Isolation and Structure Determination of the Metabolites from *Pseudomonas Putida* 39D: Oxidation of di-Substituted Aromatics.

by
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APPROVED:

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Isolation and Structure Determination of the Metabolites from *Pseudomonas Putida* 39D: Oxidation of di-Substituted Aromatics

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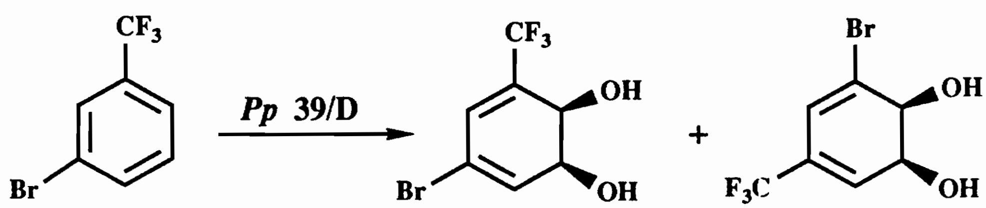
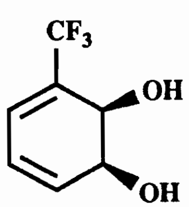
(Abstract)

m-Bromotrifluorotoluene, **1**, was subjected to microbial oxidation by *Pp* 39/D. The products from the reaction were isolated and identified by various spectral techniques. The absolute stereochemistry of the major metabolite has been determined as *cis*-ααα-trifluoromethyl 2R, 3S-dihydroxy-5-bromo-4,6-cyclohexadiene **2**.



1 **2** **3**

The absolute stereochemistry was discovered by comparison of the products from the convergent synthetic pathway of **2** and the known diol metabolite from α,α,α-trifluorotoluene, **4**.



4

To my parents

...for their love, support, guidance and wisdom

ACKNOWLEDGMENTS

I would like to thank my research advisor, Dr. Tomas Hudlicky, for his enthusiasm during my studies at VPI. Special thanks go to the members of my committee, Drs. James Tanko, Joseph Merola, David Becker and Robert White for their patience and support of my work.

It is a pleasure to acknowledge the members of Dr. Hudlicky's and Dr. Becker's groups for helpful discussions, friendship and support. Thanks to Drs. Christie Boros, Hector Luna, and Gregg Whited for training me in the process of microbial oxidation. Thanks also to Drs. Jacques Rouden and Martin Mandel for their fruitful advice.

Bill Bebout, Geno Ianaccone, and Tom Glass in Analytical Services deserve special mention for NMR help. Appreciation goes out to the undergraduates who performed numerous fermentations and extractions. I am grateful too for the help of Robert Harris in the proofreading of this manuscript.

Finally, I would like to thank all of the friends I have made at Tech, whether from organic, inorganic, polymer chemistry, biology, psychology or karate who never stopped believing in me.

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I. INTRODUCTION

The ability of several mutant bacterias to metabolize aromatic hydrocarbons via *cis* 1,2-dihydrodiols is well known.[1] Most such metabolites, enantiomerically pure cyclohexadiene *cis*-diols, or arene *cis*-diols, have proven quite useful in the total synthesis of various naturally occurring and man-made products. Synthetic targets that have been attained include: conduritols, conduramines, inositols, prostaglandin precursors, alkaloids, and terpenes.[2,3] Well over 100 of these arene *cis*-diols are known and new compounds are being isolated by application of different strains of the bacteria.[4]

The regiochemistry of the oxidation of small monocyclic arenes with *Pseudomonas Putida* 39D (*Pp* 39/D) appears predictable as the diol is introduced into the 2,3-position relative to the substituent. An exception to this observation is the oxidation of benzoic acids which furnishes 1,2-diols.[5] Studies of this oxidation have been extensive during the last twenty years[1] and include substrates ranging from monocyclic aromatic compounds to polycyclic systems such as naphthalene, phenanthrene, indole, and other heterocycles. The regiochemistry of oxidation of dichlorobenzenes has recently been reported[6] and studies are available on the oxidation of other disubstituted arenes.[7]

In an effort to determine the likely topology of the active site and to learn about the limits of the specificity of the enzyme, we initiated the study of m-disubstituted aromatics with the microbial oxidation of *m*-bromo-α, α, α-trifluorotoluene, **1**. The choice of bromine as a substituent was based on its characteristic as a potentially removable group, so that the metabolites could be compared to known monosubstituted diols. The choice of the trifluoromethyl group was based on the projected use of metabolites such as **2** in the synthesis of fluorinated derivatives of carbohydrates.

The following pages detail the biochemical origins and the structures of the known isolated cis-arene diols and a brief review of methods for determination of absolute stereochemistry.

II. HISTORICAL
1. Compilation of known cis-arene diols

The following table, a compilation of isolated diols, has been taken in part from the Ph.D. thesis of Austin S. McMordie from the School of Chemsitry, Queen's University of Belfast, Belfast, U.K. Most of the published *cis*-dihydrodiols up to 1989 were compiled by McMordie. Newly reported structures that have been identified since that date have been added to update the table which now includes over one hundred entries.

Isolation methods and the various strains of microorganisms along with the product(s) from the inital substrate have been listed. The key for the methods of identification of the various diols is shown below.

<u>Product Identification Method</u>

1. Product isolated - firm structural assignment by comparison with authentic samples or by ^{1}H NMR spectral analysis

2. Product isolated - structural assignment based on UV or MS spectral data or by identification of dehydration products

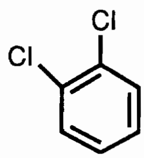
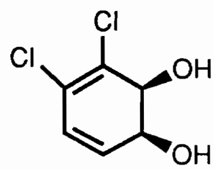
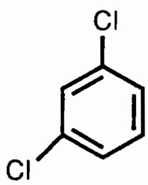
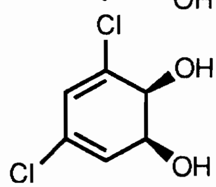
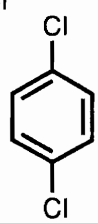
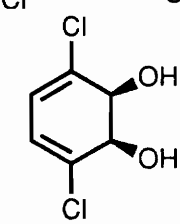
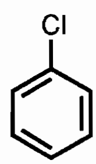
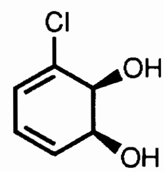
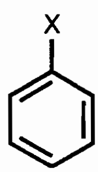
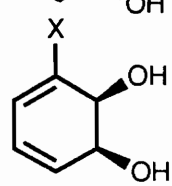
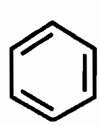
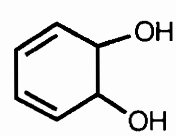
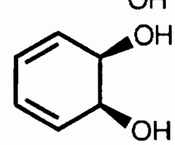
3. Product isolated - tenuous structural assignment

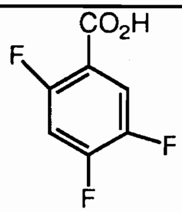
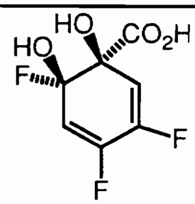
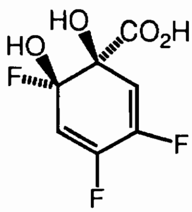
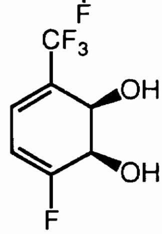
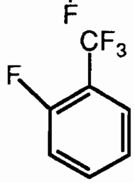
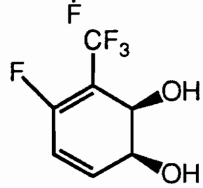
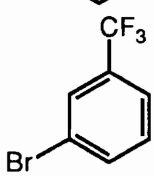
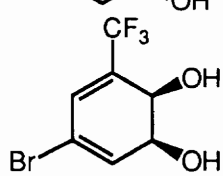
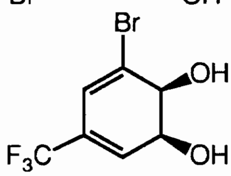
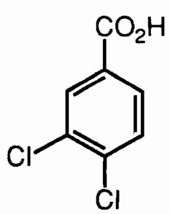
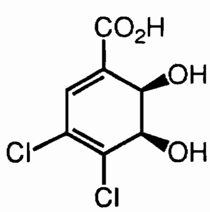
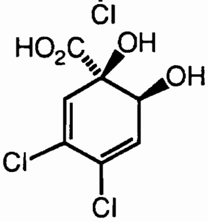
4. Product <u>not</u> isolated - structure of product proposed from nature of other degradation products

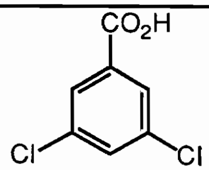
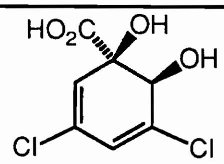
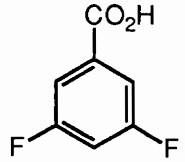
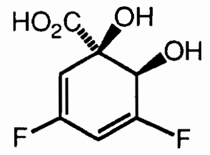
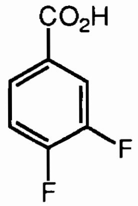
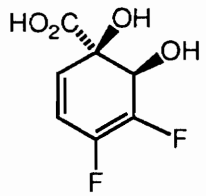
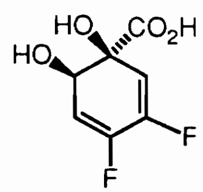
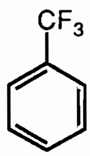
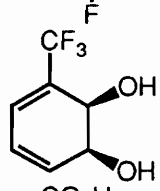
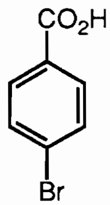
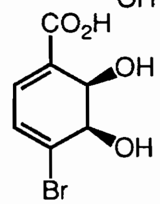
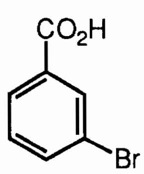
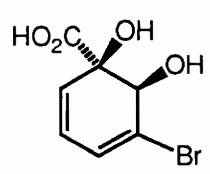
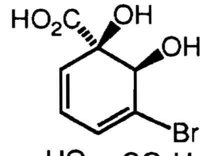
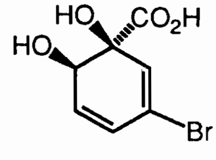
5. Product isolated - X-ray structure confirmation of diol or suitable derivative

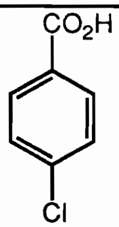
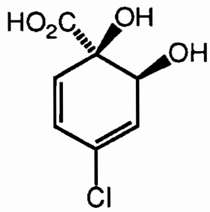
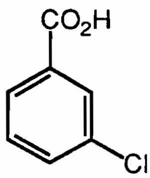
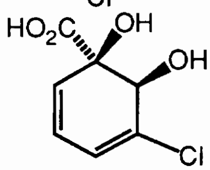
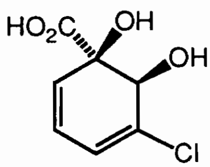
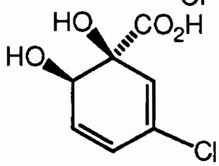
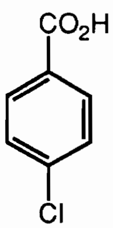
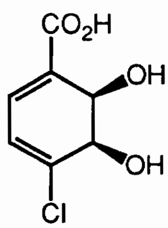
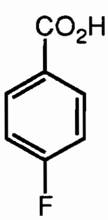
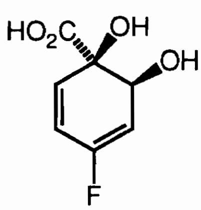
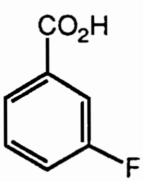
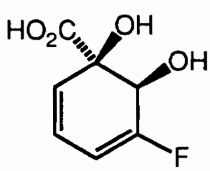
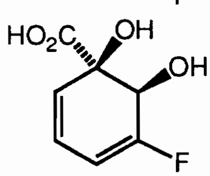
Table 1

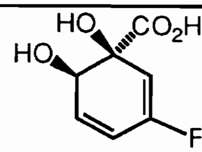
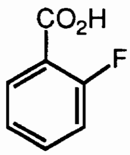
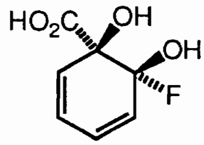
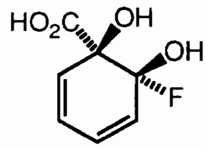
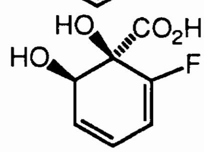
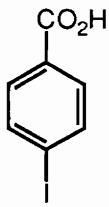
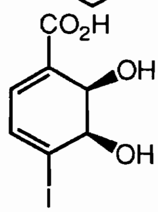
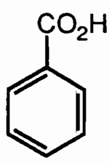
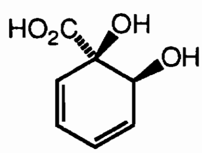
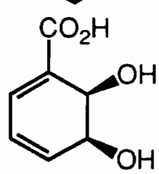
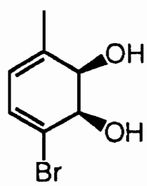
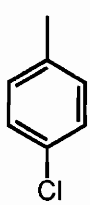
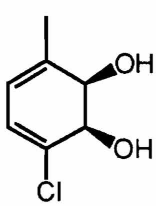
Products from the biotransformation of arenes and cyclic olefins

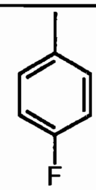
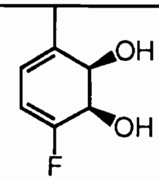
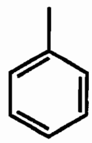
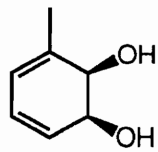
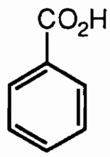
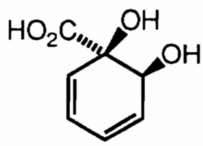
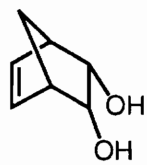
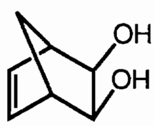
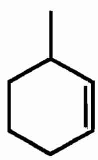
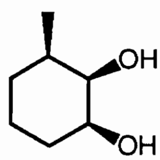
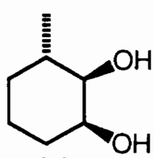
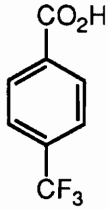
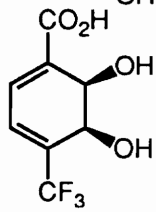
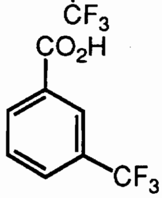
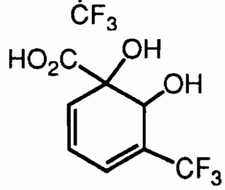
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
1 $C_6H_4Cl_2$			*Pp* F1	1	8
2 $C_6H_4Cl_2$			*Pp* F1	1	8
3 $C_6H_4Cl_2$			*Pp* F1	1	8
4 C_6H_5Cl			strain WR 1306	1	9,10,11
5 C_6H_5X	 X = Br, Cl, I, F*		*Pp* 39/D	1, 5*	12, 24*
6 C_6H_6			*Mycobacterium rhodochrous* *Pseudomonas aeruginosa*	4	13
			*P. putida* *Moraxella*	1	14,15,16

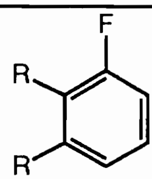
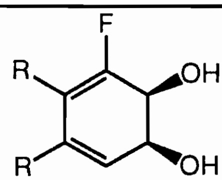
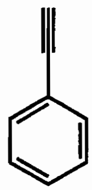
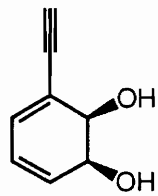
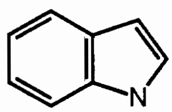
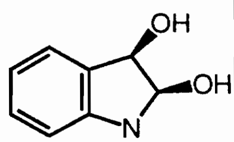
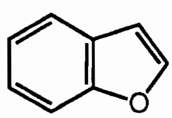
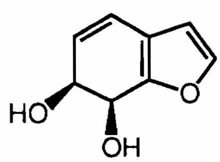
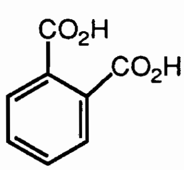
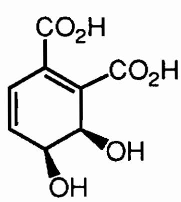
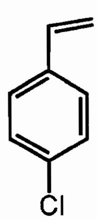
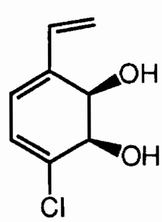
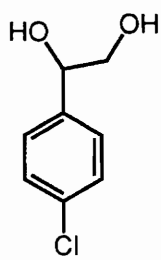
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
7 $C_7H_3F_3O_2$			*P. putida* JT 103	4	17
			*P. putida* JT 103	4	17
8 $C_7H_4F_4$			*P. putida* NCIB 12190	1	18
9 $C_7H_4F_4$			*P. putida* NCIB 12190	1	18
10 $C_7H_4BrF_3$			*Pp* 39/D	1	19
			*Pp* 39/D	1	19
11 $C_7H_4Cl_2O_2$			*P. putida* PL-pT-11/43	3	20
			*Pseudomonas sp.* B13	2	21

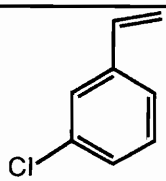
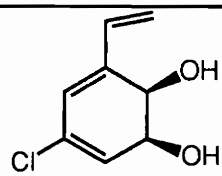
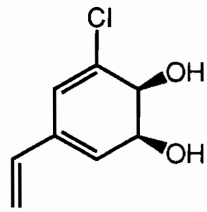
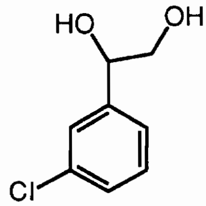
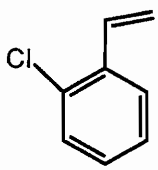
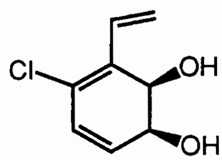
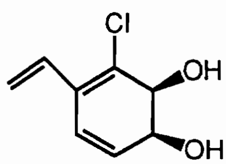
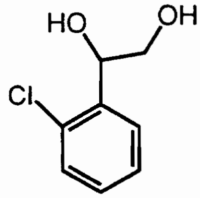
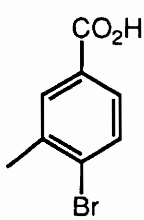
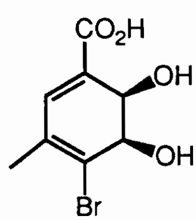
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
12 $C_7H_4Cl_2O_2$			*A. eutrophus* B9	1	21,22
13 $C_7H_4F_2O_2$			*P. putida* JT 103	1	17,23
14 $C_7H_4F_2O_2$			*P. putida* JT 103	1	23
			*P. putida* JT 103	1	23
15 $C_7H_5F_3$			*P. putida* UV4	1, 5	18,24
16 $C_7H_5BrO_2$			*P. putida* JT 107	1, 5	20,25
17 $C_7H_5BrO_2$			*A. europhus* B9	1	22
			*Pseudomonas sp.* B13	1	27
			*A. eutrophus B9*	1	22

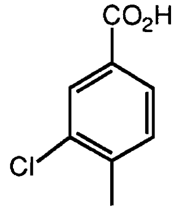
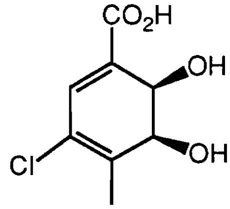
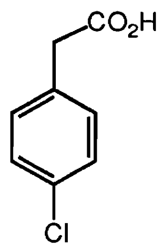
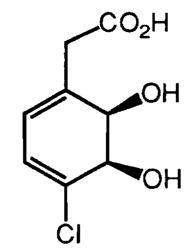
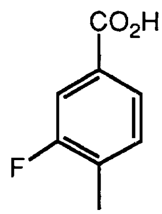
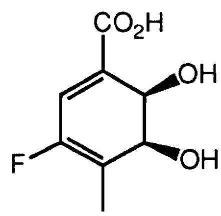
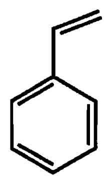
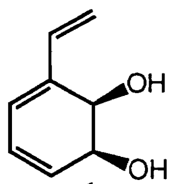
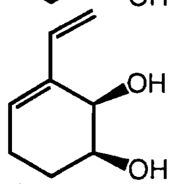
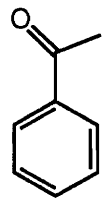
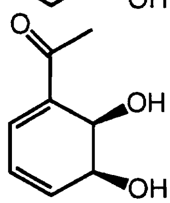
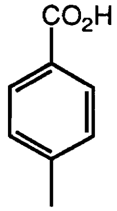
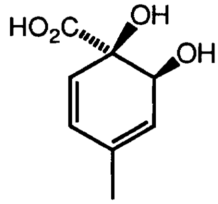
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
18 $C_7H_5ClO_2$			*A. eutrophus* B9	1	21,22
19 $C_7H_5ClO_2$			*A. eutrophus* B9 *P. putida*	1	21,22,28, 29
			*A. eutrophus* B9 *P. fluorescens*	2	21,22,30
			*Pseudomonas sp.* WR912	2	31
20 $C_7H_5ClO_2$			unidentified		32
21 $C_7H_5FO_2$			*Alcaligenes eutrophus* B 9	1	22,28
22 $C_7H_5FO_2$			*A. eutrophus* B9	1	22,28
			*Pseudomonas*	2	27

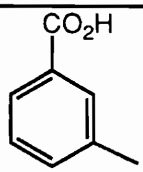
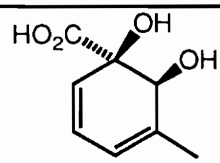
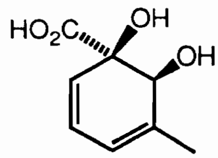
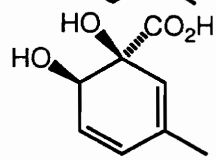
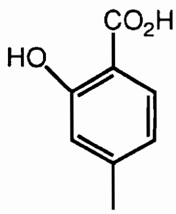
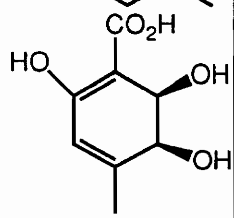
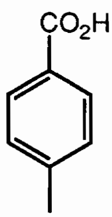
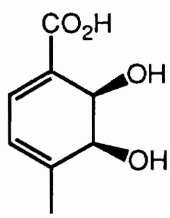
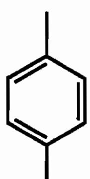
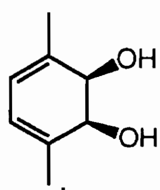
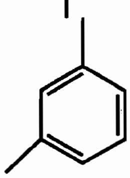
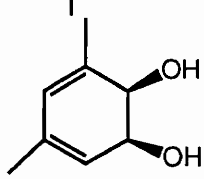
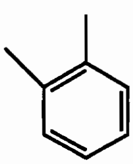
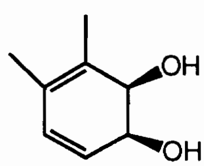
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
22 cont'd			*A. eutrophus* B9	2	22
23 $C_7H_5FO_2$			*A. eutrophus* B9	1	22
			*A. eutrophus* B9	2	33
			*Pseudomonas sp.* B39	2	34
24 $C_7H_5IO_2$			unidentified		35
25 $C_7H_6O_2$			*Alcaligenes eutrophus* B9	1	21,22,27, 28
			*P. putida* PL-pT-11/43	3	20
26 C_7H_7Br			*P. putida*	1, 5	9, 116
27 C_7H_7Cl			*P. putida*	1	9,36

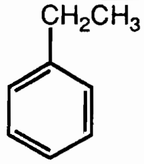
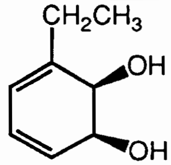
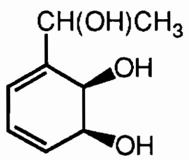
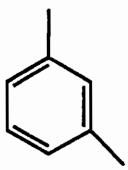
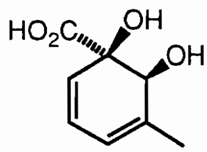
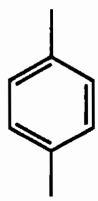
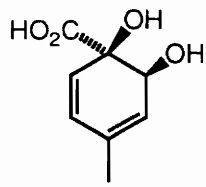
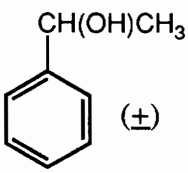
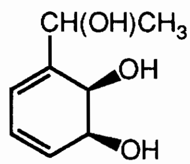
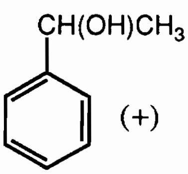
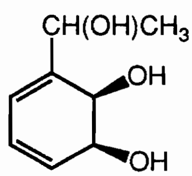
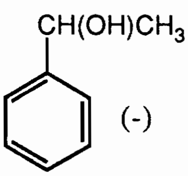
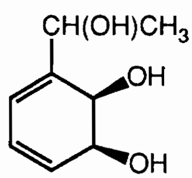
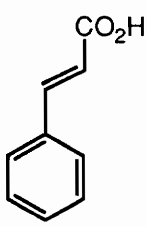
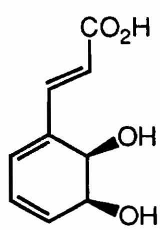
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
28 C_7H_7F			*P. putida*	1	9
29 C_7H_8			*P. putida*	1, 5	9,10,24, 37,38,39, 40
30 C_7H_8			*P. putida*	4, 5	41
31 C_7H_8			*Pp*/BM2	1	42
			*Pp*/BM2	1	42
32 C_7H_{11}			*P. putida* 39/D	1	9,40,43
			*P. putida* 39/D	1	9,40,43
33 $C_8H_5F_3O_2$			*P. putida* PL-pT-11/43	1	25,44
34 $C_8H_5F_3O_2$			*P. putida* mt-1 *A. eutrophus* B9	1	26

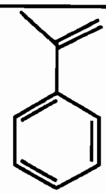
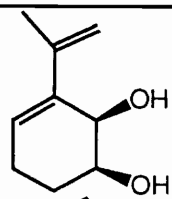
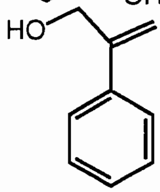
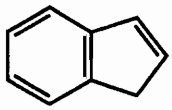
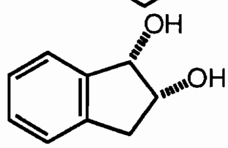
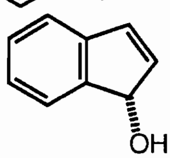
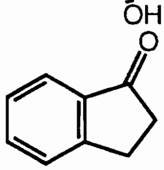
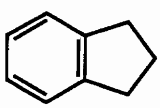
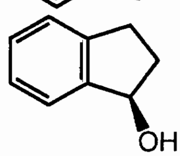
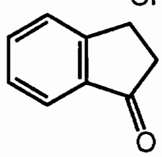
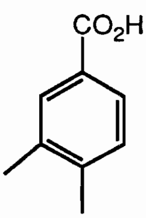
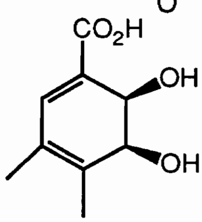
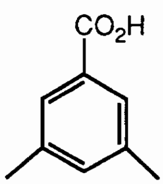
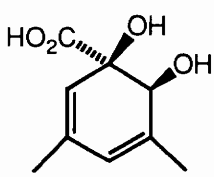
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
35 $C_8H_5FO_4$			*P. testosteroni*	1	45
	R = CO_2H				
36 C_8H_6			*P. putida* 39/D	1	10
37 C_8H_6N			*P. putida* PPG7 in *E. coli*	4	46
38 C_8H_6O			*Pp* UV4	1	47
39 $C_8H_6O_4$			*Micrococcus sp.*	4	48
40 C_8H_7Cl			*P. putida* 39/D	1	49
			*P. putida* 39/D	1	49

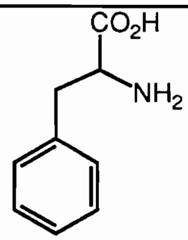
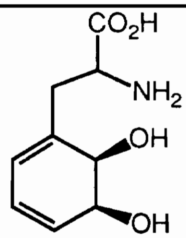
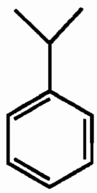
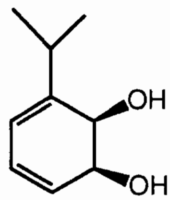
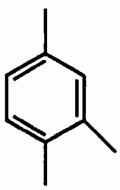
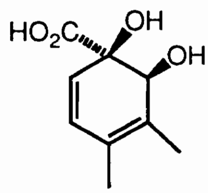
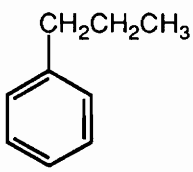
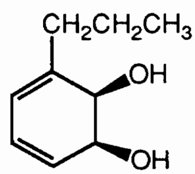
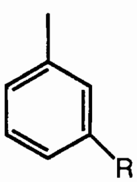
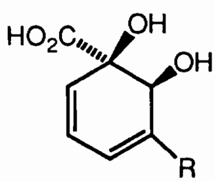
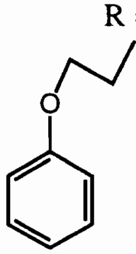
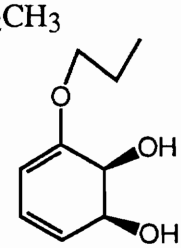
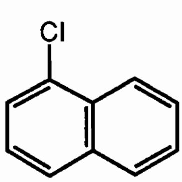
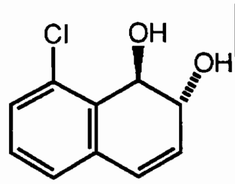
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
41 C_8H_7Cl			*P. putida* 39/D	1	50
			*P. putida* 39/D	1	50
			*P. putida* 39/D	1	50
42 C_8H_7Cl			*P. putida* 39/D	1	51
			*P. putida* 39/D	1	51
			*P. putida* 39/D	1	51
43 $C_8H_7BrO_2$			*P. putida* PL-pT-11/43	3	20

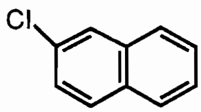
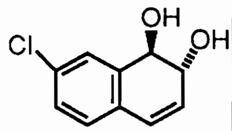
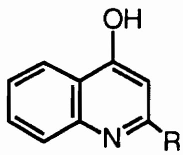
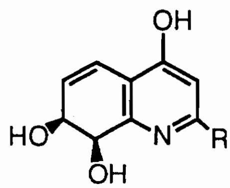
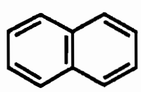
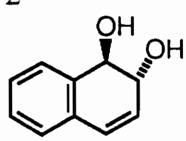
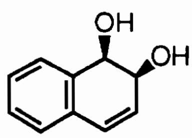
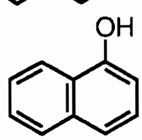
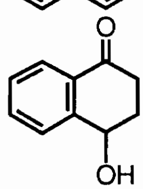
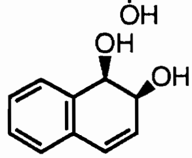
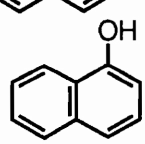
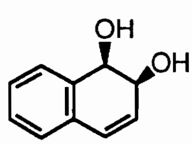
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
44 $C_8H_7ClO_2$			*P. putida* PL-pT-11/43		20
45 $C_8H_7ClO_2$			*Pseudomonas sp.* CBS 3	4	52
46 $C_8H_7FO_2$			*P. putida* PL-pT-11/34	3	20
47 C_8H_8			*P. putida* 39/D	1	10,53
			*P. putida* 39/D	1	54
48 C_8H_8O			*P. putida* 39/D	1	55
49 $C_8H_8O_2$			*A. eutrophus* B9	1	21,22

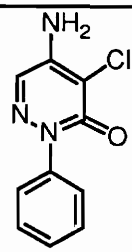
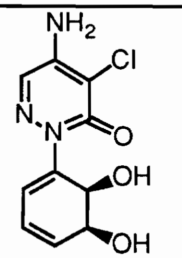
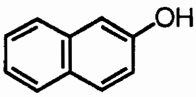
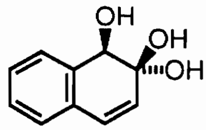
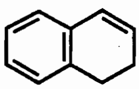
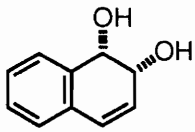
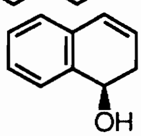
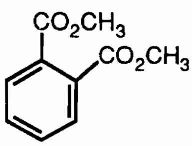
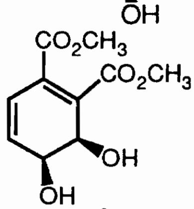
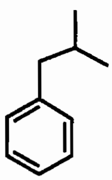
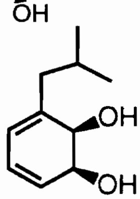
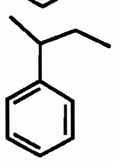
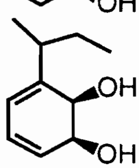
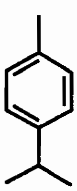
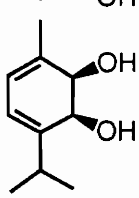
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
50 $C_8H_8O_2$			*A. eutrophus* B9	1	21,22,27
			*Pseudomonas sp. B13*	2	21,27
			*Pseudomonas sp.* B13	2	21,27
51 $C_8H_8O_3$			*P. putida* PL-pT-11/43	3	20
52 $C_8H_8O_2$			*P. putida* JT 107	1	20
53 C_8H_{10}			*P. putida* 39/D	1	56
54 C_8H_{10}			*P. putida* 39/D	2	56
55 C_8H_{10}			*Norcardia*	4	57

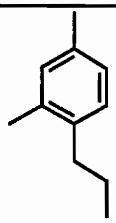
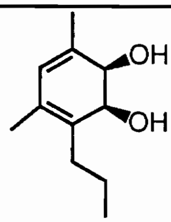
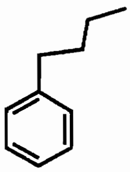
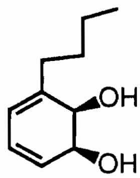
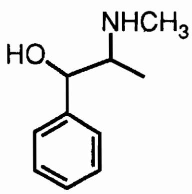
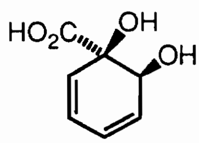
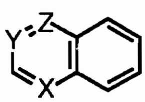
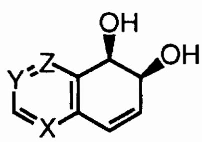
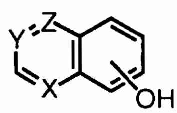
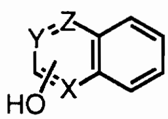
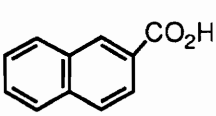
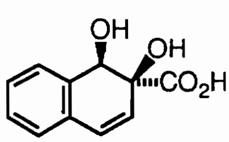
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
56 C_8H_{10}			*P. putida* 39/D	1	55
			*P. putida* 39/D	1	55
57 C_8H_{10}			*P. putida*	4	41,58
58 C_8H_{10}			*P. putida*	4	41,58
59 $C_8H_{10}O$			*P. putida* 39/D	1	55
60 $C_8H_{10}O$			*P. putida* 39/D	1	55
61 $C_8H_{10}O$			*P. putida* 39/D	1	55
62 $C_9H_8O_2$			unidentified	2	59,60

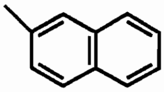
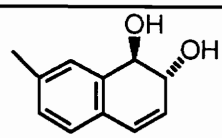
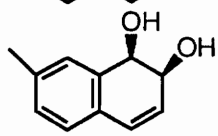
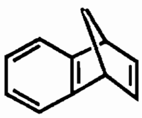
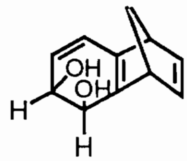
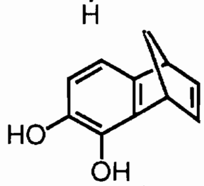
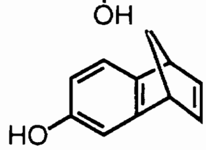
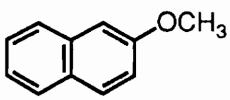
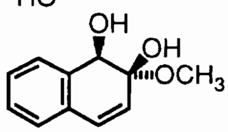
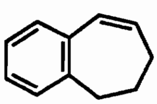
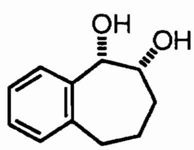
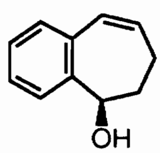
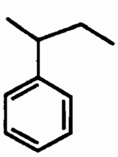
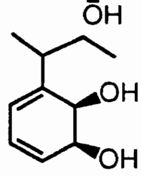
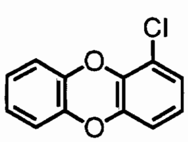
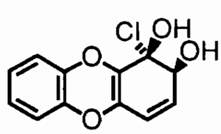
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
63 C_9H_{10}		 	*P. putida*	1	54,61
		 	*P. putida*	1	54,61
64 C_9H_8		 	*P. putida* 39/D	1	62
		 	*P. putida* 39/D	1	62
		 	*P. putida* 39/D	1	62
65 C_9H_{10}		 	*P. putida* 39/D	1	62
		 	*P. putida* 39/D	1	62
66 $C_9H_{10}O_2$	 	 	*P. putida* PL-pT-11/43	3	20
67 $C_9H_{10}O_2$	 	 	*Pseudomonas sp.* B13	2	21

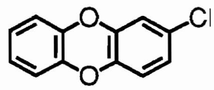
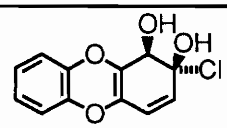
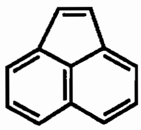
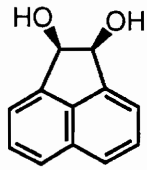
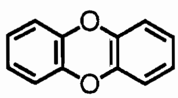
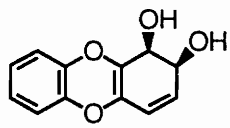
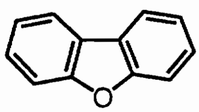
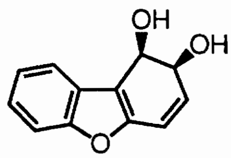
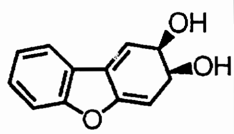
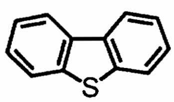
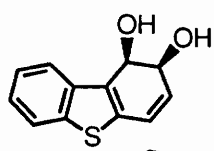
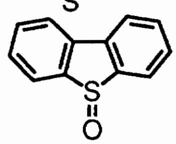
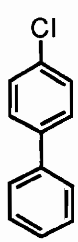
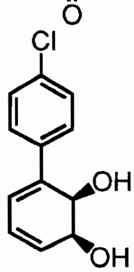
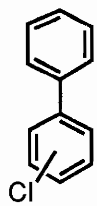
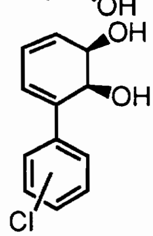
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
68 $C_9H_{11}NO_2$			unidentified	4	60,63
69 C_9H_{12}			*P. desmolytica* *P. convexa*	2	64
70 C_9H_{12}			*P. putida*	4	41
71 C_9H_{12}			*Pp* F1	1	8
72 C_9H_{12}			*P. putida*	4	51
	R = CH_2CH_3				
73 $C_9H_{12}O$			*P. putida* 39/D	1	53
74 $C_{10}H_7Cl$			soil bacteria	3	65

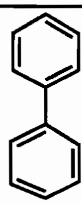
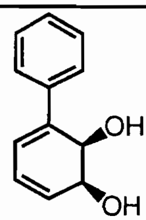
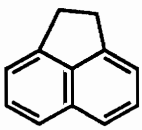
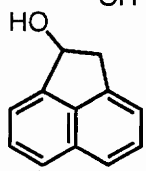
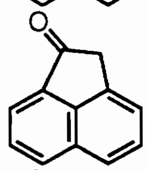
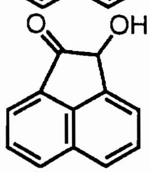
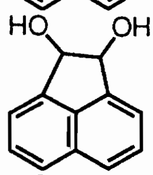
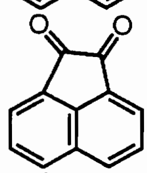
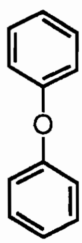
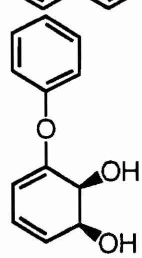
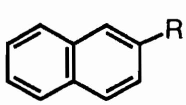
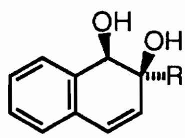
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
75 $C_{10}H_7Cl$			*P. desmolyticum* *Pseudomonas sp.* A3;C22	3	66,67
76 $C_{10}H_7O_3$			partially purified enzyme from *P. fluorescens*	2	68
	R = CO_2H				
77 $C_{10}H_8$			soil pseudomonads	3	69,70,71
			*P. putida* 119 *Pseudomonas sp.* NCIB9816	1	72,73,74
			*Oscillatoria sp.* strain JCM	2	75,76
			*Oscillatoria sp.* strain JCM	2	75,76
			*Agmenellum quadruplaticum*	2	75,77
			*Agmenellum quadruplaticum*	2	75,77
			microbial populations from two Arkansas lake ecosystems	2	78

Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
78 $C_{10}H_8ClN_3O$			unidentified	1	79
79 $C_{10}H_8O$			*P. testosteroni*	4	80
80 $C_{10}H_{10}$			*Pp*/UV4		81
			*Pp*/UV4		81
81 $C_{10}H_{10}O_4$			*Micrococcus sp.* *P. testosteroni*	4	48
82 $C_{10}H_{14}$			*P. desmolytica* *P. convexa*	2	64
83 $C_{10}H_{14}$			*P. putida* *P. acidovorans*	2	82
84 $C_{10}H_{14}$			*P. putida* PL-pT-11/43	1	20

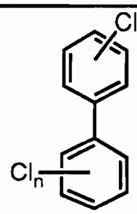
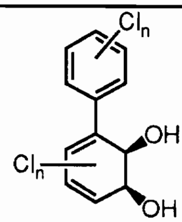
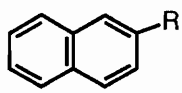
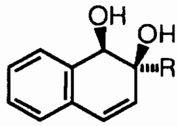
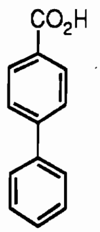
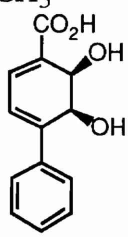
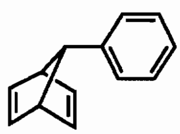
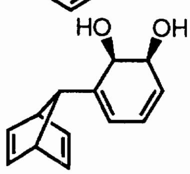
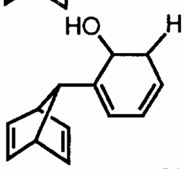
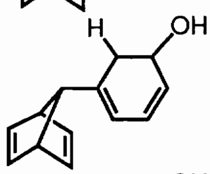
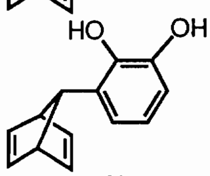
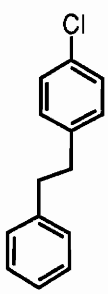
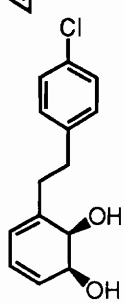
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
85 $C_{11}H_{16}$			*P. putida* PL-pT-11/43	3	20
86 $C_{10}H_{14}$			*Pp* F1		8
87 $C_{10}H_{15}NO$			*P. putida* B 1	4	82,83
			Anthrobacter globiformis		82,84
88 $C_{10}H_8$			*Pp*/39D	1	85
			*Pp*/39D	1	85
			*Pp*/39D	1	85
	X = Y = Z = CH X = N; Y = Z = CH Y = N; X = Z = CH X = Y = N; Z = CH X = Z = N; Y = CH				
89 $C_{11}H_8O_2$			*P. testosteroni*	2	80

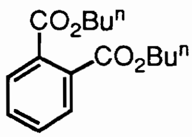
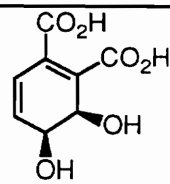
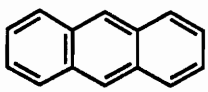
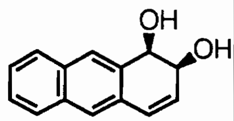
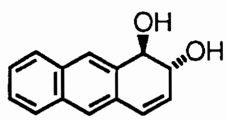
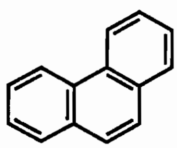
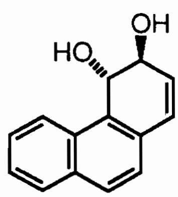
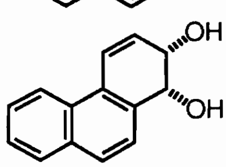
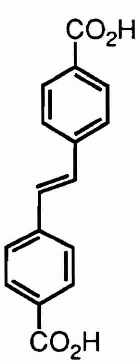
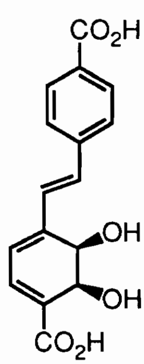
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
90 $C_{11}H_{10}$			soil bacteria	3	66
			*Pseudomonas sp.* A3;C22	4	67
91 $C_{11}H_{10}$			*Pp*/BM2	1	42
			*Pp*/BM2	1	42
			*Pp*/BM2	1	42
92 $C_{11}H_{10}O$			*Pseudomonas sp.* A3;C22	4	67
93 $C_{11}H_{11}$			*Pp*/UV4	1	81
			*Pp*/UV4	1	81
94 $C_{10}H_{14}$			*P. putida* *P. acidovorans*	2	82
95 $C_{12}H_7ClO_2$			*Beijerinckia* B836	2	86

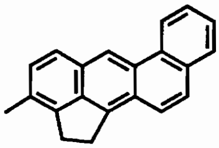
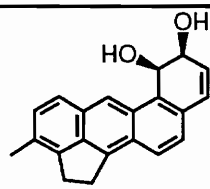
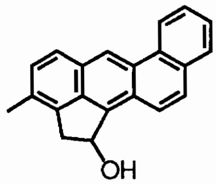
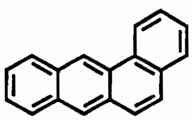
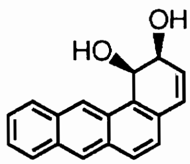
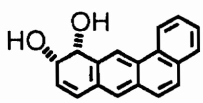
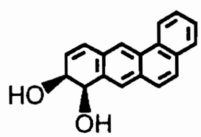
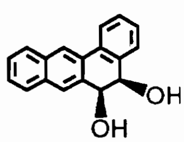
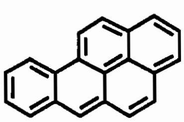
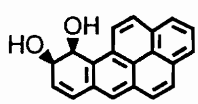
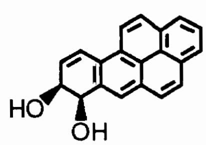
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
96 $C_{12}H_7ClO_2$			*Beijerinckia* B836	2	86
97 $C_{12}H_8$			*Beijerinckia ap.*	2	87
98 $C_{12}H_8O_2$			*Pseudomonas* NCIB9816 *Beijerinckia* B836	1	86
99 $C_{12}H_8O$			*Beijerinckia* B836	1	88
			*Beijerinckia* B836	2	88
100 $C_{12}H_8S$			*Beijerinckia* B836	1	89,90
			*Beijerinckia* B836	1	89,90
101 $C_{12}H_9Cl$			*Pseudomonas sp.*	4	57
102 $C_{12}H_9Cl$			*Pp* F1		8

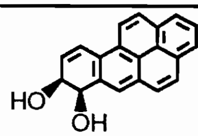
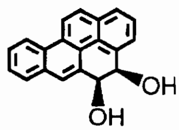
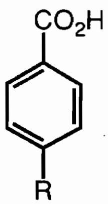
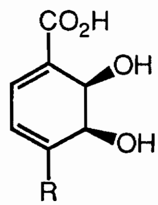
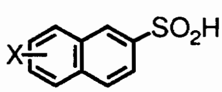
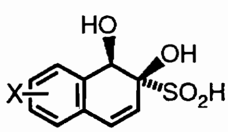
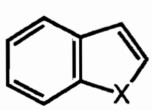
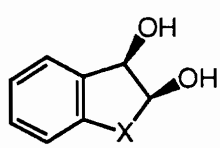
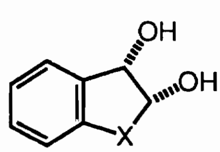
Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
103 $C_{12}H_{10}$			*Beijerinckia* B836 *P. putida*	1	9,92,93
104 $C_{12}H_{10}$			*Beijerinckia sp.*	2	87
			*Beijerinckia sp.*	2	87
			*Beijerinckia sp.*	2	87
			*Beijerinckia sp.*	2	87
			*Beijerinckia sp.*	2	87
105 $C_{12}H_{10}O$				1	91
106 $C_{12}H_{10}O_2$			*Pseudomonas sp.* A3;C22	1	45,67

$R = CO_2CH_3$

Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
107 $C_{12}H_xCl_n$			*Alcaligenes sp.* strain Y2	3	94
108 $C_{13}H_{12}O$	 R = CH_2COCH_3		*Pseudomonas sp.* A3;C22	1	67
109 $C_{13}H_{10}O_2$			unidentified		32
110 $C_{13}H_{12}$			*Pp*/BM2	1	42
			*Pp*/BM2	1	42
			*Pp*/BM2	1	42
			*Pp*/BM2	1	42
111 $C_{14}H_{13}Cl$			*Pseudomonas sp.*	4	57

Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
112 $C_{14}H_{22}O_4$			*Micrococcus sp.*	4	48
113 $C_{14}H_{10}$			*Flavobacterium*	3	68
			*P. putida* strain 119 *Beijerinckia* B836	1	95,96
114 $C_{14}H_{10}$			*Flavobactirium*	3	97
			*P. putida* strain 119 *Beijerinckia* B836	1	95,98
			*P. putida* strain 119 *Beijerinckia* B836	1	95,98
115 $C_{16}H_{12}O_4$			*P. putida* PL-pT-11/43	3	20

Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
116 $C_{21}H_{16}$			*Bejerinckia sp.*		57
			*Bejerinckia sp.*		57
117 $C_{18}H_{12}$			*Beijerinckia* B836	1	99,100
			*Beijerinckia* B836	1	99,100
			*Beijerinckia* B836	2	99,100
			*Beijerinckia* B836	1	99,100
118 $C_{21}H_{12}$			*Beijerinckia* B836	1	99
			*Beijerinckia* B836	2	99

Entry #	Substrate	Product	Bacterium	Product Id. Method	Reference
118 cont'd			*Selenastrum capricornicum* (green algae)	2	101
			*Selenastrum capricornicum* (green algae)	2	101
119			*P. putida* PL-pT-11/43	3	20
	R = H; CH_3; C_2H_5; iC_3H_7; $t\text{-}C_4H_9$; C_6H_5; $C_6H_5CH_2$; C_6H_5CO; C_2H_5O; C_3H_7O; $n\text{-}C_4H_9O$; Cl; Br; I; CH_2Br; CF_3O				
120			*P. testosteroni* *Pseudomonas sp.* A3; C22; BN6; BN9	4	67 45,80
	X = 5-NH_2; 6-NH_2; 7-NH_2; -NH_2; 5-OH; 6-OH; 7-OH; 8-OH				
121			*Pp*/UV4	1	102
			*Pp*/UV4	1	102
	X = CH_2CH_2; OCH_2; SCH_2; CH_2; O; S; NH; CH=CH				

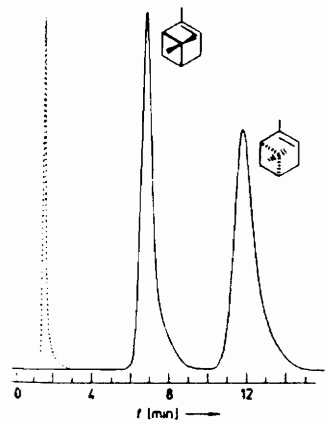
II. HISTORICAL
2. Review of methods for determination of absolute configuration

The determination of absolute configuration of new optically active products derived from natural sources remains as one of the problems in natural product chemistry. The first determination of a three-dimensional structure of a molecule was reported in 1951, seventy-five years after the tetrahedral disposition of the substituents on a carbon was recognized and proposed by van't Hoff and Le Bel.[103]

X-ray diffraction was the technique which was modified by Bijvoet in 1951 to elucidate the "depth" of photographic patterns. This technique tends to become tedious and time-consuming as it requires the expertise of a skilled crystallographer and a suitable crystal from a pure solid preferably containing elements with high electron density. This is however, to date the only method that can determine *absolute* configuration. All other methods are correlative. That is, they relate information obtained about a particular compound to that of another.

Several methods exist that determine the relative configuration of stereocenters in molecules. These include: CD/ORD, chemical degradation and NMR analysis.[104] Within the last five years, rapid progress in developing GC and HPLC methods of analysis has been apparent.[105] These methods require that the racemate of the compound be resolved into two enantiomers on the column. If only one enantiomer is available, then the method becomes ambiguous.

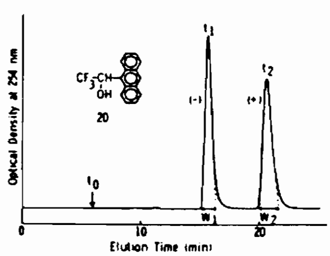
GC analysis proves useful for separating enantiomers of compounds which can be vaporized without decomposition. This method would not be useful for the cyclohexadiene diols since these compounds readily aromatize when exposed to heat. However, for better suited compounds, this method requires small sample size and generally short separation times without need for previous derivatization. As shown below, cis and trans pinane are easily separated on packed columns with a support coating of α cyclodextrin in formamide.[106]



Separation of the enantiomers of α-pinene on α-cyclodextrin in formamide solution supported on Celite at 44 °C. Packed column, 2 m × 4 mm. The chromatogram shown as a broken line was obtained on pure formamide (without cyclodextrin) supported on Celite. Carrier gas, helium, 2.75 bar [82].

Figure 1

GC methods are preferred for quality control in pharmaceutical applications, since they are more precise than NMR methods. HPLC separations are also used due to their enhanced sensitivity and improvements in colum lifetime. Several useful chiral stationary phases have been recently reported in this area. For example, microcrystalline cellulose triacetate supported on silica gel has a unique chiral recognition ability. Trifluoroethanol can be resolved in approximately twenty minutes with complete separation.[107]



Chromatographic resolution of 1-(9-anthryl)-2,2,2-trifluoroethanol (20) on a cellulose tris-(4-tolylcarbamate) (2) column. (Column, 250 × 4.6 mm I.D.; eluent, hexane–2-propanol (90:10, 0.5 ml/min); temperature, 25°C; t_0, 6 min).

Figure 2

GC /HPLC methods are still being developed. Thus, when these methods of analysis fail, the methods of CD/ORD, chemical degradation and NMR must be employed for determination of the configuration of a new compound.

CD/ORD (circular dichroism/optical rotatory dispersion) consists of analysis of light measurements. The measurment of the difference in absorption of a sample between right and left polarized light right and left is called circular dichroism. A similar experiment, ORD, involves the measurment of optical rotation, α, as a function of wavelength, λ. For compounds that absorb in the far ultraviolet region (<220 nm), α usually increases as λ decreases. If a compound absorbs in the normal region (λ=250-650 nm) then an abnormal curve is usually observed. When these measurments are obtained for an unknown along with a similarly structured compound with known configuration and overlayed on a plot of λ versus α, a powerful correlative method is revealed.

Analysis of the non-linear plots provide general trends. If the curves are of the same sign, the probability is high that the configuration is the same. However, if the curves are mirror images of each other, then the compounds are most likely of opposite configuration. This method has been used for the determination of absolute configuration for many diols. For example, the curve of methyl-naphthalene diol, **5**, (isolated in our laboratory)[108] was compared to that of naphthalene diol, **6**, which had been previously isolated by Gibson and the absolute configuration had been proven by converting naphthalene diol, **6**, via reduction to 2S-hydroxy-1,2,3,4-tetrahydro-naphthalene.[109] In the figure 1 below, the ORD plots show that the curves follow the same general trend and have the same sign. Therefore, the stereochemistry of the methyl-naphthalene diol, **5**, has been assigned as that of naphthalene diol, **6**.

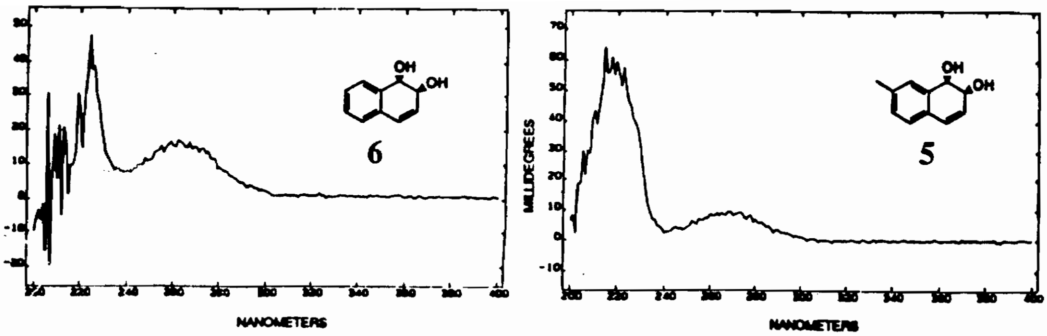
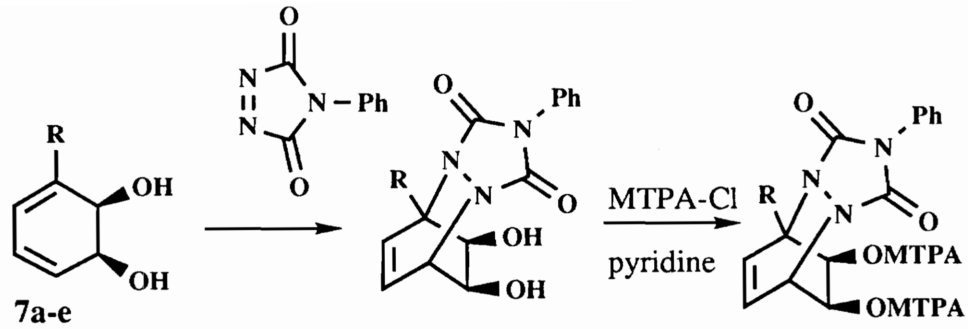


Figure 3

A rather popular method has recently been developed by Boyd for the determination of absolute configuration of *cis*-dihydrodiols. This method was first developed by Dale and Mosher,[110] and involves a correlation of configuration with NMR chemical shifts for diastereomeric α-methoxy-α-trifluromethylphenyl acetate (MTPA) derivatives of chiral alcohols.

Initial attempts by Boyd and co-workers to form MTPA derivatives of the cis dihydrodiols resulted in aromatization. Therefore, the *cis*-diols were reacted in a Diels-Alder fashion with 4-phenyl-1,2,4-triazoline-3,5-dione at ambient temperature. Treatment of this cycloadduct, **8**, which was formed as a single diastereomer (>97%), with the acid chloride of either R or S MTPA in pyridine yielded the corresponding di-MTPA ester in quantitative yield.[111]

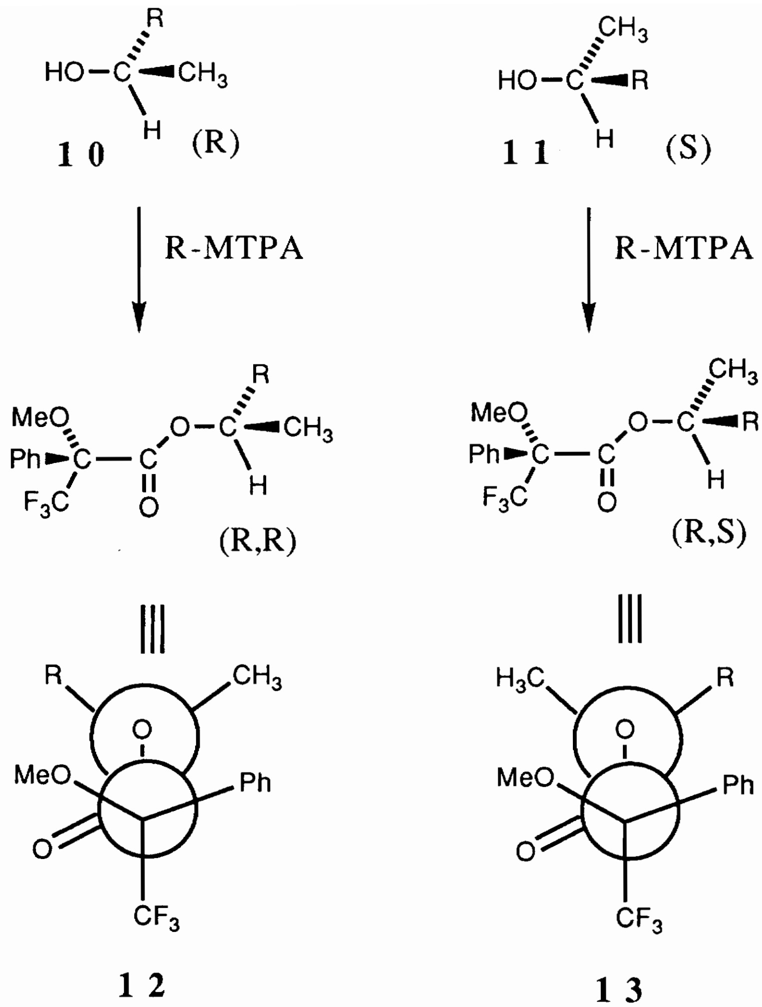


R=Cl, CH_3, CF_3, Et, F, CH_2OAc

^{1}H NMR analysis of the Mosher esters **9** with attention to the MeO and CF_3 signals, showed the sample to be homochiral. An additional structure proof using x-ray analysis, revealed that the cycloaddition reaction occurred exclusively syn to he hydroxyl groups and confirmed the R or S configuration of the chiral ceters which had been previously proven by ^{1}H NMR.

The diagnostic NMR signals, MeO and CF_3, prove either R or S configuration in the following way. A *cis*-dihydrodiol having a [1S] configuration will give a characteristic downfield MeO signal with a larger positive δ_{1H} value (3.61 to 3.65 ppm) and a smaller negative δ_{19F} value (-7.66 to -8.39) when the +R enantiomer of MTPA is used. On the other hand, the diMTPA ester from a diol with [1R] configuration shows a smaller δ_{1H} value (3.21 to 3.55 ppm) and larger negative δ_{19} value (-8.72 to -9.28) for the corresponding signals when the same +R MTPA is utilized.

This observation can be explained using the following diagram with the formation of diastereomeric esters.

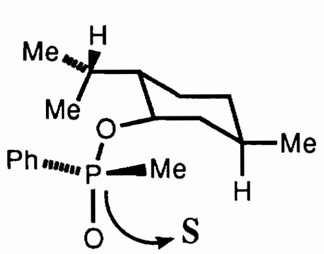


The R,R configuration causes the alkyl group to be juxtaposed with the methoxy group while the R,S configuration has the alkyl group juxtaposed with the phenyl ring. The interaction with the phenyl ring is the cause for the upfield shift of the S alcohols. The success of the method depends of course on the resolution of the signals of the diastereotopic methyls. Without adequate resolution, the method fails. It is advisable to obtain x-ray structures for unambiguous confirmation.

The method is presently under investigation for applicability to cis-dihydrodiols of monocyclic arenes bearing several substituents. It has already been extended for the determination of the absolute configuration of polycyclic arenes and heteroarenes.[112] The same trend for δ^{1}H values was observed when the determining the R or S configuration. However, in some cases the MTPA ester could not be obtained through synthesis and another method for the determination of configuration was employed. It is not foolproof, as the observed $\Delta\delta$-difference is highly structure dependent and some types of compounds may not resolve in the NMR experiment.

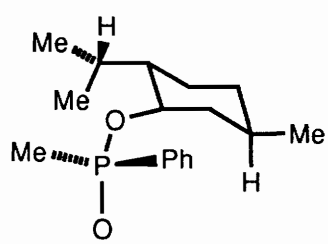
Other NMR techniques are available for the determination of absolute configuration of the chiral centers in a molecule. These methods include: analysis of phosphinate derivatives with ^{31}P NMR,[113] and the use of methylmandelate esters for the configuration of secondary alcohols.[114]

Mislow and co-workers observed that the ^{31}P NMR spectra of menthyl phosphinates were diagnostic at the configuration of the phosphorus. The S configuration at phosphorus indicated below, depicts the diastereomer where the signal for the methyls on the isopropyl group have suffered an upfield shift, (approximately 0.5 ppm). Such a shift is caused from the diamagnetic anisotropy of the phenyl ring.



-Menthyl-S-methylphosphinate

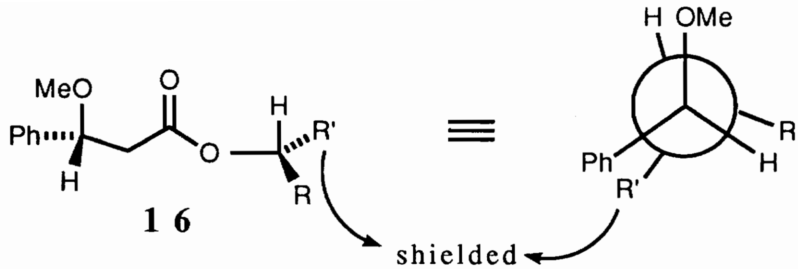
14



-Menthyl-R-methylphosphinate

15

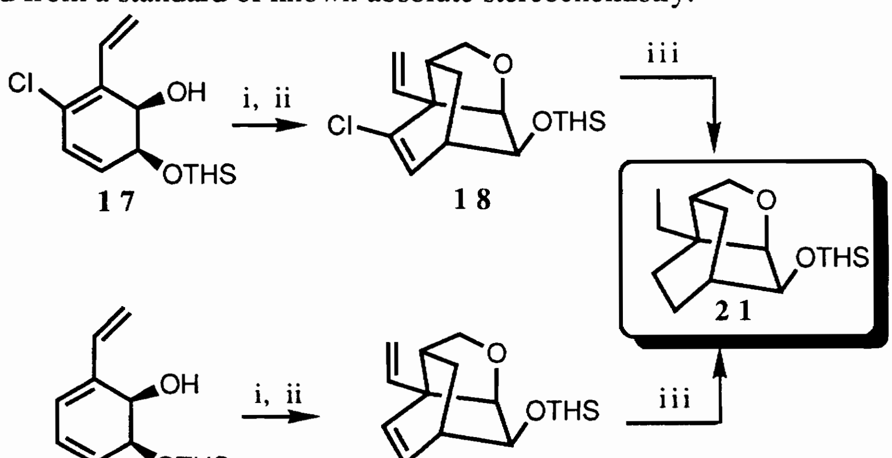
A similar trend occurs when determining the absolute configuration of secondary alcohols using methylmandelate esters. The phenyl ring again eclipses a certain substituent an causes an upfield shift due to the shilding of the phenyl ring.



16

Therefore, the configuration of the alcohol can be determined since the S mandelic acid is involved in the derivatization.

Besides NMR analysis, chemical degradation or alteration of a new chiral natural product is yet another way to determine or correlate the absolute configuration of a molecue. As shown below, a Diels-Alder strategy was employed in a few cases to convert an unknown diol metabolite into a known compound such as **21**, which is also prepared from a standard of known absolute stereochemistry.[115]



17, **18**, **19**, **20**, **21**

i. NaH, allyl bromide, THF -20°C ii. CCl_4 rt to 80°C iii H_2, Pd/C, EtOH

$[\alpha]_D^{20}$ = -32° from 18; $[\alpha]_D^{20}$ = -30° from 20

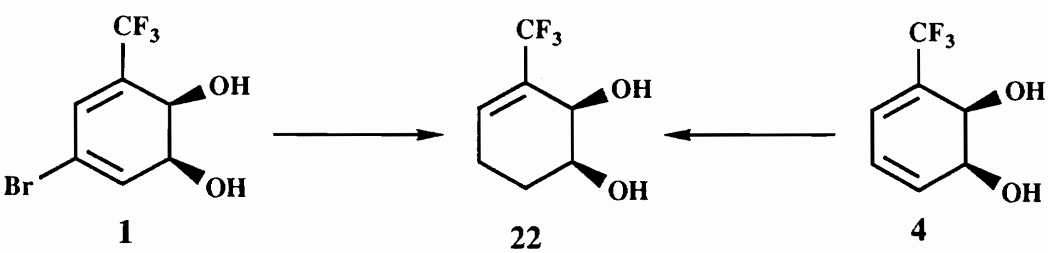
The mono-protected diol obtained from chlorostyrene, **17**, was converted to an oxatricyclodecane derivative, **21**, by a convergent synthesis and compared to the same intramolecular Diels-Alder adduct of the known metabolite from styrene. The products from the two reactions had the same optical rotation, thus establishing the absolute stereochemistry.

An example involving the hydrogenation of a newly isolated *cis*-diol to a known compound forms the topic of this dissertation. Application of this method will be disclosed in the discussion section as the method used for determining the absolute configuration of the metabolites obtained from the oxidation of *m*-bromotrifluorotoluene by *Pp* 39/D.

III. DISCUSSION
1. Introduction

Oxidation of di-substituted aromatics has been studied by Ribbons and co-workers.[116] The microbial oxidation studied involves an operant enzyme called toluene dioxygenase. Although the exact characteristics of the enzyme are not known, it is apparent that it oxidizes substituted aromatics with remarkable specificity while tolerating a wide range of stereoelectronic parameters.[117]

The microbial oxidation process involves several steps which will be discussed in detail in the next section. When the new diols are obtained from such a process, it is necessary to determine the absolute stereochemistry and enantiomeric excess of the major metabolites. In the case of disubstituted compounds, the previously mentioned method of Boyd[24] could not be used for the determination of absolute stereochemistry because this method has only been proven for mono-substituted diols. Thus, a convergent synthesis was employed for the means of structure correlation.

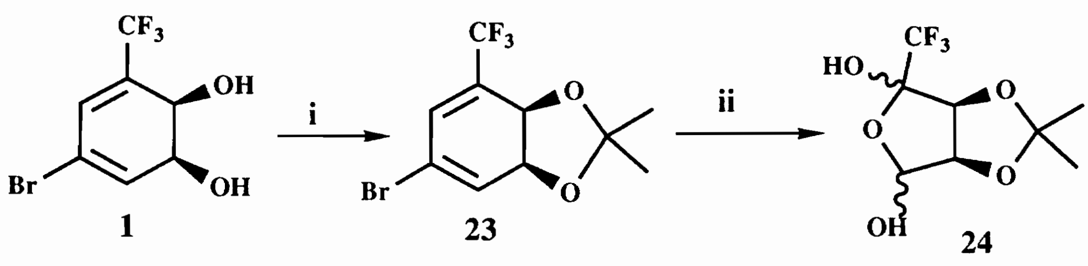


The synthesis involved the correlation of the known diol derived from trifluorotoluene, **4**, and the major diol product, **1**, from m-bromotrifluorotoluene. Optical rotations were observed from the products from each of the respective pathways, which proved the regiochemistry and enantiomeric excess as well as the absolute stereochemistry of the di-substituted diol, **1**.

The absolute stereochemistry of the minor metabolite, **2**, which was obtained in trace amounts from the *Pp* 39/D oxidations has not been established for several reasons. From a synthetic point of view, it is better to obtain a single product from such a transformation in order to avoid the tedious and difficult separation of isomers. When the biotransformation with m-bromotrifluorotoluene was run using the overexpressed recombinant organism, *E. Coli* JM109(pDTG601),[118] the minor metabolite, **2**, was not detected! This microorganism, which contains the four structural genes of toluene dioxygenase,[119] enhances the efficiency of the biotransformation. The main focus of the discussion will describe the structure correlation for the major product, **1**, from the *Pp* 39/D oxidations.

III. DISCUSSION
2. Microbial oxidation and isolation of new cis-diol metabolites

Before discussion of the microbial oxidation production process, some substantial and relevant research results regarding product isolation performed by two former co-workers will be mentioned. This project was initially investigated in 1989 by Dr. Monica DeLuca and later studies were performed by Dr. Denise Parker in 1990.[120] The first production of the major metabolite from m-bromo-trifluorotoluene was realized using a 500 ml solution of (MSB)[121] in an Erlenmeyer flask and an incubator box. The crude yield of the reaction was reported as 40 mg. After protection of the diol, **1**, as an acetonide, **23**, and ozonolysis in ethyl acetate, a hemiketal, **24**, was obtained. ^{19}F NMR studies showed the presence of fluorine in the compound with two singlets at -66 and -67 ppm which represented the CF_3 groups in the diastereomers of **24**.



i. DMP, acetone, pTSOH **ii.** O_3, Me_2S

Thus, this inital study provided information about the regiochemistry of the diol. In other words, the CF_3 group directed the oxidation in a manner similar to that of CF_3 in trifluorotoluene or the methyl group of toluene. Absolute stereochemistry of the diol at this point still remained undetermined.

When the project was continued in 1990 by Dr. Parker, the diol production process at this point was performed in a 2L fermentor with a 1.5 L working volume. With a larger fermentor, isolation of a greater quantity of material was possible.

This latter investigation revealed not one product from the reaction, but three compounds: 1. the diol, **7a**, derived from chlorobenzene from the induction period in which chlorobenzene was used, the diol that Dr. DeLuca obtained, **1**, and **2**, another regioisomeric diol from m-bromotrifluorotoluene. Separation of the two regioisomers proved difficult since they displayed only a slight difference in polarity .

Separation and purification of the products was performed by Dr. Parker using preparative TLC. However, the minor metabolite was never characterized in the 1990 study. At this stage of the project, the absolute stereochemistry of both the major and minor metabolite from the oxidation was unknown. The regiochemistry was also unknown, except for the tentative study performed by Dr. DeLuca which pointed to the major product as the compound possesing 2,3-disposition of the diol, consistant with the CF_3 group directing the microbial oxidation.

With the present study, a 15 L fermentor was used for the microbial oxidation process. This process consists of two phases: induction and production. The process of induction is necessary because the population of cells of *Pp* 39/D must build up in order to produce the operant enzyme, toluene dioxygenase.[122] The bacteria produces the toluene dioxygenase in the presence of a limited amount of an aromatic compound which is known to be an inducer of the enzyme. Several known inducers include: chlorobenzene, bromobenzene, toluene and trifluorotoluene.

In order to determine whether or not a substrate is an inducer, a color test in which indigo is produced is employed.[123] Figure 2 details the reaction pathway by which the dye is formed when the bacteria grows in the presence of an inducer. The experimental procedure for this process involves streaking a plate with *Pp* 39/D, and placing a small tube containing the substrate in the lid of the petri dish. The dish is placed in a 30°C incubator for 2 days to allow for cell growth. As the cells grow, they are exposed to the substrate and would produce toluene dioxygenase if the substance were an inducer. Crystals of indole are then placed in the lid of the petri dish and a blue color is apparent within several hours. The blue color of indigo is not apparent when the substrate does not induce the formation of the enzyme.

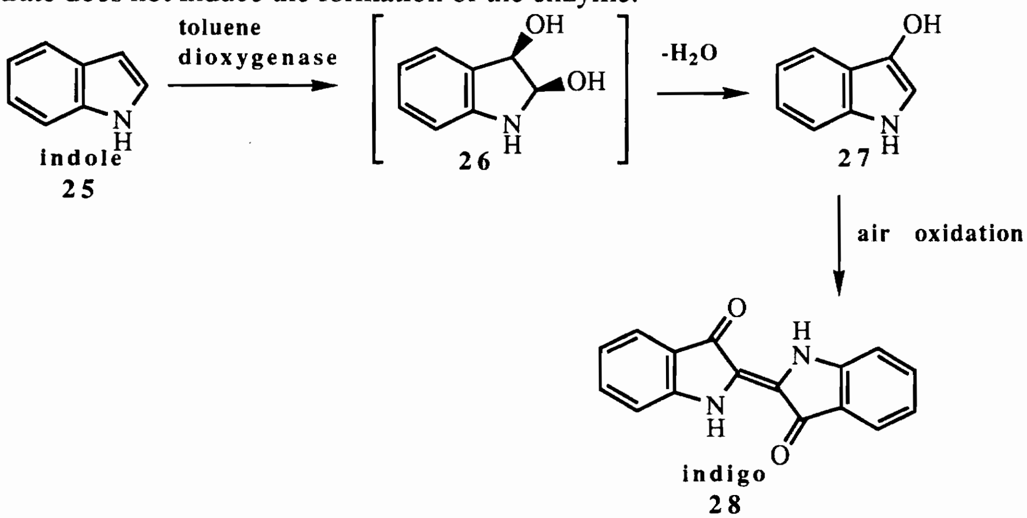


Figure 2

By this procedure, it was determined that *m*-bromo-$\alpha\alpha\alpha$–trifluorotoluene was not able to induce production of TOL_{SP} in the bacteria, and therefore toluene or chlorobenzene was used for this purpose.

Before the induction process can proceed, the innoculum must be prepared. A petri plate containing an agar-MSB-fructose medium was streaked with the cells of *Pp* 39/D. This plate was incubated at 30°C until optimum cell growth was achieved and then placed in a cold room. Single colonies from one agar plate were transferred to a 250 ml induction flask containing 50 ml of MSB. The apparatus for the induction process consisted of an erlynmeyer flask and a glass bulb sparger containing a small amount of toluene. The cell suspension in 50 ml of sterile mineral salt broth MSB solution was incubated at 30° in a rotatory shaker for 6-8 hours. The solution was then aseptically transferred in a laminar flow hood to a larger flask containing 250 ml of MSB and the induction period continued for another 10 hours at 30°C on the shaker.

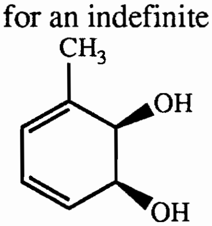
Step two of the microbial oxidation process, production, involved the transfer of the contents of the large induction flask to 6L of sterile MSB solution in a 15 L fermentor with a 12 L working volume. A 2L fructose solution which had been sterilized separately was also added. The fructose cannot be sterilized with the MSB solution because it tends to carmelize in the mixture at high temperatures. Using fructose along with arginine as the carbon source maximizes the cell growth. If the level of carbon source in the fermentor gets too low, the dissolved oxygen content will increase because the cells are not using any oxygen. Oxygen consumption only occurs when the cells have an ample supply of a food source.

Conditions for the fermentation were as follows: temperature = 30°C, pH = 7.3, stir rate = 300 rpm. For the first 6-8 hours of the process, toluene was bubbled through the solution in the form of a vapor to induce the cells. After the initial induction period, a continuous induction process was then set up. This process involved blowing the toluene vapor over the top of the broth while bubbling the substrate through the broth. In this manner, new cells were continuously exposed to toluene and would continue to produce the desired enzyme. However, the flow of both toluene and the substrate was carefully monitored because an excess of either compound will inhibit bacterial growth. The flow of air/oxygen was also kept in check. A strong flow may aid the evaporation of the substrate, while a slow stream may not provide enough oxygen required by the bacteria to carry out the oxidation.

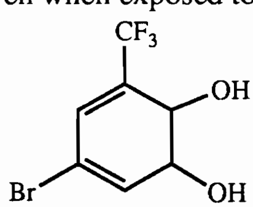
Over a 24-48 hour period, the diol accumulated in the solution. As the pH and pO_2 rose, the oxidation ceased and the process was terminated. After the process was complete, the cells and solids were separated using a continuous centrifuge to give a clear solution containing the diol. This solution was then saturated with sodium chloride to allow for a better extraction. Multiple extractions with base-washed ethyl acetate yielded the crude diol mixture.

Initial attempts to obtain the pure diol were unsuccessful and only black oil was obtained from the extractions. The oil was determined to be a mixture of the decomposition products from the dehydration of toluene diol and the concomitant catalyzed decomposition of the rest of the diol mixture to their respective phenolic compounds. Such a decomposition to occurred due to the insufficient stabilization of the supernatant with base

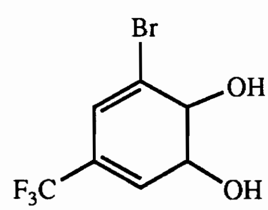
When a crude yellow oil was obtained as a result of ample base stabilization, the compounds were then separated by flash chromatography on silica (10% deactivated with water) and the pure products were identified as **7b**, expected from the induction period that used toluene, **1**, and **2**. Compounds **1** and **2** were isolated in yields of 50 mg/L and 2 mg/L respectively. Recrystallization of the metabolites from m-bromotrifluorotoluene from boiling methylene chloride/hexanes and repeated treatment with decolorizing carbon gave feathery white crystals. Slow evaporation of the solvent however, gave needles. Diols **1** and **2** prove to be remarkably stable at room temperature and survive as white crystals for an indefinite period even when exposed to air.



7 a



1



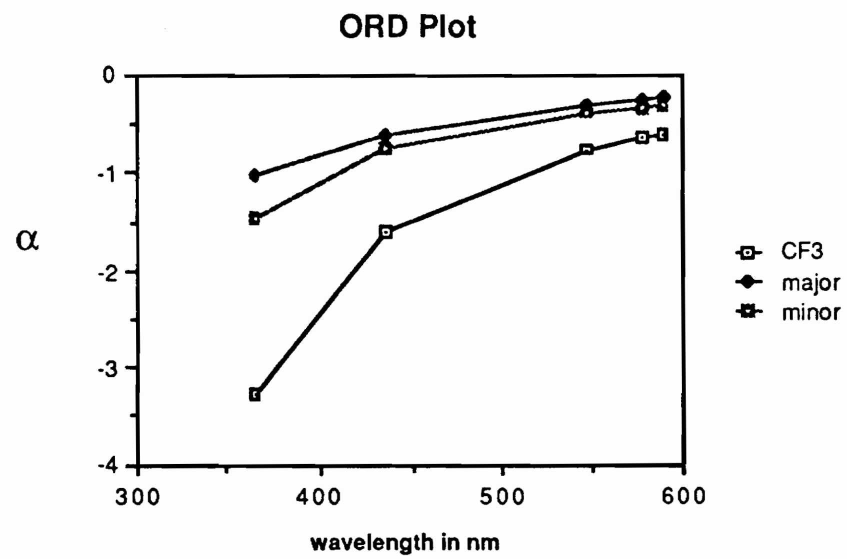
2

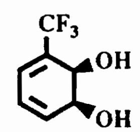
The structure determination of the major and minor metabolites along with methods for determination of absolute stereochemistry and enantiomeric excess for **1** is presented in the next section.

III. DISCUSSION
3. Stereochemical and regiochemical analysis of new diols

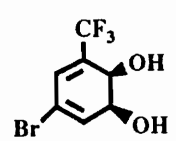
With the isolated and purified microbial oxidation products in hand, the next step of the project required a rigorous structural proof of the products. Two methods of structure proof were initially performed, but the data was not thorough enough to be unambiguous. The methods which were used included graphing a CD/ORD curve to measure absolute stereochemistry and acquiring a 2-D NMR to determine the regiochemistry.

The first method employed to approximate the absolute stereochemistry of the metabolites was the correlative method of obtaining a CD/ORD curve, as described on p. 30 section II for diol derived from naphthalene. In this case, the comparison was made with the metabolites from the oxidation of m-bromotrifluoro-toluene and the known diol from trifluorotoluene.[24] In the ORD plot below, each of the curves show the same sign. This trend hints at the similarity in the stereochemistry of the three compounds. For this reason, all of the compounds below are drawn as having the absolute stereochemistry as shown.

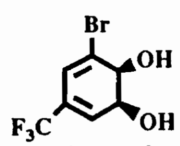




CF_3, 4



major, 1



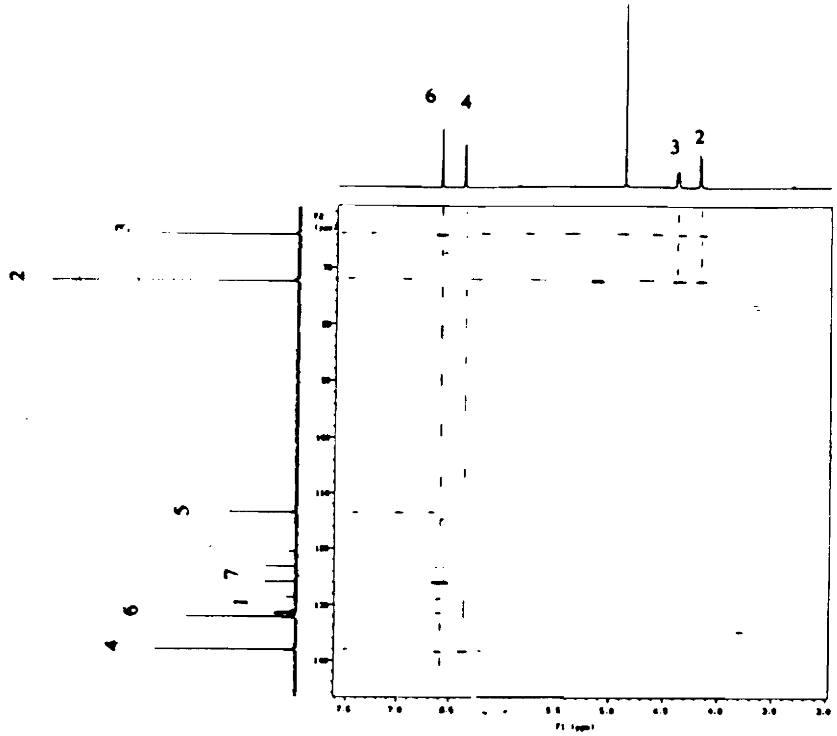
minor, 2

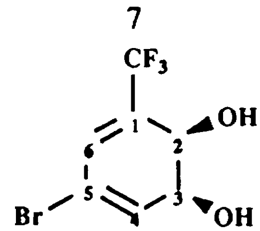
Figure 4

However, a more rigorous approach was necessary to unequivocally prove the absolute stereochemistry of the metabolites.

A second attempt at elucidation of the regiochemistry of the major product was made through the use of 2-dimensional NMR techniques.[124] Acquistion of a ^{13}C-^{1}H spectrum for diol was performed to show the relative positions of the atoms in the aromatic ring with respect to the trifluoromethyl group. This procedure is called HETCOR, or heteronuclear chemical shift correlation, and provides the carbon-hydrogen connectivity in the molecule. However, I was unable to fully identify the structure using this method. Such inability was due in part to the lack of resolution for the signals representing the two quaternary centers in the structure.

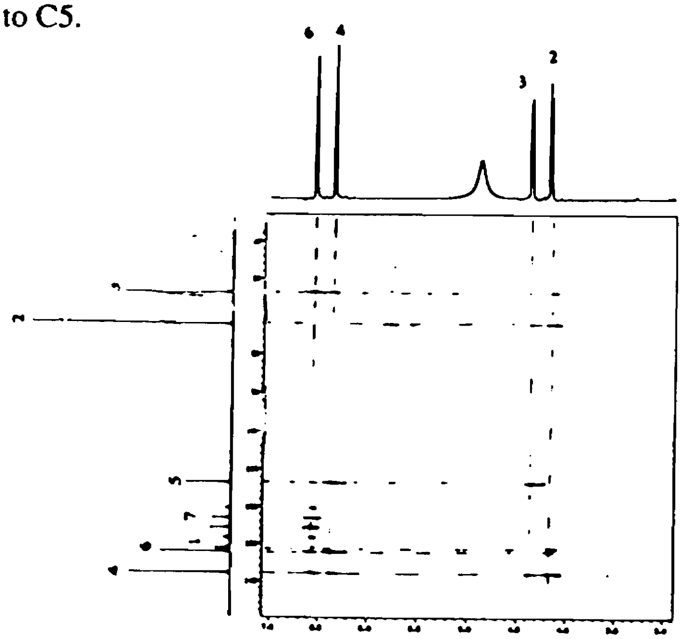
From the spectrum, one cannot obtain coupling information concerning the connectivity of carbons 1 and 2 or 3 and 5. It is obvious that the carbons labeled 2 and 3 are next to the protons with the same numbers. By following the dotted lines it can be seen that carbon two couples with both hydrogens 2 and 3. However, it is not apparent that carbon 1 is closer to the hydrogen attached to carbon 2 or to that of carbon 3. Similarly, by taking advantage of the long range coupling provided in a typical HETCOR experiment, the point where C5-H3 meet should be more intense than that of the C5-H2 relationship. As observed from the spectrum, no relationship between atoms 1 and 2 or 3 and 5 surfaces.





HETCOR Figure 5

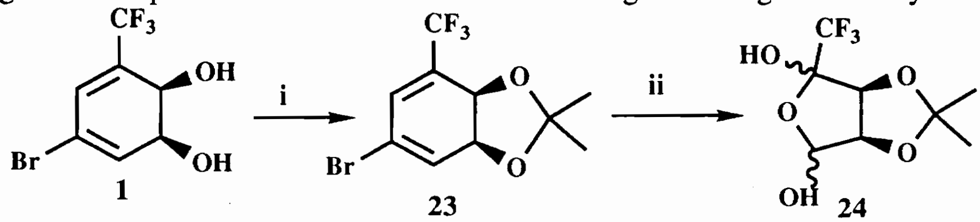
Thus, another experiment, COLOC, or a type of long-range HETCOR was performed. Anaylsis of this spectrum revealed carbon connectivity around the ring. C1 showed coupling with H2, while the vinylic protons at carbons 4 and 6 coupled strongly and weakly to C5.



COLOC Figure 6

However, the fact that the proton attached to C2 did not show arelationship with C7 reduced the validity of the previously compiled results.

The third and final attempt at structure proof involved the chemical convergence pathway. Because a previous experiment performed by Dr. Monica DeLuca had provided some information about the regiochemistry of the major diol. (Scheme I), hydrogenation experiments were carried out to investigate the regiochemistry of **2**.



1 **23** **24**

i. DMP, acetone, pTSOH **ii.** O_3, Me_2S

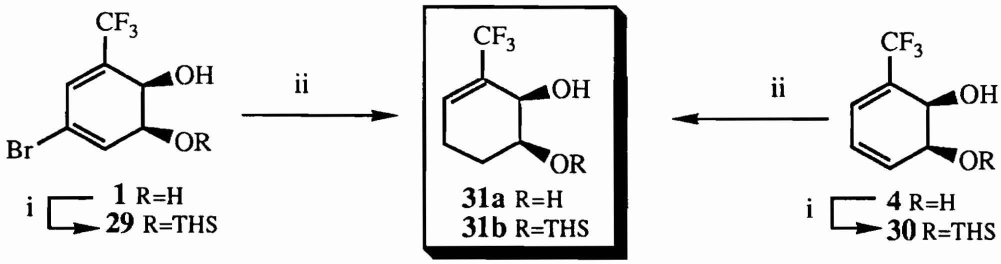
Scheme 1

The isolation of **24** as shown in scheme 1 provided the proof that the CF_3 group in the major metabolite is in the 1 position with respect to the diol in the 2,3 position. The known compound, *cis*-3-trifluoromethyl-cyclohexa-3,5-diene-1,2-diol, **4**, was chosen for comparative analysis because hydrogenation of both **1** and **4** should yield the same compound.

Hydrogenations were carried out in the following fashion. Adam's catalyst, (PtO_2), triethylamine and the methanolic solution of the diol were reacted in a pressurized autoclave. The aforementioned conditions were used for both **1** and **4**.

The products of the hydrogenation of **1** were compared with those obtained from **4** and were shown to be identical by spectral and optical criteria. Thus, the major metabolite has the structure **1**.

Now that the regiochemistry of the major product had been determined to be indisputable, the next step of the project involved the proof of absolute stereochemistry. Since the absolute stereochemistry of **4** is known to be greater than 98%, comparison of the optical rotations of its derivative **31** from each of the degradation pathways allowed determination of the enantiomeric excess in **1** as 93% (for **31a**, $[a]_D^{20}$ = -130°, c=0.9, MeOH).



i. THSCl, imidazole, CH_2Cl_2 ii. H_2, PtO_2, MeOH, Et_3N

SCHEME 2

As an added method of proof, both diols were monoprotected with dimethylsilylthexyl chloride in methylene chloride and hydrogenated according to the condtions in Scheme 2. The results shown again prove that the stereochemistry of the new metabolite is identical to that of the known cis diol (for **31b**, $[a]_D^{20}$ = -60°, c=0.7, MeOH).

IV. CONCLUSIONS

With the trifluoromethyl group directing the oxidation, the major product from the biotransformation in this study was proven to be *cis*-trifluoromethyl-2R, 3S-dihydroxy-5-bromocyclohexa-4,6-diene, **1**. Because of its remarkable stability and ease of preparation with the recombinant *E. coli* JM109(pDTG601), the major isomer, **1**, could prove useful as a chiral synthon for use in the preparation of fluorinated carbohydrates.

The minor product, **2**, from the biotransformation using *Pp* 39/D was analyzed using several spectral techniques. Its structure has been assigned as trifluoromethyl-3,4-dihydroxy-5-bromo-1,5-cyclohexadiene. Since **2** was not detected in the culture from the oxidation using the recombinant *E. coli*, the stereochemistry of this compound is assumed to be cis at this juncture.

V. EXPERIMENTALS

All nonhydrolytic reactions were carried out under an argon atmosphere with standard techniques for the exclusion of air and moisture. Glassware used for moisture sensitive reactions was flame-dried under vacuum.

Analytical TLC were performed on silica gel 60F-254 plates. Flash chromatography were performed on Kieselgel 60 (EM Reagents, 230-400 mesh). Mass spectra were recorded on a Varian MAT-112 instrument (low resolution) or on a double focusing VG 7070 E-HF instrument (exact mass). Infrared spectra were recorded on Perkin-Elmer 283B or 710B instruments. Proton NMR spectra were obtained on Bruker WP-270 or Varian UN-400 instrument. Carbon NMR spectra were recorded on a Varian UN-400. Optical rotations were recorded on a Perkin Elmer 241 digital polarimeter.

(2R,3S)-α,α,α-trifluoromethyl-2,3-dihydroxy-5-bromocyclohexa-4,6-diene (1) *P. putida* 39D was grown at 29°C in an MSB-arginine medium and the culture (50 mL) was incubated in a 250 ml Erlenmeyer flask. Toluene was supplied by a scale bulb attached to the flask by a neoprene stopper, and the culture was aerated on a reciprocal shaker. After 6 hours, the culture was aseptically transferred to a 500 mL Erlenmeyer flask containing 250 mL of the medium. This culture was aerated on the shaker for 12 hours. The contents of the 500 mL flask were transferred to a 15 L fermentor which contained 10 L of MSB-arginine-fructose medium. A stream of air/toluene was bubbled through the solution for approximately 7 hours. *m*-Bromo-α,α,α-trifluorotoluene was then bubbled through the solution for 34 hours. After adjusting the pH to 8.4 with NaOH, the cells were separated from the broth with a continuous centrifuge. The resulting clear solution was saturated with NaCl and extracted with base-washed ethyl acetate. The organic layer was dried with Na_2SO_4 and the solvent was evaporated. The crude diol mixture was purified on a flash column with 7:3

hexane/ethyl acetate (10% deactivated silica) to give 520 mg of white crystalline compound **1**, (yield 50 mg/L of culture) which was recrystallized from CH_2Cl_2/hexane. **R_f** = 0.38 (hexane/ethyl acetate 1:1) **Mp** = 112-113°C; $[\alpha]_D^{20}$ = -18.1° (c=0.53, CH_3OH); **IR** ($CHCl_3$) 3441, 1636, 1270, 1173, 1052, 1025 cm^{-1}; **^1H NMR** ($CDCl_3$) δ 6.65 (m, 1 H), 6.37 (s, 1 H), 4.46 (s, 1 H), 4.32 (d, J = 5.9 Hz, 1 H), 2.53 (br. s 2 H); **^{13}C NMR** (CD_3OD) δ138.1, (C)132.2 (q, J = 25 Hz, CH), 131.4 (q, J = 123 Hz, C), 124.5 (q, J = 1078 Hz, C), 113.6 (CH), 72.3 (CH), 63.9 (CH); **MS** (CI, 70 ev)(*m/z* (rel. int.) 259 (19, M+), 214 (100), 159 (33), 133 (70), 83 (42); **Calcd for $C_7H_6BrF_3$:** C, 32.46; H, 2.34; **Found:** C, 32.43; H, 2.34.

(3S,4S)-α,α,α-trifluoromethyl-3,4-dihydroxy-5-bromocyclohexa-1,5-diene (2) The procedure above for compound **1** was followed. The crude diol mixture was separated with a flash column using 7:3 hexane/ethyl acetate (10% deactivated silica) to give 30 mg of **5** (yield 2 mg/L of culture). Recrystallization from CH_2Cl_2/hexane resulted in pure white crystals. **R_f** = 0.45 (hexane/ethyl acetate 1:1); **Mp** = 145-146°C.; $[\alpha]_D^{20}$ = -46.5° (c=0.66, CH_3OH); **IR** ($CHCl_3$) 3440, 1620 cm^{-1}; **^1H NMR** ($CDCl_3$) δ 6.48 (d, J = 6 Hz 1 H), 6.42 (dd, J_1 = 6.2, J_2 = 1.6, Hz, 1 H), 4.51 (m, 2 H), 2.78 (s, 1 H), 2.29 (s, 1 H); **^{13}C NMR** (400 MHz, CD_3OD) δ 136.3 (CH), 129.4 (C), 128.4 (q, J = 27.6 Hz, C), 125.5 (q, J = 1077 Hz, C), 125.1 (CH), 73.4 (CH), 66.9 (CH); **MS** (EI+), *m/z* (rel. int.) 258 (M+) (20), 240 (14); **HRMS Calcd for $C_7H_6O_2BrF_3$:** 257.9503 **Found:** 257.9505 error, 0.8 ppm.

(2R,3S)-α,α,α-trifluoromethyl-2-hydroxy-3-dimethylsilylthexyl-5-bromo-4,6-cyclohexadiene (29) To a stirred solution of dienediol **(1)**, (80 mg, 0.3089 mmol) in 10 mL CH_2Cl_2 was added (26 mg, 0.3861 mmol, 1.25 eq) imidazole and (0.073 mL, 0.3707 mmol, 1.2 eq) dimethylsilylthexylchloride. The mixture was stirred at 0°C overnight. The solution was equilibrated to room temperature, dried over Na_2SO_4

and evaporated to yield 114 mg, 92% of a colorless oil. **Rf** = 0.63 (hexane/ethyl acetate 10:1) $[\alpha]_D^{20}$ = +9.15° (c=0.97, CH_3OH); **IR** (neat) 3448, 2960, 1168, 1130, 1050, 1028 cm^{-1}; **^{1}H NMR** ($CDCl_3$) δ 6.54 (m, 1 H), 6.24 (m, 1 H), 4.43 (m, 1 H) 4.20 (m, 1 H), 2.66 (d, J = 4.2 Hz, 1 H), 1.64 (m, 1 H), 0.91 (m, 6 H), 0.18 (d, J = 5.3, Hz 3 H); **^{13}C NMR** (400 MHz, $CDCl_3$) δ 134.5 (C), 131.0 (q, J = 24 Hz, CH), 129.5 (q, J = 124 Hz, C), 122.7 (q, J = 1080 Hz, C), 114.3 (CH), 71.0 (CH), 63.7 (CH), 34.1 (C), 25.2 (C), 20.2 (CH_3), 20.0 (CH_3), 18.5 (CH_3), 18.4 (CH_3), -2.54 (CH_3), -3.08 (CH_3); **MS:** *m/z* (rel int) 383 (M+) (90), **HRMS Calcd for $C_{15}H_{24}O_2F_3BrSi$:** (M-OH) 383.0653 **Found:** 383.0684 error 7.9 ppm.

(2R,3S)-α,α,α-trifluoromethyl-2-hydroxy-3-dimethylsilylthexyl-4,6-cyclohexadiene (30) To a stirred solution of dienediol (**4**), (360 mg, 2.0 mmol) in 4 mL CH_2Cl_2 was added (170 mg, 2.5 mmol, 1.25 eq) imidazole and (0.49 mL, 0.447 mmol, 1.25 eq) dimethylsilylthexylchloride. The mixture was stirred at 0°C overnight. The solution was equilibrated to room temperature, dried over Na_2SO_4 and evaporated to yield 560 mg, 90% of a colorless oil. **Rf** = 0. (hexane/ethyl acetate 1:1) $[\alpha]_D^{20}$ = -0.62° (c=0.99, CH_3OH); **IR** (neat) 3556, 2960, 1663,1558, 1366 cm^{-1}; **^{1}H NMR** ($CDCl_3$) δ 6.61 (m, 1 H), 6.10 (m, 2 H), 4.58 (m, 1 H) 4.27 (m, 1 H), 2.67 (d, J = 4.3 Hz, 1 H), 1.68 (m, 1 H), 0.91 (m, 6 H), 0.18 (d, J = 5.1, Hz 3 H); **^{13}C NMR** (400 MHz, $CDCl_3$) δ 134.1 (CH), 127.6 (q, J = 121 Hz, CH), 127.5 (q, J = 24 Hz, CH), 123.5 (q, J = 1077 Hz, C), 121.6 (CH), 70.6 (CH), 64.0 (CH), 34.3 (C), 25.1 (C), 20.3 (CH_3), 20.2 (CH_3), 18.7 (CH_3), 18.6 (CH_3), -2.5 (CH_3), -2.9 (CH_3); **MS:** *m/z* (rel int) 322 (M+), 219 (100), 143 (80); **HRMS Calcd for $C_{15}H_{25}O_2F_3Si$:** 322.1576 **Found:** 322.1582 error 2.0 ppm.

(2R, 3S) α,α,α-trifluoromethyl-2-3-dihydroxy-6-cyclohexene (31a). 100 mg (0.3861 mmol) of 2R, 3S-α,α,α-trifluoromethyl-2,3-dihydroxy-4,6-cyclohexadiene

(**4**) was dissolved in 10 mL of methanol in a bomb apparatus fitted with a pressure gauge. To this mixture was added one drop of triethylamine and a catalytic amount of PtO_2. The apparatus was pressurized to 85 psi with hydrogen and allowed to stir for 2 hours. After filtration through celite and evaporation of the solvent, 80 mg of a crude orangish oil was obtained. Purification by flash column chromatography (hexane/ethyl acetate 1:1, silica) resulted in 26 mg of a white crystalline compound (36% yield). **R_f** = 0.25 (hexane/ethyl acetate 1:1) **Mp** = 102-107°C; $[\alpha]_D^{20}$ = -130.7.0° (c=0.9, CH_3OH); **IR** (CCl_4) 3290 (br), 2930, 1670, 1275, 1110 cm^{-1}; **^1H NMR** (400 MHz, $CDCl_3$) δ 6.40 (s, 1 H), 4.08 (s, 1 H), 3.50 (dt, J_1 = 12, J_2 = 7.2 Hz, 1 H), 2.26 (m, 1 H), 2.15 (m, 1 H), 1.72 (m, 1 H), 1.58 (m, 1 H); **^{13}C NMR** (400 MHz, $CDCl_3$) δ 136.7 (q, J = 24 Hz, CH), 130.6 (q, J = 115 Hz, C), 125.3 (q, J = 1083, Hz C), 70.4 (CH), 64.9 (CH), 25.6 (CH_2), 24.7 (CH_2); **MS** *m/z* (rel. int); **HRMS Calcd for $C_7H_9O_2F_3$:** 65.0528 (M-OH) **Found:** 165.0544 error 10 ppm

(2R, 3S) α,α,α-trifluoromethyl-2-hydroxy-3-dimethylsilylthexyl-cyclohe-6-ene (31b). An autoclaved was charged with 200 mg (0.62 mmol) of 2R, 3S-α,α,α-trifluoromethyl-2-hydroxy-3-dimethylsilylthexyl-4,6-cyclohexadiene (**30**) dissolved in 10 ml of methanol. To this mixture was added two drops of triethylamine and a catalytic amount of PtO_2. The apparatus was pressurized to 85 psi with hydrogen and allowed to stir for 2.5 hours. After filtration through celite and evaporation of the solvent, 190 mg of a crude oil was obtained. Purification by column chromatography (1:1 hexane/ethyl acetate, silica) resulted in 51 mg of a colorless oil. (26% yield); **R_f** = 0.52 (hexane/ethyl acetate 10:1); $[\alpha]_D^{20}$ = -60.0° (c=0.77, CH_3OH); **IR** (neat) 3550, 2955 cm^{-1}; **^1H NMR**: (400 MHz $CDCl_3$) δ 6.52 (s, 1 H), 4.19 (s, 1 H), 3.81 (dt, J_1 = 11.2, J_2 = 7.2 Hz, 1 H), 2.73 (d, J = 2.4 Hz, 1 H), 2.34 (m, 1 H), 2.20 (m, 1 H), 1.90 (m, 1 H), 1.64 (m, 2 H), 0.89 (d, J = 2.4 Hz, 3 H), 0.91 (d, J = 2.4 Hz, 3 H), 0.87 (m, 6 H), 0.16 (s, 6 H); **^{13}C NMR** (400 MHz, $CDCl_3$) δ 135.1 (q, J = 21 Hz, CH), 129.0 (q, J = 115 Hz, C), 124.1 (q, J = 1080 Hz, C), 69.9 (CH), 64.1 (CH), 34.2 (C), 29.6 (C), 24.4 (CH_2), 23.9 (CH_2), 20.2 (CH_3), 20.1 (CH_3), 18.6 (CH_3), 18.5 (CH_3), -2.4 (CH_3), -2.9 (CH_3); **MS:** *m/z* (rel. int); 325 (M+) (100), 239 (90); **HRMS Calcd. for $C_{15}H_{27}O_2F_3Si$:** (M+) 325.1801 **Found:** 325.1780 error 3.4 ppm.

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VII. APPENDIX

A. MSB--Arginine solution

For a 10 Liter Run:

400 ml solution A

200 ml solution B

150 ml solution C

50 g arginine

Combine and dilute to 8 liters with deionized water.

Also, autoclaved separately, 200 g fructose in 2000 ml deionized water.

Solution A

1M KH_2PO_4 (136.1 g/L)

1M $Na_2HPO_4 \cdot 7H2O$ (268.1 g/L)

Solution B (Hunter's Base - Vitamin-free)

Nitrilotriacetic acid (NTA) 10 g/L E

KOH 7.5 g/L

$MgSO_4 \cdot 7H_2O$ 29.6 g/L

$CaCl_2 \cdot 2H_2O$ 3.3 g/L

$(NH_4)_6Mo_7O_{27} \cdot 4H_20$ 9.3 mg/L

$FeSO_4 \cdot 7H_2O$ 99 mg/L

Metals 44 solution 50 ml/l

Metals 44 solution

Ethylenediaminetetraacetic acid (EDTA) 250 mg

$ZnSO_4 \cdot 7H_2O$ 1.095 g

$FeSO_4 \cdot 7H_2O$ 500 mg

$MnSO_4 \cdot H_2O$ 154 mg

$CuSO_4 \cdot 5H_2O$ 39.2 mg

$Co(NO_3)_2 \cdot 6H_2O$ 24.8 mg

$Na_2B_4O_7 \cdot 10H_2O$ 17.7 mg

The solids are dissolved in 100 ml of distilled water, and 3 drops of sulfuric acid (1M) are added to retard precipitation. The solution should be aquamarine blue.

Preparation of 5 Liters of solution B:

Dilue 50 g NTA to 500 ml with deionized water and add to a solution of 37.5 g KOH diluted to 500 ml with deionized water. Slowly add a solution of 148 g of $MgSO_4 \cdot 7H_2O$ diluted to 1 liter to the NTA/KOH mixture. Stir well to avoid clouding the mixture. Next, slowly add a solution of 16.5 g $CaCl_2 \cdot 2H_2O$ diluted to 1 liter with stirring to prevent cloudiness.

Add an ammonium molybdate/iron sulfate solution to the above mixture. (46.5 mg $(NH_4)_6MoO_{24} \cdot 4H_2O$ and 495mg $FeSO_4 \cdot 7H_2O$ diluted to 1 liter). Upon mixing, a pale yellow color should remain.

Finally, add 250 ml of the Metals 44 solution, mix well, and dilute the mixture to 5 liters.

Adjust the pH to 6.8 slowly by adding 10M sodium hydroxide in 2 ml aliquots with rapid stirring. If the pH exceeds 7.0, insoluble precipitates will form and the solution will have to be made again.

This solution is to be stored in a cold room with a temperature of 4-10° C.

Solution C

$(NH_4)_2SO_4$ 200 g/L

B. Selected Spectra

(1) ααα-trifluoromethyl-2,3-dihydroxy-5-bromocyclohexa-4,6-diene

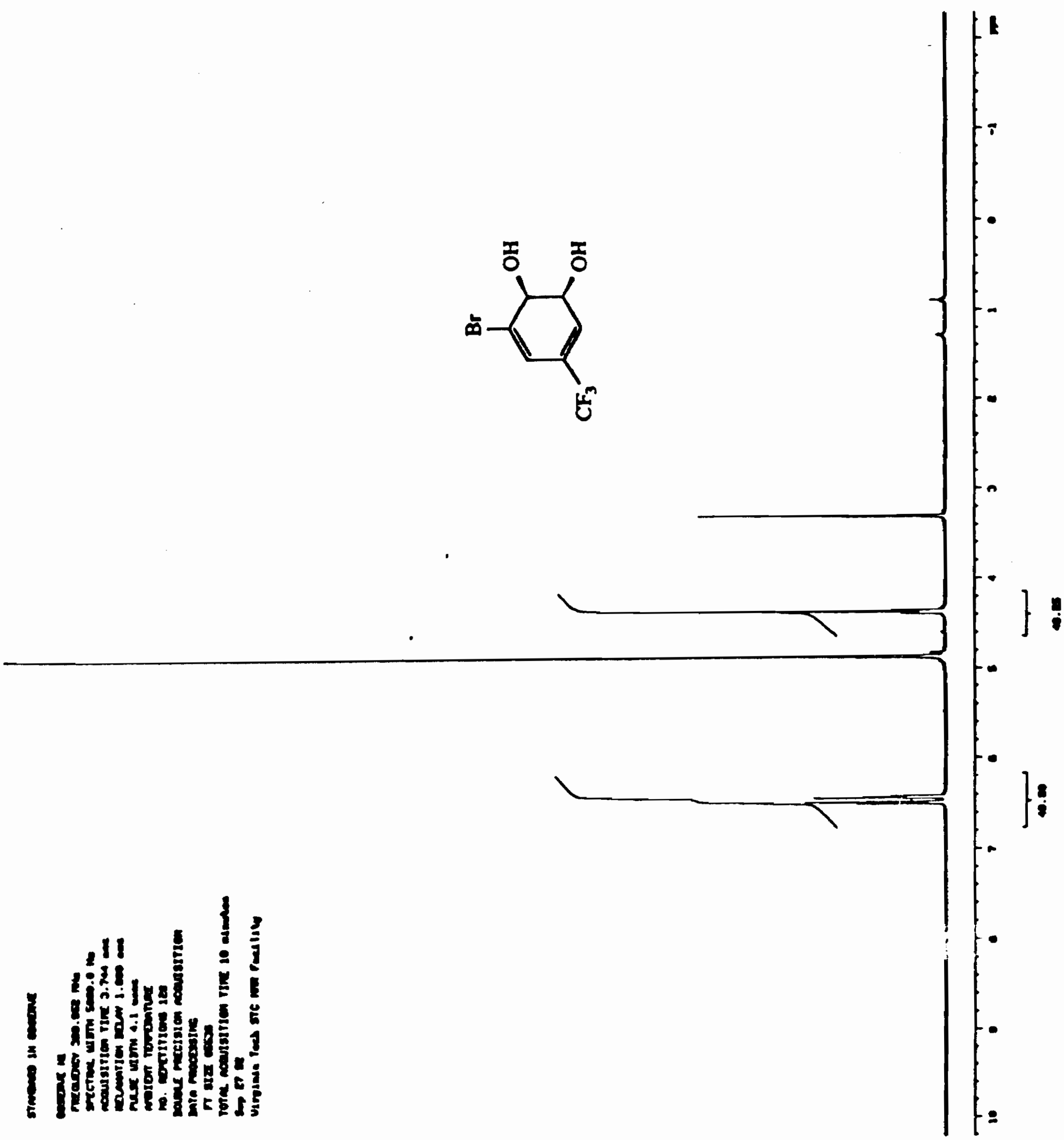
(2) ααα–trifluoromethyl-3,4-dihydroxy-5-bromocyclohexa-1,5-diene

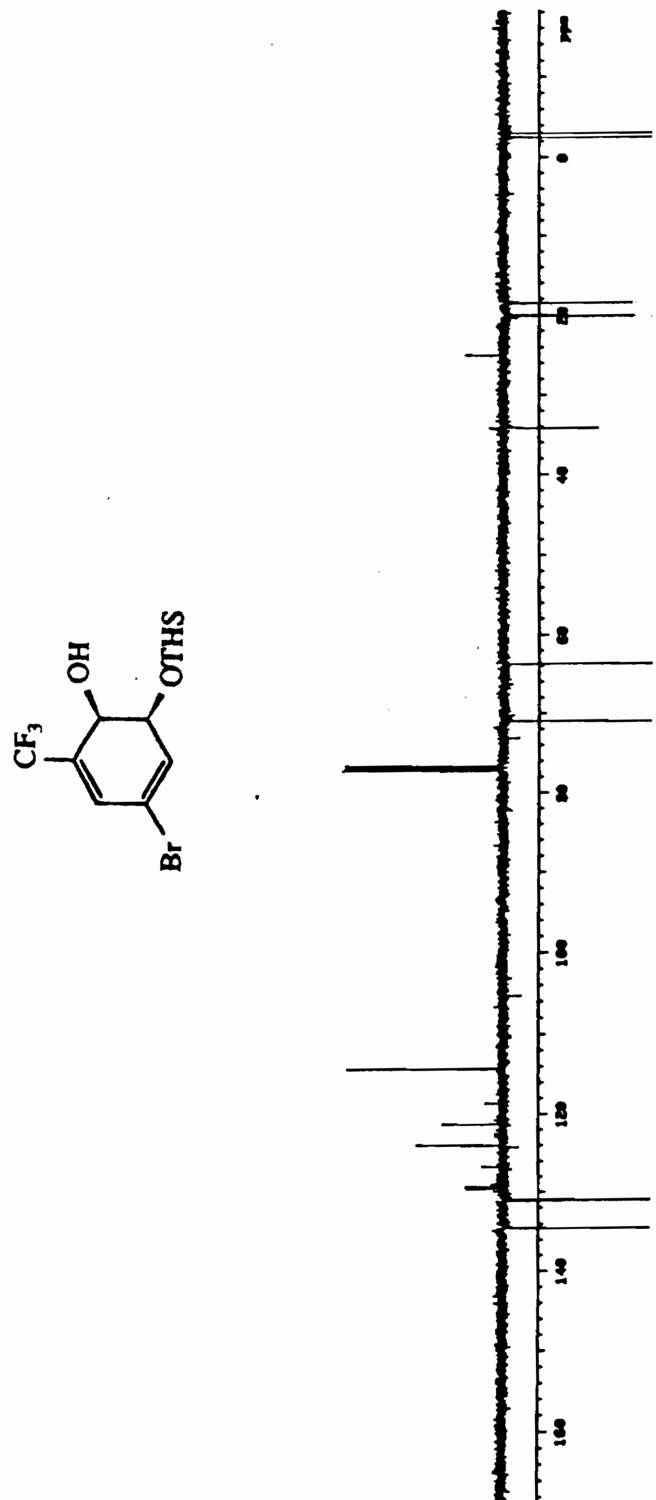
(29) ααα-trifluoromethyl-2-hydroxy-3-dimethylsilylthexyl-5-bromo-4,6-cyclohexadiene

(30) ααα-trifluoromethyl-2-hydroxy-3-dimethylsilylthexyl-4,6-cyclohexadiene

(31a) ααα-trifluoromethyl-2,3-dihydroxy-cyclohex-6-ene

(31b) ααα-trifluoromethyl-2,-hydroxy-3-dimethylsilylthexyl-cyclohex-6-ene

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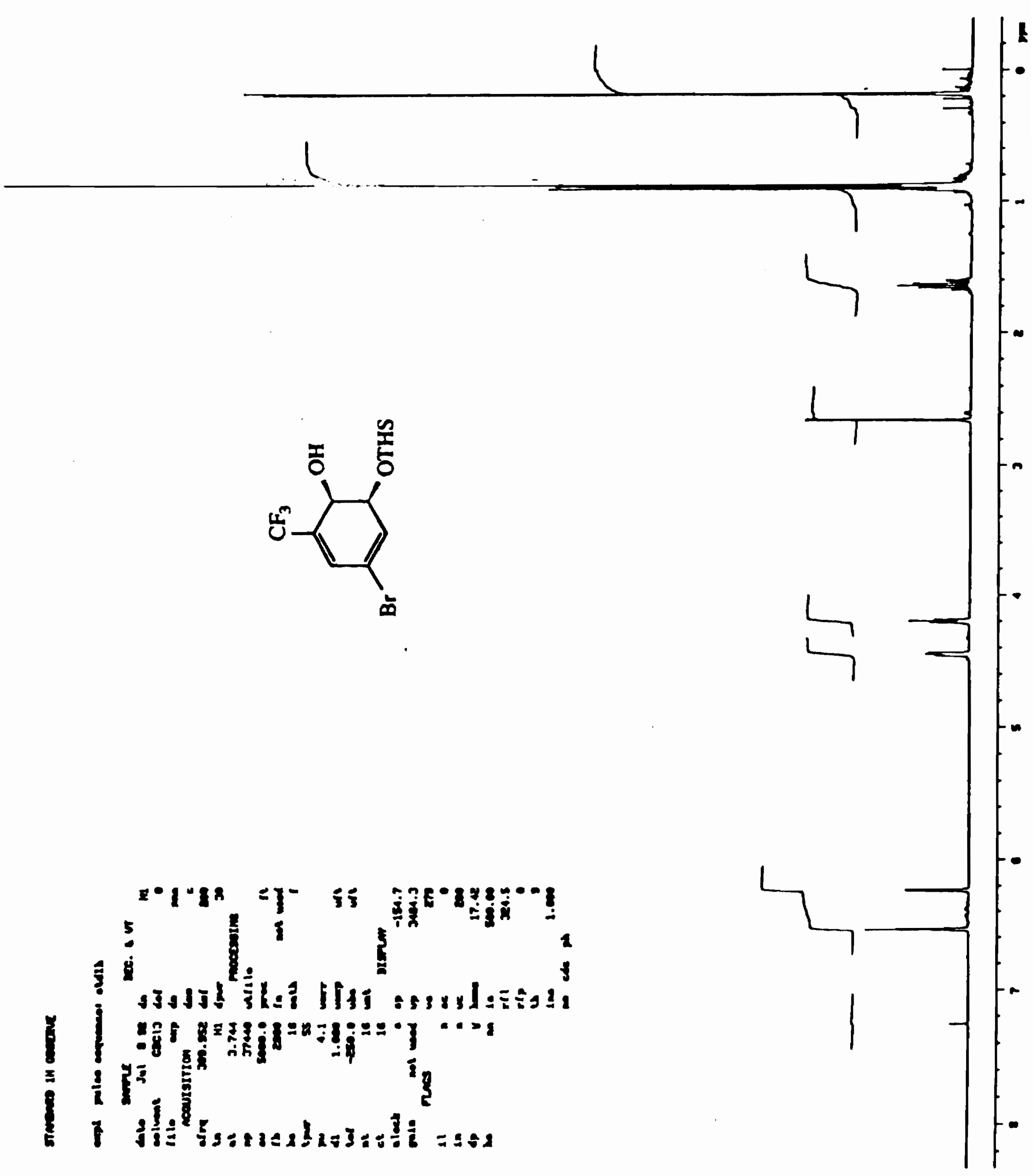
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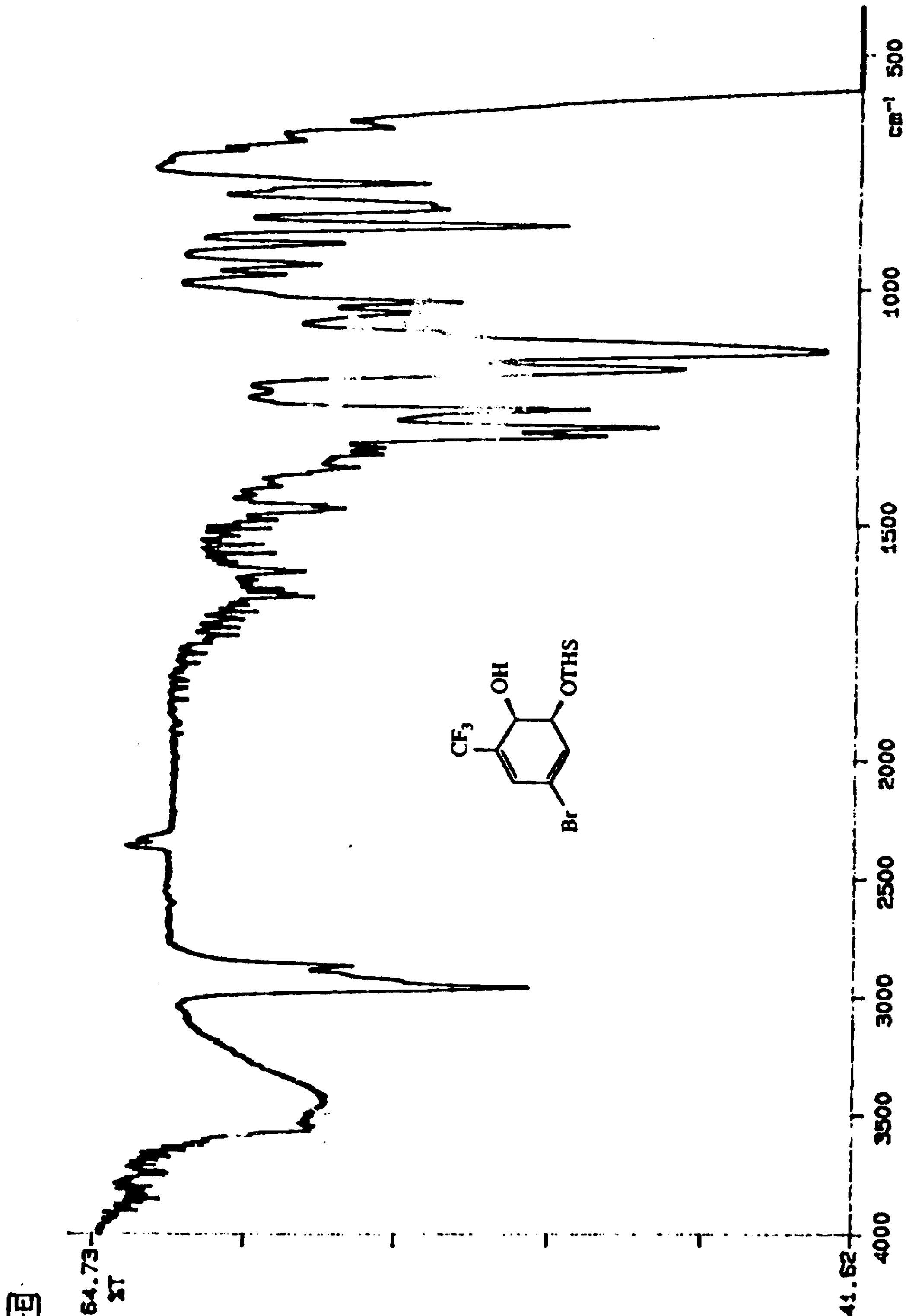
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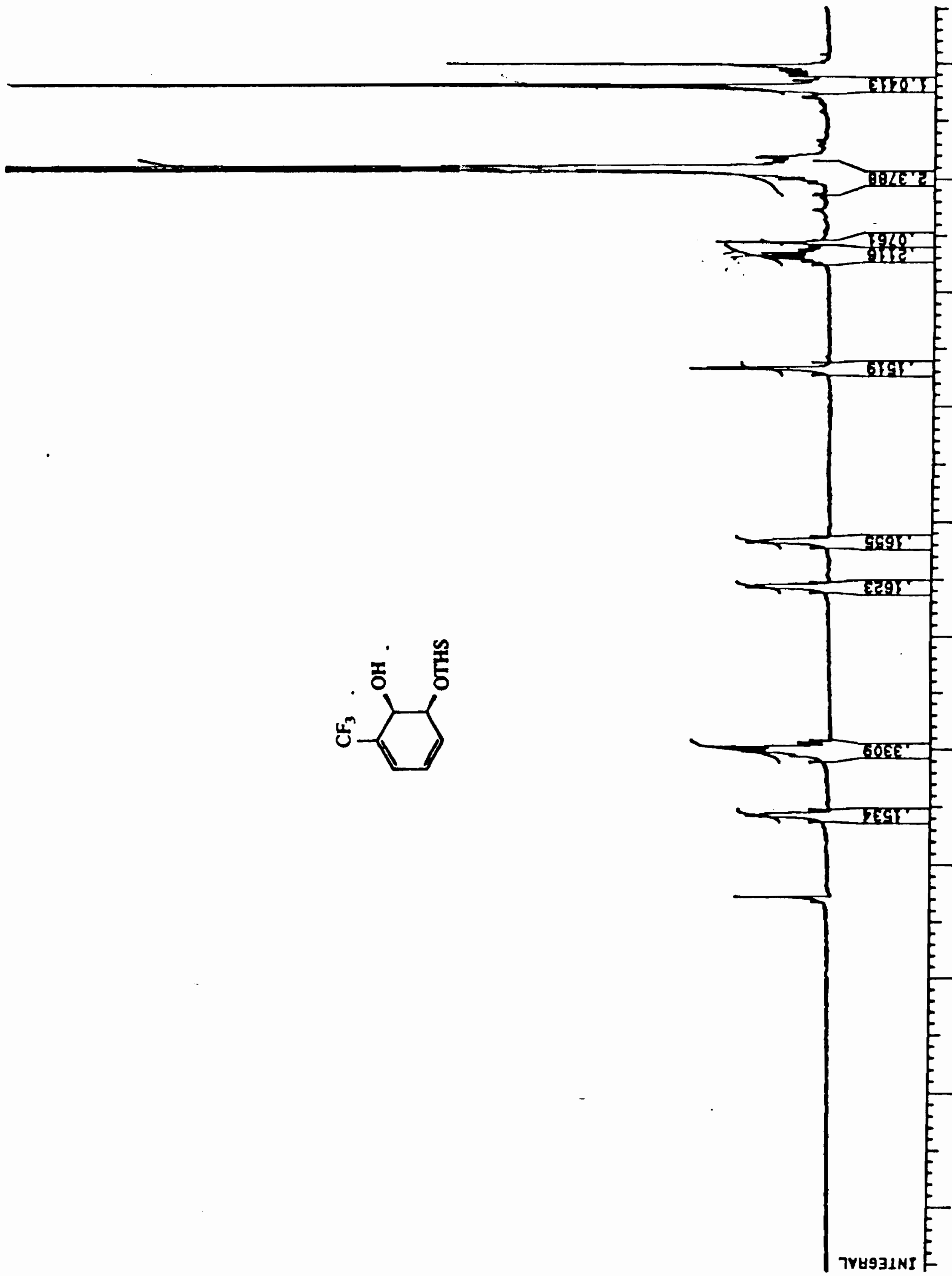
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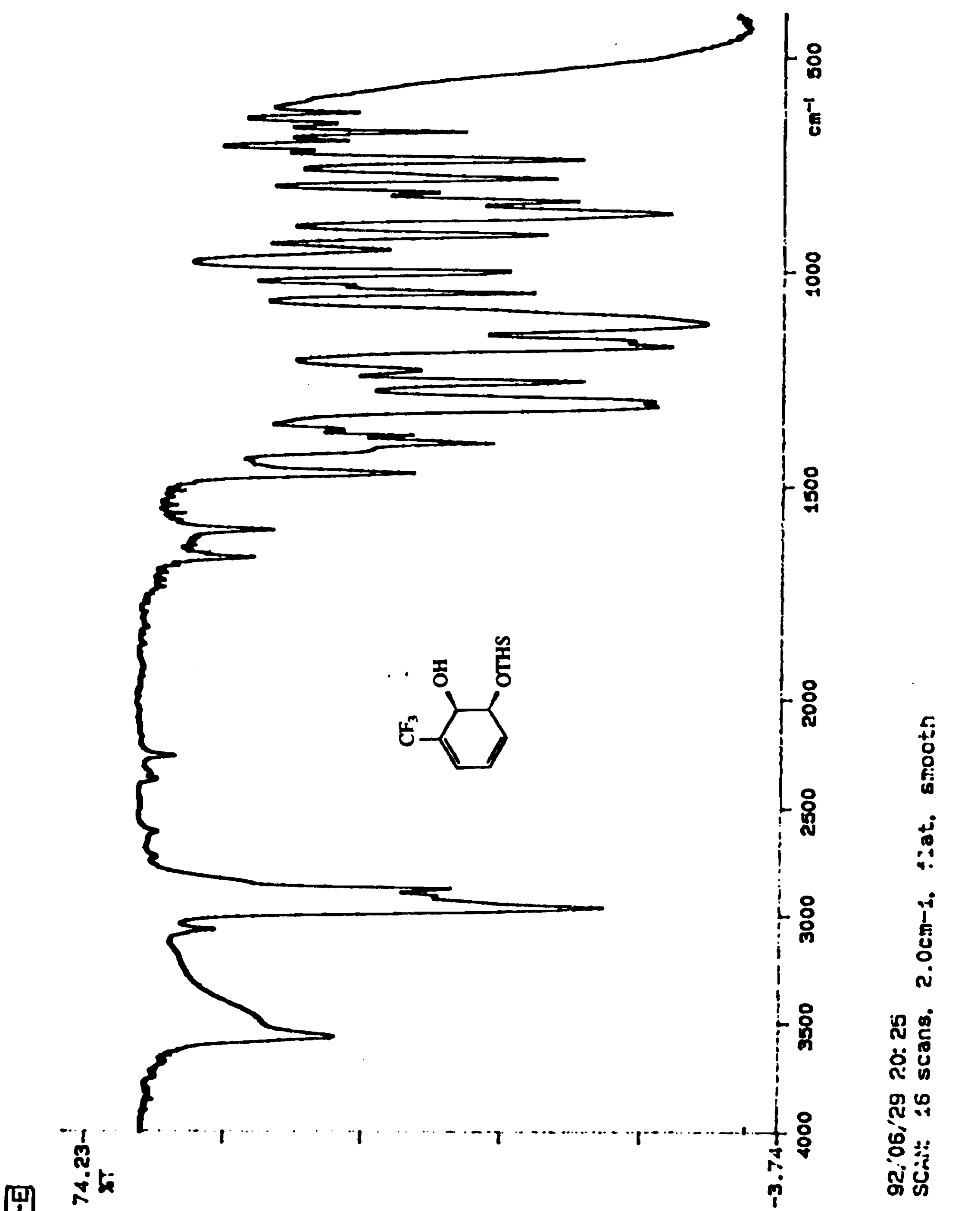
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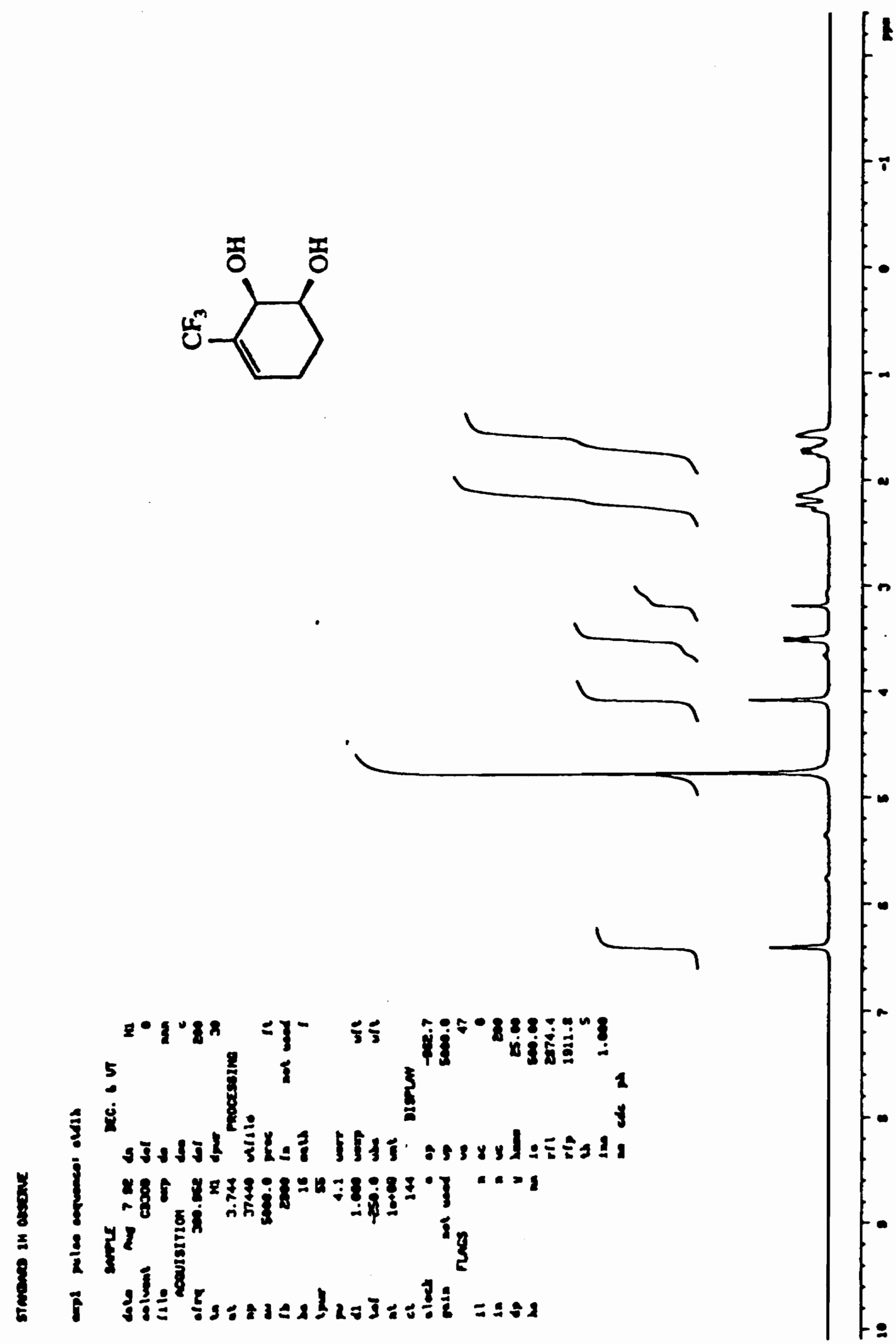

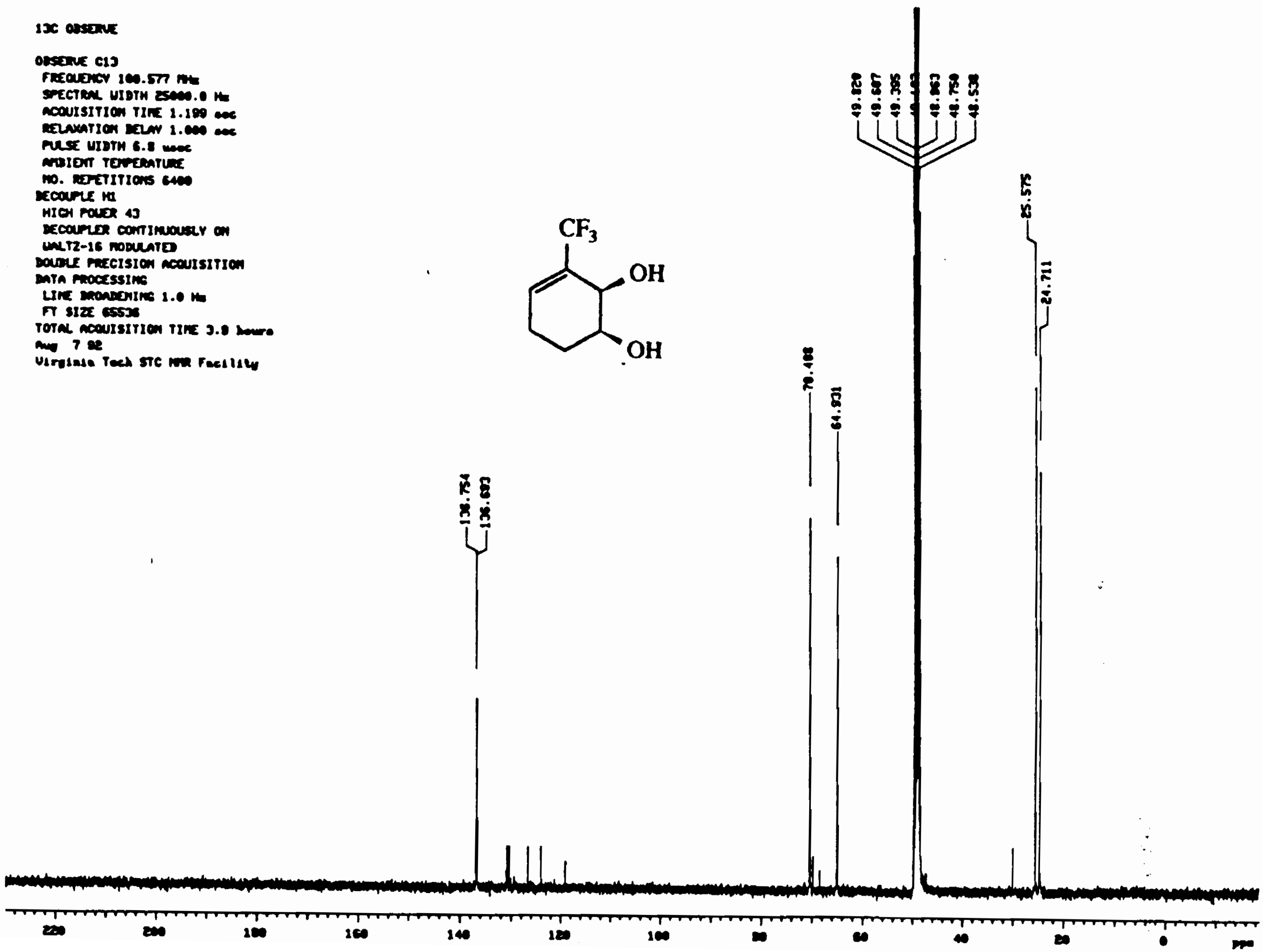
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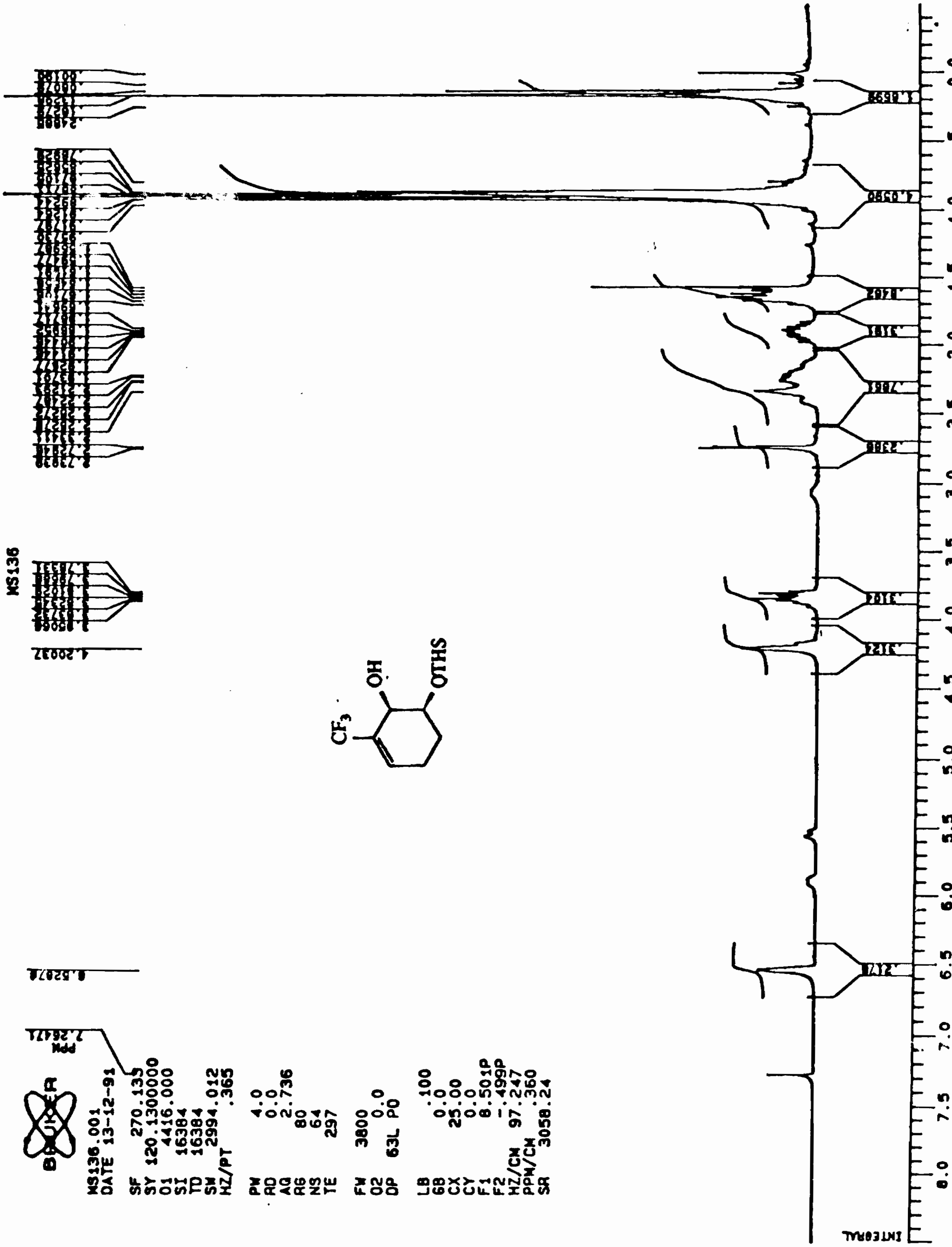
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VIII. VITA

Michele Rose Stabile was born in Pittsburgh, Pennsylvania on August 14, 1968. She graduated from Canevin High School in 1986, and entered Loyola College in Baltimore that same year. In the spring of 1990, she earned her Bachlelor of Science in Chemistry

She then continued her studies in Chemistry in the group of of Dr. Tomas Hudlicky at Virginia Tech.

Michele Stabile