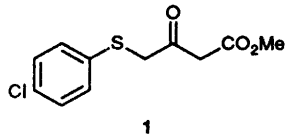
Biotransformations Using Clostridia: Stereospecific Reductions of a β-Keto Ester

Markus Christen,[a] David H. G. Crout,[a] Robert A. Holt,[b] J. Gareth Morris[b] and Helmut Simon[c]
[a] *Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK*
[b] *Department of Biological Sciences, University College of Wales, Aberystwyth, Dyfed SY23 3DA, UK*
[c] *Organische-Chemische Institut Der Technischen Universität, München, Lichtenbergstrasse 4, 8046 Garching, Germany*

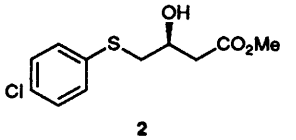
The reduction of methyl 4-(4-chlorophenylthio)-3-oxobutanoate **1** by clostridia has been studied. *Clostridium pasteurianum* ATCC 6013, *C. tyrobutyricum* DSM 1460 and *C. kluyveri* NCIB 10680 gave the D-(3*S*) reduction product **2**, whereas *C. kluyveri* DSM 555 gave the L-(3*R*) reduction product **3**. The products could be obtained in higher optical purities than by yeast reductions.

The reduction of carbonyl compounds using enzymes or whole cells has been one of the most intensively investigated areas in biotransformations.[1] For most of the reductions investigated, baker's yeast has been the organism used.[2] There are several reasons for this. Much of the work has been carried out by organic chemists to whom baker's yeast is a safe, familiar organism. Also, it can be used aerobically. However, there is great potential in the use of other microorganisms and, in particular, the clostridia. These have been studied by relatively few workers, although their ability to carry out reductions impressive both in scope and stereospecificity has been demonstrated convincingly and extensively.[3–5]

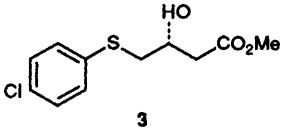
To investigate further the potential of the clostridial system in stereospecific reductions, we have studied reduction of methyl 4-(4-chlorophenylthio)-3-oxobutanoate **1**. This substrate was chosen because its reduction by yeasts has been thoroughly investigated[6] and a direct comparison of the results of clostridial and yeast reductions was therefore possible. The ester **1** was reduced with sodium borohydride to give the



1



2



3

product of biological reduction in racemic form for development of the method for chiral analysis using chiral lanthanide shift reagents. The absolute configurations of the products were determined by chemical correlation with the corresponding 3-hydroxybutanoates, which were obtained from the biotransformation products **2** or **3** by Raney nickel reduction, as described in the Experimental section.

The results obtained with various clostridia and under different conditions are given in Table 1.

The most immediately striking observation was the opposite configurations of the products obtained using *C. kluyveri*. Strain DSM 555 gave predominantly the L-(3*R*) product **3** whereas strain NCIB 10680 gave predominantly the D-(3*S*) enantiomer **2**. Reductions of carbonyl compounds with *C. tyrobutyricum* have been shown by Veschambre and his colleagues[5] to proceed with a stereospecificity dependent on growth conditions. The predominant isomer formed depended on whether cells were grown with glucose or with crotonate as the carbon source. It appeared likely that, as in the baker's yeast system, more than one dehydrogenase was acting on the substrates. The known influence of carbon source on the enzymes produced by the microorganism was expected to lead to a difference in the stereochemical course of the reductions, as was, in fact, observed. In the present case, essentially identical results were obtained, regardless of growth conditions, with the same strain of *C. tyrobutyricum* as that used by the group of Veschambre.[5]

In the experiments with *C. kluyveri* DSM 555, the essential difference between experiments 4 and 5 (Table 1) were that in experiment 4 a higher substrate concentration was used in the presence of methyl viologen (1,1′-dimethyl-4,4′-bipyridinium dichloride) and tetracycline (which were absent in experiment 5). A product of higher optical purity was obtained in experiment 5, but in a slightly lower yield.

With *C. kluyveri* NCIB 10680, as with DSM 555, better results were obtained in the absence of methyl viologen [experiments 6, 7 (Table 1)] although poor overall yields were obtained in both cases. However, yields were not optimised in these experiments and the high yields obtained in reductions of 6-methylhept-5-en-2-one show that there is no inherent limitation on yield in clostridial reductions.[5]

Using *C. kluyveri* DSM 555, experiments comparable with experiments 4 and 5 (Table 1) were carried out in Munich (experiments 8 and 9). In experiment 8, reduction was carried out under an atmosphere of hydrogen and in the presence of methyl viologen and tetracycline. In experiment 9, reducing power was made available by addition of ethanol and reductions were carried out in the absence of methyl viologen and tetracycline. The results were comparable with those of experiments 4 and 5, notably in the higher optical purity of the products obtained in the absence of methyl viologen. The high yield of product in experiment 8 is noteworthy. In part, this was because in this experiment reduction of the substrate was complete. Chromatographic purification of the product, with inevitable attendant losses, was thereby avoided.

The results obtained in these experiments clearly point to the operation of more than one reducing enzyme in this sytem. One factor responsible for shifting the balance of activities with respect to reduction of the particular substrate used in these experiments is clearly the presence or absence of methyl viologen. It can be deduced that the activity of an enzyme favouring production of the D-enantiomer is potentiated by the addition of methyl viologen to *C. kluyveri* DSM 555. However, the opposite appears to be true for *C. kluyveri* NCIB 10680,

Table 1 Reduction of the β-keto ester **1** by *Clostridium* species

Expt.	Strain	Incubation conditions (Method*)	Yield (%)	ee (%)	Config'n
1	*C. pasteurianum*	A	—	57	D
2	*C. tyrobutyricum* DSM 1460	A[a]	38	77, 79[d]	D
3	DSM 1460	A[b]	25	77, 78[d]	D
4	*C. kluyveri* DSM 555	B	38	65	L
5	*C. kluyveri* DSM 555	A	33	90	L
6	*C. kluyveri* NCIB 10680	A[c]	11	46	D
7	*C. kluyveri* NCIB 10680	A	12	92	D
8	*C. kluyveri* DSM 555	B	85[e]	77	L
9	*C. kluyveri* DSM 555	C	31[f]	85	L

*See under Experimental section. [a] Glucose grown *. [b] Crotonate grown *. [c] Methyl viologen (0.25 mg cm^{-1}) was added before addition of substrate *. [d] Repeat experiment. [e] 100% Conversion. [f] 80% Conversion. The lower isolated yields in experiments 8 and 9 reflect difficulties in effecting quantitative extraction of product.

where the presence of methyl viologen decreased the amount of D-enantiomer formed.

In the context of the use of clostridia for biotransformations on a practical scale, the most striking features of these experiments is the generally higher optical purities of products obtained when compared with the optical purities of the same products obtained under batch fermentation conditions using yeasts. For example, the L-isomer of compound **3** was obtained in 70% ee using *Saccharomyces cerevisiae* and the D-isomer in 85% ee using *Candida guilliermondii*.[6] Given that no attempts were made in these experiments to optimise either yields or optical purities of products in these experiments by methods that have been shown to be effective with yeasts (*e.g.* slow addition of substrate, immobilisation), the clostridial system is clearly susceptible of considerable further improvement.

Experimental

^1H NMR spectroscopy was carried out at 220 MHz using a Perkin-Elmer R34 spectrometer, or at 400 MHz using a Bruker WH400 spectrometer. ^{13}C NMR spectra were determined at 100.62 MHz using a Bruker WH400 spectrometer. *J*-Values are in Hz. Mass spectra were determined using a Kratos MS 80 mass spectrometer. $[\alpha]_D$ Values in 10^{-1} deg cm^2 g^{-1}.

Microorganisms and Growth Media.—The microorganisms used in this study were *Clostridium pasteurianum* ATCC 6013, *C. tyrobutyricum* DSM 1460, *C. kluyveri* NCIB 10680 and *C. kluyveri* DSM 555. The basic mineral salts growth medium contained (g dm^{-1}) KH_2PO_4 (13.6); NH_4Cl (3), $MgSO_4 \cdot 7H_2O$ (0.4), biotin (0.001), *p*-aminobenzoic acid (0.001) and trace elements solution as described by Vishniac and Santer,[7] but containing only 0.2% $ZnSO_4 \cdot 7H_2O$ (2 cm^3).

For the growth of *C. pasteurianum*, the above medium was supplemented with glucose (40 g dm^{-3}). For the growth of *C. tyrobutyricum* the medium was supplemented with glucose (25 g dm^{-3}) and yeast extract (5 g dm^{-3}), or crotonic acid (10 g dm^{-3}) and yeast extract (5 g dm^{-3}). For the growth of *C. kluyveri* (both strains) the medium was supplemented with yeast extract (1 g dm^{-3}), ethanol (15 g dm^{-3}) and sodium acetate trihydrate (17.5 g dm^{-3}). All media were adjusted to pH 6.8 before inoculation. Cultures were grown in batch mode at 35 °C without shaking under an atmosphere of 95% N_2, 5% CO_2, and harvested at the end of exponential growth phase (16–50 h of incubation, depending on the microorganism) by centrifugation.

Synthesis of Methyl 4-(4-*Chlorophenylthio*)-3-*oxobutanoate* **1**.—Freshly distilled methyl 4-chloro-3-oxobutanoate (27.5 g, 0.25 mmol) was added to a stirred solution of 4-chlorothiophenol (36.1 g, 0.25 mol) in dry pyridine (30 cm^3). The mixture was stirred for 2 h, and treated with ether (100 cm^3) and water (75 cm^3). The organic layer was washed with hydrochloric acid (1 mol dm^{-3}; 4 × 50 cm^3), dried ($MgSO_4$) and evaporated. The crude product was purified by flash chromatography (Kieselgel 60 silica (Merck, 230–400 mesh)) with ethyl acetate–light petroleum (b.p. 40–60 °C) (1:9) as eluent. The ester **1** was obtained in 85% yield, m.p. 49–50 °C; δ_H(220 MHz, $CDCl_3$) 7.32 (4 H, s, Ar), 3.83 (2 H, s, 4-H), 3.78 (3 H, s, OCH_3) and 2.69 (2 H, s, 2-H); δ_C($CDCl_3$) 197.0 (C-3), 167.0 (C-1), 133.1, 132.4, 131.1, 129.1 (C-aromatic), 52.2 (OCH_3), 46.1 (C-2) and 43.8 (C-4); *m/z* (EI) 258 (M^+), 226, 200, 184 and 157; ν_{max}/cm^{-1} 2940w, 1745s, 1715s, 1650w, 1620w, 1480m, 1325m, 1095s and 1015s; λ_{max}(MeCN)/nm (ε/dm^3 mol^{-1} cm^{-1}) 220 (7200) and 257 (10 500) (Found: C, 51.3; H, 4.35; Cl, 14.6; S, 12.3. $C_{11}H_{11}ClO_3S$ requires 51.07; H, 4.29; Cl, 13.70; S, 12.39%).

Synthesis of Methyl (±)-4-(p-*Chlorophenylthio*)-3-*hydroxybutanoate* (*as* **2**).—To a solution of the β-keto ester **1** (10 mmol) in tetrahydrofuran (100 cm^3) and methanol (25 cm^3) at −20 °C (CCl_4–CO_2) was added sodium borohydride (5 mmol). After 2 h the reaction mixture was quenched with hydrochloric acid (1 mol dm^{-3}; 50 cm^3) and extracted with ether. The ether extracts were dried ($MgSO_4$) and evaporated under reduced pressure. The product was purified by flash chromatography [ethyl acetate–light petroleum (b.p. 40–60 °C)], m.p. 40.5–41.7 °C (CH_2Cl_2–pentane or ether–pentane); δ_H(220 MHz, $CDCl_3$) 2.18 (2 H, m, 2-H_2), 3.10 (2 H, m, 4-H_2), 3.21 (1 H, d, *J* 4.8, OH), 3.78 (3 H, s, OCH_3), 4.20 (1 H, m, 3-H) and 7.45–7.35 (4 H, m, ArH); δ_C($CDCl_3$) 40.0 (C-2), 40.8 (C-4), 52.2 (OMe), 66.9 (C-3), 129.5, 131.4, 133.0, 134.3 (Ar-C) and 172.7 (C-1); *m/z* (EI) 260 (M^+), 242, 183, 158 and 143; λ_{max}(MeCN)/nm (ε/dm^3 mol^{-1} cm^{-1}) 218 (7000) and 260 (12 000); ν_{max}($CHCl_3$)/cm^{-1} 3550br, 2960w, 2930w, 1732s, 1480s, 1440s, 1390w, 1100s, 1050m and 1010m (Found: C, 50.8; H, 4.8; Cl, 13.3; S, 12.2. $C_{11}H_{13}ClO_3S$ requires C, 50.67; H, 5.03; Cl, 13.60; S, 12.30%).

Methyl (R)- *and* (S)-4-(p-*Chlorophenylthio*)-3-*hydroxybutanoates*.—These compounds were prepared by reduction with *Saccharomyces cerevisiae* or *Candida guilliermondii* respectively.[6]

Methyl (*R*)-4-(*p*-chlorophenylthio)-3-hydroxybutanoate: spectral data as for the racemate; m.p. 58.5–59.0 °C (CH_2Cl_2–pentane); $[\alpha]_D$ = 5.5° ± 1.0 (*c* 1.0, $CHCl_3$).

Methyl (*S*)-4-(*p*-chlorophenylthio)-3-hydroxybutanoate: spectral data as for the racemate; m.p. 56.0–57.0 °C (diethyl ether–pentane); $[\alpha]_D$ = −5.9 ± 1.0 (*c* 1.0, $CHCl_3$).

Reductive Desulfurisation of the Esters **2**, **3** *by Raney nickel W7: General Procedure.*—To a solution of sodium hydroxide (1.28 g, 32 mmol) in water (5 cm^3) was carefully added nickel-aluminium alloy (1 g) and the suspension was stirred for 30 min. The mixture was decanted and the Raney nickel was washed with water (5 cm^3 portions) until the washings were neutral, followed by ethanol (3 × 10 cm^3) and anhydrous methanol (1 × 10 cm^3). To the Raney nickel was added a solution of the ester (0.1 g) in methanol (10 cm^3) at 0 °C and the mixture was stirred for 4 h. The Raney nickel was filtered off and the filtrate was concentrated under reduced pressure. Flash chromatography of the residue [ethyl acetate–light petroleum (b.p. 40–60 °C) (3:7)] gave the 3-hydroxybutanoate typically in 80% yield.

Biotransformation Procedures.—Method A. Harvested cells were resuspended to a concentration of 10–30 g dry weight per dm^3 in an anaerobic buffer (potassium phosphate, 100 mmol dm^{-3}, pH, 7.0, plus $MgSO_4$, 1 mmol dm^{-3}). Of the resulting cell suspensions, 50 cm^3 volumes were dispensed into glass pressure bottles (each 160 cm^3 volume) and the tops were sealed with butyl rubber stoppers and aluminium crimps. The gas headspace was replaced with hydrogen by evacuating and flushing five times. The substrate (125 mg) was added as an acetone solution (1 cm^3) by injection through the stopper using a hypodermic needle and syringe. The hydrogen atmosphere above the cells was pressurised to 2 bar and the bottles were incubated with shaking (60 oscillations min^{-1}) at 35 °C for 48 h. Where indicated, methyl viologen was added at a concentration of 0.25 mg cm^{-3} before addition of substrate. Methyl viologen was instantly reduced by cells in the presence of hydrogen as indicated by the development of a deep blue colour.

Method B. Harvested cells (0.8 g dry weight) were resuspended in anaerobic buffer (30 cm^3) as in method A but without $MgSO_4$, together with tetracycline (0.75 mg) and methyl viologen (30 μmol) under hydrogen. The ester **1** (500 mg) was added and the mixture was incubated with shaking in a 170 cm^3 flask.

Method C. Wet packed cells (2.1 g) were suspended in potassium phosphate buffer (19.3 cm^3) as above and under a nitrogen atmosphere. The ester **1** (258 mg) was added. Ethanol (7.5 mmol) was added and the flask was incubated as in method B.

Isolation and Analysis of the Product.—Cells were removed by centrifugation and the supernatant was extracted twice with diethyl ether (equal volumes). The extracts were dried (Na_2SO_4) and evaporated to dryness under reduced pressure. The crude product was purified by chromatography over silica gel with ethyl acetate–light petroleum (b.p. 40–60 °C) (3:7) as eluent. TLC analysis of the product was carried out using silica gel 60 plates (Merck) which were developed using the solvent system ethyl acetate–hexane (1:1). Compounds were visualised by spraying the plates with phosphomolybdic acid (10% in ethanol) and heating at 170 °C. The enantiomeric purity of the product hydroxy ester was determined by ^1H NMR spectrometry using chiral lanthanide shift reagents. All commercially available shift reagents were tested in deuteriochloroform, carbon tetrachloride and deuteriobenzene at various temperatures. The most effective systems proved to be 10 mol% tris(3-trifluoromethylhydroxymethylene)-(+)-camphorato)-europium(III) or tris(3-(trifluoromethylhydroxymethylene)-(+)-camphorato)ytterbium(III) in deuteriobenzene, or a one-third molar amount of tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato]europium(III) in CCl_4–benzene (12:1). The observation signal was the methyl group of the methyl ester function. In all cases, this signal in the L-(3*R*) enantiomer was shifted further downfield than the corresponding signal in the D-(3*S*) enantiomer. The optical purity was also determined by polarimetry.

References

1 D. H. G. Crout and M. Christen, in *Modern Synthetic Methods 1989*, ed. R. Scheffold, Springer Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo, 1989, p. 1.
2 S. Servi, *Synthesis*, 1990, 1.
3 H. Simon, B. Rambeck, H. Hashimoto, H. Günther, G. Nohynek and H. Neumann, *Angew. Chem., Int. Ed. Engl.*, 1974, **13**, 608; H. Giesel, G. Machacek, J. Bayerl and H. Simon, *FEBS Lett.*, 1981, **123**, 107; S. Simon, H. Günther, J. Bader and W. Tischer, *Angew. Chem., Int. Ed. Engl.*, 1981, **20**, 861; I. Thomas and H. Simon, *Angew. Chem., Int. Ed. Engl.*, 1986, **25**, 462; H. Simon, H. White, H. Lebertz and I. Thanos, *Angew Chem., Int. Ed. Engl.*, 1987, **26**, 785; I. Thanos, J. Bader, H. Günther, S. Neumann, F. Krauss and H. Simon, *Methods Enzymol.*, 1987, **136**, 302.
4 A. Belan, J. Bolte, A. Fauve, J. G. Gourcy and H. Veschambre, *J. Org. Chem.*, 1987, **52**, 256.
5 A. Fauve and H. Veschambre, *Biocatalysis*, 1990, **3**, 95.
6 M. Christen and D. H. G. Crout, *J. Chem. Soc., Chem. Commun.*, 1988, 264.
7 W. Vishniac and M. Santer, *Bacteriol. Rev.*, 1957, **21**, 195.

Paper 1/04622E
Received 5th September 1991
Accepted 18th October 1991