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LIPASE-CATALYSED ENANTIOSELECTIVE HYDROLYSIS OF BICYCLO[3.2.0]HEPTANOL ESTERS IN SUPERCRITICAL CARBON DIOXIDE

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Abstract: The esters derived from 2-*exo*-bromo-3-*endo*-hydroxybicyclo[3.2.0]heptan-6-one were treated with Lipolase under hydrolytic conditions in supercritical carbon dioxide ($SCCO_2$). All the esters tested gave access to optically active material (e.e. 50-90%). The esters with an additional keto or hydroxy group were hydrolysed to 35-40% conversion while nonpolar lipophilic substrates gave poor conversions (3-5%) under the same conditions. © 1997 Elsevier Science Ltd.

Following the pioneering studies[1] in 1985, the use of supercritical fluids (SCF) as reaction media for enzymatic reactions has received much attention in the past few years.[2-4] With respect to organic solvents SCF as alternative low water media exhibit a number of advantages, including unique transport properties and an adjustable solvent power.[2-5]

$SCCO_2$ is considered to be a SCF of great appeal for industrial biocatalytic applications because of its natural origin, nontoxicity, nonflammability, low environmental impact, availability at low cost as well as high mass transfer and diffusion rates. Attempts to set up continuous operations as well as an integrated process of enzymatic reaction and the downstream separation of products and unreacted substances have been made.[6,7] All the features indicated above could make $SCCO_2$ useful for the enzymatic resolution of enantiomers especially for application in the pharmaceutical industry where the control of organic solvent residues is crucial.

Previous studies on the enzymatic optical resolution in $SCCO_2$ are mainly related to the use of lipases for ester synthesis[8] and transesterification.[4] The optical resolution of N-protected amino acid derivatives by protease-catalysed hydrolysis using alcalase in $SCCO_2$ has also been described.[9]

Nevertheless, the use of $SCCO_2$ as a medium for enzymatic optical resolution is restricted to a few examples. This could be related to the high efficiency and technical simplicity of the use of water and organic solvent media for carrying out lipase-catalysed reactions for the kinetic optical resolution of a very wide variety of substrates.[10-12] The use of $SCCO_2$ as a medium for enzymatic optical resolution even for the gram-scale application requires a rather complicated and tedious optimisation of the process due to a large number of partition equilibria in the system. Moreover, when carrying out a transesterification reaction one should suppose that the hydrolysis of esters can still occur because of the presence of water introduced to maintain the enzyme activity. The optimisation has usually been started from the determination of the optimum water content.[2] Afterwards the concentration of substrates as well as the content of various additives (salts[13], amines[9]) have to be optimised. Taking into account also the need for sophisticated apparatus for work under high pressure we should conclude that a well-founded motivation is needed to turn to the use of $SCCO_2$

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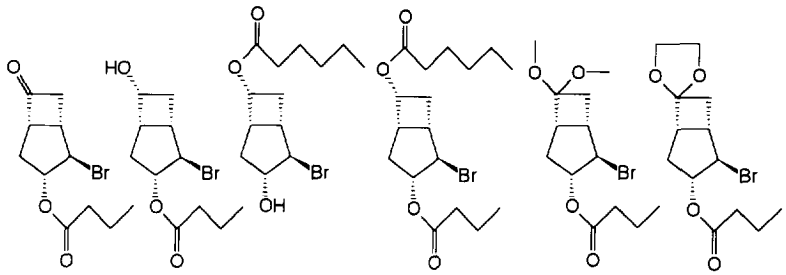
medium for optical resolution. First of all, such a motivation can arise from technological needs. Investigation of the enzymatic reactions to be stimulated or suppressed in a certain application gives guidelines for optimisation when setting up an industrial process. Another source of motivation is arising from the interest in performing lipase-catalysed reactions on an interface which could be essentially different from the interfaces present in water and organic solvent media. It can be expected that the ability of substrates to penetrate to the interface is greatly modified in $SCCO_2$. The putative modification of product distribution between the bulk phases and the interface is of great importance as well, since it may have crucial effects on the reaction equilibrium.

The present work was aimed at testing in $SCCO_2$ medium the lipase-catalysed hydrolysis of two groups of bicyclo[3.2.0]heptanol esters differing in their hydrolytic reactivity in water in order to study the role of reaction medium. A comparison of the transference of different bicyclo[3.2.0]heptanol derivatives by $SCCO_2$ aimed at separation of them thereby was expected, too. In this preliminary work there was no ambition to optimise any of the operations because the optically pure bicyclo[3.2.0]heptanol derivatives, precursors to a number of physiologically active compounds[14], can be obtained in gram-scale by using highly efficient methodologies.[15-17]



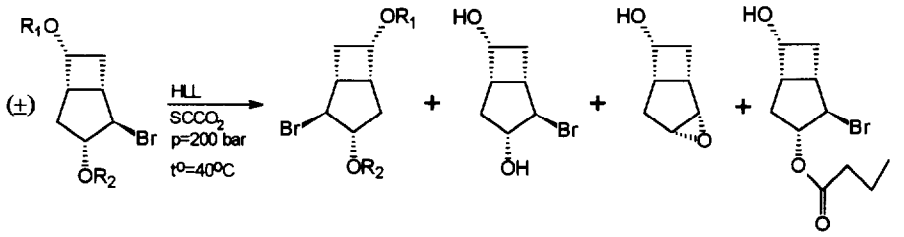
1 2 3 4 5 6

It has been reported[18] earlier that the ester (1) bearing the 6-oxo group shows a drastic difference in hydrolytic reactivity[19], from its reduced counterpart, 6-*endo*-hydroxy-ester (2). The ester (1) was hydrolysed in water by Lipolase *(Humicola lanuginosa lipase)* at a very low reaction rate together with a poor enantioselectivity only in the presence of Et_2O as a cosolvent while the hydroxyester (2) was hydrolysed smoothly and with a high enantioselectivity without the assistance of cosolvent. This difference in reactivity cannot be caused by any steric factors. The ester moieties of the substrates (3) and (4) were cleaved readily and with a high enantioselectivity by Lipolase in water.[18,19] The ester (5) is hydrolysed under the same conditions at a very low rate whereas the ester (6) appeared to be completely resistant to Lipolase either in the presence or absence of the cosolvent. In the case of the latter pair of substrates the steric hindrance of the reaction centre by the ketal group could be expected.

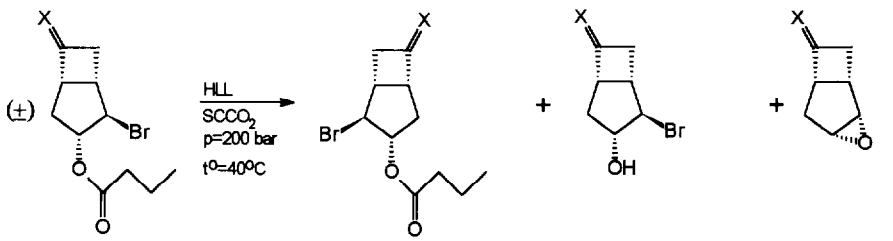
The substrates[20] (1), (2) and (4) were prepared as described earlier[18], the substrates[20] (5) and (6) were synthesised starting from (1) using conventional acetalisation methodologies[21] and the hydroxyester[20] (3) was prepared in four steps[22] using routine synthetic methods. The optically active reference compounds (-)-(1)[20], (-)-(3)[20], (-)-(10)[20] and (+)-(11)[20] were also prepared for the determination of the absolute configuration of the hydrolysis products as well as unreacted substrates starting from optically pure (+)-(9) of known configuration[23] (Table). The epoxides (+)-(12)[14], (±)-(13)[20] and (±)-(14)[14] were prepared for the chromatographic identification of the products.

The hydrolysis[24] of the substrates (1)-(6) as well as synthesis of (-)-(1)[25] catalysed by Lipolase[26] in $SCCO_2$ was carried out in a batch reactor[27] without stirring[28] under the conditions similar to those in ref. 9. The products and the unreacted substrate were collected into methanol through a flow resistor, evaporated and separated over silica. The yields of the compounds, the optical purities as well as the absolute configurations are listed in the Table.

Table



Entry					
1.	(2): R_1=H, R_2=COC_3H_7 230 mg (0.83 mmol) reaction time: 60 h conversion 35-40%[a]	(1R,2S,3S,5R,6S)-(2) 117 mg (0.42 mmol)[b]: 50.6%; $[\alpha]^{20}_{546}$ +51.6 (c 1.0, C_6H_6); e.e.=59%[c]	(1S,2R,3R,5S,6R)-(7)[18] 5.8 mg (0.028 mmol)[b]: 3.4%; $[\alpha]^{20}_{546}$ -33.9; e.e.=68%[d]	(8)[18,20] trace amount[b], detected by TLC	–
2.	(3): R_1=COC_5H_{11}, R_2=H 240 mg (0.79 mmol) reaction time: 60 h conversion 35-40%[a]	(1R,2S,3S,5R,6S)-(3) 138 mg (0.45 mmol)[b]: 57%; $[\alpha]^{20}_{546}$ +6.2 (c 5.0, C_6H_6); e.e.=50%[c]	(1S,2R,3R,5S,6R)-(7) 9.0 mg (0.043 mmol)[b]: 5.5%; $[\alpha]^{20}_{546}$ -44.1; e.e.=88.5%[d]	(8) 1.3 mg (0.01 mmol)[b]: 1.27%	–
3.	(4): R_1=COC_5H_{11}, R_2=COC_3H_7 182 mg (0.48 mmol) reaction time: 60 h conversion 3-5%[a]	(1R,2S,3S,5R,6S)-(4) 62 mg (0.17 mmol)[b]: 35.4%; e.e.<5%[c]	(7) trace amount[b], detected by TLC	(8) trace amount[b], detected by TLC	(1S,2R,3R,5S,6R)-(2)[18] 3.9 mg (0.014 mmol)[b]: 2.9%; $[\alpha]^{20}_{546}$ -82 (c0.3,C_6H_6); e.e.>95%[e]



4.	(1): X=O 390 mg (1.42 mmol); reaction time: 100 h conversion 35-40%[a]	(1R,2S,3S,5R)-(1) 230 mg (0.26 mmol)[b]: 59%; $[\alpha]^{20}_{546}$ +28.6 (c 2.0, C_6H_6); e.e.=67%[c]	(1S,2R,3R,5S)-(9) 8.2 mg (0.04 mmol)[b]: 2.8%; $[\alpha]^{20}_{546}$ +56 (c 0.5, C_6H_6); e.e.=92%[d]	(12) trace amount[b], detected by TLC
5.	(5): X=$(CH_3O)_2$ 195 mg (0.61 mmol); reaction time: 100 h; conversion 3-5%[a]	(1R,2S,3S,5R)-(5) 138 mg (0.50 mmol)[b]: 82.0%; e.e.<5%[c]	(1S,2R,3R,5S)-(10) 6.8 mg (0.021 mmol)[b]: 3.5%; e.e.=92%[e]	(13) trace amount[b], detected by TLC
6.	(6): X=$O(CH_2)_2O$ 410 mg (1.28 mmol); reaction time : 120 h; conversion 3-5%[a]	(1R,2S,3S,5R)-(6) 332 mg (1.04 mmol)[b]: 81.3%; e.e.<5%[c]	(1S,2R,3R,5S)-(11) 8.5 mg (0.034 mmol)[b]: 2.7%; e.e. 89%[e]	(14) the epoxide was not detected

a: conversion rates were estimated by the yield of isolated compounds and their e.e.-s;
b: quantities correspond to the amounts of compounds separated from the crude product released from the system through a resistor by CO_2;
c: e.e. estimated by NMR using $Yb(FOD)_3$ (e.e. estimation was supported by correlation of the optical rotation values);
d: e.e. estimated by correlation of optical rotation values;
e: e.e. estimated by HPLC of the corresponding (-)-MTPA esters.

All the substrates tested gave optically active material. The hydroxyesters (2) and (3) were hydrolysed readily to 35-40% conversion, while for the ketoester (1) the conditions had to be carefully controlled[29] to obtain the same degree of conversion. The nonpolar lipophilic substrates (4), (5) and (6) were hydrolysed only by3-5% under the same conditions. This can be explained by the low ability of the esters without polar (keto-) or hydrophilic (hydroxy-) functional groups to penetrate to the reaction interface. This interface is assumed to be formed (predominantly) by CO_2, on the one hand, and by H_2O surrounding the solid carrier of the enzyme, on the other. Increasing the pressure of $SCCO_2$ is likely to improve the hydrolytic reactivity of lipophilic substrates.[29] The effect of a steric hindrance of the reaction centre by the bulky substituents present in the molecule of substrates (4), (5) and (6) cannot be expected to play a crucial role in suppressing hydrolysis. This conclusion is supported by the fact that the hydrolysis rate[19] of the diester (4) in water is moderate. On the other hand, the substrates (5) and (6) have the reaction centre other than the diester (4) and therefore the possibility of steric hindrance for them cannot be excluded.

The enantioselectivities[30] of hydrolysis were found to be moderate: the products of e.e. 68-92% were gained, while significant amounts of optically enriched starting material have been recovered: e.e. 50%, e.e. 59% and e.e. 67% for (+)-(3), (+)-(2) and (+)-(1) were estimated, respectively. The small amounts of the hydrolysis products obtained through a resistor independently of conversion yield give hope of setting up a resolution procedure. It is interesting to note that the polar hydrophilic bromohydrin (9), introduced into the reactor as a substrate[25], was transported out from the reactor by CO_2 to a significant extent (50-60% of the total). Thus, (9) can readily be solubilized and transported by $SCCO_2$. The separation of the reaction components seems to depend on the partition of the reaction products between phases.

The extent of epoxide formation tends to depend on the partition of the parent bromohydrin between the phases. For example, the bromohydrin (7) was readily dehydrobrominated in water at pH 7 (on a pH-stat). Consequently, none of the additives (Et_3N, for instance) alone is responsible for epoxide formation, but the buffered water itself is. Anyway, the presence of more than one potential reaction centre under the hydrolytic conditions is, of course, a negative feature for the investigation of the behaviour of a substrate throughout a technically complicated procedure.

No decision can yet be made on the basis of the results of the present work about the applicability to large-scale applications of the enzymatic enantioselective hydrolysis reactions in $SCCO_2$ medium stated herein.[30] This probably needs extensive additional optimisation work. At the present level of knowledge ester synthesis[31] as well as transesterification procedures[17] in organic solvents are clearly preferable for gram-scale optical resolution of the substrates investigated because of the higher enantioselectivity and technical simplicity.

Nevertheless, the enzymatic enantioselective hydrolysis catalysed by Lipolase in $SCCO_2$ was found to be useful for deacylation of the esters being completely resistant to Lipolase in water and even in water/organic cosolvent mixture.[18]

In conclusion, it was shown that:

- unlike the reactions in water all the substrates tested gave access to optically active products upon the hydrolysis catalysed by Lipolase in $SCCO_2$ demonstrating the crucial role of the reaction medium for lipase-catalysed reactions;
- the ester substrates bearing polar (keto-) or hydrophilic (hydroxy-) functional groups in their molecule were hydrolysed to 35-40% conversion in $SCCO_2$, while only 3-5% of nonpolar lipophilic substrates was converted under the same conditions; enantioselectivity of the hydrolysis was moderate;
- the enzymatic optical resolution procedure in $SCCO_2$ medium for the preparative scale application requires extensive optimisation work.

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19. The initial rates of Lipolase-catalysed hydrolysis (μmol/min per 1.0 ml of Lipolase) in water of the substrates (1)-(6) were recorded on a pH-stat: (1) - 0, (2) - 1.36, (3) - 0.85, (4) - 0.42, (5) - very slow, (6) - 0. Details will be available in: "Lipase-Catalysed Enantioselective Hydrolysis: Interpretation of the Kinetic Results in Terms of Frontier Orbital Localisation" by O.Parve et al. Tetrahedron (in press).
20. Characterisation of substrates and products:
(1S,2R,3R,5S)-(1): ^{13}C NMR ($C_{1\text{-}7}$; $C_{(3)1\text{-}4}$) – 39.2, 54.3, 82.6, 34.3, 63.6, 209.8, 52.8; 171.7, 36.1, 18.0, 13.5; ^{1}H NMR ($H_{1\text{-}5,7}$; $H_{(3)2\text{-}4}$) – 3.36, 4.33, 5.45, 2.58x/2.29n, 3.84, 3.26x/2.98n; 2.20, 1.58, 0.91; IR – 1795, 1737, 1390, 1260, 1165, 1090, 980, 690 cm^{-1}; TLC – R_f=0.26 (C_6H_6); $[\alpha]_{546}^{20}$ -44.7 (c 2.3; C_6H_6); (1S,2R,3R,5S,6R)-(3): ^{13}C NMR ($C_{1\text{-}7}$; $C_{(6)1\text{-}6}$) – 43.8, 60.3, 83.1, 32.0, 42.4, 65.3, 33.7; 172.6, 34.1, 24.5, 31.2, 22.2, 13.8; ^{1}H NMR ($H_{1\text{-}7}$; $H_{(6)2\text{-}6}$) – 2.89, 4.17, 4.58, 2.44x/1.90n, 3.40, 5.13, 2.68x/2.30n; 2.32, 1.62, 1.28, 1.30, 0.90; IR – 3480, 2965, 2940, 2885, 2875, 1730, 1470, 1440, 1425, 1390, 1350, 1290, 1260, 1175, 1110, 1080, 1030, 980, 930, 710, 700 cm^{-1}; TLC – R_f=0.51 (C_6H_6/EtOAc 10/1); $[\alpha]_{546}^{20}$ -12.6 (c 2.0; C_6H_6); (4): ^{13}C NMR – ref. 18, ^{1}H NMR ($H_{1\text{-}7}$; $H_{(3)2\text{-}4}$; $H_{(6)2\text{-}6}$) – 2.88, 4.19, 5.46, 2.49x/2.03n, 3.39, 5.04, 2.55x/2.23n; 2.31, 1.67, 0.96; 2.25, 1.61, 1.27, 1.30, 0.90; (5): ^{13}C NMR ($C_{1\text{-}7}$; $C_{(3)1\text{-}4}$; OCH_3) – 40.2, 56.1, 84.4, 29.8, 47.1, 99.6, 35.7; 172.3, 36.2, 18.2, 13.6; 48.4, 48.2; ^{1}H NMR ($H_{1\text{-}5,7}$; $H_{(3)2\text{-}4}$; OCH_3) – 2.88, 4.17, 5.38, 2.44x/2.08n, 3.05, 2.22x/2.19n; 2.27, 1.64, 0.94; 3.08, 3.11; IR – 2970, 2885, 2840. 1735, 1470, 1425, 1300, 1255, 1180, 1145, 1035, 995, 920, 880, 850, 685 cm^{-1}; TLC – R_f=0.395 (C_6H_6/EtOAc 20/1); (6): ^{13}C NMR ($C_{1\text{-}7}$; $C_{(3)1\text{-}4}$; OCH_2) – 39.8, 56.0, 84.1, 30.0, 51.0, 105.8, 39.2; 172.4, 36.2, 18.1, 13.5; 64.2, 63.2; ^{1}H NMR ($H_{1\text{-}5,7}$; $H_{(3)2\text{-}4}$; OCH_2) – 2.89, 4.20, 5.38, 2.41x/2.15n, 3.06, 2.46xn; 2.31, 1.64, 0.91; 3.81/3.73, 3.83; IR – 2960, 2880, 1735, 1300, 1180, 1040, 1005, 985, 675 cm^{-1}; TLC – R_f=0.40 (C_6H_6/EtOAc 20/1); (1S,2S,3R,5S,6R)-(8): ref. 18; (1S,2R,3R,5S,)-(10): ^{13}C NMR ($C_{1\text{-}7}$; OCH_3) – 39.2, 59.9, 82.8, 32.5, 47.9, 100.5, 37.1; 48.4, 48.2; ^{1}H NMR ($H_{1\text{-}5,7}$; OCH_3) – 2.92, 4.15, 4.42, 2.39x/2.04n, 3.12, 2.33x/2.21n, 3.11, 3.12; IR – 3450, 2960, 2955, 2835, 1430, 1320, 1290, 1250, 1200, 1160, 1145, 1035, 910, 840, 820, 700 cm^{-1}; TLC – R_f=0.29 (C_6H_6/EtOAc 10/1); $[\alpha]_{546}^{20}$ -6.1 (c 5.0; C_6H_6; e.e.>98%); (1S,2R,3R,5S)-(11): IR – 3475, 2960, 2890, 1425, 1320, 1300, 1180, 1025, 950, 915, 840, 820, 780, 700, 680 cm^{-1}; TLC – R_f=0.254 (C_6H_6/EtOAc 10/1); $[\alpha]_{546}^{20}$ +9.4 (c 2.0; C_6H_6; e.e.>98%); (±)-(13): ^{13}C NMR ($C_{1\text{-}7}$; OCH_3) – 29.9, 61.1, 65.7, 28.0, 50.5, 100.9, 30.8; 48.3, 47.8; ^{1}H NMR ($H_{1\text{-}5,7}$; OCH_3) – 2.56, 3.48, 3.63, 1.765x/2.246n, 3.01, 2.09x/2.40n; 3.11, 3.04; IR – 3010, 2960, 2840, 1320, 1260, 1155, 1050, 990, 975, 940, 855, 845 cm^{-1}; TLC – R_f=0.32 (C_6H_6/EtOAc 10/1).
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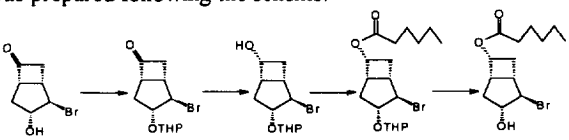
22. The substrate (3) was prepared following the scheme:



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24. **General procedure of the hydrolysis:** The reactions in $SCCO_2$ were carried out in the apparatus presented in Figure 1[27]. CO_2 (class 2.7, purity 99.7%) was supplied by AGA. The steel batch-type reactor with internal volume 10.2 cm^3 was filled with 0.48-1.42 mmol of substrate, 50 µl of 20% NH_4HCO_3 water solution, 15 µl Et_3N and 2 g of Lipolase[26] preparation. The reactor was placed in a thermostat with t°=39-40°C. Liquid carbon dioxide was pumped into the reaction vessel by a chromatographic piston pump with cooled pumpheads (DuPont Instruments) until the pressure reached the desired value (200 bar). After the reaction time was over the samples were collected into methanol through a flow resistor. The samples were evaporated, separated over silica and analyzed. Acetone washings of the reactor system were not further investigated.
25. The esterification of (±)-(9) with butyric acid (reverse process to this presented in entry 4. of the Table) was performed under the conditions described[24] for the hydrolysis except for the addition of 0.5 eq. (22 mg; 0.25 mmol) of butyric acid. The substrate amount was: 100 mg (0.5 mmol). From 66 mg of crude product obtained through the resistor, 9.0 mg (0.033 mmol; y.: 6.6%) of (-)-(1) was separated: $[\alpha]^{20}_{546}$ -39 (c 0.5; C_6H_6) corresponds to e.e.≈91%. 52 mg of enantiomerically enriched ($[\alpha]^{20}_{546}$ -6 (c 3.0; C_6H_6)) (9) was obtained also supporting the conclusion that equilibrium was reached at 7-10% conversion under the conditions used.
26. *Humicola lanuginosa* lipase preparation Lipolase 100T was kindly provided by Novo Nordisk.
27. The apparatus used for performing reactions in $SCCO_2$.

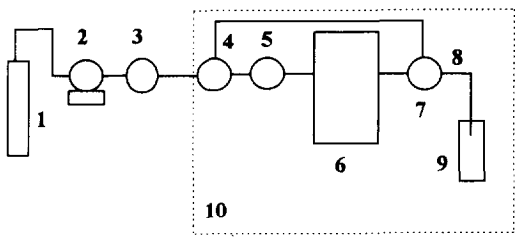

Figure 1. 1 – Liquid CO_2-tank; 2 – high pressure pump; 3 and 5 – pressure gauge; 4 and 7 – HPLC valve; 6 – reactor; 8 - resistor; 9 – sample collection; 10 – thermostat.

28. Stirring has been shown to give only a minute increase in reaction velocity in the case of enzymatic synthesis in $SCCO_2$ medium. See: Dumont, T.; Barth, D.; Corbier, C.; Branlant, G.; Perrut, M. *Biotechnol. Bioeng.*, **1992**, 39, 329-333.
29. The hydroxyesters (2) and (3) were hydrolysed readily to conv. 35-40% at p=170 bar (other conditions were kept constant), while ketoester (4) was hydrolysed to conv. 2-3% under such pressure.
30. The enantioselectivities of the hydrolysis in $SCCO_2$ of the substrates (1)-(6) were calculated: (1) E=45, (2) E=8, (3) E=30, (4) E=40, (5) E=25; (6) E=18. The calculations were based on the e.e. of the reaction products (Table) as well as conversion rates 40% and 3% estimated for the substrates (1)-(3) and (4)-(6), respectively. These conversion rates have been estimated roughly by amounts as well as by e.e. of the starting material recovered. Moreover, the E values obtained correspond to the systems in equilibrium being therefore assumed probably to change during the process as well as to differ from those corresponding to the irreversible hydrolysis. Thus, in our opinion enantioselectivity has to be reinvestigated along with the optimisation of the process.
31. The enantioselective esterification catalysed by Novozym of the parent molecule of the substrates investigated - bromohydrin (9) - in organic solvent was also tested by us: (±)-(9) (256 mg; 1.25 mmol) was dissolved in C_6H_6 (3 ml), butyric acid (115 µl; 1.25 mmol) and the lipase preparation (0.5 g) were added. After 72 h the reaction mixture was filtered, evaporated and the product was separated over silica. 69 mg of (-)-(1) was obtained (y.: 20.2%), $[\alpha]^{20}_{546}$ -44.7 (c 2.3; C_6H_6); the product was a pure enantiomer by NMR using $Yb(FOD)_3$. n-Hexane was found to be a worse medium giving a less clean product. Acetic acid and vinyl acetate as acyl donors were also successfully used.

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