Latent Inhibitors. Part 4.[1] Irreversible Inhibition of Dihydro-orotate Dehydrogenase by Hydantoins Derived from Amino Acids

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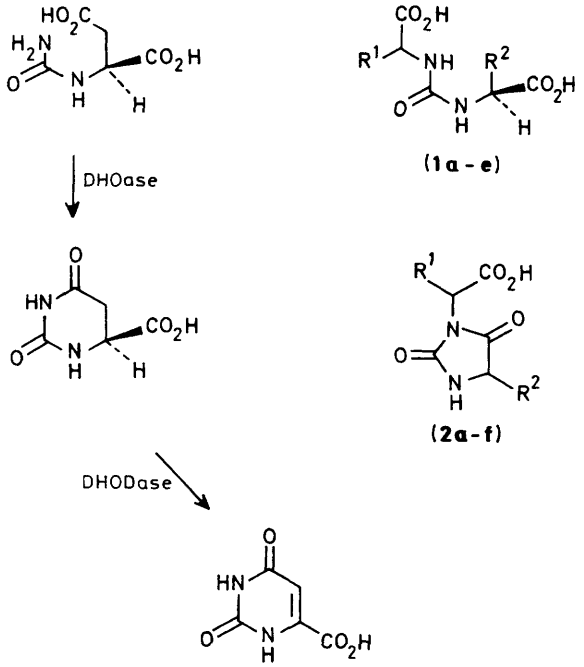
Hydantoins and ureas derived from α-amino acids are shown to interact with dihydro-orotate dehydrogenase from *Clostridium* (*Zymobacterium*) *oroticum*, chiefly as weak competitive inhibitors but that the hydantoin derived from phenylalanine, 5-benzyl-3-(1-carboxy-2-phenylethyl)hydantoin, is a time-dependent irreversible inhibitor of the enzyme. Inhibition experiments with derivatives of this hydantoin and also its intrinsic chemical reactivity lead to a hypothetical mechanism for the inhibition reaction involving benzylic oxidation of the hydantoin followed by Michael addition of an enzymic nucleophile. The relationship between this reaction and the normal substrate oxidation reaction is discussed in the light of a comparison of substrate and inhibitor structure by molecular graphics.

Rational drug design has relied upon two major strategies to achieve the goal of high selectivity of action namely the inhibition of enzymes specific to the parasite causing the disease and mechanism based inhibition of the target enzyme. In some well-characterised areas of metabolism, it is possible to apply both strategies simultaneously. We were drawn to examine the *de novo* biosynthesis of pyrimidines which has been recognised as a target of potential significance in the chemotherapy of parasitic and neoplastic diseases.[2–4] Based upon our considerable experience of pyrimidine chemistry in relation to pteridine biosynthesis,[5–8] we selected dihydro-orotate dehydrogenase (DHODase) and dihydro-orotase (DHOase) as targets for inhibition (Scheme 1). DHOase was the first target

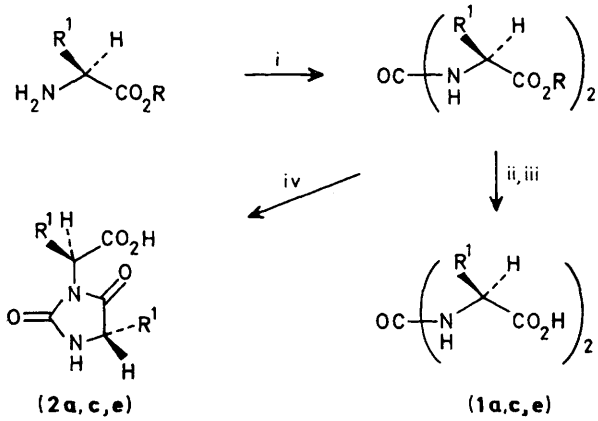


Scheme 1.

considered and ureas (carbonyldiamino acids) (**1a**—**e**) were synthesized with potential to interfere with the cyclisation reaction catalysed by the enzyme. During the synthesis, we also obtained the hydantoins (**2a**—**f**). Difficulties in assaying the enzyme prevented our evaluating DHOase inhibition but we found that both series of compounds inhibited the subsequent enzyme in the pathway, DHODase. DHODase from *Clostridium oroticum* is atypical of this enzyme because it is an NAD-linked flavoprotein whereas the flavins of enzymes from most other organisms are provided with oxidising equivalents by an electron transport chain.[2] The NAD dependence facilities assay procedures and, as will be discussed later, we have found that the inhibitory activity of the hydantoins with respect to this enzyme is mirrored by their antiparasitic activity.

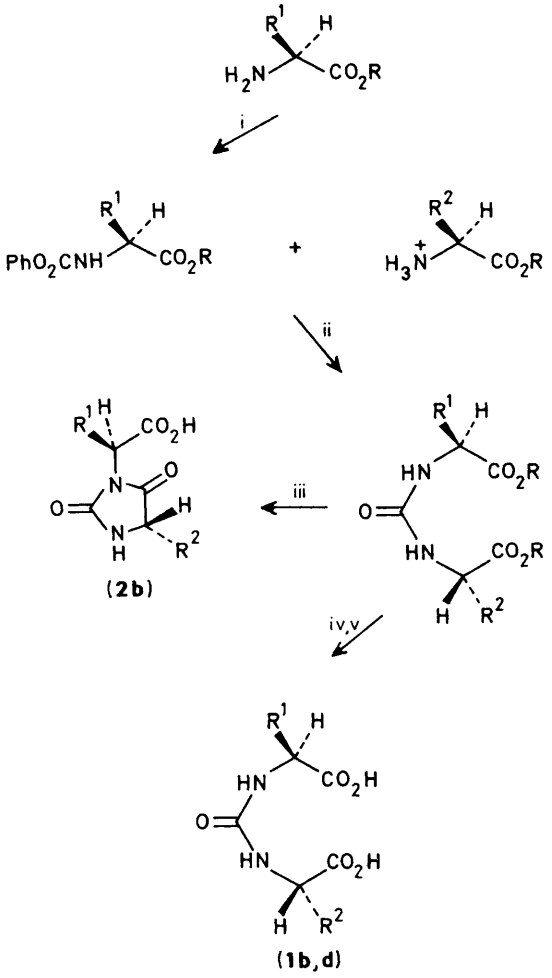
Results and Discussion

Synthetic Studies.—The symmetrical ureas, which were chiefly known compounds, were conveniently prepared by condensing the appropriate amino acid ester with *N,N'*-carbonyldi-imidazole, hydrolysing the diester with alkali, and careful precipitation of the product by acidification (Scheme 2).



Scheme 2. *Reagents:* i, carbonyldi-imidazole; ii, aq. NaOH; iii, H_3O^+; iv, HCl, HOAc

More extensive acid treatment caused cyclisation to hydantoins. N.m.r. and optical rotation measurements showed that the optical purity of the chiral compounds was retained throughout these reactions. The unsymmetrical ureas were prepared by activating one amino acid component with phenyl chloroformate and coupling the resulting *N*-carbophenoxy derivative with the ester of the second amino acid in the presence of triethylamine



Scheme 3. *Reagents:* i, PhOCOCl; ii, 3 equiv. Et_3N; iii, HCl, HOAc; iv, aq. NaOH; v, H_3O^+

(Scheme 3). Subsequent treatment as for the symmetrical compounds afforded the ureas and hydantoins. The acid catalysed cyclisation of unsymmetrical carbonyldiamino acids can lead to two products. We found, in agreement with earlier work,[9] that the cyclisation of the diester from glycine and phenylalanine led to a single product, the structure of which was confirmed as the 5-benzylhydantoin by unambiguous synthesis (Scheme 4).

Enzymic Studies.—Initial assessments of the ureas and hydantoins as inhibitors of DHODase were carried out by incubating the enzyme (0.02μM), orotic acid (0.67mM), and NADH (0.13mM) in sodium phosphate buffer (pH 6.5) containing dithiothreitol to stabilise the enzyme in the presence of a suitable concentration of the inhibitor (2—4mM). Most of the compounds were found to be typical competitive inhibitors of DHODase (Table 1) and assay reactions proceeded to completion at a slower rate than in the absence of inhibitor. However, with the hydantoin derived from (*S*)-phenylalanine, (**2c**), the assay reaction failed to reach completion (Figure 1). We subsequently found that the enantiomeric hydantoin derived from (*R*)-phenylalanine, (**2d**), showed similar behaviour. The incompleteness of these assay reactions suggested that the enzyme might be reacting directly with these inhibitors. By incubating the enzyme (0.7μM) with the hydantoins derived from phenylalanine at concentrations between 1 and 13mM,

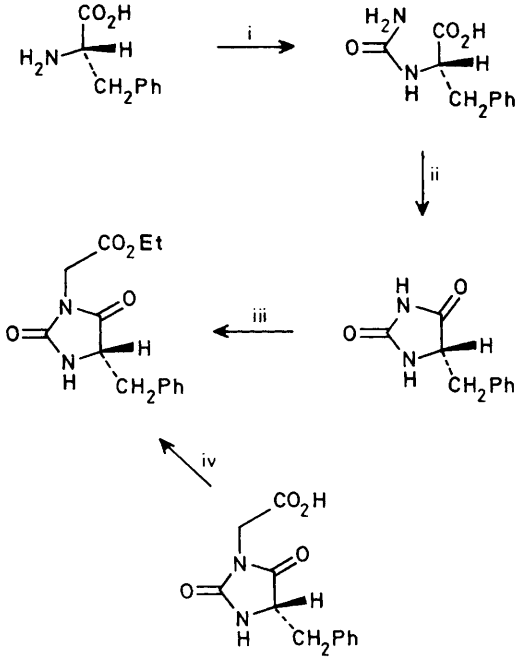
Table 1. Inhibition of DHODase by ureas (**1**) and hydantoins (**2**)

Compound	R^1	R^2	K_i/mM	% Inhibition at 4mM
(**1a**)	H	H	2.6	
(**1b**)	H	(*S*)-CH_2Ph	0.7	
(**1c**)	(*S,S*)-CH_2Ph		0.4	
(**1d**)	Me	CH_2Ph		20
(**1e**)	Me	Me		0
(**2a**)	H	H	4.5	
(**2b**)	H	(*S*)-CH_2Ph	2.3	
(**2c**)	(*S,S*)-CH_2Ph		0.7	
(**2d**)	(*R,R*)-CH_2Ph		0.9	
(**2e**)	(*S,S*)-Pr^i		3.3	
(**2f**) N^1Me	CH_2Ph	CH_2Ph	0.6	

Table 2. Properties of inhibition reaction

(a) Reversibility	% Activity before and after g.p.c.	
Control	100	69
Inhibitor (**2c**)	41	18

(b) Substrate protection	$t_{\frac{1}{2}}$ min
Inhibitor (**2c**) alone	72
+ NAD^+ (8.9mM)	390
+ NAD^+ (17.9mM)	1 350
+ orotic acid (0.56mM)	116
+ orotic acid (1.12mM)	128



Scheme 4. *Reagents:* i, NaNCO; ii, H_3O^+; iii, $ClCH_2CO_2Et$, NaOEt, EtOH; iv, EtOH/H^+

removing samples at intervals, and initiating reaction with NADH and orotic acid, a first order decrease in enzyme activity was demonstrated (Figures 2 and 3). Inhibition was not reversed by gel filtration (Table 2a) and the addition of orotic acid or NAD^+ caused partial protection of the enzyme from the inactivation (Table 2b). Such properties are characteristic of irreversible inhibition at the active site.

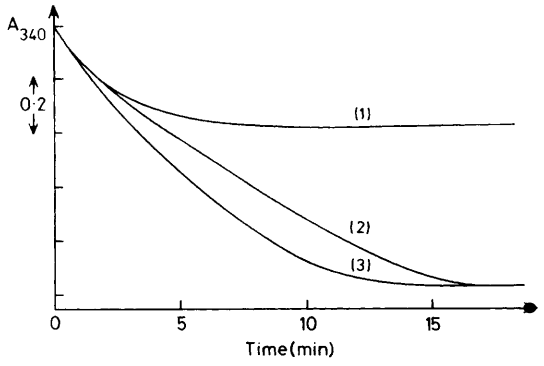


Figure 1. Time course of reduction of orotic acid by DHODase in the presence and absence of inhibitors: (1) in presence of (**2c**) 7.0 mM; (2) in presence of (**1c**) 7.3mM; (3) no inhibitor

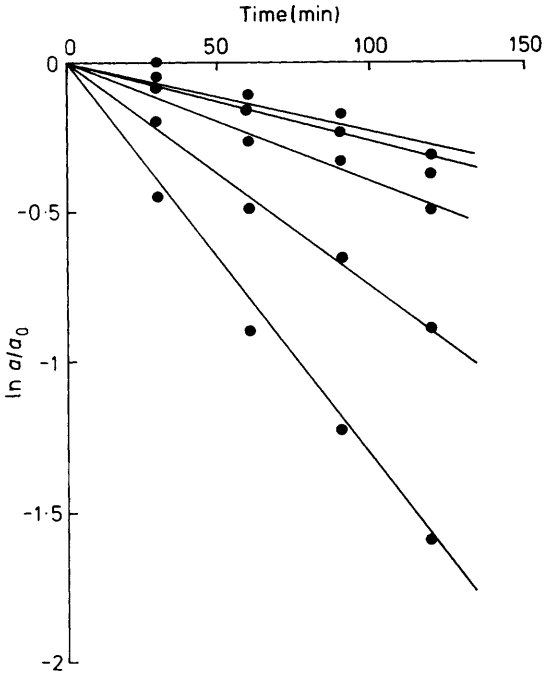


Figure 2. First-order plots for the inhibition of DHODase (0.7μM) by (**2c**) at 1.30, 1.63, 3.25, 6.50, and 13.0mM

In view of the protective anion of NAD^+, the question arose whether the hydantoin would inhibit any nicotinamide-dependent enzyme. We therefore investigated the interaction of hydantoin (**2c**) with horse liver alcohol dehydrogenase and lactate dehydrogenase. Although both enzymes were slightly inhibited by this compound, we found no evidence for time-dependent inhibition.

A Mechanism for Inhibition.—The accidental discovery of time-dependent inhibition of DHODase necessitated an investigation of the mechanism of inhibition. Inspection of the results in Table 1 shows that the benzyl group is characteristic of the better inhibitors although none of these compounds binds tightly to the enzyme. Further, it appeared that the function of a benzyl group was not simply that of a hydrophobic ligand for the enzyme because the isopropyl hydantoin, (**2e**), derived from leucine showed no time-dependent inhibition. Since DHODase is capable of catalysing a dehydrogenation reaction the

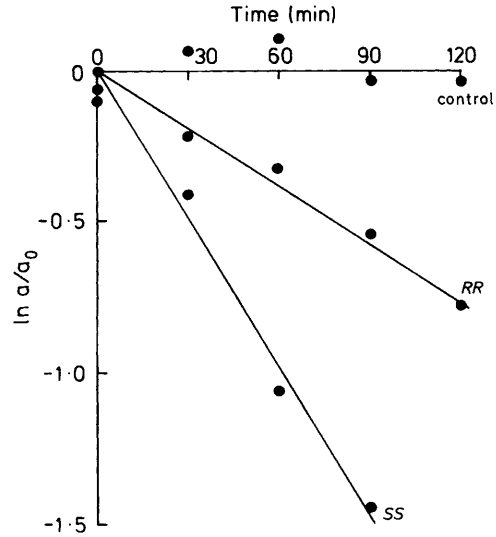
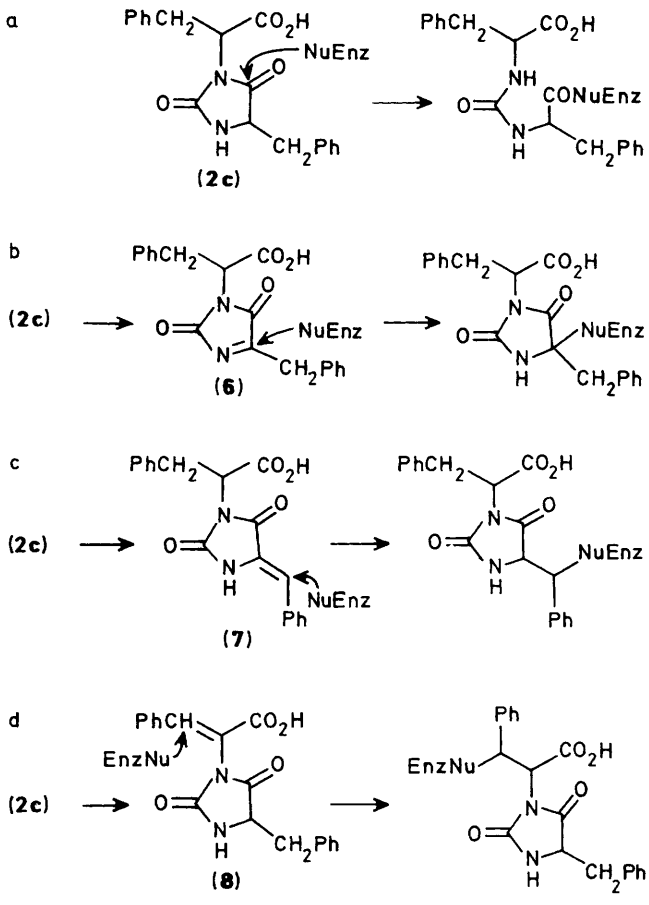


Figure 3. First-order plots for the inhibition of DHODase (0.7μMM) by (*R*,*R*)- and (*S*,*S*)-(**2c**) (13mM)



Scheme 5. Possible mechanisms of inhibition of DHODase

possibility that one of the benzyl groups was the site of an enzyme-catalysed oxidation was suggested. We therefore considered that inhibition through acylation by the hydantoin acting as an anhydride equivalent (Scheme 5a) was unlikely. Three oxidation pathways were possible. Firstly, endocyclic dehydrogenation of the hydantoin to give (**6**) by loss of the N-1 hydrogen and an adjacent hydrogen (Scheme 5b) was

conceivable although this mechanism assigns no reactive role to the benzyl groups. An alternative mechanism involving exocyclic dehydrogenation to give (**7**) at the benzylic site α to the hydantoin (Scheme 5c) seemed more plausible but the alternative exocyclic pathway in which oxidation occurs at the acidic side chain affording (**8**) (Scheme 5d) cannot be ruled out. Both enzymic and non-enzymic experiments were carried out to investigate these possibilities.

The endocyclic oxidation mechanism (**5b**) is characterised by the loss of the N-1 protons. We therefore prepared the *N*-methyl derivative (**2c**) of (**2f**) by treatment with dimethyl sulphate followed by hydrolysis of the ester. This reaction led to partial epimerisation at both chiral centres. Despite numerous attempts under a variety of conditions using methanolic sodium hydroxide, hydrogen chloride in dilute acetic acid, lithium iodide in pyridine, or trimethylsilyl chloride with sodium iodide in acetonitrile, we were unable to prepare an unracemised sample of the *N*-methylhydantoin (**2f**). It was surprising that such sensitivity to racemisation was observed. Fortunately, however, the racemisation did not prevent a telling experiment from being conducted. The mixture of diastereoisomers (**2f**) was tested as a time-dependent inhibitor of DHODase and found to be active.

Further evidence against the endocyclic mechanism came from studies of the chemical reactivity of (**2c**). We attempted to prepare the endocyclic oxidation product (**6**) by treatment of (**2c**) with oxidising agents such as chloranil, t-butyl hypochlorite, *N*-bromosuccinimide, thionyl chloride, and trityl tetrafluoroborate. In those cases in which a product could be isolated (chloranil, t-butyl hypochlorite), it was the benzylidene hydantoin (**7**) which resulted from exocyclic oxidation. If DHODase followed the natural chemical reactivity of the inhibitor (**2c**), then the benzylidene hydantoin (**7**) should be an inhibitor also. The u.v. absorption of this compound interfered with that of NADH making a precise determination of its kinetic properties as an inhibitor difficult but we were able to demonstrate that DHODase underwent time-dependent inhibition in the presence of (**7**) at a rate comparable to that of inhibition by (**2c**) under the same conditions [$t_{\frac{1}{2}}$ (**7**) 1.9mM = 135 min, $t_{\frac{1}{2}}$ (**2c**) 1.6mM = 120 min]. On the basis of these experiments, it is not possible to dismiss the pathway (**5d**) but we tentatively propose that DHODase in its oxidised form slowly oxidises (**2c**) or its enantiomer (**2d**) to a benzylidene derivative such as (**7**) which then alkylates the enzyme by Michael addition. This proposal can be tested by investigating the effect of substituents in the benzyl groups upon the rate of inhibition of DHODase and we are currently engaged in preparing the required compounds.

Interpretation of Results.—To our knowledge, only one potential suicide inhibitor of DHODase has been synthesized, the 5-ethynyl derivative of orotic acid [10] but no data for enzyme inhibition was reported. Our compounds are therefore the first irreversible inhibitors of this important enzyme. In addition, there are a number of unusual and interesting features of these results. Firstly, we find that both enantiomers of the hydantoin derived from phenylalanine are inhibitors of similar potency. Secondly, the hydantoin and its oxidation site do not relate closely to the substrate and its oxidation site in a structural sense. The latter feature is, as will be argued later, significant for the extending of strategies in rational drug design. However, both features can usefully be examined with the aid of molecular graphics even in the absence of a crystal structure for the enzyme.

If, as the evidence suggests, the hydantoin inhibitors are operating at the active site of DHODase, there should be a perceptible relationship between the binding of the substrate and that of the inhibitor. The most obvious correlation is to superimpose the amide-containing heterocyclic rings of the two

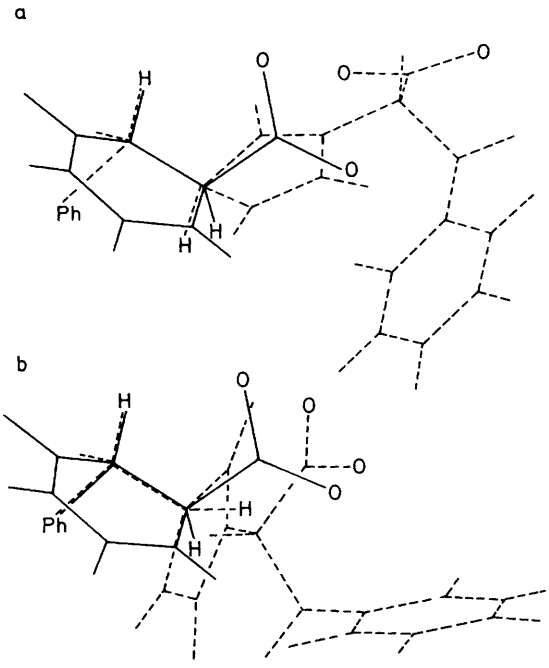


Figure 4. Computer generated superimpositions of (*a*) (*R,R*)-(**2c**) and (*b*) (*S,S*)-(**2c**) (dashed lines) on to *S*-dihydro-orotic acid (full line)

compounds but in this arrangement it is impossible to bring a plausible oxidation site for the inhibitor in conjunction with the oxidation site of the substrate. In addition, it would be expected that the carboxylate substituents of substrate and inhibitor are important in binding to the enzyme. To examine this possibility, we superimposed the presumed oxidation site of the inhibitor (**2c**) upon the known oxidation site of the substrate by means of molecular graphics (Figure 4a). It was then apparent that the carboxylate groups of inhibitor and substrate could conceivably interact with the same positive charged group at the enzyme's active site. This model must also accommodate the unusual fact that the enantiomeric hydantoin (**2d**) was also an inhibitor. When the corresponding superimposition was made (Figure 4b) the anion and oxidation site bore a similar relationship to each other. Moreover, the phenyl rings of both enantiomers could occupy the same regions of space thus suggesting the location of hydrophobic binding sites near the active site. The results therefore not only suggest a possible inhibition mechanism, but also a possible topography for the active site of DHODase from *C. oroticum*. A preliminary account of these results has been published.[11]

Since the *C. oroticum* enzyme is unusual in its method of reoxidising the active site flavin, the significance of our results in the context of the chemotherapy of parasitic or neoplastic disease can be questioned. Collaborators at Wellcome Research [12] have, however, shown that of all the compounds described, the only one with significant activity against *Plasmodium berghei* was the time-dependent inhibitor (**2c**) of *C. oroticum* DHODase. A broader discussion of the implications of our results is therefore merited. Mechanism-based drug design intrinsically generates substrate analogues. Since substrates for metabolic reactions are usually recognised elsewhere in the organism by another enzyme or receptor, side effects may still be a constitutive disadvantage of mechanism-based inhibitors as drugs. Our results highlight the notion that it is possible to maintain the mechanism based specificity in a series of compounds loosely related to the substrate. Such a concept is consistent with the belief that enzymes will bind to

molecules that present a complementary electric field to their active site and that the structural framework, in a conventional chemical sense, is of lesser significance. We therefore argue that mechanism-based drug design be expanded to transpose the catalytic mechanism of an enzyme to transform molecules related to the substrate by their electric field and not merely by their atomic framework.

Experimental

^{1}H N.m.r. spectra were recorded on Perkin-Elmer R32 (90 MHz) or Bruker WH-250 (250 MHz) spectrometers and ^{13}C n.m.r. spectra on a Jeol PFT-100 spectrometer. I.r. spectra were determined using Perkin-Elmer 397 or 257 spectrometers and u.v. spectra using a Pye-Unicam SP 800 spectrophotometer. Optical rotations were measured using a Perkin-Elmer 241 polarimeter with a 1 dm jacketted cell. H.p.l.c. was carried out on ODS reverse phase columns using acetonitrile–water (45:55) as the mobile phase and a flow rate of 30 ml h^{-1}. The detector used was a Cecil Instruments CE 2012 variable wavelength spectrophotometer.

N,N′-*Bis*(*ethoxycarbonylmethyl*)*urea*.—Anhydrous ammonia was bubbled through a suspension of ethyl glycinate hydrochloride (11.93 g, 86 mmol) in chloroform (60 ml, distilled from P_2O_5) for 30 min and the resulting precipitate of ammonium chloride was filtered off. The solution was evaporated to dryness to remove excess ammonia and the residue taken up in fresh chloroform (50 ml). This solution was cooled in an ice-bath and a solution of carbonyldi-imidazole (6.71 g, 41 mmol) in chloroform was added dropwise over 30 min. The solution was stirred at room temperature for a further 20 h, before being washed with 1M hydrochloric acid and water, dried (Na_2SO_4), and evaporated. The resulting white solid was recrystallised from chloroform to yield pure *N,N′*-bis(ethoxycarbonylmethyl)urea (4.3 g, 51%), m.p. 143—145 °C (lit.,[13] 147—148 °C) (Found: C, 46.65; H, 6.8; N, 12.0. Calc. for $C_9H_{16}N_2O_5$: C, 46.55; H, 6.9; N, 12.1%); $\nu_{max.}$(KCl) 3 340 (NH), 2 990 and 2 940 (CH), 1 750 (C=O ester), and 1 630 cm^{-1} (C=O urea); δ_H(90 MHz; [$^2H_6$]DMSO) 1.2 (3 H, t, *J* 7 Hz, CH_3), 3.8 (2 H, d, *J* 6 Hz, NCH_2), 4.1 (2 H, q, *J* 7 Hz, OCH_2), and 6.5 (1 H, t, *J* 6 Hz, NH).

N,N′-*Bis*(*carboxymethyl*)*urea* (**1a**).—*N,N′*-Bis(ethoxycarbonylmethyl)urea (2.52 g, 11 mmol) and sodium hydroxide (1.34 g, 33 mmol) were stirred in water (50 ml) at room temperature for 4 h, during which time the ester slowly dissolved as hydrolysis proceeded. The solution was evaporated to dryness and the resulting white solid was dissolved in the minimum amount of water. This solution was carefully acidified by the slow addition of hydrochloric acid until precipitation commenced and was then cooled in ice. The product was filtered off and recrystallised from water to yield the diacid (**1a**) (0.85 g, 45%), m.p. 188—189 °C (lit.,[14] 204—206 °C) (Found: C, 33.7; H, 4.55; N, 16.0. Calc. for $C_5H_8N_2O_5$: C, 34.1; H, 4.6; N, 15.9%); $\nu_{max.}$(KCl) 3 335 (NH), 2 920 (CH), 1 695 (C=O acid), and 1 625 cm^{-1} (C=O urea); δ_H (90 MHz; [$^2H_6$]DMSO) 3.7 (2 H, d, *J* 6 Hz, CH_2) and 6.4 (1 H, t, *J* 6 Hz, NH).

3-*Carboxymethylhydantoin* (**2a**).—*N,N′*-Bis(ethoxycarbonylmethyl)urea (1.75 g, 7.5 mmol) was refluxed in a mixture of 2M hydrochloric acid (25 ml) and glacial acetic acid (10 ml) for 3 h. The solution was then evaporated to dryness and the crude product was recrystallised from ethanol–ether to yield (**2a**) as white crystals (0.62 g, 52%), m.p. 190 °C (lit.,[14] 204—296 °C,[15] 190—191 °C) (Found: C, 38.1; H, 3.9; N, 18.0. Calc. for $C_5H_6N_2O_4$: C, 38.0; H, 3.8; N, 17.7%); $\nu_{max.}$(KCl) 3 270 (NH), 2 940 (CH), and 1 690br cm^{-1} (C=O); δ_H(90 MHz; [$^2H_6$]DMSO) 4.0 (2 H, s, CH_2), 4.05 (2 H, s, CH_2), and 8.1 (1 H, s, NH). Similarly prepared were the following.

(*S,S*)-*N,N′*-Bis(methoxycarbonylethyl)urea 17% from (*S*)-alanine, m.p. 183—184 °C from chloroform–light petroleum (b.p. 60—80 °C) (lit.,[13] 128 °C) (Found: C, 46.3; H, 6.9; N, 12.0. $C_9H_{16}N_2O_5$ requires C, 46.55; H, 6.9; N, 12.1%); $\nu_{max.}$(KCl) 3 335 (NH), 2 950 (CH), 1 735 and 1 745 (C=O ester), and 1 630 cm^{-1} (C=O urea); δ_H(90 MHz; [$^2H_6$]DMSO) 1.25 (3 H, d, *J* 7 Hz, CH_3), 3.6 (3 H, s, OCH_3), 4.15 (1 H, m, CH), and 6.4 (1 H, d, *J* 7 Hz, NH).

(*S,S*)-*N,N′*-Bis(1-carboxyethyl)urea (**1e**) 41% from the dimethyl ester, m.p. 170—171 °C (lit.,[16] 189—190 °C); $\nu_{max.}$(KCl) 3 350 (NH), 3 050br (OH), 1 705 (C=O acid), and 1 630 cm^{-1} (C=O urea); δ_H(90 MHz; [$^2H_6$]DMSO) 1.25 (3 H, d, *J* 7 Hz, CH_3), 4.1 (1 H, m, CH), and 6.35 (1 H, m, NH).

(*S,S*)-*N,N′*-Bis(1-methoxycarbonyl-2-phenylethyl)urea 59% from (*S*)-phenylalanine, m.p. 156—157 °C from chloroform–light petroleum (b.p. 60—80 °C); $[\alpha]_D$ +94° (*c* 1.0 in $CHCl_3$); $\lambda_{max.}$($CHCl_3$) 254 (ε 333 dm^3 mol^{-1} cm^{-1}), 260 (419), and 266 nm (333); $\nu_{max.}$(KCl) 3 350 (NH), 3 060, 3 020, and 2 945 (CH), 1 735 and 1 710 (C=O ester), and 1 630 cm^{-1} (C=O urea); δ_H(90 MHz; [$^2H_6$]DMSO) 2.95 (2 H, d, *J* 7 Hz, CH_2), 3.6 (3 H, s, CH_3), 4.4 (1 H, apparent quartet, *J* 7 Hz, CH), 6.5 (1 H, d, *J* 7 Hz, NH), and 7.1—7.3 (5 H, m, Ph); δ_C(25 MHz; [$^2H_6$]DMSO) 37.5 (t), 51.7 (q), 54.1 (d), 126.8 (d), 128.5 (d), 129.4 (d), 137.2 (d), 156.1 (s), and 173.0 (s); h.p.l.c. 17.5 min (100%).

(S,S)-N,N′-*Bis*(1-*carboxy*-2-*phenylethyl*)*urea* (**1c**) 53% from the dimethyl ester, m.p. 184—185 °C (lit.,[17] 188—188.5 °C) (Found: C, 63.4; H, 5.6; N, 7.6. Calc. for $C_{19}H_{20}N_2O_5$: C, 64.0; H, 5.7; N, 7.9%); $\nu_{max.}$(KCl) 3 390 and 3 300 (NH), 3 020 and 2 920 (CH), 1 730 and 1 710 (C=O acid), and 1 615 cm^{-1} (C=O urea); δ_H(90 MHz; [$^2H_6$]DMSO) 2.9 (2 H, m, CH_2), 4.3 (1 H, m, CH), 6.35 (1 H, d, *J* 7 Hz, NH), and 7.2 (5 H, m, Ph); δ_C(25 MHz; [$^2H_6$]DMSO) 37.8 (t), 53.9 (d), 126.7 (d), 128.4 (d), 129.7 (d), 137.7 (s), 157.2 (s), and 174.1 (s); h.p.l.c. 10.6 min (100%).

(*S,S*)-5-Benzyl-3-(1-carboxy-2-phenylethyl)hydantoin (**2c**) 62% from the dimethyl ester, m.p. 203—207 °C from aqueous acetic acid (lit.,[17] 205.5 °C, lit.,[18] 212—214 °C); $[\alpha]_D$ −198° (*c* 0.5 in MeOH) (Found: C, 67.3; H, 5.3; N, 8.15. Calc. for $C_{19}H_{18}N_2O_4$: C, 67.45; H, 5.4; N, 8.3%); $\lambda_{max.}$(EtOH) 253 (ε 384 dm^3 mol^{-1} cm^{-1}), 259 (422), 265 (328), and 269 nm (203); $\nu_{max.}$(KCl) 3 325 (NH), 3 020 (CH), 1 765, 1 735, and 1 680 cm^{-1} (C=O); δ_H(250 MHz; [$^2H_6$]DMSO) 2.4 (1 H, dd, *J* 7.7 and 13.9 Hz, HC*H*), 2.7 (1 H, dd, *J* 5 and 13.9 Hz, HC*H*), 3.1 (1 H, dd, *J* 11.2 and 13.8 Hz, HC*H*), 3.3 (1 H, dd, *J* 5 and 14.0 Hz, HC*H*), 4.2 (1 H, dd, *J* 4.9 and 7.8 Hz, CH), 4.7 (1 H, dd, *J* 5 and 11 Hz, CH), 7.0—7.4 (1 H, m, 2 × Ph), and 8.2 (1 H, s, NH); δ_C(25 MHz; [$^2H_6$]DMSO) 33.4, 37.0, 52.6, 56.8, 126.6, 128.4, 128.9, 129.5, 129.6, 135.9, 136.0, 137.7, 156.0, 170.3, and 173.3; h.p.l.c. 8.4 min (100%).

Ethyl N-*Phenoxycarbonylglycinate*.[19]—Anhydrous ammonia was bubbled through a suspension of ethyl glycinate hydrochloride (30.0 g, 0.22 mol) in chloroform (100 ml; distilled from P_2O_5) for 30 min and the resulting precipitate of ammonium chloride was filtered off. The solution was evaporated to dryness under reduced pressure to remove excess of ammonia and the residue was redissolved in fresh chloroform (100 ml). This solution was cooled to 0 °C and phenyl chloroformate (13.6 ml, 0.11 mol) was added dropwise over 30 min. The solution was maintained at 0 °C for 1 h and was then allowed to warm to room temperature and left overnight before work-up. Precipitated ethylglycinate hydrochloride was filtered off and recycled. The filtrate was washed with 0.4M hydrochloric acid, 0.1M aqueous sodium hydroxide, and water, dried (Na_2SO_4), and evaporated to dryness. Distillation of the resultant crude oil (0.01 mmHg, 130 °C) yielded *ethyl* N-*phenoxycarbonylglycinate* (23.9 g, 66%) as a clear oil which slowly

crystallised with time, m.p. 34—36 °C (Found: C, 59.9; H, 5.95; N, 6.3. $C_{11}H_{13}NO_3$ requires C, 59.2; H, 5.9; N, 6.3%); $\nu_{max.}$(liquid film) 3 340 (NH), 3 050, 2 975, and 2 930 (CH), 1 730 (C=O), and 1 590 cm^{-1} (aromatic C=C); δ_H(90 MHz; $CDCl_3$) 1.3 (3 H, t, *J* 7 Hz, CH_3), 4.0 (2 H, d, *J* 6 Hz, NCH_2), 4.2 (2 H, q, *J* 7 Hz, OCH_2), 5.8 (1 H, br s, NH), and 7.0—7.5 (5 H, m, Ph); h.p.l.c. 9.0 min (100%). Similarly prepared was the following.

Methyl *N*-phenoxycarbonyl-(*S*)-alaninate 55% from (*S*)-alanine, b.p. 130 °C, 0.01 Torr; $[\alpha]_D$ −14° (*c* 0.5 in $CHCl_3$); $\nu_{max.}$(liquid film) 3 340 (NH), 3 040, 2 990, and 2 950 (CH), 1 730br (C=O), and 1 595 cm^{-1} (aromatic C=C); δ_H(90 MHz; $CDCl_3$) 1.45 (3 H, d, *J* 7 Hz, CH_3), 3.8 (3 H, s, OCH_3), 4.45 (1 H, m, CH), 5.7 (1 H, br s, NH), and 7.1—7.4 (5 H, m, Ph); h.p.l.c. 10.5 min (100%).

Methyl N-*Phenoxycarbonyl*-(S)-*phenylalaninate*.—Methyl (*S*)-phenylalaninate hydrochloride (14.0 g, 65 mmol) was dissolved in water and neutralised by addition of sodium hydroxide (3.0 g, 75 mmol). The methyl (*S*)-phenylalaninate was extracted into chloroform (3 × 30 ml) and these chloroform extracts were combined, dried (Na_2SO_4), and filtered. To this was added a solution of phenyl chloroformate (4.1 ml, 32 mmol) in chloroform (5 ml) and the mixture was stirred at room temperature overnight; it was then washed with 1M hydrochloric acid and water, dried (Na_2SO_4), and evaporated to leave an oil. Distillation (0.2 mmHg, 180 °C) yielded methyl *N*-phenoxycarbonyl-(*S*)-phenylalaninate as an oil which crystallised with time (yield not recorded); m.p. 37—41 °C; $[\alpha]_D$ +82° (*c* 0.6 in $CHCl_3$); $\nu_{max.}$(liquid film) 3 330 (NH), 3 050, 3 020, and 2 940 (CH), 1 730 (C=O), and 1 600 and 1 590 cm^{-1} (aromatic C=C); δ_H(90 MHz; $CDCl_3$) 3.15 (2 H, dd, *J* 6 and 1 Hz, CH_2), 3.7 (3 H, s, CH_3), 4.7 (1 H, dt, *J* 8 and 6 Hz, CH), 5.55 (1 H, br d, *J* 8 Hz, NH), and 7.0—7.4 (10 H, m, 2 × Ph); h.p.l.c. 25.0 min (100%).

(S)-N-(*Ethoxycarbonylmethyl*)-N′-(1-*methoxycarbonyl*-2-*phenylethyl*)*urea*.—Methyl (*S*)-phenylalaninate hydrochloride (6.53 g, 30 mmol) and triethylamine (14.8 ml, 105 mmol) were mixed in chloroform (100 ml; freshly distilled from P_2O_5) and stirred until all the solid had dissolved. Ethyl *N*-phenoxycarbonylglycinate (5.93 g, 27 mmol) in chloroform (30 ml) was added dropwise over 10 min and the mixture was then refluxed for 4 h. The solution was washed with 0.4M hydrochloric acid, 0.1M aqueous sodium hydroxide, and water, dried (Na_2SO_4), filtered, and evaporated to dryness to leave a slightly yellow oil which slowly crystallised. Recrystallisation from chloroform–light petroleum (b.p. 60—80 °C) yielded white crystals of the *diester* (3.88 g, 47%), m.p. 120—121 °C (Found: C, 58.4; H, 6.4; N, 9.0. $C_{15}H_{20}N_2O_5$ requires C, 58.4; H, 6.5; N, 9.1%); $\nu_{max.}$(KCl) 3 340 (NH), 3 020, 2 985, 2 970, and 2 940 (CH), 1 735 (C=O ester), and 1 625 cm^{-1} (C=O urea); δ_H(90 MHz; [$^2H_6$]DMSO) 1.15 (3 H, t, *J* 7 Hz, CH_3), 2.95 (2 H, m, $CHCH_2$), 3.6 (3 H, s, OCH_3), 3.75 (2 H, d, *J* 6 Hz, $NHCH_2$), 4.05 (2 H, q, *J* 7 Hz, OCH_2), 4.45 (1 H, m, CH), 6.5 (2 H, m, 2 × NH), and 7.2 (5 H, m, Ph); δ_C(25 MHz; [$^2H_6$]DMSO) 14.0 (q), 37.7, 41.5, 51.8 (q), 54.2 (d), 60.4 (t), 126.9 (d), 128.6 (d), 129.5 (d), 137.4 (s), 157.6 (s), 171.3 (s), and 173.3 (s); h.p.l.c. 7.8 min (100%).

(S)-N-*Carboxymethyl*-N′-(1-*carboxy*-2-*phenylethyl*)*urea* (**1b**).—(*S*)-*N*-Ethoxycarbonylmethyl-*N*′-(1-methoxycarbonyl-2-phenylethyl)urea (1.00 g, 3.2 mmol) was suspended in a solution of sodium hydroxide (0.59 g, 15 mmol) in water (20 ml) and the mixture was stirred at room temperature for 5 h by which time all the solid had dissolved. The solution was evaporated to dryness on a freeze drier, and the residue was dissolved in the minimum amount of water (5 ml). 2M Hydrochloric acid (5 ml) was washed with water, and recrystallised from boiling water to yield the diacid (**1b**) (0.49 g, 57%), m.p. 176—177 °C (lit.,[9] 176—177 °C) (Found: C, 53.65; H, 5.2; N, 10.5. Calc. for $C_{12}H_{14}N_2O_5$: C, 54.1; H, 5.3; N, 10.5%); $\nu_{max.}$(KCl) 3 400 and 3 310 (NH), 3 020 and 2 940 (CH), 1 735 and 1 700 (C=O), and 1 580 cm^{-1}; δ_H(90 MHz; [$^2H_6$]DMSO) 2.9 (2 H, m, CH_2Ph), 3.7 (2 H, d, *J* 6 Hz, NCH_2), 4.4 (1 H, m, NCH), 6.4 (2 H, m, 2 × NH), and 7.2 (5 H, m, Ph); δ_C (25 MHz; [$^2H_6$]DMSO) 37.7 (t), 41.4 (t), 54.0 (d), 126.7 (d), 128.4 (d), 129.5 (d), 137.7 (s), 157.7 (s), 172.6 (s), and 174.1 (s); h.p.l.c. 6.0 min (100%).

(S)-5-*Benzyl*-3-*carboxymethylhydantoin* (**2b**).—(*S*)-*N*-Ethoxycarbonylmethyl-*N*′-(1-methoxycarbonyl-2-phenylethyl)urea (0.5 g, 1.6 mmol) was refluxed in a mixture of concentrated hydrochloric acid (20 ml) and glacial acetic acid (20 ml) for 5 h. The solvent was evaporated under reduced pressure and the resulting white solid was recrystallised from water to yield the hydantoin (**2b**) (0.23 g, 58%), m.p. 175—177 °C (lit.,[9] 184—185 °C) (Found: C, 58.1; H, 4.8; N, 11.2. Calc. for $C_{12}H_{12}N_2O_4$: C, 58.1; H, 4.9; N, 11.3%); $\nu_{max.}$(KCl) 3 350 (NH), 1 770, 1 740, and 1 690 cm^{-1} (C=O); δ_H(90 MHz; [$^2H_6$]DMSO) 3.0 (2 H, m, $CHCH_2$), 3.9 (2 H, s, NCH_2), 4.45 (1 H, m, NCH), 7.25 (5 H, s, Ph), and 8.3 (1 H, s, NH); δ_C(25 MHz; [$^2H_6$]DMSO) 36.9, 38.9, 57.6 (d), 127.0 (d), 128.5 (d), 129.8 (d), 135.9 (s), 156.3 (s), 169.0 (s), and 173.6 (s); h.p.l.c. 4.0 min (100%).

(S)-5-*Benzyl*-3-*ethoxycarbonylmethylhydantoin*.—*Method 1*. (*S*)-5-Benzyl-3-carboxymethylhydantoin (**2b**) (0.60 g, 2.4 mmol) was refluxed in ethanolic hydrochloric acid (30 ml) for 2 h. When cooled a white precipitate formed. This was collected, washed with ethanol, and dried to give the desired ester (0.48 g, 72%), m.p. 145—147 °C (mixed m.p. with product from method 2, 144—145 °C) (lit.,[20] 155 °C); $\nu_{max.}$(KCl) 3 240 (NH), 3 060, 3 030, 2 980, 2 940, and 2 905 (CH), and 1 775, 1 740, and 1 720 cm^{-1} (C=O); δ_H(90 MHz; [$^2H_6$]DMSO) 1.2 (3 H, t, *J* 7 Hz, CH_3), 3.0 (2 H, m, CH_2Ph), 4.0 (2 H, s, NCH_2), 4.1 (2 H, q, *J* 7 Hz, OCH_2), 4.5 (1 H, t, *J* 5.5 Hz, CH), 7.25 (5 H, s, Ph), and 8.4 (1 H, s, NH).

Method 2.[20] A solution of sodium ethoxide was prepared from sodium metal (0.10 g, 4.35 mmol) and ethanol (30 ml) under nitrogen. To this was added (*S*)-5-benzylhydantoin (0.81 g, 4.26 mmol) and ethyl chloroacetate (0.50 g, 4.61 mmol) and the mixture was refluxed for 4 h; on cooling a white precipitate formed. This was collected, washed with ethanol, and dried to yield the desired ester (0.56 g, 61%), m.p. 146—148 °C (lit.,[20] 155 °C); $\nu_{max.}$(KCl) 3 240 (NH), 3 060, 3 030, and 2 980 (CH), and 1 755, 1 740, and 1 720 cm^{-1} (C=O); δ_H(90 MHz; [$^2H_6$]DMSO) 1.15 (3 H, t, *J* 7 Hz, CH_3), 3.0 (2 H, m, CH_2Ph), 4.0 (2 H, s, NCH_2), 4.1 (2 H, q, *J* 7 Hz, OCH_2), 4.5 (1 H, t, *J* 5.5 Hz, CH), and 7.2 (5 H, s, Ph).

(S,S)-N-(1-*Methoxycarbonylethyl*)-N′-(1-*methoxycarbonyl*-2-*phenylethyl*)*urea*.—Triethylamine (9.5 ml, 68 mmol) was added to a stirred suspension of (*S*)-phenylalaninate hydrochloride (4.78 g, 22 mmol) in chloroform (90 ml) and, once all the solid had dissolved, a solution of methyl (*S*)-*N*-phenoxycarbonylalaninate (3.8 g, 17 mmol) in chloroform (30 ml) was added dropwise over 30 min. The solution was refluxed for 11 h, then washed with 0.4M hydrochloric acid, 0.1M aqueous sodium hydroxide, and water, dried (Na_2SO_4), filtered, and evaporated to yield an oil. Crystallisation from chloroform–light petroleum produced white crystals of the *diester* (1.56 g, 30%) (Found: C, 57.9; H, 6.5; N, 8.95. $C_{15}H_{20}N_2O_5$ requires C, 58.4; H, 6.5; N, 9.1%); $\nu_{max.}$(KCl) 3 350 (NH), 3 025, 3 000, and 2 950 (CH), 1 725 (C=O ester), and 1 630 cm^{-1} (C=O urea).

(S,S)-N-(1-*Carboxyethyl*)-N′-(1-*carboxy*-2-*phenylethyl*)*urea* (**1d**).—(*S,S*)-*N*-1-(Ethoxycarbonylethyl)-*N*′-(1-methoxycarbonyl-2-phenylethyl)urea (1.2 g, 3.9 mmol) was stirred in a solution of sodium hydroxide (0.5 g, 12.5 mmol) in water (60 ml)

at room temperature for 4 h. The solution was carefully acidified by addition of hydrochloric acid and the resultant white precipitate was collected, washed with water, and dried. Recrystallisation from water yielded the diacid (65 mg, 5%); $\nu_{max.}$(KCl) 3 415 and 3 350 (NH), 3 000br (OH), 1 725 (C=O acid), and 1 620 cm^{-1} (C=O urea).

(S,S)-5-*Benzyl*-3-(1-*methoxycarbonyl*-2-*phenylethyl*)-1-*methylhydantoin*.—Barium oxide (6.0 g, 39 mmol) and barium hydroxide octahydrate (3.5 g, 11 mmol) were added to a stirred solution of (*S,S*)-5-benzyl-3-(1-carboxy-2-phenylethyl)hydantoin (2.0 g, 6 mmol) in dimethylformamide (DMF; 80 ml) at room temperature. Dimethyl sulphate (14.0 ml, 150 mmol) was added dropwise and the mixture was stirred at room temperature for 6 h and then set aside overnight. The mixture was then partitioned between chloroform and water, and the chloroform layer was separated, repeatedly washed with water, and then evaporated. The residue was taken up in ether and this solution was repeatedly washed with water to remove last traces of DMF, dried (Na_2SO_4), filtered, and evaporated to yield the product as a viscous oil (1.85 g, 85%); $[\alpha]_D$ −114° (*c* 0.5 in MeOH); $\nu_{max.}$(liquid film) 1 771, 1 742, and 1 710 (C=O), 1 440, 1 410, 750, and 700 cm^{-1}; δ_H(250 MHz; [$^2H_6$]DMSO) 2.77 (3 H, s, NCH_3), 2.9—3.3 (4 H, overlapping dd and dd, 2 × CH_2), 3.51 (3 H, s, OCH_3), 4.27 (1 H, t, *J* 4.9 Hz, C 5-H), 4.77 (1 H, dd, *J* 10.6 and 4.8 Hz, CH), and 7.2 (10 H, m, 2 × Ph); h.p.l.c. 30.0 min (100%).

Partially Racemised 5-*Benzyl*-3-(1-*carboxy*-2-*phenylethyl*)-1-*methylhydantoin* (**2f**).—To a solution of (*S,S*)-5-benzyl-3-(1-methoxycarbonyl-2-phenylethyl)-1-methylhydantoin (214 mg, 0.6 mmol) in methanol (2 ml) was added a solution of sodium hydroxide (102 mg, 2.5 mmol) in water (2 ml). This mixture was then stirred at room temperature for 66 h, acidified by addition of hydrochloric acid, and extracted into chloroform. The chloroform extracts were dried (Na_2SO_4), filtered, and evaporated to yield the acid (**2f**) as an oil. Drying under high vacuum at 77 °C (*i.e.* above melting point) produced a glassy solid which was ground to a powder, m.p. 40—45 °C; $[\alpha]_D$ −41° (*c* 0.5 in MeOH); $\nu_{max.}$(KCl) 3 050, 3 020, and 2 915 (CH), 1 760 and 1 710 (C=O), 1 440, 1 410, 750, and 700 cm^{-1}; δ_H(250 MHz; [$^2H_6$]DMSO) 2.6 and 2.7 (3 H, both s, NCH_3), 2.6—3.4 (overlapping m, 2 × CH_2), 4.3 and 4.35 (1 H, overlapping t and t, CH), 4.6 and 4.7 (1 H, both dd, CH), and 7.2 (10 H, m, 2 × Ph); h.p.l.c. 4.7 min (100%).

(R,R)-N,N′-*Bis*(1-*methoxycarbonyl*-2-*phenylethyl*)*urea*.—Anhydrous ammonia was bubbled through a suspension of methyl (*R*)-phenylalaninate hydrochloride (13.7 g, 64 mmol) in chloroform for 30 min and the resulting precipitate of ammonium chloride was removed by washing with water. The chloroform solution was dried (Na_2SO_4), filtered, and concentrated to a volume of *ca.* 150 ml. A solution of carbonyldi-imidazole (5.0 g, 31 mmol) in chloroform (50 ml) was then added dropwise and the reaction mixture was stirred at room temperature for 24 h. The solution was washed with 1M hydrochloric acid and water, dried (Na_2SO_4), filtered, and evaporated to yield the desired urea derivative, yield not recorded; $[\alpha]_D$ −33° (*c* 0.5 in MeOH), −100° (*c* 0.5 in $CHCl_3$); $\nu_{max.}$(KCl) 3 360 (NH), 3 060, 3 020, and 2 940 (CH), 1 735 and 1 715 (C=O ester), and 1 630 cm^{-1} (C=O urea).

(R,R)-5-*Benzyl*-3-(1-*carboxy*-2-*phenylethyl*)*hydantoin* (**2d**).—The (*R,R*)-*N,N*′-bis(1-methoxycarbonyl-2-phenylethyl)urea produced above was refluxed in a mixture consisting of glacial acetic acid (50 ml), concentrated hydrochloric acid (50 ml), and water (10 ml) for 4 h. On cooling a white precipitate formed. This was filtered off, washed with water, and dried to yield the hydantoin (**2d**) (4.9 g, 47% over two stages); m.p. 209—211 °C; $[\alpha]_D$ +177° (*c* 0.5 in MeOH); $\nu_{max.}$(KCl) 3 320 (NH), 3 020 (CH), 1 760, 1 735, and 1 680 cm^{-1} (C=O).

(S,S)-N,N′-*Bis*(1-*ethoxycarbonyl*-3-*methylbutyl*)*urea*.—Anhydrous ammonia was bubbled through a suspension of methyl (*S*)-leucinate hydrochloride (10.0 g, 55 mmol) in chloroform for 45 min and the resulting suspension of ammonium chloride was filtered off. The solution of methyl (*S*)-leucinate was concentrated and a solution containing carbonyldi-imidazole (4.03 g, 25 mmol) in chloroform was added. The resulting mixture was stirred at ambient temperature for 3 h and then washed with 1M hydrochloric acid and water, dried (Na_2SO_4), filtered, and evaporated to dryness to yield a pale yellow crystalline solid. Recrystallisation of this from chloroform–light petroleum yielded the desired *product* as a white crystalline solid (3.62 g, 46%), m.p. 80—82 °C; $\nu_{max.}$(KCl) 3 350 (NH), 2 960 and 2 932 (CH), 1 751 (C=O ester), and 1 625 cm^{-1} (C=O urea); δ_H(90 MHz; [$^2H_6$]DMSO) 0.9 [6 H, dd, *J* 3 and 6 Hz, $CH(CH_3)_2$], 1.3—1.85 (3 H, m, $NCHCH_2CH$), 3.6 (3 H, s, OCH_3), 4.15 (1 H, m, $NHCH$), and 6.3 (1 H, d, *J* 8 Hz, NH); δ_C(25 MHz; [$^2H_6$]DMSO) 21.4 (q, $CHCH_3$), 22.4 (q, $CHCH_3$), 24.0 (d, $CHCH_3$), 40.8 (t, $CHCH_2CH$), 51.4 (q, OCH_3), 157.3 (s, C=O urea), and 174.1 (s, C=O ester).

(S,S)-3-(1-*Carboxy*-3-*methylbutyl*)-5-(2-*methylpropyl*)-*hydantoin* (**2e**).—(*S,S*)-*N,N*′-Bis(1-methoxycarbonyl-3-methylbutyl)urea (4.0 g, 12.7 mmol) was refluxed in concentrated hydrochloric acid (25 ml), glacial acetic acid (25 ml), and water (10 ml) for 4.5 h. The solvent was then evaporated off to leave a solid product which was recrystallised from water to yield the hydantoin (**2e**) as a white crystalline product (0.8 g, 23%), m.p. 136—136.5 °C; $\nu_{max.}$(KCl) 3 350 (NH), 2 960 and 2 930 (CH), and 1 760, 1 712, and 1 675 cm^{-1} (C=O); δ_H(90 MHz; [$^2H_6$]DMSO) 0.9 (12 H, m, 4 × CH_3), 1.1—2.1 (6 H, m, 2 × CH_2CH), 4.1 (1 H, m, CH), 4.5 (1 H, dd, CH), and 8.4 (1 H, s, NH).

(S)-5-*Benzylidene*-3-(1-*carboxy*-2-*phenylethyl*)*hydantoin* (**7**).—(*S,S*)-5-Benzyl-3-(1-carboxy-2-phenylethyl)hydantoin (1.5 g, 4.4 mmol) and choranil (2.2 g, 8.9 mmol) were refluxed in glacial acetic acid (100 ml) for 95 h. On cooling, a precipitate of chloranil formed. This was filtered off and the filtrate evaporated to dryness. The residue was taken up in chloroform and extracted into dilute aqueous sodium hydroxide. These extracts were then acidified by addition of hydrochloric acid and extracted into chloroform. The chloroform extracts were dried (Na_2SO_4), filtered, and evaporated to dryness to yield an oily product. Chromatography on silica gel with ethyl acetate–light petroleum (b.p. 60—80 °C) (1:1) as eluant yielded unchanged (*S,S*)-5-benzyl-3-(1-carboxy-2-phenylethyl)-hydantoin (0.15 g) and (S)-5-*benzylidene*-3-(1-*carboxy*-2-*phenyl*)*hydantoin* (**7**) which was recrystallised from chloroform–light petroleum (b.p. 60—80 °C), yield 0.12 g (8%), m.p. 225—227 °C (Found: C, 66.7; H, 4.6; N, 8.1. $C_{19}H_{16}N_2O_4$ requires C, 67.85; H, 4.8; N, 8.3%); $\lambda_{max.}$(MeOH) 305sh, 317.5, and 330nm sh; $\lambda_{max.}$(H_2O) 332 nm (ε 22 800 dm^3 mol^{-1} cm^{-1}); $\nu_{max.}$(KCl) 3 300 (NH), 3 030 and 2 920 (CH), 1 750 and 1 710 (C=O), and 1 655 cm^{-1} (C=C); δ_H(250 MHz; [$^2H_6$]DMSO) 3.27—3.45 (m, CH_2), 4.97 (1 H, dd, *J* 5.4 and 11.0 Hz, $CHCH_2$), 6.46 (1 H, s, =CH), 7.11—7.60 (10 H, m, 2 × Ph), and 10.82 (1 H, s, NH); h.p.l.c. 2.40 min (100%).

(*S,S*)-5-Benzyl-3-(1-carboxy-2-phenylethyl)hydantoin (1.00 g, 2.96 mmol) was refluxed in an aqueous solution of sodium hypochlorite (10 ml, 10—14% w/v available chlorine) for 1 h and then left overnight before work-up. The resultant solid was collected, washed with water and ethanol, and dried. Recrystallisation from water yielded (*S*)-5-benzylidene-3-(1-carboxy-2-

phenylethyl)hydantoin (13.5 mg, 1.4%); $\lambda_{max.}$(EtOH) 305sh, 318, and 330 nm sh; δ_H(250 MHz; [$^2H_6$]DMSO) 3.28—3.52 (m, CH_2), 4.92 (1 H, dd, *J* 5.5 and 11.0 Hz, C*H*CH_2), 6.45 (1 H, s, =CH), 7.11—7.65 (10 H, m, 2 × Ph), and 10.8 (1 H, s, NH).

Enzyme Studies.—Studies of the inhibition of dihydro-orotate dehydrogenase were carried out using the enzyme from *Zymobacterium oroticum* (Sigma, lot 32C-6850). Inhibition constants, K_i, were determined by analysing the reaction profiles of assays in the presence and absence of inhibitor following Waley.[21] Typical concentrations of reactants in the assay mixture were: sodium orotate 0.67mM, inhibitor 1—50mM, dihydro-orotate dehydrogenase 22nM, and NADH 0.18mM. Enzyme, substrate, and coenzyme were dissolved in pH 6.5 sodium phosphate buffer (0.4—1.2M containing dithiothreitol 1 mM) and inhibitors were dissolved in pH 7.5 sodium phosphate buffer 0.1M. Thus a typical assay at 25 °C following the NADH absorption at 354 nm contained the following:

	Control	Inhibition
1.2M pH 6.5 Na (P) containing 1mM DTT	0.85 ml	0.85 ml
2mM Na orotate	1.00	1.00
Inhibitor in 0.1M pH 7.5 Na (P)	–	1.00
0.1M pH 7.5 Na (P)	1.00	
4mM NADH	0.10	0.10
1 unit DHODase in 1 ml 0.4M pH 6.5 Na (P) + 1mM DTT	0.05	0.05
	3.00 ml	3.00 ml

Time-dependent assays of inhibitors were carried out by incubating the appropriate hydantoin (typically 13mM) with DHODase (0.7μM) in pH 6.5 sodium phosphate buffer at 25 °C removing samples for analysis at suitable time intervals. The concentration of active enzyme was determined by addition of the samples to an excess of NADH and orotic acid and observing the change in absorbance at 354 nm. A typical composition of a run was

Incubation	Control	Inhibition
(**2c**) 19.5mM in 0.1M pH 7.5 Na (P)	–	0.4 ml
0.1M pH 7.5 Na (P)	0.4 ml	–
1 unit DHODase in 0.4M pH 6.5 Na (P) + 1mM DTT (0.65 ml)	0.2	0.2
	0.6 ml	0.6 ml

Assay	
Sample from incubation	0.10 ml
2mM Na orotate	0.90
0.64M pH 6.5 Na (P) + 1mM DTT	1.90
4mM NADH	0.10
	3 ml

From the data obtained, first order plots were drawn to determine the observed rate constants for inhibition, $k_{obs.}$. For the reaction scheme the rate of the inhibition step can be

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{enzyme inhibitor complex}}{E, I} \xrightarrow{k_2} \underset{\text{inhibited enzyme}}{E\text{-}I}$$

$$\frac{1}{k_{obs.}} = \frac{1}{k_2} + \left(\frac{K_i}{k_2}\right)\left(\frac{1}{i}\right)$$

determined knowing the inhibition constant K_i and the inhibitor concentration i from the equation by plotting $1/k_{obs.}$ against $1/i$.

Similar experiments were carried out to determine the effect of added substrate of coenzyme upon $k_{obs.}$.

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