Reduction of Bicyclo[3.2.0]hept-2-en-6-one with Dehydrogenase Enzymes in Whole Cell Preparations of some Fungi and Yeasts

Michael J. Dawson, Gordon C. Lawrence, Gerald Lilley, Martin Todd, and David Noble
Biotechnology Department, Glaxo Group Research, Greenford, Middlesex UB6 0HE
Susan M. Green, Stanley M. Roberts, and Timothy W. Wallace
Department of Chemistry and Applied Chemistry, Salford University, Salford, Lancs. M5 4WT
Roger F. Newton,* Malcolm C. Carter, Peter Hallett, John Paton, Derek P. Reynolds, and Stuart Young
Department of Chemical Research, Glaxo Group Research, Ware, Herts. SG12 0DJ

(±)-Bicyclo[3.2.0]hept-2-en-6-one (1) was reduced using a variety of fungi and yeasts. Bakers' yeast gave 6-*exo*-(1*R*,5*S*,6*S*)-bicyclo[3.2.0]hept-2-en-6-ol (2a) and 6-*endo*-(1*S*,5*R*,6*S*)-bicyclo[3.2.0]-hept-2-en-6-ol (3b) while *Curvularia lunata* and *Mortierella ramanniana* gave only the 6-*endo*-alcohol (3b) and optically active bicycloheptenone (1a). Under slightly modified reaction conditions (±)-6-*endo*-bicyclo[3.2.0]hept-2-en-6-ol was oxidized by bakers' yeast to give (1*S*,5*R*)-bicyclo[3.2.0]hept-2-en-6-one (1b) and the *endo*-alcohol (3a).

Resolution of the bicyclo[3.2.0]heptenone (1) was required to provide both the (1*R*,5*S*)-enantiomer (1a) *and* the (1*S*,5*R*)-enantiomer (1b) (or surrogates) in order to execute enantiocomplementary syntheses of PGE_2,[1] $PGF_2\alpha$,[1] and PGA_2.[2]

Furthermore, large quantities of both enantiomers were needed to prepare optically active prostanoids for biological evaluation. Literature precedent suggested that the optically active bicycloheptenone might be obtained in at least four ways: (i) formation of an azomethine using excess of racemic ketone and a chiral amine, *via* distillation of the volatile ketone and hydrolysis of the residual Schiff's base.[3]

(ii) Reaction of the racemic ketone with a chiral amino alcohol (*e.g.* ephedrine) and separation of the diastereoisomeric oxazolidines.[4]

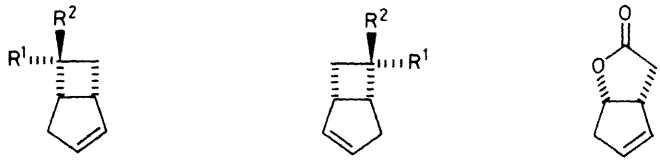
(iii) Use of a chiral ketenimine to induce asymmetry into the [2 + 2]-cycloaddition reaction leading to the bicyclic ketone.[5]

(iv) Modification of the ketone unit using an enzyme-catalysed reaction.[6]

Enzymic reduction of the ketone unit to give optically active alcohol(s) was selected for study. There are distinct advantages in using an enzyme-based resolution method. First, the cost of the chiral catalyst is relatively low. Secondly, the outcome of the reduction is predictable, *i.e.* dehydrogenase enzymes generally transform ketones into products possessing the *S*-configuration at the newly formed secondary alcohol unit.[7] The product alcohols are readily removed from the reaction mixture.

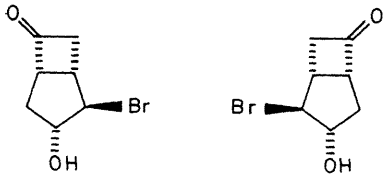
The action of dehydrogenases (principally derived from yeasts and horse liver) on prochiral cycloalkanones has been studied mechanistically using whole-cell preparations [8] and using purified enzyme with added NAD(P)H co-enzyme.[9] Several recent examples illustrate that synthetic organic chemists are turning to enzyme-catalysed reduction processes to provide small quantities of chiral alcohols for conversion into natural products or other interesting molecules.[10]

A preliminary communication [11] by the Ware group described the reduction of the racemic ketone (1) using a whole-cell preparation of bakers' yeast to give 6-*exo*-(1*R*,5*S*,6*S*)-bicyclo[3.2.0]hept-2-en-6-ol (2a) and 6-*endo*-(1*S*,5*R*,6*S*)-bicyclo[3.2.0]hept-2-en-6-ol (3b) in good chemical yield and excellent optical yields for both alcohols.† Thus the yeast



(1a) $R^1, R^2 = O$ (1b)
(2a) $R^1 = H, R^2 = OH$ (2b)
(3a) $R^1 = OH, R^2 = H$ (3b)

(4)



(5a) (5b)

enzyme system displayed low substrate enantioselectivity, *i.e.* both enantiomers were accommodated at the active site. Although the diastereoisomers (2a) and (3b) are separable by column chromatography and by careful distillation, it seemed prudent to screen other yeasts and fungi for an enzyme system which would display high substrate selectivity since this would lead to a simpler separation of optically active bicyclohepten-6-ol and the more volatile optically active bicyclohepten-6-one. The details of this search are reported in this paper together with the problems encountered in scaling up the reduction process to give large quantities of optically active materials.

Bakers' yeast (Fermipan) reduced racemic bicyclo[3.2.0]-hept-2-en-6-one (1) to give 6-*exo*-(1*R*,5*S*,6*S*)-bicyclo[3.2.0]-hept-2-en-6-ol (2a) and 6-*endo*-(1*S*,5*R*,6*S*)-bicyclo[3.2.0]hept-2-en-6-ol (3b) in the ratio 3 : 7 after 1 day (Table 1). Further incubation led to a ratio of *exo* : *endo* alcohols of 3 : 2 as the *endo*-alcohol appeared to be converted back into bicycloheptenone (Figure 1). In the presence of glucose (350 g/l) the ratio of *exo* : *endo* alcohols was 2 : 5 after 24 h and this ratio was maintained on prolonged incubation (Figure 2). Glucose appeared to enhance the extent of bicycloheptenone reduction possibly by increasing the concentration of the available

† Optical yield = [measured rotation (α) × 100]/[rotation of pure enantiomer (α_0)]; K. Drauz, A. Kleeman, and J. Martens, *Angew. Chem., Int. Ed. Engl.*, 1982, 584.

Table 1. Reduction of bicyclo[3.2.0]hept-2-en-6-one (1) with yeasts and fungi

Strain of yeast or fungus [a]	Glaxo culture collection no.	% Conversion after 1 day *endo*-Bicyclo-heptenol	% Conversion after 1 day *exo*-Bicyclo-heptenol	Ratio *endo* : *exo* alcohols [b]
NG + SF (British Fermentation products) bakers' yeast [c]		0	0	
Fermipan bakers' yeast [d]		7	3	2.3 : 1
Brewers' yeast (Boddingtons)		6	2	3 : 1
Candida albicans	C316	0	0	
Saccharomyces acidfaciens	C856	20	11	1.8 : 1
Sacch. carlsbergensis	C1738	7	3	2.3 : 1
Sacch. cerevisiae	C50	4	2	2 : 1
Sacch. cerevisiae	C1739	3	1	3 : 1
Sacch. cerevisiae	C1984	6	6	1 : 1
Sacch. cerevisiae	C1985	12	6	2 : 1
Sacch. cerevisiae	C1989	14	4	3.5 : 1
Sacch. cerevisiae	C1995	7	1	7 : 1
Sacch. elipsoideus	C846	4	1	4 : 1
Schizosaccharomyces pombe	C1747	12	Trace [e]	>10 : 1
Absidia repens	C1979	Trace	0	
Aspergillus niger	C116	0	0	
Byssachlamys fulva	C2507	4	4	1 : 1
Curvularia falcata	C2511	0	0	
C. lunata	C2100	22	Trace	>20 : 1
Mortierella ramanniana	C2506	32	0	>30 : 1
Mucor sp.	C142	8	4	2 : 1
Paecilomyces variatii	C1738	6	4	1.5 : 1
Rhizopus arrhizus	C1600	0	0	
Rh. nigricans	C2065	8	4	2 : 1
Rhodospiridium toruloides	C2391	14	6	2.3 : 1
Rhodotorula marina	C1694	Trace	Trace	
Rh. rubra	C1768	26	10	2.6 : 1

[a] Reductions were carried out under aseptic conditions as described in the Experimental section: the biomass concentration was 125 g wet weight/l. [b] Determined by h.p.l.c. [c] 125 g Wet yeast cake/l. [d] 25 g Dried yeast/l. [e] The *exo*-bicycloheptenol level increased significantly on further incubation.

reductant and/or by lowering the pH. Clearly, assuming that a nicotinamide nucleotide enzyme is involved,[12] low pH will favour bicycloheptenol formation:

$$\text{bicycloheptenone} + \text{NAD(P)H} + \text{H}^+ \rightleftharpoons \text{bicycloheptenol(s)} + \text{NAD(P)}^+$$

The successful small scale runs encouraged progression to a large scale fermentation and an assessment of the optical purity of the products.

The extent of bicycloheptenone reduction was a function of the biomass concentration up to *ca.* 150 g dried yeast/l [using 5.0 g of the ketone (1) as substrate]; above this level *ca.* 50% conversion was observed (Figure 3). On work-up of a run involving the ketone (1) (30 g), yeast (1.5 kg), and glucose (3.5 kg) by steam distillation and extraction of the distillate with a suitable solvent, 6-*exo*-bicycloheptenol (2) (24%) and 6-*endo*-bicycloheptenol (50%) (3) (yields based on consumed starting material) were obtained. Chromatography furnished the alcohols (2) and (3) in pure form. The compounds (2) and (3) were identical (t.l.c., i.r., and n.m.r.) with authentic racemic materials.[13] The absolute configuration of the dominant enantiomer of the *exo*-alcohol was shown to be (2a) by a two-stage oxidation process which gave the known, optically active lactone (4).[14]

Reaction of the *exo*-alcohol with *N*-bromosuccinimide (NBS) in aqueous acetone gave the crystalline bromohydrin (5). G.l.c. analysis of the L-menthol carbonate derivative of the crude bromohydrin enabled the ratio (5a) : (5b) to be judged as 92 : 8. One recrystallisation of the crude bromohydrin furnished optically pure material.

Similarly the *endo*-alcohol reacted with NBS in aqueous acetone to give the bromohydrin (5): the enantiomer ratio (5a) : (5b) was found to be 6 : 94 by g.l.c. analysis of the L-menthol carbonate derivative. Optically pure (5b) was obtained by recrystallisation.

These results showed that the enzymic reduction of (±)-bicycloheptenone gave the *exo*-alcohol (2a) in 84% optical yield and the *endo*-alcohol (3b) in 88% optical yield.

In order to avoid the tedious separation of the *exo*- (2) and *endo*-alcohol (3) after large scale fermentations, a substrate enantioselective enzyme system was required to effect the reduction. Also an organism that was much less sensitive to substrate concentration was required (the yeast reduction was inhibited by 50% at a ketone concentration of 15 g/l).

Thirteen strains of fungi and nineteen yeasts which have been reported in the literature [7] to reduce ketones to secondary alcohols were screened for their ability to reduce the bicycloheptenone (Table 1).

Schizosaccharomyces pombe produced only the *endo*-bicycloheptenol during 24 h, but on further incubation a substantial quantity of the *exo*-bicycloheptenol was formed. Similarly *Rhodotorula rubra* and *Saccharomyces acidfaciens* catalysed rapid reduction of both enantiomers of the bicyclohepten-6-one.

However, *Curvularia lunata* and *Mortierella ramanniana* appeared to be particularly suitable for the bicycloheptenone reduction as both gave good rates of bioconversion, and specific production of the *endo*-bicycloheptenol. These systems were investigated further.

The time courses of bicycloheptenone reduction by *M. ramanniana* and *C. lunata* were different (Figure 4). The

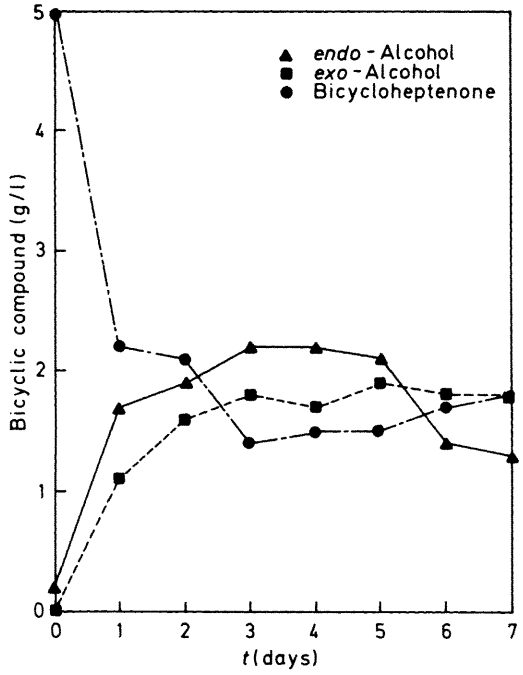


Figure 1. Reduction of bicyclo[3.2.0]hept-2-en-6-one using bakers' yeast (150 g/l)

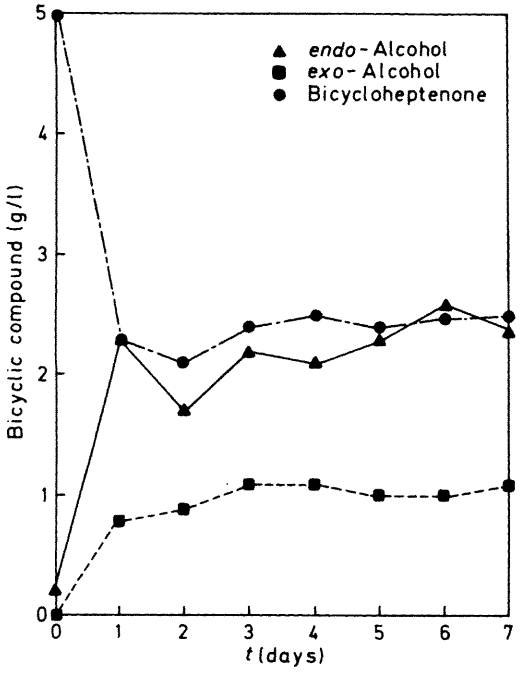


Figure 2. Reduction of bicyclo[3.2.0]hept-2-en-6-one using yeast (150 g/l) and glucose (350 g/l)

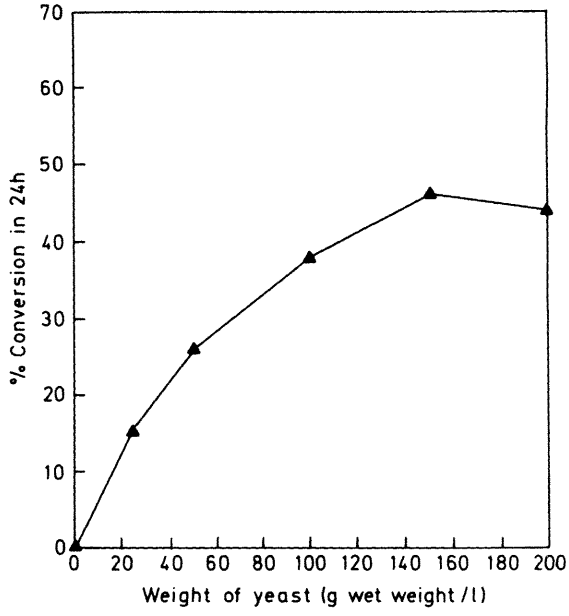


Figure 3. Reduction of bicyclo[3.2.0]hept-2-en-6-one to bicyclo-[3.2.0]hept-2-en-6-ol using different concentrations of yeast

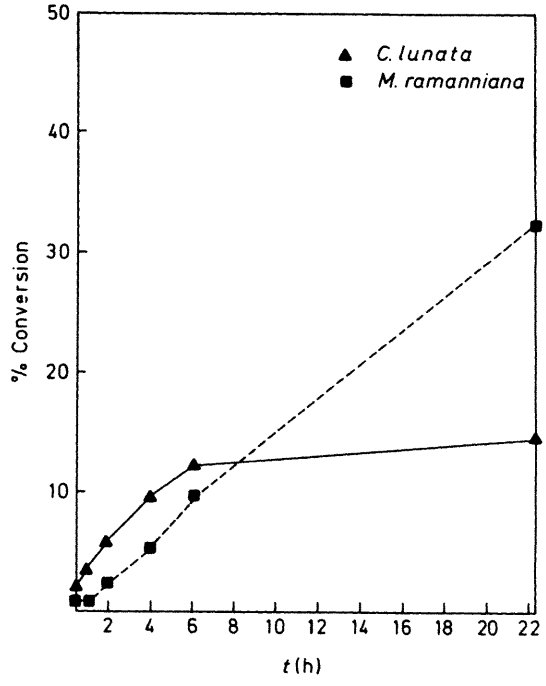


Figure 4. Rate of conversion of bicyclo[3.2.0]hept-2-en-6-one into 6-*endo*-bicyclo[3.2.0]hept-2-en-6-ol using *C. lunata* (grown in continuous culture) and *M. ramanniana* (grown in a 40-l batch)

initial rate of bicycloheptenone reduction exhibited by *C. lunata* (1.8 mg *endo*-ol/g wet weight fungus/h) was higher than that shown by *M. ramanniana* (0.5 mg *endo*-ol/g wet weight of fungus/h) but the latter system continued to reduce the bicycloheptenone at a constant rate throughout the 22 h conversion period whereas the rate of reduction exhibited by *C. lunata* fell rapidly with time and there was little further conversion after 6 h. Indeed after exposure to bicycloheptenone for 6 h, *C. lunata* was barely viable, and no viable colonies were detected after 22 h. *M. ramanniana* showed little loss of viability over the 22 h conversion period.

The recoveries and optical purities of products of the bicycloheptenone reduction catalysed by *M. ramanniana* and *C. lunata* using the shake flask system are compared to the yeast system in Table 2. No attempt was made to optimize the yields in the extraction and purification steps.

The rate of bicycloheptenone reduction catalysed by *M.*

Table 2. Optical and chemical yields of the products of the bicycloheptenone reduction using bakers' yeast, *C. lunata*, and *M. ramanniana*

Reduction system	Bicycloheptenone	6-*endo*-Bicyclo-hept-2-en-6-ol	6-*exo*-Bicyclo-hept-2-en-6-ol
C. lunata [a]			
Starting material (g)	2.5		
After bioconversion [b] (g)	1.7	0.5	0.1
After purification [c] (g)	0.8	0.3	
Optical yield (%)	11	55	
M. ramanniana [a]			
Starting material (g)	2.5		
After bioconversion [b] (g)	0.4	1.65	0.2
After purification [c] (g)	0.4	0.9	
Optical yield (%)	80	60	
M. ramanniana [d]			
Starting material (g)	25		
After bioconversion [b] (g)	*g*	12.5	
After purification [c] (g)	3.4	7.0	
Optical yield (%)	84	89	
Bakers' yeast [e]			
Starting material (g)	30		
After bioconversion [f] (g)	8.7	10.7	5.2
After purification [c] (g)	3.8	5.6	4.2
Optical yield (%)		88	84

[a] In shake flask. [b] Measured by h.p.l.c. [c] No attempt was made to optimise the purification procedure in order to minimise losses of the volatile components. [d] In fermenter with stirring and aeration. [e] In fermenter with stirring. [f] Measured by g.l.c. [g] Not determined.

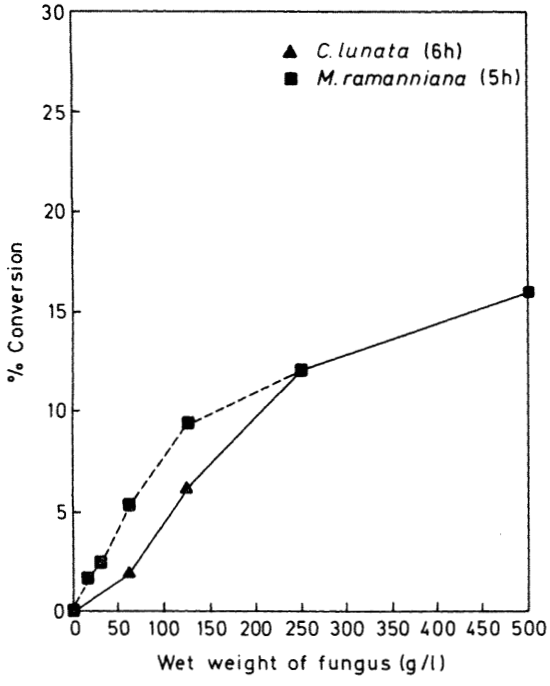


Figure 5. Effect of different concentrations of *C. lunata* and *M. ramanniana* on the reduction of bicyclo[3.2.0]heptenone to 6-*endo*-bicyclo[3.2.0]hept-2-en-6-ol during 6 h (*C. lunata*) and 5 h (*M. ramanniana*)

ramanniana increased in proportion to the biomass concentration up to 125 g wet weight/l, but above this level there was a less than proportional increase in the bioconversion rate with increasing biomass concentration, probably due to imperfect mixing (Figure 5). With *C. lunata*, both the initial rate of bicycloheptenone reduction and the final concentration of *endo*-bicycloheptenol increased with increasing biomass level; the increase became less than proportional above 250 mg wet weight/l. It proved necessary to use very high levels of biomass (>500 g wet weight/l) to obtain complete (50%) reduction of bicycloheptenone with this organism.

Glucose was found to stimulate bicycloheptenone reduction by both *M. ramanniana* and *C. lunata*; the optimum glucose concentration was 80—100 g/l for both organisms. The addition of optimum levels of glucose caused 15 and 55% stimulation of the bioconversion rate respectively. At glucose concentrations above 100 g/l bicycloheptenone reduction was inhibited, particularly in the case of *M. ramanniana*.

Bicycloheptenone reduction by *C. lunata* was found to be more successful in growth medium than in distilled water or glucose solution. In MYGP medium the extent of bioconversion achieved was approximately double that in distilled water alone. The beneficial effects of the growth medium appeared to lie in prolonging the viability of the mycelium. In growth medium, complete (50%) enantiospecific bicycloheptenone reduction was achieved in less than 24 h at a biomass level of 250 g wet weight/l. The effect of using growth medium to support bicycloheptenone reduction by *M. ramanniana* was not investigated.

Unfortunately, the bicyclic ketone inhibited *M. ramanniana* and *C. lunata* at low concentrations. 50% Inhibition of reduction was observed at ketone concentrations of *ca.* 10 g/l and *ca.* 7.5 g/l respectively. The inhibitory effect of the bicyclic ketone is probably linked to its effect on the viability of the organism. *C. lunata* will not grow on MGYP agar plates in the presence of a 0.02M-solution of the ketone in water: growth of *M. ramanniana* is inhibited by a 0.06M-aqueous solution of bicycloheptenone.

The optimum temperature for reduction by *M. ramanniana* was in the range 28—33 °C. The rate of bioreduction increased on more vigorous shaking of the reaction flask probably because of improved mixing. The effects of temperature and

agitation on bicycloheptenone reduction by *C. lunata* were not studied.

Although the rate of oxygen transfer in the shake flask bioconversion system is extremely low, this was found to be both sufficient and necessary for the reduction of the bicycloheptenone catalysed by *M. ramanniana*. The bioconversion rate was not significantly increased by increasing the oxygen transfer rate, but a definite requirement for oxygen could be demonstrated by the use of sealed flasks. The requirement for oxygen in the reduction process had not been anticipated but may reflect the need to oxidise other reduced co-enzymes in order to allow continued production of those reduced co-enzymes [probably NAD(P)H] required for the bicycloheptenone reductases.

Cell-free extracts of *C. lunata* reduced the bicycloheptenone only after the addition of NADPH (but not NADH). The extent of bicycloheptenone reduction was approximately stoicheiometric with added NADPH, and further fractionation of the cell extracts indicated the presence of soluble $NADP^+$-linked bicycloheptenone reductase activity. The reduction was specific for *endo*-alcohol but the optical purity of the alcohol was not determined. Glucose had no effect on the bioconversion by cell-free extracts while glucose 6-phosphate enhanced the extent of bicycloheptenone reduction presumably by reduction of $NADP^+$ *via* $NADP^+$-linked glucose 6-phosphate dehydrogenase.

Bioconversion by cell-free extracts was not as susceptible to inhibition by bicycloheptenone as in the whole cell system suggesting, therefore, that the principal cause of bicycloheptenone toxicity is through interaction with the cell membrane.

Any system for bicycloheptenone reduction employing cell-free extracts or purified enzymes would clearly require reduced co-enzyme regeneration and although such systems are being developed,[15] material costs would remain prohibitively high.

Following the small scale pilot runs, the reduction of the bicycloheptenone using whole-cell *M. ramanniana* was carried out in a 5-l fermenter. Air (1 l/min) was passed through a stirred aqueous solution (5 l) of ketone (25 g) and *M. ramanniana* (200 g wet weight/l) at 25 °C. After 20 h *endo*-bicycloheptenol (3b) (12.5 g) was obtained in high optical yield: optically active (1*R*,5*S*)-bicycloheptenone (1a) was recovered.

The improved optical purity in the large scale run (Table 2) may be attributed to the increased oxygen supply and/or the shorter reaction time.

One flaw remained in this excellent asymmetric reduction process, namely the inability of the *M. ramanniana* to sustain high concentrations of bicycloheptenone in the reaction mixture. Other natural isolates of *M. ramanniana* were screened but no increase in resistance to the ketone was obtained. Mutation and selection of *M. ramanniana*, including the use of continuous selection techniques in the presence of bicycloheptenone, served to provide 32 mutants but once again no increase in resistance was observed.

The final attempt to overcome the problem of substrate toxicity involved an assessment of the selectivity of racemic *exo*- (2) and *endo*-alcohols (3) to form optically active bicycloheptenone (1). A small scale reaction using a mixture of racemic 6-*exo*- (2) and 6-*endo*-bicyclohepten-6-ol (3) (ratio 1 : 5) showed promise: bakers' yeast oxidized the *endo*-alcohol (3b) with good selectivity at pH 6 to give (1*S*,5*R*)-bicycloheptenone (1b) in 85% optical yield. Racemic *exo*-alcohol was recovered unchanged, while unchanged *endo*-alcohol (3a) was isolated in 90% optical yield. Unfortunately the bicycloheptenols proved to be as toxic to the organism as the bicycloheptenone. Thus a bicycloheptenol concentration of 15 g/l inactivated the appropriate enzymes.

Conclusion

Large scale fermentations using dehydrogenase enzymes in bakers' yeast or *M. ramanniana* and the unnatural substrate, bicyclo[3.2.0]hept-2-en-6-one (1), led to good chemical yields of optically active products.

Toxicity problems due to the incompatibility of a lipophilic organic substrate and a hydrophilic enzyme system enclosed in a cell membrane were encountered.

The utility of enzyme systems to the organic chemist will be limited until cheap cell-free systems become readily available or until transport of substrate to the active site in the whole-cell system can be facilitated.

In the case of the bicycloheptenone (1) a new chemical procedure was invented to effect resolution of the enantiomers and this will be reported in due course.

Experimental

H.p.l.c. was accomplished using an LDC Constametric III pump and a Perkin-Elmer LC55 detector. G.l.c. data was acquired on a Perkin-Elmer F30 gas chromatograph. Optical rotations were measured using a Bellingham-Stanley Model A Polarimeter Type P1. Merck Kieselgel G or H was used for column chromatography. T.l.c. was carried out with Camlab 'Polygram' precoated silica gel plates. Fermipan yeast was obtained from BDH: brewers' yeast was supplied by Boddington's Brewery, Strangeways, Manchester. Solutions in organic solvents were dried using anhydrous magnesium sulphate. Ether refers to diethyl ether.

Growth of Micro-organisms.—The micro-organisms were obtained as freeze dried ampoules from the Greenford culture collection and were maintained on slopes of potato dextrose agar (fungi) or MYGP agar. [MYGP contains oxoid malt extract (3 g/l), difco yeast extract (3 g/l), oxoid peptone (5 g/l), and glucose (30 g/l)]. A variety of culture systems has been used for the growth of the micro-organisms: in all cases the growth temperature was 25 °C and the medium was MYGP.

All micro-organisms that were screened for bicycloheptenone reduction were grown in 4-l batches in 7-l LH Engineering baffled fermenters; the agitation system comprised 3 × 90 mm diameter 6-bladed turbine impellors situated 90 mm apart. The air-flow rate was 4 l/min, and the agitation speed was varied to maintain the dissolved oxygen tension above 30%. The fermenters were seeded with 400 ml of culture from a Florence flask (*vide infra*) and harvested in the stationary phase (after 3—6 days growth). The Florence flask system comprised a 2-l vessel containing 400 ml of liquid agitated at 150 rev/min on rotary shakers with a 50-mm throw. Flasks were inoculated directly from slopes (see above) and harvested after 7 days.

C. lunata was grown in Florence flasks and 7-l fermenters as described above. In addition this micro-organism was grown as a 40-l batch in a 70-l Biolafitte unbaffled fermenter fitted with a draught tube. The inoculum was 1.2 l of culture from 3 Florence flasks. The air-flow rate was 40 l/min and agitation was effected by a single 8-bladed propellor-type impellor (155 mm diam.) situated at the base of the draught tube. The agitation speed was 200—500 rev/min. The mycelium (6 g dry weight/l) was harvested after 5 days.

M. ramanniana was grown in Florence flasks and 7-l fermenters as described in the general procedure. In addition, the micro-organism was grown in a 70-l fermenter as des-

cribed above for *C. lunata* except a 70-l Biolafitte baffled fermenter was employed and the agitation system consisted of 3 × 120-mm diam. 6-bladed turbine impellors situated 220 mm apart and operated at 500 rev/min. The mycelium, including extensive wall growth (9 g dry weight/l) was harvested after 5 days.

Preparation of a Cell-free Extract of C. lunata.—A suspension of mycelium (*ca.* 500 g wet weight/l) was homogenised in 0.7M-KH_2PO_4/KOH buffer (pH 7.0) using either a motorised homogeniser or a MSE mixer–blender. The temperature was maintained at 0—4 °C. The cell-debris was removed by centrifugation at 12 000 G for 10 min and the resulting cell-free extracts, containing 1—2 mg protein/ml (estimated using the Folin–Ciocalteu reagent) [16] were stored frozen. These extracts could be fractionated into soluble and membrane fractions by high speed centrifugation (30 000 G for 2 h).

Shake-flask Reduction Procedure.—The micro-organism (*18.0—125.0* g wet weight), the bicycloheptenone (0.75 g), and glucose if required were diluted to 150 ml with distilled water in a 250 ml conical flask. The neck of the flask was restricted by a rubber bung fitted with a filter tube (5—10 mm diam.) loosely packed with cotton wool. The flasks and contents were incubated at 25 °C and agitated at 150 rev/min on a rotary shaker (50-mm throw).

Assay for Bicyclo[3.2.0]*hept-2-en-6-one* (1) *and Bicyclo*-[3.2.0]*hept-2-en-6-ols* (2) *and* (3) *using H.P.L.C.*—After incubation the biomass was removed by centrifugation and a 20-µl aliquot of the supernatant liquid (containing *ca.* 3 g bicycloheptane derivatives/l) was injected onto a column (10 cm × 4.6 mm internal diam.) packed with spherisorb S5 C18. Elution with acetonitrile in water (10% v/v) at a rate of 1.8 ml/min separated 6-*endo*-bicyclo[3.2.0]hept-2-en-6-ol (3), 6-*exo*-bicyclo[3.2.0]hept-2-en-6-ol (2), and bicyclo-[3.2.0]hept-2-en-6-one (1) (retention times 4.6, 5.3, and 6.9 min respectively), identified by comparison with authentic samples [13,17] and using a u.v. detector operating at 200 nm.

Determination of the Optical Purity of the Bicyclic Ketone (1) *and the Bicyclic Alcohols* (2) *and* (3).—The bioconversion mixture was centrifuged. Sodium chloride (240 g/l) was added and the solution was extracted with dichloromethane (2 × 300 ml). The organic extracts were combined, dried, and evaporated to a small volume (*ca.* 5 ml). The residue was chromatographed over silica gel using ethyl acetate–light petroleum (b.p. 60—90 °C) as eluant to give pure samples of the bicyclic ketone (1) and the two bicyclic alcohols (2) and (3). The pure materials were distilled prior to measurement of the optical rotations. The optical rotation of the bicyclic ketone was observed to change on storage: this was possibly due to hydration or peroxide formation. Determination of the specific rotations of the alcohols and the ketone is described in the next section.

Large Scale Reduction of Bicyclo[3.2.0]*hept-2-en-6-one using Yeast.*—Glucose (600 g), bakers' yeast (1.5 kg), yeast nutrient (6 g), and three vitamin B_1 tablets were stirred in water at 22 °C for 1.5 h. (±)-Bicyclo[3.2.0]hept-2-en-6-one (1) (30 g) was added dropwise during 30 min. Glucose (x g) in water (x ml) was added dropwise at 2 h (x = 300), 4 h (x = 600), 4.7 h (x = 400), 20 h (x = 100), 21 h (x = 500), 26.25 h (x = 500), and 27 h (x = 500). At 70 h the suspension was steam-distilled until the distillate contained no bicyclic material (t.l.c.). The distillate was saturated with sodium chloride and extracted with ether. Ether and ethanol were removed by distillation through a Vigreux column to give a residual brown oil (31.3 g). Some bicycloheptenone co-distilled with the ethanol. Quantitative g.l.c. analysis (5% Carbowax 20-m column at 95 °C using tetradecane as internal standard) showed bicycloheptenone (1) (29%), 6-*endo*-bicyclohepten-6-ol (3) (35.6%), and 6-*exo*-bicyclohepten-6-ol (2) (17.3%). Column chromatography using dichloromethane as eluant gave bicyclo[3.2.0]hept-2-en-6-one (1) (3.8 g), 6-*endo*-bicyclo[3.2.0]hept-2-en-6-ol (3) (5.6 g), $[\alpha]_D^{23}$ +60.5° (*c*, 2.8 in $CHCl_3$) and 6-*exo*-bicyclo[3.2.0]hept-2-en-6-ol (2) (4.2 g), $[\alpha]_D^{20}$ −91° (*c*, 2.6 in $CHCl_3$).

The 6-*exo*-bicycloheptenol (2) was converted into 2-oxabicyclo[3.3.0]hept-6-en-3-one (4) (81%),[2] m.p. 44 °C (two crystallisations from ether–light petroleum) $[\alpha]_D^{19.5}$ −104° (*c*, 2.85 in methanol) {lit.,[14] $[\alpha]_D^{20}$ −104° (*c*, 1.1 in methanol)}.

The 6-*exo*-bicycloheptenol (2) was converted into 2-bromo-3-hydroxybicyclo[3.2.0]heptan-6-one (5) using *N*-bromosuccinimide in aqueous acetone.[1] The L-menthol carbonate derivative was made in the standard fashion: g.l.c. analysis (3% OV 210 at 150—190 °C) indicated that the ratio of the enantiomers (5a) : (5b) of the bromohydrin was 92 : 8. The 6-*endo*-bicycloheptanol (3) was converted into 2-bromo-3-hydroxybicyclo[3.2.0]heptan-6-one (5). G.l.c. analysis of the L-menthol carbonate derivative indicated an enantiomer ratio (5a) : (5b) of 6 : 94. Optically pure (g.l.c. analysis) bromohydrins $[\alpha]_D^{20}$ ± 63° were obtained on crystallisation from carbon tetrachloride.

The *exo*-alcohol was oxidized under Collins conditions to give bicyclo[3.2.0]hept-2-en-6-one (90%), $[\alpha]_D^{22}$ −60° (*c*, 1.0 in chloroform).[2]

A reduction of bicyclo[3.2.0]hept-2-en-6-one (1) (120 g) under conditions representing a scale-up of the above process gave 6-*endo*-bicyclo[3.2.0]hept-2-en-6-ol (3) (17.3 g) and 6-*exo*-bicyclo[3.2.0]hept-2-en-6-ol (2) (10.15 g): the optical yields were 93 and 94% respectively.

Large Scale Reduction of (±)-*Bicyclo*[3.2.0]*hept-2-en-6-one* (1) *using* Mortierella ramanniana.—The bicyclic ketone (1) (25 g) and *M. ramanniana* (1 kg wet weight) in water (5 l) were charged into a 7-l LH Engineering fermenter. After the mixture had been stirred (600 rev/min) at 25 °C with a flow of air (1 l/min) for 20 h, an aliquot was centrifuged. The supernatant liquor was saturated with sodium chloride, extracted with dichloromethane, and analysed for bicycloheptenone (1) and bicycloheptenols (2) and (3) as described above. 6-*endo*-Bicyclo[3.2.0]hept-2-en-6-ol (3b) (7.0 g), $[\alpha]_D^{20}$ +61.5°, was shown to be the sole reduction product and bicyclo[3.2.0]hept-2-en-6-one (1a) (3.4 g), $[\alpha]_D^{20}$ −59.8° (*c*, 1.0 in chloroform) was recovered.

Oxidation of (±)-*Bicyclo*[3.2.0]*hept-2-en-6-ols* (2) *and* (3) *with Bakers' Yeast.*—A mixture of 6-*endo*- (3) and 6-*exo*-bicyclohept-2-en-6-ol (2) (ratio 5 : 1) (0.5 g), Fermipan yeast (10 g), and distilled water (100 ml) in a shake flask (250 ml) were adjusted to pH 6 and agitated on a rotary shaker at 25 °C for 94 h. H.p.l.c. analysis confirmed the formation of bicyclo[3.2.0]hept-2-en-6-one (1) (0.18 g), the loss of some 6-*endo*-alcohol (3) (0.15 g), and the recovery of most of the 6-*exo*-alcohol (2) (0.07 g).

From a series of similar runs, after work-up and purification as described above, the optical yield of the ketone (1b) was found to be 85%, the *endo*-alcohol (3a) was recovered in an optical yield of 90%, while the recovered *exo*-alcohol (2) was optically inactive.

The above reaction was repeated using 1.5 g of the mixture of alcohols. No bicycloheptenone (1) could be detected after 94 h.

Acknowledgements

We thank the S.E.R.C. and Glaxo Group Research (Ware) for financial support (CASE award to S. M. G.).

References

1 J. Davies, S. M. Roberts, R. F. Newton, and D. P. Reynolds, *J. Chem. Soc., Perkin Trans. 1*, 1981, 1317.
2 R. F. Newton, D. P. Reynolds, J. Davies, P. B. Kay, S. M. Roberts, and T. W. Wallace, *J. Chem. Soc., Perkin Trans. 1*, 1983, 683.
3 U. A. Huber and A. S. Dreiding, *Helv. Chim. Acta*, 1970, **53**, 495; see also H. Greuter, J. Dingwall, P. Martin, and D. Bellus, *ibid.*, 1981, **64**, 2812.
4 R. Kelly and V. VanRheenen, *Tetrahedron Lett.*, 1973, 1709.
5 C. Houge, A. M. Frisque-Hesbain, A. Mockel, L. Ghosez, J. P. Declercq, G. Germain, and M. Van Meerssche, *J. Am. Chem. Soc.*, 1982, **104**, 2920.
6 'Applications of Biochemical Systems in Organic Chemistry,' eds. J. B. Jones, C. J. Sih, and D. Perlman, Wiley, New York, 1976.
7 K. Kieslich, ' Microbial Transformations of Chemical Compounds Excluding Steroids and Non-Cyclic Structures,' G. Thieme, Stuttgart, 1976.
8 R. L. Crumbie, D. M. Ridley, and G. W. Simpson, *J. Chem. Soc., Chem. Commun.*, 1977, 315; H. Günter, C. Frank, H.-J. Schuetz, J. Bader, and H. Simon, *Angew. Chem., Int. Ed. Engl.*, 1983, **22**, 322, and references therein.
9 S.-K. Chung and S.-U. Park, *J. Org. Chem.*, 1982, **47**, 3197; T. Takemura and J. B. Jones, *ibid.*, 1983, **48**, 791; D. R. Dodds and J. B. Jones, *J. Chem. Soc., Chem. Commun.*, 1982, 1080; I. MacInnes, D. C. Nonhebel, S. T. Orszulik, and C. J. Suckling, *ibid.*, 1982, 121 and references therein.
10 H. Akita, A. Furuichi, H. Koshiji, K. Horikoshi, and T. Oishi, *Tetrahedron Lett.*, 1982, 4051; M. Hirama and M. Uei, *J. Am. Chem. Soc.*, 1982, **104**, 4251; G. Hoffmann and R. Wiartalla, *Tetrahedron Lett.*, 1982, 3887; D. W. Brooks, P. G. Grothaus, and W. L. Irwin, *J. Org. Chem.*, 1982, **47**, 2820; *Z. Chem.*, 1982, 187; G. Frater, *Helv. Chim. Acta*, 1979, **62**, 2829; Y. Takaishi, Y. L. Yang, D. DiTullio, and C. J. Sih, *Tetrahedron Lett.*, 1982, 5489; M. Nakazaki, H. Chikamatsu, and M. Taniguchi, *Chem. Lett.*, 1982, 1761; B. Wipf, E. Kupfer, R. Bertazzi, and H. G. W. Leuenberger, *Helv. Chim. Acta*, 1983, **66**, 485; M. Hirama, M. Shimizu, and M. Iwashita, *J. Chem. Soc., Chem. Commun.* 1983, 599; J. B. Jones, M. A. W. Finch, and I. J. Jakovac, *Can. J. Chem.*, 1982, **60**, 2007; C. T. Hsu, N.-Y. Wang, L. H. Latimer, and C. J. Sih, *J. Am. Chem. Soc.*, 1983, **105**, 593; H. Akita, H. Koshiji, A. Furuichi, K. Horikoshi, and T. Oishi, *Tetrahedron Lett.*, 1983, **24**, 2009; D. W. Brooks and R. P. Kellogg, *ibid.*, 1982, **23**, 4991; see also A. Kergomard, M. F. Renard, and H. Veschambre, *J. Org. Chem.*, 1982, **47**, 792 for microbiological reduction of α,β-unsaturated ketones.
11 R. F. Newton, J. Paton, D. P. Reynolds, S. N. Young, and S. M. Roberts, *J. Chem. Soc., Chem. Commun.*, 1979, 908.
12 R. N. Patel, C. T. Hou, A. I. Laskin, and P. Derelanko, *J. Appl. Biochem.*, 1981, **3**, 218.
13 J. A. Berson and J. W. Patton, *J. Am. Chem. Soc.*, 1962, **84**, 3406.
14 M. Nara, S. Terashima, and S. Yamada, *Tetrahedron*, 1980, **36**, 3161.
15 R. Wichmann, C. Wandrey, A. F. Bückmann, and M. R. Kula, *Biotechnol. Bioeng.*, 1981, **23**, 2789.
16 O. H. Lowry, N. J. Roseborough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.
17 P. A. Grieco, *J. Org. Chem.*, 1972, **37**, 2363.

Received 7th February 1983; *Paper* 3/167