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1 **Biodegradation of 2-ethylhexyl nitrate (2-EHN) by *Mycobacterium austroafricanum* IFP**

2 **2173**

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19 **ABSTRACT**

20 2-Ethyhexyl nitrate (2-EHN) is a major additive of fuel which is used to comply with the
21 cetane number of diesel. Because of its wide use and possible accidental release, 2-EHN is a
22 potential pollutant of the environment. In this study, *Mycobacterium austroafricanum* IFP
23 2173 was selected among several strains as the best 2-EHN degrader. The 2-EHN
24 biodegradation rate was increased in biphasic cultures where the hydrocarbon was dissolved
25 in an inert non-aqueous phase liquid (NAPL), suggesting that the transfer of the hydrophobic
26 substrate to the cells was a growth-limiting factor. Carbon balance calculation as well as
27 organic carbon measurement indicated a release of metabolites in the culture medium. Further
28 analysis by gas chromatography revealed that a single metabolite accumulated during growth.
29 This metabolite had a molecular mass of 114 Da as determined by GC/MS and was
30 provisionally identified as 4-ethylidihydrofuran-2(3H)-one by LC-MS/MS analysis.
31 Identification was confirmed by analysis of the chemically synthesized lactone. Based on
32 these results, a plausible catabolic pathway is proposed whereby 2-EHN is converted to 4-
33 ethylidihydrofuran-2(3H)-one, which cannot be metabolised further by strain IFP 2173. This
34 putative pathway provides an explanation for the low energetic efficiency of 2-EHN
35 degradation and its poor biodegradability.

36 INTRODUCTION

37 2-Ethyhexyl nitrate (2-EHN) is the nitric ester of 2-ethyl-1-hexanol. It is added at 0.05 % to
38 0.4 % to diesel formulations in order to boost the cetane number. As a result of the large use
39 of diesel worldwide, the 2-EHN market is about 100, 000 tons per year.

40 Although biodegradability has for a long time been regarded as a relevant characteristic of
41 chemicals, it was only recently incorporated to safety assessments. Considering fuel oils,
42 large volumes of oxygenates such as MTBE have been added to gasoline since 1992 (19).
43 Because of lack of knowledge on their biodegradability and insufficient safety regulation,
44 pollution cases resulting from accidental releases occurred in many countries. In the US for
45 example, as many as 250 000 sites may have been polluted from leaking underground fuel
46 tanks (36). Poor knowledge of the biodegradation of widely-used chemicals may also hide
47 specious concerns relating to the toxicity of metabolic products. For example, degradation of
48 chlorinated aromatics such as 4-chlorocatechol in soil gave rise to the formation of an
49 antibiotic, protoanemonin, which is detrimental to soil microcosms (6).

50 In case of accidental release of 2-EHN into the environment, the fate and impact of the
51 pollution are unpredictable because of the scarcity of data on 2-EHN biodegradation.
52 Screening tests have been recommended by both the U.S. Environmental Protection Agency
53 (35) and the OECD (24) to evaluate the biodegradability of commercial substances. In this
54 context, the so-called criterion of "ready biodegradability" requires that the tested substance
55 be biodegraded to a level of 60 % within 28 days (5). Standard degradation experiments
56 showed that 2-EHN could not be considered readily biodegradable (34). It was assumed that
57 2-EHN was poorly available to microbial communities because of its low water solubility and
58 its high volatility.

59 In fact, 2-EHN displays both a low vapour pressure corresponding to about 1.9 mg/l at 20°C
60 and a moderate solubility in water (12.6 mg/l at 20°C). Therefore, 2-EHN is expected to form
61 a separate organic phase in aqueous solution even when present in low amount. 2-EHN is also
62 a rather hydrophobic molecule as indicated by a log $K_{o/w}$ value of 5.24. Hydrophobic
63 compounds with log $K_{o/w}$ values in the range 1-5 are often toxic to cells because they insert
64 into the lipid bilayer of the cell membrane, disturbing its integrity and causing cell
65 permeabilization (13, 22).

66 The backbone of 2-EHN is a branched alkane, a type of molecules that is more resistant to
67 biodegradation than linear alkanes. The metabolism of both linear and branched hydrocarbons
68 by bacteria involves enzymes of the β -oxidation pathway (3). In the case of branched alkanes,
69 their degradation may lead to the formation of β -substituted acyl-CoA intermediates that
70 block β -oxidation (27). Such a metabolic blockage has been encountered during the
71 degradation of terpenoids such as citronellol, geraniol and nerol (10, 28). If a quaternary
72 carbon atom occurs at the end of an alkane chain, the result is a molecule quite resistant to
73 microbial attack (18).

74 In a recent study, microbial communities endowed with the ability to degrade 2-EHN were
75 obtained by enrichment from activated sludge or soil samples (33). The isolation of pure
76 strains able to utilize 2-EHN as sole source of carbon and energy proved rather difficult.
77 Nevertheless, among several strains of fast-growing Mycobacteria previously isolated on
78 other hydrocarbons, some strains, all identified as *Mycobacterium austroafricanum*, were
79 found to degrade 2-EHN.

80 In the present study, the kinetics of 2-EHN degradation by selected strains was investigated.
81 *M. austroafricanum* IFP 2173, which showed the highest rate of degradation, was chosen for
82 further investigation of 2-EHN catabolism. As a means to reduce the expected toxic effect of
83 2-EHN on bacterial cells and increase its bioavailability in aqueous media, bacterial cultures

84 were mostly carried out in biphasic media. Such biphasic cultures, including a non aqueous
85 phase liquid (NAPL) that serves as solvent for the hydrophobic substrate have already been
86 implemented to facilitate the degradation of various toxic or recalcitrant compounds (2, 4, 7,
87 12, 25, 26). A metabolite that accumulated during growth was detected in the culture medium
88 and identified by LC-MS/MS. Based on our data, a plausible pathway for 2-EHN catabolism
89 by *M. austroafricanum* IFP 2173 is proposed.

90 MATERIALS AND METHODS

91 Microorganisms and culture conditions

92 The strains used in this study were *M. austroafricanum* IFP 2173 (30), isolated on iso-octane,
93 *M. austroafricanum* IFP 2012 (11) and *M. austroafricanum* IFP 2015 (15) both isolated on
94 MTBE, *M. austroafricanum* C6 (14), *M. austroafricanum* Spyr_Ge_1 and *M.*
95 *austroafricanum* BHF 004 (J. C. Willison, unpublished data), all isolated on pyrene.

96 The culture medium consisted of a mineral salts solution (8) supplemented with 0.1 g/l of
97 yeast extract. The carbon source was added after medium sterilization (120°C for 20 min). All
98 cultures were incubated at 30°C with shaking (150 rpm).

99

100 Chemicals

101 2-EHN (CAS Number 27247-96-7), 2-ethylhexanol, 2-ethylhexanoic acid, MTBE,
102 decahydronaphthalene, 3-methyldihydrofuran-2(3H)-one, Et₂Zn, and HMN were obtained from
103 Sigma Aldrich (Saint Quentin Fallavier, France). Mineral salts were from VWR (Fontenay-
104 sous-Bois, France).

105

106 Biodegradation experiments

107 Biodegradation tests were performed in 120-ml flasks closed with Teflon-coated stoppers and
108 sealed with aluminium caps. Unless otherwise indicated, 4.8 mg of 2-EHN (or 2-ethylhexanol
109 or 2-ethylhexanoic acid) was added to 10 ml of the medium supplemented with 500 µl of
110 2,2,4,4,6,8,8-heptamethylnonane (HMN). Cultures were adjusted to an optical density
111 (O.D.₆₀₀) of 0.2 using washed pellets of centrifuged precultures grown on Tween 80 (2.5 g/l)
112 as sole source of carbon. The degradation rate was monitored by measuring at regular
113 intervals the CO₂ evolved in the headspace by gas chromatography (GC). Residual 2-EHN
114 was estimated as described below in triplicate. Abiotic controls were supplemented with

115 mercuric chloride (0.2 mg/l) and endogenous controls, lacking a carbon source but containing
116 HMN, were performed under similar conditions.

117

118 **Analyses of substrate and products**

119 Culture grown on 2-EHN were filtered on a PTFE membrane (0.45 μm) and cell biomass was
120 determined as dry weight after lyophilisation of the cell pellet. When HMN was omitted from
121 the growth medium, the total organic carbon (TOC) was measured on the filtrates using a
122 TOC-5050 carbon analyser (Shimadzu) according to the European norm NF EN 1484.
123 Residual 2-EHN in the culture filtrate, as well as derived metabolites, were extracted with 10
124 ml of methyl-*tert*-butyl ether (MTBE) containing 0.05 % (v/v) of decahydronaphthalene as
125 internal standard. After 30 min of shaking and static overnight incubation at 4°C, the solvent
126 extracts were analysed by GC with flame ionization detection (FID). A Varian 3400
127 chromatograph (Sugarland, USA) equipped with a CP-Sil Pona CB column (0.25 mm by 50
128 m) obtained from Chrompack (Raritan, NJ) was used. The carrier gas was helium. The
129 temperature of the injector and the detector were set at 250 and 280°C, respectively. The
130 column temperature was varied from 100°C to 200°C at 4°C/min, then from 200°C to 259°C
131 at 20°C/min.

132 Time courses of 2-EHN degradation and metabolite excretion were performed in flasks which
133 were sacrificed at regular time intervals. CO₂ in flask head space was measured with a Varian
134 3400 gas chromatograph (Sugarland, USA) equipped with a catharometric detector and a
135 PorapakQ (80/100 mesh, 2m) (Chrompack, Raitan, NJ). The net amount of CO₂ produced
136 was determined as the difference between the final quantity found in the test flasks and that
137 found in hydrocarbon-free flasks.

138

139 **Kinetics of O₂ consumption**

140 Continuous monitoring of substrate oxidation was carried out through measurement of O₂
141 consumption using a respirometer (Sapromat D12-S, Voith, Germany). Flasks containing 250
142 ml of culture medium and 125 µl of 2-EHN as carbon source were inoculated with *M.*
143 *austroafricanum* IFP 2173 to an optical density (O.D.₆₀₀) of 0.1. Incubation was carried out at
144 30°C with shaking in the presence or absence of HMN (12.5 ml). Cultures and substrate-free
145 controls were performed in triplicate.

146

147 **Chemical synthesis of 4-ethyltetrahydrofuran-2(3H)-one**

148 4-EDF was synthesized according to a published procedure (1). In a three-necked flask
149 containing dry toluene (5 ml), Cu(OTf)₂ (0.025 mmol) and P(OEt)₃ (0.05 mmol) were
150 successively added. The mixture was stirred for 30 min at room temperature to obtain a
151 colourless solution. After cooling to -20°C, Zinc di-ethyl (5 mmol previously dissolved in
152 hexane) was added followed by furan-2(5H)-one (5 mmol). The reaction was allowed to
153 warm to 0°C for 6 h, then incubated at room temperature and monitored by GC. After
154 completion of the reaction, the mixture was hydrolysed with aqueous 5N HCl, then extracted
155 with diethyl ether (2 x 15 ml); the organic phase was dried over MgSO₄ and concentrated *in*
156 *vacuo*. The crude product was purified by column chromatography on SiO₂ using a mixture of
157 diethyl ether / pentane: 80/20) as eluent.

158

159 **Coupled MS analyses**

160 GC-MS analysis was carried out under chromatographic conditions identical to those
161 described above for GC-FID. Mass spectra were acquired in the split mode with a time of
162 flight mass spectrometer (Tempus TOF MS, Thermo Finnigan).

163 LC-MS-MS was performed using an HPLC system (Alliance 2695, Waters, Guyancourt,
164 France) coupled to a Quattro LC triple quadrupole mass spectrometer (Micromass,

165 Manchester, UK) with an electrospray interface. Data were acquired in the positive or
166 negative ionization modes and processed with MassLynx NT 4.0 system. The electrospray
167 source voltages were: capillary 3.2 kV, extractor 2 V, cone voltage 22 and 17 V under
168 positive mode, respectively. The source block and desolvation gas were heated at 120°C and
169 350°C, respectively. Nitrogen was used as nebulisation and desolvation gas (75 and 350 l h⁻¹
170 respectively). For MS–MS, collisional induced dissociation (CID) was performed under argon
171 (2.5 10⁻³ mbar) at a collision energy set between 10 and 40 eV.

172

173 **RESULTS**

174

175 **Time course of 2-EHN biodegradation by selected strains**

176 Kinetics of 2-EHN biodegradation was studied using a few bacterial strains previously
177 selected among environmental isolates and collection strains for their ability to attack this
178 compound (33). Most of these strains were identified as members of the *Mycobacterium*
179 genus. In order to avoid growth inhibition due to 2-EHN toxicity, HMN was added as NAPL
180 to the bacterial cultures, and biodegradation time courses were monitored by measuring the
181 CO₂ production in the culture headspace. Biodegradation kinetics were found to vary widely
182 depending on bacterial strains (data not shown). *M. austroafricanum* IFP 2173 was the fastest
183 and most efficient of the microorganisms tested since it produced the largest amount of CO₂
184 (37 μmol per flask) after 13 days of incubation. *M. austroafricanum* IFP 2173 was also the
185 only strain able to grow on 2-EHN in the absence of HMN (data not shown).

186

187 **Effect of 2-EHN supply mode on the biodegradation rate**

188 The impact of NALP addition on 2-EHN biodegradation by strain IFP 2173 was studied
189 through continuous monitoring of substrate-dependent oxygen consumption by respirometry.

190 In the culture lacking HMN, O₂ uptake started after a lag phase of about one day, then
191 increased with time according to a sigmoidal curve (Fig. 1). The maximal growth rate (μ_{\max})
192 could be deduced from oxygen uptake rate assuming that the biomass yield remained constant
193 during growth. Over a 9-day period of growth, μ_{\max} was calculated to be 0.29 day⁻¹ on
194 average, corresponding to a generation time of 2.4 days. In the HMN-containing culture, the
195 lag phase was shorter and the O₂ uptake became linear after a very short exponential phase
196 ($\mu_{\max} = 0.29 \text{ day}^{-1}$). The maximal rate of O₂ uptake was 5.3 mmol/day, and the overall O₂
197 consumption reached a maximum of 2.9 mmol, compared to 2.6 mmol for cells grown
198 without HMN.

199 The effect of 2-EHN concentration on growth was studied in HMN-containing cultures (Fig.
200 2). The concentration of 2-EHN had little effect on the specific growth rate. During the linear
201 phase of growth, the O₂ uptake rate increased proportionally to the 2-EHN concentration in
202 the culture medium up to 3 g/l. This indicated that the 2-EHN diffusion rate from HMN to the
203 water phase was a limiting factor for bacterial growth. At 2-EHN concentrations higher than 3
204 g/l, bacterial growth was inhibited as indicated by both slower oxygen uptake rates and lower
205 overall O₂ consumption. For 2-EHN concentrations lower than 3 g/l, no residual substrate was
206 detected in the culture medium by the end of growth and the O₂ consumption was roughly
207 proportional to the amount of substrate supplied.

208

209 **Carbon balance of 2-EHN biodegradation by *M. austroafricanum* IFP 2173**

210 In order to determine the carbon balance of 2-EHN biodegradation, *M. austroafricanum* IFP
211 2173 was cultivated in mineral medium lacking HMN to avoid perturbation of TOC

212 measurements by HMN. The culture was stopped when no more CO₂ was released, which
213 coincided with the total consumption of 2-EHN (see Fig. 4). The biomass formed, the TOC in
214 the filtered culture medium and the amount of CO₂ released were measured. The carbon
215 recovery as metabolites and cell biomass was calculated by taking into account the elementary
216 compositions of substrate and products (Table 1). A carbon recovery rate of 92 % was
217 obtained for the 2-EHN bioconversion. Carbon converted into biomass (94 mg/l) and CO₂
218 (165 mg/l) amounted together to only 33 % of the total carbon produced. Accordingly, a high
219 proportion of the substrate-derived carbon was recovered in the clarified culture medium (67
220 %), possibly reflecting metabolite accumulation.

221

222 **Identification of a metabolite excreted in the culture**

223 GC-FID analysis of culture fluid extracts performed during 2-EHN degradation experiments
224 revealed the gradual increase in concentration of an unknown compound with a retention time
225 shorter than that of 2-EHN. This finding suggested that a metabolite might have accumulated
226 during growth and accounted for the substantial level of TOC previously detected in the
227 supernatant of 2-EHN grown cultures. High resolution mass spectral analysis of this
228 compound (Fig. 3 a) showed that it had a molecular mass of 114.07 Da and the following
229 chemical formula: C₆H₁₀O₂. The mass spectrum of this compound did not match any of the
230 spectra currently available in the databases. Nevertheless, a comparison of the LC-MS-MS
231 data of the excreted product with those of 3-methyldihydrofuran-2(3H)-one, a commercially-
232 available product, revealed several common fragment ions. The analysis also indicated that
233 the molecule did not contain any carboxylic or hydroxyl groups (Fig. 3 b). Taken together,
234 our data indicated that the product of interest might be 4-ethyldihydrofuran-2(3H)-one (4-
235 EDF), which can also be designated as β-ethyl-γ-butyrolactone. In order to confirm the
236 structure of the metabolite, the chemical synthesis of 4-EDF was undertaken as described

237 under Materials and Methods (1). The LC-MS-MS characteristics of the synthesized lactone
238 were identical to those of the metabolite, confirming that the product which accumulated in
239 cultures of *M. austroafricanum* IFP 2173 grown on 2-EHN was 4-EDF.

240 The rate of 4-EDF accumulation was assessed by GC-FID analysis of the culture fluid during
241 growth. Fig. 4 shows that 4-EDF formation and CO₂ release were concurrent with 2-EHN
242 degradation.

243

244 **Biodegradation of 2-EHN-derived compounds**

245 As a means to elucidate the biodegradation pathway of 2-EHN by *M. austroafricanum* IFP
246 2173, we tested compounds with structures derived from 2-EHN as possible substrates. 2-
247 ethylhexanol, the primary alcohol resulting from 2-EHN hydrolysis, was biodegraded,
248 yielding 2-ethylhexanoic acid and 4-EDF. 2-ethylhexanoic acid, the product resulting from 2-
249 ethylhexanol oxidation was not biodegraded, even in the presence of HMN. This compound is
250 considered to be toxic for most bacteria (21). It should be noted that 2-EHN can be used as
251 sole nitrogen source by strain IFP 2173, indicating that nitrate is formed, probably as a result
252 of an initial attack on 2-EHN by an esterase (data not shown). 2-EHN biodegradation was also
253 tested in the presence of isooctane, the compound on which *M. austroafricanum* IFP 2173
254 was selected. Diauxic growth was observed, the strain degrading isooctane first and then 2-
255 EHN into 4-EDF (data not shown).

256

257 **DISCUSSION**

258 2-EHN is a recalcitrant compound which was considered not readily biodegradable according
259 to standard procedures (34). However, we demonstrated in the present study, that selected
260 strains of Mycobacteria were able to slowly utilize 2-EHN as sole source of carbon under
261 defined culture conditions. The poor biodegradability of 2-EHN might be the consequence of

262 two factors, first the low occurrence of micro-organisms able to use it as carbon source, and
 263 second its inhibitory effect on bacterial growth even at low concentration. 2-EHN inhibition
 264 was illustrated by the experiment described in Fig. 2, and by the lack of growth of all strains
 265 tested in HMN-free cultures, except *M. austroafricanum* IFP 2173. This strain, isolated for its
 266 ability to degrade isooctane, a branched alkane (31), demonstrated wide capabilities for
 267 hydrocarbon biodegradation (16, 32). Like many members of the *Corynebacterium-*
 268 *Mycobacterium-Nocardia* (CMN) group of Gram-positive bacteria, it may be resistant to toxic
 269 hydrocarbons thanks to the properties of its cell envelope, which is highly rigid and contains
 270 mycolic acids (29). In *Mycobacteria*, mycolic acids are very long fatty acids (C₆₀-C₉₀) that
 271 contribute up to 60 % to the cell wall (9). The specific cell wall composition of the *M.*
 272 *austroafricanum* strains studied here probably accounts for their resistance to 2-EHN.
 273 However, it is unclear whether the unique ability of strain IFP 2173 to grow on 2-EHN
 274 without NAPL is due to a cell wall composition slightly different from that of other strains or
 275 to some other strain-specific trait.

276 Biphasic cultures, involving addition of an inert NAPL like HMN was found to be critical for
 277 2-EHN biodegradation and bacterial growth. In the HMN-free cultures, the dissolved fraction
 278 of 2-EHN represented only a minor part of the substrate supplied since it partitioned into three
 279 distinct phases *i.e.* the gas phase, the aqueous phase, and the bulk of insoluble 2-EHN. During
 280 the biodegradation process, the uptake of dissolved substrate was counterbalanced by the
 281 equilibrium transfer of 2-EHN from the bulk of substrate (S_{subNAPL}) to the aqueous (S_{aq})
 282 according to the following scheme:



283
 284 where S_{subNAPL} and S_{aq} represent the amounts of substrate in the bulk and in the aqueous
 285 phase, respectively, X is the cell biomass and $T_{\text{NAPL/aq}}$ is the substrate transfer rate of 2-EHN

286 to the culture medium. In HMN-containing cultures, the dissolved 2-EHN was mainly
287 confined to NAPL. Because of the high hydrophobicity of their cell walls, microbial cells
288 tightly adhered to NAPL and direct contact was thus the most probable mode of substrate
289 uptake (7, 12). Accordingly, the large NAPL volume (500 μ l of HMN versus 5 μ l of 2-EHN in
290 the case of the NAPL-free culture), which increased substrate bioavailability, probably
291 accounted for its higher efficiency of assimilation by the microorganisms. Such conditions of
292 substrate delivery were apparently required to promote growth on 2-EHN of *M.*
293 *austroafricanum* strains other than strain 2173.

294 The biodegradation of 2-EHN by *M. austroafricanum* IFP 2173 illustrates the remarkable
295 metabolic capabilities of this stain towards recalcitrant hydrocarbons. Indeed, it can degrade
296 another methyl branched alkane, 2,2,4-trimethylpentane (31), suggesting that it produces
297 enzymes specific for the degradation of anteiso-alkanes. Nevertheless, our results indicate that
298 degradation of 2-EHN by strain IFP 2173 is partial, and gives rise to the release of an acyl
299 with an ethyl substituent in the beta position. At least two reasons might explain the
300 accumulation of this metabolite: i) strain IFP 2173 lacks enzymes able to degrade it, ii)
301 because of the ethyl group in beta position, the metabolite might block the enzyme catalysing
302 the next step in the degradation of branched alkanes.

303 Considering the high biodegradation potential of strain IFP 2173, we recently observed that
304 this strain can degrade other xenobiotic compounds structurally related to 2-EHN such as
305 bis(2-ethylhexyl)phthalate (data not shown) used as plasticizer (21, 23). The biodegradation
306 of this compound by *Mycobacterium* sp. NK0301 has been reported (20). This bacterium
307 utilized phthalate as carbon and energy source and left the carbon skeleton of the 2-ethylhexyl
308 moiety intact, releasing it as 2-ethylhexanol or 2-ethylhexanoic acid. In comparison, strain
309 IFP 2173 degraded bis(2-ethylhexyl) phthalate and utilized the 2-ethylhexyl moiety, achieving
310 a higher degree of degradation (data not shown).

311 The biodegradation of 2-EHN by strain IFP 2173 gave rise to the accumulation of a lactone
312 which was identified as 4-EDF. The lactone formed by cyclization of a breakdown product, a
313 branched pentanoic acid, which was not metabolized further by the bacteria. The partial
314 degradation of 2-EHN certainly explains the observed slow growth ($\mu_{\max} = 0.29 \text{ day}^{-1}$) and
315 poor growth yield of cultures utilizing this compound as sole C source.

316 Considering the structure of the intermediate metabolite and the known degradation pathway
317 of *n*-alkanes (18), we propose for the first time a plausible metabolic pathway for 2-EHN
318 degradation (Fig. 5). The pathway would start by a simultaneous or sequential attack of the
319 molecule on both extremities, with an esterase activity hydrolyzing the nitric ester bond and
320 an oxygenase catalyzing the hydroxylation of the distal methyl group. The involvement of an
321 esterase that would release nitrate was inferred from the observation that strain IFP 2173
322 utilized 2-EHN as nitrogen source. The existence in this strain of an hydroxylase active on
323 branched alkane is expected since it grows on isooctane (31). The intermediate metabolite that
324 would form, 2-ethylpentan-1,5-diol, is proposed to be oxidized to a carboxylic acid in two
325 steps involving successively an alcohol and an aldehyde dehydrogenase. After activation by
326 coenzyme A, the resulting 5-(hydroxymethyl)heptanoic acid would undergo one cycle of
327 classical β -oxidation to give 3-(hydroxymethyl)pentanoic acid, which would spontaneously
328 convert to 4-EDF by cyclisation. Since the substrate underwent a single turn of β -oxidation
329 only two carbon atoms (out of eight in 2-EHN) could reach the TCA cycle, accounting for the
330 low percentage of carbon released as CO_2 (12%).

331 The proposed pathway now needs to be assessed experimentally by identifying enzymes
332 involved in 2-EHN degradation. To this end, we have undertaken a proteomic analysis to find
333 out the proteins that are induced upon incubation of strain IFP2173 with 2-EHN.

334

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340

341 REFERENCES

- 342 1. **Alexakis, A., J. Vastra, and P. Mangeney.** 1997. Acceleration of the conjugate
343 addition of diethyl zinc to enones by either Cu(OTf)₂ or trivalent phosphorus ligands.
344 Tetrahedron Letters **38**:7745-7748.
- 345 2. **Allen, C. C., D. R. Boyd, F. Hempenstall, M. J. Larkin, and N. D. Sharma.** 1999.
346 Contrasting effects of a nonionic surfactant on the biotransformation of polycyclic
347 aromatic hydrocarbons to *cis*-dihydrodiols by soil bacteria. Appl. Environ. Microbiol.
348 **65**:1335-1339.
- 349 3. **Alvarez, H. M.** 2003. Relationship between beta-oxidation pathway and the
350 hydrocarbon-degradation profile in actinomycete bacteria. Int. Biodeter. Biodeg.
351 **52**:35-42.
- 352 4. **Ascon-Cabrera, M., and J. M. Lebeault.** 1993. Selection of xenobiotic-degrading
353 microorganisms in a biphasic aqueous-organic system. Appl. Environ. Microbiol.
354 **59**:1717-1724.
- 355 5. **Battersby, N. S., D. Ciccognani, M. R. Evans, D. King, H. A. Painter, D. R.**
356 **Peterson, and M. Starkey.** 1999. An 'inherent' biodegradability test for oil products:
357 description and results of an international ring test. CONCAWE Biodegradation Task
358 Force. Chemosphere **38**:3219-3235.
- 359 6. **Blasco, R., R. M. Wittich, M. Mallavarapu, K. N. Timmis, and D. H. Pieper.**
360 1995. From xenobiotic to antibiotic, formation of protoanemonin from 4-

- 361 chlorocatechol by enzymes of the 3-oxoadipate pathway. *J. Biol. Chem.* **270**:29229-
362 29235.
- 363 7. **Bouchez-Naitali, M., H. Rakatozafy, R. Marchal, J. Y. Leveau, and J. P.**
364 **Vandecasteele.** 1999. Diversity of bacterial strains degrading hexadecane in relation
365 to the mode of substrate uptake. *J. Appl. Microbiol.* **86**:421-428.
- 366 8. **Bouchez, M., D. Blanchet, and J. P. Vandecasteele.** 1995. Degradation of polycyclic
367 aromatic hydrocarbons by pure strains and by defined strain associations: inhibition
368 phenomena and cometabolism. *Appl. Microbiol. Biotechnol.* **43**:156-164.
- 369 9. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. *Annu. Rev.*
370 *Biochem.* **64**:29-63.
- 371 10. **Fall, R. R., J. L. Brown, and T. L. Schaeffer.** 1979. Enzyme recruitment allows the
372 biodegradation of recalcitrant branched hydrocarbons by *Pseudomonas citronellolis*.
373 *Appl. Environ. Microbiol.* **38**:715-722.
- 374 11. **Francois, A., H. Mathis, D. Godefroy, P. Piveteau, F. Fayolle, and F. Monot.**
375 2002. Biodegradation of methyl tert-butyl ether and other fuel oxygenates by a new
376 strain, *Mycobacterium austroafricanum* IFP 2012. *Appl. Environ. Microbiol.* **68**:2754-
377 2762.
- 378 12. **Goswami, P., and H. D. Singh.** 1991. Different modes of hydrocarbon uptake by two
379 *Pseudomonas* species. *Biotechnol. Bioeng.* **37**:1-11.
- 380 13. **Heipieper, H. J., F. J. Weber, J. Sikkema, H. Keweloh, and J. A. M. de Bont.**
381 1994. Mechanisms of resistance of whole cells to toxic organic solvents. *Trends*
382 *Biotechnol.* **12**:409-415.
- 383 14. **Jouanneau, Y., J. C. Willison, C. Meyer, S. Krivobok, N. Chevron, J. L.**
384 **Besombes, and G. Blake.** 2005. Stimulation of pyrene mineralization in freshwater

- 385 sediments by bacterial and plant bioaugmentation. Environ. Sci. Technol. **39**:5729-
386 5735.
- 387 15. **Lopes Ferreira, N., H. Maciel, H. Mathis, F. Monot, F. Fayolle-Guichard, and C.**
388 **W. Greer.** 2006. Isolation and characterization of a new *Mycobacterium*
389 *austroafricanum* strain, IFP 2015, growing on MTBE. Appl. Microbiol. Biotechnol.
390 **70**:358-365.
- 391 16. **Lopes Ferreira, N., H. Mathis, D. Labbe, F. Monot, C. W. Greer, and F. Fayolle-**
392 **Guichard.** 2007. n-Alkane assimilation and tert-butyl alcohol (TBA) oxidation
393 capacity in *Mycobacterium austroafricanum* strains. Appl. Microbiol. Biotechnol.
394 **75**:909-919.
- 395 17. **Mc Carty, P. L.** 1972. Energetics of organic matter degradation. In R. Mitchell (ed.),
396 Water Pollution Microbiology, Wiley Interscience, New York.
- 397 18. **Mc Kenna, E. J.** 1972. Microbial metabolism of normal and branched chain alkanes,
398 In Degradation of synthetic organic molecules in the biosphere, Proceedings of the
399 San Francisco Conference, Academy of science, Washington D.C.
- 400 19. **Moran, M.** 2007. Occurrence of methyl tert-butyl ether and other fuel oxygenates in
401 source water and drinking water in the United States. In O. Hutzinger (ed.), The
402 handbook of environmental chemistry: fuel oxygenates, vol. 5, Water pollution.
403 Springer Verlag, Berlin Heidelberg.
- 404 20. **Nakamiya, K., S. Hashimoto, H. Ito, J. S. Edmonds, A. Yasuhara, and M. Morita.**
405 2005. Microbial treatment of bis (2-ethylhexyl) phthalate in polyvinyl chloride with
406 isolated bacteria. J. Biosci. Bioeng. **99**:115-119.
- 407 21. **Nalli, S., D. G. Cooper, and J. A. Nicell.** 2002. Biodegradation of plasticizer by
408 *Rhodococcus rhodochrous*. Biodegradation **13**:343-352.

- 409 22. **Neumann, G., N. Kabelitz, A. Zehnsdorf, A. Miltner, H. Lippold, D. Meyer, A.**
410 **Schmid, and H. J. Heipieper.** 2005. Prediction of the adaptability of *Pseudomonas*
411 *putida* DOT-T1E to a second phase of a solvent for economically sound two-phase
412 biotransformations. *Appl. Environ. Microbiol.* **71**:6606-6612.
- 413 23. **Nishioka, T., M. Iwata, T. Imaoka, M. Mutoh, Y. Egashira, T. Nishiyama, T.**
414 **Shin, and T. Fujii.** 2006. A mono-2-ethylhexyl phthalate hydrolase from a *Gordonia*
415 sp. that is able to dissimilate di-2-ethylhexyl phthalate. *Appl. Environ. Microbiol.*
416 **72**:2394-2399.
- 417 24. **OECD.** 1993. Guidelines for the testing of chemicals. OCDE editions, Part 3, Paris.
- 418 25. **Ortega-Calvo, J. J., and M. Alexander.** 1994. Roles of bacterial attachment and
419 spontaneous partitioning in the biodegradation of naphthalene initially present in
420 nonaqueous-phase liquids. *Appl. Environ. Microbiol.* **60**:2643-2646.
- 421 26. **Sandrin, T. R., W. B. Kight, W. J. Maier, and R. M. Maier.** 2006. Influence of a
422 nonaqueous phase liquid (NAPL) on biodegradation of phenanthrene. *Biodegradation*
423 **17**:423-435.
- 424 27. **Schaeffer, T. L., S. G. Cantwell, J. L. Brown, D. S. Watt, and R. R. Fall.** 1979.
425 Microbial growth on hydrocarbons: terminal branching inhibits biodegradation. *Appl.*
426 *Environ. Microbiol.* **38**:742-746.
- 427 28. **Seubert, W., and E. Fass.** 1964. Studies on the bacterial degradation of isoprenoids.
428 Iv. The purification and properties of beta-Isohexenylglutaconyl-CoA-hydratase and
429 beta-hydroxy-beta-isohexenylglutaryl-CoA-lyase. *Biochem. Z* **341**:23-34.
- 430 29. **Sokolovska, I., R. Rozenberg, C. Riez, P. G. Rouxhet, S. N. Agathos, and P.**
431 **Wattiau.** 2003. Carbon source-induced modifications in the mycolic acid content and
432 cell wall permeability of *Rhodococcus erythropolis* E1. *Appl. Environ. Microbiol.*
433 **69**:7019-7027.

- 434 30. **Solano-Serena, F., R. Marchal, S. Casaregola, C. Vasnier, J. M. Lebeault, and J.**
435 **P. Vandecasteele.** 2000. A *Mycobacterium* strain with extended capacities for
436 degradation of gasoline hydrocarbons. *Appl. Environ. Microbiol.* **66**:2392-2399.
- 437 31. **Solano-Serena, F., R. Marchal, S. Heiss, and J. P. Vandecasteele.** 2004.
438 Degradation of isooctane by *Mycobacterium austroafricanum* IFP 2173: growth and
439 catabolic pathway. *J. Appl. Microbiol.* **97**:629-639.
- 440 32. **Solano-Serena, F., R. Marchal, J. M. Lebeault, and J. P. Vandecasteele.** 2000.
441 Selection of microbial populations degrading recalcitrant hydrocarbons of gasoline by
442 monitoring of culture-headspace composition. *Lett. Appl. Microbiol.* **30**:19-22.
- 443 33. **Solano-Serena, F., E. Nicolau, G. Favreau, Y. Jouanneau, and R. Marchal.** 2008.
444 Biodegradability of 2-ethylhexyl nitrate (2-EHN), a cetane improver of diesel oil.
445 Biodegradation, in press.
- 446 34. **The American Chemistry Council Petroleum additives panel.** 2006. High
447 Production Volume Challenge program Final Submission for Nitric Acid, 2-
448 Ethylhexyl Ester.
- 449 35. **US Environmental Protection Agency.** 1982. Chemical Fate Test Guidelines, EPA
450 560/6-82-003, NTIS PB82-23308.
- 451 36. **Waul, C. K., E. Arvin, and J. E. Schmidt.** 2007. Microbial degradation of MTBE in
452 reactors. In O. Hutzinger (ed.), *The handbook of environmental chemistry: fuel*
453 *oxygenates*, vol. 5, Water pollution. Springer Verlag, Berlin Heidelberg.
- 454

455 **Table 1:** Carbon balance of 2-EHN biodegradation by *M. austroafricanum* IFP2173

456 Cultures (10 ml) were performed at 30°C in 120-ml flasks.

457

Substrate or product	Mass change ^a (mg/l)	Carbon balance	
		Carbon change ^a (mg/l)	Carbon recovery (%)
2-EHN	482	269	0
Cell biomass	94	50 ^b	19
CO ₂ ^c	115	31	12
TOC ^d	165	165	61
Total products			92

458 ^a Considering the whole content of the culture flasks.

459 ^b Carbon to dry biomass ratio was assumed to be 52 % (17). Dry biomass was determined
460 from 100 ml cultures grown in 1-L flasks.

461 ^c CO₂ was determined after acidification of the culture

462 ^d Total organic carbon (TOC) measured in the culture fluid after filtration through a 0.22 µm
463 membrane.

464

465 **Figure legends**

466

467 **Fig 1.** Effect of a non aqueous liquid phase (HMN) on the rate of oxygen consumption by *M.*

468 *austroafricanum* IFP 2173.

469 Cultures (250 ml) were grown in the flasks of a respirometer and contained 125 µl of 2-EHN

470 as carbon source. Cultures were incubated in the presence (black line) or absence (grey line)

471 of HMN (12.5 ml).

472

473 **Fig. 2.** Effect of 2-EHN concentration on oxygen consumption by *M. austroafricanum* IFP
474 2173.

475 Biphasic cultures contained a variable concentration of 2-EHN and 12.5 ml of HMN.

476 Maximal rates of O₂ uptake or V_{max} (■) and overall O₂ consumption (◆) were determined.

477

478 **Fig. 3.** MS characterization of the metabolite produced by strain IFP 2173 upon degradation
479 of 2-EHN.

480 **a.** High resolution electron impact mass spectrum of the accumulated metabolite as obtained
481 by GC-MS analysis.

482 **b.** CID/MS/MS product ion spectrum of the protonated molecule (MH⁺) obtained by LC-
483 MS/MS analysis at a collision energy of 10 eV.

484

485 **Fig 4.** Accumulation of 4-EDF during 2-EHN biodegradation.

486 Parallel cultures were carried out in 120-ml flasks and removed at the times indicated for
487 extraction and measurements of 2-EHN (◆) and 4-EDF (▲). CO₂ (■) was determined in a
488 separate culture flask. Residual 2-EHN is the fraction of hydrocarbon which stayed bound to
489 the flask wall and stopper, and remained inaccessible to bacteria.

490

491 **Fig 5.** Proposed pathway for 2-EHN biodegradation by *M. austroafricanum* IFP 2173.

492

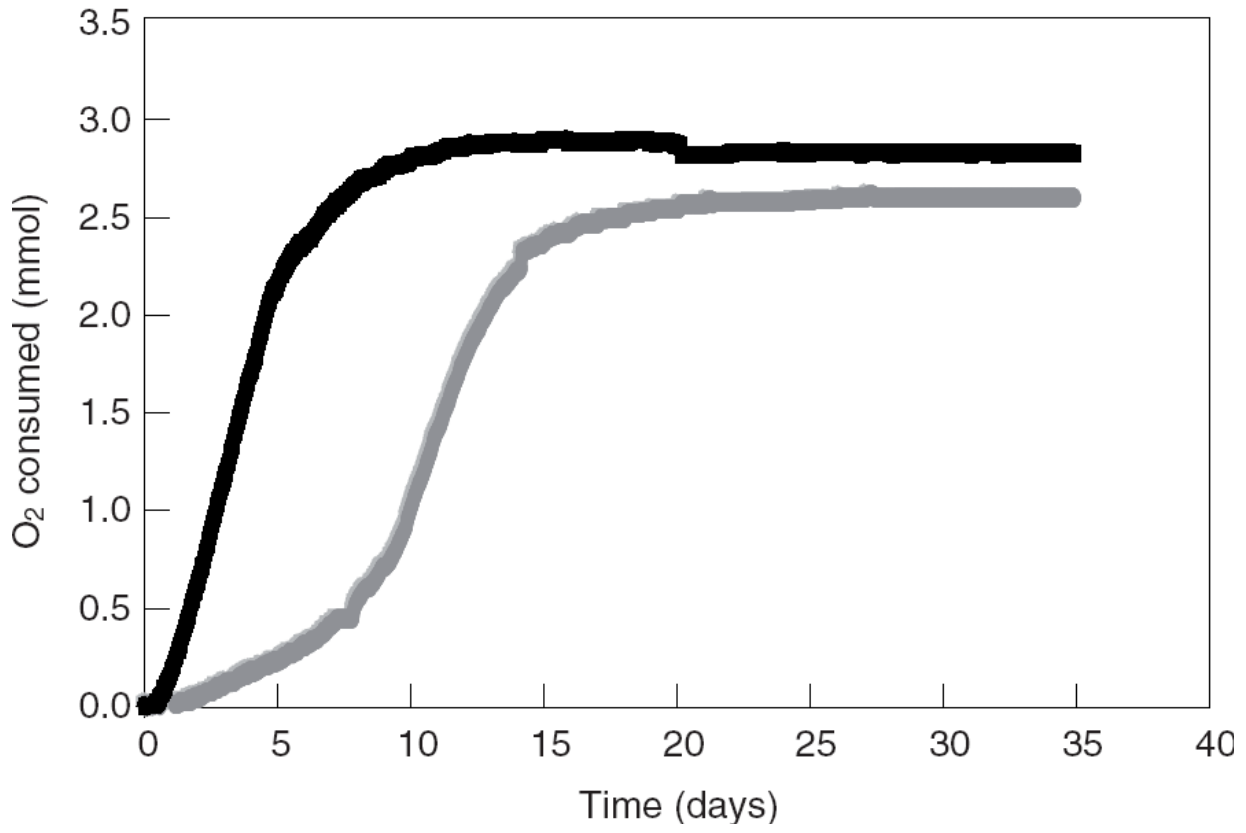


Fig. 1

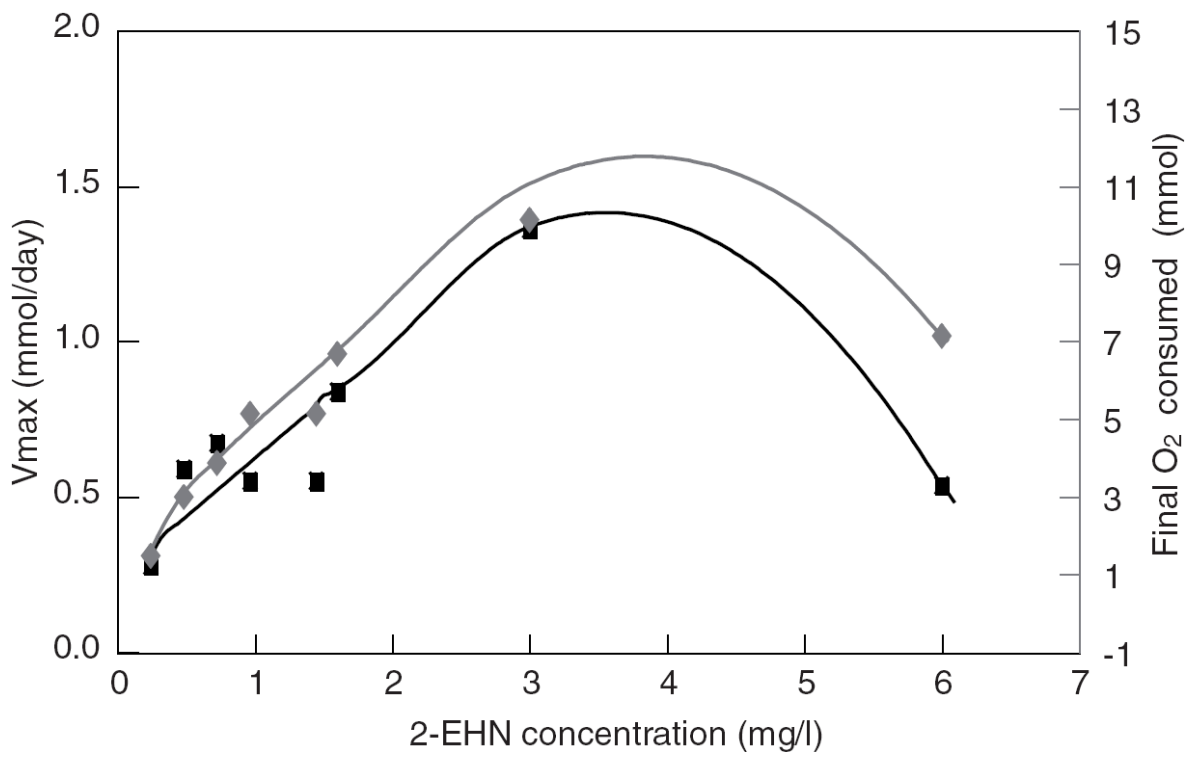


Fig. 2

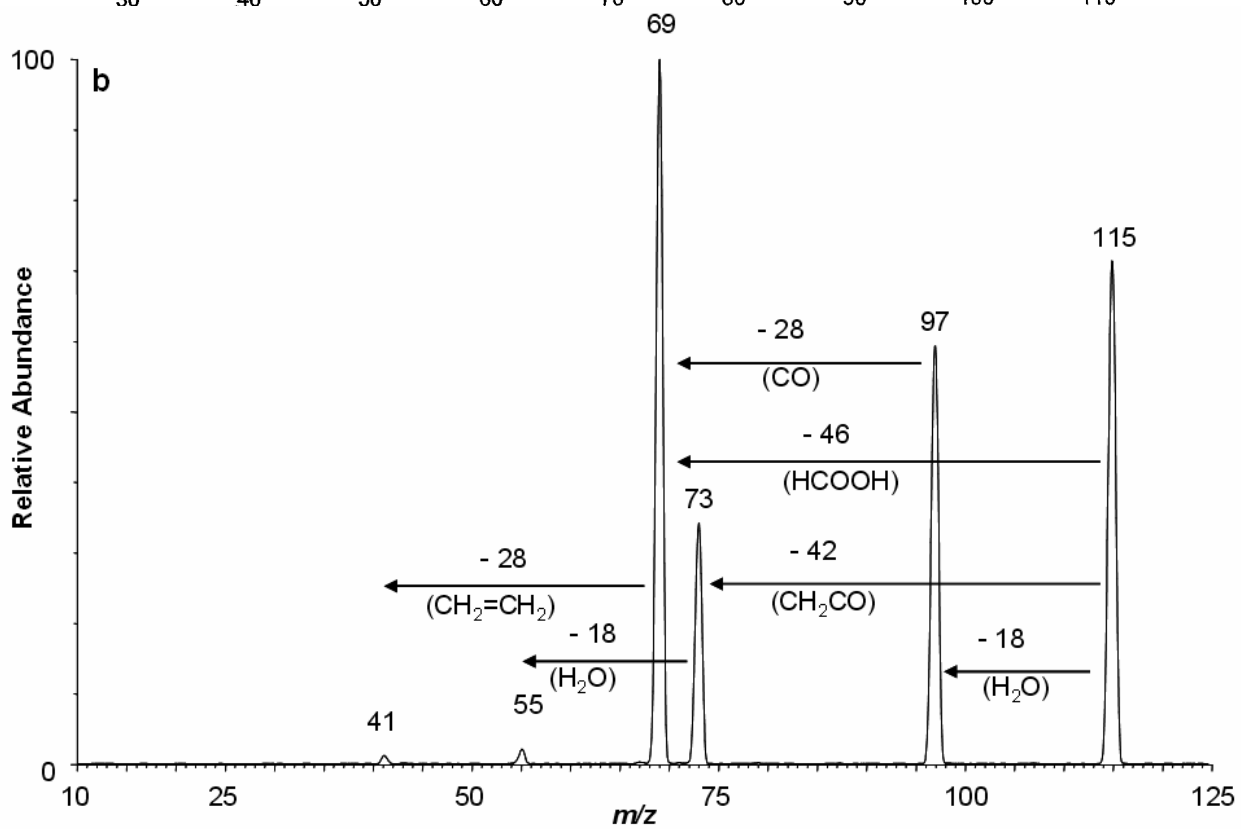
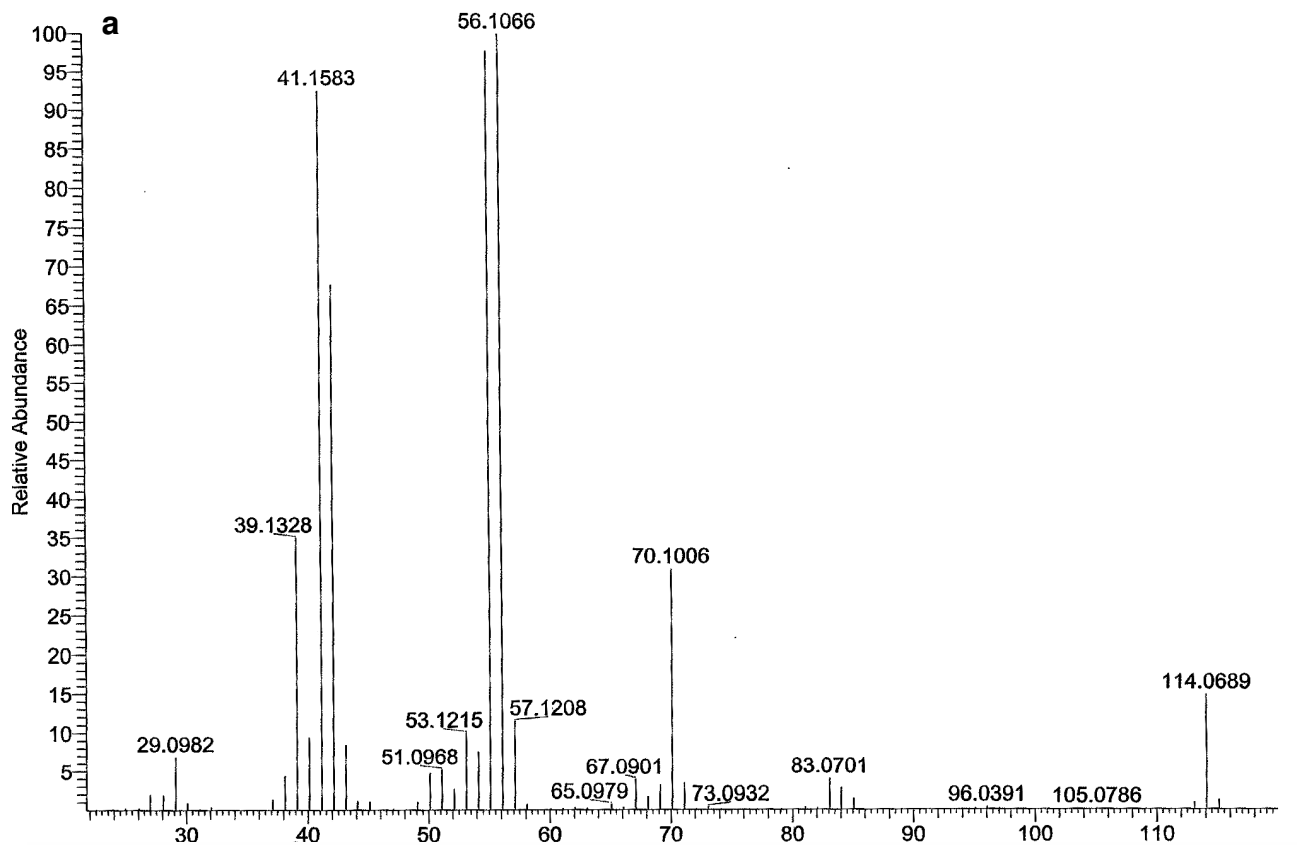


Fig. 3

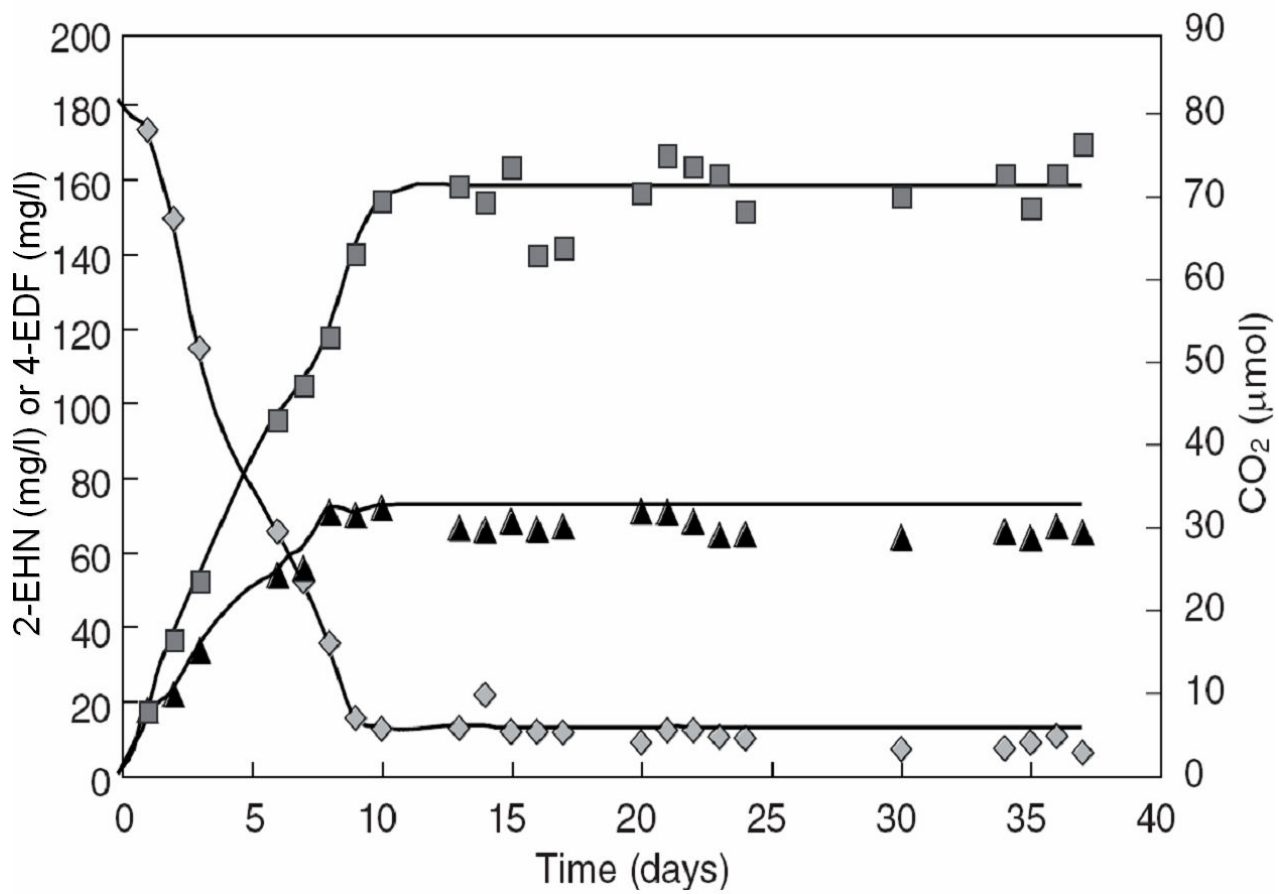


Fig. 4

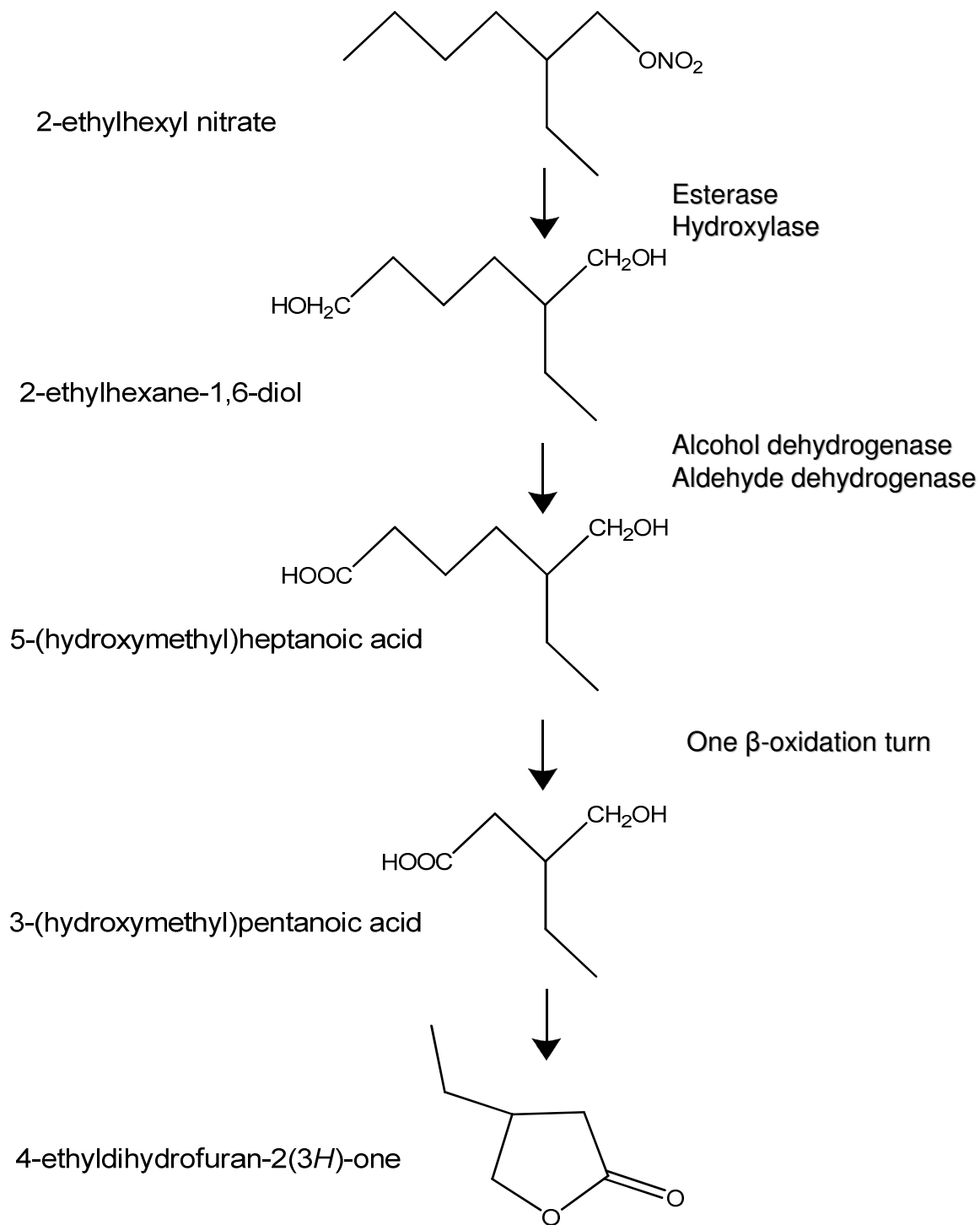


Fig. 5