strain MC1 (C). Spectrum of wild-type extract at 17.23 min (B) matches that of authentic **1**. Spectrum of Strain MC1 extract at 17.19 min (D), kinafluorenone, does not match spectra of any of the known kinamycins.

color on soybean/glucose agar and dark green in the soybean/glucose liquid seed medium, whereas the wild type is dark brown in both instances.

Survivors of the mutagenesis treatment were routinely screened by TLC of ethyl acetate extracts of 5-mL fermentations in glycerol/asparagine broth. Under these conditions, strain MC1 showed no detectable production of kinamycins or of murayaquinone, a polyketide-derived *o*-phenanthraquinone that is also normally produced by the wild strain of *S. murayamaensis*.[14]

Ethyl acetate extracts of strain MC1 grown in different production media were further examined by HPLC with photodiode array detection. The UV/vis spectra thus obtained provided a valuable fingerprint for identifying various metabolites. The production profile of strain MC1 was compared to that of the wild type strain in the soybean/glucose seed medium and in the baby oatmeal production medium (Figure 1). This approach has proven to be more sensitive than TLC, and one major colored metabolite, which had not been previously observed in the wild strain, was produced by MC1. Chromatography of an extract from a large-scale fermentation also gave a small amount of **3** which was identified by photodiode array HPLC. This indicated that the strain could still produce the early enzymes of the kinamycin pathway. In addition to the colored metabolites, a large quantity of aliphatic, oily material was produced but not investigated.

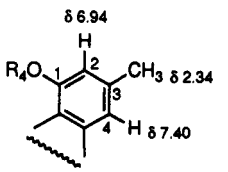
Isolation and Characterization of Kinafluorenone. A survey of five production media indicated that the best yield of the major product was obtained in shake flask fermentations in 2% baby oatmeal. The colored metabolites were obtained from the mycelium by sequential extraction with ethyl acetate and acetone. Initial attempts to purify the major metabolite by flash chromatography on Silicar CC-4 gave low yields due to the relative insolubility of the material. However, ^{1}H NMR analysis of the sample thus obtained revealed the presence of aromatic hydrogens, an *O*-methyl, and one apparent aryl methyl group.

In order to improve the solubility, a partially purified sample was acetylated. Reaction with acetic anhydride under acidic conditions was incomplete, whereas in pyridine the reaction proceeded rapidly to yield a single bright yellow, fluorescent product. Thereafter, crude organic extracts were routinely treated with acetic anhydride in pyridine prior to chromatography. Initial separation was carried out on a column of buffered flash-grade silica gel in ethyl acetate/hexane, and the fraction enriched in acetylated kinafluorenone was then chromatographed on the same support with dichloromethane as the eluent. Recrystallization from ether/chloroform yielded pure product as light orange needles.

The infrared spectrum of this material, with strong absorptions at 1770, 1716, and 1631 cm^{-1}, was somewhat misleading, since it was highly reminiscent of the naphthoquinone of the kinamycins plus the presence of phenolic acetates.[15] Indeed, the typical ^{1}H NMR pattern of the kinamycin A-ring was also discerned, although the resonance at 6.86 ppm for H-8 was significantly shifted upfield (H-7 of **1**, δ 7.22). This was clarified when the molecular formula of $C_{25}H_{20}O_8$ was obtained from the high-resolution FAB mass spectrum, which required a hydroquinone rather than a quinone. Thus, assignment of part structure **6** was complete.

The ^{1}H NMR spectrum of acetylated kinafluorenone also contained seven additional resonances. Five were methyl singlets: an aryl methyl, a methoxyl, and three acetates. In addition, there were two one-proton aromatic singlets. A ^{1}H COSY spectrum revealed the aryl methyl (2.34 ppm) and the two aromatic hydrogens (6.94 and 7.40 ppm) were coupled, and with higher digital resolution the ^{1}H NMR spectrum revealed these couplings, as well. Single-frequency decoupling provided confirmation of this analysis. Since the 6.94 ppm hydrogen was clearly ortho to a phenolic substituent, part structure **7** was defined.

6



7

These two part-structures and the *O*-methyl accounted for all the hydrogen-bearing carbons. The positions of attachment of one remaining carbonyl group, the methoxyl group, and the three acetates were still unclear. A long-range C–H correlation of H-8 to the methoxyl carbon in the LR HETCOSY[14] spectrum indicated the latter was located on C-7. Similarly, a long-range correlation from H-4 to the remaining carbonyl carbon allowed its assignment to C-5 (**8a**). Thus, kinafluorenone triacetate has structure **8**, rather than the isomeric structure **9**. As further confirmation, difference NOE experiments yielded the enhancements indicated in **8b**. Lastly, we were able to obtain a single-crystal X-ray diffraction analysis that provided the same structure. The ORTEP plot is shown in Figure 2. The natural product, **10**, has thus been named kinafluorenone and appears to be the first identified naturally occurring benzo[*b*]fluorenone.

The recent observation of **5** in an extract of *S. murayamaensis* mutant MC2[16] is consistent with our hypothesis[7] that a benzo[*b*]phenanthridine is an intermediate in kinamycin biosynthesis. This would suggest that strain MC1 is blocked between **3** and **5**. A rationale for the formation of kinafluorenone is provided in Scheme II. In the uninterrupted pathway, the acid–aldehyde **11** would undergo nitrogen insertion followed by decarboxylation

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(15) Seaton, P. J.; Gould, S. J. *J. Antibiot.* **1989**, *42*, 189.

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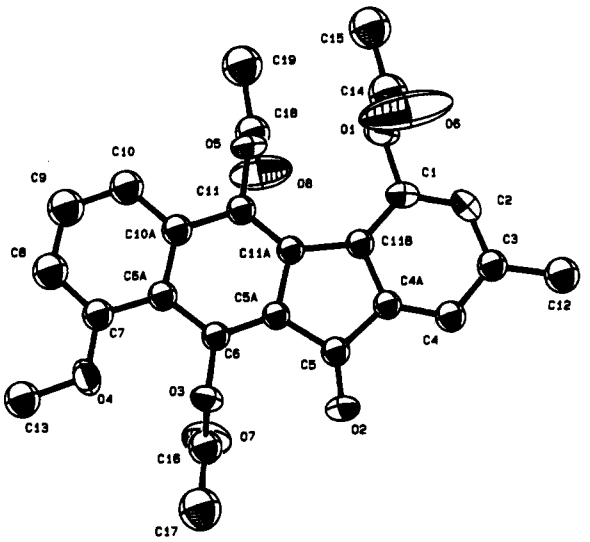
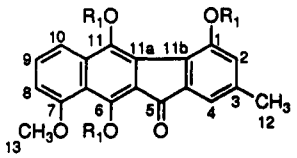
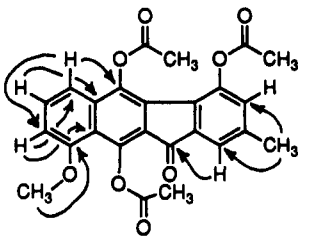


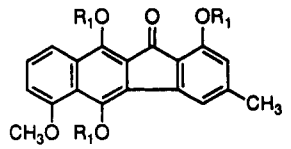
Figure 2. ORTEP drawing from the single-crystal X-ray structure determination of 8. Hydrogens have been omitted for clarity.



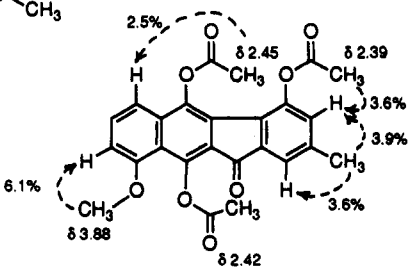
8, R_1 = Ac
10, R_1 = H



8a



9, R_1 = Ac



8b

of the nonenolizable β-keto acid thus generated and ring closure. In our synthetic studies,[17,18] we have observed these three processes. If strain MC1 is blocked at the nitrogen insertion step, the hydroquinone could attack the aldehyde, again yielding a nonenolizable β-keto acid. The remaining steps to **10** would then be unexceptional. The ease with which the naphthoquinone moiety seems able to accept electrons is fully consistent with the "electron-sink" mechanism we recently proposed for the required ring contraction in the kinamycin biosynthetic pathway.[17]

Bioactivity. Unlike the kinamycins to which it is related, kinafluorenone triacetate had no detectable antibiotic activity at a concentration of 64 μg/mL against

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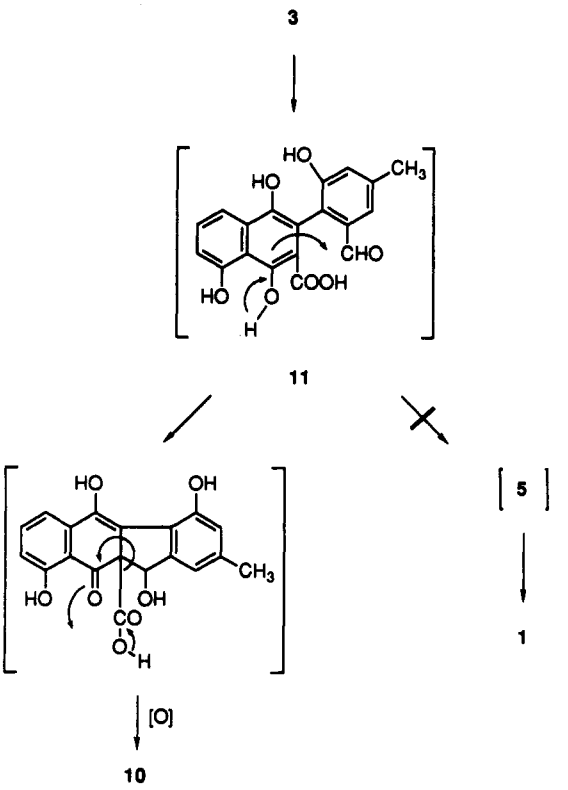
Scheme II



the following bacteria: *Escherichia coli* ATCC 10536, *Serratia marcescens* ATCC 13880, *Pseudomonas aeruginosa* ATCC 25619, *Klebsiellia pneumoniae* "A" AD (Lederle Laboratories), *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Streptococcus faecalis* ATCC 29212, and *Micrococcus luteus* ATCC 9341. The unacetylated material was not completely purified and, therefore, was not tested directly for antibiotic activity. However, it is probable that **10** is not very active, at best, because the fermentation broth in which it is a major component had no detectable antibiotic activity against *B. subtilis* ATCC 6633, which is very sensitive to the kinamycins.

Experimental Section

Bacterial Strains and Culture Media. Except as noted below, the bacterial strains and culture media are those specified in ref 11.

Mutagenesis and Screening of Cultures. A spore suspension of wild-type *S. murayamaensis* was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) in Trizma-maleate buffer (pH 8.7).[19] The survival rate of the spores was between 1% and 10% for the samples which were chosen for further screening. The surviving spores, washed free of the mutagen, were plated on WB agar (Difco yeast extract, 0.1%; BBL beef extract, 0.08%; ICN NZ Amine-A, 0.2%; glycerol, 1.0%; pH 7.3; 1.5% Difco Bacto agar; recipe modified from basal agar of ref 20). The isolated colonies were transferred with sterile wooden picks to 96-well plates (Corning) containing oatmeal agar (OM-TM).[11] A sample for bioassay was removed from each well with a sterile 1-in. section of a plastic milk straw and placed on a layer of *B. subtilis* ATCC 6633 in 0.5% peptone agar. The cultures

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were replicated to glycerol/asparagine agar in 96-well plates and tested again.

For metabolite screening, an agar plug of each strain was used to inoculate a 5-mL seed broth culture (Kinako soybean/glucose).[11] The seed cultures were incubated in test tube slant racks in a rotary shaker at 26–27 °C, 290 rpm, for 3 d and then used to inoculate oatmeal–trace metals (OM–TM) broth (5% inoculum). These cultures were incubated further for 3 d, acidified (pH 2.5–3.0), and extracted with EtOAc and the extracts dried using a Speedvac centrifuge (Savant). The dried extracts were taken up in 100 μL of 10% MeOH/CH_2Cl_2, and 10 μL of each were applied to two silica gel TLC plates. One plate was then developed in CH_2Cl_2, the other in 10% MeOH/CH_2Cl_2.

Diode-Array HPLC. Samples of further interest were analyzed by diode array HPLC, using a Waters 600E gradient HPLC equipped with Waters 990+ diode array detector. The metabolites were separated on a Waters NovaPak C_{18} radial compression column (0.8 × 10 cm) 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. A reference library of spectra of the kinamycins and all other characterized metabolites of *S. murayamaensis* was constructed under the same chromatographic conditions.

Growth of Cultures for Large-Scale Isolation of Kinafluorenone. Seed cultures in Kinako soybean/glucose broth were inoculated with agar plugs of strain MC1 preserved at −80 °C. The seed cultures were incubated on a rotary shaker under the standard conditions for 3–5 d, during which time the medium turned deep green in color. The seed cultures were used to inoculate 1-L flasks containing 200 mL of 2% baby oatmeal cereal (Gerber), pH 7.2 (5% v/v inoculum). The production of the major metabolite was monitored over 72–96 h by HPLC of EtOAc extracts of 5-mL samples. At the end of the fermentation, the dark purple mycelium was separated from the light orange broth using a Sharples Super centrifuge.

Kinafluorenone Triacetate, 8. The mycelium from 5 L of culture was suspended in 600 mL of deionized water and the thick slurry adjusted to pH 2.5. The slurry was filtered through Celite and the 800-mL total residue sequentially extracted three times with acetone and once with acetone/EtOAc (1:1, 500 mL). After the combined filtrates were concentrated in vacuo, this residue was partitioned between EtOAc (500 mL) and H_2O (500 mL). The organic layer was then dried and concentrated under high vacuum to yield a purple oil (3.68 g). Extraction of the original culture broth with EtOAc yielded an additional 267 mg.

Freshly distilled Ac_2O (11 mL) was added dropwise to a vigorously stirred solution of the crude extract (2.68 g) and DMAP (5 mg) in dry pyridine (27 mL) under Ar. The color changed from purple to yellow and the reaction was monitored by HPLC. Upon completion of the reaction, the mixture was poured onto a mixture of ice and EtOAc. The organic layer was dried and concentrated in vacuo to yield a brownish-yellow oil (2.64 g).

Buffered silica gel was prepared by adding potassium phosphate buffer (200 mL, 0.1 M, pH 7.0) to a slurry of flash grade silica gel (200 g in 100 mL deionized water). Most of the water was removed by rotary evaporation, followed by drying in a 100 °C oven for 24 h and sieving (100 mesh). The crude acetylated mixture was dissolved in CH_2Cl_2 and adsorbed onto a small quantity of buffered silica. This was added to the top of a column of buffered silica (21 × 5 cm) equilibrated with 20% EtOAc/hexane. The column was then eluted with 30% EtOAc/hexane to yield the acetate of the major product (75.7 mg). This was further purified by flash chromatography on a buffered silica gel column (1 × 21 cm) in CH_2Cl_2 to yield 27 mg of material, which was recrystallized from ether/$CHCl_3$: mp 273–275 °C; IR (KBr) 1770, 1616, 1631 cm^{-1}; UV_{max} ($CHCl_3$) 436 (5600), 419 (5650), 364 (4250), 329 (sh), 292 (57 700), 281 (39 900), 270 (48 400), 236 nm (sh 20 400); ^1H NMR ($CDCl_3$/MeOH-d_4 = 2/1) δ 2.34 (3 H, s), 2.39 (3 H, s), 2.42 (3 H, s), 2.45 (3 H, s), 3.88 (3 H, s), 6.86 (1 H, d, J = 8.6 Hz), 6.94 (1 H, s), 7.39 (1 H, d, J = 8.6 Hz), 7.40 (1 H, s), 7.47 (1 H, t, J = 8.6 Hz); ^{13}C NMR ($CDCl_3$/MeOH-d_4 = 2/1) δ 20.50 (q), 20.71 (q), 20.85 (q), 20.83 (q), 56.21 (q), 108.48 (d), 114.76 (d), 120.23 (s), 122.22 (s), 123.01 (d), 128.33 (s), 130.28 (d), 130.89 (d), 131.37 (s), 134.84 (s), 137.76 (s), 138.68 (s), 142.18 (s), 144.64 (s), 145.54 (s), 158.60 (s), 168.38 (s), 168.91 (s), 169.27 (s), 188.77 (s); mass spectrum (positive FAB) m/z 449 $[M + H]^+$, 407, 364, 322; HRMS (positive FAB) calcd for $C_{25}H_{21}O_8$ 449.1236; found 449.1240.

Bioassay for MIC Determination. MIC values were determined in 96 well plates in Mueller–Hinton broth (Difco) according to the procedure given in ref 21.

X-ray Crystallography of $C_{25}H_{20}O_8$ (8).[22] A crystal of 8 of dimensions 0.2 × 0.2 × 0.4 mm was secured on a glass fiber mount, the data were collected at 23 °C on a Rigaku AFC6R single-crystal diffractometer with graphite-monochromated Mo Kα radiation from a 12-kW rotating anode generator. The P-1 space group and unit cell parameters (triclinic, Z = 2) were determined from 19 reflections in the range $26.13 < 2q < 30.27°$. Three reflections measured every 300 reflections throughout data collection demonstrated crystal stability.

The structure was solved and refined with the use of the TEXSAN[23] crystallographic software package, and the positions of all non-hydrogen atoms were determined with the direct methods program SHELXS based on 2239 observed reflections ($2q_{max}$ = 55.0°). No absorption correction was applied, and all hydrogens were placed in calculated positions. Final cycle anisotropic refinement of C and O atoms gave an R value of 0.055 with a p-factor of 0.03.

Acknowledgment. This research was supported by a U.S. Public Health Service Grant GM 31715 to S.J.G. Professor S. Ōmura of Kitasato University and Professor U. Hornemann of the University of Wisconsin are thanked for cultures of *S. murayamaensis* and samples of kinamycins. D. Borders, Lederle Laboratories (Pearl River, NY), is thanked for a culture of *K. pneumoniae* "A" AD. We are grateful to J. Rain, C. Loch, and T. Vu for excellent technical assistance. P. Chapman of the EPA (Guld Breeze, FL) is also thanked for helpful discussions. The Bruker AM 400 NMR spectrometer was purchased in part through grants from the National Science Foundation (CHE-8216190) and from the M. J. Murdock Charitable Trust to Oregon State University, and the Bruker AC 300 spectrometer was purchased in part though grants from the Public Health Service Division of Research Resources (RR04039-01) and the National Science Foundation (CHE-8712343) to Oregon State University.

Supplementary Material Available: ^1H NMR spectrum of compound 8 (1 page). 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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(22) The authors have deposited atomic coordinates for this structure with the Cambridge Crystallographic Data Centre. The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK.

(23) Molecular Structure Corporation. TEXSAN, 1988; MSC, 3200A Research Forest Drive, The Woodlands, TX, 77381.