The 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase reaction in the anaerobic degradation of naphthalene and identification of downstream metabolites

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25 **Running title:** THNCoA reductase and downstream reactions

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Abstract

Anaerobic degradation of polycyclic aromatic hydrocarbons has been mostly investigated with naphthalene as a model compound. Naphthalene degradation by sulphate-reducing bacteria proceeds via carboxylation to 2-naphthoic acid, formation of a coenzyme A thioester and subsequent reduction to 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA), which is further reduced to hexahydro-2-naphthoyl-CoA (HHNCoA) by tetrahydronaphthoyl-CoA reductase (THNCoA reductase), an enzyme similar to class I benzoyl-CoA reductases. When analysing THNCoA reductase assays with crude cell extracts and NADH as electron donor via LC-MS, scanning for putative metabolites, we could show that small amounts of the product of an HHNCoA hydratase are formed in the assays, but the downstream conversion by an NAD^+-dependent β-hydroxyacyl-CoA dehydrogenase was prevented by the excess of NADH present in those assays. Experiments with alternative electron donors indicated that 2-oxoglutarate can serve as an indirect electron donor for the THNCoA-reducing system via a 2-oxoglutarate:ferredoxin oxidoreductase. With 2-oxoglutarate as electron donor, THNCoA was completely converted and further metabolites resulting from subsequent β-oxidation-like reactions and hydrolytic ring-cleavage were detected. These metabolites indicate a downstream pathway with water addition to HHNCoA and ring fission via a hydrolase acting on a β'-hydroxy-β-oxo-decahydro-2-naphthoyl-CoA intermediate. Formation of the downstream intermediate *cis*-2-(carboxycyclohexyl)acetyl-CoA, which is the substrate for the previously described lower degradation pathway leading to the central metabolism, completes the anaerobic degradation pathway of naphthalene.

52 **Importance**

53 Anaerobic degradation of polycyclic aromatic hydrocarbons is poorly investigated
54 despite its significance in anoxic sediments. Using alternative electron donors for the
55 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase reaction, we observed intermediary
56 metabolites of anaerobic naphthalene degradation via in vitro enzyme assays with cell
57 extracts of anaerobic naphthalene degraders. The identified metabolites provide
58 evidence that ring reduction terminates at the stage of hexahydro-2-naphthoyl-CoA and
59 a sequence of β-oxidation-like degradation reactions starts with a hydratase acting on
60 this intermediate. The final product of this reaction sequence was identified as *cis*-2-
61 carboxycyclohexylacetyl-CoA, a compound for which a further downstream degradation
62 pathway has recently been published (see reference 33). The current manuscript reveals
63 the first ring-cleaving reaction in the anaerobic naphthalene degradation pathway. It
64 closes the gap between the reduction of the first ring of 2-naphthoyl-CoA by 2-napthoyl-
65 CoA reductase and the lower degradation pathway starting from *cis*-2-
66 carboxycyclohexylacetyl-CoA, where the second ring cleavage takes place.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) can derive from mineral oil products or incomplete combustion processes and are omnipresent in nature(1). Aerobic degradation of PAHs involving ring-opening dioxygenases is well described (2, 3), whereas the extremely slow, anaerobic degradation of PAHs is less well understood and even the degradation pathway of the simplest PAH, naphthalene, is still not fully elucidated (4, 5). Anaerobic bacteria cannot rely on oxygen (O_2) as reactive co-substrate and, after the initial activation reaction, channel aromatic substrates to central intermediates such as benzoyl-CoA (6) for monocyclic aromatic compounds such as toluene and 2-naphthoyl-CoA for naphthalene and 2-methylnaphthalene (5, 7). In contrast to aerobic pathways, the resonance energy of the aromatic ring system is overcome by reduction via de-aromatising aryl-CoA reductases (8-10). Three different strategies for aromatic ring reduction are known to date:

Class I benzoyl-CoA reductases consist of four different subunits and their catalytic activities are ATP-dependent and oxygen sensitive (11). They are usually found in facultative anaerobic bacteria like *Thauera aromatica* (formerly known as *Pseudomonas* strain K172) (11-13), *Azoarcus* strain CIB (14) or *Rhodopseudomonas palustris* (15). All class I benzoyl-CoA reductases hydrolyse one ATP to ADP + P_i per electron transferred and use ferredoxin as natural electron donor (16). The reduced ferredoxins delivering the required low-potential electrons are generated by electron-donor:ferredoxin oxidoreductases and genes coding for an oxidoreductase and a ferredoxin are typically found within the gene clusters containing the benzoyl-CoA reductase genes (14, 15, 17).

91 Class II benzoyl-CoA reductases are also sensitive towards oxygen and, in contrast to the
92 class I reductases, are ATP-independent W-enzymes (18). The vast majority of strict
93 anaerobes that can utilise aromatic compounds employ a class II reductase (19). These
94 enzymes were, for example, studied in the sulphate-reducer *Desulfococcus multivorans*
95 (20), the iron reducer *Geobacter metallireducens* (21, 22) and the fermenting bacterium
96 *Syntrophus aciditrophicus* (23). Class II reductases are huge enzyme complexes (ca.
97 1 MDa) that are proposed to have a modular composition of eight different subunits
98 (18). ATP-independent reduction of the aromatic system is most likely achieved via an
99 electron bifurcation mechanism (24) in which one low-potential electron from
100 ferredoxin is transferred to the substrate and a second is transferred from ferredoxin to
101 the high-potential electron acceptor $NAD(P)^+$ making the overall process exergonic (18,
102 25).

103 A third type of aryl-CoA reductase was recently discovered in sulphate-reducing
104 naphthalene degraders and is only known for anaerobic PAH degradation (26). These
105 class III reductases belong to the 'old yellow enzyme' (OYE) family and are ATP-
106 independent and insensitive towards oxygen. In the initial steps of the anaerobic
107 degradation pathway of naphthalene (Figure 1), two distinct OYE-like enzymes catalyse
108 the reduction of 2-naphthoyl-CoA to 5,6-dihydro-2-naphthoyl-CoA and the subsequent
109 reduction of the latter to 5,6,7,8-tetrahydro-2-naphthoyl-CoA (27).

110 The subsequent reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) in this
111 pathway is probably catalysed by enzymes similar to the class I benzoyl-CoA reductases
112 of the *Azoarcus*-type that were identified in the anaerobic naphthalene-degrading
113 strains *Deltaproteobacterium* strain NaphS2 and *Desulfobacterium* strain N47 (28, 29).
114 In cell free extracts of enrichment culture N47, THNCoA was reduced to a hexahydro-2-

115 naphthoyl-CoA with an as yet unknown positioning of the diene moiety (30). This
116 reaction was dependent on ATP and an electron donor, preferably NADH. However, a
117 putative oxidoreductase as well as a ferredoxin are encoded directly downstream of the
118 genes coding for the proposed THNCoA reductase subunits in the genome of strains
119 NaphS2 and N47 (28, 29). These enzymes might be involved in NAD(P)H-dependent
120 electron transfer to THNCoA via ferredoxin in analogy to the electron generating
121 systems of benzoyl-CoA reduction described above.

122 It remains an open question whether the hexahydro-2-naphthoyl-CoA (HHNCoA)
123 observed in THNCoA reductase assays (30) is the final product of the reductase reaction
124 or if the reduction proceeds to octahydro-2-naphthoyl-CoA (OHNCoA). The latter would
125 be analogous to cyclohex-1-ene-1-carboxyl-CoA, which is known as intermediate of the
126 benzoyl-CoA pathway in *R. palustris* (15). The similarity of the identified THNCoA
127 reductase to benzoyl-CoA reductases of the *Azoarcus*-type might be an indication for
128 HHNCoA as final product of the reductase reaction because known reductases of this
129 type most likely transfer two electrons to their substrate (14, 31). However, the number
130 of electrons transferred by the reductase cannot be unequivocally deduced from
131 sequence homologies alone because the two electrons transferring benzoyl-CoA
132 reductase from *T. aromatica* (32) and the four electrons transferring reductase from
133 *R. palustris* (6) are very similar at the protein sequence level (31).

134 Given the uncertainties described above, the objective of the present work was to
135 identify the final product of THNCoA reduction in anaerobic naphthalene catabolism.
136 Furthermore, we set out to identify subsequent metabolic intermediates of the pathway
137 in order to elucidate the underlying enzyme reactions. Unlike the aerobic degradation
138 pathways, metabolic pathways of anaerobic degradation of mono- and polycyclic

aromatic compounds do not converge at the same central intermediate. The downstream degradation pathway of naphthalene, which can serve as an exemplar for polycyclic aromatic hydrocarbons, does not proceed via benzoyl-CoA, but via metabolites with a cyclohexane skeleton (7). Our research aimed to elucidate the pathway further and to close the gap between the THNCoA reductase and the recently described downstream pathway starting from 2-carboxycyclohexylacetyl-CoA (33).

This research was conducted by Philip Weyrauch in partial fulfilment of the requirements for a doctoral degree from the University of Duisburg-Essen (34).

Results

THNCoA reductase reaction in cell free extract of culture N47

In order to further study the THNCoA reductase, we measured the reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) to hexahydro-2-naphthoyl-CoA (HHNCoA) with NADH as electron donor as reported previously (30) (Figure 2 panels A and B).

When NAD^+ was added to the assay mixture after a pre-incubation time of 30 min, further conversion of the previously formed HHNCoA could be detected (Figure 2 panels C and D). Already after 30 min, more than 50% of the initially added THNCoA (m/z = 926, retention time 13.6 min) was converted to HHNCoA (m/z = 928, peak at retention time 14.1 min) (Figure 2 panel C). The HHNCoA/THNCoA ratio slightly increased during further incubation and a new peak with m/z = 946 (retention time 11.3 min) appeared (Figure 2 panel B). The mass of m/z = 946 indicates that this metabolite could either represent the product of a hexahydro-2-naphthoyl-CoA

161 hydratase, β-hydroxyoctahydro-2-naphthoyl-CoA (compound 7, Figure 7), or a
162 metabolite resulting from water addition to octahydro-2-naphthoyl-CoA and a
163 subsequent β-hydroxyacyl-CoA dehydrogenase reaction, namely β-oxodecahydro-2-
164 naphthoyl-CoA. After further incubation with NAD^+, the metabolite with m/z = 946 was
165 completely converted and two new peaks arose (m/z 962, Figure 2 panel D). NAD^+
166 therefore seems to be an essential co-substrate for an enzyme, most likely a β-
167 hydroxyacyl-CoA dehydrogenase, involved in the conversion steps downstream of the
168 THNCoA reductase. Since the metabolite with m/z = 946 only accumulated in absence of
169 NAD^+ (Figure 2 panel B), it is presumably the substrate for a β-hydroxyacyl-CoA
170 dehydrogenase rather than the product of this enzyme reaction, which points to β-
171 hydroxyoctahydro-2-naphthoyl-CoA (compound 7, Figure 7). This also indicates that the
172 β-oxidation-like downstream pathway starts with a hydratase reaction on hexahydro-2-
173 naphthoyl-CoA (HHNCoA pathway).

174 Among the downstream metabolites formed after addition of NAD^+ were two
175 compounds each with m/z = 962 (pair of peaks in the LC-MS chromatogram, Figure 2
176 panel D). This mass-to-charge ratio corresponds to the substrate of a ring-opening
177 hydrolase in an HHNCoA pathway, i.e. β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA
178 (compound 9, Figure 7), present as two diastereoisomers (in this case and for other
179 chiral compounds the configuration at a stereogenic center is not defined). Alternatively,
180 this product pair could be a CoA thioester of 2-(3-carboxyallyl)cyclohexane-1-carboxylic
181 acid or 3-(2-(carboxymethyl)cyclohexyl)acrylic acid (compounds A1 and A2 in Figure 8)
182 derived by the action of a hydrolase in a β-oxidation-like downstream pathway starting
183 from octahydro-2-naphthoyl-CoA (OHNCoA pathway). The other peak (retention time
184 5.8 min, m/z = 936) arising from NAD^+ action was identified as a CoA thioester of *cis*-2-

185 (carboxymethyl)cyclohexane-carboxylic acid (compound 12, Figure 7) by comparison
186 with a chemically synthesised reference. This compound was previously identified as an
187 intermediate in anaerobic naphthalene degradation and a downstream degradation
188 pathway starting from this metabolite was recently described (7, 33).

189 In reductase assays with culture N47 cell free extracts that contained only ATP and
190 NADH as co-substrates, the reduction of THNCoA to HHNCoA typically stopped at a
191 conversion rate of ca. 60% and the metabolite with m/z = 946 was the only downstream
192 metabolite that could be detected. Subsequent experiments with an increased
193 concentration of THNCoA indicated that the reduction of the latter was not limited to a
194 certain conversion rate but to a fixed amount of THNCoA that could be reduced under
195 the given assay conditions, so most likely a co-substrate necessary for the reduction was
196 depleted. However, when 2-oxoglutarate was added as electron donor instead of NADH,
197 a complete conversion of THNCoA within 45 min was observed (Figure 3).

198 HHNCoA was only transiently detected and was obviously further converted to products
199 giving rise to peaks with m/z = 962 and m/z = 936, which were also observed when
200 NAD^+ was added to reductase assays with NADH as electron donor after a pre-
201 incubation time of 30 min (see above). These observations indicate that 2-oxoglutarate
202 can indirectly serve as electron donor for the THNCoA reductase, most likely because
203 reduced ferredoxin can be regenerated via a 2-oxoglutarate:ferredoxin oxidoreductase
204 comparable to the ferredoxin-reducing system reported previously for *T. aromatica*
205 (35). Surprisingly, the reductase reaction with 2-oxoglutarate as electron donor did not
206 require the addition of CoA-SH as would be expected for such a system (a 2-
207 oxoglutarate:ferredoxin oxidoreductase forms succinyl-CoA and CO_2 from 2-
208 oxoglutarate and CoA-SH) and supplementation with free CoA-SH did not affect the

209 product pattern observed in those assays. It appears that culture N47 cell extract either
210 contains high levels of free CoA-SH or can regenerate CoA-SH from acetyl-CoA, succinyl-
211 CoA or via non-specific thioesterases hydrolysing acyl-CoA compounds in the assay
212 mixture. This is in accordance with results for 2-naphthoate:CoA ligase assays conducted
213 in our group, where up to 100 µM 2-naphthoyl-CoA were produced from 2-naphthoate
214 in culture N47 cell free extract supplemented with ATP, but without external addition of
215 CoA-SH (unpublished results). Only when the THNCoA reductase assays were performed
216 with cell free extracts of strain NaphS2 or with culture N47 extracts that were harvested
217 during a later growth phase, the THNCoA reductase reaction with 2-oxoglutarate as
218 electron donor indeed required coenzyme A as additional co-substrate, confirming the
219 proposed oxidoreductase reaction.

220

221 **Oxidoreductase assays with different electron donors and acceptors**

222 As demonstrated above, different compounds can serve as electron donor for the
223 THNCoA reductase reaction in cell free extracts. Assuming that the reductase itself
224 should be specific for one kind of electron donor, the observed versatility might be a
225 result of oxidoreductase activities in the extracts which can mediate electron transfer
226 between different combinations of electron donors and acceptors. Therefore,
227 spectrophotometric oxidoreductase assay were performed in order to elucidate electron
228 transfer capabilities of the extracts (Table 1).

229 No electron transfer could be observed from NADH to methyl viologen, which served as
230 a proxy for ferredoxin, or from 2-oxoglutarate to NAD^+. In contrast, a 2-oxoglutarate
231 dependent reduction of methyl viologen occurred at a rate of 0.223 µM min^{-1}, proving

232 the activity of a 2-oxoglutarate:ferredoxin oxidoreductase. An even higher
233 oxidoreductase rate of 1.85 µM was observed when 2-oxoglutarate, methyl viologen and
234 NAD^+ were combined so methyl viologen would serve as a mediator for the indirect
235 electron transfer from 2-oxoglutarate to NAD^+ via 2-oxoglutarate:ferredoxin and a
236 presumed ferredoxin: NAD^+ oxidoreductase, resulting in the production of NADH.

237

238 **THNCoA reductase assays with variation of NADH and NAD^+ concentrations**

239 The results from THNCoA reductase assays described above indicated that levels of
240 NADH and NAD^+ need to be balanced to allow for both the NADH-dependent THNCoA
241 reductase reaction and a NAD^+-dependent β-hydroxyacyl-CoA dehydrogenase reaction
242 to take place. We therefore conducted further THNCoA reductase assay supplemented
243 with different ratios of NADH and NAD^+ (Figure 4).

244 In assays with 5 mM of both NADH and NAD^+ (Figure 4 panel A), some downstream
245 metabolites were produced but conversion of THNCoA was incomplete and HHNCoA
246 accumulated. Less THNCoA remained unconverted and more HHNCoA accumulated in
247 assays with 5 mM NADH and 0.2 mM NAD^+ (Figure 4 panel B). Furthermore, no
248 downstream metabolites were detected under those conditions, indicating that this
249 NAD^+/NADH ratio did not allow for any downstream reaction to occur and as a
250 consequence accumulation of HHNCoA inhibited further conversion of THNCoA. In
251 contrast, an almost complete conversion of 50 µM THNCoA and production of the
252 downstream metabolites could be achieved with 0.2 mM NADH and 5 mM NAD^+ (Figure
253 4 panel C). The lower NADH concentration was apparently sufficient for THNCoA
254 reduction and the elevated NAD^+/NADH ratio enabled further conversion of HHNCoA.

255

256 **THNCoA reductase rates with different electron donors**

257 After observing that compounds other than NADH can also be used as electron donors
258 for THNCoA reduction in cell free extracts of anaerobic naphthalene degraders, the
259 conversion rates of THNCoA in standard THNCoA reductase assays were determined as
260 a function of the electron donor (Figure 5).

261 Within the first 30 min of the reaction, about 40 µM THNCoA was converted for the
262 three electron donors tested: NADH, 2-oxoglutarate, and sodium citrate. However, with
263 NADH as electron donor the consumption of THNCoA was accompanied by an
264 accumulation of HHNCoA, whereas in assays with 2-oxoglutarate or citrate no
265 accumulation of HHNCoA was observed. The HHNCoA concentration in assays with
266 NADH reached a maximum of ca. 25 µM after 45 – 60 min and remained almost constant
267 during further incubation. Coinciding with the accumulation of HHNCoA, the conversion
268 rate of THNCoA decreased after 45 min of incubation and less than 60 µM THNCoA were
269 consumed after 120 min. In contrast, with 2-oxoglutarate or citrate as electron donor no
270 decrease of the THNCoA conversion rate after 45 min was observed and a total of 80 µM
271 THNCoA could be converted during 120 min incubation time without any accumulation
272 of HHNCoA.

273

274 **THNCoA reductase reaction in cell free extract of strain NaphS2**

275 In contrast to cell free extract of culture N47, which showed a clear preference for NADH
276 over NADPH as electron donor for the THNCoA reducing system, strain NaphS2 extract
277 produced almost the same THNCoA conversion rates with either of the two co-

278 substrates. The maximum conversion rate was, however, only ca. 30% which is
279 significantly lower than the rate obtained with culture N47 extract. Again, a metabolite
280 with m/z = 946 appeared at a retention time of 11.3 min in the LC/MS chromatograms
281 which had already been detected in THNCoA reductase assays with culture N47 extract.
282 Since the reductase reaction with NADPH was not inhibited by the presence of NAD^+, the
283 two co-substrates could be added simultaneously to the assay mixture at a
284 concentration of 5 mM each. This led to a further conversion of the HHNCoA formed
285 during THNCoA reduction and the production of metabolites with m/z = 962 and
286 m/z = 936 which had the same retention times as the respective compounds formed in
287 assays with culture N47 extract. Hence, the downstream pathways in strains N47 and
288 NaphS2 most likely proceed via the same intermediates.

289 THNCoA reductase assays with other electron donors in strain NaphS2 cell free extract
290 gave similar results to the ones with culture N47 extract (data not shown). The best
291 THNCoA conversion with strain NaphS2 extract was obtained when 2-oxoglutarate was
292 added as electron donor matching the results obtained with culture N47 extract, albeit
293 with the difference that the 2-oxoglutarate dependent reduction in strain NaphS2 cell
294 free extract indeed required CoA-SH as additional co-factor as expected for a 2-
295 oxoglutarate:ferredoxin oxidoreductase. Without addition of CoA-SH, no significant
296 conversion of THNCoA took place. Only if both 2-oxoglutarate (5 mM) and coenzyme A
297 (1 mM) were added to the standard assay mixture, was THNCoA converted giving
298 HHNCoA, as well as the metabolites with m/z = 962 and m/z = 936. A similar pattern
299 was observed if the assays were additionally dosed with NAD^+. In this case no HHNCoA,
300 but only residual THNCoA and the downstream metabolites, were detectable after
301 90 min incubation. In contrast, addition of NADH to assays containing 2-oxoglutarate

and CoA-SH caused an accumulation of HHNCoA and seemed to prevent further conversion of this intermediate. The two latter observations indicate that the further conversion of THNCoA depends on the oxidation of an intermediate by a β-hydroxyacyl-CoA dehydrogenase which requires NAD^+. In presence of excess of NADH, the equilibrium of this reaction shifts towards the reduced substrate which prevents further conversion and leads to the accumulation of upstream metabolites.

Identification of the downstream metabolites

In a previous study, a β-oxidation-like downstream pathway was proposed starting from octahydro-2-naphthoyl-CoA with a CoA thioester of 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid (compound A1) or 3-(2-(carboxymethyl)cyclohexyl)acrylic acid (compound A2) as intermediate (7). The expected mass-to-charge ratio of these coenzyme A thioesters is 962, which is identical to the pair of downstream metabolites that occurred in THNCoA reductase assays (see above). LC-MS chromatograms of samples from these assays were compared under two different pH conditions with CoA thioesters of chemically synthesised reference compounds A1 and A2 (Figure 6).

At pH 7.0, the unknown metabolite appeared as two close peaks with retention times 7.4 and 7.7 min. CoA thioesters of compound A1 eluted significantly different showing two peaks with retention times 7.6 and 7.9 min, whereas the retention times of the two peaks of CoA thioesters of compound A2 (7.5 min and 7.7 min) was identical in one case and similar in the other to those observed for the unknown metabolite. In the case of the reference compounds, the two peaks represent isomers differing in the position of the CoA thioester on the dicarboxylic acid. At pH 5.5, the distance between the two isomer

peaks of the reference compounds was increased (10.3 and 11.0 min for compound A1 and 10.1 and 10.4 min for compound A2). In contrast, the unknown metabolite appeared as a single peak at pH 5.5 (retention time 10.4 min). The elution patterns of the unknown metabolite and the reference compounds were confirmed by analyses of several independent samples and by spiking of biological samples with reference standards. Thus, we can tentatively conclude that neither compound A1 nor compound A2 CoA thioesters represent the naturally occurring metabolite with m/z = 962.

The CoA thioesters of the two reference compounds were also tested for conversion in cell free extracts of N47 or NaphS2 cultures grown with naphthalene or 2-naphthoate, respectively. The naturally occurring metabolite should be converted by an enoyl-CoA hydratase which is assumed not to require any co-substrate but water. LC-MS analysis of the assay mixtures revealed no conversion of either of the two compounds during incubation in cell free extracts (data not shown), which indicates that the metabolite with m/z = 962 has a different structure from the two reference compounds, despite the similarities in elution patterns described above. Hence, it is unlikely that octahydro-2-naphthoyl-CoA as well as CoA thioesters of 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid or 3-(2-(carboxymethyl)cyclohexyl)acrylic acid are intermediates of the naphthalene degradation pathway. Although we propose (see Discussion section) that the unknown metabolite is a β-keto CoA thioester, the uncertainty in the precise identity of the metabolite pair giving rise to m/z = 962 requires further investigation. The observed pH-dependent chromatographic behaviour of metabolite and reference compounds could be explained by the different ionisation states of the CoA moiety as

348 well as, in the case of the unknown metabolite, of a pH-dependent shift in the
349 equilibrium between the keto-enol pair of tautomers.

350

351

352 ## Discussion

353 ### HHNCoA is the final product of the THNCoA reductase

354 The reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) to a hexahydro-2-
355 naphthoyl-CoA (HHNCoA) was previously demonstrated with cell free extracts of the
356 N47 enrichment culture. The reaction was catalysed by an enzyme similar to the class I
357 benzoyl-CoA reductases and NADH was described as the best working electron donor
358 (30). However, a complete conversion of THNCoA could not be achieved in these assays
359 and no further metabolites downstream of HHNCoA were detected. THNCoA conversion
360 stopped at approximately 50% conversion in conjunction with an accumulation of
361 HHNCoA. The high excess of NADH in those assays presumably shifted the equilibrium
362 of an NAD^+-dependent β-hydroxyacyl-CoA dehydrogenase involved in the downstream
363 pathway backwards, leading to an accumulation of the upstream metabolites. A better
364 conversion of THNCoA was achieved by the further addition of NAD^+, the supposed
365 dehydrogenase co-substrate. We observed a novel downstream metabolite with
366 m/z = 946, presumably β-hydroxyoctahydro-2-naphthoyl-CoA (compound 7, Figure 7).
367 Thus, we propose a downstream pathway involving a hydratase reaction on HHNCoA
368 and a subsequent β-hydroxyacyl-CoA dehydrogenase which acts analogously to
369 cyclohexa-1,5-diene-1-carboxyl-CoA hydratase (36) and 6-hydroxycyclohex-1-ene-1-
370 carboxyl-CoA dehydrogenase (37) from the benzoyl-CoA pathway in *T. aromatica*.

371 Despite the fact that downstream reactions were inhibited in THNCoA reductase assays
372 with high concentrations of NADH whereas complete conversion of THNCoA and
373 production of downstream metabolites could be achieved in assays with 2-oxoglutarate
374 or citrate, our data still suggest that NADH is the natural electron donor for THNCoA
375 reductase. This is based on the following two observations: Firstly, THNCoA reductase
376 assays with reduced NADH levels and a high NAD^+/NADH ratio allowed for both
377 THNCoA reduction as well as downstream reactions to take place (Figure 4). Secondly,
378 indirect electron transfer from 2-oxoglutarate to NAD^+ took place in oxidoreductase
379 assays but in turn NADH did not serve as electron donor for the reduction of methyl
380 viologen which was used as a proxy for ferredoxin (Table 1). Therefore, the observed
381 NADH dependent reduction of THNCoA should only be possible if NADH rather than
382 ferredoxin was the natural electron donor for the reductase. Citrate or 2-oxoglutarate
383 dependent THNCoA reduction was most likely an artefact of the assay conditions where
384 2-oxoglutarate served as indirect electron donor via oxidoreductase mediated electron
385 transfer yielding NADH. *Desulfobacterium* strain N47 operates a modified citric acid
386 cycle in which the ordinary 2-oxoglutarate dehydrogenase (38, 39) is replaced by a 2-
387 oxoglutarate:ferredoxin oxidoreductase (28). A similar oxidoreductase was also
388 identified in strain NaphS2 (29). Such enzymes can generate reduced ferredoxin upon
389 conversion of 2-oxoglutarate and CoA-SH to succinyl-CoA and CO_2 (35). Another
390 oxidoreductase would then use the reduced ferredoxin as electron donor for the
391 reduction of NAD^+ to NADH (35). As a result, NADH can be continuously regenerated
392 from the pool present in cell free extracts without requirement for external addition of
393 NADH. Notably, the observed rate of 1.85 µM would be sufficient to supply the NADH
394 required for the reduction of 100 µM THNCoA within 90 min. This continuous
395 regeneration of NADH presumably resembles intracellular conditions much better than

external addition of excess NADH and does therefore not interfere with downstream reactions.

Earlier studies with the THNCoA reductase could not detect any metabolites downstream of HHNCoA, which was surprising because the comparable reactions of the benzoyl-CoA reductase and the subsequent cyclohexa-1,5-diene-1-carboxyl-CoA hydratase had previously been measured in one assay (32). In fact, a peak presumably representing the HHNCoA hydratase product was detected in our assays, albeit in very low concentrations, even with NADH as electron donor. Due to its very low concentration, this peak could only be detected via LC-MS in single ion mode and was overlooked in previous studies that used HPLC for analysing the *in vitro* assays (30). Our results indicate that if the equilibrium of the subsequent β-hydroxyacyl-CoA dehydrogenase is shifted due to high NADH concentrations, mainly HHNCoA rather than its hydratase product accumulated as upstream metabolite. Hence, the equilibrium of the hydratase reactions seems to be far on the side of the educt HHNCoA. Since the co-substrate of a hydratase – water – is present in vast excess in aqueous assays, the equilibrium of such a reaction is usually expected to be in favour of the product. For the cyclohexa-1,5-diene-1-carboxyl-CoA hydratase, equilibrium concentrations of substrate and product were, however, reported to be almost equal (36), which makes the very low concentration of HHNCoA hydratase product observed in our assays therefore appear realistic. Assuming that water addition occurs in the β,γ'-position (i.e. 1,4- or 3,8a-) by analogy with cyclohexa-1,5-diene-1-carboxyl-CoA hydratases, the HHNCoA hydratase could be handicapped sterically by the already reduced ring, depending on the nature of HHNCoA.

420 **Identification of downstream metabolites**

421 Further novel metabolites identified in our assays (m/z = 962 and m/z = 936)
422 presumably represented the CoA thioesters of compounds that were identified earlier in
423 culture extracts of the N47 enrichment culture (7). While m/z = 936 matches the mass of
424 2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA thioester (compound 12 in Figure
425 7), the metabolite with m/z = 962 is most likely not a CoA thioester of the earlier
426 postulated 3-(2-(carboxymethyl)cyclohexyl)acrylic acid or 2-(3-
427 carboxyallyl)cyclohexane-1-carboxylic acid but rather a β'-hydroxy-β-oxodecahydro-2-
428 naphthoyl-CoA (compound 9). This was deduced from the chromatographic behaviour
429 (Figure 6) of this metabolite which indicated a structure that is (i) different from the
430 previously postulated structures and can (ii) undergo keto-enol tautomerism. In the
431 benzoyl-CoA pathway, the analogous β'-hydroxy-β-oxo-intermediate is normally not
432 detected as a free metabolite since the Oah enzyme, which acts on 6-oxocyclohex-1-ene-
433 1-carboxyl-CoA in this pathway, has both enoyl-CoA hydratase and ring hydrolysing
434 activity (37, 40). As described before, no Oah-like enzyme is encoded by the *thn*-operon
435 that codes for enzymes of the downstream pathway of anaerobic naphthalene
436 degradation (4). We assume that β-oxooctahydro-2-naphthoyl-CoA hydratase and β'-
437 hydroxy-β-oxodecahydro-2-naphthoyl-CoA hydrolase are two separate enzymes in this
438 pathway (Figure 7), which is in accordance with the detection of β'-hydroxy-β-
439 oxodecahydro-2-naphthoyl-CoA as free metabolite.

440 In this pathway, up to five acetyl-CoA are formed via β-oxidation-like reactions and can
441 be fed into a modified citric acid cycle or the Wood-Ljungdahl pathway, which are both
442 expressed during growth with naphthalene (28).

443

444 **Implications for the downstream pathway**

445 Previous studies identified an intermediate of the β-oxidation-like downstream pathway
446 in cell extracts that was interpreted as 3-(2-(carboxymethyl)cyclohexyl)acrylic acid or 2-
447 (3-carboxyallyl)cyclohexane-1-carboxylic acid according to its *m/z* in GC-MS analyses
448 (7). Since the further downstream metabolite 2-(carboxy-methyl)cyclohexane-1-
449 carboxylic acid was found to occur only as the *cis*-isomer (7) and β-oxidation-like
450 reactions usually proceed via *E*-unsaturated CoA thioester intermediates (41), we tested
451 the chemically synthesised CoA thioesters of the *cis*-(*E*)-isomers of the two compounds
452 for conversion in cell free extracts of culture N47 and strain NaphS2. However, none of
453 the tested CoA thioesters was metabolised. Although it cannot be completely excluded
454 that the pathway proceeds via the *trans*-isomers or that the double-bond occurs in *Z*-
455 rather than in *E*-configuration, the observed persistence of *cis*-(*E*)-3-(2-
456 (carboxymethyl)cyclohexyl)acrylyl-CoA and *cis*-4-(2-carboxycyclohexyl)-crotonyl-CoA
457 (i.e. CoA thioesters of 3-(2-(carboxymethyl)cyclohexyl)acrylic acid and 2-(3-
458 carboxyallyl)cyclohexane-1-carboxylic acid) in cell free extracts of sulphate-reducing
459 naphthalene degraders casts doubt on the previous interpretation of the identified mass.

460 In the work referred to, metabolites were extracted as free acids (hydrolysis of CoA
461 thioesters via NaOH treatment) from N47 cultures and the acids were derivatised with
462 trimethylsulfonium hydroxide, which methylates carboxyl groups (42). High-resolution
463 GC-MS could confirm the elemental composition of the respective metabolite as $C_{11}H_{16}O_4$
464 (non-methylated form) but could not prove the presence of two methylated carboxyl
465 groups. Thus, the observed metabolite could also represent β'-hydroxy-β-oxodecahydro-
466 2-naphthoic acid which exhibits the same elemental composition (Figure 8).

467 Assuming hexahydro-2-naphthoyl-CoA as the final product of reductive dearomatisation
468 (30), β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA could result from a second water-
469 addition to the remaining double-bond in β-oxooctahydro-2-naphthoyl-CoA prior to first
470 ring-cleavage (Figure 7). This would represent a reaction scheme similar to the 6-
471 oxocyclohex-1-enecarboxyl-CoA hydratase/hydrolase from anaerobic benzoate
472 degrading bacteria (40). A hydrolytic ring-cleavage at β'-hydroxy-β-oxodecahydro-2-
473 naphthoyl-CoA would result in the same downstream metabolite as a hydratase reaction
474 at 3-(2-(carboxymethyl)cyclohexyl)-acrylyl-CoA or 4-(2-carboxycyclohexyl)crotonyl-
475 CoA, respectively (Figure 8). Regarding the apparent absence of an enoyl-CoA hydratase
476 activity towards cyclohexane-derivatives with unsaturated C_3- or C_4-side-chains, we
477 postulate that the previously proposed CoA thioester of 3-(2-
478 (carboxymethyl)cyclohexyl)acrylic acid or 2-(3-carboxyallyl)cyclohexane-1-carboxylic
479 acid is not an intermediate of anaerobic naphthalene degradation. In fact, our data
480 suggest that β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA (compound 9 in Figure 7) is
481 the true intermediate.

482 This is in perfect agreement with our analysis of downstream metabolites in THNCoA
483 reductase assays (see above). The postulated pathway via HHNCoA and β'-hydroxy-β-
484 oxodecahydro-2-naphthoyl-CoA (Figure 7) is therefore supported by the results of two
485 independent experimental approaches (metabolite analysis and negative conversion
486 tests with previously postulated metabolites), indicating a β-oxidation-like downstream
487 pathway starting from HHNCoA rather than from OHNCoA as proposed previously.

488

Material and Methods

Growth of bacterial cells

The enrichment culture N47 and *Deltaproteobacterium* strain NaphS2 (DSMZ 14454) were grown anaerobically in artificial freshwater (N47) or seawater (NaphS2) medium with naphthalene (N47) or 2-naphthoate (NaphS2) as sole source of carbon and electrons as described previously (33). A freshly harvested 6 weeks (N47) or 4 weeks (NaphS2) old 1.6 L culture was used for each set of assays.

Anaerobic preparation of cell free extracts

Cultures were transferred into air-tight centrifuge beakers (4 × 400 mL) in an anaerobic LABstar Glove Box Workstation (M. Braun, Garching, Germany) with a N_2-atmosphere and cells were harvested by centrifugation for 30 min at 17,700 × *g* and 4 °C. The supernatant was discarded, the cell pellets were washed once with enzyme test buffer (see below), and then suspended in the same buffer (1 mL buffer per 1 L initial culture). The cells were opened by using a French press (Thermo Electron, Waltham, USA) operated at 6.9 MPa. To obtain the cell free extract, cell debris was afterwards segregated by centrifugation for 15 min at 20,800 x *g* and 4 °C. Protein concentrations in cell free extract were typically 5 – 10 mg/mL. To keep the extract anoxic, the following steps were conducted inside a glove box: resuspension and washing of cell pellets, loading of French Press mini cell and transfer of crude extract into air-tight screw cap microcentrifuge tubes after French Press passage.

511 **Synthesis of potential downstream metabolites**

512 Racemic (1*S*,2*S*/1*R*,2*R*)-2-((*E*)-3-carboxyallyl)cyclohexane-1-carboxylic acid (**A1**): Two
513 consecutive reductions (43) of commercially available 1,2-*cis*-cyclohexanedicarboxylic
514 anhydride with $NaBH_4$ followed by di-isobutylaluminium hydride yielded an
515 intermediate hemiacetal (see Figure 9). Wittig reaction of the hemiacetal with
516 (methoxymethyl)triphenylphosphonium chloride (44) and subsequent acidic hydrolysis
517 of an intermediate methyl enol ether led to the homologous hemiacetal, which was
518 subjected to a Wittig reaction with methyl triphenylphosphoranylideneacetate. The
519 resulting hydroxy-ester was oxidised to a carboxylic acid, which was saponified to give
520 the desired dicarboxylic acid **A1** after acidification (Figure 9).

521 Racemic (*E*)-3-((1*R*,2*R*/1*S*,2*S*)-2-(carboxymethyl)cyclohexyl)acrylic acid (**A2**): The
522 hemiacetal (Figure 10) was reacted with methyl triphenylphosphoranylideneacetate to
523 give a hydroxy-ester, which was converted into the corresponding triflate for
524 subsequent S_N2 reaction with cyanide to afford an intermediate nitrile. Acidic
525 methanolysis of the nitrile followed by basic hydrolysis (1 M LiOH in THF/water) of the
526 methyl ester group afforded the desired dicarboxylic acid **A2** (Figure 10) after
527 acidification.

528 **Synthesis and purification of CoA thioesters**

529 5,6,7,8-Tetrahydro-2-naphthoyl-CoA (THNCoA) and the CoA thioesters of 2-(3-
530 carboxyallyl)cyclohexane-1-carboxylic acid and 3-(2-(carboxymethyl)cyclohexyl)acrylic
531 acid were synthesised from the free acid via their succinimidyl esters and purified as
532 described earlier (33). A Strata® Phenyl SPE column (Phenomenex, Aschaffenburg,
533 Germany) was used for purification of THNCoA and a HyperSep™ C18 SPE column

534 (Thermo Fisher Scientific, Ulm, Germany) was used for the purification of the CoA
535 thioesters of 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid and 3-(2-
536 (carboxymethyl)cyclohexyl)acrylic acid. Identity of the synthesised CoA thioester
537 compounds was verified by LC-MS analysis.

538

539 **Discontinuous reductase assays**

540 THNCoA reductase activity was measured under strictly anoxic conditions in 100 mM
541 MOPS/KOH buffer, pH 7.3 with 15 mM $MgCl_2$ as described previously (30). The 200 µL
542 reaction mixture typically contained 60 – 100 µL of anaerobic cell free extract of
543 enrichment culture N47 or *Deltaproteobacterium* strain NaphS2, 5 mM ATP, 5 mM
544 electron donor (NADH, NADPH, sodium citrate or 2-oxoglutarate) and 50 µM THNCoA.
545 Assays with 2-oxoglutarate as electron donor were conducted with and without addition
546 of 1 mM CoA-SH. Some assays additionally contained 0.2 – 5 mM electron acceptors like
547 NAD^+ or $NADP^+$, or a combination of two electron donors (see results section for detailed
548 composition of respective assays). The reactions were started by the addition of
549 THNCoA and samples for HPLC analysis (see below) were taken at different time points
550 as described elsewhere (33).

551

552 **Spectrophotometric oxidoreductase assays**

553 Cell extract mediated electron transfer between different donors and acceptors was
554 tested at 30 °C under anoxic conditions in air-tight glass cuvettes using a Specord 200
555 photometer (Analytik Jena, Jena, Germany) according to literature procedures (35, 45).
556 The 1 mL reaction mixture in 100 mM MOPS/KOH buffer, pH 7.3 with 15 mM $MgCl_2$

557 contained 250 µL of anaerobic cell free extract of enrichment culture N47, 0.5 mM CoA-
558 SH and 0.025 mM sodium dithionite. NADH (1 mM) or 2-oxoglutarate (2.5 mM) were
559 used as electron donors and methyl viologen (1 mM) or NAD^+ (1 mM) as electron
560 acceptors. The assays were monitored at 600 nm (methyl viologen reduction) or 365 nm
561 (NAD^+ reduction) and reaction rates were calculated using the following molar
562 absorption coefficients (ε): NADH, ε_{365} = 3.4 mM^{-1} cm^{-1}; methyl viologen, ε_{600} = 13 mM^{-1} cm^{-1}. Control assays were performed without the respective electron donor.

564

565 **Assays with potential downstream metabolites**

566 The CoA thioesters of the chemically synthesised compounds *cis*-2-((*E*)-3-carboxy-
567 allyl)cyclohexane-1-carboxylic acid and *cis*-(*E*)-3-(2-(carboxymethyl)cyclohexyl)acrylic
568 acid, which had earlier been proposed as possible intermediates of the anaerobic
569 naphthalene degradation pathway in culture N47 (7), were tested for conversion by
570 enoyl-CoA hydratases in cell free extracts of culture N47 and strain NaphS2 as described
571 previously (33). Due to the two carboxyl groups present in these compounds, a mixture
572 of two isomers differing in the attachment sites of the CoA thioester was obtained upon
573 synthesis of the respective CoA thioesters. The complete mixture was used as substrate
574 for the hydratase tests. However, only the isomers with a double-bond next to the
575 carboxyl-CoA residue, namely *cis*-(*E*)-3-(2-(carboxymethyl)-cyclohexyl)acrylyl-CoA and
576 *cis*-4-(2-carboxycyclohexyl)crotonyl-CoA, were expected to be converted in a β-
577 oxidation-like manner.

578

LC-MS analysis

LC-MS analyses (liquid-chromatography coupled to mass-spectrometry) were performed with a LC-2040C system coupled to a LCMS-2020 single quadrupole mass-spectrometer (Shimadzu Deutschland, Duisburg, Germany). Samples were separated via a Nucleodur C18 Gravity-SB column, 100 × 3 mm, 5 µm particle size or a Nucleodur C18 Pyramid column, 100 × 3 mm, 5 µm particle size (both Macherey-Nagel, Dueren, Germany). The column oven was set to 35 °C. Eluent A was water with 0.1% (w/v) ammonium acetate, eluent B was acetonitrile. Eluent B increased from 5% up to 35% over 15 min at a flow rate of 0.7 mL/min. Mass spectrometric analysis was carried out with an ESI system in positive mode. The voltage of the ESI system was set to 4.5 kV, the temperature to 350 °C. Nebulising gas flow was 1.5 L/min, drying gas flow 12 L/min. Heat block temperature was 200 °C and the desolvation line was operated at 0 V and 250 °C.

Mass-to-charge ratios (m/z) of expected metabolites (Table 2) were detected in single ion mode with ionisation conditions as optimised for 5,6,7,8-tetrahydro-2-naphthoyl-CoA. For the detection of all these ions, the desolvation line was operated at 0 V and 250 °C. Settings for the Qarray™ ion guide were as follows: DC voltage 0 V, RF voltage 105 – 120 V (compound specific).

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602

603

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605

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744

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746

747 # Tables

748

749 **Table 1** Oxidoreductase rates observed in cell free extracts of culture N47 for different combinations of
750 electron donors and acceptors (n.d.: not detected).

Electron donor	Electron acceptor	Oxidoreductase rate in µM min^{-1}
NADH	Methyl viologen	n.d.
2-oxoglutarate	NAD^+	n.d.
2-oxoglutarate	Methyl viologen	0.22
2-oxoglutarate and methyl-viologen as mediator	NAD^+	1,85

751

752

753 **Table 2** Mass-to-charge ratios of metabolites expected in 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase
754 assays. Depending on the final product of the reductase reaction – hexahydro-2-naphthoyl-CoA (HHNCoA)
755 or octahydro-2-naphthoyl-CoA (OHNCoA) – and on the position of the first ring-opening – between C1 and
756 C2 or between C2 and C3 – different downstream metabolites should emerge.

m/z	Metabolite of putative HHNCoA pathway	Metabolite of putative OHNCoA pathway
926	5,6,7,8-tetrahydro-2-naphthoyl-CoA	5,6,7,8-tetrahydro-2-naphthoyl-CoA
928	hexahydro-2-naphthoyl-CoA	hexahydro-2-naphthoyl-CoA
930		octahydro-2-naphthoyl-CoA
946	β-hydroxyoctahydro-2-naphthoyl-CoA	β-oxodecahydro-2-naphthoyl-CoA
944	β-oxooctahydro-2-naphthoyl-CoA	
948		β-hydroxydecahydro-2-naphthoyl-CoA
962	β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA	3-(2-(carboxymethyl)cyclohexyl)acrylyl-CoA or 4-(2-carboxycyclohexyl)crotonyl-CoA
980	3-(2-(carboxymethyl)cyclohexyl)-3-hydroxypropionyl-CoA or 4-(2-carboxycyclohexyl)-3-hydroxybutyryl-CoA	3-(2-(carboxymethyl)cyclohexyl)-3-hydroxypropionyl-CoA or 4-(2-carboxycyclohexyl)-3-hydroxybutyryl-CoA
978	3-(2-(carboxymethyl)cyclohexyl)-3-oxopropionyl-CoA or 4-(2-carboxycyclohexyl)-3-oxobutyryl-CoA	3-(2-(carboxymethyl)cyclohexyl)-3-oxopropionyl-CoA or 4-(2-carboxycyclohexyl)-3-oxobutyryl-CoA
936	2-(carboxymethyl)cyclohexanecarboxyl-CoA 2-(2-carboxycyclohexyl)-acetyl-CoA	2-(carboxymethyl)cyclohexanecarboxyl-CoA 2-(2-carboxycyclohexyl)-acetyl-CoA

757

758

Figure legends

Figure 1 First steps of the anaerobic naphthalene degradation pathway. Naphthalene (1) is initially carboxylated to 2-naphthoate (2) followed by the formation of 2-naphthoyl-CoA (3). The latter is stepwise reduced to 5,6-dihydro-2-naphthoyl-CoA (4) and 5,6,7,8-tetrahydro-2-naphthoyl-CoA (5) via ATP-independent and oxygen-insensitive class III aryl-CoA reductases. The subsequent reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA via an ATP-dependent and oxygen-sensitive class II aryl-CoA reductase yields a hexahydro-2-naphthoyl-CoA (6) with the diene moiety being one of the possibilities shown.

Figure 2 LC-MS chromatograms of reductase assays with cell free extracts of culture N47 and 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) as substrate. Standard assays contained 50 µM THNCoA, 5 mM ATP and 5 mM NADH. (A): 0 min incubation time. (B): 90 min incubation time. In a second experiment, 5 mM NAD^+ was added to the standard assay after a pre-incubation time of 30 min. (C): assay directly after addition of NAD^+ (30 min). (D): 60 min after addition of NAD^+. Note different y-scales in panels C and D. LC-MS chromatograms obtained in single ion mode show accumulated ion counts of expected metabolites.

Figure 3 LC-MS chromatograms of a 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) reductase assay with cell free extract of culture N47. The assay contained 50 µM THNCoA, 5 mM ATP and 5 mM 2-oxoglutarate as electron donor. Samples were analysed via LC-MS in single ion mode scanning for expected metabolites. Chromatograms show accumulated ion counts for those metabolites.

783

784 **Figure 4** LC-MS chromatograms (accumulated ion counts of single ion mode scans) of 5,6,7,8-tetrahydro-
785 2-naphthoyl-CoA (THNCoA) reductase assays in cell free extract of culture N47. The assays contained
786 50 µM THNCoA, 5 mM ATP and different ratios of NADH and NAD^+ (A): 5 mM NADH and 5 mM NAD^+, (B):
787 5 mM NADH and 0.2 mM NAD^+, (C): 0.2 mM NADH and 5 mM NAD^+. Samples were taken after 90 min and
788 analysed via LC-MS in single ion mode scanning for expected metabolites.

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790

791 **Figure 5** Rates of THNCoA consumption or HHNCoA accumulation in THNCoA reductase assays with cell
792 free extracts of culture N47 using NADH, 2-oxoglutarate or citrate as electron donor (5 mM each). Error
793 bars indicate standard deviations of triplicate incubations for each electron donor.

794

795

796 **Figure 6** Comparison of the LC-MS chromatograms of the downstream metabolite with m/z = 962
797 observed in THNCoA reductase assays with 2-oxoglutarate (5 mM) as electron donor and chemically
798 synthesised reference compounds (A1 and A2, both obtained as a mixture of *cis*-isomers with the CoA
799 thioester formed on either carboxyl group). LC-MS analyses were conducted at pH 5.5 and pH 7.0 for each
800 compound. Grey lines indicate the retention times of peaks of the unknown metabolite. The compounds
801 with m/z = 962 were detected via the MS-unit in single ion mode.

802

803

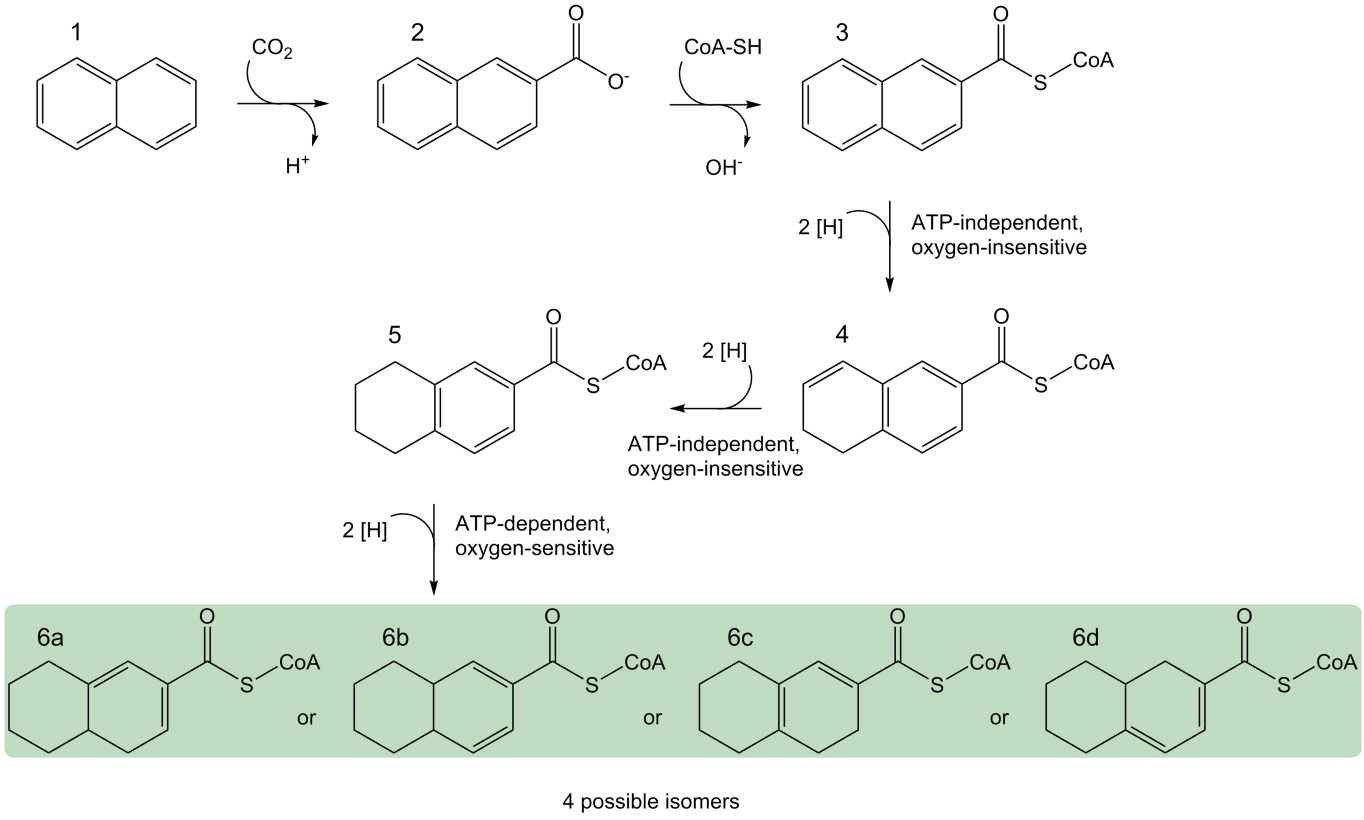
804 **Figure 7** Proposed degradation pathway of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA, compound 5,
805 m/z = 926) assuming the product of the THNCoA reductase to be a hexahydro-2-naphthoyl-CoA (HHNCoA,
806 m/z = 928) with double bonds in α,β- or β',γ'-position (6). The subsequent β-oxidation-like pathway
807 proceeds via β-hydroxyoctahydro-2-naphthoyl-CoA (7) (m/z = 946), β-oxooctahydro-2-naphthoyl-CoA (8)

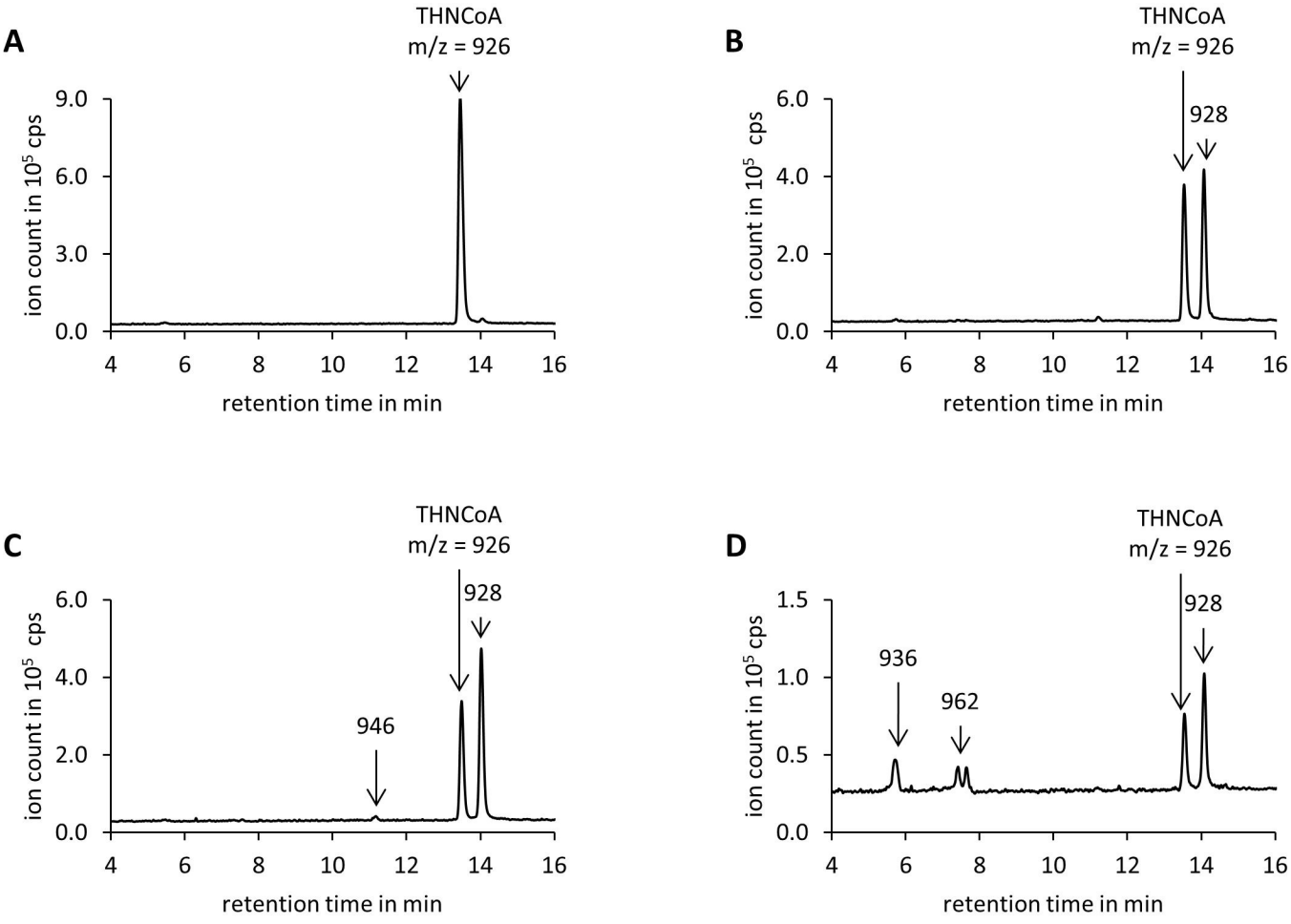
(m/z = 944), and β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA (9) (m/z = 962). Hydrolytic opening of ring I gives 4-(2-carboxycyclohexyl)-3-hydroxybutyryl-CoA (10a) or 3-(2-(carboxymethyl)cyclohexyl)-3-hydroxypropionyl-CoA (10b) (both m/z = 980). Another β-oxidation-like sequence then continues via 4-(2-carboxycyclohexyl)-3-oxobutyryl-CoA (11a) or 3-(2-(carboxymethyl)cyclohexyl)-3-oxopropionyl-CoA (11b) (both m/z = 978), and 2-(2-carboxycyclohexyl)acetyl-CoA (12a) or 2-(carboxymethyl)cyclohexane-1-carboxyl-CoA (12b) (both m/z = 936), from where a previously described downstream pathway starts.

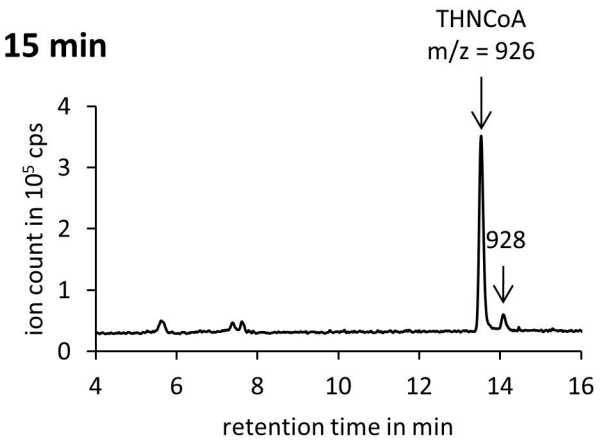
Figure 8 Potential intermediates (as free acids) of anaerobic napthalene degradation with m/z = 962 (as CoA thioester) correlating with the previously observed elemental composition $C_{11}H_{16}O_4$ (A and B) and their downstream metabolites (C). (A1): 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid. (A2): 3-(2-(carboxymethyl)cyclohexyl)acrylic acid. (B1): 3-hydroxy-1-oxodecahydro-2-naphthoic acid. (B2): 1-hydroxy-3-oxodecahydro-2-naphthoic acid. (C1): 4-(2-carboxycyclohexyl)-3-hydroxybutyric acid. (C2): 3-(2-(carboxymethyl)cyclohexyl)-3-hydroxypropionic acid.

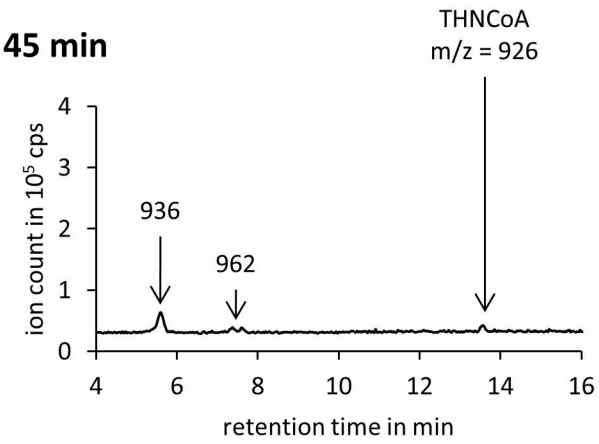
Figure 9 Synthesis of putative metabolite A1 (only one enantiomer shown; DIBAL-H = di-isobutylaluminium hydride; DCM = dichloromethane; THF = tetrahydrofuran; PCC = pyridinium chlorochromate).

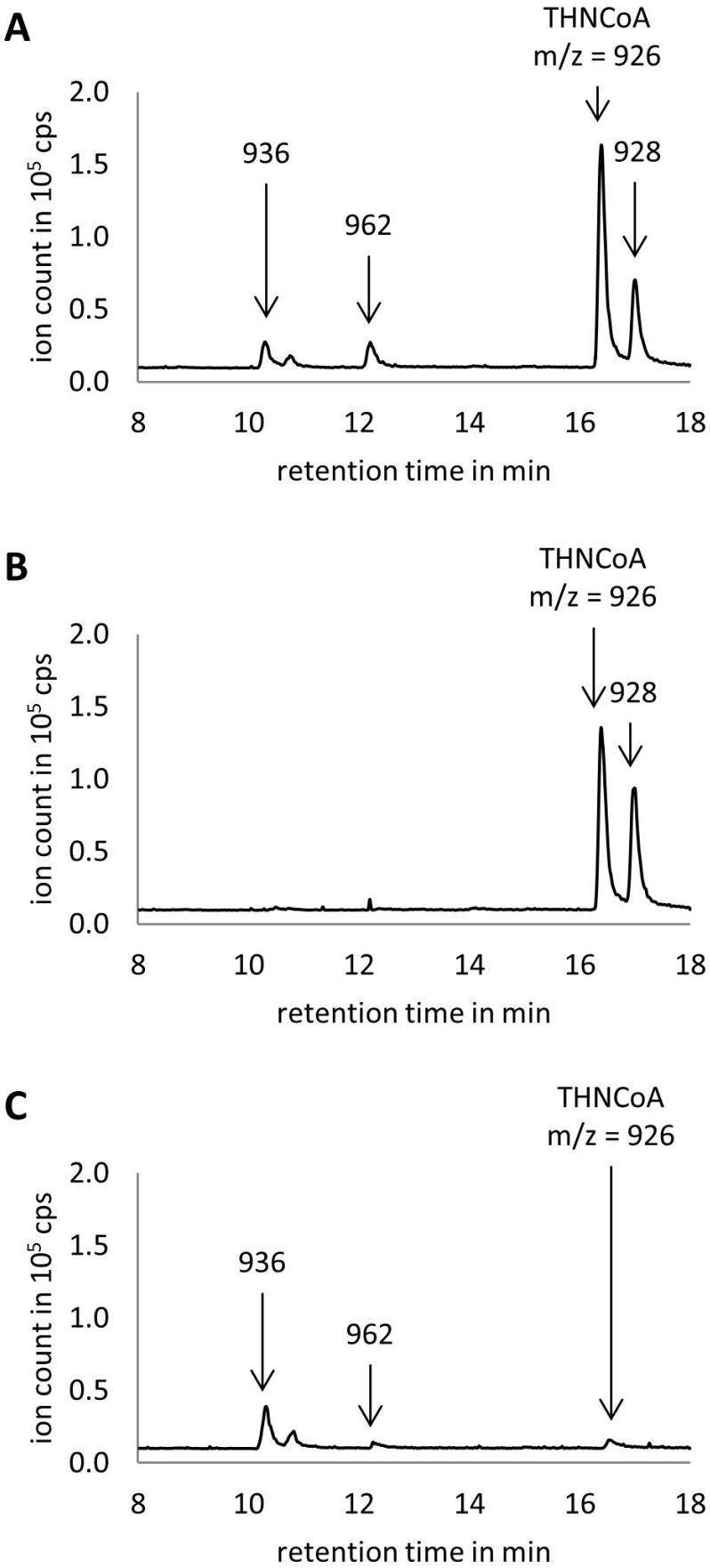
Figure 10 Synthesis of putative metabolite A2 (only one enantiomer shown; Tf = CF_3SO_2).

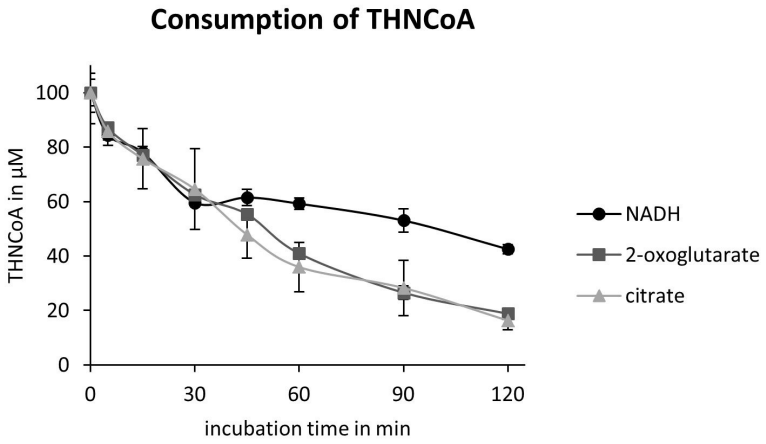


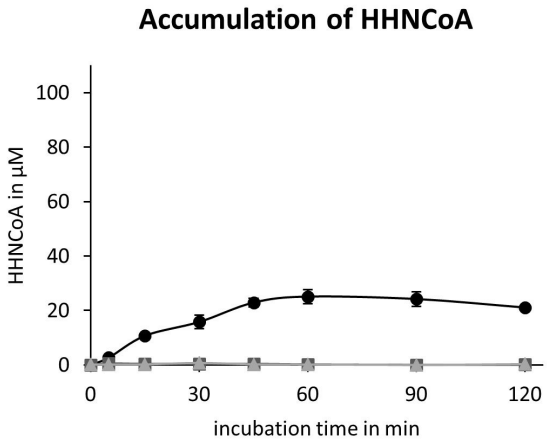












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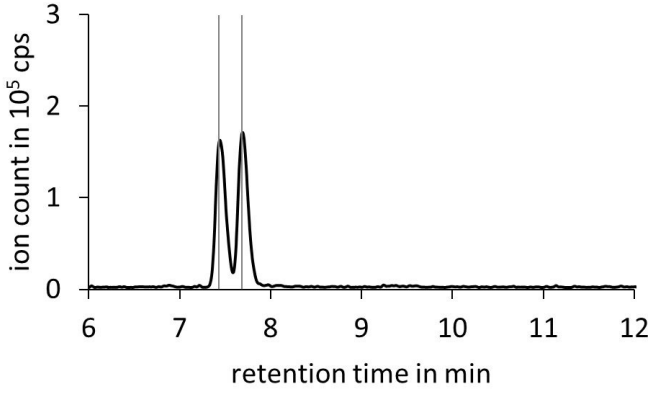
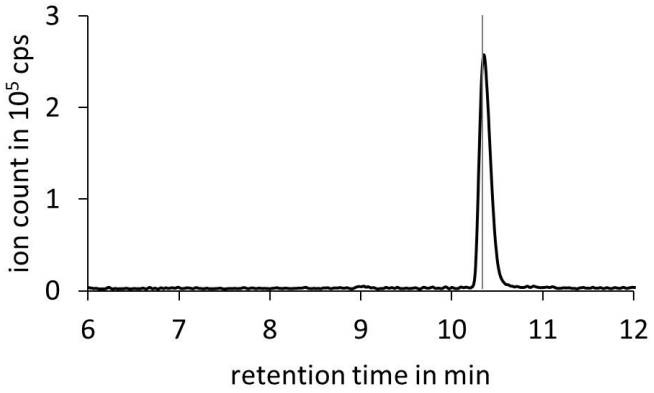
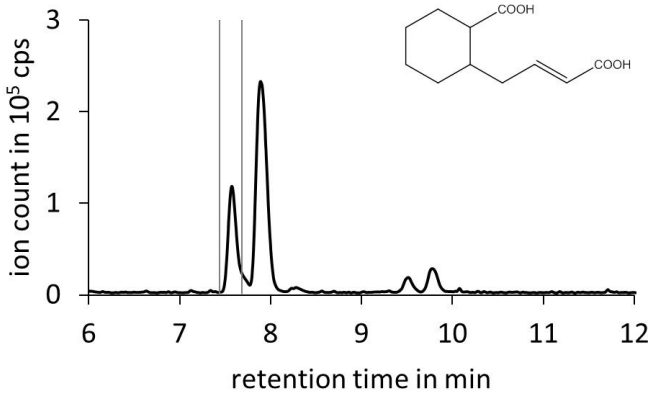


unknown metabolite, pH 5.5



CoA ester of compound A1, pH 7.0



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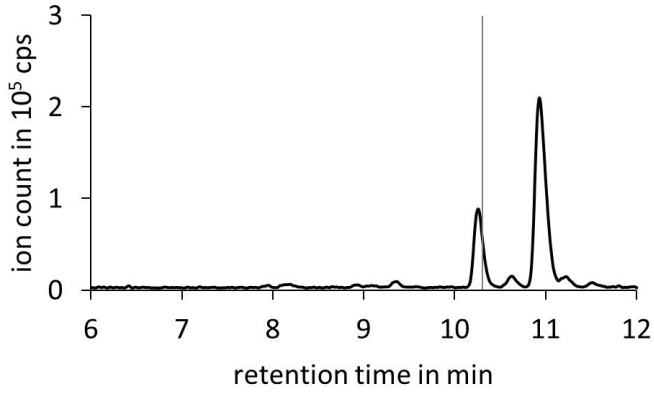

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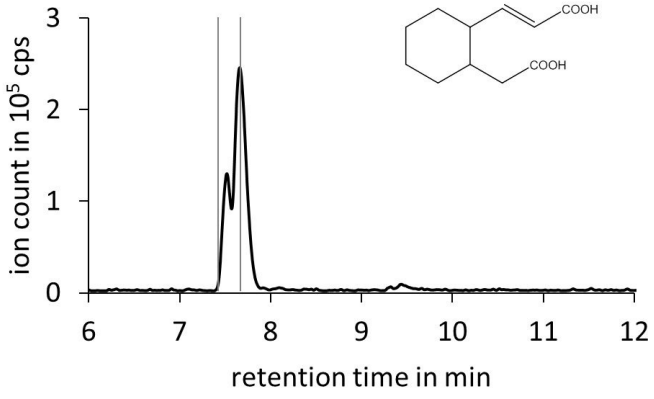
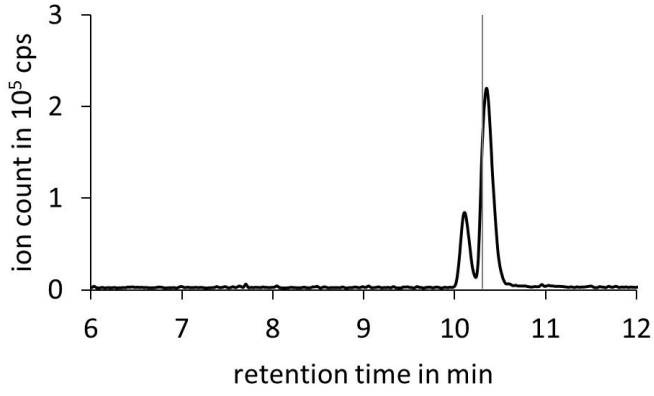
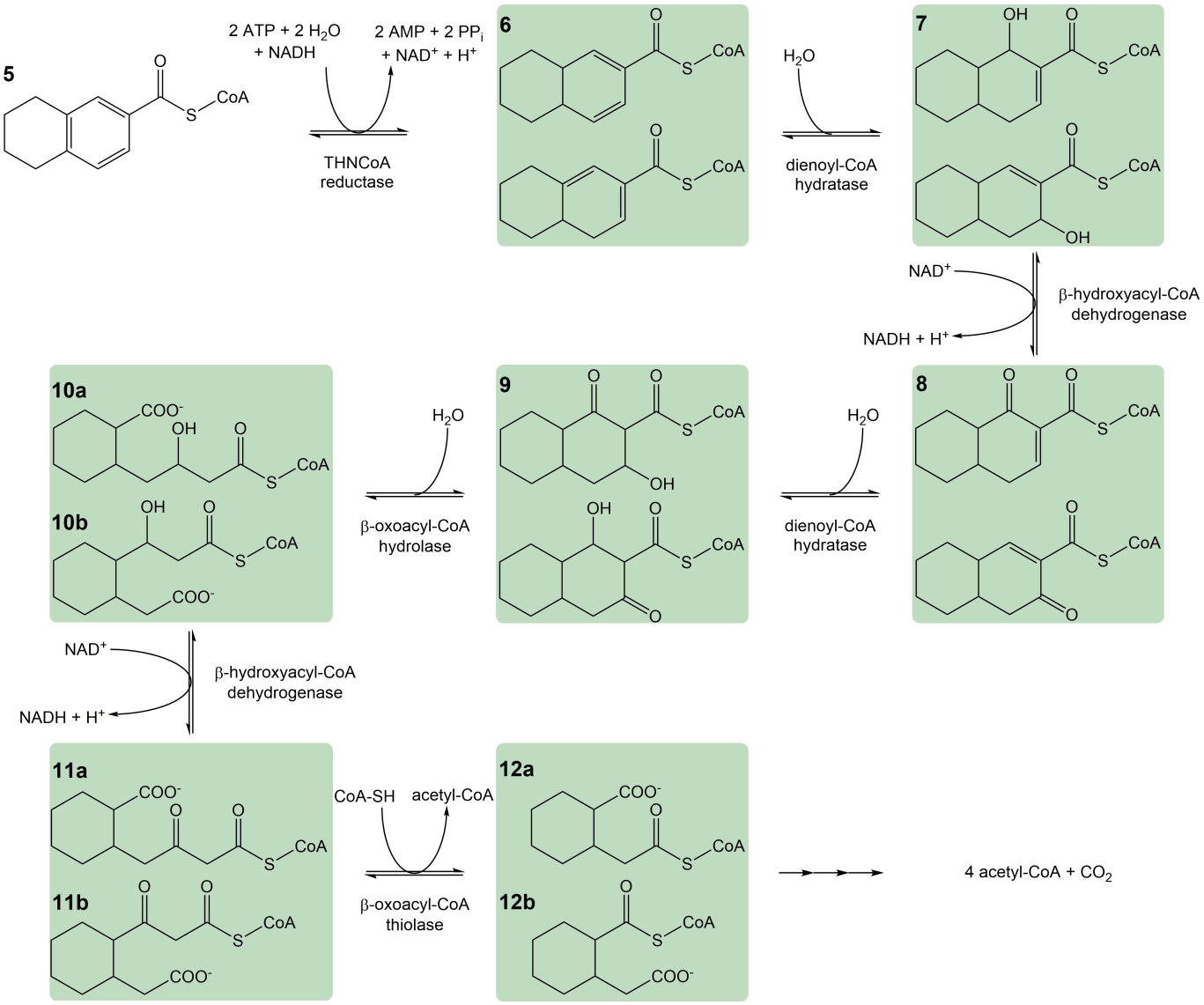
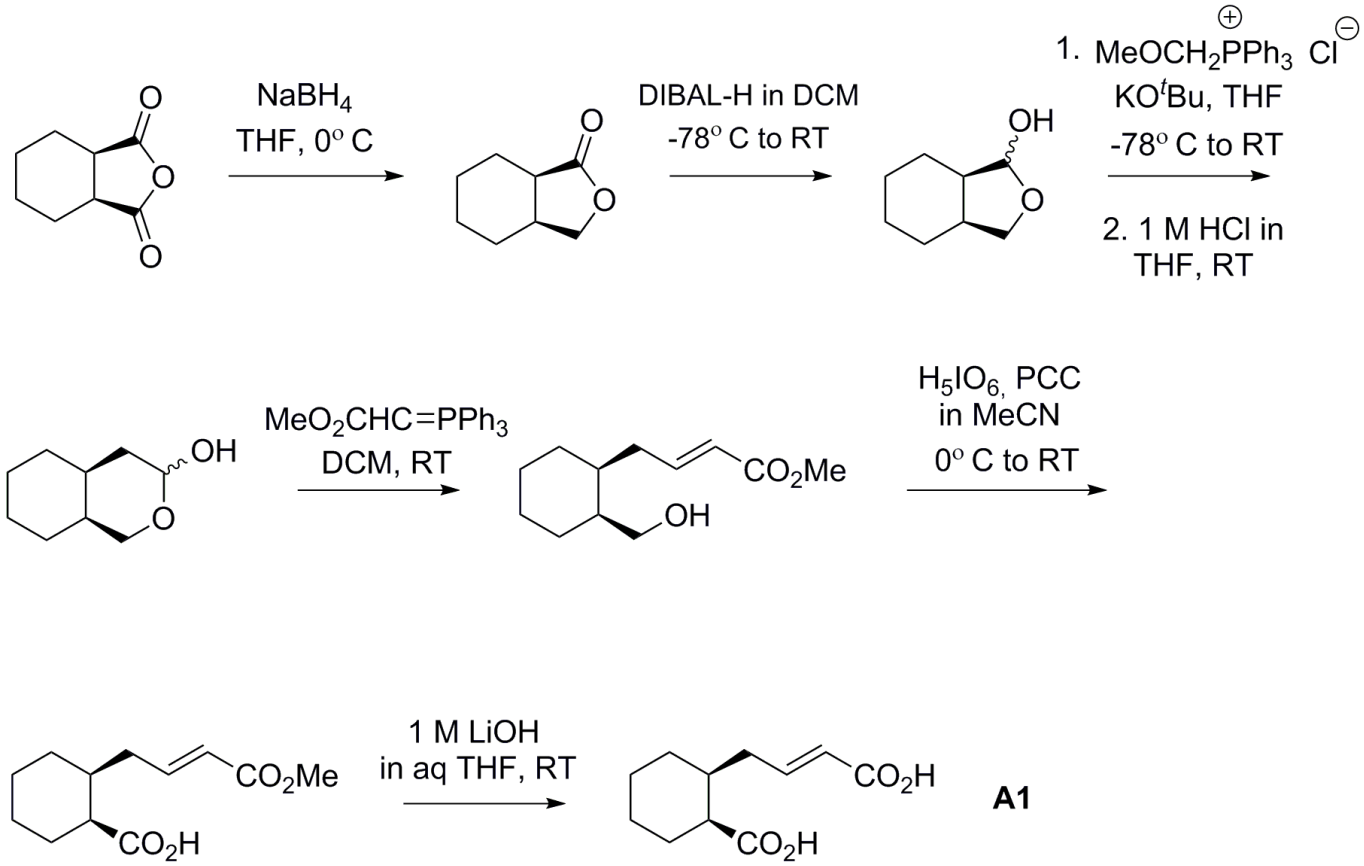


CoA ester of compound A2, pH 5.5







A2