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Control of the Stereoselectivity of Pig Liver Esterase by Different Reaction Conditions in the Hydrolysis of *cis*-*N*-Benzyl-2,5-bismethoxycarbonylpyrrolidine and Structurally Related Diesters

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The enantiotopic selectivity of pig liver esterase in the enzyme-catalyzed hydrolysis of *cis*-*N*-benzyl-2,5-bismethoxycarbonylpyrrolidine (**1**) was investigated under different reaction conditions. Optically pure monoester **2** (≥98% enantiomeric excess) was obtained in Tris-buffered incubations at pH 7.5 when the reaction medium contained 25% dimethyl sulfoxide or 10% methanol. Reactions run in Tris buffer were faster and gave higher stereoselectivity than reactions performed in a pH-stat. Similar observations were made with structurally related diesters (**3, 5, 7, 9, 11–13**). Tris acts as an alternative nucleophile in the pig liver esterase-catalyzed hydrolysis of diester **1** and the Tris-amide formed lowers the chemical yield of monoester **2**.

INTRODUCTION

The ability of many enzymes to act with enantiotopic selectivity on prochiral substrates and to distinguish between enantiomers has made them accepted as valuable tools in asymmetric synthesis (*1, 2*). Comparatively cheap hydrolases like chymotrypsin, lipases, and carboxylesterases are particularly useful in this context since they are easy to handle and do not need any cofactor.

For enzymes with a known tertiary structure like α-chymotrypsin, knowledge has accumulated regarding the reaction mechanism, the active site dimensions, and other important features (*3*). Consequently qualitative predictions of the stereoselectivity for such an enzyme are sometimes possible (*4*). Enzymes with a well known structure are not always available to provide the desired products. The enzyme pig liver esterase has never been crystallized due to its complex microheterogeneity (*5, 6*). Nevertheless pig liver esterase accepts a wide range of substrates and the products are often formed with high enantiomeric excess (*7–10*). Structure/activity relationships have been extensively investigated with this enzyme but further studies of how the stereoselectivity can be influenced by control of the reaction conditions are needed (*11, 12*).

The pig liver esterase catalyzed hydrolysis of *cis*-*N*-benzyl-2,5-bismethoxycarbonylpyrrolidine (**1**) has previously been reported to give 80% enantiomeric ex-

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cess (e.e.)[1] of product monoester **2** (*13*) (Scheme 1). Based on results from our earlier work on the effect of dimethyl sulfoxide on the stereoselectivity in pig liver esterase-catalyzed hydrolyses (*14, 15*), we recently developed a procedure for the preparation of optically pure **2** (*16*). Highly functionalized 2,5-disubstituted pyrrolidines of this type are useful intermediates in the synthesis of pyrrolidine (*17*), pyrrolizidine (*18*), or indolizidine alkaloids (*19*). Furthermore the optically pure monoester **2** should be an attractive precursor to pyrrolidine-based chiral auxiliaries for asymmetric synthesis. In this paper we report on the influence of different reaction conditions on the pig liver esterase catalyzed hydrolysis of diester 1 and structurally related diesters (Scheme 2).

EXPERIMENTAL PROCEDURES

^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker WP200 or a Bruker AM400 FT instrument in $CDCl_3$ with $(CH_3)_4Si$ as internal standard except where otherwise stated. Infrared spectra were recorded on a Pye Unicam sp4000 spectrophotometer. A Perkin–Elmer 241 polarimeter was used for optical rotation measurements. Melting and boiling points were uncorrected. Boiling points were determined as air bath temperatures in a Büchi GKR50 glass tube oven. The high resolution mass spectrum of compound **14** was obtained on a Kratos MS50 mass spectrometer. Merck Kieselgel 60 (40–63 μm) and Waters Preparative C18 gel (55–105 μm) were used for liquid chromatography separations.

Enzyme. Pig liver esterase (EC 3.1.1.1) was purchased as a suspension in aqueous $(NH_4)_2SO_4$, from Sigma (batches No. 34F-8110 and 45F-8130). The suspension was centrifuged and the enzyme pellet was dissolved in the medium used for each experiment.

Buffered incubations. Reactions were typically carried out in 20-ml buffered batches containing dimethyl sulfoxide, 0.25 M Tris–HCl, pH 7.5, pig liver esterase (0.1–1 mg), and substrate (12 mM) at the temperature indicated for each experiment. The conversion of diester was monitored by taking samples. These were extracted with diethyl ether and were thereafter analyzed in the presence of an internal standard by GLC on a DB-WAX30N (J&W Scientific, Inc.) fused silica capillary column (200–220°C). This also gave figures for the determination of the total rate of substrate cleavage. In the case of *trans*-diester hydrolysis the reaction was stopped at a conversion of 41–46% by a fast extraction of remaining diester with diethyl ether.

pH-stat incubations. Reactions were carried out in a Radiometer pH-stat under N_2 at 22–25°C and maintained at pH 7.5 by the addition of 1 M NaOH. The reaction mixture contained dimethyl sulfoxide, $CaCl_2$ (75 mM), substrate (60 mM), and pig liver esterase (0.1–1 mg), in a total volume of 6–10 ml.

Enzymatic and base-catalyzed rates of hydrolysis. Reaction velocities were determined in the pH-stat under N_2 at 30°C. By addition of 10 mM NaOH, pH was maintained at 7.5. The reaction mixture contained dimethyl sulfoxide (25%), KCl

[1] Abbreviation used: e.e., enantiomeric excess.

(75 mM), substrate (10 mM), and pig liver esterase (0.04 mg) in a total volume of 4 ml. The enzyme was desalted on Sephadex G-25 prior to use. The apparent rate constant for the base-catalyzed reaction (k_{HO^-}) was determined by measuring the rate of hydrolysis at pH 10.5 under the same conditions as above but without enzyme.

Enantiomeric excess determinations. The e.e.'s of monoacids 2, 4, 6, 8, and 10 were determined by ^{1}H NMR analyses of their *in situ* prepared salts with enantiomerically pure (*R*)-(+)-phenylethylamine (compounds **2** and **6**) or (*S*)-(−)-phenylethylamine (compounds **4, 8,** and **10**). The e.e.'s of recovered diesters **3, 7,** and **9** were determined by ^{1}H NMR in the presence of the chiral shift reagent tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato]europium (III) [Eu(hfc)$_3$]. Relevant diastereoisotopic signals for the e.e. determinations were in all cases the methyl singlets arising from the methyl ester groups. Absolute configurations of compounds **2** and **4** were determined as previously described (*16*).

Preparation of dimethyl ester substrates. Diesters **1, 3, 5, 7, 9,** and **11–13** were prepared as mixtures of *cis*- and *trans*-isomers from α,α'-dibromodimethyladipate (*20*) (**1, 3, 5, 7**) or α,α'-dibromodimethylpimelate (*21*) (**9, 11–13**) using the method of Cignarella and Nathanson (*22*). Pyrrolidine-based diesters were synthesized from the *meso* (method A) or the *dl* (method B) form of the adipate (*23*). Piperidine-based diesters were prepared using a mixture of *meso*- and *dl*-pimelate (*24*). Assignments of the relative configuration of all diesters were based on their ^{1}H NMR spectra (appearance of NCH_2R methylene protons (*24, 25*) and chromatographic behavior). The yield of isolated product was determined only for the extensively employed diester **1.** All other diesters were synthesized in small amounts and the chromatographic effort was not aimed at a quantitative separation of isomers. Crude yields of mixtures of *cis*- and *trans*-diesters were in the 40–75% range and the isomers were separated by gradient elution liquid chromatography on silica gel (hexane–EtOAc).

Workup of monoesters. Monoesters **2, 4, 6, 8,** and **10** were isolated from crude reaction mixtures by a repetitive extractive workup procedure or by preparative reversed phase chromatography (H_2O–MeOH elution). Isolated yields are given for compounds **2** and **4.** Esters **6, 8,** and **10** were obtained in low yields (ca. 5–20 mg) from incubations of 100 mg or less of the corresponding diester and were purified only to a degree allowing accurate ^{1}H NMR determination of e.e. values. Optical rotation figures and physical properties are only given for ester **2,** where reliable workup procedures were elaborated to remove all impurities. In the case of esters **4, 6, 8,** and **10** the sign (+/−) of optical rotation (589 nm, 25°C, $CHCl_3$) is merely reported.

cis-N-Benzyl-2,5-bismethoxycarbonylpyrrolidine (***1***). Isolated yield 53% (method A), 33% (method B).[2] bp 140–150°C/0.075 mm Hg (lit. (*26*) 115°C/0.02

[2] Method A typically gave a 90/10 *cis/trans* ratio in the crude product, whereas crude diester obtained by method B showed a 70/30 *cis/trans* relationship (GLC, OV101 30-m fused silica capillary column, 185°C). The stereochemistry of this cyclization has been discussed previously (*27*). The lower yield when d,l-adipate was employed (method B) is due largely to impurities in this material, manifested by the presence of a by-product (16% of crude product (GLC)) in the cyclization reaction preliminarily identified as *N*-benzyl-6-oxo-2-piperidinecarboxylic acid methyl ester.

mm Hg), crystallized on standing, mp 42–44°C. ^{1}H NMR (200 MHz) δ 2.05–2.10 (4H,m), 3.4–3.5 (2H,m) 3.57 (6H,s), 3.92 (2H,s), 7.20–7.35 (5H,m). ^{13}C NMR (100.61 MHz) δ 28.6, 51.7, 57.6, 65.3, 127.2, 128.0, 129.6, 137.1, 173.8. ir (film) 3140–2750 (m), 1750 (s) cm^{-1}.

cis-(2S,5R)-(+)-N-Benzyl-5-methoxycarbonylpyrrolidine-2-carboxylic acid (**2**). Isolated yield 85% (pH-stat incubation), 39% (buffered inc.). Crystallized on standing, mp 63–64°C (lit. (*26*) 67–68°C) $[\alpha]_D^{21}$ = +16.6°.[3] ^{1}H NMR (200 MHz) 1.75–2.00 (1H,m) 2.05–2.35 (3H,m), 3.60–3.75 (2H,m) 3.64 (3H,s), 3.90 (2H,s), 7.20–7.35 (5H,m). ^{13}C NMR (100.61 MHz) δ 30.3, 30.5, 52.5, 58.9, 66.3, 66.6, 128.2, 128.8, 129.3, 135.9, 174.8, 174.9. ir (film) 3140–2780 (m), 1745 (s), 1650 (m) cm^{-1}.

trans-N-Benzyl-2,5-bismethoxycarbonylpyrrolidine (**3**)[4] (*lit. 29*). bp 120–130°C/0.095 mm Hg. ^{1}H NMR (200 MHz) δ 1.80–2.05 (2H,m), 2.20–2.45 (2H,m), 3.64 (6H,s), 3.78 (1H,d,J=13.2 Hz), 3.80–3.90 (2H,m), 3.96 (1H,d,J=13.2 Hz), 7.20–7.35 (5H,m). ^{13}C NMR (100.61 MHz) δ 28.4, 51.5, 54.1, 63.4, 127.2, 128.2, 129.0, 138.4, 174.6. ir (film) 3120–2800 (m), 1745 (s) cm^{-1}.

trans-(2S,5S)-(−)-N-Benzyl-5-methoxycarbonylpyrrolidine-2-carboxylic acid (**4**) (*lit. 29*). Isolated yield 39% (pH-stat incubation, 42.5% conversion = theor. yield). ^{1}H NMR (200 MHz) δ 1.85–2.25 (3H,m), 2.35–2.65 (1H,m), 3.68 (3H,s) 3.70–4.00 (4H,m), 7.20–7.35 (5H,m).

cis-N-Ethyl-2,5-bismethoxycarbonylpyrrolidine (**5**). bp 110–120°C/0.12 mm Hg. ^{1}H NMR (200 MHz) δ 1.03 (3H,t), 2.00–2.10 (4H,m), 2.81 (2H,q), 3.35–3.50 (2H,m), 3.72 (6H,s). ^{13}C NMR (100.61 MHz) δ 12.0, 29.0, 48.3, 52.0, 65.5, 174.3. ir (film) 3080–2780 (m), 1745 (s) cm^{-1}.

cis-(+)-N-Ethyl-5-methoxycarbonylpyrrolidine-2-carboxylic acid (**6**). ^{1}H NMR (200 MHz) δ 1.14 (3H,t), 1.80–2.05 (1H,m), 2.10–2.40 (3H,m), 2.90 (2H,q), 3.55–3.75 (2H,m), 3.81 (3H,s).

trans-N-Ethyl-2,5-bismethoxycarbonylpyrrolidine (**7**). bp 105–112°C/0.11 mm Hg. ^{1}H NMR (200 MHz) δ 1.09 (3H,t), 1.80–2.05 (2H,m), 2.20–2.45 (2H,m), 2.55–2.90 (2H,m), 3.71 (6H,s), 3.80–3.95 (2H,m). ^{13}C NMR (100.61 MHz) δ 13.8, 28.2, 44.3, 51.5, 63.5, 174.7. ir (film) 3100–2800 (m), 1740 (s) cm^{-1}.

trans-(−)-N-Ethyl-5-methoxycarbonylpyrrolidine-2-carboxylic acid (**8**). ^{1}H NMR (200 MHz) δ 1.26 (3H,t), 2.00–2.70 (4H,m), 3.00–3.25 (2H,m), 3.77 (3H,s), 3.95–4.10 (1H,m), 4.25–4.40 (1H,m).

trans-N-Benzyl-2,6-bismethoxycarbonylpiperidine (**9**) (*lit. 24*). bp 150–160°C/0.10 mm Hg, mp 56–57°C. ^{1}H NMR (200 MHz) δ 1.45–1.65 (2H,m), 1.70–1.95 (4H,m), 3.58 (1H,d,J=13.5 Hz), 3.69 (6Hs,), 3.76 (1H,d,J=13.5 Hz), 3.80–3.95 (2H,m), 7.20–7.35 (5H,m). ^{13}C NMR (100.61 MHz) δ 19.2, 28.7, 51.4, 56.9, 59.2, 127.2, 128.2, 129.0, 138.1, 174.5. ir (KBr) 2960 (w), 2870 (w), 1750 (s), 1735 (s) cm^{-1}.

trans-(+)-N-Benzyl-6-methoxycarbonylpiperidine-2-carboxylic acid (**10**). ^{1}H NMR (200 MHz) δ 1.55–1.75 (2H,m), 1.80–2.15 (4H,m), 3.74 (3H,s), 3.80–4.15 (4H,m), 7.25–7.45 (5H,m).

[3] Calculated from measurements on a purified sample of 79% e.e. $[\alpha]_D^{21}$ = +13.1° (*c* 2.69, $CHCl_3$).

[4] Also available via isomerization of **1** by treatment with NaOMe (*28*).

cis-N-Benzyl-2,6-bismethoxycarbonylpiperidine (***11***) (*lit. 24*). bp 135–145°C/0.13 mm Hg, mp 60–61°C. ^{1}H NMR (200 MHz) δ 1.25–1.50 (1H,m), 1.70–1.95 (5H,m), 3.15–3.30 (2H,m), 3.60 (6H,s), 3.86 (2H,s), 7.15–7.35 (5H,m). ^{13}C NMR (100.61 MHz) δ 20.6, 28.8, 51.5, 59.0, 62.5, 127.2, 128.0, 129.5, 137.1, 173.6. ir (KBr) 3260–2780 (m), 1745 (s) cm^{-1},

cis-N-Ethyl-2,6-bismethoxycarbonylpiperidine (***12***). bp 110–115°C/0.12 mm Hg. ^{1}H NMR (200 MHz) δ 0.98 (3H,t), 1.25–1.50 (1H,m), 1.65–1.95 (5H,m), 2.73 (2H,q), 3.20–3.30 (2H,m), 3.73 (6H,s). ^{13}C NMR (100.61 MHz) δ 8.6, 22.1, 29.3, 47.3, 51.8, 63.0, 173.4. ir (film) 2960 (m), 2870 (w), 1750 (s) cm^{-1}.

trans-N-Ethyl-2,6-bismethoxycarbonylpiperidine (***13***). bp 105–115°C/0.14 mm Hg. ^{1}H NMR (200 MHz) δ 107 (3H,t), 1.50–1.65 (2H,m), 1.70–2.00 (4H,m), 2.57 (2H,q), 3.71 (6H,s), 3.90–4.00 (2H,m). ^{13}C NMR (100.61 MHz) δ 12.5, 18.9, 28.6, 47.0, 51.4, 59.1, 174.4. ir (film) 2960 (s), 2880 (m), 1750 (s) cm^{-1}.

(+)-*cis-5-[N-(1,1-Bishydroxymethylethan-2-ol)-carbamyl]-1-benzylpyrrolidine-2-carboxylic acid* (***14***). mp 221–223°C (dec.). $[\alpha]_D^{25} = +15.0°$ (*c* 1.0, HCl (aq), 1 M). ^{1}H NMR (400 MHz, CF_3COOD) δ 2.25–2.40 (1H,m), 2.45–2.60 (1H,m), 2.85–3.00 (2H,m), 3.88 (6H,bs), 4.60 (1H,d,J=12.6 Hz), 4.70–4.95 (3H,m), 7.45–7.70 (5H,m). ^{13}C NMR (100.61 MHz, CF_3COOD) δ 30.9, 32.3, 62.6 (3C), 62.7, 65.1, 70.1, 70.7, 129.6, 132.0 (2C), 132.6 (2C), 133.5, 171.3, 173.7. ir (KBr) 3600–2300 (s), 1675 (s), 1640 (s), 1600 (s) cm^{-1}. High resolution MS (70 eV), *m/e* (%rel intensity) 352 (0.18) (M^+); calcd for $C_{17}H_{24}N_2O_6$ M = 352.1634, found M = 352.1533.

RESULTS

The e.e. of the monoester **2** obtained from pig liver esterase-catalyzed hydrolysis of *meso-cis-N*-benzyl-2,5-bismethoxycarbonylpyrrolidine (**1**) (Scheme 1) was increased by addition of dimethyl sulfoxide to the reaction medium (Fig. 1). The resulting e.e. values were higher when incubations were made in the presence of Tris buffer as compared to incubations in the pH-stat without buffer. When **1** was incubated with 25% (v/v) dimethyl sulfoxide in Tris buffer optically pure monoester **2** was obtained. The remarkably enhanced e.e. values of the monoester product achieved upon addition of dimethyl sulfoxide to the medium were not observed in the hydrolysis of *cis-N*-ethyl-2,5-bismethoxycarbonylpyrrolidine (**5**). From pH-stat incubations of **5** with 0 or 25% (v/v) dimethyl sulfoxide we isolated product monoester **6** of 71% e.e. and 65% e.e., respectively. With *trans-N*-benzyl-2,5-bismethoxycarbonylpyrrolidine (**3**), *trans-N*-ethyl-2,5-bismethoxycarbonylpyrrolidine (**7**), and *trans-N*-benzyl-2,5-bismethoxycarbonylpiperidine (**9**) a posi-

1 2

SCHEME 1

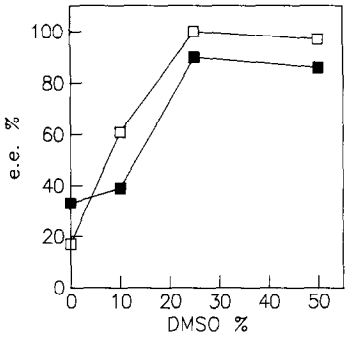


FIG. 1. Enantiomeric excess of *cis*-monoester **2** obtained by pig liver esterase hydrolysis of **1** in the presence of different concentrations of dimethyl sulfoxide. ■, Incubations in pH-stat at pH 7.5 without buffer, 22–25°C; □, incubations in 0.25 M Tris–HCl, pH 7.5, 25–30°C.

tive effect of dimethyl sulfoxide on the e.e. of the corresponding monoester products was again observed (Fig. 2a). Figure 2b emphasizes the pronounced improvement of the enantiomeric ratio *E* (*30*) from Tris buffer incubations as compared to reactions in the pH-stat. A good fit between *E*-values calculated on the basis of e.e. for formed monoester and *E*-values calculated on the basis of e.e. for remaining diester was obtained.

The effect of other reaction conditions on the enantiotopic selectivity of pig liver esterase in the hydrolysis of **1** was investigated by using other co-solvents, pH, type of buffer, buffer concentrations, or temperature. Of these variables all but the buffer concentration (ionic strength) seemed to influence the e.e. that could be reached for the *cis*-monoester **2** (Table 1).

Optically pure monoester **2** was also obtained using a reaction medium containing 10% methanol, but only if Tris was present. In incubations with 10% methanol

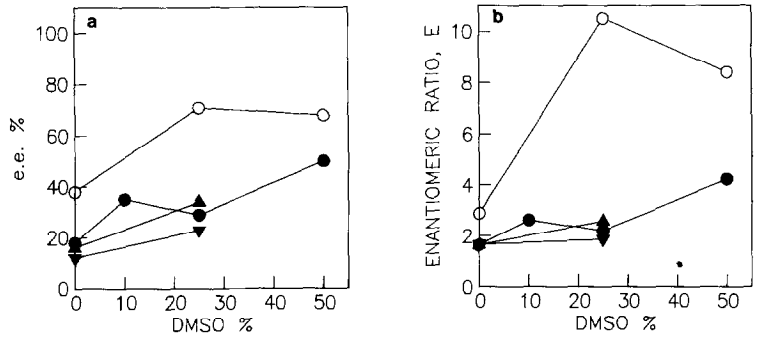


FIG. 2. Enantiomeric excess (a) and enantiomeric ratio, *E* (b), of *trans*-monoesters **4**, **8**, and **10** obtained by pig liver esterase hydrolysis of **3**, **7**, and **9** in the presence of different concentrations of dimethyl sulfoxide. ●, Incubations of **3** in pH-stat at pH 7.5, 22–25°C, conversion 42–46%; ○, incubations of **3** in 0.25 M Tris–HCl, pH 7.5, 22–25°C, conversion 41.6–45%; ▲, incubations of **7** in pH-stat at pH 7.5, conversion 42%; ▼, incubations of **9**, in pH-stat at pH 7.5, conversion 42%.

TABLE 1

Effect on the Enantiomeric Excess of Monoester **2** by Reaction Conditions in the Enzymatic Hydrolysis

Organic solvent	Deviations from normal conditions[a]	Monoester e.e.%
0%	No	17
0%	Phosphate buffer, 0.1 M	11
10% dimethyl sulfoxide	No	61
10% dimethyl sulfoxide	Enzyme batch 45F-8130	54
10% dimethyl sulfoxide	pH 8.0	49
25% dimethyl sulfoxide	No	100
25% dimethyl sulfoxide	Enzyme batch 45F-8130	86
25% dimethyl sulfoxide	t = 22°C	68
25% dimethyl sulfoxide	t = 22°C, 1.5 M Tris buffer	69
10% acetonitrile	No	39
10% acetone	pH-stat	61
10% methanol	No	100
10% methanol	pH-stat	−24[b]

[a] Normal reaction conditions: 20 ml 0.25 M Tris–HCl, pH 7.5, at 30°C and 1–2 mg pig liver esterase batch 34F-8110.

[b] The other enantiomer was formed.

in the pH-stat an e.e. of 24% was reached for the opposite enantiomer. The use of a second pig liver esterase preparation gave **2** of somewhat lower e.e. If the reaction was carried out at a lower temperature or at pH 8.0 instead of pH 7.5 lower e.e. values were obtained.

As can be seen in Fig. 3 the pig liver esterase catalyzed hydrolysis proceeded with the highest rate at a concentration of 25% of dimethyl sulfoxide with both the *cis*- and *trans*-diester, **1** and **3**, respectively. The specific enzymatic activity in Tris buffer at the 25% dimethyl sulfoxide level with *trans*-diester **3** was almost three-

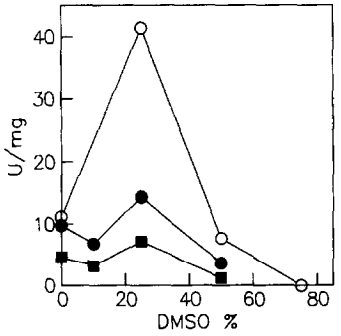
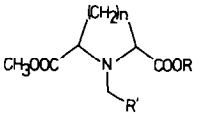


FIG. 3. Total reaction rate of pig liver esterase incubations with *cis*-diester **1** and *trans*-diester **3** at different concentrations of dimethyl sulfoxide. ■, Incubations of **1** in pH-stat at pH 7.5, 25°C; ●, incubation of **3** in pH-stat at pH 7.5, 25°C; ○, incubation of **3** in 0.25 M Tris–HCl, pH 7.5, 25°C.

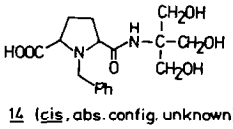
Cpd.	R	R'	n	rel. conf.	abs. conf.
1	CH_3	Ph	2	cis	
2	H	Ph	2	cis	(2S,5R)
3	CH_3	Ph	2	trans	
4	H	Ph	2	trans	(2S,5S)
5	CH_3	CH_3	2	cis	
6	H	CH_3	2	cis	n.d.
7	CH_3	CH_3	2	trans	
8	H	CH_3	2	trans	n.d.
9	CH_3	Ph	3	trans	
10	H	Ph	3	trans	n.d.
11	CH_3	Ph	3	cis	
12	CH_3	CH_3	3	cis	
13	CH_3	CH_3	3	trans	



SCHEME 2.

fold higher than for the corresponding reaction in the pH-stat. A comparison of the rate constants for the pig liver esterase-catalyzed and the base-catalyzed hydrolyses of the structurally related diesters **1, 3, 5, 7, 9,** and **11–13** (Scheme 2) showed that both the enzymatic rate constant and the ratio k_{cat}/k_{HO^-} had the largest values for the *trans*-pyrrolidine diester derivatives **3** and **7** (Table 2). The piperidine diesters **9, 11, 12,** and **13** are hydrolyzed very slowly.

Absolute configurations of product monoesters were not determined except for compounds **2** and **4** (cf. Experimental Procedures). All isolated *cis*-monoesters gave (+)-values in measurements of optical rotation, whereas *trans*-monoesters gave (−)-values indicating a common preference for the hydrolysis of the pro-S ester group. The chemical yields of monoester **2** were lower in Tris-buffered incubations than in the corresponding reactions in the pH-stat (39% vs 85%). A by-product that was formed in the enzyme-catalyzed hydrolysis of diester **1** when the reaction was carried out in Tris buffer was isolated. High resolution MS, proton NMR, and ^{13}C NMR demonstrated that this by-product was the dicarboxylic acid mono-Tris amide **14.**



14 (cis, abs. config. unknown)

TABLE 2

Rate of Enzymatic and Base-Catalyzed Hydrolysis of Diesters in the pH-stat

Substrate	k_{cat} (s^{-1})	k_{HO^-} (s^{-1} M^{-1})	k_{cat}/k_{OH^-} (M)
1	15.5	0.058	270
3	100	0.041	2440
5	5.8	0.17	34
7	81.0	0.032	2530
9	1.4	0.0069	200
11	0.12	n.d.	
12	1.4	n.d.	
13	2.2	0.0050	440

Note. Analyses were performed in the pH-stat in 25% dimethyl sulfoxide, 75 mM KCl at 30°C. The pig liver esterase-catalyzed reaction was maintained at pH 7.5 and the base-catalyzed reaction at pH 10.5. The k_{cat} values were calculated from the initial enzymatic activities with 10 mM substrate using the molecular weight of 180,000 for pig liver esterase (*5*) and one active site per enzyme molecule.

DISCUSSION

In a recent investigation we demonstrated the effect of dimethyl sulfoxide on the pig liver esterase-catalyzed hydrolysis of dialkylated propanedioic acid diesters (*14*). A more detailed investigation showed a remarkable enhancement of the enantiotopic selectivity for the hydrolysis of the pro-S ester group of the above-mentioned diesters with increasing dimethyl sulfoxide concentration in the reaction medium (*15*). This effect was interpreted as resulting from a distortion of the interaction of a hydrophobic substrate side chain with a hydrophobic binding site that has certain steric limitations. Earlier we noticed that the enantiomeric ratio, *E*, was much higher for the pig liver esterase-catalyzed hydrolysis of the *trans*-diethyl ester of 1,2-cyclohexanedicarboxylic acid when the reaction was carried out in Tris buffer than the values of *E* calculated from kinetic measurements in the absence of buffer in the pH-stat (*31*). Results in the present investigation were in accordance with these earlier findings. It has recently been pointed out that pig liver esterase-catalyzed reactions were effected by co-solvents, temperature, pH, or addition of albumin (*11, 32*). Another point to consider is that commercial preparations of pig liver esterase most probably are mixtures of several isoenzymes (*5, 6*). The isoenzyme composition of individual pig livers might differ significantly, but commercial preparations based on many livers should show a less pronounced variation. The isoenzymes have been shown to differ in properties such as substrate specificities, pH dependence, deacylation of acyl-enzyme

with methanol, and inhibition or activation by organic solvents or other substances (*33, 34*). The isoenzymes have been reported to react with similar stereospecificities (*35*).

The pronounced enhancement of the e.e. for the monoesters obtained in Tris-buffered pig liver esterase-catalyzed hydrolysis of *cis-* and *trans-N*-benzyl-2,5-bismethoxycarbonylpyrrolidine, respectively (Figs. 1 and 2a), can be explained assuming isoenzymes with similar stereoselectivities. The fact that a mono-Tris-amide **14** of the diacid corresponding to substrate diester **1** was isolated, indicates that Tris deacylates the acyl-enzyme intermediate formed in the reaction. Similar reactions have previously been shown for chymotrypsin (*36*) and alkaline phosphatase (*37*). In the latter case, however, the hydroxyl groups of Tris are the attacking nucleophiles. The observation that the enantioselectivity of pig liver esterase was higher in the presence of Tris suggests that the binding of Tris affected the deacylation of the two product-forming diastereomeric acyl-enzyme intermediates in different proportions. If Tris is able to bind prior to the formation of the acyl-enzyme the effect could of course also be a differentiated interference with this formation. As can be seen in Figs. 1 and 2a the increase of e.e. in Tris-buffered incubations compared to pH-stat incubations was higher when the concentration of dimethyl sulfoxide was increased. A partial explanation could be the increased concentration of uncharged Tris or increased nucleophilicity of Tris as the medium becomes less polar. Another possibility is increased binding of the rather polar Tris nucleophile when the environment becomes more hydrophobic. If such a binding site was saturated with 0.25 M Tris at a 25% dimethyl sulfoxide concentration this explains why the e.e. was not increased when the concentration of Tris was increased (Table 1). The higher rate of cleavage of the *trans*-diester **3** in the presence of Tris can only be explained by Tris being a better nucleophile than water for the deacylation of acyl-enzyme (Fig. 3). In the same way as with the enantioselectivity, the rate enhancement by Tris was most pronounced at the 25% dimethyl sulfoxide level. In this case the effect was probably also caused by the increased solubility of the substrate. No substrate cleavage was seen at a concentration of 75% dimethyl sulfoxide.

Some other factors that also influenced the e.e. obtained for monoester **2** were temperature, pH, and choice of organic co-solvent (Table 1). The enantiospecific reaction with 10% methanol in Tris buffer is interesting since it is known that methanol also deacylates the acyl-enzyme (*6*) and thus, with a dimethyl ester, substrate will inhibit the reaction. Incubations in the pH-stat with 10% methanol resulted in the predominant formation of the opposite enantiomer. If methanol preferentially attacked the same intermediate that was normally preferred by water the normal hydrolysis would be inhibited and the other enantiomer would dominate. Different binding sites for amino- and hydroxy-nucleophiles on acyl-enzymes are known (*38*), supporting the idea that both Tris and methanol can affect the stereoselectivity of pig liver esterase. Since methanol can also influence the enantioselectivity by altering hydrophobic enzyme–substrate interactions, as suggested for dimethyl sulfoxide, the nature of its effect is not clear.

The result that somewhat lower e.e. values were obtained with another enzyme batch supports the idea that different isoenzymes have different enantioselectivi-

ties. The strong influence of many incubation parameters on the e.e. values obtained shows how important the reaction conditions are when results obtained in different laboratories are compared.

Unfortunately we have not yet been able to determine the absolute configuration of the Tris-amide formed, when pig liver esterase-catalyzed hydrolysis is carried out in Tris buffer. We hope that this will be possible in the near future. A full understanding of the e.e. enhancing effect of an alternative nucleophile may provide new possibilities of preparative interest in asymmetric organic synthesis.

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REFERENCES

1. WHITESIDES, G. M., AND WONG, C.-H. (1985) *Angew. Chem. Int. Ed. Eng.* **24,** 617–718.
2. JONES, J. B. (1986) *Tetrahedron* **42,** 3351–3403.
3. JONES, J. B., AND BECK, J. F. (1976) *Tech. Chem. (N.Y.)* **10,** 107–401.
4. BJÖRKLING, F., BOUTELJE, J., HULT, K., KRAULIS, P., NORIN, T., AND SZMULIK, P. (1987) *Biocatalysis* **1,** 83–95.
5. HEYMANN, E., AND JUNGE, W. (1979) *Eur. J. Biochem.* **95,** 509–518.
6. FARB, D., AND JENCKS, W. P. (1980) *Arch. Biochem. Biophys.* **203,** 214–226.
7. MOHR, P., WAESPE-SARCEVIC, N., TAMM, C., GAWRONSKA, K., AND GAWRONSKI, J. K. (1983) *Helv. Chim. Acta* **66,** 2501–2511.
8. BJÖRKLING, F., BOUTELJE, J., GATENBECK, S., HULT, K., NORIN, T., AND SZMULIK, P. (1985) *Tetrahedron* **41,** 1347–1352.
9. CROUT, D. H. G., GAUDET, V. S. B., LAUMEN, K., AND SCHNEIDER, M. P. (1986) *J. Chem. Soc. Chem. Commun.*, 808–810.
10. RAMASWAMY, S., HUI, R. A. H. F., AND JONES, J. B. (1986) *J. Chem. Soc. Chem. Commun.*, 1545–1546.
11. LAM, K. P. L., HUI, R. A. H. F., AND JONES, J. B. (1986) *J. Org. Chem.* **51,** 2047–2050.
12. BOUTELJE, J., HJALMARSSON, M., SZMULIK, P., NORIN, T., AND HULT, K. (1987) *in* Biocatalysis in Organic Media (Laane, C., Tramper, J., and Lilly, M. D., Eds.), pp. 361–368, Elsevier, Amsterdam.
13. KURIHARA, M., KAMIYAMA, K., KOBAYASHI, S., AND OHNO, M. (1985) *Tetrahedron Lett.* **26,** 5831–5834.
14. BJÖRKLING, F., BOUTELJE, J., GATENBECK, S., HULT, K., AND NORIN, T. (1985) *Tetrahedron Lett.* **26,** 4957–4958.
15. BJÖRKLING, F., BOUTELJE, J., GATENBECK, S., HULT, K., NORIN, T., AND SZMULIK, P. (1986) *Bioorg. Chem.* **14,** 176–181.
16. BJÖRKLING, F., BOUTELJE, J., HJALMARSSON, M., HULT, K., AND NORIN, T. (1987) *J. Chem. Soc. Chem. Commun.*, 1041–1042.
17. PINDER, A. R. (1986) *Natl. Prod. Rep.* **3,** 171–180.
18. ROBINS, D. J. (1986) *Natl. Prod. Rep.* **3,** 297–305.
19. GRUNDON, M. F. (1985) *Natl. Prod. Rep.* **2,** 235–243.
20. BUCHMAN, E., REIMS, A., SKEI, T., AND SCHLATTER, M. (1942) *J. Amer. Chem. Soc.* **64,** 2696–2700.

21. McDonald, R. N., and Reitz, R. R. (1972) *J. Org. Chem.* **37,** 2418–2423.
22. Cignarella, G., and Nathanson, G. (1961) *J. Org. Chem.* **26,** 1500–1504.
23. Guha, P. C., and Sankaran, D. K. (1955) Organic Syntheses, Vol. 3, pp. 623–625, Wiley, New York.
24. Solladie-Cavallo, A., Bouchet, M. J., and Wermuth, C. G. (1983) *Org. Magn. Resonance* **21,** 6, 367–370.
25. Hill, R. K., and Chan, T. H. (1965) *Tetrahedron* **21,** 2015–2019.
26. Della, E. W., and Kendall, M. (1973) *J. Chem. Soc. Perkin Trans. I,* 2729–2730.
27. Blackman, S. W., and Baltzly, R. (1961) *J. Org. Chem.* **26,** 2750–2755.
28. Lowe, G., and Ridley, D. D. (1973) *J. Chem. Soc. Perkin Trans. I,* 2024–2029.
29. Morimoto, Y., Terao, Y., and Achiwa, K. (1987) *Chem. Pharm. Bull.* **35**(6), 2266–2271.
30. Chen, C. S., Fujimoto, Y., Girdaukas, G., and Sih, C. J. (1982) *J. Amer. Chem. Soc.* **104,** 7294–7299.
31. Björkling, F., Boutelje, J., Gatenbeck, S., Hult, K., and Norin, T. (1985) *Appl. Microbiol. Biotechnol.* **21,** 16–19.
32. Guanti, G., Banfi, L., Narisano, E., Riva, R., and Thea, S. (1986) *Tetrahedron Lett.* **27,** 4639–4642.
33. Junge, W., and Heymann, E. (1979) *Eur. J. Biochem.* **95,** 519–525.
34. Farb, D., and Jencks, W. P. (1980) *Arch. Biochem. Biophys.* **203,** 227–235.
35. Jones, J. B. (1985) *in* Proceedings, FECS Third International Conference, Sofia, Bulgaria, Sept. 1985, Vol. 1, pp. 18–39.
36. Kasche, V., and Zöllner, R. (1982) *Hoppe-Seyler's Z. Physiol. Chem.* **363,** 531–534
37. Snyder, S. L., and Wilson, I. O. I. (1972) *Biochemistry* **11,** 3220–3223.
38. Kasche, V. (1986) *Enzyme Microb. Technol.* **8,** 4–16.