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Enhanced Stereoselectivity in Pig Liver Esterase Catalysed Diester Hydrolysis. The Role of a Competitive Nucleophile

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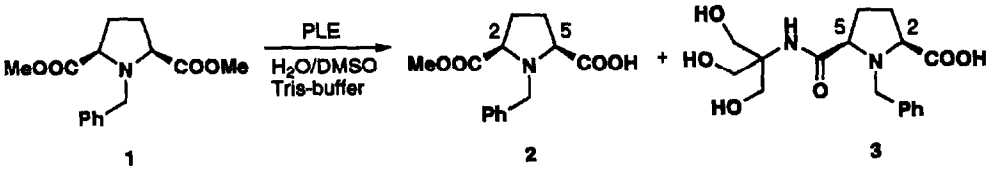
Abstract—The enantioselectivity of pig liver esterase catalysed hydrolysis of *cis*-N-benzyl-2,5-bis(methoxycarbonyl)pyrrolidine (**1**) has previously been shown to be very dependent on the reaction conditions.[1,2] Hydrolysis performed in media buffered with tris(hydroxymethyl)aminomethane (Tris) afforded a monoester with much higher optical purity than hydrolysis in media without Tris. Detailed product studies in a Tris-buffered medium have been performed using NMR-techniques and a ^{13}C-labelled ester. The NMR-studies revealed the presence of (2*S*,5*R*)-N-benzyl-2-methoxycarbonyl-5-[[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]carbonyl]pyrrolidine (**4**) as an intermediate, which together with the isolated product (2*S*,5*R*)-N-benzyl-2-carboxy-5-[[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]carbonyl]pyrrolidine (**3**) suggested Tris as a competitive nucleophile to water. The increased enantioselectivity seen in the produced (2*R*,5*S*)-N-benzyl-2-methoxycarbonyl-5-carboxypyrrolidine (**2**) was explained by the preference of Tris to react faster with one of the diastereomeric acyl enzymes over the other.

Introduction

The activity and selectivity of biocatalysts are highly dependent on reaction conditions. A firm knowledge of this dependence is essential for the successful employment of enzymes in synthetic chemistry. Among commercially available enzymes, pig liver esterase (PLE, EC 3.1.1.1), has received much attention as a synthetically useful hydrolase that accepts a broad range of prochiral or racemic substrates and operates with a high degree of stereoselectivity.[3] Several models for the prediction of the enantioselectivity of PLE-catalysed reactions have been published (see ref.[4]). The most extensive model is based on the presence of two hydrophobic binding pockets, a small and a large one.[4] It has been suggested that the two different binding pockets exist on different subunits of the isoenzyme mixture of PLE.[5] The optimisation of reaction conditions to achieve higher stereoselectivity in PLE-catalysed reactions has been studied. Careful examination of how reaction conditions influence the stereochemical outcome of PLE-catalysed diester hydrolysis is rewarding and provides new unexpected clues to the kinetics and mechanism of the reaction.

We have previously reported the enzyme-catalysed hydrolysis of *cis*-N-benzyl-2,5-bis(methoxycarbonyl)-pyrrolidine (**1**).[1,2] Conditions for an enantiospecific PLE-catalysed hydrolysis were found in a medium buffered with tris(hydroxymethyl)aminomethane (Tris).[1] A drawback was the moderate chemical yield of 39 %. In our initial studies of the reaction, high stereoselectivity was of primary importance, and the loss of isolated yield was attributed to work-up problems. Surprisingly, a much higher yield (80 %) of the (2*R*,5*S*)-ester-acid **2**[6] could be obtained, when the reaction was performed using a pH-stat. Evidence for different reaction pathways under the two hydrolytic conditions was provided with the isolation of the (2*S*,5*R*)-acid-amide **3** from Tris-buffered incubations.[2] This observation triggered the present study of Tris acting as a nucleophile.

There is a pronounced enhancement of the enantiomeric excess (*e.e.*) of the (2*R*,5*S*)-ester-acid **2** in Tris-buffered



Scheme I.

Dedicated to Professor Bryan Jones on the occasion of his 60th birthday.

incubations versus reactions performed using a pH-stat. We rationalised this effect as a result of nucleophilic attack by Tris on the acyl enzyme intermediate with subsequent formation of Tris-amide.[2,7] Although similar reactions of Tris with chymotrypsin[8] and alkaline phosphatase[9] have been reported previously, the isolation of this type of Tris-amide was unprecedented.

In this paper, we present direct evidence of Tris acting as a competitive nucleophile in the PLE-catalysed hydrolysis of diester **1**. The relative and absolute configuration of the isolated (2*S*,5*R*)-acid-amide **3** was determined from X-ray diffraction data and synthetic transformations, respectively (see Results). ^{13}C NMR spectroscopy was used to follow the reaction. Our results allow for a mechanistic interpretation of earlier studies and provide kinetic information, which is important in the understanding of stereochemical control of PLE-catalysed reactions.

Results

The determination of the gross structure of the acid-amide **3** has been reported earlier on the basis of ^1H NMR, ^{13}C NMR and high resolution mass spectroscopy.[2] The previously assigned *cis*-configuration of **3** has now been confirmed by a single-crystal X-ray structure determination. However, it was not possible to conclude whether the absolute configuration of isolated **3** was 2*S*,5*R* or 2*R*,5*S* from crystallographic data.

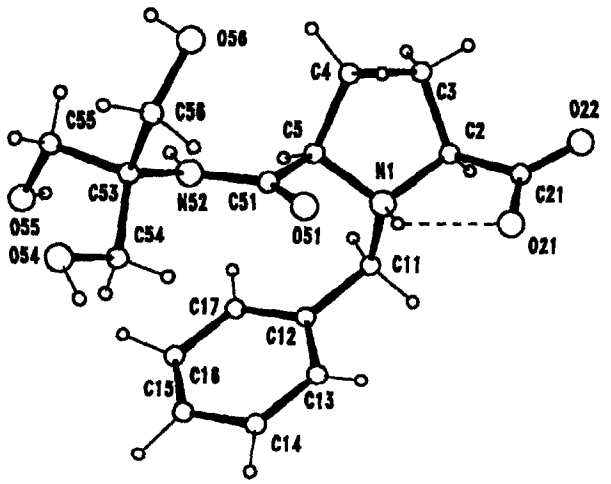


Figure 1. Perspective view of the acid-amide **3**. The pyrrolidine nitrogen atom (N1) is protonated and has a short intramolecular hydrogen bond to the carboxyl O21 atom. Solid and dashed lines represent covalent and hydrogen bonds, respectively. In the figure the absolute configuration is drawn as (*S*)-C2 and (*R*)-C5. This configuration was, however, not possible to conclude from X-ray data.

Several attempts to prove the absolute configuration of **3** via synthesis of (2*R*,5*S*)-ester-amide **4** from (2*R*,5*S*)-ester-acid **2** and Tris failed. This is probably due to the poor solubility of Tris in organic solvents. To circumvent these problems, we prepared compound **5**, the TMS-derivative of Tris. This O-protected Tris was isolated as a clear oil in 38 % yield after distillation.[10] Using the TMS-Tris derivative **5**, the synthesis of ester-amide product (–)-(2*R*,5*S*)-**4** was accomplished in a single operation from (+)-(2*R*,5*S*)-ester-acid **2**, the absolute configuration of which has been determined previously.[1] In agreement with earlier observations by Della and Kendall[11] it was not possible to prepare the acyl chloride ester from **2**. Instead, amide formation was best performed via the mixed anhydride of (+)-(2*R*,5*S*)-ester-acid **2** prepared *in situ* with ethyl chloroformate. The intermediate **6** was deprotected directly using citric acid in methanol[12] affording the ester-amide (–)-(2*R*,5*S*)-**4** in 46 % isolated yield. This correlation of (–)-**4** to (+)-**2** establishes the (2*R*,5*S*)-configuration of (–)-**4**. Furthermore, esterification of (+)-(2*S*,5*R*)-acid-amide **3** with methanol under acid-catalysed conditions[13] gave a low yield (14 %) of (+)-(2*S*,5*R*)-**4** along with other products, among these (+)-(2*R*,5*S*)-ester-acid **2** (Scheme II). On the basis of these transformations, we assigned the (2*S*,5*R*)-configuration to the acid-amide (+)-**3**, which was originally isolated from Tris-buffered incubations of diester **1** with PLE.

The isolation of (+)-(2*R*,5*S*)-ester-acid **2** during the esterification of (+)-(2*S*,5*R*)-acid-amide **3** deserves a brief comment. The formation of **2** seems to be the result of methanolysis of the amide bond. Evans *et al.*[14,15] have observed that proximal hydroxyl groups facilitate the hydrolysis of amide bonds in prolinol amides via acid-catalysed N→O acyl transfer[16] giving an intermediate ester function which is then slowly transesterified. Applying this mechanistic rationale to the methanolysis of **3**, where the hydroxyl groups are likewise placed in γ-positions to the amide carbonyl, would involve a transesterification as the product-forming step (Scheme III). The (+)-(2*R*,5*S*)-ester-acid **2** obtained in this experiment was found to be of high optical purity (> 98 % *e.e.*) by ^1H NMR analysis.[1] This result lends further support to the above assignment of the absolute configuration of (+)-(2*S*,5*R*)-**3** and, still more important, shows that crystalline (+)-(2*S*,5*R*)-acid-amide **3**, isolated from Tris-buffered incubations of **1** with PLE, is of, at least, an equally high enantiomeric purity (> 98 % *e.e.*). This fact clearly establishes that the isolated (+)-(2*S*,5*R*)-amide **3** was formed in an enzymatic reaction.

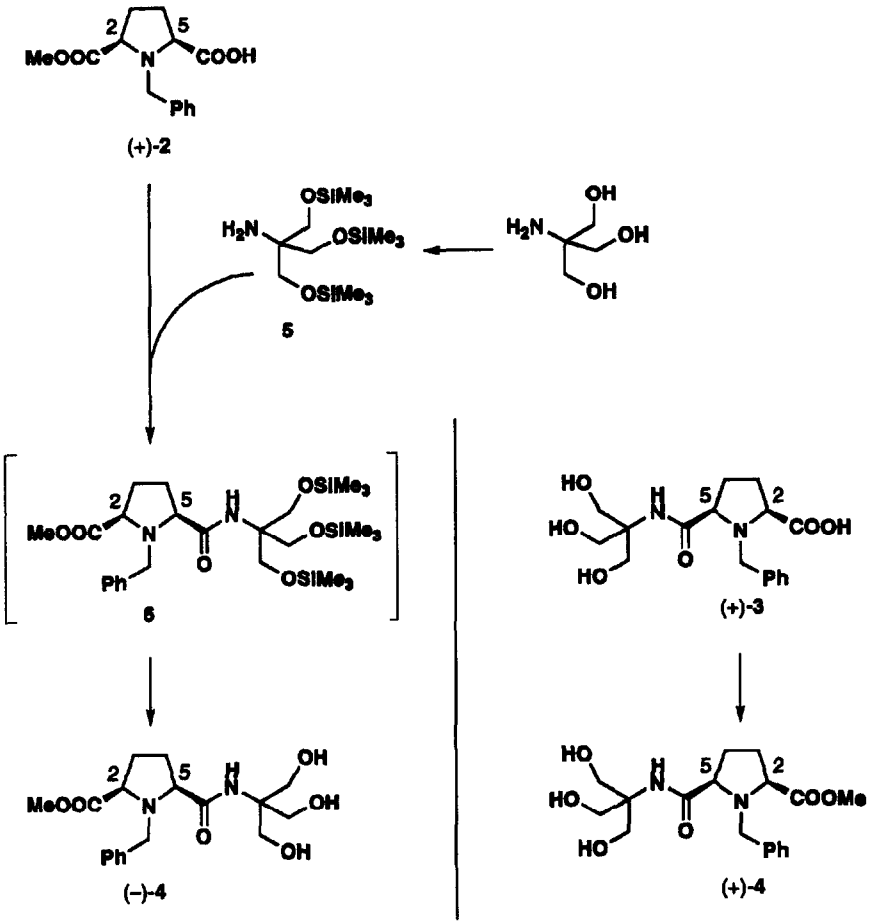
The diester **1**, which has been ^{13}C-labelled in the methoxy groups, was used in the PLE-catalysed hydrolysis giving (+)-(2*R*,5*S*)-ester-acid **2**. The reaction was monitored by ^{13}C NMR in an ordinary NMR tube and proceeded smoothly in a Tris-buffered medium under the same conditions as previously reported[1] to yield (+)-(2*R*,5*S*)-ester-acid **2** of > 98% *e.e.* (only one enantiomer was observed). Figure 2 shows the relative amounts of the ^{13}C-enriched reaction species **1**, **2**, **4** and ^{13}C-enriched methanol. Unfortunately, **1** and **2** had coinciding chemical shifts. Their peak areas could not be separately integrated and the combined area is indicated in Figure 2.

No reaction was seen until the reaction was initiated, after which two signals quickly appeared in the ^{13}C spectra. They were identified as methanol (δ 50.4) and the intermediate **4** (δ 53.9). A qualitative interpretation of the results from this experiment is given in the Discussion. In order to prove the assignment of the NMR peaks belonging to the product **2** and the intermediate **4**, ^{13}C

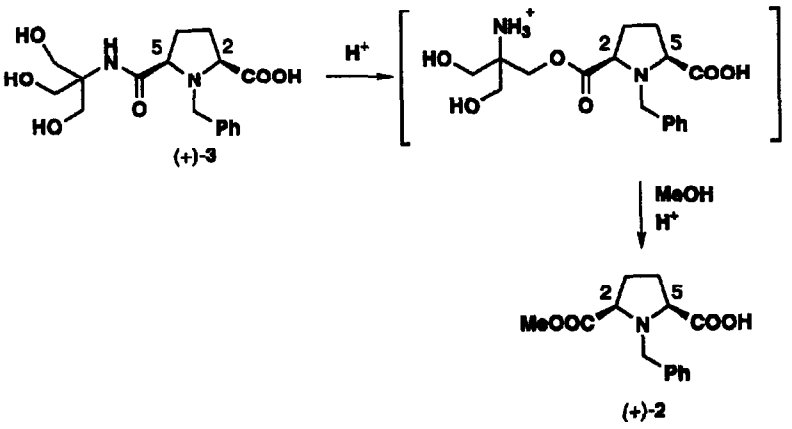
NMR spectra were recorded of the unlabelled synthetic samples under incubation conditions excluding the enzyme. The methoxy signal of **4** appeared at δ 53.7, which was roughly identical to the position (δ 53.9) already assigned to **4** in the hydrolysis reaction.

Discussion

The isolation of the (+)-(2*S*,5*R*)-acid-amide **3** as a reaction product, and the detection by ^{13}C NMR measurements of the ester-amide **4** as a reaction intermediate during the PLE-catalysed hydrolysis of the diester **1**, confirmed earlier observations that Tris can compete effectively with water as a nucleophile attacking the acyl enzyme intermediate. It is well known that nucleophiles other than water react with acyl enzyme intermediates in serine hydrolase-catalysed reactions to give new products, e.g. amides[17] and esters.[18]



Scheme II.



Scheme III.

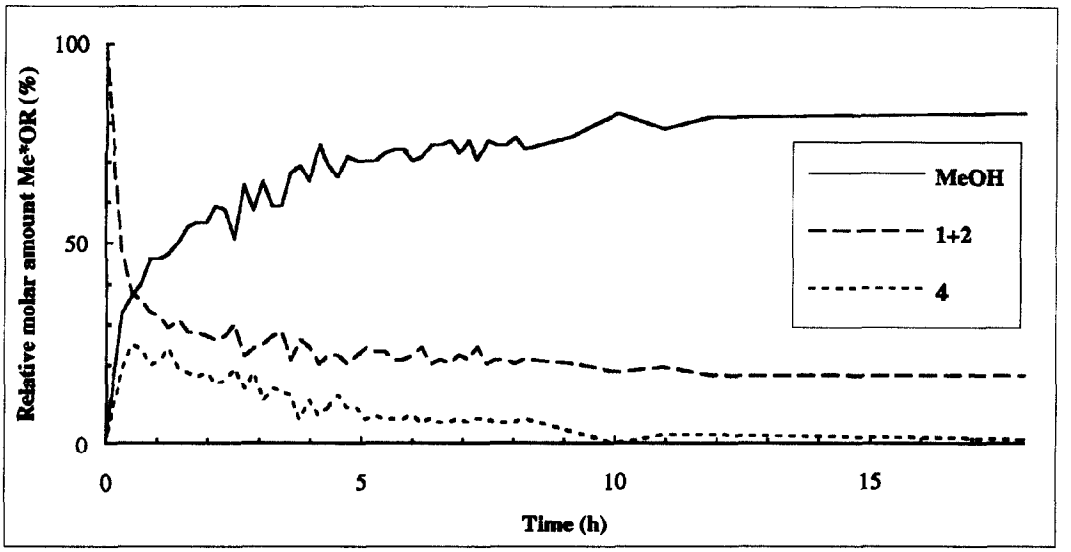


Figure 2. Enzymatic hydrolysis of ^{13}C-labelled diester **1** monitored by ^{13}C NMR. Automatically integrated peak areas were corrected for the lower NMR sensitivity of the methyl carbon atom in MeOH when compared to the methyl carbon atoms of the ester group (correction factor 3.65). Relative amounts of MeOH, the sum of diester **1** and ester-acid **2**, and ester-amide **4** are plotted against reaction time. The traces are based on data from 67 consecutive spectra.

The diester substrate **1** can form two diastereomeric acyl enzymes (Scheme IV).[19] The *e.e.* (90 %) of the product **2**, obtained by hydrolysis in the absence of any alternative nucleophile other than water,[2] shows that the two diastereomeric forms of the acyl enzyme can be formed and hydrolysed. The high enantiomeric excess of the 2*R*,5*S*-enantiomer of the produced ester-acid shows that the (*S*)-ester group of **1** reacts more easily with the enzyme than the (*R*)-ester group. The effect of Tris, to increase the *e.e.* of the product **2**, could be explained by the fact that Tris reacted preferentially with the acyl enzyme formed by attack on the (*R*)-ester affording the (+)-(2*S*,5*R*)-ester-amide **4**, which in turn was enzymatically hydrolysed to (+)-(2*S*,5*R*)-acid-amide **3**. This drained the acyl enzyme to the alternative product (+)-(2*S*,5*R*)-acid-amide **3**. The result was seen as an increased enantiomeric excess in the (+)-(2*R*,5*S*)-ester-acid **2**.

Further reaction of Tris with the intermediate amide acyl enzyme would lead to the formation of the *meso* diamide **7**. This compound was synthesised and it was shown that it was not a substrate to the enzyme. As no accumulation of the diamide in the reaction mixture was detected during the enzymatic hydrolysis of **1**, its involvement in the reaction was ruled out.

Unfortunately, the ^{13}C NMR experiments could not be used for the detailed quantification of reaction rates or concentrations of intermediates due to a combination of the inherent problems in quantification by ^{13}C NMR and the identical chemical shifts of the diester **1** and the ester-acid **2**.

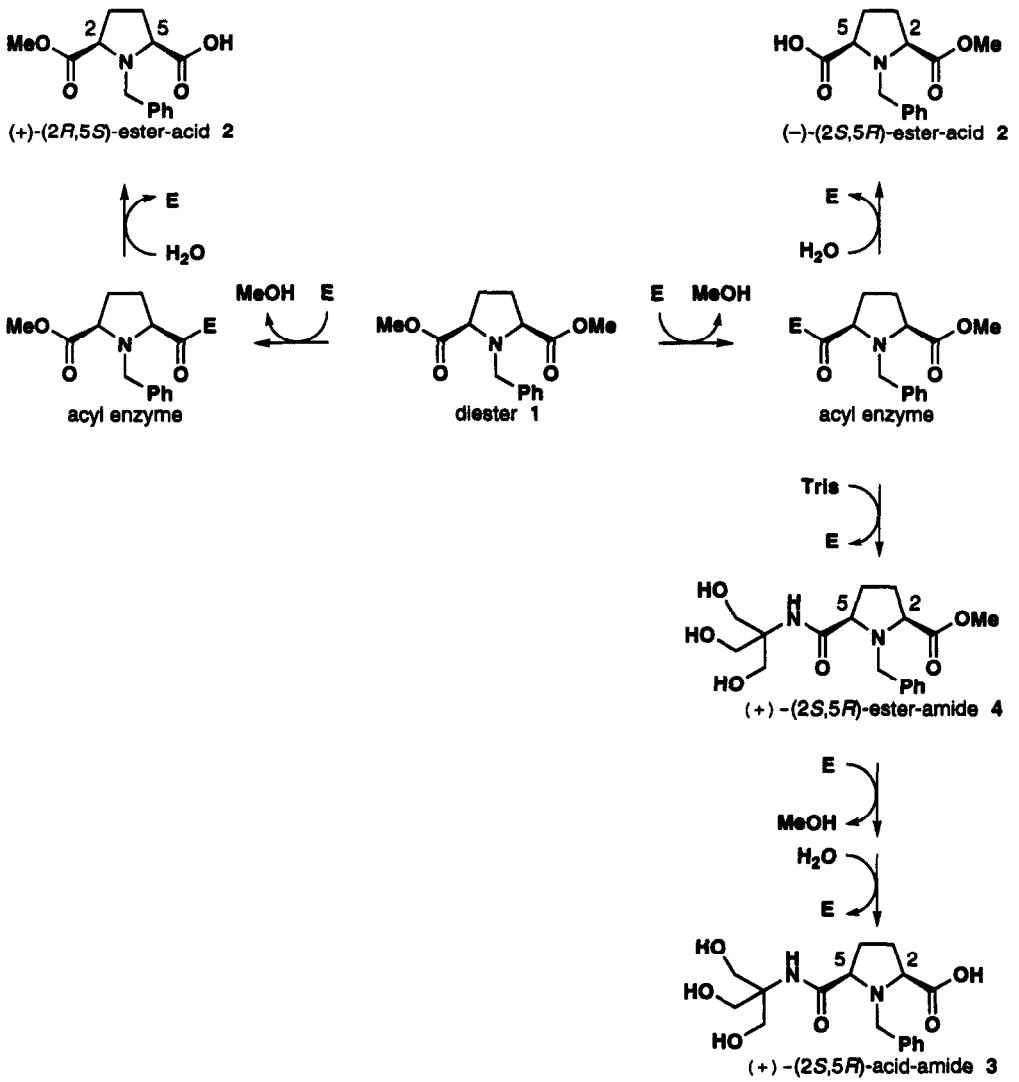
Considering that the concentrations of water and Tris were ≈ 42 M and 0.5 M, respectively, the reactivity of Tris in comparison to water could be estimated. A mass balance calculated from the enantiomeric excess of **2**, in presence and absence of Tris (90 % *e.e.* and 98 % *e.e.*, respectively), and **3** (98 % *e.e.*) results in a reactivity of Tris that is approximately 350 times that of water in the deacylation of the acyl enzyme yielding (+)-(2*S*,5*R*)-**3**. On the other hand, the reactivity of water was calculated to be about 30 times that of Tris in the deacylation of the acyl enzyme yielding (+)-(2*R*,5*S*)-**2**. The calculated reactivities are very sensitive to the values of *e.e.* Since it was not possible to determine an enantiomeric excess higher than 98 % with accuracy, the calculated reactivity values should be seen as estimates.

The present investigation demonstrates the importance of reaction conditions in asymmetric organic synthesis using hydrolytic enzymes. The results of our study provide information of general interest. Thus, a nucleophile like Tris can drastically influence the enantiomeric excess of the product in a PLE-catalysed hydrolytic reaction of a prochiral substrate, where the alternative nucleophile competes with water in the enzyme-catalysed hydrolysis. The observed effect of a competing nucleophile should be general and applicable also to other prochiral or racemic substrates and to other hydrolytic enzymes, which form acyl enzyme intermediates.[20]

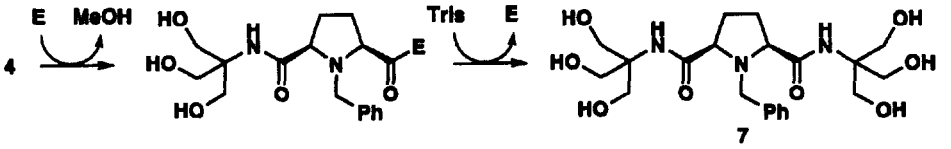
Experimental Section

General procedures

^{1}H and ^{13}C NMR spectra were recorded on Bruker WP200 (200 MHz for ^{1}H) and AM400 (400 MHz for ^{1}H, 100 MHz for ^{13}C) FT instruments. Chemical shifts are given in ppm using tetramethylsilane (Me_4Si) as internal standard. The signal from DMSO-d_6 (δ 39.5) was used as internal reference in experiments with ^{13}C-enriched substrate. Infrared spectra were recorded on a Perkin–Elmer 1420 spectrophotometer. A Perkin–Elmer 241 polarimeter was used for optical rotation measurements. Boiling points are given as air bath temperatures in a Büchi GKR 50 glass tube oven. Melting and boiling points are uncorrected.



Scheme IV.



Scheme V.

General materials

Pig liver esterase (PLE, EC 3.1.1.1) was purchased as a suspension in aqueous $(NH_4)_2SO_4$ from Sigma (batch no. 34F-4110). The suspension was centrifuged and the enzyme pellet was dissolved in the medium used for each experiment. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Sigma (85F-5608) and it was dried *in vacuo* before use in the synthesis of compound **5**. Triethylamine (Et_3N , Merck z. Synth.) was distilled from calcium hydride prior to use. Ethyl chloroformate was purified by fractional distillation before use. *cis*-N-Benzyl-2,5-bis(methoxycarbonyl)pyrrolidine (**1**) was prepared as previously described.[2] All other chemicals were used as obtained from commercial sources.

cis-N-Benzyl-2,5-bis(methoxycarbonyl)(methoxy-^{13}C)-pyrrolidine

cis-N-Benzylpyrrolidine-2,5-dicarboxylic acid (obtained from hydrolysis of diester **1**[21] in 1 M HCl, 65 % yield after 2 recryst.; cryst. from H_2O/EtOH; mp 242–244 °C, lit.:[22] 247–250 °C) was esterified upon reflux (6 h) in $^{13}CH_3OH/C_2H_4Cl_2$ (1:7) with acid catalysis (H_2SO_4). Solvent evaporation and filtration of the crude product through a short column of silica gel gave pure ^{13}C-

labelled diester **1** (purity and identity were checked by ^{1}H NMR, GLC and TLC).

^{13}C NMR Measurements of the transformation of diester 1 to ester-acid 2

Diester **1** (methoxy-^{13}C-labelled, 1.4 mg, 5 μmol), DMSO-d_6 (0.25 mL) and Tris–HCl (0.5 M, pH 7.5, 0.725 mL) were added to an NMR tube (5 mm, Wilmad 507PP). Following thorough mixing, a ^{13}C spectrum was recorded to establish the shift of the methoxy group carbon of **1** in this medium. The reaction was initiated by addition of PLE (12.5 μL of a 0.8 mg/mL solution in 50 mM 2-(N-morpholino)propanesulfonic acid–KOH, pH 6.0) and H_2O (12.5 μL) to the NMR tube, which was then vigorously shaken. Acquisition of ^{13}C NMR data was started within 2 min after this addition. The experiments were performed at 31 °C. The shifts were very sensitive to minor changes of the pH-value of the medium. The FIDs were automatically recorded. Each of 67 recorded FIDs required a total time of 11 min including data acquisition (NS = 2000, AQ = 0.33 s, 16K data points, 37° pulse angle) and storage. After Fourier transformation, the integrated area under each peak was measured. The peak area corresponding to the amount of MeOH was corrected for the lower NMR sensitivity of MeOH using a correction factor (see below; the sensitivities for **1**, **2**, and **4** are assumed to be identical). The peaks from the ^{13}C-enriched species, which were observed in this experiment, were positioned at δ 53.9 (**4**), δ 53.5 (**1** + **2**) and δ 50.4 (MeOH). The compounds **2** and **4**, as well as methanol, have been dissolved separately in the medium used for enzyme-catalysed hydrolysis in order to confirm the observed shifts in the hydrolysis reaction. The results of the NMR measurements are presented in Figure 2.

Control experiment in the absence of enzyme

Diester **1** (methoxy-^{13}C-labelled, 1.4 mg, 5 μmol), DMSO-d_6 (0.25 mL) and Tris–HCl (0.5 M, pH 7.5, 0.725 mL) were added to an NMR tube (5 mm, Wilmad 507PP) and thoroughly mixed. No reaction was detected by NMR within 3 h, i.e. no formation of methanol.

Determination of the correction factor for the ^{13}C NMR integrated peak area of MeOH

Diester **1** (1.4 mg, 5 μmol), DMSO-d_6 (0.25 mL) and Tris–HCl (0.5 M, pH 7.5, 0.725 mL) were added to an NMR tube (5 mm, Wilmad 507PP). MeOH (in Tris–HCl 0.5 M, pH 7.5; 25 % DMSO) was added in portions of ~0.2 equivalents. Two FIDs were recorded after each addition; (1) NS = 2000, AQ = 0.33 s, 16K data points, and 37° pulse angle, (2) NS = 700, AQ = 0.66 s, 32K data points, 90° pulse angle, and 120 s relaxation delay between each pulse. The ratio between the peaks corresponding to MeOH and **1**, respectively, from the first spectrum was compared to the same ratio obtained from the second experiment. Totally, 16 comparisons gave an average correction factor for MeOH of 3.65 ± 0.52 (95 % significance).

Structure determination of compound 3 by X-ray diffraction

The selected needle shaped single crystal, grown from a DMSO–water (1:3) mixture, had the approximate dimensions 0.11×0.38×0.07 mm. Crystal data: $C_{17}H_{24}N_2O_6$, M_w = 352.386, orthorhombic, ($P2_12_12_1$), a = 9.7587(2), b = 10.2901(3), c = 17.2608(3) Å, V_c = 1733.30(7) Å^3, Z = 4, D_c = 1.350 g/cm^3, F(000) = 752, and μ = 8.16 cm^{-1}. The intensities of 2817 reflections, of which 1728 were unique and non-zero, were collected at room temperature (CuK_α radiation, λ = 1.54183 Å, θ_{max} = 70°) and corrected for background, Lorentz and polarisation effects. The unit cell dimensions were refined against the angular settings of 40 reflections with 18° < 2θ < 72°.

The structure was solved by application of direct methods (SHELXS[23]) and refined by full-matrix least-squares calculations (SHELX[24]). The hydrogen atoms were located from difference electron density maps and were held riding on their parent atoms during the subsequent calculations. In the last stage of the refinement, the non-hydrogen atoms were treated anisotropically, and isotropic vibrational parameters were refined for the hydrogens.

The molecule comprises two chiral carbon atoms, C2 and C5 (*cf.* Figure 1). According to the X-ray study, the absolute configuration of the molecule is either 2*S*,5*R* or 2*R*,5*S*. The final refinement of 250 variables against 1357 unique reflections with $I/\sigma(I) > 2.5$ was carried out for both possible mirror-symmetry-related diastereoisomers. The two refinements, however, converged to identical agreement factors, R = 0.040 and ωR = 0.063 (SHELX[24] weighting scheme with g = 0.006414), indicating that the crystal contained only 'light' atoms, which do not yield observable anomalous dispersion effects with the radiation used. Accordingly, only the relative configuration could be deduced from the present X-ray diffraction study.

The ring-puckering coordinates Φ = –63.3(7)° and Q = 0.3405 Å, calculated according to Cremer and Pople,[25] show that the non-planar pyrrolidine ring is intermediate between envelope and half-chair.

Tris(trimethylsiloxymethyl)aminomethane, 5

Tris (tris(hydroxymethyl)aminomethane; 3.63 g, 30 mmol) and trimethylsilyl chloride (22.7 mL, 180 mmol) were placed in a 100 mL round-bottom flask under argon atmosphere. A solution of imidazole (16.3 g, 240 mmol) in dimethylformamide (30 mL) was added under stirring at room temperature. The flask was then provided with a drying tube. The resulting clear solution was stirred overnight at room temperature, and the formation of a white precipitate was observed. Excessive trimethylsilyl chloride was carefully removed *in vacuo* and the solid material was then filtered off. The filtrate was diluted with CH_2Cl_2 (50 mL) and washed successively with H_2O (50 mL) and $NaHCO_3$ (aq., sat., 50 ml). Rotary evaporation yielded an oil that was purified by distillation affording

3.85 g (38 %) of the colourless O-silylated Tris **5**; bp 70–80 °C, 0.04 mm Hg; IR (cm^{-1}): 2960 (s), 2920 (m), 1250 (s), 1085 (s), 875 (s), 840 (s), 750 (m); ^1H NMR ($CDCl_3$, 400 MHz): δ 0.07 (27H, s), 1.50 (2H, bs), 3.37 (6H, s); ^{13}C NMR ($CDCl_3$, 100 MHz): δ –0.6 (q), 56.7 (s), 63.8 (t). Anal. calcd for $C_{13}H_{35}NO_3Si_3$: C, 46.24; H, 10.45; N, 4.15; O, 14.21; Si, 24.95. Found: C, 46.04; H, 10.26; N, 4.28; Si, 25.10.

(–)-(2R,5S)-N-Benzyl-2-methoxycarbonyl-5-[[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]carbonyl]pyrrolidine, (–)-(2R,5S)-ester-amide 4

(+)-(2*R*,5*S*)-N-Benzyl-2-methoxycarbonyl-5-carboxypyrrolidine **2** [79 mg, 0.3 mmol, 79 % *e.e.*,[26] $[\alpha]_D^{21}$ = +13.1° (*c* 2.69, $CHCl_3$)] was dissolved in CH_2Cl_2 (1 mL) and stirred at 0 °C under argon. Following addition of Et_3N (73 mg, 0.72 mmol) in CH_2Cl_2 (1 mL), a solution of ethyl chloroformate (36 mg, 0.33 mmol) in CH_2Cl_2 (1 mL) was injected via a syringe. After 1 h at 0 °C, compound **5** (111 mg, 0.33 mmol) in CH_2Cl_2 (1 mL) was added and the mixture was allowed to reach ambient temperature. Stirring was continued (1 h) and the solvent subsequently evaporated to yield a semi-crystalline oil. This residue was partly dissolved in diethyl ether (10 mL). Filtration and concentration of the ethereal solution *in vacuo* gave 152 mg of a viscous colourless oil. This oil was dissolved in MeOH (5 mL). Citric acid (69 mg of the monohydrate, 0.33 mmol) was added and the mixture stirred for 15 min at room temperature. MeOH was removed under reduced pressure. The residue was then partly dissolved in CH_2Cl_2 (5 mL), and Et_3N (36 mg, 0.36 mmol) was added. After 5 min of stirring, the mixture was filtered and concentrated to give 75 mg of crude **4**. Chromatography on a column of silica gel using gradient elution (hexane/EtOAc/EtOH) finally yielded 51 mg (46 %) of (–)-(2*R*,5*S*)-ester-amide **4** as a colourless oil; $[\alpha]_D^{22}$ = –5.75 ° (*c* 1.67, $CHCl_3$); ^1H NMR ($CDCl_3$, 200 MHz): δ 1.58 (4H, bs, 3OH + NH), 1.65–1.90 (1H, m), 2.10–2.30 (3H, m), 3.50–3.85 (10H, m), 3.61 (3H, s), 7.30 (5H, s); ^1H NMR ($CDCl_3$ + D_2O, 200 MHz): δ 1.65–1.90 (1H, m), 2.10–2.30 (3H, m), 3.45–3.70 (11H, m + s + s), 3.84 (2H, s), 7.30 (5H, s); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 30.5, 30.6, 52.4, 60.0, 61.5, 63.9 (3C), 67.1, 68.0, 127.9, 128.6 (2C), 129.4 (2C), 137.2, 175.6, 176.4. Anal. calcd for $C_{18}H_{26}N_2O_6$: C, 59.00; H, 7.15; N, 7.65; O, 26.20. Found: C, 60.4; H, 6.79; N, 7.39; O, 25.4.

(+)-(2S,5R)-N-Benzyl-2-methoxycarbonyl-5-[[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]carbonyl]pyrrolidine, (+)-(2S,5R)-ester-amide 4

Crystalline (+)-(2*S*,5*R*)-acid-amide **3** [155 mg, 0.44 mmol; $[\alpha]_D^{22}$ = +15.0° (*c* 1.0, 1 M HCl, aq.)][2] was stirred in a 1:3 (v/v) mixture (25 mL) of MeOH and 1,2-dichloroethane. After addition of a few drops of H_2SO_4 (conc.), the resulting solution was refluxed (7.5 h) and then neutralised with $NaHCO_3$ (s). Drying ($MgSO_4$), filtration and concentration of the reaction mixture yielded 161 mg of crude product. This material was divided into a CH_2Cl_2-soluble part (fraction I) and a remaining non-soluble, semi-crystalline part (fraction II).

Fraction I was concentrated and purified by flash chromatography on silica gel using gradient elution (hexane/EtOAc/CH_2Cl_2/MeOH) affording polar products A–C. Product A was the desired (+)-(2*S*,5*R*)-ester-amide **4** (23 mg, 14 %). Due to the limited quantity of material available, the exact optical rotation figures are not given. (The (+)-sign of rotation refers to measurement in $CHCl_3$ solution). NMR, GC and TLC data were in accordance with those of (–)-(2*R*,5*S*)-ester-amide **4**. Product B (16 mg) was unidentified. Product C (6 mg) was identified as (+)-(2*R*,5*S*)-ester-acid **2** and ^1H NMR analysis of the corresponding *in situ* prepared salt with (+)-(*R*)-1-phenylethylamine indicated this acid to be of > 98 % *e.e.*.

Fraction II was dissolved in MeOH (10 mL), H_2SO_4 (cat.) was added, and the solution was brought to gentle reflux and stirred overnight. Work-up as for fraction I gave diester **1** (7 mg) and a small amount of ester-acid **2**.

cis-*N-Benzyl-2,5-bis[[[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino]carbonyl]pyrrolidine, diamide 7*

Benzylpyrrolidine-2,5-dicarboxylic acid (280 mg, 1.12 mmol, obtained from hydrolysis of diester **1** in 1 M HCl), was dissolved in CH_2Cl_2 (2 mL) and stirred at 0 °C under argon. Following addition of Et_3N (352 mg, 3.48 mmol) in CH_2Cl_2 (2 mL), a solution of ethyl chloroformate (366 mg, 3.38 mmol) in CH_2Cl_2 (2 mL) was injected via syringe. The mixture was allowed to reach room temperature and then stirred overnight. After extraction with $NaHCO_3$ (aq., sat., 10 mL) and filtration, the crude intermediate product was purified by chromatography on a silica gel column using gradient elution (hexane/EtOAc) giving 158 mg of a colourless oil.[27] The oil was dissolved in THF (5 mL). Following addition of Et_3N (475 mg, 5 mmol) in THF (10 mL), a solution of **5** (TMS-derivative of Tris; 1.62 g, 5 mmol) in THF (10 mL) was added. The mixture was refluxed for 6 h. The crude mixture was concentrated *in vacuo* and 20 mL of CH_2Cl_2 was added. After filtration the solution was washed with water and the organic phase concentrated *in vacuo*. The yellow oil was dissolved in MeOH (25 mL). Citric acid (1 g of the monohydrate, 5 mmol) was added, and the solution was stirred for 20 min followed by concentration *in vacuo*. The crude product was purified through a short silica gel column and eluated with EtOAc/EtOH 1:1 yielding 39 mg (9 %) of **7**; ^{13}C NMR (DMSO-d_6, 100 MHz): δ 30.0, 58.2, 60.3, 61.6, 67.9, 127.2, 128.2, 129.5, 137.7, 174.2.

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Supplementary Material Available

Further crystallographic data for the acid-amide **3** including, stereoscopic packing diagram, the list of fractional atomic coordinates of the non-hydrogen atoms with equivalent isotropic temperature factors, distances and angles of hydrogen bonds, bond distances and bond angles involving the non-hydrogen atoms, fractional atomic coordinates of the hydrogen atoms, bond distances and angles involving the hydrogen atoms and anisotropic thermal parameters of the non-hydrogen atoms as well as the list of the observed and calculated structure factors (10 pages) are available on request.

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