(2*R*,3*S*)- and (2*S*,3*R*)-2-Benzyl-3,4-epoxybutanoic acid as highly efficient and fast acting pseudomechanism-based inactivators for carboxypeptidase A:[1,2] design, asymmetric synthesis and inhibitory kinetics

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2-Benzyl-3,4-epoxybutanoic acid (BEBA) was studied as an irreversible inhibitor for the zinc-containing protease, carboxypeptidase A. Of four possible stereoisomers, those having a 2*R*,3*S*- and a 2*S*,3*R*-configuration inhibited carboxypeptidase A in a time-dependent manner. The latter compound that belongs to the D series is more effective with a k_{inact}/K_i value of 139.5 $dm^3\ mol^{-1}\ s^{-1}$ than the former having a K_{inact}/K_i value of 53.9 $dm^3\ mol^{-1}\ s^{-1}$. Partition ratios for (2*R*,3*S*)- and (2*S*,3*R*)-BEBA were determined as 1.01 and 0.53, respectively. The observed kinetic parameters reveal that both are highly efficient and fast acting pseudomechanism-based inactivators for carboxypeptidase A. Details of the kinetic analyses, design principles and asymmetric syntheses of these inactivators are described.

Carboxypeptidase A (CPA, EC 3.4.17.1) is a much studied zinc-containing metalloprotease which selectively hydrolyses the C-terminal amino acid residue having a hydrophobic side chain.[3] Its ternary structure including that of the active site has been well characterized, although the catalytic mechanism at the molecular level has yet to be fully elucidated.[3] Nevertheless, successful designs of mechanism-based inactivators for the enzyme have been reported[4] and CPA has been used as a model target enzyme for designing therapeutically valuable inhibitors of physiologically important metalloenzymes.[5]

2-Benzyl-3,4-epoxybutanoic acid (BEBA) is conceived as an irreversible inhibitor for CPA on the basis of the established topology of the active site and the known catalytic mechanism of the enzyme.[3,6] The rationale used in designing BEBA as a CPA inhibitor is illustrated in Fig. 1.

In binding of BEBA to the enzyme, the carboxylate of the inhibitor would interact with the guanidium moiety of Arg-145 with accommodation of the phenyl group by the hydrophobic recognition pocket. The oxirane of BEBA would then rest at a position proximal to the active site zinc ion, thus enabling the oxygen of the oxirane to coordinate to the metal ion with resultant activation of the oxirane ring for nucleophilic attack. The carboxylate of Glu-270 that attacks the scissile peptide carbonyl carbon of the substrate in the proteolytic process would interact with the activated oxirane. Such an interaction will result in a covalent attachment of BEBA to the enzyme with concurrent opening of the oxirane ring (Fig. 1). Permanent inactivation of the enzyme then ensues.

A kinetic analysis of the CPA which is exposed to BEBA, indeed, demonstrated that it inhibits irreversibly CPA with 1:1 molecularity.[2] A single-crystal X-ray crystallographic study of the inactivated enzyme confirmed the conclusion reached from the kinetic analysis, showing that the carboxylate of Glu-270 is alkylated by the inhibitor.[1g] Furthermore, to our surprise, the bound BEBA was shown to have a 2*S*,3*R*-configuration. On the basis of the established mode of the inactivation coupled with the design principle involved, the inhibitor was referred to as a pseudomechanism-based inactivator for CPA. This report describes the stereoselective syntheses of (2*S*,3*R*)-BEBA as well as its enantiomer, and the evaluation of the two as inactivators of CPA. The inhibitory stereochemistry of BEBA for CPA and its implication with respect to the catalytic mechanism of CPA are also discussed.

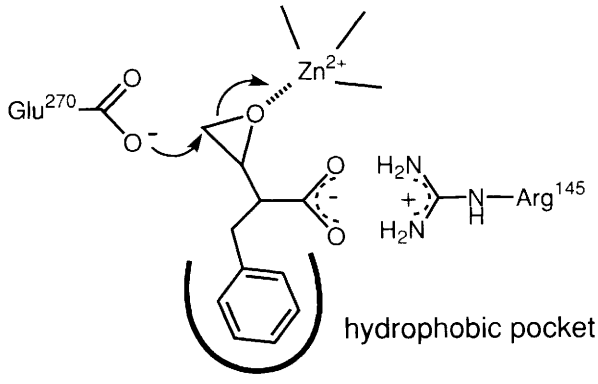
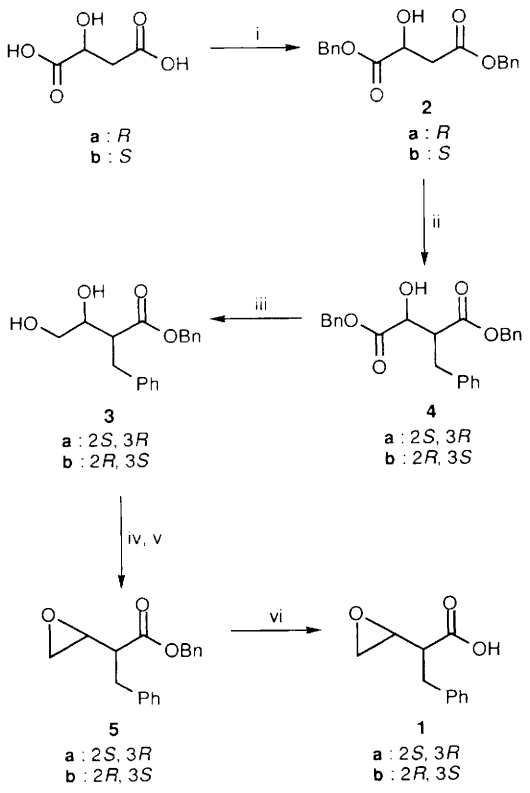


Fig. 1 Schematic representation of the active site of carboxypeptidase A, which is occupied by 2-benzyl-3,4-epoxybutanoic acid. The nucleophilic attack of the carboxylate of Glu-270 on the oxirane ring would result in the alkylation of the carboxylate with a concomitant ring cleavage, leading to an inactivation of the enzyme.

Results and discussion

Synthesis of (2*S*,3*R*)- and (2*R*,3*S*)-BEBA

Dibenzyl D-malate **2a** was stereoselectively benzylated by following the procedure reported by Seebach and Wasmuth[7] to give **3a**. No diastereoisomeric impurity was detected when the optical purity of the product was analysed by the NMR method. The conversion of **3a** into **4a** was effected in 76% yield by regioselective reduction with borane–dimethyl sulfide complex in the presence of a catalytic amount of sodium boranuide in tetrahydrofuran.[8] Treatment of **4a** with mesyl chloride effected selective mesylation of the primary hydroxyl group. Subsequent treatment of the mesylate thus obtained with potassium carbonate in methanol afforded **5a**, catalytic hydrogenation of which in the presence of palladium-on-charcoal catalyst yielded (2*S*,3*R*)-BEBA in an overall yield of 31% (Scheme 1). Analogously, (2*R*,3*S*)-BEBA was synthesized starting from L-malic acid in an overall yield of 29%.



Scheme 1 *Reagents and conditions:* i, benzyl alcohol, cat. *p*-TsOH, reflux; ii, LHMDS, benzyl bromide, −78 °C to −50 °C; iii, borane–dimethyl sulfide, cat. $NaBH_4$, room temp.; iv, MsCl, pyridine; v, K_2CO_3, MeOH; vi, H_2, Pd-C

Inhibitory kinetics

Kinetic parameters were determined by a competitive substrate assay[9] in which CPA was incubated with the inhibitor in the presence of hippuryl-L-Phe, a chromogenic substrate. In this assay, the irreversible inhibitor and the substrate compete for the same enzyme active site as illustrated in Scheme 2, and the rate of the enzymic reaction can be expressed by eqn. (1)

$$E + I \overset{K_i}{\rightleftharpoons} E \cdot I \xrightarrow{k_{inact}} E-I$$

$$E + S \overset{K_m}{\rightleftharpoons} E \cdot S \xrightarrow{k_{cat}} E + P$$

Scheme 2

$$\ln\left(\frac{v}{v_0}\right) = \frac{-k_{inact}K_m[I]_0}{K_i \cdot K_m + [S]_0K_i + [I]_0K_m} \cdot t = -k_{obs}t \quad (1)$$

that was derived by Main.[10] For rapidly inactivating irreversible inhibitors such as BEBA the competitive substrate assay was found to be preferred to the Kitz and Wilson method[11] since the concentration of BEBA changes significantly during the initial observation interval, and thus accurate measurements of the initial velocity were difficult.

Fig. 2 shows progress curves for the inactivation of CPA by (2*S*,3*R*)-BEBA at different concentrations, from which the k_{obs} values for each concentration were obtained. Very similar progress curves were obtained for inactivation of the enzyme with (2*R*,3*S*)-BEBA. The time-dependent loss of the enzymic activity by (2*S*,3*R*)-BEBA shown in Fig. 3 suggests strongly that the CPA inhibition occurs in an irreversible fashion. The time-dependent loss of the CPA activity was also observed

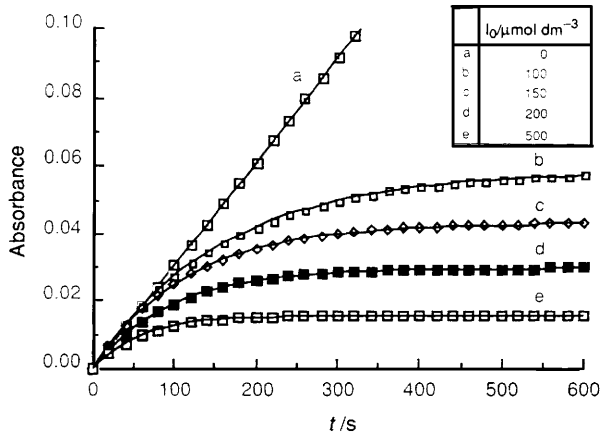


Fig. 2 Progress curves for the hydrolysis of hippuryl-L-Phe by CPA in the presence and absence of (2*S*,3*R*)-BEBA

with (2*R*,3*S*)-BEBA. Eqn. (2) obtained by the rearrangement of Main's rate expression shows that a double reciprocal plot of k_{obs} *vs.* the initial concentration of the inhibitor will give a straight line with a *y* intercept of $1/k_{inact}$ and the slope of $K_i/k_{inact}\{1 + ([S]_0/K_m)\}$ where $[S]_0$ represents the initial concentration of the substrate and K_m its Michaelis–Menten constant, as demonstrated by Fig. 4. Values of K_i and k_{inact} for both inactivators thus calculated from Fig. 4, are summarized in Table 1. The improved kinetic data over those obtained from the previous studies[1d,1i] appear to be due to the higher optical purity of the inhibitors used coupled with the improved assay method[9] employed in the present study.

$$\frac{1}{k_{obs}} = \frac{1}{k_{inact}} + \frac{K_i}{k_{inact}}\left(1 + \frac{[S]_0}{K_m}\right)\frac{1}{[I]_0} \quad (2)$$

The competitive inhibitory experiment with benzyl succinate, a reversible and competitive inhibitor of CPA that is known to bind to the active site of the enzyme, exhibited diminished inhibitory rate; this demonstrated that BEBAs bind exclusively at the active site. The present kinetic study strongly suggests that the covalent modification at the catalytic carboxylate that is expected from the design principle does, indeed, occur during the inactivation of the enzyme by BEBAs. This has been confirmed by the X-ray crystallographic analysis[1g] of the BEBA-inactivated CPA. We found that the ester linkage thus formed on the carboxylate of Glu-270 is quite stable. Prolonged dialysis up to 48 h of the inactivated enzyme at pH 9.56 failed to regenerate the enzymic activity, suggesting that the ester moiety is shielded by the bulk of the enzyme molecule. These observations further suggest that the water molecule that is ligated to the zinc ion is displaced by the oxirane, because, otherwise, the ester would be readily hydrolysed by it. Apparently, the carboxylate of Glu-270 of CPA attacks exclusively the epoxide ring at the 4-position in BEBA. It is worth mentioning that the present observation challenges the prevailing view that side-chain carboxylates are generally so unreactive in enzymic reactions that they are not modified by simple epoxides.[12] Because BEBA is not activated through CPA-induced chemical transformation but, rather, activated by the non-covalent interaction of its oxirane oxygen with the active site zinc ion, BEBA is not a mechanism-based inactivator in the strict sense;[13] the proposed description of the inhibitors as pseudomechanism-based inactivators is, therefore, justifiable.

The observation that the carboxylate of Glu-270 is readily alkylated by BEBA, a substrate analogue is of considerable

Table 1 Kinetic parameters for the inactivation of CPA

Inactivator	K_i/μmol dm^{-3}	k_{inact}/min^{-1}	(k_{inact}/K$_i$)/dm^3 mol^{-1} s^{-1}
(2*S*,3*R*)-BEBA	190	1.59	139.5
(2*R*,3*S*)-BEBA	343	1.11	53.9

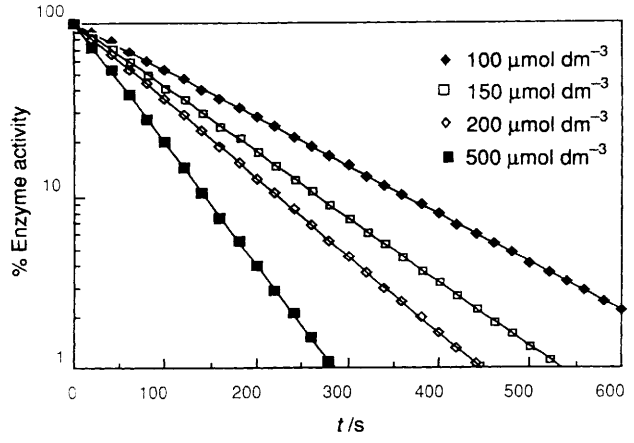


Fig. 3 Time-dependent inactivation of CPA by (2*S*,3*R*)-BEBA of different concentrations

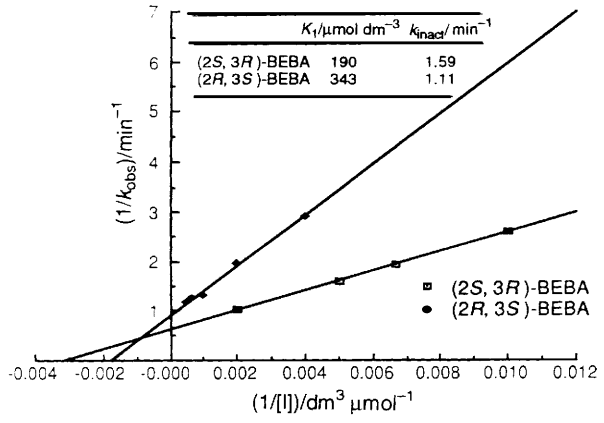


Fig. 4 Double reciprocal plots of k_{obs} for the inactivation of CPA by (2*S*,3*R*)-and (2*R*,3*S*)-BEBA *vs.* the inhibition concentration

importance with respect to the enzymic mechanism of CPA. There have been two principal schools of thought concerning the catalytic mechanism of carboxypeptidase A. In one, the carboxylate is thought to attack directly the scissile carboxamide carbon atom of the substrate to form an anhydride intermediate (anhydride pathway).[14] In the other, the carboxylate is thought to function merely as a base which activates a nearby zinc-coordinated water molecule, thus enabling it to serve as a nucleophile and attack the scissile carboxamide carbon. This generates a tetrahedral intermediate (general base pathway).[15] The results of the present study are consistent with the anhydride pathway for the catalytic action since no covalent modification is expected to occur at the Glu-270 if the general base pathway is in operation. Hence, the present observation may count as experimental evidence in support of the anhydride pathway for the CPA catalytic mechanism. However, there is an alternative explanation for the experimental observation that BEBAs effectively displace the zinc-bound water molecule upon binding to the enzyme, the result of which is that the catalytic carboxylate no longer functions as a base but as a nucleophile which attacks the oxirane ring. Although this interpretation which is supportive of the general base mechanism appears unlikely to us, it cannot, at present, be excluded.

The parameter most characteristic of a mechanism-based inactivator is the partition ratio which reflects the efficiency of the mechanism-based inactivator.[13] The partition ratios of 0.53 and 1.01 for (2*S*,3*R*)- and (2*R*,3*S*)-BEBA, respectively, determined by plotting $[I]_o/[E]_o$ *vs.* the fraction of the activity remaining from a series of inactivation experiments (Fig. 5),[13a] demonstrate that they are highly efficient inactivators approaching the ideal. This means that nearly every turnover results in inactivation of the enzyme. This is important, since in the design of enzyme-activated substrate analogues every attempt is made to optimize the inactivators by decreasing the partition ratio and increasing the rate of its turnover.

The value of k_{inact}/K_i referred to inactivation potency reflects a measure of the effectiveness of the inactivator. Thus, in a comparison of the inactivation potency of the two inactivators (2*S*,3*R*)-BEBA is seen to be more than twice as potent as its enantiomer. This is surprising because (2*S*,3*R*)-BEBA has D stereochemistry at the 2-position, the reverse of that of the substrate. The other two stereoisomers, (2*R*,3*R*)- and (2*S*,3*S*)-BEBA were found to have no noticeable irreversible inhibitory activity against CPA.[1i] Presumably, they fail to bind to the enzyme, their molecular configurations being incompatible with the geometry of the active site of CPA.

It is intriguing that both stereoisomers in a mirror-image relationship are potent inhibitors for CPA, although one is more potent than the other. This kind of stereochemical behaviour is rarely observed in enzymic catalysis. The observation may be understood, however, by envisioning that the active site zinc ion and carboxylate of the catalytic Glu-270 of CPA are probably situated in the region of the active site, which can be reached equally well by the oxirane ring of both isomers when they bind CPA. Molecular models of the two inhibitory BEBA isomers when brought together with superimposition of their benzyl and carboxylate groups show that the two oxirane rings are separated symmetrically. Hence, it can be envisioned that the epoxide oxygens of the two isomers are within the zinc coordinating sphere, being equidistant from the Lewis acid and the carboxylate when the inhibitors bind the enzyme. The inference of the present study is that in enzymic reactions the inhibitory stereochemistry does not necessarily agree with the stereospecificity of substrates. In fact, on many occasions the inhibitory stereospecificity is shown to be the reverse of the stereochemistry displayed by substrates. In this connection it is noteworthy that a recent redetermination with refinement of the X-ray diffraction data of the complex of benzylsuccinic acid with thermolysin has shown that the complexed benzylsuccinic acid has D[16] rather than L stereochemistry[17] as was previously thought. Thermolysin is a zinc-containing metalloendopeptidase whose catalytic mechanism, active site topology and substrate stereospecificity are very similar to those of CPA.[18] Interestingly, however, an X-ray structural analysis of the crystalline complex of CPA with benzylsuccinic acid demonstrated that the L isomer is preferentially bound.[19] It is known that D-Phe is a competitive inhibitor[20] of CPA with K_i = 2 mmol dm^{-3}, and Christianson

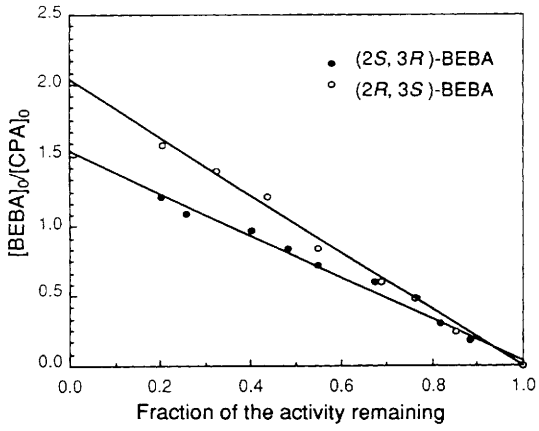


Fig. 5 Determination of partition ratio. $[BEBA]_0/[CPA]_0$ is plotted against the fraction of the activity remaining after dialysis of the incubated enzyme and inactivator mixture for 24 h

et al. reported that the amino group of D-Phe forms a salt link with the carboxylate of Glu-270.[21]

Experimental

^{1}H NMR spectra were recorded on Bruker 300 MHz FT-NMR spectrometer in deuteriochloroform and chemical shifts are expressed in ppm relative to tetramethylsilane. IR spectra were recorded on BOMEM FT-IR M100-C15 spectrometer. Low resolution mass spectra were obtained with a KRATOS MS 25 RFA instrument. High resolution mass spectra were recorded by Korea Basic Science Center, Daejeon, Korea. Elemental analyses were performed at Kyushu University (Fukuoka, Japan). Optical rotations were measured on a RUDOLPH RESEARCH AUTOPOL III digital polarimeter. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. All compounds described in this report showed a single spot on TLC plates. Specific optical rotations reported for oily compounds may not be valid because the purity of the sample was not rigorously proved by elemental analysis; the values are recorded in units of 10^{-1} deg cm^2 g^{-1}. These compounds are very sensitive to heat and could not be vacuum distilled. Enzyme assays were monitored using a Hewlett Packard 8452 A Diode Array spectrometer fitted with a cell-temperature controller.

All chemicals were of reagent grade obtained from Aldrich. Dichloromethane, toluene, and hexane were distilled over calcium hydride and stored under nitrogen. Tetrahydrofuran was distilled over sodium–benzophenone before use.

Synthesis

Dibenzyl (*R*)-2-hydroxysuccinate 2a. A mixture of D-malic acid (13.4 g, 100 mmol), benzyl alcohol (21.6 g, 200 mmol) and toluene-*p*-sulfonic acid monohydrate (0.19 g, 1.0 mmol) in dried toluene (150 cm^3) was heated under reflux in a Dean-Stark apparatus with azeotropic removal of water. When no more water appeared in the distillate, the mixture was allowed to cool to room temperature when it was washed with saturated aqueous sodium hydrogen carbonate and brine, dried ($MgSO_4$) and evaporated. The residue was purified by flash column chromatography on silica gel with 20% ethyl acetate–hexane as eluent to give the dibenzyl ester **2a** as a colourless oil (29.8 g, 95%), $[\alpha]_D$ +18.2 (*c* 2.0 in $CHCl_3$) {lit.,[22] $[\alpha]_D$ +18.0 (*c* 2.0 in $CHCl_3$)}, ν_{max}(neat)/cm^{-1} 3450 (OH) and 1725 (C=O); δ_H 2.78 (2 H, t, 3-H), 3.27 (1 H, br s, OH), 4.46 (1 H, t, 2-H), 5.01 (2 H, s, CO_2CH_2Ph) and 7.23 (10 H, br s, Ph).

Dibenzyl (*S*)-2-hydroxysuccinate **2b**. This compound was obtained in 96% yield by the same procedure described above starting with L-malic acid; $[\alpha]_D$ −18.6 (*c* 2.0 in $CHCl_3$) {lit.,[22] $[\alpha]_D$ −19.3 (*c* 1.9 in $CHCl_3$)}.

Dibenzyl (2*S*,3*R*)-2-benzyl-3-hydroxysuccinate 3a. Butyllithium (2.5 mol dm^{-3} solution in hexane; 66.0 cm^3, 164 mmol) was added dropwise into a solution of diisopropylamine (23.0 cm^3, 164 mmol) in dried tetrahydrofuran (THF; 150 cm^3) at 0 °C under nitrogen atmosphere, and the mixture was stirred for 40 min. Compound **2a** (25.1 g, 80.0 mmol) in dried THF (50 cm^3) was added dropwise to the solution of lithium diisopropylamide thus prepared at −78 °C and the mixture stirred for 1 h. It was then treated with benzyl bromide (9.50 cm^3, 80.0 mmol) and stirred at −78 °C for 1 h and at −50 °C for 5 h. Saturated aqueous ammonium chloride (100 cm^3) was then added to the mixture which was then extracted with ethyl acetate. The extract was washed with brine, dried ($MgSO_4$) and evaporated and the residue was purified by column chromatography on silica gel. Elution with 10% ethyl acetate–hexane gave **3a** as a white solid (16.8 g, 52%), mp 66–67 °C (Found: C, 74.2; H, 5.9. $C_{25}H_{24}O_5$ requires C, 74.24; H, 5.98%); $[\alpha]_D$ +17.5 (*c* 1.0 in MeOH); ν_{max}(KBr pellet)/cm^{-1} 3400 (OH) and 1720 (C=O); δ_H 3.00 (1 H, m, 2-H), 3.14–3.24 (2 H, m, CH_2Ph), 3.30 (1 H, br s, OH), 4.16 (1 H, dd, 3-H), 5.01 (4 H, s, CO_2CH_2Ph) and 7.16–7.30 (15 H, m, Ph); *m*/*z* 404 (M^+).

Dibenzyl (2*R*,3*S*)-2-benzyl-3-hydroxysuccinate **3b**. This compound was obtained in 47% yield by the same procedure described above starting with **2b**; mp 66–67 °C (Found: C, 74.2; H, 5.9. $C_{25}H_{24}O_5$ requires C, 74.24; H, 5.98%); $[\alpha]_D$ −17.2 (*c* 1.0 in MeOH).

Benzyl (2*S*,3*R*)-2-benzyl-3,4-dihydroxybutanoate 4a. Borane–methyl sulfide complex (2.0 mol dm^{-3} solution in THF; 19.8 cm^3, 39.6 mmol) was added dropwise to a solution of **3a** (16.0 g, 39.6 mmol) in dried THF (200 cm^3) at 0 °C under a nitrogen atmosphere, and the mixture was stirred for 1 h. Sodium boranuide (0.1 g) was added to the mixture and stirring continued for 30 min at room temperature. The mixture was then quenched by dilution with methanol (20 cm^3), stirred for a further 30 min and then evaporated. The residue was purified by column chromatography on silica gel eluting with 50% ethyl acetate–hexane to give **4a** as a colourless oil (9.0 g, 76%), $[\alpha]_D$ −24.7 (*c* 1.0 in MeOH); ν_{max}(neat)/cm^{-1} 3350 (OH) and 1720 (C=O); δ_H 2.23 (1 H, br s, OH), 2.90 (1 H, m, 2-H), 2.94–3.02 (2 H, m, CH_2Ph), 3.19 (1 H, br s, OH), 3.53–3.68 (2 H, m, 4-H), 3.80 (1 H, m, 3-H), 5.02 (2 H, s, CO_2CH_2Ph) and 7.13–7.30 (10 H, m, Ph); *m*/*z* 300 (M^+, 3%) and 282 (M^+ − 18) (Found: *m*/*z* 300.1368. $C_{18}H_{20}O_4$ requires 300.1362).

Benzyl (2*R*,3*S*)-2-benzyl-3,4-dihydroxybutanoate **4b**. This compound was obtained in 74% yield by the same procedure described above starting with **3b**; $[\alpha]_D$ +24.5 (*c* 1.0 in EtOH); *m*/*z* 300 (M^+, 4%) and 282 (M^+ − 18) (Found: *m*/*z* 300.1357. $C_{18}H_{20}O_4$ requires 300.1362).

Benzyl (2*S*,3*R*)-2-benzyl-3,4-epoxybutanoate 5a. Freshly distilled pyridine (4.3 cm^3, 53.4 mmol) and methanesulfonyl chloride (2.1 cm^3, 26.7 mmol) were added to a solution of **4a** (8.0 g, 26.7 mmol) in dried dichloromethane (150 cm^3) at 0 °C under a nitrogen atmosphere. The mixture was stirred for 48 h and then acidified with 3 mol dm^{-3} aqueous hydrochloric acid (pH 3–4). The organic layer was washed with water and brine, dried ($MgSO_4$) and evaporated to give the monomesylate as a semi-solid. This was stirred in methanol (100 cm^3) and treated with potassium carbonate (3.5 g, 25.4 mmol) whilst being chilled in ice. After 30 min, the mixture was quenched with water and extracted with ethyl acetate. The extract was washed with brine, dried (Na_2SO_4) and evaporated and the residue was purified by column chromatography on silica gel. Elution with 20% ethyl acetate–hexane gave **5a** as a colourless oil (6.7 g, 89%); $[\alpha]_D$ +1.5 (*c* 1.0 in EtOH); ν_{max}(neat)/cm^{-1} 1725 (C=O) and 1270 (C–O); δ_H 2.29 (1 H, m, 2-H), 2.50 (1 H, q, CH_2Ph), 2.69 (1 H, t, CH_2Ph), 3.04–3.11 (2 H, dq, 4-H), 3.21 (1 H, m,

3-H), 5.13 (2 H, s, CO_2CH_2Ph) and 7.12–7.35 (10 H, m, Ph); *m/z* 282 (M^+, 3%) and 251 ($M^+ - 31$) (Found: *m/z* 282.1259. $C_{18}H_{18}O_3$ requires 282.1256).

Benzyl (2*R*,3*S*)-2-benzyl-3,4-epoxybutanoate **5b**. This compound was obtained in 92% yield by the same procedure described above starting with **4b**; $[\alpha]_D$ −1.5 (*c* 1.0 in EtOH); *m/z* 282 (M^+, 3%) and 251 ($M^+ - 31$) (Found: *m/z* 282.1248. $C_{18}H_{18}O_3$ requires 282.1256).

(2*S*,3*R*)-2-Benzyl-3,4-epoxybutanoic acid 1a. Palladium-on-activated carbon (10% Pd; 0.2 g) was added to a solution of **5a** (5.0 g, 17.7 mmol) in methanol (50 cm^3), and the solution stirred under a hydrogen atmosphere (1 atm) for 1 h. When the hydrogenolysis was complete, the catalyst was filtered off and concentrated under reduced pressure to afford the title compound as a colourless oil (3.2 g, 95%); $[\alpha]_D$ −10.8 (*c* 1.0 in EtOH); ν_{max}(neat)/cm^{-1} 3028 (OH), 1725 (C=O), and 1270 (C–O); δ_H 2.29 (1 H, m, 2-H), 2.50 (1 H, q, CH_2Ph), 2.69 (1 H, t, CH_2Ph), 2.89–3.17 (2 H, dq, 4-H), 3.20 (1 H, m, 3-H) and 7.18–7.32 (5 H, m, Ph); *m/z* 192 (M^+, 8%) and 174 ($M^+ - 18$) (Found: *m/z* 192.0786. $C_{11}H_{12}O_3$ requires 192.0786).

(2*R*,3*S*)-2-Benzyl-3,4-epoxybutanoic acid **1b**. This compound was obtained in 94% yield by the same procedure described above starting with **5b**; $[\alpha]_D$ +9.8 (*c* 1.0 in EtOH); *m/z* 192 (M^+, 9%) and 174 ($M^+ - 18$) (Found: *m/z* 192.0777. $C_{11}H_{12}O_3$ requires 192.0786).

Kinetics

All solutions were prepared by mixing doubly distilled, deionized water. Stock assay solutions were filtered prior to use. Carboxypeptidase A (CPA, EC 3.4.17.1) was obtained from Sigma Chemical Co. (catalogue no. C-0386, Allan form, twice crystallized, from bovine pancreas, aqueous suspension). CPA stock solutions were prepared by dissolving the enzyme in 0.05 mol dm^{-3} Tris–0.5 mol dm^{-3} NaCl, pH 7.5; Tris buffer solution and the concentrations were determined from the absorbance at 278 nm (E_{278} = 6.42 × 10^4 l dm^{-3} cm^{-1}).[23] Hippuryl-L-Phe was obtained from Sigma Chemical Co. All the kinetics were performed at 25 °C in 0.05 mol dm^{-3}, pH 7.5, Tris buffer, containing 0.5 mol dm^{-3} NaCl and concentrations shown in the text are final concentrations after all the incubation solutions are combined.

Determination of K_i and k_{inact}. A 2 cm^3 cuvette was charged with 1620 mm^3 of 0.05 mol dm^{-3} Tris/0.5 mol dm^{-3} NaCl, pH 7.5, buffer solution, 180 mm^3 of a 5.0 mol dm^{-3} solution of hippuryl-L-Phe in the Tris buffer (final concentration is 450 μmol dm^{-3}), 100 mm^3 of 2–10 mmol dm^{-3} solutions of the inactivator in the Tris buffer (final concentrations are 100, 150, 200 and 500 μmol dm^{-3}, respectively). To this cuvette was added 100 mm^3 of a 0.75 μmol dm^{-3} solution of CPA in the Tris buffer (final concentration 0.0375 μmol dm^{-3}) and the change in absorbance at 254 nm was recorded over a time interval of 0–600 s. Values of k_{obs} were calculated from the progress curves using the computer-assisted spectrophotometer and values of K_i and k_{inact} were calculated, respectively, from the slope and the *y* intercept of straight lines in Fig. 4 and eqn. (2). In the calculation the K_m value of 170 μmol dm^{-3} that was reported by Jacobsen and Bartlett[24] was used.

Determination of the partition ratios. The partition ratio was evaluated by the titration method.[12] A series of solutions (500 mm^3 each) containing 32.2 μmol dm^{-3} CPA and various molar equivalents of inactivator were prepared to give $[I]_o/[E]_o$ ratios of 0–2 in 0.05 mol dm^{-3} Tris/0.5 NaCl, pH 7.5, and were stirred gently at 4 °C for 16 h. The mixture was dialysed in the same buffer solution at 4 °C for 24 h, after which a 100 mm^3 sample of each solution was added to 1900 mm^3 of the assay mixture ($[S]_o$ = 750 μmol dm^{-3}) and the activity was measured immediately. Control experiments were carried out in the same manner in the absence of the inactivator.

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