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RESEARCH ARTICLE

Biocatalysed reductions of α-ketoesters employing Cyrene™ as cosolvent

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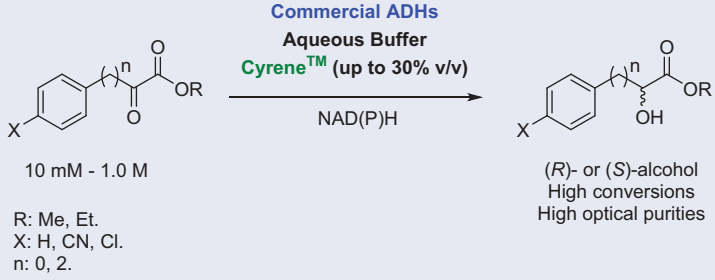
ABSTRACT

The search for novel reaction media with environmental friendly properties is an area of great interest in enzyme catalysis. Water is the medium of biocatalysed processes, but due to its properties, sometimes the presence of organic (co)solvents is required. Cyrene™ represents one of the newest approaches to this *medium engineering*. This polar solvent has been employed for the first time in biocatalysed reductions employing purified alcohol dehydrogenases. A set of α-ketoesters has been reduced to the corresponding chiral α-hydroxyesters with high conversions and optical purities, being possible to obtain good results at Cyrene contents of 30% *v/v* and working at substrate concentrations of 1.0 M in presence of 2.5% *v/v* of this solvent. At this concentration, the presence of Cyrene has a beneficial effect in the bioreduction conversion.

GRAPHICAL ABSTRACT



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Biobased solvents; alcohol dehydrogenases; biocatalysis; solvent engineering; hydroxyesters; bioreductions

1. Introduction

In the last few years, the development of processes catalyzed by biological systems as purified enzymes or whole cells has experienced a great development (de Gonzalo and Domínguez de María 2017; Devine 2018; Sheldon and Woodley 2018). Water is the natural medium in which these reactions are performed. But this solvent can present some drawbacks for the application of biocatalysts in organic reactions. Thus, water is a very polar solvent, which generates solubility issues. In addition, some side-reactions (hydrolysis) can occur in aqueous media. By these reasons, since the seminal discoveries by Klibanov et al. in the 80 s of the last century, organic solvents have appeared as a valuable reaction medium for carrying out biocatalyzed procedures (Zaks and Klibanov 1985; Carrea and Riva 2000). Environmental concerns have pushed for the developed of greener alternatives to the classical organic solvents, including for instance the use of neoteric or supercritical solvents (Hernáiz 2010; Itoh 2017; Gotor-Fernández and Paul 2019). But these two approaches do not represent a proper alternative and a requirement of organic solvents with the same properties and a much lower environmental footprint as the biobased solvents, that is those solvents obtained from natural resources (Lomba 2019; Sheldon 2019), has appeared in the recent years. The use of 2-methyltetrahydrofuran (2MeTHF) (Pace 2012) or cyclopentyl methyl ether (CPME) (de Gonzalo 2019) in biocatalyzed reactions has been documented in several examples, as well as other biobased solvents with interesting applications in Green Chemistry (Jerome and Luque 2017; Iemhoff et al. 2018). One recent example of this biobased approach is dihydrolevoglucosenone (Cyrene™), a polar aprotic solvent similar to *N*,*N*-dimethylformamide (Sherwood 2014). Cyrene can be obtained from natural sources, as it can be produced by pyrolysis and/or hydrogenation of cellulose (Kudo 2017). Cyrene presents a high solubility in water, forming a set of derivatives in this solvent whose

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proportions will modify the polarity of the reaction media, being able by this reason to solve a wide set of compounds (de Bruyn 2019). This fact can also have effect on the catalytic processes in which Cyrene is employed as (co)solvent. Until nowadays, very few examples of the Cyrene application as (co)solvent in biocatalysis have been shown. In 2020, Guajardo and Domínguez de María have developed the lipase-catalyzed esterification of benzoic acid and glycerol in presence of Cyrene concentrations up to 40% *v/v* (Guajardo and Domínguez de María 2020), but no examples of its use in reductive processes catalyzed by alcohol dehydrogenases (ADHs) have been described. ADHs, also called ketoreductases (KREDs) are able to catalyze the reversible reduction of carbonyl compounds to the corresponding chiral alcohols in presence of nicotinamides as cofactors (Musa and Phillips 2011; Zheng 2017; de Gonzalo and Lavandera 2020). ADHs have been widely used in synthetic methodologies for the preparation of alcohols with different structures. Thus, we have analysed the application of Cyrene as cosolvent in the bioreduction of a set of α-ketoesters to the corresponding optically active α-hydroxyesters, which are versatile products that find application in the chemical, food, and pharmaceutical industries, as for instance, antibiotics and bioactive compounds (Desage-El Murr 2003; Mallinger 2008).

2. Experimental section

2.1. Materials and methods

Purified alcohol dehydrogenases (Codex® KRED Screening Kit) were purchased from Codexis Inc. Cyrene™ and α-ketoesters **1-5a** were commercially available at Sigma-Aldrich. The rest of reagents and solvents were products from Sigma-Aldrich and TCI. Racemic α-hydroxyesters (±)-**1-5 b** were obtained by reduction of the α-ketoesters employing sodium borohydride in methanol with high yields (75-92%), and exhibited physical and spectral properties in accordance with those reported for (±)-**1-2 b** (Zhang 2014), (±)-**3-4b** (Francesco 2008) and (±)-**5b** (Chadha and Baskar 2002).

GC/MS analyses were performed with a GC Hewlett Packard 7890 Series II equipped with a Hewlett Packard 5973 chromatograph MS (Agilent Technologies) using a HP-5MS cross-linked methyl siloxane column (30 m × 0.25 mm × 0.25 μm, 1.0 bar N_2). To monitor levels of conversion, substrates and products were quantified by use of calibration curves. The following temperature program was employed: 50 °C (5 min), 10 °C/min to 200 °C (7 min). t_R (**1a**): 14.9 min; t_R (**1b**); 15.3 min; t_R (**2a**): 14.3 min; t_R (**2b**): 14.8 min; t_R (**3a**): 18.1 min; t_R (**3b**): 18.8 min; t_R (**4a**): 16.8 min; t_R (**4b**): 17.3 min; t_R (**5a**): 14.3 min; t_R (**5b**): 14.5 min.

HPLC analyses were performed on a Waters 2695 Instrument equipped with a Waters 996 Photodiode Array Detector. To determine the enantiomeric excesses of chiral α-hydroxyesters **1–5b**, a Chiralcel OD (25 × 0.46 cm, Daicel) column was employed, with the following conditions: *n*-hexane:IPA 95:5, 1.0 mL/min, 30 °C. t_R (*S*)-**1b**: 7.8 min; t_R (*R*)-**1b**: 13.2 min; t_R (*S*)-**2b**: 8.6 min; t_R (*R*)-**2b**: 12.9 min; t_R (*S*)-**3b**: 15.8 min; t_R (*R*)-**3b**: 18.1 min; t_R (*S*)-**4b**: 12.7 min; t_R (*R*)-**4b**: 15.3 min; t_R (*S*)-**5b**: 9.7 min; t_R (*R*)-**5b**: 13.8 min. The configuration of the α-hydroxyesters were established by comparing the HPLC chromatograms with the previously described (Chadha and Baskar 2002).

2.2. General procedure for the bioreduction of ketoesters 1–5a catalyzed by ADHs using isopropanol as cosubstrate

Unless otherwise stated, α-ketoesters **1-5a** (10–1000 mM) were dissolved in IPA (100 μL) and Cyrene (25-300 μL). The corresponding ADH from Codex® KRED Screening Kit (10 mg) was dissolved in KRED Recycle Mix P pH 7.0 phosphate Buffer (600–875 μL) and the substrate solution was added to the enzyme solution. Reactions were stirred at 30 °C and 220 rpm for 24 hours. Reactions were then extracted with EtOAc (2 × 0.5 mL), dried onto Na_2SO_4 and the samples were directly analysed by GC/MS and HPLC in order to determine the level of conversion as well as the enantiomeric excesses of α-hydroxyesters (*S*)- or (*R*)-**1-5b** depending on the biocatalyst employed.

2.3. General procedure for the bioreduction of ketoesters 1-5a catalyzed by ADHs using glucose/glucose dehydrogenase as cofactor recycling system

Unless otherwise stated, ketoesters **1-5a** (10–50 mM) were dissolved in KRED Recycle Mix N Buffer pH 7.0 phosphate containing Cyrene (2.5–10% *v/v*) up to a final volume of 1.0 mL. The suspension was added to the corresponding ADH from Codex® KRED Screening Kit (10 mg) and the resulting mixture was stirred at 30 °C and 220 rpm for 24 hours. Reactions were then extracted with EtOAc (2 × 0.5 mL), dried onto Na_2SO_4 and the samples were directly analyzed by GC/MS and HPLC in order α-hydroxyesters (*R*)-**1-5b**.

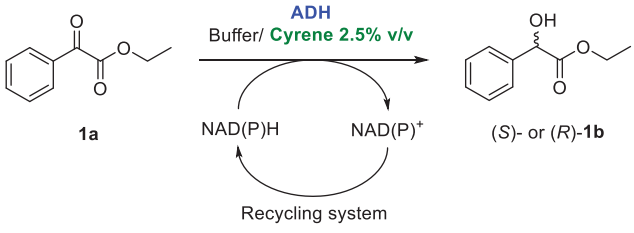
2.4. Bioreduction of ethyl benzoylformate to ethyl 2-hydroxy-2-phenylacetate at multimilligram scale catalysed by P2-D03 in buffer containing 2.5% v/v cyrene

Ethyl benzoylformate **1a** (1.0 M, 178.1 mg) was dissolved in Cyrene (25 μL) and IPA (100 μL) and added into a suspension of P2-D03 (10 mg) in KRED Recycle Mix P pH 7.0 phosphate buffer (875 μL). The reaction was stirred for 24 hours at 30 °C and 220 rpm. Water (3 mL) was added to the reaction mixture and it was extracted with EtOAc (6 × 2.0 mL). The organic phases were dried onto Na_2SO_4 and solvent was removed under reduced pressure. The crude mixture was purified by column chromatography employing *n*-hexane:EtOAc 8:2 as eluent to obtain 127.9 mg of (*S*)-ethyl 2-hydroxy-2-phenylacteate (**1b**, 71% yield) with 90% enantiomeric excess.

3. Results and discussion

Initial experiments were devoted to analyse the effect of Cyrene at 2.5% *v/v* content on the bioreduction of a α-ketoester as ethyl benzoylformate (**1a**). This process was catalyzed by different commercially available ADHs (Codex® KRED Screening Kit from Codexis Inc.). Reactions were carried out using two different nicotinamide cofactor [NAD(P)H] recycling systems. As most of these commercial biocatalysts were tolerant to isopropanol (IPA), a substrate-coupled approach for NADPH regeneration in presence of 10% *v/v* of this compound was employed. For the rest of biocatalysts tested, nicotinamide was recycled by an enzyme-coupled approach, using glucose dehydrogenase (GDH) and glucose as secondary enzymatic system (Scheme 1).

All the KREDs tested in presence of 2.5% *v/v* Cyrene afforded optically active ethyl 2-hydroxy-2-phenylacetate (**1 b**) with complete conversion after 24 hours, indicating that this solvent does not present any negative effect on the biocatalysts activities. Regarding the selectivities achieved, most of IPA-dependent KREDs led to (*S*)-**1b** with optical purities from moderate to high. The best results were achieved in the bioreductions catalyzed by P2-D03 and P2-D12, being possible to obtain the final product with complete conversion and enantiomeric excesses around 90% (entries 1 and 3, respectively). The use of the IPA-dependent dehydrogenases P2-C11, P2-G03 and P1-B02 (entries 2, 4, and 7) led to the (*R*)-enantiomer, but with low or moderate enantioselectivities. Those KREDs that required a secondary enzymatic system for cofactor regeneration afforded the *R* enantiomer of the α-hydroxyester, in most cases with optical purities higher than 80% (entries 12, 14, and 15). The best result was obtained with NADH 101, recovering a 98% of (*R*)-**1b** with 88% *ee*. Thus, by proper selecting the biocatalyst, it was possible to obtain both enantiomers of **1b** with high optical purities and complete conversions in presence of 2.5% *v/v* Cyrene (Table 1).



Scheme 1. Biocatalyzed reduction of ethyl benzoylformate (**1a**) in buffer containing 2.5% v/v Cyrene.

In view of the results obtained, P2-D03 and NADH 101 were further tested in the bioreduction of α-ketoester **1a** modifying some reaction parameters. It has to be established that for these two biocatalysts no Cyrene reduction products were observed after 24 hours, as this solvent contains a keto group in its structure that could be reduced by the ADHs. In a first set of experiments the effect of Cyrene content in the enzymatic reactions when working at 10 mM substrate concentration was analyzed. When employing KRED P2-D03, bioreductions can be performed using up to 30% *v/v* Cyrene. At these conditions (Table 2, entry 5), (*S*)-**1b** was still obtained with good conversion (73%) and only a slight decrease in the product optical purity (83% *ee*) after 24 hours, demonstrating the good performance of this KRED. As shown in Table 2, the use of solvent contents from 5 to 20% *v/v* allowed obtaining **1b** with conversions around 90%, with no effect in the optical purity of (*S*)-**1b** at 5–10% *v/v* cosolvent (entries 2–3) and with 86% *ee* at 20% *v/v*

Table 1. Bioreductions of ethyl benzoylformate (**1a**) catalyzed by commercial alcohol dehydrogenases from Codexis.[a].

Entry	ADH	Recycling	*c* (%)[b]	*ee* (%)[c]	*Configuration*
1	P2-D03	IPA	≥97	90	*S*
2	P2-C11	IPA	≥97	13	*R*
3	P2-D12	IPA	≥97	92	*S*
4	P2-G03	IPA	≥97	50	*R*
5	P2-D11	IPA	≥97	31	*S*
6	P1-C01	IPA	≥97	66	*S*
7	P1-B02	IPA	≥97	47	*R*
8	P1-B05	IPA	≥97	35	*S*
9	P1-H08	IPA	≥97	25	*S*
10	P1-B10	IPA	≥97	22	*S*
11	P2-C02	IPA	≥97	73	*S*
12	KRED 119	Glucose/GDH	≥97	82	*R*
13	NADH 110	Glucose/GDH	≥97	13	*R*
14	KRED 130	Glucose/GDH	≥97	87	*R*
15	NADH 101	Glucose/GDH	96	88	*R*

[a]For reaction details, see experimental section.
[b]Determined by GC/MS.
[c]Determined by HPLC.

Table 2. Effect of Cyrene percentage and **1a** concentration in the bioreductions catalysed by P2-D03, NADH 101 and KRED 130.[a]

Entry	ADH	[1a] (mM)	Cyrene (%)	*c* (%)[b]	*ee* (%)[c]	*Config.*
1	P2-D03	10	2.5	≥97	90	*S*
2	P2-D03	10	5	≥97	90	*S*
3	P2-D03	10	10	96	89	*S*
4	P2-D03	10	20	91	86	*S*
5	P2-D03	10	30	73	83	*S*
6	NADH 101	10	2.5	≥97	88	*R*
7	NADH 101	10	5	68	81	*R*
8	NADH 101	10	10	56	60	*R*
9	NADH 101	20	2.5	53	59	*R*
10	NADH 101	50	2.5	39	41	*R*
11	KRED 130	10	2.5	≥97	87	*R*
12	KRED 130	10	5	≥97	87	*R*
13	KRED 130	10	10	95	80	*R*
14	KRED 130	20	2.5	88	86	*R*
15	KRED 130	50	2.5	84	83	*R*

[a]For reaction details, see experimental section.
[b]Determined by GC/MS.
[c]Determined by HPLC.

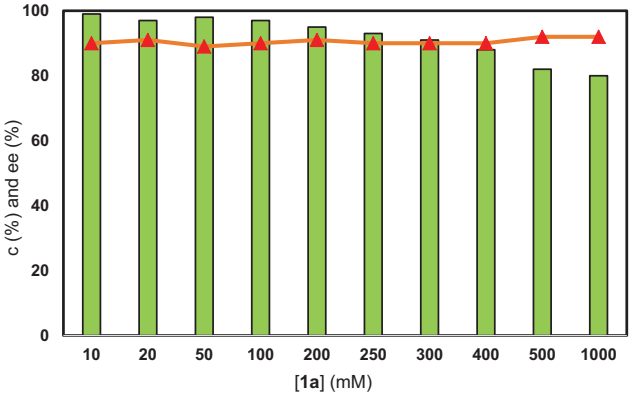


Figure 1. Effect of ethyl benzoylformate (**1a**) concentration in conversion and the optical purity of (*S*)-**1b** in the bioreductions catalyzed by P2-D03 in buffer containing 2.5% *v/v* Cyrene.

(entry 4). Cyrene concentration has a more pronounced effect in the bioreductions catalysed by KRED NADH 101, as shown in entries 7 and 8. Thus, when using only 5% *v/v* of the cosolvent, there is an important drop in the conversion (68% after 24 hours), whereas the optical purity also decreased to 81% *ee*. Further increase in the cosolvent concentration led to the recovery of (*R*)-**1b** with 56% conversion and a much lower enantiomeric excess (60% *ee*).

Substrate concentration was also a parameter to analyse in the bioreduction of **1a** in presence of 2.5% *v/v* of Cyrene. As shown in Figure 1, P2-D03 is a very stable biocatalyst at high substrate concentrations in these conditions. The presence of Cyrene led to excellent conversions (higher than 90%) up to **1a** concentrations of 300 mM. The use of ethyl benzoylformate at 500 mM concentration afforded (*S*)-**1b** with 83% conversion, whereas it is possible to perform the

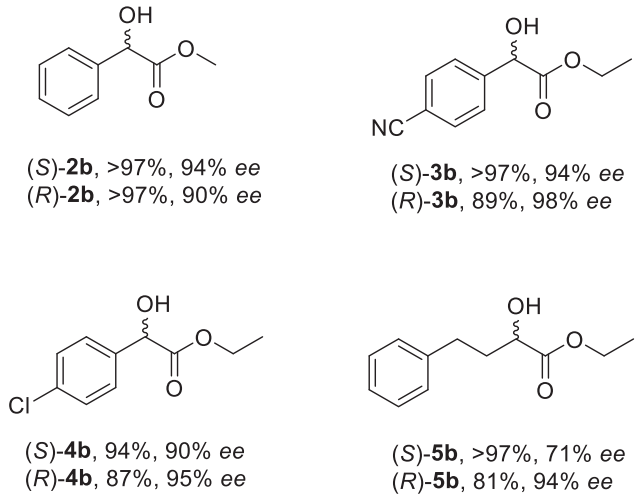


Figure 2. Bioreductions of ketoesters **2-5a** catalyzed by P2-D03 and KRED 130 in buffer containing 2.5% *v/v* Cyrene.

bioreduction of **1a** at 1.0 M with excellent results. Thus, (*S*)-**1b** is recovered at these conditions with 90% *ee* and 80% conversion (71% isolated yield) after 24 hours, with a productivity of 144.0 grams of **1 b**/L day. The conversion and productivity values were higher than those achieved in absence of Cyrene (67% conversion, 120.6 g/L day, 90% *ee*), indicating the beneficial effect of this cosolvent at 2.5% *v/v* in the enzymatic system at high substrate concentration. In view of this result, the reaction was performed using a higher amount of Cyrene in the reaction medium. Thus, the bioreduction of 1.0 M of **1a** in 10% Cyrene was carried out. After 24 hours, (*S*)-**1b** was recovered with 90% *ee* and 60% conversion (108.0 g/L day), lower values than in absence of this solvent. When working with 2.5% v/v Cyrene at lower substrate concentrations (250 or 500 mM), very similar conversions and optical purities were achieved in presence and in absence of Cyrene, with productivities between 40 and 70 g/L day, much lower than in 1.0 M of **1b**. Finally, as can be observed in Figure 1, the presence of 2.5% *v/v* Cyrene in the reductions has no effect on the optical purity of the final product at all **1a** concentrations studied.

The same study was carried out in the bioreductions catalyzed by NADH 101, but as shown in entries 9 and 10 of Table 2, the use of 20 or 50 mM of **1a** led to a fast loss in the biocatalytic properties of this ADH. In view of the low performance of NADH 101 when increasing both **1a** concentration or the amount of Cyrene in the reaction medium, bireductions were also tested in presence of KRED 130, which also led to (*R*)-**1b** with good results (Table 2, entry 11). This biocatalyst seems to be more tolerant to Cyrene, as 10% *v/v* of this cosolvent can be employed with a small loss in

the conversion, but with a more pronounced effect in the optical purity of (*R*)-**1b**, as the α-hydroxyester was recovered with 80% *ee* (entry 13). When **1a** concentration was increased to 20 and 50 mM, (*R*)-**1b** was recovered with conversions and optical purities around 85%, which indicates a slight loss regarding the bioreduction at 10 mM. KRED 130 presents a higher performance than NADH, but it is still sensitive to the cosolvent amount and the substrate concentration.

Bioreductions carried out in 2.5% *v/v* Cyrene catalysed by P2-D03 or KRED 130 were extended to other prochiral ketoesters, as shown in Figure 2. Thus, methyl benzoylformate (**2a**) was selectively reduced to (*S*)-**2b** by P2-D03 in presence of this cosolvent with a better result than its ethyl analogue, as after 24 hours, a complete conversion was achieved for the (*S*)-hydroxyester with 94% *ee*, whereas the bioreduction catalysed by KRED 130 afforded (*R*)-methyl 2-hydroxy-2-phenylacetate with 90% *ee* and excellent conversion. The reactions catalysed by P2-D03 of ethyl benzoylformate derivatives presenting substituents at the aromatic ring, as ethyl 4-cyanobenzoylfomate (**3a**) or ethyl 4-chlorobenzoylformate (**4a**) also afforded the corresponding (*S*)-hydroxyesters with high conversions and excellent selectivities, as shown in Figure 2. When KRED 130 was tested as biocatalyst, both (*R*)-**3b** and (*R*)-**4b** were recovered with conversions close to 90% and high optical purities. The enzymatic processes were also tested with an α- ketoester in which the aromatic ring is not conjugated to the carbonyl moiety, as of ethyl 4-phenyl-2-oxobutanonate (**5a**). This compound led to the (*S*)-hydroxyester with moderate selectivity, as (*S*)-**5b** was recovered with a moderate optical purity (71% *ee*). When the reaction was performed in presence of KRED 130, the (*R*)-enantiomer was recovered with much better optical purity (95% *ee*) and 81% conversion after 24 hours.

Bioreduction of α-ketoesters **2-5a** catalysed by P2-D03 was also tested in buffer containing 2.5% *v/v* Cyrene at 1.0 M substrate concentration. After 24 hours, it was possible to recover all the α-hydroxyesters with conversions between 70 and 80%. Optical purities of compounds (*S*)-**2-5b** showed similar values to those obtained in the bioreductions carried out at 10 mM, demonstrating the applicability of this cosolvent for developing the processes at high substrate concentrations.

4. Conclusion

In the present paper, Cyrene™, a biobased solvent obtained from renewable sources, has demonstrated to be a valuable cosolvent in the bioreduction of α-ketoesters to the corresponding optically active α-hydroxyesters catalyzed by different commercially available alcohol dehydrogenases. When this solvent was employed in 2.5% *v/v*, it is possible to obtain both the (*S*)- and the (*R*)-hydroxyesters with complete conversion and high optical purities depending on the ADH employed. Bioreductions catalyzed by the IPA-tolerant alcohol dehydrogenase KRED P2-D03 can be carried out up to 30% *v/v* of Cyrene with only a small loss in the biocatalyst properties, whereas substrate concentration can be increased up to 1.0 M, obtaining a higher conversion in presence of this cosolvent than in its absence and a maximum productivity of 144.0 grams of chiral alcohol produced per litre and day. These results indicate that this biobased solvent is a suitable option for developing further enzymatic bioreductions catalyzed by isolated ADHs. The search for novel biocatalysts (both wild types or mutants) or processes able to be conducted in sustainable solvents must be an area of high interest for the research groups focussed on enzyme technologies, with the aim of developing more efficient biocatalytic procedures compatible with the Green Chemistry principles.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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