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Hexabromocyclododecanes Are Dehalogenated by CYP168A1 from Pseudomonas aeruginosa Strain HS9

(Article begins on next page)

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1 **Hexabromocyclododecanes are dehalogenated by CYP168A1 from a**

2 ***Pseudomonas* strain HS9**

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16   **Abstract**

17   Hexabromocyclododecanes (HBCDs) are widely used brominated flame retardants,

18   which cause antidiuretic hormone syndrome and even induce cancer. However, little

19   information is available about the degrading mechanisms of HBCDs. In this study,

20   genomic, proteomic analyses, RT-qPCR and gene knockout assays reveal that a

21   cytochrome P450 encoding gene is responsible for the HBCD catabolism in

22   *Pseudomonas aeruginosa* HS9. CO-difference spectrum of the enzyme CYP168A1

23   was matched to P450 character and proved by western blot analysis and UV-visible.

24   We demonstrate that the reactions of debromination and hydrogenation are carried out

25   one after another based on detection of the metabolites pentabromocyclododecanols

26   (PBCDOHs), tetrabromocyclododecadiols (TBCDDOHs) and Br^- iron. In the ^{18}O

27   isotope experiments, $PBCD^{18}OHs$ were only detected in the $H_2^{18}O$ group, proving that

28   the added oxygen is derived from H_2O not from O_2. This study elucidates the

29   degrading mechanism of HBCDs by *Pseudomonas*.

30   **Importance**

31   Hexabromocyclododecanes (HBCDs) are environmental pollutants, which are wildly

32   used in industry. In this study, we identified and characterized a novel key

33   dehalogenase CYP168A1 responsible for the HBCDs degradation from a

34   *Pseudomonas aeruginosa* strain HS9. This study provides new insights into

35   understanding biodegradation of HBCDs.

Introduction

Hexabromocyclododecanes (HBCDs) are the second most widely used brominated

flame retardants (BFRs), and are utilized in building materials, electronics, textiles,

and plastics (1). They are a threat to human health due to causing antidiuretic

hormone syndrome and even inducing cancer. Microorganisms play important roles in

degradation and detoxification of pollutants of HBCDs (2). However, little

information is available about molecular and biochemical mechanisms, particularly

how functional proteins relate to debromination. Only two dehalogenases, LinA and

LinB from the hexachlorocyclohexane transformation strain *Sphingobium indicum*

B90A, can convert HBCD to different debrominated products. LinA selectively

catalyzes   the   transformation   of   *β*-HBCDs   to

1E,5S,6S,9R,10S-pentabromocyclododecene (PBCDE), while LinB transforms all *α-,*

β-, and *γ*-HBCD isomers to pentabromocyclododecanols (PBCDOHs) and even

tetrabromocyclododecadiols (TBCDDOHs) (3-4). The kinetics and stereochemistry of

LinB-catalyzed *γ*-HBCD transformation have been described in detail, with *Km*, *kcat*,

and *kcat*/*Km* values at 1.82 ± 0.60 μmol/L, 0.25 ± 0.10 μmol/L/h and 13.0 ± 6.2

L/mol/s. The results suggest that LinB has a high capability to dehalogenate *γ*-HBCD

(5).

Catalytic enzyme resources from bacteria are abundant, and cytochrome P450

enzymes (CYPs) are the key enzymes responsible for the degradation of numerous

endogenous compounds. CYPs are involved in the degradation and detoxification of

multiple toxicants, such as herbicides, xenobiotic poly aromatic hydrocarbons,

58 halogenated aromatics, and polychlorinated biphenyls (6-10). Hydroxylation is the

59 typical metabolic reaction of xenobiotics catalyzed by CYPs. Transformed CYP81As

60 from *Echinochloa phyllopogon* decreased—the susceptibility of *Arabidopsis* to

61 clomazone (11-12). Mammalian CYPs (CYP1 family) degrade dibenzo-*p*-dioxins

62 (PCDDs) with efficient activity, and the rat CYP1A1 family also showed high activity

63 towards 2,3,7-trichloro-dibenzo-*p*-dioxin, with the detection of hydroxylated products,

64 8-hydroxy-2,3,7-trichloro-dibenzo-*p*-dioxins   (13).   CYP101   dehalogenates

65 hexachlorobenzene with a different metabolic method, in which the halogen atoms are

66 replaced by hydroxyl groups (14). CYP2E1 from *Nicotiana tabacum*, CYP3A4 from

67 human liver and CYPs (CYP71C3v2, CYP71C1, CYP81A1 and CYP97A16) from

68 maize can metabolize HBCDs, and the hydroxylated metabolites OH-HBCDs,

69 OH-PBCDs and OH-TBCDs have been detected (15-20). However, the substitution

70 reaction of HBCDs by CYPs is rarely reported.

71   Previous work by our research group on *Pseudomonas aeruginosa* HS9 indicated

72 that HBCDs could be degraded to PBCDOHs. Strain HS9 was reported to be a

73 HBCD-metabolizing bacterium based on its ability to convert HBCDs to PBCDOHs

74 or tetrabromocyclododecene (TBCDe), dibromocyclododecadiene (DBCDi), and

75 cyclododecatriene (CDT) (21). In this study, the whole-genome sequence of strain

76 HS9 was sequenced and analyzed, and putative genes for HBCD degradation were

77 elucidated.   By   combining   metabolite   analysis   with   real-time   fluorescence

78 quantification experiments (RT-qPCR), the cytochrome P450 enzyme CYP168A1 was

79 considered as the initial dehalogenase in HBCD metabolism. The gene *cyp168A1* was

80   cloned and expressed in *Escherichia coli*. The subsequent enzymatic properties were

81   investigated on the purified CYP168A1.

82

83   **Materials and methods**

84   **Chemicals.** 1, 2, 5, 6, 9, 10-Hexabromocyclododecanes (HBCDs, $\geq$ 95%) were

85   purchased from Anpel (New Jersey, USA). Hexachlorobenzene (HCB, $\geq$ 95%) was

86   purchased from AccuStandard (Connecticut, USA). Ethyl acetate, methanol, and all

87   the other regents and solvents used in this study were of analytical grade.

88

89   **Strains and culture media.** *Pseudomonas aeruginosa* HS9 was isolated by our

90   research group in previous work, and it can be obtained from the China Center for

91   Type Culture Collection (CCTCC) under accession M 2019094 (20). *Escherichia coli*

92   DH5α and BL21(DE3) (Novagen, Inc. USA) were used for plasmid construction and

93   protein expression, respectively. Lysogeny broth (LB), containing 5 g/L yeast

94   extraction, 10 g/L tryptone and 5 g/L NaCl, or LB agar (1.5% wt/vol) plates with

95   appropriate antibiotics were used to culture *E. coli* (22). *E. coli* harboring each of the

96   constructed plasmids was grown at 37°C, 200 rpm with 50 mg/L kanamycin or 100

97   mg/L ampicillin for pET28a or pETduet-1 vectors. Strain HS9 was grown at 30°C in

98   mineral salt medium (MSM) containing 5.0 g/L K_2HPO_4, 3.7 g/L KH_2PO_4, 1.0 g/L

99   Na_2SO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L NH_4Cl and 0.5 mL 2,000-times trace elements

100   solution. The trace elements solution consisted of 0.3 g/L $FeCl_2 \cdot 4H_2O$, 0.038 g/L

101   $CaCl_2 \cdot 6H_2O$, 0.02 g/L $MnCl_2 \cdot 4H_2O$, 0.014 g/L $ZnCl_2$, 0.0124 g/L H_3BO_3, 0.04 g/L

102 Na$_2$MoO$_4$·2H$_2$O and 0.0034 g/L CuCl$_2$·2H$_2$O (21).

103

104 **Genome sequencing and proteomic assay of strain HS9.** The genomic DNA of

105 strain HS9 was extracted using a Wizard genomic purification kit A1125 (Promega,

106 USA). Genome sequencing was performed on the Illumina Hiseq-2000 platform.

107 Functional genes were predicted and annotated with the Rapid Annotations using

108 Subsystems Technology (RAST) annotation server (23). This whole genome sequence

109 project was submitted to GenBank under accession GCA_003319235.1. Proteomic

110 analysis comparing the protein expression of cells incubated in the presence or

111 absence of 1 mg/L HBCDs MSM media was carried out as follows. Strain HS9 was

112 cultured in 2 L flasks containing 1 L HBCDs-MSM. As a control group, strain HS9

113 was grown in sodium citrate medium. A total of 10 L of culture were collected during

114 the exponential phase. Both groups were detected with three biological replicates (22).

115

116 **Quantitative RT-qPCR.** Total RNA was isolated from strain HS9 incubated in the

117 presence or absence of 1 mg/L HBCD MSM media, using a total RNA kit (Tiangen,

118 China). Total cDNA was synthesized using a SuperScript III reverse transcriptase

119 (Invitrogen, USA). The 20 μL reverse transcription reaction system contained 1.0 μg

120 total RNA, 0.5 mM dNTP mix, 200 U transcriptase, and 12.5 ng random primers. The

121 reactions were performed according to the manufacturer's protocols. RT-qPCR was

122 then carried out using the CEX96 real-time PCR detection system (Bio-Rad) with a

123 SYBR green I Real Master Mix (TianGen, China). All the data of candidate genes was

124   normalized to the expression level of 16S rRNA and presented as relative to the

125   expression level in cells growing in the absence of HBCDs. All detections were

126   performed with three replicates (23−25).

127

128   **Expression and purification of heterologous expressed His-CYP168A1.** The DNA

129   fragment of *cyp168A1* was amplified by pfu DNA polymerase (New England Biolabs,

130   Ipswich, MA) with primers Fcyp168A1

131   (CCG<u>GAATTC</u>CTACTCGCAGGTCTTCTGAG) and Rcyp168A1

132   (CCC<u>AAGCTT</u>ATGGACGACGCATTCAGCGA), in which the enzyme digestion

133   sites (*Eco*RI and *Hin*dIII) are underlined. The double enzyme digested DNA

134   fragments were ligated into expression vector pET28a, which incorporates 6 ×

135   Histidine tags. Then, the constructed plasmid pET28a-*cyp168A1* was transferred into

136   *E. coli* (BL21) for heterologous expression. The culture was induced by adding 0.6

137   mM isopropyl β-D-thiogalactopyranoside (IPTG) after the optical density at 600 nm

138   (OD_{600}) reached 0.6 to 0.8. Then, the culture was incubated at 30°C for 10 h. *E. coli*

139   was harvested by centrifuging at 4,000 rpm for 20 min, and the pellet was

140   re-suspended with nickel column balance buffer (20 mM NaH_2PO_4-Na_2HPO_4, 300

141   mM NaCl, 10 mM imidazole, 6 M urea, pH 8.0); urea was used to denature the

142   proteins to enhance solubility (26). The cell suspension was broken by repetitive

143   sonication at 4°C, and the cell debris was removed by centrifugation at 10,000 rpm for

144   40 min. The his-CYP168A1 was loaded into the nickel column, and then washed by

145   gradient imidazole buffers from 10, 40, 70, 100 to 300 mM (27). The residual

146   imidazole in the eluted buffer was removed by gradient dialysis from buffer I (20 mM

147   KH_2PO_4-K_2HPO_4, 4 M urea, 5% glycerol, 1% glycine, 1‰ mercaptoethanol, pH 8.0)

148   for 2 h, to buffer II (20 mM KH_2PO_4-K_2HPO_4, 2 M urea, 5% glycerol, 1% glycine,

149   1‰ mercaptoethanol, pH 8.0) for 2 h, and then to buffer III (20 mM KH_2PO_4-K_2HPO_4,

150   5% glycerol, 1% glycine, 1‰ mercaptoethanol, pH 8.0) for 3 h. CYP168A1 was

151   successfully refolded *in situ* through a gradient of decreased urea concentrations (26).

152

153   **Western blot analysis and UV-vis characterization of purified CYP168A1.** The

154   purified CYP168A1 was determined by western blot analysis, using an anti-6 × His

155   tag® antibody (Abcam, China). The purified CYP168A1 was diluted 10, 100, and

156   1,000 times, and 10 μL was transformed to PVDF film, respectively. The carbon

157   monoxide (CO)-difference spectrum was performed in buffer (50 mM

158   KH_2PO_4-K_2HPO_4, 1‰ mercaptoethanol, 5% glycerol, pH 8.0) at 20°C, respectively,

159   in a 0.5-mL quartz cuvette with a 1-mm path length. Protein CYP168A1 was reduced

160   by adding 10 mM dithionite, and the CO complex was performed by slow bubbling

161   with CO gas for 1 min 30 s (27).

162

163   **Construction of electron-supplying system and enzyme activity.** To test the *in vitro*

164   activity of CYP168A1, sufficient electrons must be supplied to the reaction system.

165   Therefore, an electron-supplying system (named as FdFNR) was constructed by

166   combining a 4Fe-4S ferredoxin (HS1040) (Fd) and a NAD(P)H-dependent ferredoxin

167   reductase (HS6332) (FNR) with a glycine linker (GGGGG). The combined DNA

168   fragment was ligated to expression vector pETduet-1 at the second multiple cloning

169   site (MCS). The protein was induced by adding 0.2 mM IPTG after the OD_{600} reached

170   0.6 to 0.8; then, the culture was incubated at 16°C for 10 h. Ultimately, the cells were

171   broken in PBS buffer with the same method in the paragraph of CYP168A1

172   purification, and it was used as the electron-supplying cell free system. The electron

173   supplying ability was determined with potassium ferricyanide ($K_3[Fe(CN)_6]$) as the

174   receptor of the free donor, and NADPH as the source of donor. Absorbance of

175    $K_3[Fe(CN)_6]$ at 340 nm was measured and the color feature (yellow) of $K_3[Fe(CN)_6]$

176   was captured. To test the enzyme activity of CYP168A1 with HBCDs, 1 mg/L

177   HBCDs, 0.4 mM NADH, 5 μg purified CYP168A1 and 1 mL cell-free system were

178   mixed, and the reaction system was incubated under different reaction conditions. The

179   decrease in HBCD concentration was used to calculate enzyme activity. To test the

180   effect of metal irons on enzyme activity, 10 mM chloride salts ($NiCl_2$, $CoCl_2$, $CaCl_2$,

181    $CuCl_2$, $MnCl_2$, $ZnCl_2$, $MgCl_2$, KCl, $FeCl_2$ and $NaMoO_4$) were separately added to

182   reaction system.

183

184   **^{18}O isotope experiments and analysis.** To confirm the source of the oxygen atom

185   incorporated into the HBCD degradation products, $^{18}O_2$ and $H_2^{18}O$ were used to

186   supply oxygen atoms for CYP168A1 reactions. The $^{18}O_2$ labeling reaction and

187   anaerobic assay were performed in an anaerobic workstation AW200SG (Electrotek

188   Ltd, UK). After excluding air for 1 h by N_2 atmosphere, all the liquid (1 mL FdFNR

189   buffer, 5 μg purified CYP168A1) was exposed to an N_2 atmosphere for 30 min to

190 remove O_2. An activity assay system (equal to the system for enzyme activity

191 detection) that was cell free containing enzyme and NADH was dried and dissolved in

192 $H_2^{18}O$. All reactions were carried out at 30°C for 6 h. After terminating the reaction by

193 adding 10 μL HCl (11.64 M) to the 1 mL reaction system, HBCDs were extracted by

194 using an equal volume of ethyl acetate. Then, samples were mixed using vortex

195 oscillation for 30 s. Before detection, the upper organic phase was concentrated 30

196 times. Samples were analyzed using ultra-high-performance liquid

197 chromatography/time-of-flight mass spectrometry (UPLC-TOF/MS).

198   HBCDs or its products in activity assay experiments and the ^{18}O isotope

199 experiments were quantified by UPLC-TOF/MS, equipped with an Eclipse XDB C18

200 analytical column (5 μm, 4.6 × 150 μm, Keystone Scientific, Agilent). HBCDs in

201 samples used for products detection were extracted as the above described, and then,

202 the organic phase was concentrated about 1,000 times. A mobile phase of water and

203 methanol at a flow rate of 0.25 mL/min was applied for the target compounds. The

204 proportional gradient of the mobile phase was started at 95% methanol, and increased

205 linearly to 100% over 25 min, then decreased directly to 95% for 10 min. For mass

206 spectrometric analysis, the ionization source was run in negative mode, and MS

207 detection was set from 0 to 1,700 *m/z*. All target compounds were extracted based on

208 their hydrogen adduct ions [M+H]- at *m/z* and characterization of bromine isotope

209 (28).

210

211 **Bromide detection.** The detection of bromide was conducted on an ion

212 chromatograph coupled with an AS11-HC negative ion column (ICS-5000+, Thermo

213 Fisher, Germany). The samples were prepared by terminating the reaction by adding

214 10 μL HCl to the 1 mL reaction system, followed by centrifugation-at 12,000 rpm for

215 5 min to remove the proteins.

216

217 **Gene deletion and complementation.** The suicide vector pK18*mobsacB-Gm* used

218 for gene deletion was derived from pK18*mobsacB* by replacing the kanamycin

219 resistance gene with a gentamicin resistance gene (from plasmid pUCTn7T).

220 Upstream (600 bp) and downstream (600 bp) fragments close to the target gene were

221 amplified and aligned using fusion PCR, the primers and corresponding PCR

222 functions used in this study were listed in Table 1. The constructed plasmid

223 pK18*mobsacB-Gm-cyp168A1AB* was transferred from the *E. coli* donor strain S17-1

224 to *P. aeruginosa* HS9 by conjugal transfer. Donor strain S17-1 and recipient strain

225 HS9 were mixed with a volume rate at 5:1 - 10:1 and cultured on LB solid media

226 without antibiotics at 37°C for 4 h, and 30°C for 20 h, then the mixture was spread on

227 M9 solid plates and incubated at 30°C for 48 h with 50 mg/L gentamicin, after it was

228 resuspended and washed using saline solution. Finally, the correct transconjugants

229 were washed and plated on LB-sucrose agar medium for plasmid elimination (29).

230 The gene engineered groups were obtained by inserting a *lacZ* promoter with the

231 genes *FdFNR* before *cyp168A1* (PLAC-HS9), or inserting a single *lacZ* promoter with

232 the genes *FdFNR* followed *cyp168A1* (HS9-DW), or combined the operations of

233 PLAC-HS9 and HS9-DW to get PLAC-DW. The *lacZ* promoter sequence was

234   amplified from clone vector pMD18T.

235   The expression vector pUC18k was used for gene complement in *P. aeruginosa*.

236   The new expression plasmid pUC18k-*cyp168A1* was electrotransferred to strain HS9.

237   Electrocompetent cells were prepared as follows: first, the strain was cultured in LB

238   medium at 30°C after OD600 reached 0.6, and then the cells were pelleted at 4,000

239   rpm for 10 min, after incubating on ice for 20 min; finally, the cell pellets were

240   washed twice using electroporation buffer (10% glycerol) (30). Correct transformants

241   were verified by PCR and the cells were further incubated in LB medium with

242   corresponding antibiotics.

243

244   **Results**

245   **Genomic and proteomic profiles of strain HS9.** Whole genome sequencing was

246   preformed, and the sequence was assembled into a single circular chromosome

247   without gaps (Fig. S1A). The circular chromosome is 6,876,988 bp in size, with a G +

248   C content of 66.2% and 6,421 coding sequences (CDSs). Further analysis indicated

249   that there were 157 CDSs annotated as related to metabolism of aromatic compounds

250   (Fig. S1B). To explore functional genes involved in HBCD degradation, a proteomic

251   analysis was carried out to compare the expression of proteins from cells incubated in

252   the presence or absence of 1 mg/L HBCDs MSM media. A total of 1,770 proteins

253   were identified, accounting for 27.6% of the genomic putative CDSs in strain HS9.

254   Normalization was performed to average the abundance of all peptides. Differentially

255   expressed proteins were filtered if their fold changes were over 2.0-fold with

256   significance > 20 (PEAKS Significance B Algorithm, p-value < 0.01) and if they had

257   two unique peptides.

258   The expression of 277 proteins was significantly changed ($\geq$ 2-fold change,

259   P-value < 0.01), of which 190 proteins were up regulated and 87 were down-regulated

260   (Fig. S1C). The up regulated proteins were divided into 25 categories by Clusters of

261   Orthologous Groups (COGs) analysis (Fig. S1D). To narrow the search, the most

262   significantly changed proteins ($\geq$ 10-fold change, P-value < 0.01) are summarized in

263   Fig. S2, and the HBCD-induced proteins are listed in Table S1. No annotated

264   dehalogenases were identified among the 277 up regulated proteins, while the

265   expression of the NADH reductase (HS5738), heme d1 biosynthesis protein (NirF)

266   (HS1898) and iron (III) dicitrate transport protein (FecA) (HS1283) were up regulated

267   with fold changes of 5.81, 217.80, and $+\infty$, respectively. As many up regulated genes

268   were related to electron donating, the functional genes that cooperated with electron

269   donor were considered as possible HBCD degrading genes.

270   Many genes related to heavy metal were up regulated, including zinc and

271   mercury transporting ATPase (EC 3.6.3.3), heavy metal sensor histidine kinase,

272   copper resistance protein (CopC), Na^+/alanine symporter, iron (III) dicitrate transport

273   protein (FecA), and zinc protease. Moreover, genes correlated with basic bioactivity,

274   such as D-lactate dehydrogenase, L-lactate dehydrogenase (EC 1.1.2.3), L-lactate

275   permease, and succinate dehydrogenase were significantly up regulated.

276

277   **Identification of HBCD degrading genes.** To identify the possible genes involved in

278   HBCD degradation in cooperation with Ferredoxin-NADP (+) reductase, three

279   cytochrome P450 (CYP) proteins were selected as candidates. RT-qPCR assays were

280   carried out to further detect the mRNA expression levels of the potential genes (Fig.

281   1A). The cytochrome P450 coding gene *cyp168A1* was up regulated in response to

282   HBCDs with a fold change of 9.1. The HBCD consumption curve of strain HS9 was

283   determined in a resting cell reaction system. The wild type of strain HS9 (WT) could

284   degrade 1 mg/L HBCDs within 8 h. In addition, we also deleted or complemented the

285   gene *cyp168A1*, and HBCD consumption capability of the mutant strain M*cyp168A1*

286   was eliminated. When the *cyp168A1* gene was complemented in M*cyp168A1*, the

287   HBCD degradation capability of strain W*cyp168A1* recovered to the same value as

288   strain HS9 (Fig. 1B).

289

290   **Verification of electron donor capability of cell free system-FdFNR.** To confirm

291   the donor supplying ability of FdFNR, potassium ferricyanide ($K_3[Fe(CN)_6]$) was

292   used as the receptor of the free donor. Compared to the control group, the absorbance

293   of $K_3[Fe(CN)_6]$ at A340 decreased to zero over 5 min, and the color feature (yellow)

294   of $K_3[Fe(CN)_6]$ disappeared (Fig. 2A). Results showed that the cell free system was

295   able to oxidize NADH to NAD^+ with an electron accepter present.

296

297   **CYP168A1 is an efficient debromination enzyme.** The gene *cyp168A1* was

298   amplified and expressed in pET28a in *E. coli* BL21(DE3). The heterologously

299   expressed 6 × His-CYP168A1 was successfully purified, with a molecular mass of 50

300 kDa (Fig. 2B), and the western blot analysis demonstrated the purified protein (Fig.

301 S3A). The CO-difference spectrum showed that the purified protein has a strong

302 absorbance at 450 nm (Fig. 2C). Results of enzyme activity detection showed that

303 CYP168A1 degraded HBCDs in the presence of NADH in the FdFNR system. The

304 optimal temperature for CYP168A1 activity was at 30°C (Fig. S3B), The effect of

305 temperature on CYP168A1 stability was monitored by circular dichroism

306 spectroscopy (CDS) (JASCO, Japan), which showed that CYP168A1 began to

307 degenerate at temperatures above 35°C (Fig. S3C). Kinetic analysis revealed that the

308 *Vmax* and *Km* were 0.73 U/mg and 0.35 mM (Fig. 2D), respectively. Most metal ions,

309 including Ca^{2+}, Co^{2+}, Cu^{2+}, MoO_4^{2+}, enhanced enzyme activity, while Zn^{2+} and K^+ did

310 not (Fig. S3D).

311

312 **Product analysis and ^{18}O isotope experiments.** The products of the reaction

313 catalyzed by CYP168A1 were identified using LC-TOF-MS, based on the mass

314 spectra (*m/z*) of the target products. Products with molecular weights at [M-H]⁻

315 612.7000, 614.7000, and 616.7000, or 576.7240 and 578.7219 were detected. The

316 results were matched to the previously reported standard compounds PBCDOHs and

317 two tetrabromocyclododecadiols (TBCDDOH$_S$) (Fig. 3AB). Products with molecular

318 weights at [M-H]⁻ 450.8949, 388.9792, 325.0679 and 263.1655 were also detected

319 (Fig. 3C-F). The bromide was detected by ion chromatography analysis (Fig. S4).

320 These products suggest that CYP168A1 can degrade HBCDs through a debromination

321 and hydrogenation process, and one oxygen atom was added to the product in each

322   step of the reaction. To determine the source of the oxygen that participates in the

323   reaction, ^{18}O isotope experiments were carried out as mentioned above. Products in

324   ^{18}O$_2$ group had molecular weights of [M-H]$^-$ 611.5232, 612.6262, and 613.5282,

325   which match to PBCD^{16}OHs (Fig. 4A). In the ^{16}O-Not lyophilized and

326   ^{16}O-Lyophilized groups, PBCD^{16}OHs were detected (Fig. 4B, 4C). Thus,

327   lyophilization would not inactivate the enzyme activity. In contrast, products

328   containing ^{18}O, with molecular weights of [M-H]$^-$ 612.7000, 614.7000, 616.7000, and

329   617.4908, were only detected in the H$_2$^{18}O group (Fig. 4D). Comparing the results of

330   Fig. 4A, B, and C with Fig. 4D, ^{18}O from H$_2$^{18}O was added to PBCDOHs, and no ^{18}O

331   labeled products formed in the ^{18}O$_2$ group. The results confirmed that the oxygen in

332   PBCD^{18}OHs was derived from H$_2$^{18}O. All the corresponding TIC spectrums for

333   products detection are shown in Fig. S5 and Fig. S6. The proposed pathway of HBCD

334   degradation catalyzed by CYP168A1 is shown in Fig. 5.

335

336   **Enhancement of degrading capacity of strain HS9.** To enhance the degrading

337   capacity of strain HS9, the expression of gene cyp168A1 and the combined donor

338   supplying system FdFNR were increased with a promoter lacZ was added as Fig. 6A.

339   The gene engineering modes and comparison of the HBCDs degrading ability of the

340   wild-type HS9 (WT), mutants PLAC-HS9, HS9-DW, and PLAC-DW are shown in

341   Fig. 6B. The cell growth and degrading rates of the mutants were detected in the

342   MSM-HBCDs system, and the results showed that the degrading rates of strain

343   HS9-DW were improved, compared to WT. However, increase the gene *cyp168A1*

344   expression cannot improve the degrading rate of HBCDs.

345

346   **Phylogenetic analysis**. Several amino acid sequences of dehalogenases CYPs, such

347   as CYP7A11, CYP81A3v2 (17), CYP1A1, CYP2C11, CYP26B1 (13), CYP2E1 (15),

348   CYP3A4 (17), CYP101 (14) and P450BM-1 (10), were compared, and the results

349   showed that CYP168A1 was most closely related to CYP101 (Fig. 7).

350

351   **Discussion**

352   Hexabromocyclododecanes (HBCDs) have become a global research focus, due to

353   their widespread pollution and serious harm to human health, such as inducing cancer

354   (31), disrupting liver and thyroid hormones (32-33), and causing reproductive

355   disorders (34). Several bacteria have been discovered from natural environments that

356   can degrade HBCDs, such as *Pseudomonas* sp. HB01, *Bacillus* sp. HBCD-sjtu,

357   *Achromobacter* sp. HBCD-1, *Achromobacter* sp. HBCD-2, and *P. aeruginosa* strain

358   HS9 (35-38). Corresponding pathways have been proposed for these strains, but the

359   specific molecular mechanisms of the degradation have not been revealed. In this

360   study, the functional enzymes for HBCDs degradation from *P. aeruginosa* strain HS9

361   was characterized.

362   Proteomic analysis comparing the expression of proteins of the cells incubated

363   was carried out with MSM medium in the presence or absence of 1 mg/L HBCDs, and

364   the results showed that environmental stress response genes like lactate

365   dehydrogenases (LDH) were up regulated in the HBCD group. *Enterococcus faecalis*

366   has general resistance to very different environmental stresses, depending on the

367   ability to maintain redox balance via LDH (39). In addition, decreases and increases

368   in salinity concentrations sharply increase the LDH activity of *Neanthes*

369   *arenaceodentata* (40). Moreover, succinate dehydrogenase was up regulated, which

370   could catalyze succinate to fumarate when a FADH was formed. Based on the above

371   information, we propose that the resistance of strain HS9 to HBCD stress occurs due

372   to maintaining the balance of reducing power in vivo, coupled with HBCD

373   degradation. HBCD could induce the expression of *cyp168A1* of strain HS9, while the

374   CYP168A1 cooperation with electron donors, and the electron transport and stress

375   resistance reactions (related to lactate dehydrogenases or succinate dehydrogenase)

376   were used to balance the electron supply in vivo.

377   In nature, cytochrome P450 (CYP) enzymes participate in degrading large

378   amounts of environmental pollutants. Typically, CYP monooxygenases introduce a

379   single oxygen atom into their substrates (41-43). However, there are few reports about

380   CYP enzymes simultaneously catalyzing debromination and hydrogenation reactions

381   (44). In this study, a novel CYP (CYP168A1) was shown to be the initial

382   dehalogenase enzyme in HBCD biodegradation. The gene *cyp168A1* was cloned and

383   expressed in *E. coli* and the enzymatic properties of the purified CYP168A1 were

384   investigated. The *Km* of CYP168A1 for HBCDs was 0.35 mM, while the *Km* of

385   γ-HBCD for LinB was 1.82 ± 0.60 μM, the affinity for HBCD to LinB is almost 1,000

386   times than that to CYP168A1. However, biochemistry information for the other two

387   major HBCD isomers to LinB was still limited. The affinity of CYP168A1 to HBCDs

388 was lower than that of LinA/B, it matched the lower degrading rate of strain HS9,

389 compared to other HBCD degraders.

390   Considering the toxicity of HBCDs to the environment and humans, comparative

391 metabolism studies and *in vitro* activity tests indicated that the human liver CYP3A4,

392 maize CYPs, and male rat CYPs can degrade different HBCD isomers. To trace the

393 source of gene *cyp168A1*, phylogenetic analysis was carried out. Several mono- and

394 dihydroxylated metabolites of HBCDs are formed through catalyzing with the human

395 liver CYP3A4, maize CYPs, and male rat CYPs, with mono-OH-HBCDs detected as

396 the major metabolites (6, 17, 43). However, the products of HBCDs catalyzed by

397 CYP168A1 were PBCDOHs and TBCDDOHs, generated from the debromination and

398 hydrogenation processes of HBCDs, of which PBCDOHs were also identical to that

399 produced by strain HS9 in HBCDs-MSM medium (21). This result revealed the

400 difference of HBCDs biodegradation between eukaryotic cells and prokaryotic

401 microorganisms.

402   In the reactions of 1,2-halododecanoic acids oxidation catalyzed by both CYP4A

403 (44) and CYP52A (45), oxygen in 1,2-Hydroxydodecanoic acids derives from water,

404 not from molecular oxygen, which was introduced by hydrolysis of an initially formed

405 oxohalonium $(R–X^+–O^-)$ metabolite. The results of ^{18}O isotope labeling reactions

406 showed that H_2O serves as the source of the oxygen atom incorporated into

407 PBCDOHs (Fig. 4). Mechanism of the oxygen addition was the same as oxidation of

408 1,2-halododecanoic acids by CYP4A and CYP52A. The present study revealed a

409 novel mechanism of CYP to catalyze the brominated organic compounds.

410 In summary, this study reveals a new catalytic mechanism of CYP168A1 for the

411 degradation of HBCDs, in which the debromination and hydrogenation reactions are

412 carried out one after another. The ^{18}O isotope experiments show that the oxygen

413 added into hydrated products were from H_2O. Engineering mutants of strain HS9 not

414 only supplies new insights into biochemical properties of protein CYP168A1, but also

415 serves as a model for enhancing the abilities of this strain in bioremediation.

416

417 **Competing interests**

418 All the authors declare no competing interests.

419

420 **Authors' contributions**

421 LH and HT outset and designed experiments. LH and WW performed experiments.

422 HT and PX contributed reagents and materials. LH, HT, ZG, and PX wrote the paper.

423 All Authors discussed and revised the manuscript. All Authors commented on the

424 manuscript before submission. All authors read and approved the final manuscript.

425

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594

Table 1. The primers used in this study. (The underline represents homologous sequences to the constructed vector)

Names	Sequence (3' - 5')	Function
GmF	<u>CCCAAGCTT</u>ATGTTACGCAGCAGCAACGA	Replacement of resistance gene
GmR	<u>CTAGCTAGC</u>TTAGGTGGCGGTACTTGGGT	Replacement of resistance gene
Fcyp168A1	CCG<u>GAATTC</u>ATGGACGACGCATTCAGCGA	Construction of pET28a-*cyp168A1*
Rcyp168A1	CCC<u>AAGCTT</u>CTCGCAGGTCTTCTGAGCGT	Construction of pET28a-*cyp168A1*
AFcyp168A1	<u>TATGACATGATTACGAATTC</u>ATGGACGACGCATTCAGCGA	Gene knockout
ARcyp168A1	<u>GTTATAAATTTGGAGTGTGA</u>GCACGGCGTCGGGGCCGAAG	Gene knockout
BFcyp168A1	<u>TCACACTCCAAATTTATAAC</u>GCGGCGAACGCGGTGGAGGA	Gene knockout
BRcyp168A1	<u>AGGTCGACTCTAGAGGATCC</u>CTCGCAGGTCTTCTGAGCGT	Gene knockout
Uplac-A1F	<u>TGACATGATTACGAATTC</u>CGAATACCAGAACCAGGGCA	Construction of PLAC-HS9/PLAC-DW
Uplac-A1R	<u>TGAGTGAGCTAACTCACATT</u>GGCCCTTGCTCCGCTGGGTT	Construction of PLAC-HS9/PLAC-DW
UplacF	AATGTGAGTTAGCTCACTCA	Construction of PLAC-HS9/PLAC-DW
UplacR	<u>TCGCTGAATGCGTCGTCC</u>ATGGCGTAATCATGGTCATAGC	Construction of PLAC-HS9/PLAC-DW
Uplac-B1F	ATGGACGACGCATTCAGCGA	Construction of PLAC-HS9/PLAC-DW
Uplac-B1R	<u>GCAGGTCGACTCTAGAGGATCC</u> CCCGGCATCGCCGTGGCTGG	Construction of PLAC-HS9/PLAC-DW
Dplac-A2F	<u>TGACATGATTACGAATTC</u> CCAGCCACGGCGATGCCGGG	Construction of HS9-DW/PLAC-DW
Dplac-A2R	<u>TGAGTGAGCTAACTCACATT</u>CTACTCGCAGGTCTTCTGAG	Construction of HS9-DW/PLAC-DW
Dplac-F	AATGTGAGTTAGCTCACTCA	Construction of HS9-DW/PLAC-DW
Dplac-R	<u>TCCAGCACGACGAAGGT</u>CATGGCGTAATCATGGTCATAGC	Construction of HS9-DW/PLAC-DW
DFERF	ATGACCTTCGTCGTGCTGGA	Construction of HS9-DW/PLAC-DW
DFERR	<u>GGCAGCCGGCTGATCCTGC</u>GTCACTTCTCGACGAAGGCGC	Construction of HS9-DW/PLAC-DW
Dplac-B2F	CGCAGGATCAGCCGGCTGCC	Construction of HS9-DW/PLAC-DW
Dplac-B2R	<u>GCAGGTCGACTCTAGAGGATCC</u>GAGGCCGACGACTTCATGGA	Construction of HS9-DW/PLAC-DW
cyp168A1cF	<u>GGTCGACTCTAGAGGATCCC</u>ATGGACGACGCATTCAGCGA	Gene complementation

cyp168A1cR   AGGTCGACTCTAGAGGATCCCTCGCAGGTCTTCTGAGCGT   Gene complementation

Figure legends

596	**Fig. 1. Identification of functional proteins.** (A) RT-qPCR verification of the

597	proposed functional genes in degrading HBCDs. In RT-qPCR assays, the treatment

598	group used HBCDs as the sole carbon source and the control group used sodium

599	citrate. HS651: putative cytochrome P450 hydroxylase; HS1037: cytochrome P450;

600	HS6073 (*cyp168A1*): putative cytochrome P450 hydroxylase (B) Comparison of the

601	HBCD degrading ability of the wild type HS9 (WT), *cyp168A1* deleted mutant strain

602	(M*cyp168A1*) and *cyp168A1* complemented strain (W*cyp168A1*).

603

604	**Fig. 2. Characterization of CYP168A1.** (A) Verification of the electron donor

605	capability of the cell free system-FdFNR. The protein expression of FdFNR was

606	measured and shown by SDS-PAGE, the color feature (yellow) of $K_3[Fe(CN)_6]$ was

607	captured, and the full wavelength scanning shows the concentration of $K_3[Fe(CN)_6]$ in

608	the cell free system. (B) SDS-PAGE analysis of CYP168A1. M: protein marker; lane

609	1, supernatant of the sonicated Bl21-pET28a-*cyp168A1*; lane 2, column effluent; lane

610	3, 10 mM imidazole buffer washed effluent; lane 4, 45 mM imidazole washed effluent;

611	lane 5, 70 mM imidazole washed effluent; lane 6, 100 mM imidazole washed effluent;

612	lane 7, 150 mM imidazole washed effluent. (C) The CO-difference spectrum of

613	CYP168A1. (D) Kinetic analysis of CYP168A1 (fitted to the Michaelis-Menten

614	kinetics).

615

616	**Fig. 3. Identification of intermediates of HBCD degradation by LC-TOF-MS.** (A)

617	Mass spectra of PBCDOHs. (B) Mass spectra of TBCDDOHs. (C)-(F) Corresponding

618 mass spectra of molecular weights (*m/z* 450.8949) (*m/z* 388.9792) (*m/z* 325.0679) and

619 (*m/z* 263.1655).

620

621 **Fig. 4. ^{18}O labeled products of HBCD degradation.** (A) Mass spectra extracted

622 from the ^{18}O group (PBCD^{16}OHs). (B) Mass spectra extracted from the ^{16}O-Not

623 lyophilized group (PBCD^{16}OHs). (C) Mass spectra extracted from the

624 ^{16}O-Lyophilized group (PBCD^{16}OHs). (D) Mass spectra extracted from the

625 ^{18}O-Lyophilized group (PBCD^{18}OHs).

626

627 **Fig. 5. Proposed pathway for HBCD degradation.** HBCDs were dehalogenated by

628 CYP168A1, with a serial of hydroxy added. The undetected oxohalonium metabolites

629 have been drawn in frame.

630

631 **Fig. 6.** Schematic for the gene engineering (A), and comparison of the HBCDs

632 degrading ability of the wild-type HS9 (WT) and genome edited mutants PLAC-HS9,

633 HS9-DW, and PLAC-DW (B).

634

635 **Fig. 7.** Phylogenetic tree analysis of CYP168A1 with reported cytochrome P450

636 enzymes that function in dehalogenation.
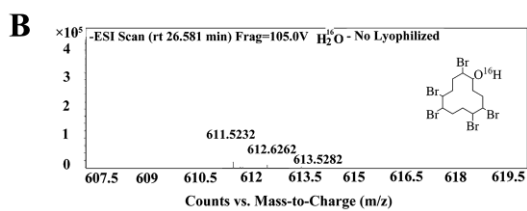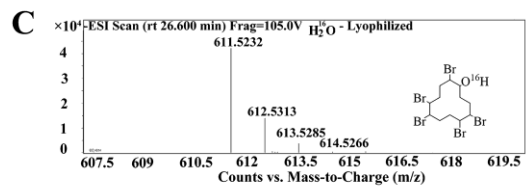
637   **Fig. 1**

638
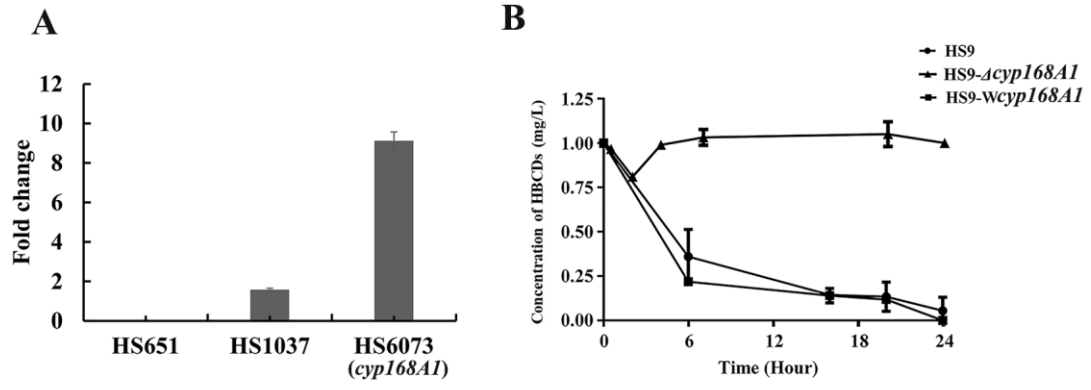
639

640   **Fig. 2**
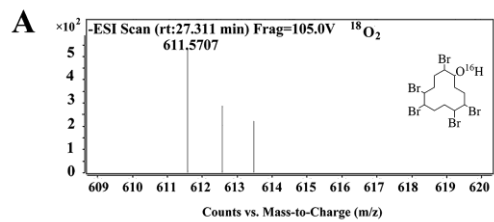
641
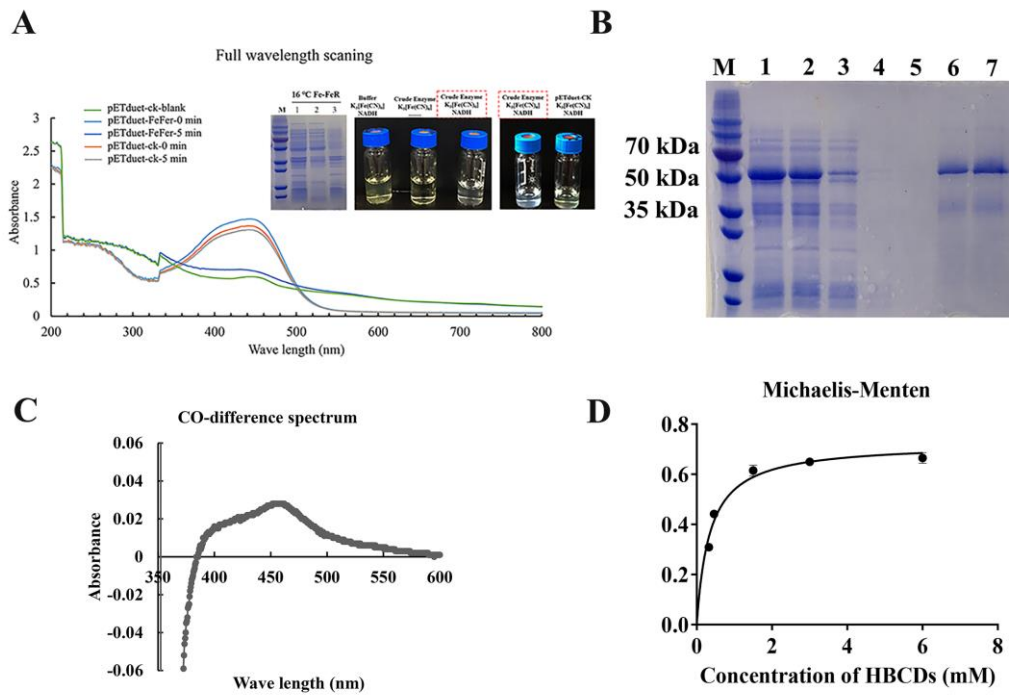
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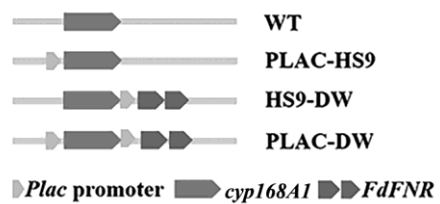
643   **Fig. 3**
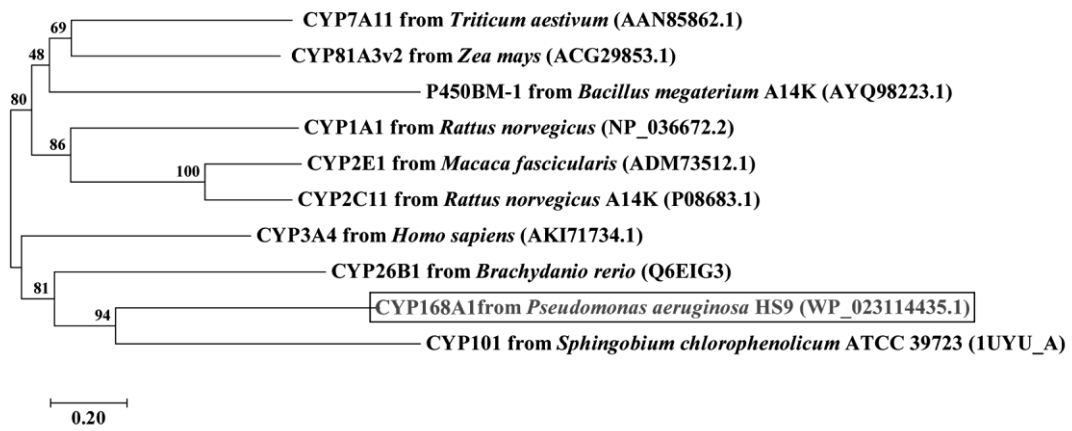
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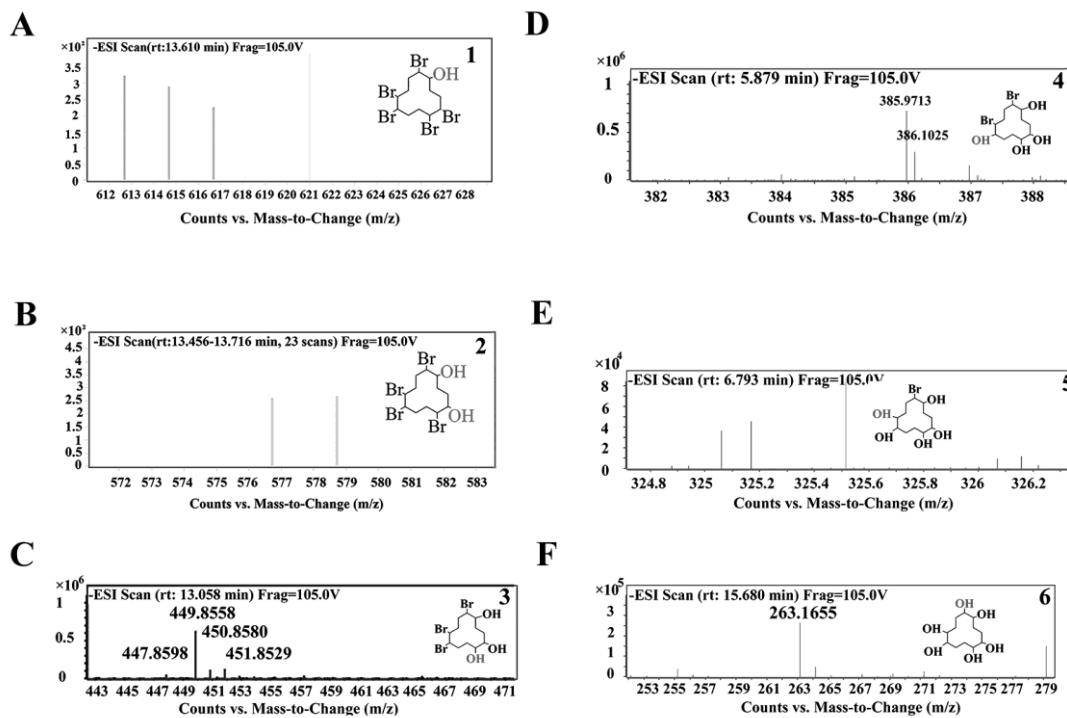
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646   **Fig. 4**

647
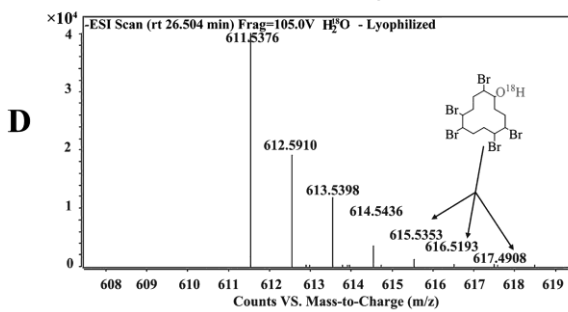
648

649   **Fig. 5**

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652   **Fig. 6**

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655 **Fig. 7**

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