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Enantiospecific enzyme-catalysed resolution of novel *N*,*N*-disubstituted α-amino acid phenolic ester derivatives using pig liver esterase

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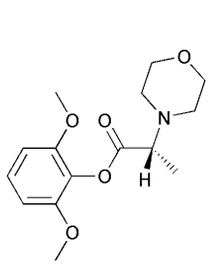
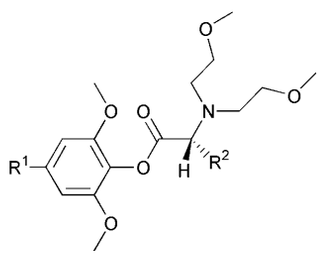
R-Amino acid esters **1**, **2** and **3** are novel compounds possessing hypnotic activity. On attempting an asymmetric synthesis of these molecules, racemisation was observed when reacting bis(2-methoxyethyl)amine with α-bromo intermediate **4**. *In vitro* plasma stability studies showed that the *R* enantiomers had much greater resistance to esterase-mediated degradation than the corresponding *S* enantiomers. This observation led to the use of commercially available pig liver esterase to prepare **1**, **2** and **3** on a multigram scale. The crystal structures of **1** and **2** are reported and confirm *R* configuration.

Introduction

N,*N*-Disubstituted α-amino acid ester derivatives have been shown to be useful intermediates for the synthesis of novel β-lactams.[1] In addition, there have been a number of reports describing interesting biological activity of this class of compounds. Puhl *et al.*[2] recently described *N*,*N*-disubstituted α-amino acid esters as endothelin inhibitors with potential therapeutic uses in the treatment of cardiovascular and other disorders. Previous reports outlined the local anaesthetic activity of aryl ester and amide derivatives[3] as well as compound **A**



A



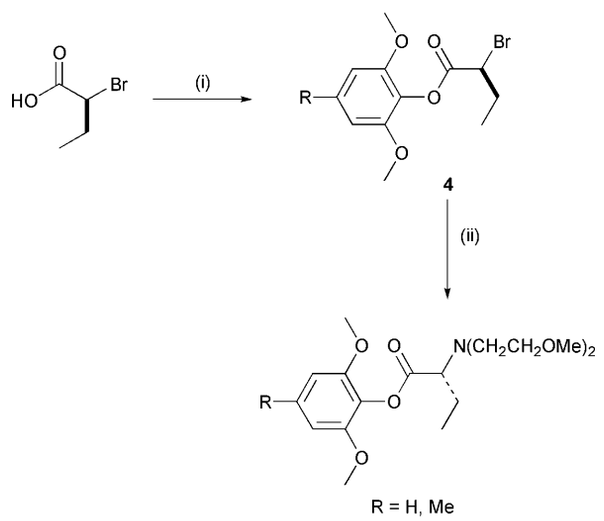
B

1 R^1 = H, R^2= Et,
2 R^1= Me, R^2= Et,
3 R^1= H, R^2= ^{n}Pr,

which was shown to possess general anaesthetic activity.[4] More recently, phenolic esters of general structure **B** have also been shown to possess this activity.[5] Compared with compound **A**, these new leads have improved stability and anaesthetic profiles such as quick onset, with smooth, short sleeps and fast recovery. As a result of this research, compound **2** was examined in clinical trials as a potential new intravenous anaesthetic.

Results and discussion

In order to perform extensive pharmacological testing of target molecules **1**–**3**, it was necessary to prepare gram-scale quantities of enantiopure material. Generally, the 'unnatural' *R* enantiomer of this series is the more active anaesthetic, so a synthesis starting from commercially available (*S*)-2-bromobutyric acid was envisioned as the most direct route to the desired targets **1** and **2** (Scheme 1).



Scheme 1 Attempted synthesis of (*R*)-**1** and (*R*)-**2** from (*S*)-2-bromobutyric acid. *Reagents and conditions*: (i) PyBroP, DCM, ^{i}Pr$_2$EtN, DMAP, 2,6-dimethoxyphenol or 2,6-dimethoxy-4-methylphenol, RT, 18 h; (ii) bis(2-methoxyethyl)amine, PhMe, 100 °C, 2 d.

Whilst the PyBroP (bromotripyrrolidinophosphonium hexafluorophosphate)-mediated esterification[6] worked well, racemisation was observed during subsequent displacement of the α-bromo substituent on compound **4** with bis(2-methoxyethyl)amine. Relatively high temperatures were required for the reaction to proceed and despite varying the reaction conditions, we were unable to prevent loss of chiral integrity. This result was disappointing since minimal racemisation was observed when 1,2,3,4-tetrahydroisoquinoline was used as the nucleophile. It was therefore decided to examine alternative routes to our desired targets. Preparative chiral HPLC proved useful only for **2**. Inadequate separation of the component enantiomers of **1** and **3**, as well as lengthy run times and the large volumes of solvent waste, rendered this approach unviable to provide bulk quantities of material.

During a series of stability studies of racemic **2** in blood plasma at 37 °C, a number of interesting observations were made. Different rates of degradation of **2** occurred in mouse, rat, dog and human plasma. The degradation was thought to be due to esterases present in the plasma, with rodent plasma

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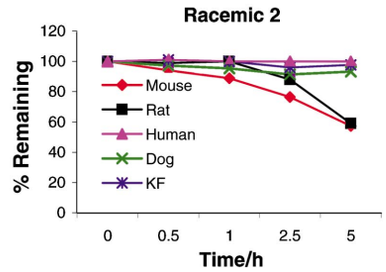
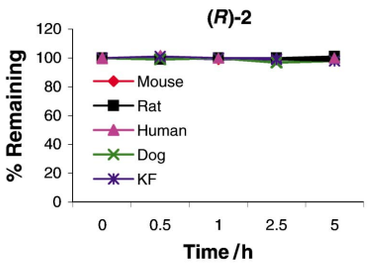
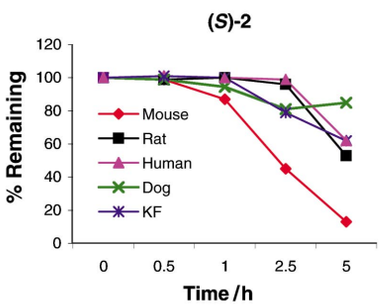


Racemic 2					
Time/h	Mouse	Rat	Human	Dog	KF
0	100	100	100	100	100
0.5	94.1	98.9	101	97.3	101
1	89	100	100	95.4	100
2.5	76.5	88	100	91.4	96
5	57.4	59.1	100	93.3	97.8

Fig. 1 Degradation of racemic **2** in different blood plasma types over 5 h.



(*R*)-2					
Time/h	Mouse	Rat	Human	Dog	KF
0	100	100	100	100	100
0.5	100	99	101	99	101
1	99	100	100	100	100
2.5	98.9	100	100	96.8	99
5	99	101	100	98	98



(*S*)-2					
Time /h	Mouse	Rat	Human	Dog	KF
0	100	100	100	100	100
0.5	99	98.9	101	99	101
1	87	100	100	94.6	100
2.5	45	96	99	81	79
5	13	53	62.2	85	62

Fig. 2 Degradation of (*R*)-**2** and (*S*)-**2** in different blood plasma types over 5 h.

esterases being the most aggressive (Fig. 1). This theory of esterase-catalysed degradation was given further credence by the effect of potassium fluoride (KF), a known inhibitor of esterase activity,[7] which dramatically slowed the rate of breakdown.

When the separate enantiomers of **2** were subjected to the same study, a large difference in degradation rate was observed, particularly in mouse plasma. As can be seen in Fig. 2, the *R* enantiomer was shown to be extremely stable in all plasma types over a 5 h time-course with essentially no degradation occurring. In contrast, the 'natural' *S* enantiomer underwent extensive degradation with only 13% remaining in mouse plasma after 5 h. The inclusion of potassium fluoride (at 80 mM) reversed the effect, but only for the first 2.5 h. Subsequent degradation suggested an element of non-specific breakdown or the presence of some KF-insensitive enzyme. However the possible contribution of spontaneous breakdown was discounted since on incubation of (*S*)-**2** at pH 7.4 in Tris buffer at 37 °C for 5 h, negligible compound loss was observed.

Based on these results, we considered the possibility of using a commercially available esterase to effect an enantioselective ester hydrolysis. Pig liver esterase (ple) was chosen since it is readily available and able to tolerate a wide range of structurally diverse racemic, prochiral and *meso* substrates.[8] However, the vast majority of substrates reported to be hydrolysed in high enantioselectivity were methyl or ethyl esters, so we were interested to see if the bulky substituted phenolic esters would be tolerated by the enzyme. There are few reports of enzyme-mediated hydrolyses of phenolic esters in the literature; Parmar's work with lipase-catalysed polyphenolic acetates[9] is the most noteworthy, although the products mentioned are not optically active.

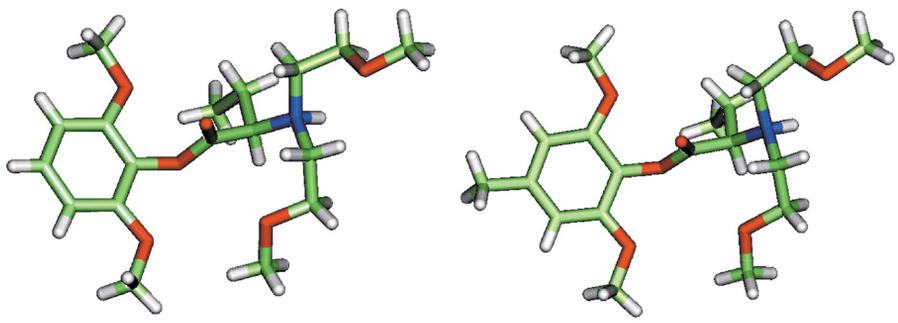
As shown in Table 1, racemates of **1**–**3** are hydrolysed enantioselectively with the desired *R* enantiomers being recovered in >99.5% ee within 90 h at RT in pH 7 phosphate buffer. Each hydrolysis was monitored by chiral HPLC with work-up occurring once all the *S* enantiomer had been consumed. Yields of the recovered *R* ester were routinely between 25–30% (max. 50%) after chromatography on alumina to remove the substituted phenol released during the hydrolysis of the *S* enantiomer.

Addition of 10–20% DMSO is known to improve enantioselectivity in ple-catalysed reactions[10] and a slight improvement with substrate **1** was observed as well as an increase in reaction rate. However, when DMSO is replaced with acetone, the reaction rate is slowed to such an extent that only a 21% ee is observed after 90 h. Compound **2** was hydrolysed much more slowly than **1** or **3** such that an ee of 85.6% was observed after 13 d reaction time. However, by increasing the amount of enzyme used from ~2000 to ~4000 units per mmol substrate and by performing the reaction with DMSO present, complete enantioselective hydrolysis was observed within 90 h. Since both **1** and **2** possessed good solubility in aqueous media, differential solubility was not thought to be the cause of the slower hydrolysis of **2**. Hence this result indicates either an unfavourable inductive effect arising from the *p*-methyl group of **2**, or that this group may be interacting unfavourably with amino acid residues lining the hydrophobic pocket occupied by the bulky phenolic ester in the enzyme active site. No enzyme crystal structure is available to confirm this latter hypothesis, although a widely accepted model for the prediction of enzyme-enantiomer complementarity has been proposed by Jones *et al.*[11] Whilst this cubic-space model pays particular attention to the dimensions of hydrophobic and polar pockets available for side chain binding, the precise positioning of the ester group, other than it being in close proximity to a key serine residue responsible for the hydrolysis, has not been examined. There may therefore be an additional hydrophobic pocket available for accommodating ester groups bulkier than methyl or ethyl.

Table 1 Ple-mediated enantioselective hydrolysis of racemic **1**–**3**

Compound	Substrate/ mmol	Enzyme/units per mmol substrate	Vol. phosphate buffer/ml	Enantiomeric ratio/h	Ee of product (%)
1	7.66	1538	40	90.9 : 9.1 (42)	>99.5[a]
1	3.83	1538	35 + 5 DMSO	100 : 0 (66)	>99.5[b]
1	3.83	1538	30 + 10 Me_2CO	60.3 : 39.7 (66)	21.0[a]
2	5.23	2216	75	57.8 : 42.2 (24)	85.6[c]
2	1.41	4042	40	88.9 : 11.1 (24)	99.4[a]
2	1.41	4042	35 + 5 DMSO	90.0 : 10.0 (24)	>99.5[a]
3	7.40	2054	100	—	>99.5[d]

[a] After 90 h reaction time. [b] After 66 h. [c] After 312 h. [d] After 72 h.



1 **2**

Fig. 3 X-Ray crystal structures of (*R*)-1 and (*R*)-**2**.

Further refinements to Jones' model would assist in the explanation of these results. Also a more in-depth study of other substituted phenolic ester substrates may help in establishing these activated esters, in conjunction with ple, as general tools for inducing chiral discrimination.

In order to verify that the *R* enantiomer was the configuration of the recovered ester from the enzyme hydrolysis reaction, and indeed the enantiomer which invokes the greater general anaesthetic activity, crystals of **1** and **2** were grown and submitted for X-ray analysis. The structures are shown below (Fig. 3) and confirm *R* configuration.

The 3D structure of **1** is represented by an orthorhombic crystal system with a $P2_12_12_1$ space group. In contrast, the *p*-methyl group of **2** forces the crystal into a monoclinic system with a $P2_1$ space group. The asymmetric units of both compounds consist of two independent complexes of a protonated parent molecule and a chloride counterion, which is hydrogen bonded to a water molecule.

Conclusion

Using commercially available pig liver esterase, we have performed enantioselective hydrolyses on a series of substituted phenolic esters. The active *R* enantiomers are recovered in 25–30% yield (max. 50%) and in >99.5% ee. These reactions have been scaled up and have been used to obtain multigram quantities of target compounds **1**, **2** and **3**, required for further testing as general anaesthetics. The Jones' model for predicting enzyme–substrate complementarity does not take into account any additional stabilisation provided by the binding of large ester groups such as the substituted phenolic esters used here. Further studies are recommended to investigate whether these, or more activated phenolic esters, can be used to probe suitability of substrates for chiral discrimination by esterases.

Experimental

General

The racemates of compounds **1**, **2** and **3** were prepared by a three step procedure[5] comprising addition of a commercially available phenol to an appropriate 2-bromoalkylacyl chloride, displacement of the halide with bis(2-methoxyethyl)amine and subsequent hydrochloride salt formation.

The racemate of **2** was separated into its component enantiomers by chiral HPLC. The sample was run on a Chiracel™ OJ Daicel column 25 × 2 cm using hexane–isopropyl alcohol–diethylamine (95 : 5 : 0.1) as eluant, a flow rate of 10 cm^3 min^{-1} at 30 °C with detection at 230 nm. For enzymatic studies and analytical chiral separation of the racemates of **1**, **2** and **3**, samples were run on a Chiralpak™ AD Daicel column 25 × 0.46 cm using hexane–ethanol–diethylamine (97 : 3 : 0.1) as eluant, a flow rate of 1 cm^3 min^{-1} at 30 °C with detection at 230 nm.

For the plasma studies, Tris buffer (39.6 mg Tris HCl, Sigma-Aldrich) was dissolved in 1 l ultrapure water (Milli-Q quality) to give a concentration of 250 mM. The pH was adjusted to 7.4 with 1 M sodium hydroxide. A 1 mg cm^{-3} stock solution of **1**, **2** or **3** in 50 : 50 acetonitrile–water was also prepared. For the assay, the concentration of compound is diluted to 10 μg cm^{-3} in plasma.

742.5 μl of plasma was pre-warmed for 10 minutes in a shaking water bath at 37 °C, then 7.5 μl of the pre-warmed stock solution of compound was added giving a final concentration of 10 μg cm^{-3}.

The plasma was sampled at 0, 0.5, 1 and 2.5 h. The sample was analysed by taking 100 μl plasma from the incubation tube into 300 μl acetonitrile containing 10 μg cm^{-3} internal standard. This was extracted then analysed by chiral HPLC as described above.

For the enzymatic resolutions, pig liver esterase was purchased as a crude, lyophilised powder from Sigma-Aldrich. The reactions were performed in pH 7 phosphate buffer made up by mixing 30.5 cm^3 0.2 M disodium hydrogen orthophosphate (Na_2HPO_4) and 19.5 cm^3 0.2 M sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$). 2 M Sodium hydroxide solution was added during the course of the reaction to maintain the pH at 7.0. Aliquots were taken from the reaction mixture at regular intervals. To these were added hexane (1 cm^3), the layers agitated and then passed down a short plug of anhydrous sodium sulfate. The samples were then analysed by chiral HPLC as described above.

Melting points were taken with either a Gallenkamp capillary melting point apparatus or a Reichert hot plate apparatus and are uncorrected. Optical rotations were determined at room temperature on an Optical Activity Ltd. AA-1000 polarimeter. ^{1}H NMR (200 or 400 MHz) spectra were obtained using a Bruker AM200 or a Bruker DRX400 instrument; chemical shifts (δ) are relative to tetramethylsilane as internal standard. Only discrete or characteristic signals are reported. IR spectra were obtained with a Perkin-Elmer 16PC FT-IR spectrometer. Accurate masses were measured on a PerSeptive Biosystems Mariner™ Biospectrometry Workstation using TOF-MS analysis.

Crystal data

$C_{18}H_{29}O_6N{\cdot}HCl{\cdot}H_2O$ (**1**), $M = 409.91$, monoclinic, $P2_1$, $a = 7.7225(1)$, $b = 27.3326(1)$, $c = 10.6961(1)$ Å, $\beta = 103.669(1)°$, $V = 2193.75(4)$ Å^3, $Z = 4$, $D_c = 1.2411(1)$ g cm^{-3}, λ(Cu K_α) = 1.54184 Å, $\mu = 18.6$ cm^{-1}, $F(000) = 880$, $T = 294$ K, $R = 0.054$ for 3561 reflections with $I > 2\sigma(I)$.

$C_{19}H_{31}O_6N{\cdot}HCl{\cdot}H_2O$ (**2**), $M = 423.93$, orthorhombic, $P2_12_12_1$, $a = 12.0201(1)$, $b = 14.7647(1)$, $c = 25.6491(1)$ Å, $V = 4552.03(5)$ Å^3, $Z = 8$, $D_c = 1.2372(1)$ g cm^{-3}, λ(Cu K_α) = 1.54184 Å, $\mu = 18.1$ cm^{-1}, $F(000) = 1824$, $T = 294$ K, $R = 0.090$ for 5595 reflections with $I > 2\sigma(I)$.

CCDC reference numbers 153401 and 153402.

See http://www.rsc.org/suppdata/p1/b0/b008000o/ for crystallographic files in .cif format.

(*R*)-2-[*N*,*N*-Bis(2-methoxyethyl)amino]butyric acid, 2′,6′-dimethoxyphenyl ester hydrochloride (1)

Pig liver esterase (0.62 g, 19 units mg^{-1}) was added to a stirred solution of racemic **1** as its hydrochloride salt (3.00 g, 7.66 mmol) in phosphate buffer (35 cm^3) and DMSO (5 cm^3). After 90 h, the system was stirred with MTBE (3 × 100 cm^3) for 2 h and the layers separated. Any gel that formed was dispersed by filtration through a glass sinter. The combined organic layer was dried (Na_2SO_4), filtered and solvent removed. The residue was then purified by chromatography on alumina using petroleum ether (40 : 60)–ethyl acetate (2 : 1) as eluant. This gave the desired optically pure *R* enantiomer as its free base (693 mg, 25.5%). The hydrochloride salt was then prepared by passing dry hydrogen chloride gas through a solution of **1** in diethyl ether.

$[a]_D$ +4.62 (*c* 0.52 in $CHCl_3$); mp 53.5–54.5 °C; ν_{max}/cm^{-1} (KBr) 3507, 2946, 2531, 1762, 1606, 1483, 1264, 1174, 1112; δ_H($CDCl_3$ + C_5D_5N) 1.13 (t, 3H), 1.96–2.09 (m, 2H), 3.08–3.17 (m, 2H), 3.20–3.30 (m, 2H), 3.38 (s, 6H), 3.56–3.65 (m, 2H), 3.66–3.75 (m, 2H), 3.76–3.89 (m, 7H), 6.60 (d, 2H), 7.14 (t, 1H); *m*/*z* (ES) $[M + H]^+$ 356; Found: 356.2060. $C_{18}H_{30}NO_6$ requires 356.2068.

The following compounds were prepared similarly.

(*R*)-2-[*N*,*N*-Bis(2-methoxyethyl)amino]butyric acid, 2′,6′-dimethoxy-4′-methylphenyl ester hydrochloride (2)

$[a]_D$ +7.03 (*c* 0.77 in $CHCl_3$); mp 108–109 °C; δ_H($CDCl_3$ + C_5D_5N) 1.13 (t, 3H), 1.92–2.13 (m, 2H), 2.34 (s, 3H), 3.10–3.21 (m, 2H), 3.23–3.35 (m, 2H), 3.37 (s, 6H), 3.56–3.65 (m, 2H), 3.68–3.81 (m, 8H), 3.82–3.91 (m, 1H), 6.42 (s, 2H); ν_{max}/cm^{-1} (KBr) 2944, 2278, 1768, 1607, 1470, 1248, 1196, 1119 cm^{-1}; *m*/*z* (ES) $[M + H]^+$ 370; Found: 370.2196. $C_{19}H_{32}NO_6$ requires 370.2227.

(*R*)-2-[*N*,*N*-Bis(2-methoxyethyl)amino]pentanoic acid, 2′,6′-dimethoxyphenyl ester hydrochloride (3)

$[a]_D$ −5.88 (*c* 0.20 in MeOH); mp 85.5–86.5 °C; δ_H($CDCl_3$ + C_5D_5N) 1.00 (t, 3H), 1.53–1.67 (m, 2H), 1.88–1.99 (m, 2H), 3.05–3.14 (m, 2H), 3.17–3.28 (m, 2H), 3.37 (s, 6H), 3.55–3.72 (m, 4H), 3.80 (s, 6H), 3.90 (t, 1H), 6.60 (d, 2H), 7.12 (t, 1H), 7.21 (br s, 1H); ν_{max}/cm^{-1} (KBr) 2934, 2113, 1750, 1607, 1484, 1309, 1265, 1174, 1114 cm^{-1}; *m*/*z* (ES) $[M + H]^+$ 370; Found: 370.2196. $C_{19}H_{32}NO_6$ requires 370.2227.

Acknowledgements

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