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ISOLATION AND CHARACTERIZATION OF (+)-1,1a-DIHYDROXY-1-HYDROFLUOREN-9-ONE FORMED BY ANGULAR DIOXYGENATION IN THE BACTERIAL CATABOLISM OF FLUORENE

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Transformation of fluorene by washed cells of fluorene-grown *Pseudomonas* sp. F274 yielded 1,1a-dihydroxy-1-hydrofluoren-9-one (up to 100 mg/l) as the stable product of angular dioxygenation of 9-fluorenone. Structural identity of the angular keto-diol was established by ^{13}C- and ^1H-NMR, gas chromatography- and direct probe-mass spectrometry. Definitive assignment of 1,1a-dioxygenation, but not 4,4a-, was based on the isolation and rigorous identification of 1-hydroxyfluoren-9-one as the exclusive product of acidic dehydration. Chiral ^1H-NMR analysis and optical rotation of isolated 1,1a-dihydroxy-1-hydrofluoren-9-one ($[\alpha]_D$=+132.1°) are indicative of a single enantiomer with an inferred *cis*-stereochemistry of the hydroxyl groups. This compound is evidently an intermediate of fluorene catabolism by this strain and not a dead-end product because its formation is transient in washed cell incubations and ultimately it is completely consumed with the formation of acidic metabolites.

Fluorene is an abundant constituent of coal tar and creosote and one of the simplest polycyclic aromatic hydrocarbons (PAHs) classified as a priority pollutant (1). Compared to many other aromatic compounds whose bacterial biodegradation has been intensively studied for decades, catabolism of fluorene is little investigated. Only recently have a few preliminary attempts been made to elucidate its fate in microbial systems (2,3). Meanwhile, little is known of the biochemical mechanisms by which microorganisms in PAH-contaminated soils and other environments degrade this chemical. Such information is relevant to the development of bioremediation technologies. Possessing two aromatic rings along with a benzylic methylenic group in a central five-membered ring, fluorene is a potential substrate for bacterial arene-dioxygenases generally known to initiate catabolic pathways by forming vicinal *cis*-dihydrodiols (4-7) or by oxidizing adjacent methylenic groups to *sec*-alcohols (8-10). The naphthenoaromatic structure of fluorene presents several options for initial oxidation by bacteria, allowing predictions of alternative catabolic sequences based on known strategies of aromatic hydrocarbon degradation (4,11). Productive microbial catabolism of

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many aryl ethers (including p-dibenzodioxin and dibenzofuran) has been shown to occur via specific "angular" dioxygenation of these compounds thereby converting aryl ether bond to hemi-ketals with subsequent formation of catechols to be cleaved by *meta*-fisson dioxygenases (12-16). The extent to which this type of dioxygenase activity leads to productive utilization of benzophenones and fluorene has been discussed (17,18), although only few published experimental data are available. A recent attempt to isolate a product of angular dioxygenation of fluorene by a gram-positive organism, *Staphylococcus auriculans* DBF63, grown with either fluorene or dibenzofuran was not successful (19). Formation of an angular dioxygenation product from fluorenone by *Brevibacterium* sp. DPO1361 has been reported. Assignment of 1,1a-dioxygenation, however, was proposed, not on the basis of detailed experimental data, but rather on a presumed analogy in this strain of the reactions in degradation pathways of fluorene and of diaryl ethers, such as dibenzofuran (20).

In this paper we present complete experimental evidence for the formation of a stable angular 1,1a-dioxygenation product as an intermediate of fluorene catabolism by a bacterial strain *Pseudomonas* sp. F274 isolated from creosote-contaminated soil.

MATERIALS AND METHODS

Isolation of 1,1a-dihydroxy-1-hydrofluoren-9-one. Cells of fluorene-degrading strain *Pseudomonas* sp. F274 were harvested by centrifugation of eight liters of a 48-hour culture (OD_{650}=0.7) grown in mineral salts medium (21) with fluorene (1g/l) as sole carbon source. Collected cells were washed twice with 50 mM sodium-potassium phosphate buffer pH 7.2 and resuspended in 2 l of the same buffer. Incubation of the washed cell suspension with added fluorene (1g/l) was conducted during 18h on a rotary shaker at 20 °C (180 rpm). After incubation, cells and remaining fluorene were removed by centrifugation. The supernatant was brought to 5% (w/vol) NaCl by addition of solid, and extracted three times with equal volumes of ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4 and stored in a freezer (-70 °C) without evaporation or concentration. Portions of this extract were evaporated under vacuum at 30 °C immediately before column chromatography. The oily yellow-brown residue obtained by concentration of one half of the ethyl acetate extract was promptly dissolved in 20 ml of methylene chloride and applied to a silica-gel (J.T.Baker, Inc, N.J., No. 3405-01, 60-200 mesh) column (1.0 x 17 cm) pre-equilibrated with methylene chloride. After development with 200 ml CH_2Cl_2, and 50 ml of 10% (vol/vol) EtOAc in CH_2Cl_2, a yellowish crystalline solid, **I** (98 mg), was obtained by elution with 50 ml of ethyl acetate and evaporation of the solvent under vacuum without excessive heat. This material was chromatographically pure (TLC, HPLC) and had a very intense green fluorescence under UV light (366 nm). The compound showed the presence of two major UV-absorption maxima (228 and 316 nm in MeOH). The purified compound (overall recovery was approx. 200 mg from two liter-scale incubations) was analyzed by physico-chemical methods and used in further studies.

Analytical methods. All NMR spectra were recorded in $CDCl_3$ with a General Electric QE 300 PLUS NMR Spectrometer using, as references, TMS for ^1H- and the central signal of $CDCl_3$ at 77.0 ppm for ^{13}C-spectra. ^1H- and ^{13}C-Spectra were obtained at 300.65 MHz and 75.61 MHz respectively.

Chiral proton NMR experiments for compound **I** and its acetylated derivative **III** were performed by monitoring the H-a proton signal of the *sec*-alcohol or *sec*-acetoxy- groups in the presence of the chiral shift reagent, *tris*[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato]-europium(III) (Eu(hfc)$_3$), the ratio of I:Eu(hfc)$_3$ was from 1:1 to 1:20 (w/w), or in the presence of the chiral solvent, (R)-(-)-2,2,2-trifluoro-1-(9-anthryl)ethanol (TFAE), the ratio of I:TFAE was 1:10 (w/w).

Direct probe mass-spectrometry employed a Hewlett Packard 5988A instrument at 70 eV electron ionization using a temperature gradient of 10 °C/min of the sample source from 30 to 200 °C. Gas chromatography-mass spectrometry (GC-MS) was performed with a HP 5971 ion selective detector connected to GC chromatograph HP 5890A with a capillary column (HP-5, 0.32mm x 25m, 0.25 μm thickness, cross-bonded 5% diphenyl-95% dimethyl polysiloxane) programmed at 10 °C from 50 to 290 °C. Injector, transfer line and ion-selective detector were set at 270, 300 and 300 °C respectively.

HPLC analyses were performed on an HP 1090 liquid chromatograph equipped with a UV-VIS diode array detector. A reversed phase (C_{18}/ODS 5μ Hypersil column 2.1 x 100 mm) was eluted (1.5 ml/min) with a linear gradient of acetonitrile (10-79%) in 50 mM potassium phosphate buffer pH 3.5. Samples of metabolites were dissolved in methanol and injected by autosampler (10 μl).

Thin-layer chromatography was performed on 0.25 mm thickness silica-gel plates (Merck, Silica gel 60 F254) using methylene chloride or ethyl acetate as developing solvents. Compounds were visualized under a UV-lamp (254 and 366 nm).

Derivatization methods. Dehydration of compound **I** was achieved by heating (100 °C, 15 min) its solution (6.1 mg in 5 ml of 10 N H_2SO_4), resulting in the formation of a single dehydration product **II** which was extracted with hexane (5.6 mg recovered, UV-VIS λ_{max} (nm) in MeOH: 212, 246, 284, 294, 306, 354) and identified by physico-chemical methods. Acetylation of compound **I** (6.4 mg in 0.2 ml of methylene chloride) was performed by addition of acetic anhydride (150 μl) and pyridine (50 μl). Routine work-up of the reaction mixture after 1 h of incubation yielded 8.1 mg of the chromatographically pure product of acetylation **III** (UV-VIS λ_{max} (nm) in MeOH: 222, 244, 314). Esterification of compound **I** was also attempted using acetyl chloride and oxalyl chloride. Formation of isopropylidene derivatives with 2,2-dimethoxypropane in the presence of *p*-toluenesulfonic acid was examined according to previously published protocols (22,23). All reactions during the course of derivatization were routinely monitored by TLC. Obtained derivatives were analyzed by GC-MS and NMR.

Chemicals. All chemicals were of highest purity commercially available. Fluorene, fluorenone, 2-hydroxy-9-fluorenone, 4-hydroxy-9-fluorenone, TFAE and Eu(hfc)$_3$ were purchased from Aldrich Chemical Co. (Milwaukee, WI).

RESULTS AND DISCUSSION

Direct probe mass spectrometry of compound **I** revealed the presence of a low-intensity molecular ion with m/z 214 (1%) (Fig. 1) indicative of an empirical formula $C_{13}H_{10}O_3$. Observed ion peaks are indicative of several different fragmentation pathways (m/z, % of intensity, proposed composition of prominent fragment ions): 212 (3.5, $[M-H-H]^+$), 197 (28, $[M-OH]^+$), 196 (100, [M-

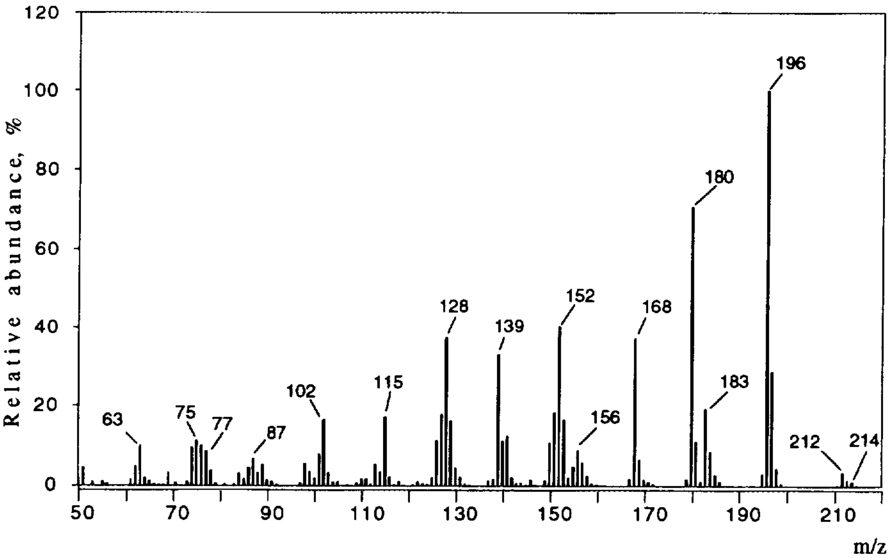

Fig. 1. Direct probe mass-spectrum of compound I isolated as an intermediate of fluorene catabolism by *Pseudomonas* sp. F274.

$H_2O]^+$), 183 (19, $[M\text{-}H\text{-}HCOH]^+$), 180 (71, $[M\text{-}OH\text{-}OH]^+$), 168 (37, $[M\text{-}H_2O\text{-}CO]^+$), 156 (8.8, $[M\text{-}HCOH\text{-}CO]^+$), 152 (40, $[M\text{-}OH\text{-}OH\text{-}CO]^+$), 139 (33), 128 (38), 115 (17), 102 (16). When chromatograpically pure (TLC, HPLC), compound **I** was analysed by GC-MS, it underwent thermal decomposition in the GC-injector, giving along with the major (approx. 80%) eluting compound [R_t 20.23 min , m/z (% of intensity): 214 (11.3, $[M]^+$), 197 (34, $[M\text{-}OH]^+$), 196 (100, $[M\text{-}H_2O]^+$), 183 (16, $[M\text{-}H\text{-}HCOH]^+$), 180 (6.9, $[M\text{-}OH\text{-}OH]^+$), 168 (38, $[M\text{-}H_2O\text{-}CO]^+$), 157 (11, $[M\text{-}COH\text{-}CO]^+$), 152 (11, $[M\text{-}OH\text{-}OH\text{-}CO]^+$), 139 (22), 128 (31), 115 (23), 102 (12)] two minor compounds (approx. 8 and 10% with retention times of 17.26 and 18.31 min, respectively). The two latter compounds have retention times and mass-spectra identical, respectively, to those of authentic samples of 9-fluorenone, ($[M]^+$ 180), and the product of acid-catalyzed dehydration ($[M]^+$ 196) shown below as compound **II**. In comparison to the direct-probe-MS, the fragmentation observed in GC-MS experiments showed significantly less dehydrogenation of the molecular ion (fragment ion m/z 212(1.2%, $[M\text{-}H\text{-}H]^+$)) and decreased formation of a fluorenone ion fragment (m/z 180) by elimination of two hydroxyl group. The obtained MS-fragmentation data of **I** were similar to those reported for the product of angular dioxygenation of fluorenone by *Brevibacterium* sp. DPO 1361 (20) indicative of: (i) microbial introduction of three oxygen atoms into the fluorene molecule, (ii) ready elimination of two hydroxyl groups typical of non-phenolic hydroxy-derivatives, (iii) dehydration as a more important fragmentation route than elimination of hydroxy-groups, (iv) high-temperature dehydration (GC-injector) resulting in the formation of rearomatized compounds, (iv) ketonic character of one atom of oxygen appearing as the elimination of C=O from the fluorenone fragment ion of m/z 180 with formation of a biphenylene fragment ion (m/z 152).

The broad-band ^{13}C-NMR decoupled spectrum of **I** revealed thirteen different carbon atom signals, including seven tertiary sp^2, one keto- , three quaternary sp^2 , one tertiary sp^3 and one quaternary sp^3, suggesting a fluorenone-derived structure with one aromatic ring and one non-aromatic (cyclohexadiene) ring with an angular dihydroxylation pattern. A deconjugated carbonyl carbon atom signal (203.33 ppm) is indicative of a 9-fluorenone-related structure dearomatized adjacent to the carbonyl group (approx. 10 ppm downfield compared to that of 9-fluorenone at 193.3 ppm, determined under the same conditions).

Assignment of carbon and proton NMR signals was made on the basis of separate C-H COSY (two-dimensional heteronuclear C-H correlation), ATP, DEPT and H-H COSY experiments (numbering scheme is given in Fig. 2,A).

Carbon atom signals (ppm) were assigned as follows: tertiary sp^3 - 70.57 (C-1); quaternary sp^3 - 71.14 (C-1a); tertiary sp^2 - 118.16 (C-4), 122.11 (C-5), 123.74 (C-3), 124.98 (C-8); 129.49 (C-7); 133.41 (C-2); 136.09 (C-5); quaternary sp^2 - 135.49 and 135.82 (8a and 4a, not assigned), 147.38 (C-5a); carbonyl - 203.33 (C-9).

Proton NMR analysis showed the presence of ten fully resolved proton signals. Part of the ^1H-NMR spectrum showing signals of aromatic, olefinic and sp^3-CH(OH) protons is given in fig. 2,A. The proton signals have been measured as follows (ppm): 4.749 (ddd, H-a), 5.938 (dd, H-b), 6.201 (ddd, H-c), 6.575 (d, H-d), 7.425 (ddd, H-g), 7.690 (ddd, H-f), 7.694 (dd, H-e) 7.813 (dd, H-h), one proton each. Two hydroxyl protons were observed as broad singlets at 3.62 and 4.10 ppm.

^1H-NMR analysis showed the presence of only one proton carried by an sp^3 carbon atom (*sec*-alcohol group) while two different hydroxyl (non-phenolic) group proton signals were observed. Based on analysis of H-H couplings by ^1H-COSY we have concluded that the seven observed aromatic and olefinic protons are distributed between two separate groups: four signals of

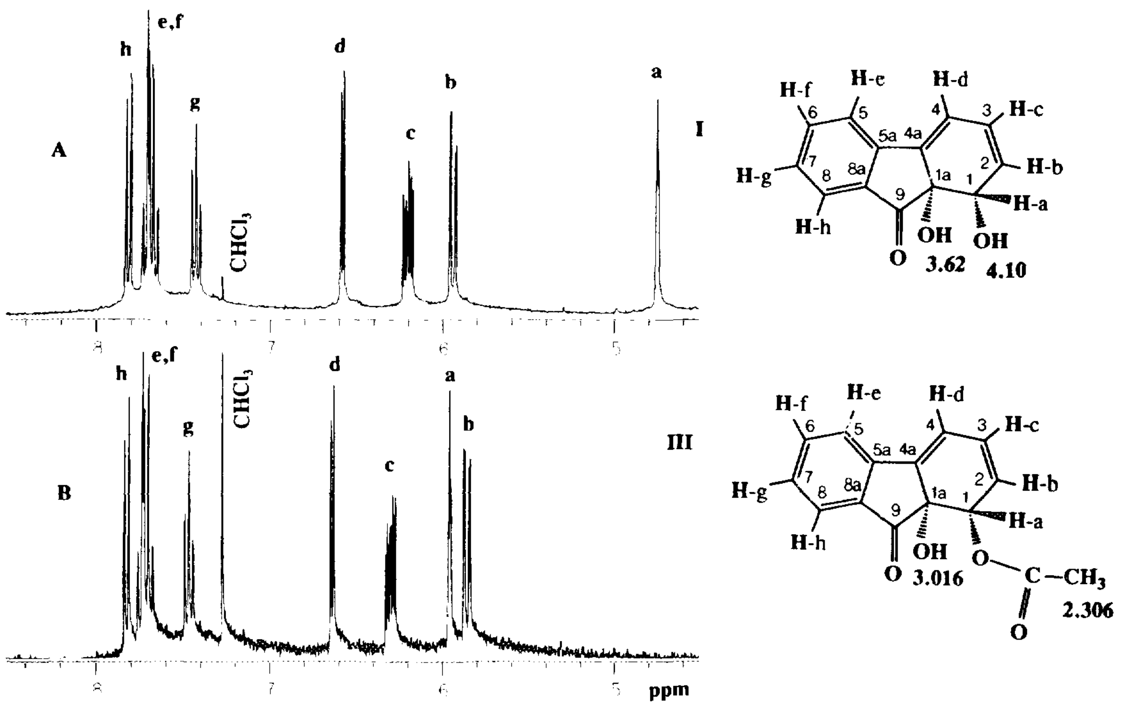

Fig. 2. ^{1}H-NMR spectra of compound **I** (40 mg/ml), (A), its acetylated derivative **III** (6 mg/ml), (B), and their chemical structures.

the first intercorrelated group are assigned to the non-oxidized aromatic ring of fluorenone; the three remaining belong to an intercorrelated group due to a dearomatized (cyclohexadiene) ring. No signals typical of isolated aromatic or olefinic protons were observed. H-H Coupling constant J_{bc}= 9.6 Hz indicated the position of the olefinic double bond between H-b and H-c, while J_{cd}= 5.1 Hz showed that olefinic proton H-d is the *cis*-relative to H-c. Coupling constants J_{ab}= 1.9, J_{ac}= 2.9 Hz were similar to values reported for the angular diol formed by *Brevibacterium* sp. DPO1361 and are indicative of an axial orientation of H-a; therefore, *cis*-stereochemistry of the angular hydroxylation is inferred from the inevitable axial orientation of 1a-hydroxyl and equatorial orientation of 1-hydroxyl groups.

From MS and NMR data the structure of compound **I** has been established as vicinal angular dihydroxyhydrofluoren-9-one with hydroxyl groups suggested at positions 1 and 1a. However, a 4,4a-dioxygenated structure cannot be excluded on the basis of MS or NMR data. From previously published data of Engesser *et al* (20) , it was noted that the MS-fragmentation data and ^{1}H-NMR proton signals of an angular diol of fluorenone formed by *Brevibacterium* sp. DPO1361 were indeed similar to those of **I**. However, contrary to the almost coincidental ^{13}C NMR signals of sp^3 carbon atoms C-1 and C-1a (given as C-10 by the authors) reported at 70.85 and 70.87 ppm, the corresponding signals of C-1 and C-1a of the isolated product **I** were completely resolved and observed at 70.57 and 71.14, respectively. Moreover, these values were reproducible in several separate NMR experiments with different concentrations. Unfortunately, the lack of complete published ^{13}C-NMR data for the product of angular dioxygenation of fluorenone formed by the

Brevibacterium sp. DPO1361 does not allow a critical comparison of its identity to that of **I**. Considering the differences of ^{13}C-NMR data, unambiguous evidence for 1,1a-dioxygenation or 4,4a-dioxygenation by the strain *Brevibacterium* sp. DPO1361 has yet to be published.

Further evidence of 1,1a-dihydroxylation, but not 4,4a-, 1,2- or 3,4-dihydroxylation, has been obtained by dehydration experiments. We have found that the purified **I** is very resistant to acid-catalyzed dehydration, known to occur readily with many other arene *cis*-dihydrodiols (22,23). No spontaneous dehydration of **I** was observed during the course of its purification and storage at room temperature. No dehydration occured in the presence of dilute (0.1 N) sulfuric or hydrochloric acid. On dissolution of **I** in 10 N sulfuric acid and incubation at room temperature for 30 min no detectable dehydration was observed. However, after heating at 100 ^{o}C for 15 min in 10 N H_2SO_4 dehydration was complete and yielded a yellow precipitate of chromatograpically pure **II**. GC-MS analysis of the dehydration product **II** [R_t 18.33 min, m/z of M^+ 196 (100%)] showed a molecular mass indicative of the loss of one molecule of water from compound **I** as the result of acid-catalyzed dehydration. The presence of prominent fragment peaks at m/z 168 (40%, $[M\text{-}CO]^+$), 139 (31.5%, $[M\text{-}CO\text{-}COH]^+$), 113(3.5%, $[M\text{-}CO\text{-}COH\text{-}HC{=}CH]^+$) supports the phenolic character of the hydroxyl group in structure **II** whose fragmentation is typical of a monohydroxylated 9-fluorenone. ^{1}H-NMR analysis has shown seven aromatic protons distributed in two separated intercorrelated systems (4 and 3, no isolated protons found) assigned as shown in Fig.3. The proton of the phenolic hydroxyl group was shifted downfield to 8.446 ppm. ^{13}C-NMR analysis showed the signals of 7 different tertiary sp^2 aromatic carbon atoms (112.96, 118.32, 121.16, 124.21, 129.23, 134.82, 137.62 ppm), 5 different low intensity signals of quarternary sp^2 carbon atoms (121.01, 134.35, 143.96, 144.27, 157.49 ppm) and a weak signal of the carbonyl carbon atom (208 ppm). Based on the obtained MS and NMR data by comparison of its properties with those of 2- and 4-hydroxyfluorenones, the dehydratation product **II** is established as 1-hydroxy-9-fluorenone. Comparison of MS and ^{1}H-NMR data of **II** with those published in the literature for 1-hydroxy-9-fluorenone (19) confirmed this assignment.

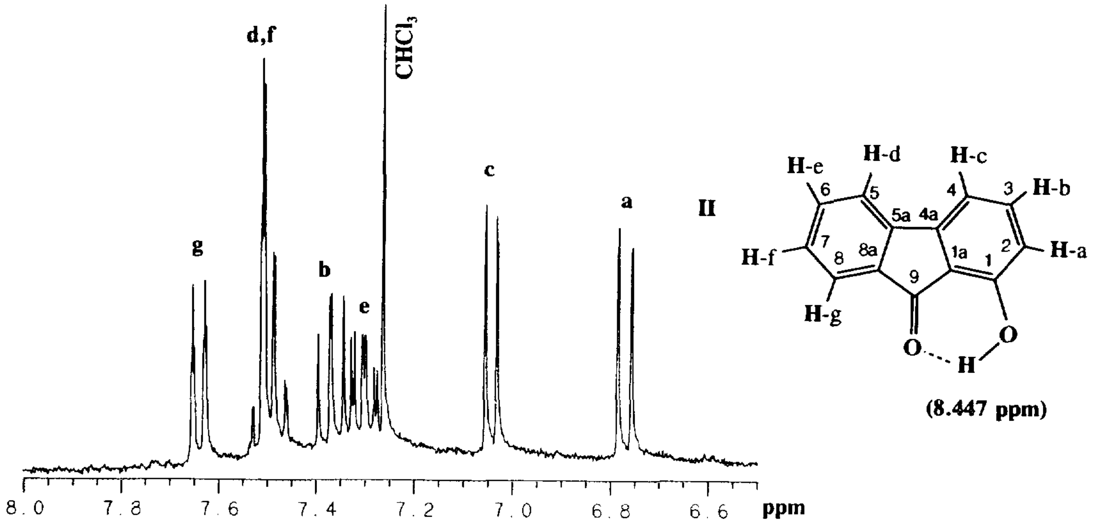

Fig. 3. ^{1}H-NMR spectrum and chemical structure of dehydration compound **II** (5 mg/ml) formed from 1,1a-dihydroxy-1-hydro-9-fluorenone (**I**).

Authentic 4-hydroxy-9-fluorenone showed mass-spectrum with m/z of 196(100%), 168(42), 139(46), 113(8) remarkably similar to the MS obtained for compound **II**. However, the UV-visible spectrum of 4-hydroxy-9-fluorenone (λ_{max},nm: 212, 246, 288, 300, 313, 332, 424, in MeOH) was different from that of **II** (see in Materials and Methods). Moreover, TLC analysis showed that **II** has a higher R_f value (0.8 in EtOAc) than 4-hydroxy- (R_f =0.54) or 2-hydroxy-9-fluorenone (R_f =0.38). Capillary GC retention times under conditions used for GC-MS analysis were 18.33, 19.66 and 21.25 min for **II**, 4-hydroxy- and 2-hydroxyfluorenone respectively. The ^{1}H-NMR spectrum of authentic 4-hydroxyfluorenone revealed aromatic proton signals and H-H coupling constants similar to those of compound **II** since it also has two intercorrelated proton systems (4 and 3 protons, respectively). The only evident distinction between ^{1}H-NMR spectra of these two compounds was the signal due to a phenolic proton in the spectrum **II** (8.446 ppm) shifted downfield almost 4 ppm compared to that of the 4-hydroxy-derivative. However, shifts of these signals are concentration dependent and vary with the nature and moisture of NMR solvents. Therefore, MS and ^{1}H-NMR data alone do not allow unambiguous assignment of **II** as 1-hydroxy-9-fluorenone. Herein, 3,4- or 4,4a-dioxygenation patterns are eliminated by rigorous identification of **II** using a combination of NMR analysis, chromatographic data and UV-visible spectro-photometry.

Monna *et al.* (19) have proposed the structure of 1,1a-dihydroxy-1-hydro-9-fluorenone for metabolite FN-P2 formed from fluorene by *S. auriculans* by identification of 1-hydroxy-9-fluorenone as a product of its spontaneous dehydration. The marked stability of **I** noted in the present work and its accumulation unaccompanied by any detectable (TLC, HPLC) 1-hydroxy- (or other hydroxy-) 9-fluorenone in extracts of fluorene incubations, suggest that an alternative diol, 1,2-dihydro-1,2-dihydroxy-9-fluorenone, may account for the observations of Monna *et al.* These authors excluded this structure as possible metabolite because of a failure to detect 2-hydroxy-9-fluorenone as a second product of dehydration. Lacking 1,2-dihydro-1,2-dihydroxy-9-fluorenone for study, it can only be suggested that the possibility of internal hydrogen bonding in such a structure between the 1-hydroxy- and 9-keto-groups may provide sufficient stability for predominant, even exclusive, elimination of the C-2 hydroxyl, leading to 1-hydroxy-9-fluorenone formation. That such hydrogen bonding, between 9-keto- and 1-hydroxyl functions, occurs can be inferred from the fact that 1-hydroxy-9-fluorenone is the least polar compound among other monohydroxy-9-fluorenones based on their TLC and HPLC properties.

It is also of note that 1-hydroxy-9-fluorenone has been reported as an HPLC-detectable product of fluorene oxidation by *Brevibacterium* sp. DPO 1361 (20).However rigorous identification to distinguish it from 4-hydroxyfluoren-9-one was not provided. Based on the stability of **I** shown here it appears unlikely that the detected hydroxy-9-fluorenone originates by facile dehydration of an angular 1,1a-diol as suggested by Engesser and coauthors. Its formation may be as readily explained by the long recognized 1,2-dioxygenation (4,24) as discussed above for the metabolite FN-P2 observed by Monna *et al* .(19). In fact, 1,2-dioxygenation of naphthalene by cells of *Brevibacterium* sp. DPO 1361 was also reported by Engesser *et al* .(20).

Confirmation of a *cis*- stereochemistry inferred by ^{1}H-NMR for the vicinal hydroxyl groups of compound **I** could not be obtained by traditional methods (22,23) based on the formation and characterization of a cyclic isopropylidene derivative. All attempts to obtain a cyclic isoropylidene derivative from 1,1a-dihydroxy-1-hydro-9-fluorenone were unsuccessful resulting only in formation of 2-(9-keto-1a-hydroxy-1-hydro-1-fluorenoxy)-2-methoxypropane (MS ion peaks, m/z(% of intensity): 286(4.1, $[M]^+$), 271(0.8, $[M-CH_3]^+$), 213(1.3, $[M-CH_3OC(CH_3)_2]^+$), 196(100), 186(28), 180(4), 168(43), 157(7), 152(5), 139(23). Formation of the dehydration product, 1-hydroxy-9-fluorenone, was also observed over time with 2,2-dimethoxypropane in the presence of trace amounts of *p*-toluenesulfonic acid. Attempts to obtain a cyclic diester derivative with oxalyl dichloride resulted in rapid formation of the corresponding 1-monoester of **I** and oxalylmonochloride; the former was tentatively identified as the corresponding methyl ester after quenching of a small aliquot of the reaction mixture with water and methylation of extracted material with diazomethane (GC-MS: R_t=17.40 min; 213(1.3, $[M\text{-}CO\text{-}COOCH_3]^+$), 196(100), 180(35), 168(36), 157(6), 152(30), 139(30), 128 (20), 115(18). During prolonged reaction with oxalyl chloride, formation of the dehydration product, 1-hydroxy-9-fluorenone, was observed instead of the expected cyclic diester.

Acetylation of **I** with acetic anhydride resulted in the formation of a monoacetylated derivative **III** in nearly quantitative yield. GC-MS analysis of **III** (R_t=22.4 min) showed the presence of molecular ion m/z (% of intensity) of 256(0.2) and fragment ions similar to those of **I**: 214(1.2, $[M+H\text{-acetyl}]^+$), 213(0.7, $[M\text{-acetyl}]^+$), 196(100), 185(1.6), 180(9), 168(40), 152(15), 139(23), 128(11), indicative of a monoacetylated derivative. ^{1}H-NMR spectrum of **III** showed the presence of acetyl protons (2.306 ppm, 3H, singlet) along with one hydroxyl group proton (3.016 ppm, 1H, broad singlet). Although signals of aromatic and olefinic protons of **III** and their coupling constants were remarkably similar to those of non-acetylated **I**, resonance of the proton (H-a) attached to sp^3 C-1 carbon carrying the acetoxy-group has been shifted from 4.749 ppm to 5.950 ppm (Fig. 2,B). Vicinal and allylic coupling constants (J_{ab}=2.1 and J_{ac}=2.7 Hz) were also indicative of the axial orientation of H-a.

No diacetyl derivative was found, and no dehydration products were formed in the reaction with acetic anhydride. Attempts to obtain a diacetyl derivative from **I** or **III** using acetyl chloride resulted in rapid loss of the 1a-tertiary hydroxyl group as occured in the presence of oxalyl chloride or in attempts to obtain a cyclic isopropylidene derivative. It is important to note, that even though it was impossible to obtain a cyclic oxalate diester of **I**, or its diacetyl derivative (the latter might be expected in the case of either *cis*- or *trans*- stereochemistry), absence of any reaction may be explained not by steric hindrance, but rather by insufficient reactivity of the tertiary 1a- hydroxyl group with ester forming reagents, and by a competing dehydration of the tertiary alcohol group resulting in rearomatization of the structure. Therefore, failure to obtain cyclic isopropylidene ether with 2,2-dimethoxypropane neither supports nor contradicts a *cis*-stereochemistry for **I**.

Bacterial dioxygenation of many aromatic compounds yields vicinal *cis*-dihydrodiols, usually in very high enantiomeric excess (3,9,25), while eukaryotic systems form *trans*-dihydro-diols as the result of monooxygenase reactions and hydrolysis of unstable epoxides (4). However, the methods developed to establish their absolute configuration cannot be applied here in a straightforward way to the product of angular dioxygenation because of insufficient reactivity of the tertiary alcohol group and of limited options for application of different chiral NMR techniques. Analysis of optical activity revealed dextrorotatory properties of **I** ($[\alpha]_D$=+132.1°±14.2°, c=1.68, MeOH). Thus, the isolated 1,1a-dihydroxy-1-hydro-9-fluorenone is the product of enantioselective angular bacterial dioxygenation because it is formed from non-chiral starting material. Chiral NMR analysis of **I** and **III**, as described in Material and Methods, failed to reveal any detectable split of the C-1 proton signal in the presence of $(+)Eu(hfc)_3$ or (-)TFAE suggesting that isolated compound **I** is a single enantiomer.

Due to the absence of any chemical approach allowing independent synthesis of either authentic racemic or chiral, *cis*- or *trans*-1,1a-dihydroxy-1-hydro-9-fluorenone and the difficulty of predicting the structure from sign of optical rotation, establishing the absolute configuration of **I** will probably require arbitrary methods such as X-ray diffraction analysis.

It should be noted that transient formation of 1,1a-dihydroxy-1-hydro-9-fluorenone by *Pseudomonas* sp. F274 is evidently the result of several sequential reactions including monooxygenation of the methylenic group, its dehydrogenation to the ketone and angular reductive dioxygenation at positions 1-1a. Details of the bacterial catabolic pathway leading to the formation of I and its further utilization will be given in a separate publication. Although discussion of the entire biodegradation pathway for fluorene is beyond the scope of the present communication, formation of an angular dioxygenation product as an intermediate indicates the need for detailed studies of the catabolic pathways for other naphthenoaromatic compounds. The simple fact that such angular diols cannot be rearomatized to catechols by dehydrogenation (4) or by spontaneous reaction (as in the case of aryl ethers, ref. 12-16) should be noted. Catechols are frequently found as substrates for *meta*- or *ortho*-cleavage dioxygenase enzymes known to function in many "traditional" or "typical" pathways for bacterial catabolism of aromatic compounds (4). The existence of alternative biochemical strategies employed by bacteria for complete catabolism of simple naphthenoaromatic structures such as fluorene reiterates (11) how little is known of the biodegradation mechanisms for more complex aromatic hydrocarbons. Any bioremediation technology for the treatment of complex aromatic hydrocarbons must be firmly based on a knowledge and application of the most effective biological systems.

Such novel mechanisms and strains may also be effectively employed in specialty chemical technologies to approach inaccessible precursors of chirally functionalized materials. A number of the products of bacterial dioxygenation of aromatic compounds have been recognized as valuable synthons and are being commercially produced (26). The ability of bacterial strains such as *Pseudomonas* sp. F274 to perform angular dioxygenation of aromatic rings of a variety of fluorenone analogs and napthenoaromatic compounds is currently being examined in conjunction with studies of subsequent reactions of angular diols.

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