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ORIGINAL PAPER

Aerobic biotransformation of decalin (decahydronaphthalene) by *Rhodococcus* spp.

Kathlyn M. Kirkwood · Paul Chernik · Julia M. Foght · Murray R. Gray

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Abstract Mixed bacterial cultures aerobically transformed decalin (decahydronaphthalene) dissolved in an immiscible carrier phase (heptamethylnonane; HMN) in liquid medium. Conversion was enhanced in the presence of decane, a readily degraded *n*-alkane, and/or HMN. Four *Rhodococcus* spp. isolates purified from one of the mixed cultures were active against decalin in the presence of *n*-decane, but their ability to use decalin as a sole carbon source for growth could not be sustained. Isolate Iso 1a oxidized decalin under co-metabolic conditions with decane vapours as the primary carbon source. Mass spectrometry and comparison to authentic standards showed that the oxidized products of decalin biotransformation were 2-decahydronaphthol and 2-decalone. Some evidence of ring-opening was obtained, but the possible ring-opened product was not definitively identified. These results are consistent with co-metabolic oxidation of decalin by enzymes active toward *n*-alkanes.

K. M. Kirkwood · P. Chernik · M. R. Gray (✉)
Department of Chemical and Materials Engineering, University of Alberta, Edmonton, AB, Canada T6G 2G6
e-mail: murray.gray@ualberta.ca

J. M. Foght
Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada

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Introduction

Crude petroleum contains a range of alkyl cycloalkanes, with one to five cyclic fused rings (Strausz and Lown 2003). Catalytic hydrogenation of crude oil fractions can increase the concentration of these components considerably, particularly in diesel fuel where hydrogenation of aromatics is used to increase the cetane index, a measure of fuel quality.

In general these cycloalkanes are more recalcitrant to aerobic biodegradation than linear alkanes (Prince et al. 2007). The monocyclic cyclohexane and alkyl cyclohexanes typically are degraded by co-metabolism with *n*-alkanes, by oxidation either of the cyclic ring or the attached side chain (Beam and Perry 1974a, b). The polycyclic hopanoids can be biodegraded slowly with other crude oil components (Frontera-Suau et al. 2002; Moldowan et al. 1995), but the specific pathways of biodegradation have not been studied. Partially hydrogenated aromatic compounds such as tetralin (1, 2, 3, 4-tetrahydronaphthalene) can undergo hydroxylation of the alicyclic ring (Schreiber and Winkler 1983) or oxidation of the aromatic ring via dioxygenase activity (Sikkema and de Bont 1993).

The dicycloalkanes in the diesel oil fraction have received much less attention. Decalin (decahydronaphthalene), like cyclohexane and tetralin, is both

volatile and toxic to cells (Sikkema et al. 1995). In mammals, decalin is oxidized to decahydronaphthol (Bernhard 1939; Dill et al. 2003a, b; Elliott et al. 1966; Olson et al. 1986) and decalone (Dill et al. 2003a, b; Olson et al. 1986). To our knowledge, only five reports have been published investigating microbial transformation of decalin and none on alkyl decalins. Only one of these (Cundell and Traxler 1974) reported growth on decalin as a primary carbon source: 14 out of 15 dodecane-degrading isolates (13 bacteria and 1 yeast) grew on basal medium plates with decalin. Pelz and Rehm (1971) did not identify any activity towards decalin in more than 50 bacterial strains. Soli and Bens (1973) found that decalin present in a mixture of 28 hydrocarbons was poorly degraded by four of 10 unidentified pure bacterial cultures (7–25% loss), and not by two mixtures of the isolates. Ko and Lebeault (1999) observed that decalin was consumed in the presence of hexadecane and pristane by a co-culture of *Pseudomonas aeruginosa* K1 and *Rhodococcus equi* P1; similarly, Vitale and Viale (1994) observed that decalin was consumed in the presence of decane by a co-culture of *Pseudomonas* sp. strains D1 and D2, as well as by strain D1 alone.

Bacterial activity towards dicycloalkanes, such as decalin, is important for two reasons: first as a possible initial step in biodegradation and eventual mineralization to bioremediate petroleum contamination; and second as a potential biocatalytic pathway to open saturated rings and improve fuel quality. In this communication we describe the isolation of a strain of *Rhodococcus* sp. capable of biotransforming decalin.

Materials and methods

Chemicals and microbiological media

Chemicals were purchased from Sigma-Aldrich (Milwaukee, WI), BDH (Toronto, ON), Caledon (Georgetown, ON), Fisher Scientific (Fairlawn, NJ), or Supelco (Bellefonte, PA) in the highest purity available. Plate Count Agar (PCA), R2A agar, Trypticase Soy Broth (TSB), and Agar Noble were Difco brand from BD (Franklin Lakes, NJ). Bushnell-Haas (BH) medium contained (g l^{-1}): $MgSO_4$ (0.2), $CaCl_2$ (0.02), KH_2PO_4 (1.0), K_2HPO_4 (1.0), NH_4NO_3 (1.0), $FeCl_3$ (0.05); pH = 7. To prepare BH plates, 15 g of Agar Noble was added before autoclaving. All media were prepared with doubly-distilled water.

Screening mixed cultures for activity against decalin

Mixed inocula were taken from eight liquid cultures enriched from soils and wastewaters from Canada, Antarctica and Germany that had been maintained by monthly transfers in mineral medium with crude oil fractions or petroleum products. Twenty millilitres of each enrichment culture was transferred into 200 ml of BH medium in screw-cap 500 ml Erlenmeyer flasks with PFTE-lined caps. 2, 2, 4, 4, 6, 8, 8-Heptamethylnonane (HMN) containing 5 wt% decalin with or without 5 wt% decane was filter-sterilized (Millipore Millex-FG; 0.22 μm) and 1 ml was added to each flask. The HMN served as a non-toxic, water-immiscible carrier, creating a two-phase liquid culture system to reduce toxicity and volatility of the decalin. Successive transfers were used to dilute crude oil components from the cultures. Further enrichment was carried out on the two most turbid cultures by successive transfer with addition of one of the following to 200 ml of BH medium: 100 μl of pure decalin; 100 μl of HMN; 1 ml of HMN containing 5 wt% decalin; 100 μl of decalin plus 100 μl of decane; or 1 ml of HMN with 5 wt% decalin and 5 wt% decane. Parallel abiotic (uninoculated) experiments were included for each condition. The cultures were incubated on a rotary shaker (200 rpm) for 21 days at 28°C, observed visually for relative growth, then extracted and analyzed for residual hydrocarbons by gas chromatography (GC) as described below. The most active culture was selected as a source of pure isolates.

Isolation of pure cultures

Vapour plates were used for strain isolation by streaking the most active mixed culture onto BH plates that were then placed in large plastic canisters along with beaker(s) containing glass wool soaked with several millilitres of decalin or decane individually, or in combination with 50 μl HMN spread on the plates prior to inoculation. The canisters were sealed and stored in separate locations at room temperature (approximately 20°C). Colonies arising on the plates were transferred to R2A agar to confirm their purity, and archived in glycerol at −80°C. Four

pure isolates were selected for partial (300–400 bp) 16S rRNA gene sequencing using the methods and primers described by Cheng and Foght (2007) and tentatively identified using BLAST searches of GenBank (Altschul et al. 1997) (searches performed January 8 2008, http://www.ncbi.nlm.nih.gov/BLAST). Identity was confirmed using conventional taxonomic tests including Gram stain, motility, cell morphology, catalase activity and optimum growth temperature.

Vapour plates (BH medium) were also used to test for growth of pure isolates on decane or decalin. Inoculated negative control plates were not exposed to any hydrocarbon or other carbon source and were stored at room temperature separately from the vapour plate containers. To check potential toxicity of the vapours, duplicate PCA and R2A plates were streaked with an isolate and stored in the vapour plate containers or without hydrocarbons at room temperature and the amount of colony growth was visually compared after 4 days incubation.

Metabolite analysis of pure cultures

To collect metabolites from pure cultures for qualitative analysis, cultures were grown in 250 ml side-arm flasks with PTFE-lined screw-caps prepared with 50 ml BH medium. The volatile substrates decane and decalin were filter-sterilized and added to the side-arm (500 µl each) to provide substrate in vapour phase only. To prepare replicate inocula for the flasks, pure isolates were grown in TSB for $\sim$24 h. The culture was harvested by centrifugation, washed three times and resuspended in a volume of 10 mM potassium phosphate buffer (pH 7.0) equal to the volume of the initial culture aliquot, and 250 µl of this suspension was used to inoculate each flask. The final suspension was streaked on R2A to check for purity. Flasks were incubated on a rotary shaker (160 rpm) at 28°C for 10 days.

Analytical methods

To quantify hydrocarbon losses from mixed cultures, 50 µl of a surrogate standard (dodecane) and 50 ml of pentane was added to the liquid culture. After mixing on a rotary shaker at 200 rpm for 10 min, a sample of the hydrocarbon phase was removed for analysis using a Hewlett Packard 5890 series gas chromatograph equipped with a 25-m HP-1 capillary column (Agilent Technologies, Wilmington, DE) and a flame ionization detector (GC–FID). The initial oven temperature of 50°C was increased at 6°C/min to a final temperature of 200°C. Peak areas of parent hydrocarbons were normalized to the surrogate standard to calculate total % loss, and peak areas for both *cis*- and *trans*-isomers of decalin were summed. This extraction method was not exhaustive, but rather was used to rapidly screen the mixed cultures to assess their relative activity.

For extraction of metabolites, the culture medium was acidified with 2 ml concentrated HCl to pH < 1. The cultures were extracted three times with 10 ml of ethyl acetate using a separatory funnel. An extra 10 ml of solvent was added to help break emulsions as necessary. The organic phase was dried by passage through anhydrous sodium sulfate into a round bottom flask. The extract was concentrated to near dryness at reduced pressure on a rotary evaporator, then transferred to a vial and dried completely under a nitrogen stream. The sample was then redissolved in 2 ml of solvent and split between two autosampler vials for analysis with and without derivatization using *N*,*O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) to generate trimethylsilyl (TMS) derivatives of alcohols and carboxylic acids. Extracts in ethyl acetate were dried under a nitrogen stream and suspended in 1 ml of dichloromethane before derivatization. Metabolites were identified by mass spectrometry (GC–MS) using an Agilent model 6890N gas chromatograph with a model 5973 mass selective detector following a 30-m HP-5MS capillary column (Agilent Technologies). The temperature program was 50°C for 1 min, then 10°C/min to 280°C final temperature. In preliminary tests the extraction and GC–MS protocols were verified using two model compounds (salicylic acid and 1, 2-cyclohexanedicarboxylic acid) similar to the expected metabolites of decalin, and found to be satisfactory.

Results and discussion

The toxicity of compounds like decalin necessitates a fine balance between providing sufficient substrate for growth in liquid culture and inhibiting the growth of cells. Incubating with vapour phase can help reduce the toxicity associated with direct contact of liquid decalin with the cells, but this cultivation

method makes it difficult to quantify substrate losses due to biodegradation. Therefore we used two different cultivation methods to reduce abiotic losses and toxicity yet provide sufficient substrate for biodegradation and metabolite analysis. For quantitative studies of parent compound losses, we used a two-liquid-phase culture system, and for qualitative metabolite analysis we provided vapour phase hydrocarbons to single-liquid-phase cultures. We selected the highly branched *iso*-alkane HMN as the non-toxic immiscible carrier phase because of its effectiveness as a carrier solvent for biotransformation of hydrophobic compounds (Efroymson and Alexander 1991; Kirkwood et al. 2007). Also, although Rontani and Giusti (1986) showed oxidation of HMN to a ketone by a mixed culture and evidence for further breakdown of the molecule, HMN is generally considered resistant to biodegradation (Schaeffer et al. 1979). Because we also anticipated the need for a co-metabolic substrate and/or inducer, we provided the readily degradable *n*-alkane decane to some cultures to assess its effect on decalin biodegradation. Therefore, cultures contained decalin, decane and HMN in various combinations, with parallel uninoculated flasks serving as controls for abiotic losses.

Eight mixed cultures enriched from a range of hydrocarbon-contaminated sites and wastewaters were tested for biodegradation of decalin provided in the carrier phase HMN, either with or without the co-metabolic substrate decane. After 21 day incubation, the disappearance of decalin ranged from 15% to 51% in HMN and 6% to 99% in HMN plus decane (not shown).

The culture with the most consistent activity toward decalin, enriched from a hydrocarbon-contaminated site in Antarctica (Lake Vanda former research station site) by transfer with the aliphatic fraction of a crude oil, was selected for further screening. The losses of pure decalin and pure HMN incubated with this mixed culture were small (<10%) and equivalent to abiotic control flasks, indicating that the individual compounds were not oxidized by the mixed culture. In the case of pure decalin, this could be attributed to toxicity and/or lack of co-metabolic substrate for enzyme induction, whereas the pure HMN was expected to be recalcitrant to biodegradation. To discriminate between these possibilities, parallel flasks of the Antarctic mixed culture were analyzed after incubation with three combinations of decalin in HMN carrier, with or without decane. (Results presented are average ± standard deviation, $n = 3$ unless otherwise specified.) In the presence of HMN, decalin degradation was 33.7 ± 3.5 % ($n = 5$). This increase compared to degradation of pure decalin is possibly due to reduced toxicity afforded by the carrier phase and/or a correspondingly higher bioavailability through contact of the culture with the liquid (rather than vapour) carrier phase. The presence of decane also increased decalin loss, presumably due to co-metabolism, although only two of three cultures grew with 80.7% and 85.1% loss of decalin. Decalin loss was 88.3 ± 6.3% in cultures with HMN plus decane. No difference was observed in the extent of degradation of the *cis*- and *trans*-decalin isomers in any cultures. In contrast, the co-culture of *Pseudomonas* sp. strains D1 and D2 degraded the *trans*-isomer more rapidly than the *cis*-isomer (Vitale and Viale 1994). The co-metabolic substrate decane was consumed in the presence of decalin, both in the absence (93.2% and 97.1% loss in the two cultures that grew) and presence (95.7 ± 0.2% loss) of HMN. Average abiotic losses were 0.2 ± 3.6% ($n = 9$) for decalin and 16.4 ± 3.1% ($n = 6$) for decane in these experiments.

Interestingly, although HMN is usually considered to be recalcitrant, significant disappearance from the mixed culture was measured when decalin was present (38.3 ± 3.9% loss, $n = 5$) and when decalin and decane were both present (83.9 ± 3.7% loss). This suggests that co-metabolic oxidation of HMN may be occurring in the presence of the other hydrocarbons, particularly in the presence of decane which stimulated significant culture growth. However, the single-step extraction protocol used was not exhaustive and was selected to allow rapid screening for loss of hydrocarbons rather than to extract acidic or polar metabolites. The contribution of non-metabolic mechanisms (for example, adsorption to the biomass) to the apparent loss of HMN cannot therefore be estimated, nor can loss of HMN be correlated to appearance of metabolites. Because the focus of this study was on decalin, the observation of HMN disappearance in the mixed culture was not further investigated, but is an interesting avenue for future work.

Four strains of bacteria were isolated from the Antarctic mixed culture using BH plates incubated

with decalin vapour, designated Iso 1a, Iso 2aCl, Iso 2cO and Iso 2aCr. All four grew well on decane vapour plates and were identified as *Rhodococcus* spp. using conventional biochemical tests and by partial (~300–650 bp) 16S rRNA gene sequencing. The first three sequences showed high (≥98%) similarity to *Rhodococcus* sp. GW-2006 (GenBank accession number DQ453477), *Rhodococcus* sp. ON2 (GenBank accession number EU310929), and numerous other *Rhodococcus* isolates. Strain Iso 2aCr had the best match with *Rhodococcus* spp. but at lower similarity (95%) because of short sequence length for analysis. Although these strains gave encouraging initial results by appearing to grow on vapour plates incubated with pure decalin and in BH medium with decalin in HMN, the same activity was not observed with cultures prepared from cryopreserved glycerol stocks. Instead, the resuscitated isolates showed marginal growth on decalin vapour plates, equivalent to that on substrate-free control plates, and in liquid medium showed growth (increased turbidity) and loss of decalin only in the presence of decane. Attempts to induce the strains by incubation with decane prior to exposure to decalin were not successful. Toxicity was not considered to be a factor in these tests because inoculated R2A and PCA plates incubated with decalin vapours showed growth comparable to duplicate plates incubated without exposure to decalin vapours. Consequently, we concluded that decalin was co-metabolized when the strains were grown with decane as a carbon source, thus resulting in loss of the parent compound in the liquid cultures as determined by GC (not shown), but the ability of any of the pure strains to grow on decalin as a sole carbon source has not been demonstrated repeatably. Similar inconsistent activity was reported for bioconversion and degradation of cyclohexane by Cheng et al. (2002).

Because decalin was degraded, but did not serve as a primary carbon source, we conducted experiments to identify the partial oxidation products of decalin biotransformation. Based on the published pathways for cyclohexane (Brzostowicz et al. 2005), an analogous pathway for decalin biotransformation would include a cyclic alcohol, a cyclic ketone and ring-opened metabolites having either an alcohol plus a carboxylic acid group, or two carboxylic acids. Experiments were performed in liquid culture to detect and identify metabolites from the biotransformation of decalin by the most active isolate, *Rhodococcus* sp. Iso 1a, either as sole carbon source or under co-metabolic conditions with decane as the primary substrate. The following substrate combinations were provided solely as vapour phase: decalin alone, decane alone, or decalin plus decane vapours. No growth of *Rhodococcus* sp. Iso 1a was observed when decalin was the sole carbon source and no new peaks were observed by GC–MS analysis with or without derivatization. In contrast, good growth was observed in the cultures incubated with decane alone or with decane plus decalin, and several new peaks were present in the GC chromatograms of these extracts. At least four peaks were present in the decalin plus decane culture extract that were not in the decane alone extract, indicating that they were possible metabolites of decalin (Fig. 1, peaks A–D).

Peak A, at 12.19 min, was present in both the derivatized and underivatized samples, and the mass spectrum (Fig. 2) was an excellent match for both library spectra and the authentic standards of 1- and 2-decalone. Based on the relative abundances of fragments at *m/z* 95 and 97 and on comparison of the retention time to the authentic standards, this compound was identified as 2-decalone.

Peaks B and C, at 12.83 and 13.08 min respectively, were detected only in the derivatized sample, consistent with either an alcohol or a carboxylic acid. The mass spectra for both peaks (Fig. 3) matched the TMS derivatives of authentic *cis*-1-, *cis*-2- and *trans*-2-decahydronaphthol (Fig. 4), with a better match to the *cis*- and *trans*-isomers of 2-decahydronaphthol due to the relative abundances of the fragments at *m/z* 136, 183, and 211. The retention times also matched those of the TMS derivatives of authentic *cis*- and *trans*-2-decahydronaphthol, but since the authentic standards were a mixture of the *cis*- and *trans*-isomers, the specific identities of each could not be determined. Thus, whereas growth of *Pseudomonas stutzeri* AS39 on tetralin yielded 1-tetralol and 1-tetralone, presumably by arylhydrocarbon oxygenase attack on the alicyclic ring (Schreiber and Winkler 1983), no evidence was found in the current study for oxidation of decalin at the 1-position.

The presence of alcohol and ketone oxidation products is consistent with the known mechanisms for oxidation of cycloalkanes such as cyclohexane via monooxygenases (Brzostowicz et al. 2005). Metabolites were only found in the culture with decalin plus decane, and not in the culture with decalin alone,

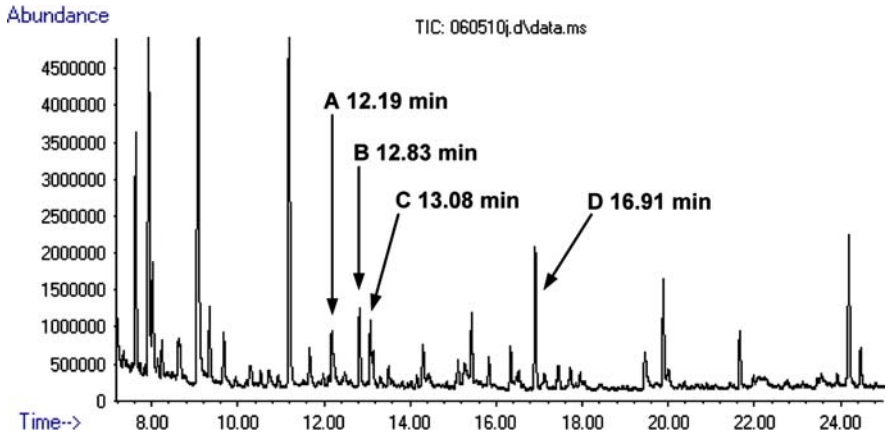

Fig. 1 Total ion chromatogram from GC–MS analysis of the BSTFA-derivatized ethyl acetate extract from *Rhodococcus* sp. Iso 1a grown with decalin plus decane. The labelled peaks, absent from cultures incubated with decane only, are discussed in the text, and the corresponding mass spectra are shown in Figs. 2, 3 and 5

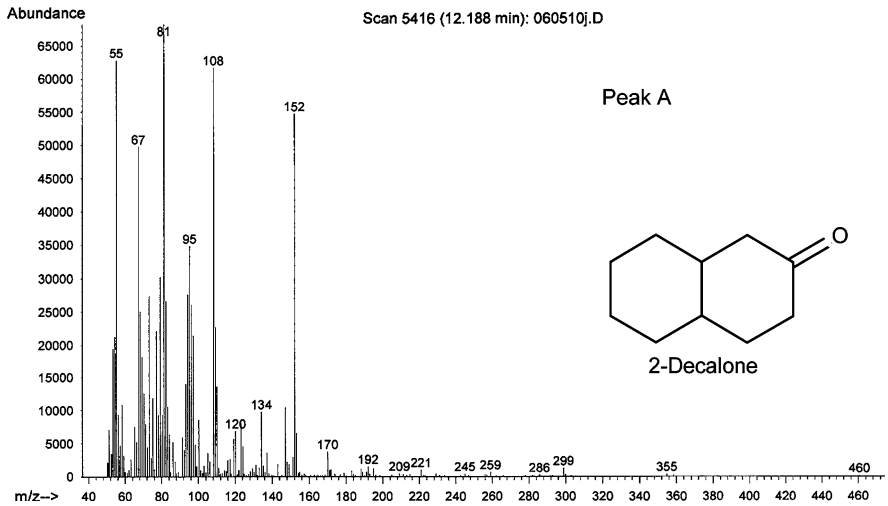

Fig. 2 Mass spectrum for metabolite peak A (retention time of 12.19 min in Fig. 1), proposed to be 2-decalone (structure shown)

consistent with non-specific oxidations catalysed by enzymes expressed during growth on decane. Furthermore, *Rhodococcus* sp. Iso 1a was unable to grow on any of the authentic standards of decalone or decahydronaphthol provided as sole carbon source in BH liquid medium (not shown). The ability of strain Iso 1a to oxidize decalin may account for the significant loss of this compound observed in the initial enrichment cultures, giving dead-end metabolites that did not support growth of strain Iso 1a but could be degraded by other organisms in mixed cultures.

Peak D, at 16.91 min, was also present only in the derivatized sample. The mass spectrum showed fragmentation consistent with double TMS derivatives, and indicated a molecular weight of 330 (Fig. 5), consistent with a ring-opened product with both an alcohol and a carboxylic acid group. The structure suggested in Fig. 5 would arise from ring-opening of a lactone yielding alcohol and carboxylic acid groups, with no further oxidation reactions. No evidence of dicarboxylic acids was found (i.e., no peaks unique to decalin or peaks with mass spectra containing the expected *m/z* values for potential dicarboxylic acids) although the experimental protocol was demonstrated to be effective for such compounds using analogous compounds. We cannot rule out the possible use of the compound represented by Peak D as a carbon source by strain Iso 1a, although its accumulation in the medium relative to the other metabolites (Fig. 1) argues strongly against its efficient use by the isolate.

The results of this study suggest that cycloalkanes with two or more rings can be transformed by the same mechanism as cyclohexanes; that is,

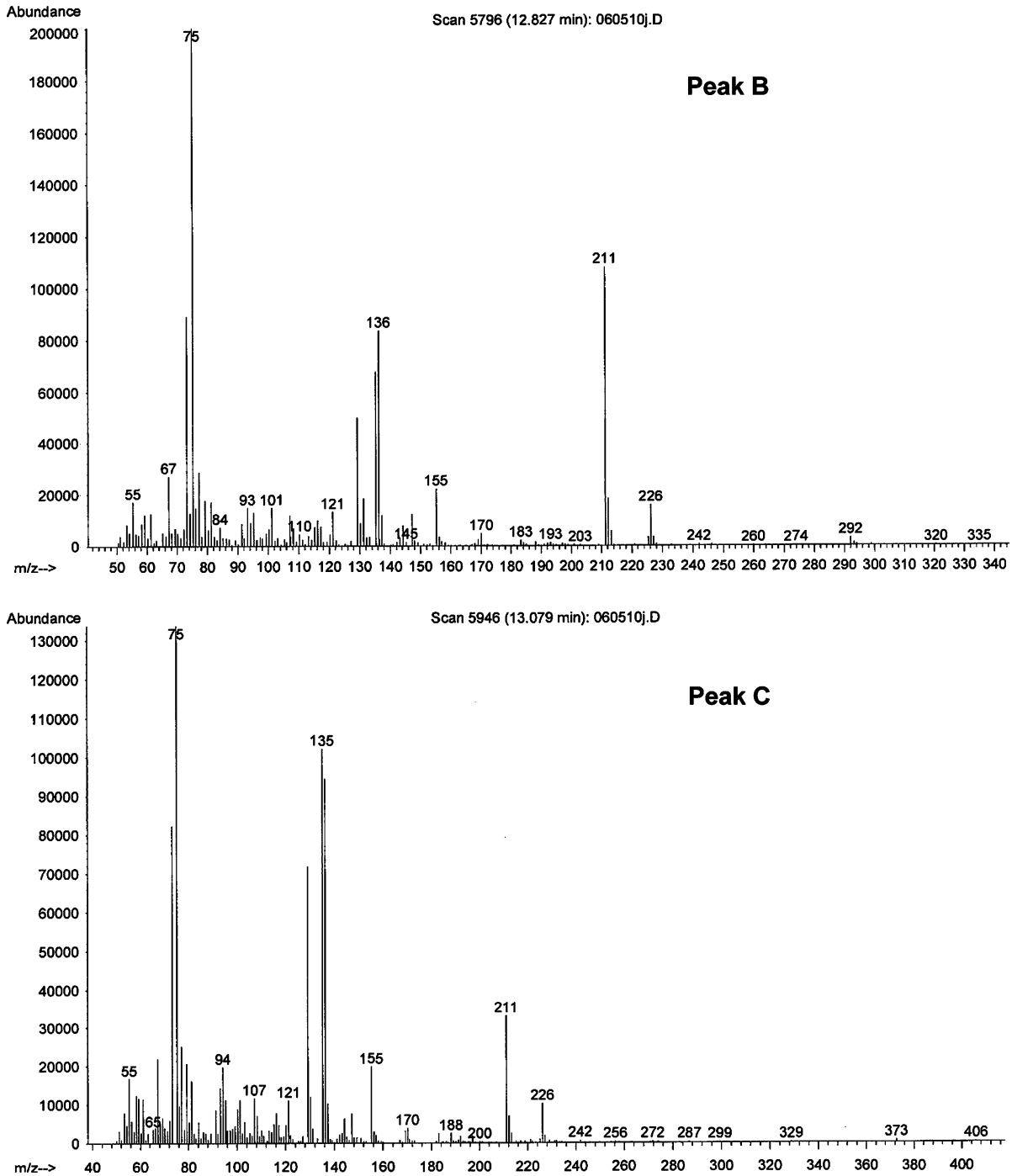

Fig. 3 Mass spectra for metabolite peaks B and C (retention times of 12.83 and 13.08 min, respectively, in Fig. 1), proposed to be the TMS derivatives of either *cis*- or *trans*-2-decahydronaphthol (structures and spectra of authentic standards shown in Fig. 4)

non-selective oxidation presumably achieved by enzymes induced by and active against *n*-alkanes, to yield alcohols, ketones, and potential ring-opened products. This oxidation appears to require the presence of suitable co-metabolic substrates or inducers in both pure culture and mixed cultures.

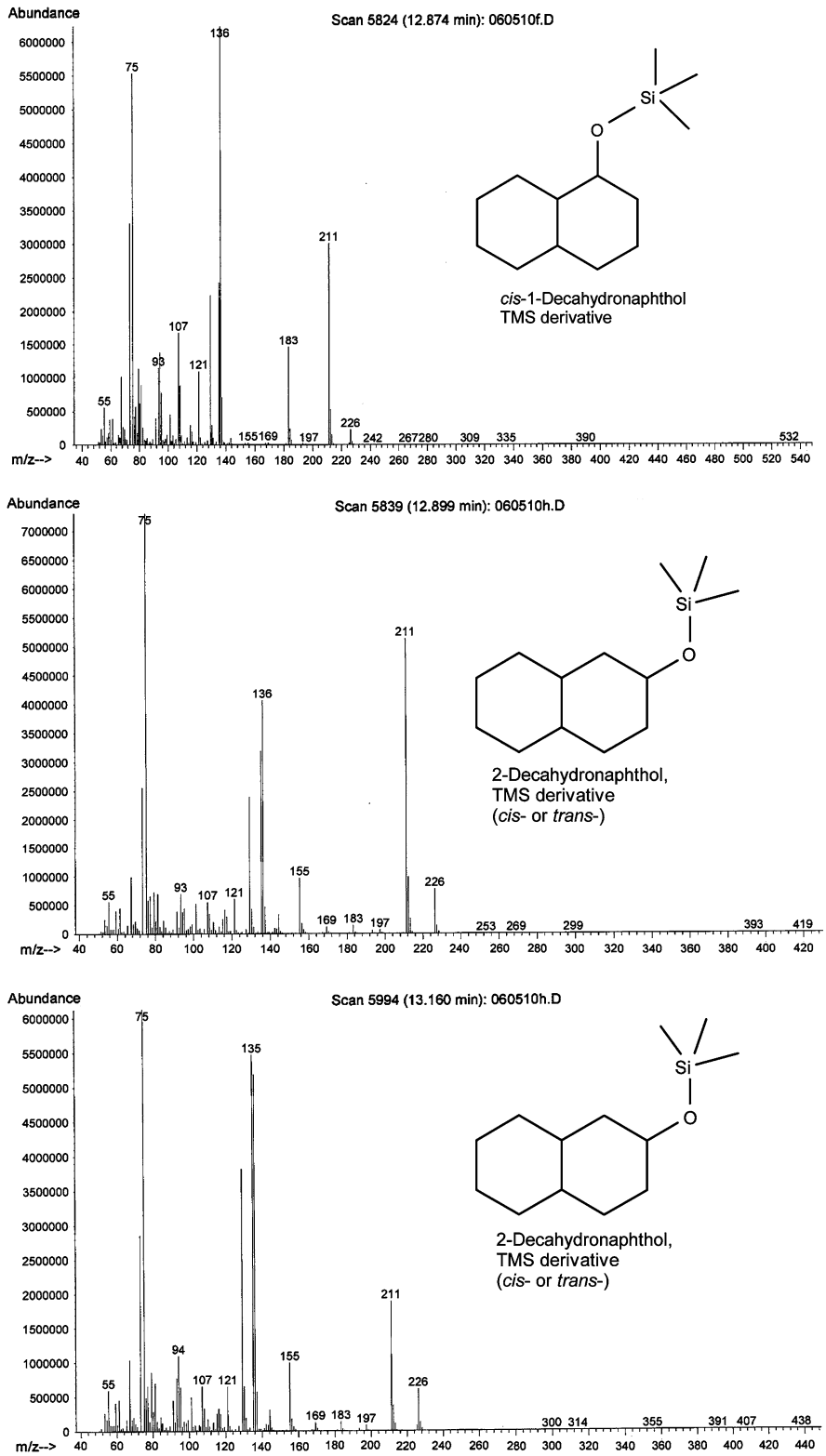

Fig. 4 Mass spectra for the TMS derivatives of *cis*-1-decahydronaphthol and the *cis*- and *trans*-isomers of 2-decahydronaphthol (specific identity of each isomer is unknown)

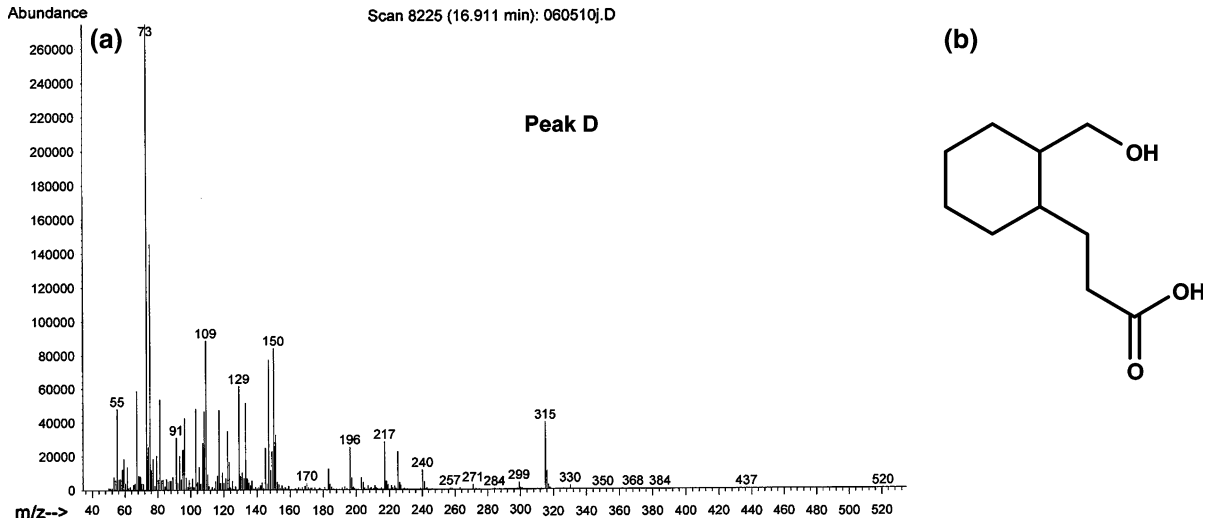

Fig. 5 (**a**) Mass spectrum for metabolite peak D (retention time of 16.91 min in Fig. 1). (**b**) An example of a ring-opened product that would yield a double TMS derivative with a molecular weight of 330 and *m*-15 peak of 315

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