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STUDIES TOWARDS A HYDROPHOBIC SERINE PROTEASE MODEL

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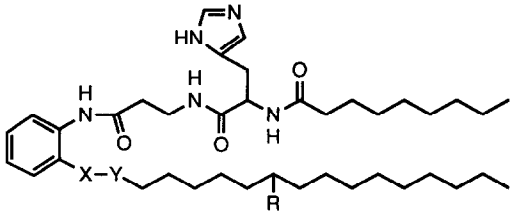
Summary: The synthesis is described of a series of 1,2-disubstituted benzenes, designed to mimic the 'active site' of the serine proteases and have recognition properties for hydrophobic substrates; the kinetics of transacylation using fatty acid 4-nitrophenyl esters in their presence indicate cooperation between imidazole and hydroxyl functionality to produce rate accelerations enhanced with increase in substrate hydrophobicity.

Transferring enzyme-specific characteristics to more accessible molecules has been a valuable strategy in studies directed towards the evolution of new catalytic systems.[1] Enzymes display a conformational preference in solution giving rise to sites which allow specific binding of substrates at or near *active sites* that contain a conformationally defined array of functional groups correctly positioned to facilitate catalysis.[2] We chose to construct an enzyme mimic of the serine proteases,[3] a group of natural transacylation catalysts.[4] Our proposed 'active site' unit centred on the general structure **1** which combines the required nucleophilic (hydroxyl) and general acid-base (imidazole) functionality in a hydrophobic environment to provide a recognition site for non-polar substrates in aqueous media,[5] linked through a 1,2-disubstituted benzene support.[6] We report the synthesis and properties of a first generation of such molecules.

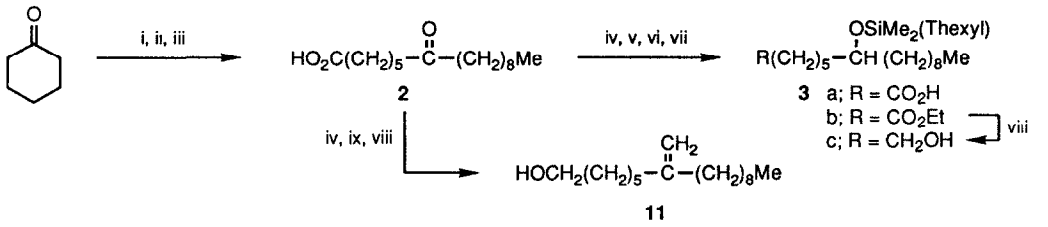


1 a; X–Y = NH–CO, R = OH
b; X–Y = O–CH_2, R = OH
c; X–Y = O–CH_2, R = CH_2OH
d; X–Y = O–CH_2, R = H
e; no 'lower' side-chain

The syntheses followed the general strategy developed for the tetra-amide **1a**. The 'lower' side-chain was easily prepared from cyclohexanone (Scheme 1) *via* acylation of the morpholine enamine [morpholine (1.75 mol equiv.), benzene reflux, 72%; decanoyl chloride, Et_3N, then 1M aq. HCl; 86%] and cleavage of the 2-decanoylcyclohexanone (1.3M aq. NaOH reflux; 94%) to afford 7-oxohexadecanoic acid **2**.[7,8] Esterification (MeOH, c.H_2SO_4, reflux; 98%), reduction ($NaBH_4$, MeOH; 99%), protection of the hydroxy-group as the thexyldimethylsilyl ether[9] ($Me_2CHCMe_2SiMe_2Cl$, imidazole, DMF; 92%) and ester hydrolysis (2.5M aq. NaOH reflux; 82%) gave the acid **3a** suitable for coupling to a 1,2-disubstituted benzene.

Acid **3a** was coupled with 2-nitroaniline (Scheme 2) using a mixed anhydride procedure (EtO_2CCl, Et_3N, -8°C) under unexpectedly severe conditions (EtOAc reflux, 18 h) to give the amide **4** (49%) and ethyl ester **3b** (40%);[10] recycling of the latter (1M aq. NaOH reflux; 85%) raised the yield of **4** to 74% based on recovered starting acid **3a**. Reduction of the nitro group (H_2, 1 atm, 10% Pd–C, EtOAc; 86%) afforded the amino-amide **5** ready for the construction of the 'upper' side-chain.

After a number of sequences were tried,[11] the 'upper' side-chain was assembled *via* the following protocol. N-Benzyloxycarbonyl-β-alanine as a mixed anhydride (EtO_2CCl, Et_3N, -5°C) was coupled with **5**

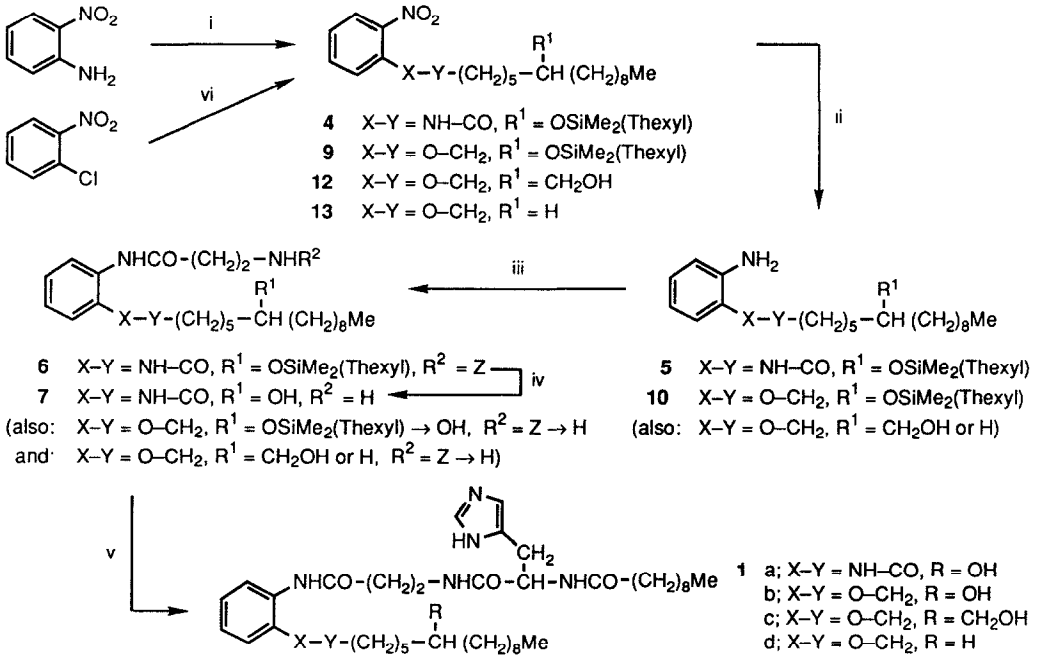


Scheme 1

Reagents: i, morpholine, benzene reflux; ii, $Me(CH_2)_8COCl$, Et_3N, then 1M aq. HCl; iii, 1.3M aq. NaOH reflux; iv, MeOH, c.H_2SO_4; v, $NaBH_4$, MeOH; vi, $Me_2CHCMe_2SiMe_2Cl$, imidazole, DMF; vii, 2.5M aq. NaOH reflux; viii, $LiAlH_4$, Et_2O; ix, $Ph_3P^+MeBr^-$, BuLi, THF.

to afford the diamide **6** (93%). Hydrogenolysis under acidic conditions (H_2, 1 atm, 10% Pd–C, MeOH–c.HCl) removed both the urethane and silyl ether protecting groups to afford, after base treatment (NH_3–$CHCl_3$), the free amine **7** that was used directly in an azide coupling with N^{α}-nonanoyl-L-histidine hydrazide **8** ($NaNO_2$, aq. HCl–EtOAc, 0°→20°C) to generate the tetra-amide **1a** (75% from **6**).[12] Hydrazide **8** was prepared from L-histidine methyl ester hydrochloride by reaction with nonanoyl azide, generated *in situ* from nonanoyl chloride and sodium azide, to give the N^{α}-nonanoyl amide (67%), followed by treatment with hydrazine hydrate (EtOH, 20°C; 75%).

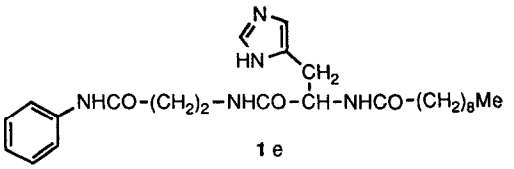
The same general strategy was used for synthesis of the remaining targets **1b-e**. Reduction of the acid **3a** ($LiAlH_4$, Et_2O; 91%) and S_NAr reaction of the product alcohol **3c** with 1-chloro-2-nitrobenzene (KOH,



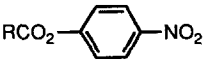
Scheme 2

Reagents: i, **3a**, EtO_2CCl, Et_3N, -8°C, then EtOAc reflux, 18 h; ii, H_2 1 atm, 10% Pd–C, EtOAc ; iii, N-benzyloxycarbonyl-β-alanine, (for **6**) EtO_2CCl, Et_3N, -5°C: otherwise DCC, CH_2Cl_2; iv, H_2 1 atm, 10% Pd–C, MeOH–c.HCl, then NH_3–$CHCl_3$; v, **8**, $NaNO_2$, aq. HCl–EtOAc, 0°C→20°C; vi, (for **9**) **3c**, KOH, DMSO: (for **12**) **11**, KOH, DMSO; $BH_3.Me_2S$; H_2O_2, aq. NaOH: (for **13**) 1-hexadecanol, KOH, DMSO.

DMSO; 81%) afforded the aryl ether **9**.[13] The upper side-chain was assembled by nitro-group reduction as above (81%), coupling of the amine **10** to N-benzyloxycarbonyl-β-alanine (DCC, CH_2Cl_2; 92%),[14] followed by double deprotection by hydrogenolysis and direct coupling with the hydrazide **8** as before to afford the triamide-ether **1b** (89%). A primary alcohol was incorporated into the lower side-chain by Wittig reaction of methyl 7-oxohexadecanoate ($Ph_3P^+Me\ Br^-$, BuLi, THF, inverse addition)[15] followed by reduction ($LiAlH_4$, Et_2O) to afford the alkenol **11** (71% overall). Reaction with 1-chloro-2-nitrobenzene as before (80%) and hydroboration–oxidation ($BH_3.Me_2S$, hexane; H_2O_2, aq. NaOH; 88%) produced the alcohol **12** from which elaboration to the target **1c** proceeded efficiently by the sequence used for **1b** (Scheme 2). The final targets were compounds **1d** and **1e** having no hydroxy-function in the lower side-chain, and no lower side-chain, respectively. Reaction of 1-hexadecanol with 1-chloro-2-nitrobenzene as usual gave the nitro ether **13** (61%) and the remaining steps followed the now established sequence (Scheme 2) to afford **1d**. Elaboration of aniline, again by the same upper side-chain sequence, led straightforwardly to the single-chain derivative **1e**.



1 e



14 a; R = Me
b; R = $(CH_2)_4Me$
c; R = $(CH_2)_6Me$
d; R = $(CH_2)_8Me$

The kinetic tests for acceleration of transacylation by the compounds **1a-e** were carried out using 4-nitrophenyl esters **14a-d** as convenient first generation substrates;[16] the acetate **14a** is commercially available and the hexanoate, octanoate and decanoate derivatives **14b-d** were prepared from the corresponding alcohols (4-nitrophenol, DCC, CH_2Cl_2; 87, 91, and 87%, respectively) as probes for the recognition of hydrophobic substrates. A typical experiment was performed[3b] in pH 10.2 sodium carbonate buffer (3.0 cm^3) at 25.5°C and the reaction initiated by addition of the ester substrate (*ca.* 110 nmol in MeCN); where appropriate the additive (*ca.* 110 nmol) in DMSO was added immediately prior to the substrate. Reactions were followed spectrophotometrically by appearance of the 4-nitrophenolate anion absorption at 400 nm, and the results cited are averages of multiple determinations. Data were analysed by the Swinbourne procedure,[17] using computerised graphical analysis, to generate first-order rate coefficients for the cleavage of the esters **14a-d**. Solubility difficulties with **1a** and **1d** restricted the kinetic investigations to additives **1b**,**c**,**e** and imidazole, and the substrates showed good first-order kinetics. The results, presented as a ratio k_{obs}/k_o (k_{obs} = pseudo-first order rate constant for deacylation in the presence of the additive; k_o = pseudo-first order rate constant for deacylation in buffer alone), are shown in Table 1.

The following conclusions may be drawn. From the reactions of 4-nitrophenyl acetate **14a**, 'control' compound **1e** behaves essentially as an elaborated imidazole, and secondary alcohol **1b** shows only marginal rate improvement. Apparent cooperation between the primary alcohol and imidazole residues in **1c** produces

Table 1

Substrate	Additive			
	Imidazole	**1e**	**1b**	**1c**
14a	1.00	1.10	1.40	3.05
14b	0.97	1.02	3.58	5.67
14c	1.58	1.38	3.41	4.01
14d	1.32	1.40	4.30	5.49

a 3-fold acceleration of deacylation. The reactions of the esters **14b-d** show **1e** again as a modified imidazole, whereas with the increased hydrophobic nature of the substrate, secondary alcohol **1b** shows a marked rate improvement over that observed with the acetate. Increased substrate hydrophobicity also further increases the acceleration observed with the primary alcohol **1c**. The primary alcohol **1c** afforded the largest rate enhancements and so was assessed at less than equimolar quantities against substrate; the results suggested that deacylation was not catalytic but stoichiometric, possibly giving acylation of the additive.[18]

We have thus developed a new range of molecules that accelerate the deacylation of esters, with limited recognition for non-polar substrates; attempts to refine these molecules to improve the accelerations and to find catalytic systems are under way. The support of SERC (studentship to M.T., fellowship to A.M.H.) is gratefully acknowledged.

References and Footnotes:

1. See, for example: V.T. D'Souza and M.L. Bender, *Acc. Chem. Res.*, 1987, **20**, 146.
2. M.L. Bender, 'Mechanisms of Homogeneous Catalysis from Proteins to Protons,' Wiley-Interscience, New York, 1971, p.476.
3. For leading references to other studies of serine protease models, see: (a) K.D. Kramer and S.C. Zimmerman, *J. Am. Chem. Soc.*, 1990, **112**, 3680; (b) F.M. Menger and M. Ladika, *J. Am. Chem. Soc.*, 1987, **109**, 3145; (c) 'Principles of Enzyme Activity,' eds. J.F. Liebman and A. Greenberg, VCH, New York, 1988, vol. 9, p. 86; and citations in refs. 3a-c.
4. A. Fersht, 'Enzyme Structure and Mechanism,' 2nd edn., W.H. Freeman, New York, 1985, p.405. C. Walsh, 'Enzymatic Reaction Mechanisms,' W.H. Freeman, San Francisco, 1979, p.53.
5. Molecular modelling using molecular mechanics and the COSMIC force field (J.G. Vinter, A. Davis, and M.R. Saunders, *J. Comput.-Aided Mol. Design*, 1987, **1**, 31) indicated the imidazole and hydroxy-group to be within *ca.* 5Å of each other in one of the minimum energy conformations. Based on a 'charge-relay' type mechanism for ester deacylation (ref. 3), a low energy intermediate was indicated for 4-nitrophenyl acetate and decanoate as substrates; extension and alignment of the decanoate alkyl chain with the apolar side-chains of the models was observed.
6. Elaboration of the benzene ring would allow future incorporation into polystyrene-like structures.
7. S. Hünig and W. Lendle, *Chem. Ber.*, 1960, **93**, 913.
8. All new compounds gave spectral data (IR, UV, NMR, MS) in accord with the assigned structure, and satisfactory combustion analysis or accurate mass measurement.
9. H. Wetter and K. Oertle, *Tetrahedron Lett.*, 1985, **26**, 5515; direct reduction of acid **2** ($NaBH_4$, MeOH) gave low yields of 7-hydroxydecanoic acid, and various procedures for protection of the keto-group as a dimethyl acetal led merely to the methyl ester.
10. The low nucleophilicity of the amino-group of 2-nitroaniline is related to its low basicity (pK_a -0.28), and allows the ethanol released from the mixed anhydride on coupling to compete for remaining mixed anhydride; attempts to remove the ethanol were unsuccessful. Coupling of keto-acid **2** with 2-nitro-aniline directly gave low yields.
11. Amongst other sequences tried, assembly of the complete upper side-chain before linkage to benzene derivative **5** was thwarted by low yields in couplings of β-alanine benzyl ester to hydrazide **8** by the azide method, and to N-nonanoyl-L-histidine by the mixed anhydride technique. Conditions could not be established for coupling of N-benzyloxycarbonylhistidyl-β-alanine with amine **5**.
12. No separation of diastereoisomers was observed in **1a-c**.
13. R.A.W. Johnstone and M.E. Rose, *Tetrahedron*, 1979, **35**, 2169.
14. Application of the mixed anhydride protocol that had been suitable for the preparation of amide **6**, gave only the ethyl carbamate of amine **10**, indicating that the mixed anhydride of N-benzyloxycarbonyl-β-alanine was attacked at the carbonate carbonyl in this case.
15. 'Normal' addition gave considerable quantities of 2-decanoylcyclohexanone, the Claisen condensation product of methyl 7-oxohexadecanoate.
16. See ref. 3b for a critique of the selection of 4-nitrophenyl esters as artificially reactive substrates to use when modelling systems that are primarily amidases; nevertheless their rapid reaction times and the convenience of monitoring the reactions favours their continued use as first generation test 'substrates'.
17. E.S. Swinbourne, *J. Chem. Soc.*, 1960, 2371.
18. Lack of 'turnover' has been observed in many serine protease mimics (ref. 16). Purification of the reaction products has so far proved elusive.