



Stereochemical control in diastereoselective reduction of α -substituted- β -ketoesters using a reductase purified from *Kluyveromyces marxianus*

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Abstract

An NADPH-dependent carbonyl reductase that shows (3*R*)-selective reducing activity for α -substituted- β -ketoesters was purified from *Kluyveromyces marxianus* and racemic alkyl 2-substituted-3-oxobutanoates were reduced to the corresponding (2*S*,3*R*)- or (2*R*,3*R*)-2-substituted-3-hydroxybutanoates with enantiomeric purity (> 99%) and diastereoselectivity (24 ~ 98%).

Introduction

Asymmetric synthesis with biocatalysts is becoming more important because of its potential in environmentally-benign organic synthesis. Of many types of transformations with biocatalysts (Klibanov 1990, Faber 1995, Rensburg *et al.* 1997), asymmetric reduction of carbonyl compounds with microorganisms, such as bakers' yeast (*Saccharomyces cerevisiae*), is useful to prepare optically active alcohols (Servi 1990, Csuk & Glanzer 1991). Although the bakers' yeast whole-cell reduction is inexpensive and convenient, stereoselectivity is often incomplete owing to the several oxidoreductases contained in the yeast cells. On the other hand, enzymatic reduction using a purified reductase usually shows high stereoselectivity (Nakamura *et al.* 1995, Ema *et al.* 1998). Complete stereoselectivity, as frequently observed in enzymatic reactions, is one of the requirements for modern asymmetric synthesis.

Many researchers in this area have examined the bakers' yeast reduction of numerous α -substituted- β -ketoesters, where the substituents were alkyl, benzyl, allyl, or thioalkyl derivatives (Servi 1990). In most cases, the reduction of the ketone carbonyl group is completely enantioselective, in accordance with Prelog's rule, giving rise to secondary alcohols with (*S*)-absolute configuration (Larcheveque & Henrot 1987). The absolute configuration of the second stereogenic center, i.e., the C-2 position, is much less well defined, and is dependent on the substituent. In this paper, we report an uncommon enantioselective and diastereoselective reduction of α -substituted- β -ketoesters to secondary alcohols with (*R*)-absolute configuration.

We have been looking for reductases that can provide secondary alcohols of (*R*)-configuration from α -substituted- β -ketoesters, and have found that the yeast, *Kluyveromyces marxianus*, gives in part the alcohol of desired configuration. From the microorganism we have now purified an enzyme that showed

an appropriate reducing character. The enzyme assay for the cell-free extract of the yeast using UV spectroscopy revealed that the reductase requires NADPH as a coenzyme.

Material and methods

Purification method of enzyme

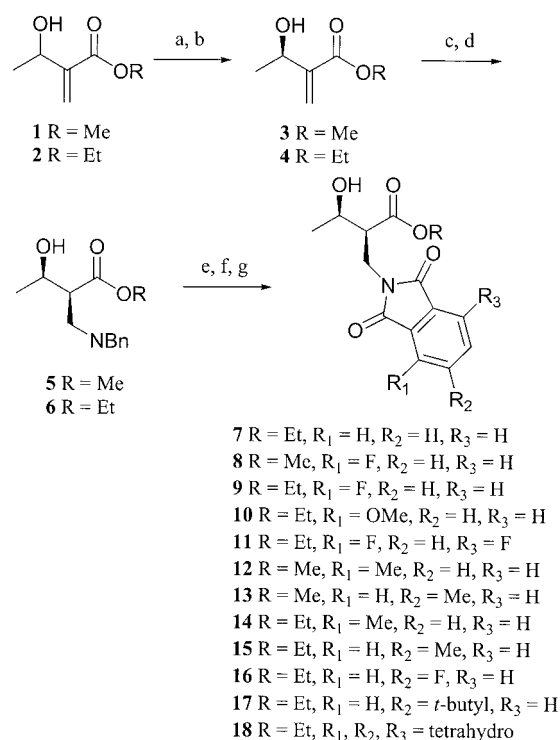
Kluyveromyces marxianus was harvested from 8 l medium by centrifuging at 3000 g for 10 min. Wet cells (64 g) were subjected to French Press and then sonication on Branson sonifier (Model 450). The crude lysate was purified through sequential treatment with Q-Sepharose Fast Flow, phenyl Sepharose, HiTrap Blue column, Red Sepharose, and gel-filtration column chromatography (Superdex 75). The fractions of active enzyme from Superdex 75 were collected and further purified by the native gel electrophoresis.

Synthesis of the authentic compounds

Authentic compounds were synthesized to identify the products from the enzymatic reduction (Scheme 1). Acylation of alkyl 2-(1-hydroxyethyl)acrylates **1** and **2**, the Baylis–Hillman products (Basavaiah *et al.* 1996), and enzymatic hydrolysis (Masayoshi *et al.* 1990) of the resulting acetates provided (*R*)-2-(1-hydroxyethyl)acrylates **3** and **4**, respectively. Silylation of the hydroxyl group and conjugate addition of benzylamine to the acrylates **3** and **4** in methanol at room temperature afforded the adducts **5** and **6** with virtually complete anti-diastereoselectivity (Perlmutter & Tabone 1995). Hydrogenolytic removal of the benzyl group and phthalimide formation of the resulting amines with various phthalic anhydrides followed by deprotection of tertiarybutyldimethylsilyl group using *p*-toluenesulfonic acid gave the authentic compounds **7–18**.

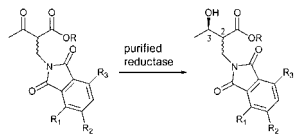
General method for enzymatic reaction

To an aqueous solution of the purified reductase (1.5 U with respect to the substrate), NADPH (2.1 mg, 2.5 mmol) in 10 mM phosphate buffer (pH 6.8, 25 ml) was added the substrate (0.25 mmol). The mixture was shaken at 30 °C for the appropriate reaction time (11–24 h). The progress of the reaction was monitored by HPLC and TLC. After saturating the solution with NaCl, the crude product was extracted with ethyl acetate (5 × 1 ml). The combined



Scheme 1. Reagents and conditions: (a) acetyl chloride, pyridine, dichloromethane, 0 °C, 1 h, 95%. (b) 'Lipase P' (*Pseudomonas* sp., Amano pharmaceutical Co.), phosphate buffer pH 6.8, 23 °C, 55 h, 22%. (c) Tertiarybutyldimethylsilyl chloride, *N,N*-dimethylformamide, *N,N*-dimethylaminopyridine, triethylamine, 0 °C, 10 h, 89%. (d) Benzylamine, methanol, 23 °C, 72 h, 82%. (e) Hydrazine monohydrate, ethanol, reflux, 1 h, 72%. (f) Phthalic anhydride chloroform, reflux. (g) Acetone/water (v/v, 5/1), *p*-toluenesulfonic acid (catalytic amounts).

organic phase was dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was separated by HPLC using a chiral column (Daicel chiral OD) to give the product (syn/anti). **7a** (syn); ¹H NMR (600 MHz, CDCl₃) 7.80–7.78 (m, 2H), 7.68–7.66 (m, 2H), 4.09–3.92 (m, 5H), 2.66–2.60 (m, 1H), 1.19 (d, *J* = 6.2 Hz, 3H), 1.12 (t, *J* = 7.2 Hz, 3H); HRMS FAB (M+H)⁺, calcd. for C₁₅H₁₇NO₅ 291.1107, found 291.1109; **7b** (anti); ¹H NMR (600 MHz, CDCl₃) 7.80–7.79 (m, 2H), 7.68–7.66 (m, 2H), 4.09–4.05 (m, 3H), 3.93–3.88 (m, 2H), 2.78–2.75 (m, 1H), 1.25 (d, *J* = 6.5 Hz, 3H), 1.08 (t, *J* = 7.2 Hz, 3H); HRMS FAB (M+H)⁺, calcd. for C₁₅H₁₇NO₅ 291.1107, found 291.1110.

Table 1. Asymmetric reduction of α -substituted- β -ketoesters using the purified reductase.


Entry	Substrates				Product a(syn) b(anti)	Purified reductase		
	R	R ₁	R ₂	R ₃		Time (h)	Syn/anti (2 <i>S</i> ,3 <i>R</i>)/(2 <i>R</i> ,3 <i>R</i>)	CR ^a (%)
1	C ₂ H ₅	H	H	H	7a : 7b	18	20 : 80	98
2	CH ₃	F	H	H	8a : 8b	19	76 : 24	87
3	C ₂ H ₅	F	H	H	9a : 9b	11	64 : 36	94
4	C ₂ H ₅	OCH ₃	H	H	10a : 10b	20	62 : 38	99
5	C ₂ H ₅	F	H	F	11a : 11b	14	87 : 13	93
6	CH ₃	CH ₃	H	H	12a : 12b	24	22 : 78	99
7	CH ₃	H	CH ₃	H	13a : 13b	24	31 : 69	99
8	C ₂ H ₅	CH ₃	H	H	14a : 14b	24	18 : 82	67
9	C ₂ H ₅	H	CH ₃	H	15a : 15b	24	23 : 77	99
10	C ₂ H ₅	H	F	H	16a : 16b	24	28 : 72	99
11	C ₂ H ₅	H	C(CH ₃) ₃	H	17a : 17b	24	>1 : 99	64
12	C ₂ H ₅	3,4,5,6-tetrahydro			18a : 18b	24	>1 : 99	36

^aConversion rate.

Analytical techniques

HPLC

Analysis of reference compounds and products were carried out using a HPLC with a Chiralcel OD 5 μm , 25 cm \times 4.6 mm Daicel chromatography column using *n*-hexane 2-propanol (98/2, v/v) at 1 ml min⁻¹ for elution at 40 °C. The mobile phase was degassed by helium for 30 min. Compounds were detected at 220 nm.

NMR spectroscopy techniques

¹H NMR and ¹³C NMR were carried out on Bruker DPX-300 or 600 MHz spectrometer with tetramethylsilane as internal standard. Samples of products (1 mg) and reference samples (10 mg) were dissolved in chloroform, CDCl₃.

GC-MS and MS

High resolution mass spectra were determined with a JEOL, JMS-AX505WA using chemical ionization (CI+) and FAB mode. GC-MS was carried out on a HP 5890 Series II.

Results and discussion

Firstly the purified reductase was used to reduce ethyl 2-phthalimidomethyl-3-oxobutanoate (Table 1, entry 1). The reductase successfully reduced the β -keto group of the substrate to (*R*)-alcohol, whereas baker's yeast gave the corresponding (*S*)-alcohol. The products were identified by HPLC on a chiral column. The peaks were assigned by comparing with the authentic compounds prepared above and their racemates prepared by sodium borohydride reduction. To the best of our knowledge, this is the first example of the enzyme giving (*R*)-alcohol in this substrate system. In the case of entry 5, (2*S*,3*R*)-compound, **11a**, can be used for the synthesis of (3*S*,1'*R*)-3-(1'-hydroxyethyl)azetidino-2-one as a key intermediate of carbapenem antibiotics (Perlmutter & Tabone 1995, Fuganti *et al.* 1993).

For the investigation on diastereoselectivity, we carried out the reduction with purified enzyme varying C-2 substituents and esters. The results are summarized in Table 1.

First of all, methyl and ethyl groups of the ester did not significantly alter the diastereomeric selectivity of the reduction either by the purified enzyme or by baker's yeast. For the baker's yeast whole-cell reduction,

R₁, R₂ or R₃ on the phenyl ring of phthalimido group at the C-2 position did not greatly affect the diastereomeric ratio of the product. In case of the purified reductase, fluorine substitution at the phenyl ring was critical. Comparing the results of the entries 2, 3, 5 and 10, fluorine substituents as R₁ and/or R₃ remarkably showed reversed syn diastereoselectivity, while fluorine as R₂ (entry 10) gave little effect. Interestingly, the methoxy substituent as R₁ (entry 4) also showed syn selectivity to the extent comparable to the monofluorinated substrates (entries 2 and 3), while the substrates bearing methyl group (entries 6–9) retained the syn/anti ratio of 20:80. The stereochemical outcomes seemed to have resulted from some other factor than the electronic effect of the substituents. The difluorinated substrate (entry 5) showed diastereoselectivity most favorable to the syn (2*S*,3*R*)-product.

In particular, the substrates **17** and **18** bearing *t*-butyl and 3,4,5,6-tetrahydro (entries 11 and 12) showed exclusive anti-diastereoselectivity for the enzymatic reaction. We can expect that this diastereoselective reduction may be a highly effective method to obtain (2*R*,3*R*)-2-substituted-butanoates.

Further applications of this enzymatic reduction are currently under investigation.

Acknowledgements

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