bis-cis-Dihydrodiols: A New Class of Metabolites Resulting from Biphenyl Dioxygenase-Catalyzed Sequential Asymmetric *cis*-Dihydroxylation of Polycyclic Arenes and Heteroarenes

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The biphenyl dioxygenase-catalyzed asymmetric *mono-cis*-dihydroxylation of the tetracyclic arenes chrysene **1A**, benzo[*c*]phenanthridine **1B**, and benzo[*b*]naphtho[2,1-*d*]thiophene **1C**, has been observed to occur exclusively at the bay or pseudo-bay region using the bacterium *Sphingomonas yanoikuyae* B8/36. The *mono-cis*-dihydrodiol derivatives **2A** and **2C**, obtained from chrysene **1A** by oxidation at the 3,4-bond **(2A)** and benzo[*b*]naphtho[2,1-d]thiophene **1C** by oxidation at the 1,2-bond **(2C),** respectively, have been observed to undergo a further dioxygenase-catalyzed asymmetric *cis*-dihydroxylation at a second bay or pseudo-bay region bond to yield the corresponding *bis-cis*-dihydrodiols (*cis*-tetraols) **4A** and **4C**, the first members of a new class of microbial metabolites in the polycyclic arene series. The enantiopurities and absolute configurations of the new *mono-cis*-dihydrodiols **2B**, **2C**, and **3B** were determined by ^{1}H NMR analyses of the corresponding (*R*)- and (*S*)-2-(1-methoxyethyl)benzeneboronate (MPBA) ester derivatives. The structure and absolute configurations of the *bis-cis*-dihydrodiols **4A** and **4C** were unambiguously determined by spectral analyses, stereochemical correlations, and, for the metabolite **4C**, X-ray crystallographic analysis of the *bis*-acetonide derivative **7C**. These results illustrate the marked preference of biphenyl dioxygenase for the *cis*-di- and tetra-hydroxylations of polycyclic arenes, at the more hindered bay or pseudo-bay regions, by exclusive addition from the same (si:si) face, to yield single enantiomers containing two and four chiral centers.

Introduction

Identification of new metabolites and biodegradation pathways is of particular importance in the context of carcinogenic activity of polycyclic aromatic hydrocarbons (PAHs) and their aza-, thia-, and oxa-analogues, all of which are found to be prevalent in the environment. The initial metabolic step for PAHs in eucaryotic systems (animals, plants, fungi) frequently involves a monooxygenase-catalyzed epoxidation followed by isomerization to yield phenols or by epoxide hydrolase-catalyzed hydrolysis to yield *trans*-dihydrodiols; it is exemplified by the mammalian metabolism of the PAH chrysene **1A**[1] and the thiaarene benzo[*b*]naphtho[2,1-*d*]thiophene **1C**[2] having a similar carcinogenic potency.[3] The enzyme-catalyzed epoxidation steps in arene **1A** and thiaarene **1C** were found to occur at both non-K (1,2-/7,8- in **1A**, 3,4- and 9,10- in **1C**), bay (3,4- and 9,10- in **1A**), and pseudo-bay (1,2- and 7,8- in **1C**) region bonds. The processes of epoxidation/aromatization (phenol formation) or epoxidation/hydrolysis (*trans*-dihydrodiol formation) occurring within one arene ring followed by a second epoxidation of a different ring or sequential *bis*-epoxidation of two different rings[4] has been reported as an example of multiple site oxidation of PAHs during mammalian metabolism. The monooxygenase-catalyzed epoxidation in two different arene rings may thus account for the formation of *bis-trans*-dihydrodiol,[5] arene oxide *trans*-dihydrodiol,[6] arene oxide phenol,[7] and phenol *trans*-dihydrodiol metabolites.[8] Metabolite instability and/or insufficient quantities of bioproducts have generally precluded an unequivocal determination of relative and absolute stereochemistry of the products formed by multiple site oxidation of PAHs in mammalian systems.

In procaryotic systems (bacteria), the dioxygenase-catalyzed dihydroxylation of a PAH to yield a *mono-cis*-dihydrodiol is generally the first step in a metabolic pathway leading to mineralization. Earlier research from these laboratories, on the dioxygenase-catalyzed *cis*-dihydroxylation of PAHs and heterocyclic analogues of

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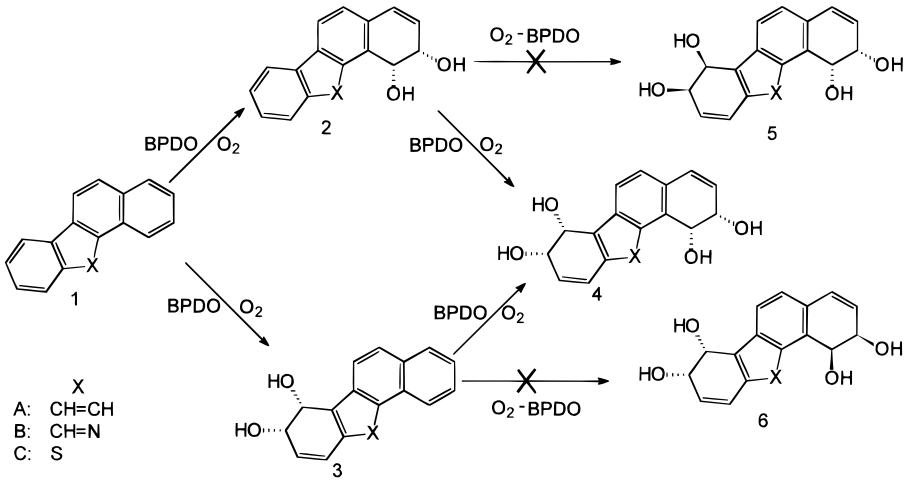
Scheme 1



PAHs,[9–19] showed a strong preference for oxidation at the bay or pseudo-bay region.

In a recent bacterial study using a mutant strain of *Sphingomonas yanoikuyae* (B8/36, deficient in *cis*-dihydrodiol dehydrogenase activity), we reported the metabolism of chrysene **1A**,[9] a tetracyclic PAH. The study has been extended to include a tetracyclic azaarene, benzo[*c*]phenanthridine **1B**, and a thiaarene, benzo[*b*]naphtho[2,1-*d*]thiophene **1C**, both of which also contain two bay regions or pseudo-bay regions. Since the monooxygenase-catalyzed multiple site oxidation of larger PAHs in mammals has been reported, [5–8] the possibility of similar dioxygenase-catalyzed multiple site oxidation of PAHs also occurring in bacterial biodegradation is of particular interest. The results contained herein indicate that a previously unobserved reaction sequence i.e., *bis-cis*-dihydrodiol or *cis*-tetraol formation, is available to the *cis*-dihydrodiols of PAHs and thia-PAHs which contain bay regions or pseudo-bay regions.

Results

Biotransformation of Chrysene 1A To Yield (+)-(3*S*,4*R*)-3,4-Dihydroxy-3,4-dihydrochrysene, 2A, and (+)-(3*S*,4*R*,9*S*,10*R*)-3,4,9,10-Tetrahydroxy-3,4,9,10-tetrahydrochrysene, 4A. Biotransformation of the weakly carcinogenic PAH chrysene **1A**, in the presence of growing cultures of *S. yanoikuyae* B8/36 under the conditions reported,[9] again gave the *cis*-dihydrodiol **2A** (ca. 1% isolated yield, $[\alpha]_D$ +112, THF) as the major metabolite whose structure, enantiopurity (>98%), and (3*S*,4*R*) absolute configuration had earlier been confirmed by synthesis, stereochemical correlation, and reaction with (−)-(*S*)- and (+)-(*R*)-2-(1-methoxyethyl)phenylboronic acid (MPBA)[20] to yield the corresponding MPBA esters ($2A_S$ and $2A_R$).[9] In the earlier study [9] a more polar minor metabolite was isolated by PLC but could not be identified due to the lack of sufficient material. Repeated biotransfomations of chrysene **1A** provided a larger pooled sample from which the more polar metabolite was isolated by PLC (silica gel, MeOH:$CHCl_3$ 1:9), (R_f 0.15; 0.002 g, ca. 0.3% isolated yield). The new metabolite could be assigned structure **4A**, **5A**, or **6A** (Scheme 1**)** on the basis of chromatographic behavior, NMR spectral characteristics (^{1}H and ^{13}C NMR spectroscopy including δ values, coupling constants, COSY and NOE correlations), and MS analysis. Measurement of the optical rotation, $[\alpha]_D$ +135, MeOH, and CD spectrum of the minor metabolite allowed the *cis-anti-cis* structures **5A** and **6A** to be eliminated since both contain a center of symmetry. The relative (*cis-syn-cis*) and absolute (3*S*,4*R*,9*S*,10*R*) stereochemistry of the *bis-cis*-diol **4A** was confirmed by addition of the major (+)-(3*S*,4*R*)-*cis*-dihydrodiol metabolite **2A** ($[\alpha]_D$ +112, THF), as a substrate (0.003 g) to *S. yanoikuyae* B8/36, and isolation of the (+)-(3*S*,4*R*,9*S*,-10*R*)-*bis-cis*-dihydrodiol **4A** (0.0015 g**,** $[\alpha]_D$ +135, (MeOH). The *bis-cis*-dihydrodiol **4A** proved to be less stable than the corresponding *mono-cis*-dihydrodiol precursor **2A** and readily aromatized during isolation and workup, yielding a mixture of *bis*-phenols (by NMR and GC-MS analysis).

Biotransformation of Benzo[*c*]phenanthridine 1B To Yield (+)-(3*S*,4*R*)-3,4-Dihydroxy-3,4-dihydrobenzo[*c*]phenanthridine, 2B, and (+)-(9*S*, 10*R*)-9,10-Dihydroxy-9,10-dihydrobenzo[*c*]phenanthrene, 3B. Addition of benzo[*c*]phenanthridine **1B** (0.4 g) to cultures of *S. yanoikuyae* B8/36 and biotransformation over an 18 h period using conditions similar to those employed for chrysene **1A**,[9] followed by ethyl acetate extraction of the aqueous bioextracts, gave a mixture of two isomeric *cis*-dihydrodiols, **2B** and **3B**. The two diols were separated by PLC on silica gel ($CHCl_3$:MeOH 1:9) and

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Table 1. Application of ^{1}H NMR Chemical Shift Values for the MeO Signals of the Boronate Derivatives $2A_R$, $2A_S$, $2B_R$, $2B_S$, $3B_R$, $3B_S$, $2C_R$, and $2C_S$ in the Determination of Enantiomeric Excess and Absolute Configuration

compd	δ_{MeO}(RMPBA)	δ_{MeO} (S-MPBA)	$\Delta\delta_{MeO}$	% ee	config
(+)-2A	2.97 (**$2A_R$**)	3.11 (**$2A_S$**)	0.14	>98%	3*S*,4*R*
(+)-2B	3.15 (**$2B_R$**)	3.26 (**$2B_S$**)	0.11	>98%	3*S*,4*R*
(+)-3B	3.08 (**$3B_R$**)	3.23 (**$3B_S$**)	0.15	>98%	9*S*,10*R*
(+)-2C	3.06 (**$2C_R$**)	3.109 (**$2C_S$**)	0.49	>98%	1*R*,2*S*

identified from spectral (IR, NMR, MS) data as *cis*-dihydrodiol **2B** (0.036 g, R_f 0.23, $[\alpha]_D$ +114, $CHCl_3$; 8% isolated yield) and the less polar *cis*-dihydrodiol **3B** (0.030 g, R_f 0.36, $[\alpha]_D$ +94, $CHCl_3$; 7% isolated yield). Both *cis*-dihydrodiols **2B** and **3B** were found to be enantiopure by ^{1}H NMR analyses of the corresponding MPBA derivatives (**$2B_R$**, **$2B_S$**, **$3B_R$**, and **$3B_S$**) using (+)-(*R*)- and (−)-(*S*)-MPBA. Furthermore, on the basis of the general trend,[20] shown to be applicable in the bay region *cis*-dihydrodiols of the phenanthrene[18] and chrysene[9] series, that MPBA derivatives formed from (+)-(*R*)-MPBA (**$2B_R$** and **$3B_R$**) and a bay region *cis*-dihydrodiol having a benzylic (*R*) and an allylic (*S*) configuration will have a smaller δ value for the methoxyl signal in the ^{1}H NMR spectrum (Table 1), the bioproducts were identified as (+)-(3*S*,4*R*)-3,4-dihydroxy-3,4-dihydrobenzo[*c*]phenanthridine **2B** and (+)-(9*S*,10*R*)-9,10-dihydroxy-9,10-dihydrobenzo[*c*]phenanthridine **3B**. It is noteworthy that both *cis*-dihydrodiols were formed in similar proportions due to *cis*-dihydroxylation in a bay region. When individual *cis*-dihydrodiols **2B** or **3B** were added as substrates to *S. yanoikuyae* B8/36, several minor unidentified peaks were observed by HPLC analysis but no unequivocal evidence of the *bis-cis*-dihydrodiol structure **4B** could be obtained.

Biotransformation of Benzo[*b*]naphtho[2,1-d]-thiophene, 1C, To Yield (+)-(1*R*,2*S*)-1,2-Dihydroxy-1,2-dihydrobenzo[*b*]naphtho[2,1-*d*]thiophene, 2C, and (+)-(1*R*,2*S*,7*R*,8*S*)-1,2,7,8-Tetrahydroxy-1,2,7,8-tetrahydrobenzo[*b*]naphtho [2,1-d]thiophene, 4C. Metabolism of benzo[*b*]naphtho[2,1-*d*]thiophene **1C**, using *S. yaniokuyae* B8/36 under conditions similar to those used for substrates **1A** and **1B**, followed by ethyl acetate extraction and PLC separation ($CHCl_3$:MeOH 9:1) of the aqueous bioextracts, yielded a major, less polar (R_f 0.33) *mono-cis*-dihydrodiol ($[\alpha]_D$ +146, MeOH; 7% isolated yield) and a minor, more polar (R_f 0.18) metabolite ($[\alpha]_D$ +270, MeOH; 3% isolated yield). On the basis of NMR and MS data, and comparison with the spectral data reported for the mammalian metabolite, *trans*-1,2-dihydroxy-1,2-dihydrobenzo[*b*]naphtho[2,1-*d*]thiophene,[2] the *mono-cis*-dihydrodiol metabolite was identified as (+)-(1*R*,2*S*)-1,2-dihydroxy-1,2-dihydrobenzo[*b*]naphtho[2,1-*d*]-thiophene **2C**. The enantiopurity of compound **2C** (>98%) and the absolute configuration of (+)-(1*R*,2*S*) were determined by formation and ^{1}H NMR analyses of the corresponding MPBA esters, **$2C_R$** and **$2C_S$**.

The second more polar metabolite was identified as a *bis-cis*-dihydrodiol having structure **4C**, **5C,** or **6C (**Scheme 2**)**. Time course studies of the biotransformation of thiaarene **1C** using *S. yanoikuyae* showed that *cis*-dihydrodiol **2C** was the initial product and that the proportion of this metabolite decreased in concert with an increasing proportion of the *bis-cis*-dihydrodiol metabolite. The latter bioproduct, formed from either the thiaarene **1C** or the *cis*-dihydrodiol **2C**, was indistinguishable. To determine the relative and absolute stereochemistry of the *bis-cis*-dihydrodiol metabolite, it was converted to the crystalline *bis*-acetonide **7C** ($[\alpha]_D$ +466, $CHCl_3$). An X-ray crystal structure analysis of compound **7C** established the (1*R*,2*S*,7*R*,8*S*) configuration for the acetonide **7C** and hence for the parent *bis-cis*-diol **4C** (Figure 1). A minor byproduct, from the synthesis of the *bis*-acetonide **7C**, was found to be the phenolic acetonide **8C**, presumably derived from the corresponding *cis*-diol **9C** formed by selective acid-catalyzed dehydration of the *bis-cis*-dihydrodiol **4C**.

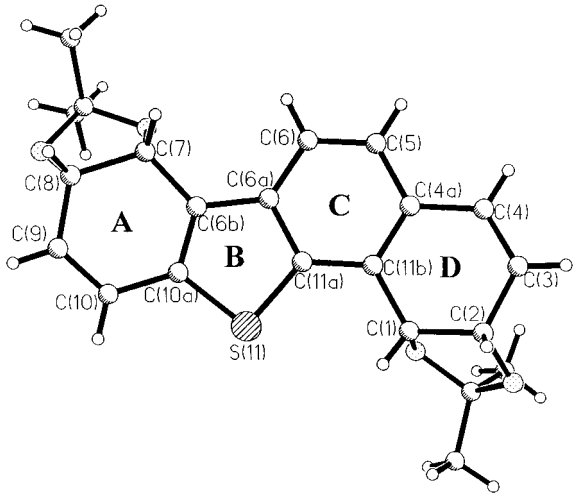


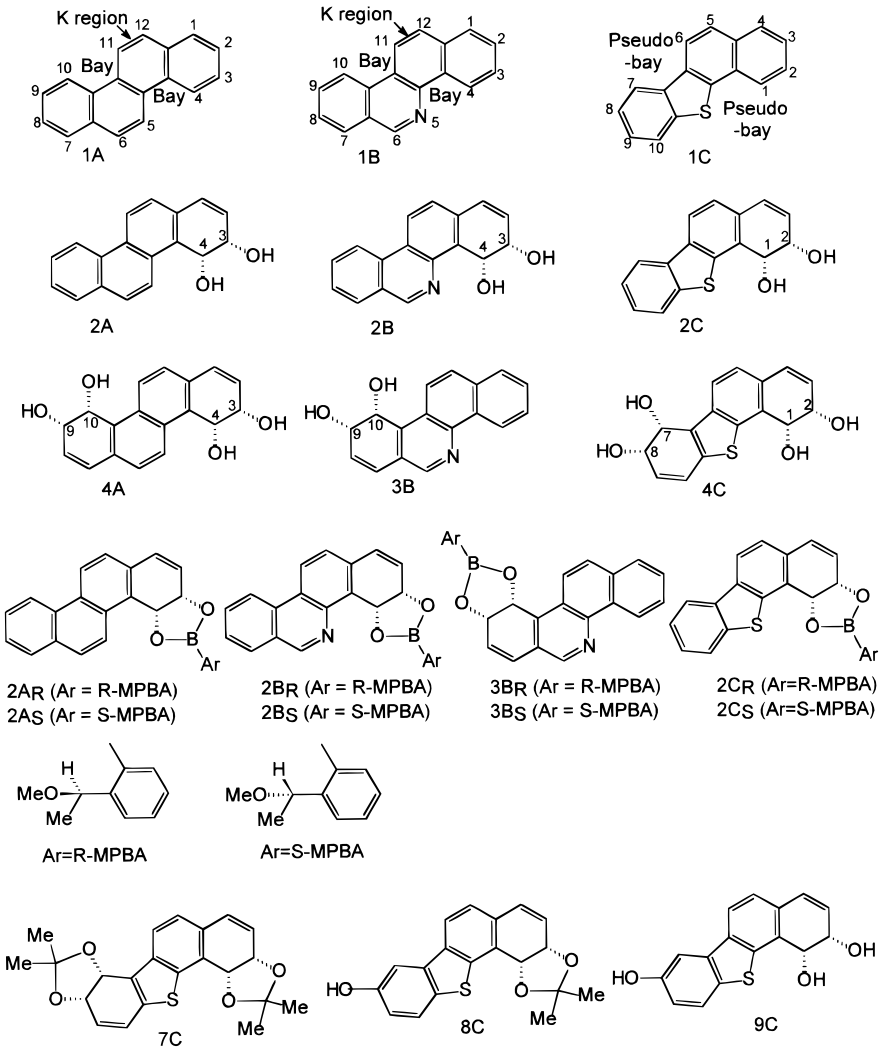
Figure 1. X-ray crystal structure analysis of compound **7C** indicating the (1*R*,2*S*,7*R*,8*S*) absolute configuration.

Discussion

Studies of the biodegradation of larger members of the PAH series by bacterial systems, and identification of metabolites, have been restricted by their low aqueous solubility resulting in the low bioavailability of such substrates and thus the isolation of metabolites in very low yields. A further limitation has been the inability of bacterial systems willing to accept larger PAHs as substrates. The biphenyl dioxygenase (BPDO) present in *S. yanoikuyae* B8/36 (previously described as *Beijerinckia* strain B8/36) has however proved to be exceptional; it has the ability to accept tetracyclic PAHs, benz[*a*]-anthracene[16] and chrysene,[9] and pentacyclic PAHs, benzo[*a*]pyrene,[15] as substrates for *cis*-dihydroxylation. Use of strain B8/36, a mutant deficient in *cis*-dihydrodiol dehydrogenase activity, has allowed the initial *cis*-dihydrodiol metabolites to be isolated, albeit in low yield. Regioselectivity for a bay region bond has emerged as a common feature of BPDO-catalyzed *cis*-dihydroxylation of these larger PAHs. The increased water solubility of tetracyclic arenes, containing a heteroatom, allied to the presence of two bay or pseudo-bay regions, prompted the investigation of the azaarene, benzo[*c*]phenanthridine **1B**, and thiaarene, benzo[*b*]naphtho[2,1-*d*]thiophene **1C**, as substrates for BPDO.

Chrysene **1A** is the smallest member of the PAH series to contain two bay regions. When used as a substrate for the bacterium *S. yanoikuyae* (B8/36), enzyme-catalyzed *cis*-dihydroxylation occurs exclusively at a bond proximate to a bay region.[9] In principle, the 3,4-, 5,6-, 9,10-, and 11,12-bonds are all adjacent to a bay region and might be expected to form *cis*-dihydrodiol metabolites

Scheme 2



preferentially. However, in practice, dioxygenase-catalyzed *cis*-dihydroxylation of chrysene (and other PAHs containing a bay region, including phenanthrene and benz[*a*]anthracene) occurs almost exclusively at the positions of lower electron density (3,4- or 9,10-bonds in **1A**) rather than the K region bonds (5,6- or 11,12-bonds in **1A**). During the course of the earlier study,[9] the *cis*-dihydrodiol **2A** was found to be the major metabolite, accompanied by a very minor unidentified metabolite isolated by PLC in quantities insufficient for characterization. In continuation of this investigation, the extracts, obtained from several identical biotransformation runs, were combined and the minor bioproduct isolated by PLC was spectrally identified as 3,4,9,10-tetrahydroxy-3,4,9,-10-tetrahydrochrysene, **4A**. This is the first example of a novel type of dioxygenase metabolite formed by sequential bay region *cis*-dihydroxylation of PAHs, i.e., the *bis-cis*-dihydrodiol series (*cis*-tetrahydrotetraols). The symmetrical nature of compound **4A** resulted in a relatively simple NMR spectrum in which the number of signals was halved. The proximity of protons H-6 and H-7 (H-12 and H-1) in structure **4A** was evident from the NOE signal enhancement. While the NMR data did not allow a distinction to be made between the *cis-syn-cis* (**4A**) and *cis-anti-cis* (**5A**/**6A**) tetraol isomers (Scheme 1), the presence of optical activity ($[\alpha]_D$ +135, MeOH) and a circular dichroism spectrum can only be consistent with the *cis-syn-cis* isomer.

The enantiopurity and absolute configuration of the *mono-cis*-diol metabolite **2A**[9] has been unequivocally assigned by stereochemical correlation and the formation of MPBA esters (Table 1). When *mono-cis*-diol **2A**, the initial metabolite from chrysene **1A**, was in turn added as a substrate to *S. yanoikuyae* B8/36, the *bis-cis*-diol **4A** was isolated as the only metabolite. This sample proved to be spectrally indistinguishable (NMR, CD) from that originally obtained from chrysene **1A**. The formation of *bis-cis*-diol **4A** as a metabolite of chrysene **1A** is, to our knowledge, a new reaction sequence for *mono-cis*-dihydrodiol derivatives of PAHs **(**Scheme 1**)**. Enzyme-catalyzed dehydrogenation (*cis*-diol dehydrogenase) to yield a catechol in wild type bacterial strains or spontaneous dehydration to yield a phenol appear to be the only reported reactions of *mono-cis*-diols that occur during the bacterial biodegradation of PAHs.[17−19]

The *bis-cis*-dihydrodiol **4A** was found to be less stable than the precursor *cis*-diol **2A**; it readily aromatized to the corresponding *bis*-phenols during attempted purification. This instability is consistent with the loss of aromaticity across two benzene rings when chrysene is tetrahydroxylated and the concomitant gain in resonance energy upon dehydration. The successful interception of the first *bis-cis*-tetrahydrodiol metabolite **4A** appears to have been facilitated by having (i) two bay regions in the PAH, (ii) a mutant strain deficient in *cis*-diol dehydrogenase activity, thus preventing dehydrogenation of the

cis-dihydrodiol, and (iii) a bay region *bis-cis*-diol metabolite of sufficient stability.

Benzo[*c*]phenanthridine **1B**, having bay region bonds in the naphthalene (3,4-bond) and isoquinoline (9,10-) portions of it, was synthesized by the literature method[21] and used as a substrate for the BPDO enzyme present in *S. yanoikuyae* B8/36. Earlier studies of the dioxygenase-catalyzed *cis*-dihydroxylation of naphthalene and isoquinoline individually have shown that the former is a much better substrate. Thus, it was anticipated that *cis*-dihydroxylation would occur preferentially at the 3,4-bond to yield *mono-cis*-dihydrodiol **2B**, but surprisingly the *mono-cis*-dihydrodiol **3B** was also found in almost equal proportions. The new *cis*-dihydrodiols **2B** and **3B** were identified by spectral comparison with the *mono-cis*-diol **2A** derived from chrysene **1A**. NOE interactions between protons H-6 ↔ H-7 (2%) in *cis*-diol **2B** and H-6 ↔ H-7 (3%) in *cis*-diol **3B** were consistent with the assigned structures. Formation of the MPBA derivatives $\mathbf{2B_R}$, $\mathbf{2B_S}$, $\mathbf{3B_R}$, and $\mathbf{3B_S}$ from *cis*-diols **2B** and **3B** indicated that both metabolites were enantiopure (>98% ee). The chemical shift positions of the OMe signals (Table 1) in each case allowed the absolute configurations to be assigned as (+)-(3*S*,4*R*)-3,4-dihydroxy-3,4-dihydrobenzo[*c*]phenanthridine, **2B**, and (+)-(9*S*,10*R*)-9,10-dihydroxy-9,10-dihydrobenzo[*c*]phenanthrene, **3B**. The formation of single enantiomers (**2B** and **3B**) by oxidation of bay region bonds (3,4- and 9,10-) from the si:si face of benzo[*c*]phenthridine **1B** is in accord with similar results obtained during the *cis*-dihydroxylation of chrysene **1A**. When small samples of enantiopure *cis*-diol metabolites **2B** and **3B** were in turn used as substrates for *S. yanoikuyae* B8/36, HPLC analysis showed that each was being biotransformed and several minor products appeared to be formed. Due to the insufficient quantities of metabolites, neither the anticipated *bis-cis*-dihydrodiol **4B** nor any of the other metabolites could be identified. It was assumed that the presence of an isoquinoline ring in benzo[*c*]phenthridine **1B** would make the putative *bis-cis*-dihydrodiol **4B** even less stable than the analogous metabolite **4A** obtained from chrysene. Thus, the possibility that *bis-cis*-dihydrodiol **4B** was indeed formed as an unstable metabolite, which decomposed to a range of phenolic products, cannot at present be eliminated.

Benzo[*b*]naphtho[2,1-*d*]thiophene **1C** is a tetracyclic thiaarene containing two regions each of which has geometry similar to a bay region. The term "*pseudo-bay region*" has been adopted for benzo[*b*]naphtho[2,1-*d*]-thiophene **1C** as the terminology "*bay region*" has generally been applied to alternant members of the PAH series. Earlier studies[2] on the metabolism of benzo[*b*]naphtho-[2,1-*d*]thiophene **1C**, using rat liver enzymes, have resulted in the detection of *trans*-dihydrodiols and phenols due to initial monooxygenase-catalyzed epoxidation at pseudo-bay region (1,2-, and 7,8-) and non-bay region (3,4-, and 9,10-) bonds. Using the bacterium *S. yanoikuyae* B8/36, the initial metabolic step appears to be *cis*-dihydroxylation at the pseudo-bay region (1,2-bond) of the naphthyl ring of thiaarene **1C** rather than the pseudo-bay region (7,8-bond) of the phenyl ring which would yield the *cis*-dihydrodiol **3C** (Scheme 1). The structure of the *mono-cis*-dihydrodiol **2C** was determined by ^{1}H NMR analysis. The coupling constant ($J_{1,2}$ = 5.1 Hz) was indicative of a *cis*-diol moiety, and its location at the 1,2-bond was inferred from the NOE interactions between H-4 and H-5 (5%). The enantiopurity was determined to be >98% by formation of the corresponding MPBA esters $\mathbf{2C_R}$ and $\mathbf{2C_S}$. Applying the empirical method earlier applied to the *mono-cis*-diols **2A**, **2B**, and **3B**, based upon the chemical shift values of the OMe signals in the ^{1}H NMR spectra of MPBA esters $\mathbf{2C_R}$ and $\mathbf{2C_S}$ (Table 1), the absolute configuration (+)-(1*R*,2*S*) was assigned to *cis*-diol **2C**.

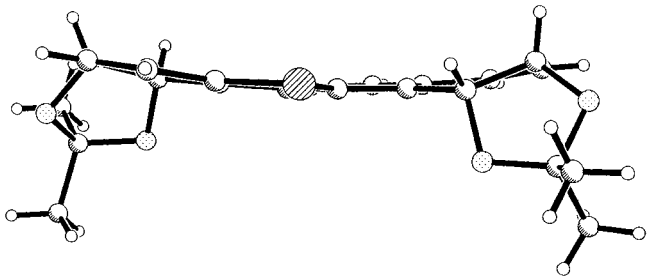


Figure 2. X-ray crystal structure analysis of compound **7C** indicating the overall curvature of the molecule.

A second more polar metabolite from benzo[*b*]naphtho-[2,1-*d*]thiophene **1C** was identified from spectral data as the *bis-cis*-diol **4C** rather than the isomers **5C** or **6C** (Scheme 1). Thus, coupling constants of 5.2 Hz ($J_{1,2}$) and 6.0 Hz ($J_{7,8}$) are consistent with the presence of *cis*-diol groups at the 1,2- and 7,8-bonds. The formation of an identical sample of the *bis-cis*-diol **4C** when the (+)-(1*R*,2*S*)-*mono-cis*-diol **2C** (>98% ee) was used as substrate for *S. yanoikuyae* B8/36 allowed the enantiopurity of compound **4C** to be established as >98% and the absolute configuration at two of the four chiral centers to be confirmed as (1*R*,2*S*). To establish that the relative and absolute configuration of the *bis-cis*-diol metabolite was consistent with structure **4C**, and to obtain a more stable derivative, the *bis*-acetonide **7C** was prepared. Fortunately the *bis*-acetonide **7C** turned out to be a crystalline compound, mp 136−137 °C, [α_D] +466 ($CHCl_3$). The crystal structure analysis of compound **7C**, employing anomalous dispersion methods, established the relative stereochemistry of the hydroxyl groups in metabolite **4C** as *cis-syn-cis* and the absolute configurations at the four chiral centers as (1*R*,2*S*,7*R*,8*S*), Figure 1. It also confirmed that the *cis*-dihydroxylation steps had occurred at the pseudo-bay region bonds (1,2- and 7,8-) and on the same (si:si) face to yield a single enantiomer of tetraol **4C**. X-ray crystallography indicated that rings A, B, and C are essentially planar but ring D is puckered, with the tetrahedral carbons C1 below (−0.22 Å) and C2 above (+0.15 Å) the mean plane defined by the atoms of the diene moiety C3, C4, C4a, C11b. This diene is itself skewed, with torsion angle −7.6°. There is very little deviation from planarity in ring A, with the tetrahedral carbons C7 and C8 only +0.08 and −0.03 Å, respectively, out of the mean plane defined by the planar diene C6b, C10a, C10, C9 (Figure 2). At each ring fusion along the A−D sequence there is a slight fold, 5.8°at junction A/B, 5.4° at B/C, and 2.8° at C/D (where the D plane is defined by atoms C1, C11b, C4a, C4 only). This produces an overall curvature, shown in Figure 2. The differences in the conformations of rings A and D may result from the differing geometries at the adjacent pseudo-bay regions. *cis*-Tetrahydrodiol **4C** is thus the second identified member of this new class of bacterial metabolites from the corresponding polycyclic arenes.

(21) Hall, G. E.; Walker, *J. Chem. Soc.* **1968**, 2237.

The isolation of the phenolic acetonide **8C** as a byproduct from the reaction mixture of the *bis*-acetonide **7C** demonstrates the difference in stability between the two *cis*-dihydrodiol moieties. The *cis*-dihydrodiol formed in the benzothiophene ring appears to be more susceptible to acid-catalyzed dehydration, thus forming the phenolic *cis*-diol **9C** and its acetonide derivative **8C**. The chemoenzymatic formation of the trihydroxylated compound **9C** suggests that similar phenolic *cis*-dihydrodiol metabolites could also be isolated directly from the bacterial metabolism of other polycyclic arenes containing two bay or pseudo-bay regions. This type of metabolite would be analogous to the reported examples of phenolic *trans*-dihydrodiols formed during biotransformation of PAHs using monooxygenase enzymes.[8] The isolation of compounds **4A** and **4C** provides the first unequivocal evidence that procaryotic (dioxygenase-catalyzed) metabolism of PAHs and HPAHs occurs by multiple site oxidation of different rings to yield polyoxygenated metabolites if two bay regions or pseudo-bay regions are present in the substrate.

Conclusion

Evidence is presented for the formation of a new class of metabolites (*bis*-*cis*-dihydrodiols) during the bacterial biodegradation of carcinogenic PAHs. *mono*-*cis*-Dihydrodiols (from chrysene, benzo[*c*]phenanthridine, and benzo[*b*]naphtho[2,1-*d*]thiophene), and *bis*-*cis*-dihydrodiols from chrysene and benzo[*b*]naphtho[2,1-d]thiophene), have been isolated and stereochemically assigned. The formation of *bis*-*cis*-dihydrodiols as procaryotic metabolites from the exclusively *cis*-tetrahydroxylation of chrysene and benzo[*b*]naphtho[2,1-*d*]thiophene) is complementary to earlier reports of *bis*-*trans*-dihydrodiol formation as a result of multiple site oxidation of PAHs by eucaryotic systems. It is probable that a much larger number of *bis*-*cis*-dihydrodiol metabolites have previously gone unreported during bacterial metabolism of PAHs due to increased instability and water solubility. Recent investigations from these laboratories have already identified two further members of this new family of *bis*-*cis*-dihydrodiol metabolites but from the tricyclic azaarene series (acridine and phenazine). The structure and reactivity of these *bis*-*cis*-dihydrodiol metabolites of acridine and phenazine will be reported elsewhere.

Experimental Section

Biphenyl Dioxygenase-Catalyzed *cis*-Dihydroxylation of Chrysene 1A To Yield (+)-(3*S*,4*R*)-3,4-Dihydroxy-3,4-dihydrochrysene 2A and (+)-(3*S*,4*R*,9*S*,10*R*)-3,4,9,10-Tetrahydroxy-3,4,9,10-tetrahydrochrysene 4A. A biotransformation of chrysene **1A** (0.5 g) was carried out using *S. yanoikuyae* strain B8/36 in the manner described previously.[9] The reaction mixture was incubated in the dark at 30 °C with shaking (220 rpm) for 18 h. The cells were subsequently removed by centrifugation, and the supernatant was extracted with ethyl acetate after saturation with sodium chloride. The dried ethyl acetate extract was concentrated and the residue subjected to PLC on silica gel ($CHCl_3$:MeOH 9:1), to yield two compounds. The less polar (R_f 0.6) compound was identified as *cis*-dihydrodiol metabolite **2A** (0.005 g, ca. 1% yield): mp 241−243 °C (dec) as colorless needles ($CHCl_3$−MeOH), $[\alpha]_D$ +112. ($c = 0.5$, THF; lit.[9] $[\alpha]_D$ +112). The enantiopurity of the dihydrodiol metabolite **2A** (0.0005 g) was established by treatment with (−)-(*S*)- and (+)-(*R*)-2-(1-methoxyethyl)phenyl boronic acid (0.00034 g) separately in chloroform solution. The boronates $\mathbf{2A_S}$ and $\mathbf{2A_R}$ were dried (Na_2SO_4), concentrated, and analyzed by ^{1}H NMR spectroscopy ($CDCl_3$, see Table 1). The diagnostic OMe signals of the boronate esters formed with (−)-(*S*)-MPBA, $\mathbf{2A_S}$ (δ_H 3.11), and (+)-(*R*)-MPBA, $\mathbf{2A_R}$ (δ_H 2.97), suggested an (*R*)-configuration for the benzylic C-4 position. Thus, the *cis*-dihydrodiol metabolite **2A** was predicted to have the (3*S*,4*R*) absolute configuration. Since only one OMe signal was observed in the ^{1}H NMR spectrum of either the (*R*)- **($\mathbf{2A_R}$)** or the (*S*)-MPBA ester **($\mathbf{2A_S}$)**, the *cis*-dihydrodiol metabolite **2A** was assumed to be enantiopure.

The more polar metabolite (R_f 0.15) was isolated as a semisolid and was characterized as the *bis*-*cis*-diol **4A** (0.002 g, 0.3% yield): $[\alpha]_D$ +135 ($c = 0.4$, MeOH); ^{1}H NMR (500 MHz, $(CD_3)_2CO$) δ 3.62 (2H, bs, OH), 4.02 (2H, bs, OH), 4.62 (2H, ddd, $J_{1,3} = J_{7,9} = 2.8$, $J_{2,3} = J_{8,9} = 1.6$, $J_{3,4} = J_{9,10} = 5.2$, 3-H and 9-H), 5.24 (2H, d, $J_{3,4} = J_{9,10} = 5.2$, 4-H and 10-H), 5.97 (2H, dd, $J_{2,1} = J_{7,8} = 9.6$, $J_{2,3} = J_{8,9} = 1.6$, 2-H and 8-H), 6.53 (2H, dd, $J_{1,2} = J_{7,8} = 9.6$, $J_{1,3} = J_{7,9} = 2.8$, 1-H and 7-H), 7.42 (2H, d, $J_{6,5} = J_{11,12} = 8.6$, 6-H and 12-H), 8.18 (2H, d, $J_{5,6} = J_{11,12} = 8.6$, 5-H and 11-H); ^{13}C NMR (125 MHz, CD_3COCD_3) δ 65.88, 70.91, 124.69, 126.44, 126.76, 130.04, 131.00, 132.33, and 133.26; *m*/*z* 295($M - H)^-$ (16%); CD 368.1 nm $\Delta\epsilon$ +0.416, 348.9 nm $\Delta\epsilon$ +0.481, 272.60 nm $\Delta\epsilon$ +4.24, 262.9 nm $\Delta\epsilon$ +1.91, 243.80 nm $\Delta\epsilon$ −0.775, 211.4 $\Delta\epsilon$ −3.603.

Biphenyl Dioxygenase-Catalyzed *cis*-Dihydroxylation of Benzo[*c*]phenanthridine 1B To Yield (+)-(3*S*,4*R*)-3,4-Dihydroxy-3,4-dihydrobenzo[*c*]phenanthridine, 2B, and (+)-(9*S*,10*R*)-9,10-Dihydroxy-9,10-dihydrobenzo[*c*]phenanthrene, 3B. A biotransformation of benzo[*c*]phenanthridine **1B** (0.40 g, 1.75 mmol, 18 h) was carried out using *S. yanoikuyae* strain B8/36 in the manner described for chrysene **1A**.[9] The ethyl acetate extract was dried (Na_2SO_4), and the residue obtained after removal of solvent was separated by PLC on silica gel using $CHCl_3$:MeOH (90:10). A small proportion of the substrate **1B** (0.10 g) was recovered from the biomass. The more polar metabolite proved to be **(+)-(3*S*,4*R*)-3,4-dihydroxy-3,4-dihydrobenzo[*c*]phenanthridine**, **2B** (0.036 g, 8%; R_f 0.23): mp 63−64 °C (from $CHCl_3$/MeOH); $[\alpha]_D$ +114 ($c = 0.2$, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.49 (1H, dd, $J_{3,2} = 5.4$, $J_{3,4} = 5.0$ 3-H), 5.64 (1H, d, $J_{3,4} = 5.0$, 4-H), 6.37 (1H, dd, $J_{2,1} = 9.5$, $J_{2,3} = 5.4$, 2-H), 6.77 (1H, d, $J_{1,2} = 9.4$, 1-H), 7.50 (1H, d, $J_{12,11} = 8.3$, 12-H), 7.74 (1H, dd, $J_{8,7} = 7.8$, $J_{8,9} = 7.0$, 8-H), 7.90 (1H, dd, $J_{9,8} = 7.0$, $J_{9,10} = 8.2$, 9-H), 8.07 (1H, d, $J_{7,8} = 7.9$, 7-H), 8.49 (1H, d, $J_{11,12} = 8.3$, 11-H), 8.59 (1H, d, $J_{10,9} = 8.2$, 10-H), 9.20 (1H, s, 6-H); ^{13}C NMR (125 MHz, CD_3COCD_3) 65.60, 70.54, 121.89, 122.11, 124.10, 125.57, 126.83, 127.85, 128.81, 129.19, 129.89, 130.78, 131.78, 132.59, 132.91, 143.26, 151.53; *m*/*z* 263 (35), 245 (40), 256 (100) (found M^+ 263.094901, $C_{10}H_{12}O_2$ requires 263.094621); CD 317.43 nm $\Delta\epsilon$ +1.844, 285.60 nm $\Delta\epsilon$ −2.21, 264.60 nm $\Delta\epsilon$ +1.965, 234.80 nm $\Delta\epsilon$ −0.681, 226.20 nm $\Delta\epsilon$ +4.027, 207.60 nm $\Delta\epsilon$ −3.619.

The less polar metabolite **(+)-(9*S*,10*R*)-9,10-dihydroxy-9,10-dihydrobenzo[*c*]phenanthridine**, **3B** (0.030 g, 7%; R_f 0.36): mp 83−85 °C (from $CHCl_3$/MeOH); $[\alpha]_D$ +94 ($c = 0.2$, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.77 (1H, ddd, $J_{9,10} = 5.2$, $J_{9,8} = 1.5$, $J_{9,7} = 2.6$, 9-H), 5.38 (1H, d, $J_{10,9} = 5.2$, 10-H), 6.12 (1H, dd, $J_{8,7} = 9.6$, $J_{8,9} = 1.5$, 8-H), 6.68 (1H, dd, $J_{7,8} = 9.7$, $J_{7,9} = 2.6$, 7-H), 7.72 (2H, m, 2-H and 3-H), 7.87 (1H, d, $J_{12,11} = 9.2$, 12-H), 7.89 (1H, d, $J_{1,2} = 9.1$, 1-H), 8.03 (1H, d, $J_{11,12} = 9.2$, 11-H), 8.77 (1H, s, 6-H), 9.22 (1H, d, $J_{4,3} = 7.1$, 4-H); ^{13}C NMR (125 MHz, CD_3COCD_3) δ 64.90, 70.18, 120.14, 124.16, 124.19, 124.65, 125.03, 127.42, 127.81, 128.45, 129.07, 131.69, 132.75, 133.19, 137.24, 146.59, 147.34; *m*/*z* 263 (40), 245 (100) (found M^+ 263.094743, $C_{10}H_{12}O_2$ requires 263.094621); CD 369.80 nm $\Delta\epsilon$ +0.657, 324.80 nm $\Delta\epsilon$ −1.410, 281.00 nm $\Delta\epsilon$ +8.593, 232.80 nm $\Delta\epsilon$ −5.197, 213.80 nm $\Delta\epsilon$ −9.347.

Biphenyl Dioxygenase-Catalyzed *cis*-Dihydroxylation of Benzo[*b*]naphtho[2,1-*d*]thiophene 1C To Yield (+)-(1*R*,2*S*)-1,2-Dihydroxy-1,2-dihydrobenzo[*b*]naphtho[2,1-*d*]thiophene 2C and (+)-(1*R*,2*S*,7*R*,8*S*)-1,2,7,8-Tetrahydroxy-1,2,7,8-tetrahydrobenzo[*b*]naphtho[2,1-*d*]thiophene 4C. A biotransformation of benzo[*b*]naphtho[2,1-*d*]thiophene **1C** (0.20 g, 0.85 mmol, 18 h) was carried out using *S. yanoikuyae* strain B8/36 in the manner described for

chrysene **1A**.[9] The ethyl acetate extract was dried, and the residue obtained after removal of solvent was purified by PLC on silica gel using $CHCl_3$:MeOH (90:10) to yield the less polar metabolite **(+)-(1*R*,2*S*)-1,2-dihydroxy-1,2-dihydrobenzo[*b*]-naphtho[2,1-*d*]thiophene 2C** (0.016 g, 7%; R_f 0.32): mp 103−105 °C (dec, from $CHCl_3$) $[\alpha]_D$ +146 (c = 0.7, MeOH); ^{1}H NMR (500 MHz, $(CD_3)_2CO$) δ 4.18 (1H, d, $J_{2,OH}$ = 5.6, OH), 4.33 (1H, d, $J_{1,OH}$ = 6.8, OH), 4.46 (1H, dd, $J_{2,3}$ = 3.8, $J_{1,2}$ = 5.1, 2-H), 4.93 (1H,d, $J_{1,2}$ = 5.1, 1-H), 6.09 (1H, dd, $J_{3,4}$ = 9.7, $J_{3,2}$ = 3.8, 3-H), 6.64 (1H, dd, $J_{4,3}$ = 9.7, $J_{4,2}$ = 1.3, 4-H), 7.31 (1H, d, $J_{5,6}$ = 7.9, 5-H), 7.47 (2H, m, 8-H and 9-H), 7.94 (1H, m, 7-H), 8.19 (1H, d, $J_{6,5}$ = 7.9, 6-H), 8.26 (1H, m, 10-H); ^{13}C NMR (125 MHz, $(CD_3)_2CO$) 67.79, 70.46, 121.41, 121.87, 122.95, 124.49 126.30, 127.25, 128.16, 130.46, 130.98, 131.49, 135.55, 136.27, 139.28, 140.89; *m*/*z* 268 (57), 250 (100), 222 (82), 196 (20), 149 (25), 111 (34) (found M^+ 268.056348, $C_{16}H_{12}SO_2$ requires 268.055802); CD 308.8 nm $\Delta\epsilon$ +2.506, 285.1 nm $\Delta\epsilon$ +2.429, 234.1 nm $\Delta\epsilon$ +1.022, 216.9 nm $\Delta\epsilon$ −2.514, 200.9 nm $\Delta\epsilon$ +4.334.

The more polar bioproduct was identified as **(+)-(1*R*,2*S*,7*R*,8*S*)-1,2,7,8-tetrahydroxy-1,2,7,8-tetrahydrobenzo [*b*]naphtho[2,1-*d*]thiophene 4C.** A solid (6 mg, 3%), R_f 0.18 (10% MeOH/$CHCl_3$): mp 65−66 °C (dec, from $CHCl_3$−MeOH); $[\alpha]_D$ +270 (c = 0.3 in MeOH); ν_{max}/cm^{-1} 3432.9 (4 × OH); δ_H (500 MHz, $(CD_3)_2CO$) 4.26 (1H, ddd, $J_{2,1}$ = 5.2, $J_{2,3}$ = 4.3, $J_{2,4}$ = 1.1, 2-H), 4.45 (1H, ddd, $J_{8,7}$ = 5.8, $J_{8,9}$ = 3.4, $J_{8,10}$ = 2.7, 8-H), 4.75 (1H, d, $J_{1,2}$ = 5.2, 1-H), 4.78 (1H, dd, $J_{7,8}$ = 6.0, $J_{7,9}$ = 0.9, 7-H), 5.87 (1H, dd $J_{2,3}$ = 4.1, $J_{3,4}$ = 9.7, 3-H), 5.93 (1H, ddd, $J_{9,10}$ = 9.8, $J_{9,8}$ = 3.4, $J_{9,7}$ = 0.9, 9-H), 6.41 (1H, dd, $J_{10,9}$ = 9.8, $J_{10,8}$ = 2.7, 10- H), 6.47 (1H, dd, $J_{4,3}$ = 9.7, $J_{4,2}$ = 1.0, 4-H), 7.07 (1H, d, $J_{5,6}$ = 8.1, 5-H), 7.65 (1H, d, $J_{6,5}$ = 8.0, 6-H); ^{13}C NMR (125 MHz, CD_3OCD_3) δ 69.92, 67.25, 70.42, 70.52, 119.88, 120.89, 124.40, 1218.33, 128.74, 129.04, 130.09, 130.77, 133.67, 136.98, 138.78, 139.41; *m*/*z* = 266 (100), 237 (27), 208 (12), 183 (22), 138 (54); CD 276.9 nm $\Delta\epsilon$ 1.114, 260.5 nm $\Delta\epsilon$ −0.7359, 232.5 nm $\Delta\epsilon$ 0.1888, 210.9 $\Delta\epsilon$ −0.7390.

3,4:9,10-*bis*-Acetonide of (1*R*,2*S*,7*R*,8*S*)-1,2,7,8-Tetrahydroxy-1,2,7,8-tetrahydrobenzo [*b*]naphtho[2,1-*d*]thiophene 7C. A catalytic quantity of *p*-toluenesulfonic acid was added to a suspension of the *bis-cis*-diol **4C** (0.008 g) in 2,2-dimethoxypropane (0.4 mL), and the reaction mixture was stirred for 2 h at room temperature. After removal of the solvent from the reaction mixture, two compounds were isolated by PLC on silica gel (diethyl ether:hexane 1:1). The less polar (R_f 0.57) compound was identified as the *bis*-acetonide **7C** (0.005 g, 50%) as colorless crystals: mp 136−137 °C (methanol); $[\alpha]_D$ +466 (c = 0.36, $CHCl_3$) (found M^+ 382.123881, $C_{22}H_{22}SO_4$ requires 382.123540).

The more polar compound (R_f 0.24) was found to be the monoacetonide derivative of 1,2,8-trihydroxy-1,2-dihydrobenzo-[*b*]naphtho[2,1-*d*]thiophene **8C** (0.003 g, 35%): mp 168−70 °C (dec, from CH_2Cl_2−hexane); $[\alpha]_D$ +184 (c = 0.25, $CHCl_3$) (found M^+ 324.082016, $C_{19}H_{16}SO_3$ requires 324.080887).

X-ray Crystal Structure Analysis of the (+)-*bis*-Acetonide 7C. Crystal Data: $C_{22}H_{22}O_4S$; M = 382.46, monoclinic, a = 9.524(7), b = 7.379(5), and c = 14.253(14) Å, β = 104.73(7)°, V = 968.7(14) Å^3, space group $P2_1$ (No. 4), Z = 2, D_x = 1.311 Mg/m^3, μ(Cu Kα) = 1.69 mm^{-1}, F(000) = 404.

Data Collection. Diffraction data were collected on a Siemens P3 four-circle diffractometer using graphite-monochromated Cu Kα radiation (λ = 1.54184 Å). Unit cell parameters were determined by least-squares refinement on the setting angles for 10 automatically centered reflections in the range 8 < θ < 20°. Data were collected using the ω-scan mode, ω scan range 2°, for a unique monoclinic set (+h, +k, ±l) and for their Friedel opposites, for 3 < θ < 55°. The intensity of one control reflection, measured after every 100 reflections, showed a deterioration to 57% of its original value, and the measured intensities were rescaled accordingly. A total of 2844 reflections were measured, of which 2426 were independent. Friedel pairs were not averaged. Lorentz and polarization factors were applied.

Analysis and Refinement. The structure was determined by direct methods and refined by full-matrix least-squares on F^2. Except for some methyl hydrogens, all hydrogen atoms were located in a difference Fourier synthesis, but in the final refinement all hydrogens were included at positions calculated from the geometry of the molecule and riding on the carbon atoms to which they were attached, with isotropic displacement parameters kept at 120% of the equivalent isotropic displacement parameters of the parent carbons for tertiary and aromatic C−H and at 150% for methyl C−H atoms. Methyl hydrogen atoms were placed using a rotating group refinement for the methyl group, and it was confirmed that the resulting positions agreed with those originally located in the difference Fourier. Final R1 = 0.056 for 1953 reflections with $I > 2\sigma(I)$, wR2 (all data) = 0.134. Goodness of fit = 1.04. Maximum/minimum residual electron density was +0.17/−0.16 e Å^{-3}. The Flack absolute structure parameter x = 0.02(4) established the configuration of compound **7C** as (1*R*,2*S*,7*R*,8*S*). [Refinement of the inverse structure gave x = 0.97(4).] (See Supporting Information.) All crystallographic computations were carried out using the SHELX-97 suite of programs.[22]

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Supporting Information Available: Additional experimental details, ^{1}H and ^{13}C NMR spectral data for compounds **2A**, **7C**, and **8C,** and crystallographic details for **7C** (coordinates, anisotropic displacement parameters, bond lengths angles, and torsion angles). This material is available free of charge via the Internet at http://pubs.acs.org.

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