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Resolution of (±)-*trans*-Sobrerol by Lipase PS-Catalyzed Transesterification and Effects of Organic Solvents on Enantioselectivity

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ABSTRACT. Resolution of the mucolytic drug (±)-*trans*-sobrerol (**1**) was achieved by transesterification with vinyl acetate in organic media, catalyzed by free or immobilized Lipase PS. The enantioselectivity of the enzyme was markedly influenced by the nature of the organic solvent, but there was no correlation between enantiomeric ratio values (70-500) and either the hydrophobicity or the dielectric constant of the medium. With the enzyme immobilized onto Hyflo Super Cell and *t*-amyl alcohol as the solvent, the selectivity of Lipase PS for (-)-**1** was extremely high and, at 50 % conversion both (-)-*trans*-sobrerol and (+)-*trans*-sobrerol monoacetate were obtained in practically 100 % optical purity

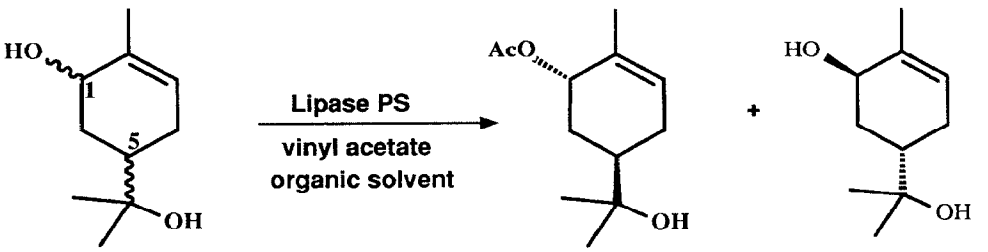
INTRODUCTION

In recent years the use of lipases, esterases, and proteases for resolution of racemic alcohols, acids, and esters (carried out in aqueous or organic media) has been continuously growing[1]. Medicinal chemistry has been, and is, an ideal area for this biocatalytic method, since enantiomers may have different biological activities[2] and, therefore, a racemic drug may not act as an individual compound but as a combination of drugs.[3]

(±)-*trans*-Sobrerol (**1**) [(±)-*trans*-5-(1-hydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-ol] [4] is a mucolytic drug[5] and, in spite of differences in the pharmacological activity between the (+)- and (-)-form,[6,7] it is produced and marketed as a racemate. The two enantiomers have been isolated by asymmetric synthesis[8] or by resolution of the racemate through crystallization of diastereomeric salts.[7] However, both methods, besides being complex and time consuming, afford low yields.

In the present report, we describe the preparative-scale resolution of (±)-**1** by enantioselective acylation catalyzed by free or immobilized Lipase PS in organic media (Scheme). Under proper experimental conditions the method was very effective and at 50 % conversion yielded the two enantiomers with practically 100 % optical purity.

SCHEME



(±)-*trans*-sobrerol (-)-(1S,5R)-2 (+)-(1R,5S)-1

RESULTS AND DISCUSSION

Screening of Enzymes and Solvents.

For the enantioselective acylation of (±)-*trans*-sobrerol with vinyl acetate (Scheme), several enzymes and organic solvents were tested. Of the eight hydrolases investigated, three (Lipase PS, lipase from *Chromobacterium viscosum*, and subtilisin) were able to catalyze the acetylation of (±)-**1**. However, Lipase PS was much more active than the other two enzymes and, therefore, it was selected for further study.

The selectivity of the enzymatic reaction was routinely determined by chiral GLC of the reaction mixture. With the CP-cyclodextrin-β-column, the enantiomers of both the substrate and the product were base line separated, in the same run, without any derivatization (Figure 1A).

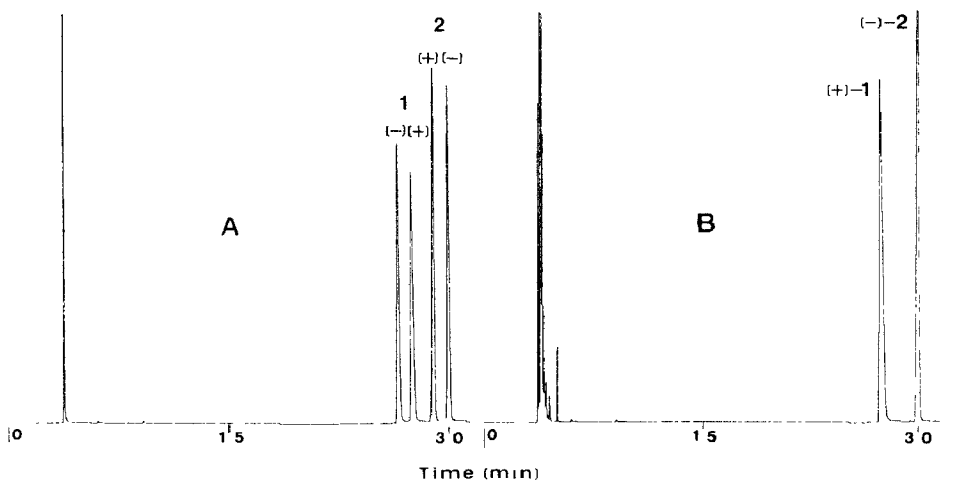


Figure 1 : Chiral gas chromatography of : A (±)-1 and (±)-2 ; B reaction product of (±)-1 with vinyl acetate, in *t*-amyl alcohol, using immobilized Lipase PS.

Note that there is an inversion in the elution order of the enantiomers of the product, with (+)-**2** eluting before (-)-**2**, compared to the substrate where (-)-**1** elutes before (+)-**1** (Figure 1A).

Table I shows the effects of the nature of the organic solvent on the enantioselectivity (expressed as enantiomeric ratio, E) and activity of Lipase PS. It can be seen that both parameters were markedly influenced by the solvent, with *t*-amyl alcohol giving the best results. However, there was no correlation between the hydrophobicity (log P) or dielectric constant of the solvents and the enantioselectivity or activity of Lipase PS. There was, instead, some positive correlation between activity and selectivity, except when vinyl acetate was used as both solvent and acylating agent. More hydrophobic solvents were not tested because the substrate was not very soluble in them.

Table I : Effects of the nature of the organic solvents on the enantioselectivity (E) and activity of Lipase PS[a].

solvent	log P[b]	dielectric constant[c]	E[d]	relative rate
dioxane	-1.14	2.2	178	20
acetone	-0.23	20.6	142	20
vinyl acetate	0.31	---	89	100
tetrahydrofuran	0.49	7.6	69	14
3-pentanone	0.80	17.0	212	35
t-amyl alcohol	1.45	5.8	518	80[e]

a) 10 ml of organic solvent containing (±)-**1** (117 mM), vinyl acetate (216 mM), Lipase PS (800 mg), and 3 A molecular sieves (1 g), were shaken at 250 rpm, at 45°C. The reaction progress and the e.e values were measured by GLC (see Methods).

b) Log P values, which are an index of solvent hydrophobicity, were calculated according to Rekker, R.F.; De Kort, H.M. Eur.J.Med.Clin.Therapeut, 1979, 14, 479.

c) Dielectric constant values were taken from : Reichardt, C. Solvents and Solvent Effects in Organic Chemistry, 2nd ed.; VCH : Weinheim, Germany. 1988, pp. 408-10.

d) E (enantiomeric ratio) values were calculated from the degree of conversion and the e.e. of the product according to Chen, C.H.; Fujimoto, Y.; Girdaukas, G ; Sih, C.J. J.Am.Chem.Soc., 1982, 104, 7294. Each value was the average of at least three E values calculated for conversions ranging from 10 to 50 %.

e) The reaction was carried out also with 70 and 650 mM vinyl acetate, and the relative rates were 63 and 97 % respectively.

About the nature of organic solvents and enzyme enantioselectivity, the results reported so far are rather contradictory. In fact, for subtilisin Carlsberg, there was an inverse correlation between selectivity and solvent hydrophobicity with N-acetylalanine chloroethyl ester as the substrate,[9] an inverse correlation between selectivity and dielectric constant of the solvent with *sec*-phenethyl alcohol,[10] and (like Lipase PS with sobrerol) no correlation with either hydrophobicity or dielectric constant with chiral amines.[11] With

Candida cylindracea lipase and 2-hydroxy acids, instead, the enantioselectivity increased as solvent hydrophobicity increased.[12] Thus the formulation of a single rationale for all these cases is a difficult task, since the effects of solvents on enantioselectivity depend not only on the nature of the enzyme but, for a given enzyme, also on the nature of the substrate.

Probably the rates reported in Table I are not maximal, as suggested by the 20 % increase in activity caused by a three-fold increase of concentration of vinyl acetate (solvent *t*-amyl alcohol, see footnote *e* in Table I). The addition of molecular sieves to the medium caused a 20-40 % increase in the reaction rate, depending on the solvent. This result is in agreement with previous findings reported by us[13] and by others[14] on the increase of reactivity of Lipase PS on lowering the content of water.

As for the acylating agent, vinyl acetate was preferred to isopropenyl acetate as well to trichloroethyl or trifluororethyl butanoate because of greater reactivity and because of the irreversibility of the reaction[15].

The enantioselectivity of *Chromobacterium viscosum* lipase and subtilisin was also tested. Using 3-pentanone as the solvent, the E value of the lipase was 81 (conversion 18 %, e.e. 97 %, (-)-**1** was acetylated preferentially) and the E value of the protease was 87 (conversion 23 %, e.e. 97 %, (+)-**1** was acetylated preferentially).

Preparative Scale Resolution of (±)-1.

Resolution of (±)-**1** was carried out with Lipase PS under different conditions. *t*-Amyl alcohol was used throughout because of the extremely high enantioselectivity of the enzyme in this solvent. It should also be noted that the reaction was completely regioselective, yielding, as expected, *trans*-sobrerol acetylated at the secondary hydroxyl.

With free Lipase PS, the e.e. of the product (-)-**2** and of the remaining substrate (+)-**1** were 98-99 %, at 50 % conversion. Because of the high enantioselectivity, the reaction tended to stop spontaneously after consumption of the (-)-enantiomer, thus making the resolution process handy. The enzyme retained 40 % of its original activity after about three weeks of use. The decay of activity is ascribable, at least in part, to the reaction of acetaldehyde, formed in the transesterification of vinyl acetate (Scheme), with the amino groups of the enzyme. Indeed, after prolonged use the enzyme became slightly orange, which is an indication of Schiff-base formation.

Lipase PS immobilized onto Hyflo Super Cell was 8-9 times more active than free. This is most likely due to the distribution of the protein onto the large surface of the support, which improves enzyme-substrate interaction over that of free powdered lipase. Contrary to what happened with the free enzyme, not only did addition of molecular sieves to the reaction mixture not increase the reaction rate, but, after prolonged shaking, it caused a reduction of enzyme activity, ascribed to mechanical shearing of the protein off the suppport. The immobilized enzyme contained 2 % (w/w) water. However, as suggested by Reslow *et al.*, who worked with chymotrypsin,[16] water has to be mostly bound to the hydrophilic support, which makes water removal by molecular sieves unnecessary.

The best results in terms of enantioselectivity and enzyme reusability were obtained with the fixed-bed reactor and substrate recirculation. The reaction, carried out at 25°C, stopped spontaneously at 50 % conversion and the optical purities of the ester product (-)-**2** and of remaining substrate (+)-**1** were absolute, as judged by chiral GLC (Figure 1B).

Resolution of (±)-**1** was also attempted by enzymatic hydrolysis of the acetate (or butanoate) of

(±)-*trans*-sobrerol in aqueous buffer. Quite surprisingly, because hydrolytic enzymes are generally much more active in aqueous than in organic media,[1b,15b] the rates of hydrolysis were too slow with the three enzymes tested (Lipase PS, *Chromobacterium viscosum* lipase, and subtilisin) for practical use. In the hydrolysis of (±)-**2** with Lipase PS, the e.e of (-)-**1** and (+)-**2** were , at 50 % conversion, quite high (94 and 95 %), in spite of the concomitant small spontaneous hydrolysis that occurred in the medium because of the long incubation time (the rate of hydrolysis was at least 30 times slower than the rate of transesterification). This indicates that the enantioselectivity of Lipase PS in the aqueous buffer is still very high. To our knowledge, this is the first report of an enzymatic transesterification that is much faster than the corresponding hydrolysis. Comparable rates of acylation and hydrolysis have been found by Guo *et al.*[17] for Lipase AK and 2,4-pentendiol; in that case, however, the enantioselectivity in water was very low.[17]

CONCLUSIONS

Lipase PS, especially in the immobilized form, has proven to be a very effective and selective catalyst for the resolution of (±)-*trans*-sobrerol by transesterification with vinyl acetate. This, together with the satisfactory stability of the enzyme under working conditions, makes the approach highly promising for large-scale application. The proper choice of the organic medium was found to be crucial, since both the activity and selectivity of the enzyme were highly dependent on the nature of the solvent. A rationale for this phenomenon of notable practical and theoretical importance has not as yet been found.

EXPERIMENTAL

Materials. Lipases were obtained as follows : *Pseudomonas sp.* lipase (Lipase PS), *Penicillium cyclopium* lipase and *Humicola lanuginosa* lipase from Amano Pharmaceutical Co.., *Candida cylindracea* lipase and porcine pancreatic lipase from Sigma, *Mucor miehei* lipase from Biocatalyst Ltd., and *Chromobacterium viscosum* lipase from Finnsugar. Subtilisin (protease from *Bacillus licheniformis*) was obtained from Sigma; prior to use, this enzyme was dissolved in H_2O, and the solution adjusted to pH 7.8 and freeze-dried.

(±)-*trans*-Sobrerol was bought from Aldrich-Chemie. (±)-*trans*-Sobrerol acylated (with acetyl or butanoyl groups) at the secondary hydroxyl, was prepared by reacting (±)-**1** (6 mmol), dissolved in 10 ml of dry pyridine, with 6 mmol of acetyl chloride or butanoyl chloride for 3 h at room temperature. The products were purified by flash chromatography on silica gel and their identities confirmed by ^{1}H-NMR and elemental analysis.

Hyflo Super Cell was purchased from Fluka. Organic solvents were dried over 3 A° molecular sieves.

General Methods. ^{1}H-NMR spectra were obtained on a Varian XL-200 (200 MHz) instrument in $CDCl_3$. Melting points are uncorrected and were determined on a Köfler hot-stage microscope. Enzymatic

acetylation of (±)-**1** was followed by gas-chromatography with a 25 m Hp-1 capillary column coated with methylsilicone gum (Hewlett Packard) with H_2 as carrier gas.

Enantiomeric Excess. Enantiomeric excesses were determined by measuring optical rotations with a Perkin-Elmer 241 polarimeter and (or) by chiral gas chromatography with a CP-Cyclodextrin-β-2,3,6-M-19 column (50 m, 0.25 mm ID, Chrompack) under the following conditions : oven temperature from 130°C (initial time 10 min) to 160°C with a heating rate of 0.5°C/min; H_2 as carrier gas.

Immobilization of Lipase PS. Lipase PS (3g) was mixed accurately with Hyflo Super Cell(10 g). Then, 10 ml of 0.1 M potassium phosphate buffer, pH 7, were added, the mixture was shaken vigorously, and dried over vacuum pump (24 h, 0.02 mbar). The water content, determined by the optimized Fischer method[18] was 2 % (w/w).

Resolution of (±)-1 with free Lipase PS. To a solution of (±)-**1** (1 g, 5.87 mmol) in *t*-amyl alcohol (50 ml) containing vinyl acetate (10.8 mmol), Lipase PS (1.5 g) and molecular sieves (3 A°, 3g) were added. The suspension was shaken at 250 rpm, at 45°C, and the reaction progress followed by GLC. After 5 h (approssimately 50 % conversion), the enzyme and the molecular sieves were filtered off and the solution evaporated under reduced pressure. The residue was then flash-chromatographed on a silica-gel column (eluent : n-hexane/ethyl acetate 3:1) to separate unreacted (+)-**1** from (-)-**2**.

(+)-**1** (390 mg) : mp 150°C (lit.[8] 148-9°C); $[\alpha]_D^{20}$ + 149.3 (c 5.1, EtOH)(lit.[8] + 150 , c 10.0, EtOH); e.e (determined by chiral GLC) > 99 %.

(-)-**2** (480 mg) : mp 70-2°C; $[\alpha]_D^{20}$ -120.7 (c 4.5, EtOH); e.e. (determined by chiral GLC) 97.5 %. ^{1}H-NMR δ : 5.62 (1H, br.d, J=6 Hz, H-3), 5.13 (1H, br.s, H-1), 1.97 (3H, s, OOC-Me), 1.58 (3H, br.s), 1.07 (3H, s), 1.05 (3H, s).

(-)-**2** (400 mg) was treated, at room temperature, with 15 ml of methanol/water (10:5) containing 2 % KOH, up to complete hydrolysis. The solution was evaporated, and the residue dissolved in 8 ml of water and extracted with CH_2Cl_2. The organic extract was washed with water, desiccated end evaporated to give (-)-**1** (300 mg) : mp 149°C (lit[8]148-9°C), $[\alpha]_D^{20}$ -147.0 (c 5.6, EtOH)(lit.[8] -150, c 10.0, EtOH); e.e. (determined by chiral GLC) 97.5 %. The enzyme was reused several times and, after 20 days of continuous working, it retained 40 % of its original activity.

Resolution of (±)-1 with immobilized Lipase PS.

(A). To a solution of (±)-**1** (2 g, 11.7 mmol) in *t*-amyl alcohol (100 ml) containing vinyl acetate (21.6 mmol), immobilized Lipase PS (3.8 g, equivalent to 880 mg of free lipase) was added. The suspension was shaken at 250 rpm, at 45°C, and the reaction progress followed by GLC. After 2 hours (approssimately 50 % conversion), the enzyme was filtered off and (+)-**1** and (-)-**2** recovered as described above. The e.e. of both (+)-**1** and (-)-**2**, determined by chiral GLC, were 98-99 %. The immobilized enzyme was reused several times and, after 14 days of continuous working, it retained about 50 % of its original activity.

(B). Immobilized Lipase PS (1.25 g, equivalent to 290 mg of free enzyme) was put into a reactor consisting of a column (1 cm diameter) provided in the upper part with a reservoir (50 ml). A solution of (±)-**1** (1 g, 5.87 mmol) in *t*-amyl alcohol (50 ml) containing vinyl acetate (10.8 mmol) was put into the reactor

and recirculated at a flow rate of 400 ml/h, at 25°C. After approximately 15 h, the conversion was 50 %. The solution was separated from the immobilized enzyme, and the e.e. of both (+)-**1** and (-)-**2**, determined by chiral GLC, were practically 100 %. (+)- and (-)-*trans*-sobrerol were then recovered as previously described. (+)-**1** (385 mg) : mp 150°C ; $[\alpha]_D^{20}$ + 149.5 (c 6.3, EtOH). (-)-**1** (310 mg) : mp 150°C ; $[\alpha]_D^{20}$ - 149.4 (c 5.4, EtOH).The reactor containing immmobilized Lipase PS was reused several times and, after 13 days of continuous working, the enzyme retained about 65 % of its original activity.

Hydrolysis of (±)-2 with Lipase PS. (±)-**2** (900 mg, 4.24 mmol) dissolved in 4.5 ml of acetone was added to 40 ml of 0.1 M potassium phosphate buffer, pH 7, and treated with Lipase PS (3 g), under stirring, at 25°C. After 4 days (approximately 50 % conversion), the reaction mixture was extracted with ethyl acetate and the organic extract was flash-chromatographed to separate *trans*-sobrerol ((-)-**1**) from its acetate ((+)-**2**). The e.e. of (-)-**1** and (+)-**2**, determined by chiral GLC, were 94 % and 95 %.

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