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Biotransformation of aliphatic and aromatic ketones, including several monoterpenoid ketones and their derivatives by five species of marine microalgae

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Abstract

The biotransformation of a series of aliphatic and aromatic ketones by five cultures of photosynthetic microalgae is reported. The test substrates include the monoterpenoid ketones carvone and menthone and a series of aromatic ketones related to and including acetophenone. All of the test organisms show some degree of specificity and stereoselectivity in the biotransformation of substrates.

1. Introduction

Microalgae are photoautotrophic and therefore do not require organic substrates for energy. Consequently the large scale culture of microalgae is theoretically simpler and cheaper than that of bacteria, yeasts or fungi. This is an important advantage when considering the potential of micro-algae in the biotransformation of readily available chemicals eg. terpenoids, into compounds of greater potential value to the food and perfume industries (Borowitzka, 1988; van der Werf et al., 1997; Berger et al., 1999). As part of an ongoing biotechnological study into the biotransformation and metabolism of organic substrates by marine microalgae we have recently shown that a range of aromatic aldehydes are biotransformed to their corresponding primary alcohols (Hook et al., 1999). In continuation of this study we now report on an investigation into the ability of these same microalgae (Table 1) to biotransform substrates including cyclohexanone (**1**), cyclohexenone (**2**), the monoterpenes, *4S*-(+)- and *4R*-(−)-carvone (**3**, **4**), *2RS,4R*-(−)-*trans* and *2RS,4R*-(−)-*cis* carvyl acetate (**11**, **12**), *2RS,4R*-(−)-*trans* and *2RS,4R*-(−)-*cis* carvyl propionate (**13**, **14**), 2*RS,4R*-(−)-*trans* and *2RS,4R*-(−)-*cis* carveol (**15**, **16**), *1S,4R*-(−)-menthone (**17**) and (±)-menthyl acetate (**8**). The biotransformation of a number of organic ketones (**19**–**23**) including and related to acetophenone was also investigated. The findings of this study are similar to those reported for *Euglena* (Noma et al., 1991; Shimoda et al., 1998; Noma and Asakawa, 1998), *Dunaliella parva* (Noma and Asakawa, 1992, 1994) and the non chlorophyllous cell line *Astasia longa* (Shimoda and Hirata, 2000). The trend of the biotransformation of these substrates is now firmly established.

2. Results and discussion

The optimal growth patterns for the five micro-algae were established previously (Hook et al., 1999). Due to their growth requirements biotransformations catalysed by *Chlorella minutissima*, *Nannochloris atomus*, *D. parva* and *Porphyridium purpureum* were examined under shake conditions while those of *Isochrysis galbana* were carried out under static conditions.

Detailed toxicity studies to determine the correlation between test volume and the observed toxicity of substrate and solvent control were carried out using a selection of substrates. For all microalgae substrate toxicity increased with time and after 5 days exposure all cultures showed a marked decrease in cell viability. When incubation volumes of 100 ml were used the toxicity of the solvent control (MeOH) toward the cell cultures was not as great as when 10 ml volumes were

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used, although substrate toxicity was still a factor, in particular at concentrations >200 ppm. Cultures of *D. parva* and *P. purpureum* and *I. galbana* were most susceptible. Therefore in the biotransformation study substrate incubations were carried out at concentrations of 100 ppm in conical flasks containing 100 ml medium.

In an initial biotransformation experiment cyclohexanone (**1**) was incubated with the test cultures. In all cases a single reduction product, cyclohexanol, was recovered although the different microalgae reduced the substrate to different degrees (Table 2). After 5 days *P. purpureum* and *N. atomus* had only reduced circa 50 and 60% of substrate respectively while *C. minutissima* and *D. parva* reduced greater than 70%. *I. galbana* was the most efficient organism reducing some 90% of substrate within the 5 day period. When cyclohexenone (**2**) was incubated with the test microalgae no substrate biotransformation was catalysed by any of the cultures.

The biotransformation of the α,β-unsaturated ketone *4S*-(+)-carvone (**3**) was also investigated. Incubation of (**3**) with the five test microalgae was investigated over a period of 5 days. In all cases the test organisms reduced **3** to yield the same four products, *1S,4S*-(−)-dihydrocarvone (**4**), *1S,2R,4S*-(−)-neodihydrocarveol (**5**), *1R,4S*-(+)-isodihydrocarvone (**6**) and *1R,2R,4S*-(−)isodihydrocarveol (**7**) (Table 3, Fig. 1). *P. purpureum* was the most effective microalga for the biotransformation of **3** and *I. galbana* was the least effective. The stereospecificity of the reduction of **3** is in keeping with the results observed for *Euglena* (Noma et al., 1991; Shimoda et al., 1998; Noma and Asakawa, 1998) but differs from *D. tertiolecta* which does not produce **5** and **6** in its metabolism of **3**, but does produce *1R,2S,4S*-(+)-neoisodihydrocarveol (Noma and Asakawa, 1992, 1994). These results also differ from those observed for *A. longa* where the stereospecificity in the reduction of the C=C bond is quite high and only **4** and **5** are produced (Shimoda and Hirata, 2000). No products derived from the reduction of the methylethenyl group were observed for any test organism.

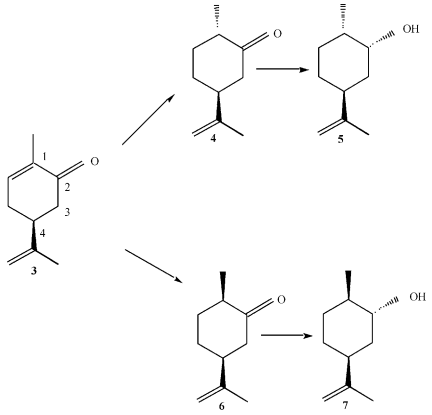

Fig. 1. Biotransformation products of **3**.

4R-(−)-Carvone (**8**) was transformed to a far lesser extent by the test organisms. For all algal strains the metabolites recovered were *1R,4R*-(+)-dihydrocarvone (**9**) and *1R,2S,4R*- (+)-neodihydrocarveol (**10**) (Table 4, Fig. 2). These results are in keeping with the results observed for *D. tertiolecta* (Noma and Asakawa, 1994) and *A. longa* (Shimoda and Hirata, 2000). No 8-hydroxy derivatives were found in any strain although such products have been reported in the biotransformation of **3** by *Euglena*. (Noma and Asakawa, 1994). As in the case of 3 the alga that gives the most efficient biotransformation of *4R*-carvone is *P. purpureum*, *C. minutissima* (2%) was the least effective at reducing the substrate. When the biotransformation of *4S*-(+)-carvone (**3**) and *5R*-(−)-carvone (**8**) are compared greater biotransformation of the *S*-substrate occurs in all algal strains.

In subsequent experiments it was established that *1RS,4R*-(−)-*cis* and (−)-*trans* carvyl acetates (**11**, **12**)

Table 1
Microalgae used in biotransformation study

Organism	Family	Description of alga	References
Chlorella minutissima	Chlorophyta	Non-motile, green	Bold and Wynne, 1978
Nannochloris atomus	Chlorophyta	Non-motile, green	Bold and Wynne, 1978; Lee, 1989
Dunaliella parva	Chlorophyta	Motile, green,	Bold and Wynne, 1978; Lee, 1989
Porphyridium purpureum	Rhodophyta	Non-motile, red	Bold and Wynne, 1978; Lee, 1989
Isochrysis galbana	Chrysophyta	Motile, red	Bold and Wynne, 1978; Lee, 1989

Table 2
Biotransformation (%) of substrates **1**, **2**, **17** and **18** by test microalgae after a 5 day incubation period

Organism/(%) compound biotransformed	*C. minutissima*	*N. atomus*	*D. parva*	*P. purpureum*	*I. galbana*
Cyclohexanone (**1**)	28	37	26	48	10
Cyclohexenone (**2**)	0	0	0	0	0
Menthone (**17**)	18	29	31	14	37
Menthyl acetate (**18**)	100	100	100	100	100

(mixture 37% *cis* and 63% *trans*) and *1RS,4R*-(−)-*cis* and (−)-*trans* carvyl propionates (**13**, **14**) (mixture 62% *cis* and 38% *trans*) were totally hydrolysed to yield the *1RS,4R*-(−)- *trans* and (−)-*cis* carveols (**15**, **16**).

Diastereoselective oxidation of *1RS,4R*-(−)-*trans* carveol (**15**) to 4*R*-(−)-carvone (**8**) was then demonstrated by all algal species (Table 5, Fig. 2) with *1RS,4R*-(−)-*cis* carveol (**16**) not being reduced to any significant extent (Table 5). These results are in keeping with those observed for *Euglena* (Noma and Asakawa, 1994).

Thus it was established that in the biotransformation of *4S*-(+)- and *4R*-(−)-carvone (**3**, **8**) the initial reduction is that of the α,β-unsaturated double bond followed by that of the carbonyl group itself leaving the exocyclic C=C bond of the 1-methylethenyl group unaffected. It is also interesting to note that similar substrate selectivity is demonstrated by all of the test algae with *4S*-(+)-carvone (**3**) undergoing the greatest degree of reduction by all strains but with lower stereospecificity than that demonstrated by some other microalgae (Noma and Asakawa, 1992; Noma and Asakawa, 1994; Shimoda and Hirata, 2000). All of the test algae show diastereoselectivity in the biotransformation of (−)-*trans* and (−)-*cis* carveol (**15**, **16**) with only *(−)-trans* carveol being converted to *R*-(−)-carvone (**8**) thus leading to isomeric resolution of the mixture. No selectivity was observed in the hydrolysis of (−)-*trans* and (−)-*cis* carvyl acetate (**11**, **12**) and carvyl propionate (**13**, **14**) derivatives to their corresponding alcohols. These results are similar to those observed for the biotransformation of carvone related monoterpenes in the algae *Euglena* (Noma and Asakawa, 1994), *D. tertiolecta* (Noma and Asakawa, 1994), *A. longa* (Shimoda and Hirata, 2000) and the plant and cell cultures *Nicotiana* (Hirata et al., 1986).

Table 3
Biotransformation (%) of **3** by test microalgae and the products formed after a 5 day incubation period

Organism		*C. min.*	*N. atom*	*D. parva.*	*P. purp.*	*I. galbana*
Substrate	4S-(+)-Carvone (**3**)	85	63	74	35	93
Products	1S,4S -(−)-Dihydrocarvone (**4**)	Trace	Trace	Trace	Trace	Trace
	1S,2R,4S -(−)-Neodihydrocarveol (**5**)	1	3	1	9	1
	1R 4S -(+)-Isodihydrocarvone (**6**)	14	30	22	53	6
	1R,2R,4S-(−)-Isodihydrocarveol (**7**)	Trace	4	3	3	Trace

Table 4
Biotransformation (%) of **8** by test microalgae and the products formed after a 5 day incubation period

Organism		*C. min.*	*N. atom*	*D. parva.*	*P. purp.*	*I. galbana*
Substrate	*4R*-(−)-Carvone (**8**)	98	96	97	92	96
Products	*1R,4R*-(+)-Dihydrocarvone (**9**)	0	1	1	5	1
	1R,2S,4R-(−)-Neodihydrocarveol (**10**)	2	3	2	3	3

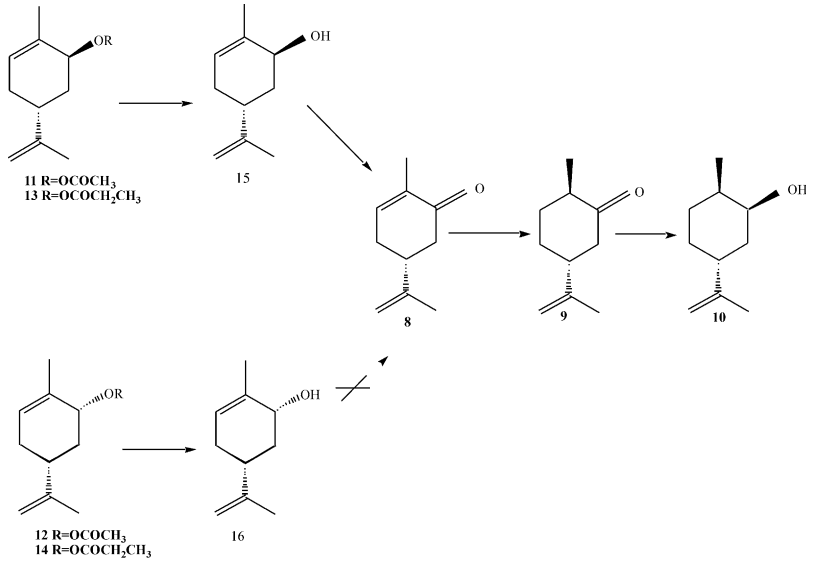

Fig. 2. Biotransformation products of **8** and **11**–**16**.

It is interesting to note that limited non-selective reduction of the unconjugated carbonyl group of *1S, 4R*-(−)-menthone (**17**) occurred in all cultures over a 5 day period yielding *1RS, 2S,4R*-(−)-menthol (**18**) as the sole product (Table 2). The most efficient reduction was catalysed by *I. galbana*, *P. purpureum* was the least effective. The non-selective biotransformation of the diastereomeric mixture (±)-menthyl acetate (**19**) to (±)-menthol was completed by *C. minutissima*, *N. atomus* and *P. purpureum* within a 24 h period. Cultures of *I. galbana* effected its biotransformation by the third day while *D. parva* took 4 days to completely biotransform the substrate. These results are in keeping with those observed for the biotransformation of (±)-menthyl acetate by the duckweed *Spirodela oligorrhiza* (Pawlowicz and Siewinski, 1987). However for this species reduction to menthol was selective producing only (−)-menthol within 14 days. This is in contrast with the failure of *Euglena* to metabolise this substrate to any degree (Noma and Asakawa, 1994).

The reduction of a series of aromatic ketones related to acetophenone (**20**) was also investigated. Acetophenone (**20**) was reduced to the corresponding alcohol by all of the test cultures within the five day period with *P. purpureum* and *N. atomus* being the most effective and *D. parva* being the least effective (Table 6). In the case of *P. purpureum* the biotransformation was 90% completed after 2 days. *N. atomus*, *C. minutissima* and *I. galbana* had undergone 50% reduction of substrate after 4 days. For *D. parva* a maximum reduction of only 21% was obtained after 5 days however, this organism exhibited the greatest stereoselectivity in the reduction of the substrate with an enantiomeric excess of 29.5% of the the *S*-(−)-isomer being produced. *C. minutissima* also demonstrated stereoselectivity in the reduction of this substrate but with maximum excess of 14.8% of the *S*-(−)- isomer being produced. The enantiomeric excess of the product produced by the other algal species was <1%. The 4-chloro and 4-methyl derivatives of acetophenone (**21**, **22**) were also reduced to the corresponding alcohols with **21** being reduced most effectively by *I. galbana*. 4-Methyl acetophenone **22** was most effectively reduced by *P. purpureum*. Examination of the products of reduction of this series of compounds showed that some stereoselectivity was demonstrated in the reduction of **21** and **22** by *D. parva* with excess of the *S*-(−)-isomer being shown (Table 6). It is interesting to note that neither 4-hydroxy acetophenone **23** or benzophenone **24** were reduced by any of these cultures. A study of the biotransformation of similar substrates by *Euglena* has also shown stereoselective reduction of many aromatic keto substrates with an excess of the *S*-(−)-isomer being produced. In most cases there was no reduction of hydroxyacetophenones, however benzophenone was biotransformed to benzhydrol (87%) within 4 days (Shimoda et al., 1998).

In conclusion this study demonstrates the ability of the five test microalgae to reduce, oxidise and hydrolyse substrates in keeping with those reported in the literature for the related organisms *Euglena* (Noma and Asakawa, 1998a), *D. tertiolecta* (Noma and Asakawa, 1994), *A. longa* (Shimoda and Hirata, 2000) and *S. oligorrhiza* (Pawlowicz and Siewinski, 1987). All organisms show some degree of specificity and stereoselectivity in the biotransformation of substrates. The most selective reaction observed for all of the microalgae was in the biotransformation of (−)-*trans* carveol (**15**). However, despite the potential of this reaction to allow for the separation of (−)-*trans* and (−)-*cis* carveols (**15**, **16**) and in theory the preparation of *R*-(−)-carvone (**8**) the highest levels of biotransformation of (**15** and **16**) were observed in cultures of *C. minutissima* with a maximum conversion of 44% of

Table 5
Biotransformation (%) of **15** by test microalgae and the products formed after a 5 day incubation period

Organism		*C. min.*	*N. atom*	*D. parva.*	*P. purp.*	*I. galbana*
Substrate	(−)-*Trans* carveol (**15**)	56	67	80	79	92
Products	*4R*-(-)-Carvone (**8**)	36	14	6	2	2
	1R,4R-(+)-Dihydrocarvone (**9**)	6	13	9	9	3
	1R,2S,4R-(+)-Neodihydrocarveol (**10**)	2	6	5	10	3

Table 6
Biotransformation (%) of **20**–**24** by test microalgae and the enantiomeric excess (e.e) of products formed after a 5 day incubation period

Organism (%) compound biotransformed	*C. minutissima*	*N. atomus*	*D. parva*	*P. purpureum*	*I. galbana*
Acetophenone (**20**)	65 (14.88 e.e.)	90	21 (29.52 e.e.)	97	64
4-Cl-Acetophenone (**21**)	36	28	21	53	99
4-CH_3-Acetophenone (**22**)	60	66	35	75	4
4-OH-Acetophenone (**23**)	0	0	0	0	0
Benzophenone (**24**)	0	0	0	0	0

substrate. The further reduction of **8** to **9** and **10** reduces the yield of **8** and therefore would not compete with success of the model system developed for the selective conversion of (−)-*trans* –carveol (**15**) to *R*-(−)-carvone (**8**) using whole cells of *Rhodococcus erythropolis* DCL14 (Tecelao et al., 2001).

3. Experimental protocols

3.1. General methodology

^{1}H NMR: Brüker MSL 300 at 300 MHz, ^{13}C NMR at 75.47 MHz. in $CDCl_3$ with SiMe4 as internal standard. Ir: Nicolet 205 FT-IR. UV: on a Varian Carey 3E UV–visible spectrophotometer. TLC's were run on commercially pre-coated plates (Merck, Kieselgel 60F_{254}). Column chromatography [Merck Kieselgel 60 (9385)]. GC:-OV-17 or a CP cyclodextrin β 236M-19 (0.25 mm×25 m) capillary column using He as carrier gas (split column head pressure, 10 psi). A temperature programme of 70 °C for 3 min, 70–250 °C at 10 °C/min for 18 min, then 250 °C for 4 min. GC/MS: a Hewlett-Packard 5890 GC with a 5970 mass selective detector by EI mode at 70 eV using α-methylbenzene methanol (Rt 8.54) and 2,6-bis (1,1-dimethylethyl)-4-methylphenol (Rt 14.60) as internal refs. MS: at 70 eV on an AEI MS 30 instrument.

3.2. Algal cultures

The algae used in this experiment were *Chlorella minutissima* Fott & Novakova; *Nannochloris atomus* Butcher *Isochrysis galbana (T-Isochrysis)* (Supplied by Regional Technical College, Galway, Ireland), *Dunaliella parva*; Lerche; *Porphyridium purpureum* (Supplied by Carolina Biological Supply Company, USA). The stock cultures were maintained in an artifical sea water (ASW) culture medium and cultured for 30 days (Mockler, 1992). The algae were grown at a constant temperature of 25 °C, and illuminated by cool white fluorescent tubes kept on a 6/18 h dark/light cycle at light intensity of 20 W m^{-2}. At subculture 1:10 or 1:5 dilutions were made of algal broth into sufficient fresh ASW to give a 40% flask fill. All procedures were carried out using strict aseptic protocols. All determinations were carried out in triplicate. Growth of algal cultures under standard static and shake conditions was measured after 7, 14, 21 and 28 days in the manner previously described (Hook et al., 1999).

3.3. Substrates

Cyclohexanone, cyclohexenone, *1S,4R*-(−)-menthone (90%, contains 5% isomenthone), (±)-menthyl acetate, *3RS,4R*-(−)-carveol [(−)-*cis* carveol 37.5%, (−)-*trans* carveol 59.55%, (−)carvone 2.9%], *2RS,4R*-(−)carvyl acetate [(−)-*trans* 63%, (−)-*cis* 37%], *2RS,4R*-(−)carvyl propionate [(−)-*cis* 38%, (−)-*trans* 62%], *1R,4R*-(+)-dihydrocarvone [(+)-isodihydrocarvone 28%, *4S*-(+)-carvone 5% , (+)-dihydrocarvone 67%], *1R,2R,4R*-(−)-dihydrocarveol [(−)-dihydrocarveol 66%, (+)-isodihydrocarveol 18%, (+)-neoisodihydrocarveol 17%] were supplied by the Aldrich Co. *4S*-(+)-Carvone (>99%) and *4R*-(−)-carvone (>99%) were supplied by Fluka, *2RS,4R*-(−)-*trans* carveol (>98%) and *2RS,4R*-(−)-*cis* carveol (>99% contains 0.8% (−)-*trans* carveol) were obtained from Tokoshima Bunri Univ, Yamashiro-cho, Japan.

3.4. Toxicity measurements

3.4.1. Method 1

Each algal type (1:5) dilution was set up in 10 ml test tubes (1 ml algal broth in 4 ml ASQ) and toxicity was measured using a modification of the method of Noma et al. (1991). Growth under standard static conditions was allowed for 7 days. Substrates were dissolved in MeOH (10%) and 1 ml of this solution was added to the algae to bring final substrate concentrations to 100 ppm. In addition two types of control were used: Control 1: ASW + algae only; and Control 2: ASW + algae + 1 ml MeOH (i.e. substrate free). For toxicity measurement, aliquots (0.5 ml) were removed aseptically from the culture tubes at intervals of 24, 48, 72, 96 and 120 h. Survival of all green algae was monitored by measuring the absorbance of whole cells at 440 nm. Survival of *Isochrysis* and *Porphyridium* was determined by cell count using a haemocytometer.

3.4.2. Method 2

Each algal type (1:5) dilution was set up in 250 ml conical flasks (20 ml algal broth in 80 ml ASW, 40% flask fill). Growth under standard static conditions was allowed for 7 days. Cyclohexanone in MeOH (1 ml) and 1 ml of this solution was added to the algae to bring final substrate concentrations to 100, 200 and 500 ppm. All concentrations and controls were set up in triplicate.

3.5. Biotransformations

The growth culture conditions are as outlined above. Dilutions of microalgae (1:5) were made in conical flasks (250 ml) to provide a 40% flask fill (i.e. 20 ml algal broth into 80 ml ASW). Algae were allowed to grow statically for 7 days at which stage a solution of substrate in MeOH (1 ml) was added. Aliquots (10 ml) were taken from the culture flasks in triplicate at intervals of 24, 48, 72, 96 and 120 h. These were extracted with equal volumes of distilled Et_2O. The ether layer was separated, dried over Na_2SO_4, filtered and evaporated to dryness in vacuo. The residue was re-dissolved in Et_2O (1 ml) and analyzed under standard GC condi-

tions. The three replicates for a given data point of the time course experiment for each alga were combined. The algal cells were removed by filtration and the aqueous medium was extracted with Et_2O. The components of the diethyl ether extracts were separated by column chromatography on silica gel. Products were visualized by UV light or by spraying with vanillin in conc. H_2SO_4 (1%) or freshly prepared iodoplatinate spray. Compounds were characterized by spectroscopic analysis and by comparison with authentic samples or literature values.

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