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NOVEL INHIBITORS OF ENKEPHALIN-DEGRADING ENZYMES IV: STRUCTURE-ACTIVITY RELATIONSHIPS WITHIN THE PENICILLINS AS ENKEPHALINASE INHIBITORS

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A range of penicillins have been examined as competitive reversible inhibitors of enkephalinase (neutral endopeptidase; EC 3.4.24.11). Carfecillin ($K_i = 0.18\ \mu M$) was the most potent inhibitor in the series, whereas cloxacillin (27.5 μM), ampicillin (41.0 μM), nafcillin (58.7 μM) and carbenicillin (158 μM) had moderate potency and benzyl penicillin (885 μM), mezlocillin (473 μM) and azlocillin (556 μM) were weak inhibitors. Structure-activity relationships within the series have been rationalised from a consideration of molecular graphics analysis of the match between receptor binding groups with thiorphan as well as log P values.

KEY WORDS: Enkephalinase, neutral endopeptidase, penicillins.

INTRODUCTION

Enkephalins are endogenous pentapeptidases and act as neurotransmitters in the central nervous system by stimulating opioid receptors. Rapid termination of the enkephalinergic signal occurs by enzymic degradation and the effects of the enkephalins are weak and transient.[1,2] At the synapse, aminopeitidase M and enkephalinase (neutral endopeptidase, EC 3.4.2.11) are considered to account for 90% of the degradation,[3] with enkephalinase playing the dominant role.[4–8]

Enkephalinase inhibitors have been developed as potential antinociceptive agents[9–12] by preserving the effects of the enkephalins in the brain and, more recently, as potential anti-hypertensive agents[13] by preserving the naturally occurring atrial naturetic factors (ANF) from the enzyme's degradative process.[14,15] In a previous communication[16] we reported that certain penicillins, from a small number examined, were moderately potent inhibitors of enkephalinase and exhibited proantinociceptive properties. Here, we have extended this study to a much wider range of penicillins and examined the structure-activity relationships within the series.

MATERIALS

The penicillins were obtained as sodium salts: ampicillin, nafcillin and oxacillin from Sigma; carbenicillin, carfecillin and cloxacillin from Beecham; mezlocillin and

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azlocillin from Bayer; benzyl penicillin from Glaxo. 6-Aminopenicillanic acid (as free acid) was obtained from Sigma. [Leu]-enkephalin was obtained from Sigma, (^{3}H-tyrosyl)-[Leu]-enkephalin (48.5 Ci, mol^{-1}) from Amersham International and Cocktail T from BDH. TLC plates (plastic, silica gel 60 Merck 5748) were obtained from BDH (U.K.).

METHODS

"Particulate fraction". A procedure based on the technique used by Hudgin *et al.*[17] as adapted from the original method of Malfroy *et al.*[5] was employed as previously described.[16]

Enkephalinase

50 μl of "particulate fraction" was preincubated in a shaking water bath for 15 min at 25°C with puromycin (0.1 mM), captopril (1 μM) and putative enkephalinase inhibitors at appropriate concentrations each added in 10 μl volumes of 50 mM Tris HCl buffer (pH = 7.4).

Incubations were started by the addition of ^{3}H-[Leu]-enkephalin (40 nM final concentration) added in 10 μl of buffer and a suitable concentration of unlabelled [Leu]-enkephalin in 10 μl of buffer (K_m determination) and 10 μl of untreated buffer (for K_m and IC_{50} determinations). This gave a final incubation volume of 100 μl.

Incubations lasted 15 min at 25°C and were terminated by placing the incubation tubes in a boiling water bath for 10 min. After termination of incubations 10 μl of a mixture of unlabelled [Leu]-enkephalin and its metabolites, Tyr, Tyr-Gly, Tyr-Gly-Gly was added (final concentration of each in 120 μl 0.1–0.5 mg/ml) to the incubation mixture.

50 μl of incubation mixture and suitable reference compounds were then applied in 10 μl aliquots to a TLC plate (plastic, silica gel 60 Merck 5748) and thoroughly dried. Plates were developed in ethyl acetate:propan-2-ol:water:acetic acid (40:40:19:1), full development taking approximately 2.5 h. After development the plates were removed and allowed to dry, sprayed with ninhydrin reagent (0.5% in acetone) and heated at 55°C for 15 min.

Spots corresponding to [Leu]-enkephalin ($R_f = 0.86$), Tyr ($R_f = 0.71$), Tyr-Gly ($R_f = 0.62$) and Tyr-Gly-Gly ($R_f = 0.51$) were cut out and placed in plastic scintillation vials. To each vial 1 ml of water was added followed by 15 ml of Cocktail T (BDH). The vials were sealed, well agitated, left to stand for 2 h and then placed in LKB 1217 Rackbeta liquid scintillation counter and the amount of tritiated compound associated with each vial calculated.

Inhibition studies

Non-enzymatic degradation of ^{3}H[Leu]-enkephalin during the assay procedure was accounted for by conducting the assay in the absence of the enzyme. This background count was deducted from each value obtained for the individual products in the

normal assay procedure to give corrected values. The percentage inhibition of the enzyme by inhibitors was given by the expression, [1-(DPM fragment/DPM total (test))/(DPM fragment/DPM total (control))] × 100, where the control value was obtained in the absence of inhibitor.

The Michaelis-Menten constant (K_m) was determined graphically from a Lineweaver-Burk plot[18] (not shown) where v = ((DPM (Product)/DPM (total)) × (S)/(t × P) (μM/min/mg), S = substrate concentration (μM), DPM = disintegrations per min, t = incubation time (min) and P = protein concentration[19] (mg/ml). A substrate concentration range of 5–80 μM was used where each concentration contained 40 nM labelled [Leu]-enkephalin and the remainder consisted of unlabelled substrate. Each value on the plot was the mean for three determinations.

The IC_{50} values were determined from plots (not shown) of % inhibition vs log inhibitor concentration using a single ^{3}H[Leu]-enkephalin substrate concentration (40 nM final concentration) with an inhibitor concentration range of 10 nm–100 μM. Each point on the plot represented the mean of 3 determinations. Since the substrate concentration used in the assay is in the nanomolar range and the K_m value for enkephalinase is also in this range then since $IC_{50} = K_i(1 + S/K_m)$[20] then the IC_{50} value and K_i value are practically equivalent.

Aminopeptidase

The assay of aminopeptidase activity in the particulate fraction was determined by the method previously described.[21]

Log P values

The partition in octanol-water of the penicillins was calculated using the MEDCHEM software supplied by Daylight Chemical Information Systems Inc., Claremont, USA. Experimentally determined values supplied by the software for certain penicillins are also quoted. The programme could not calculate data for azlocillin and mezlocillin. Log P values in propylene glycol dipelargonate (PGDP) were calculated for the 6-acyl amino group from fragment values.[22]

Molecular Graphics

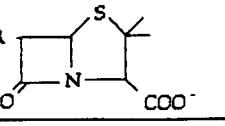
The Chem-X (Chemical Design Ltd., Oxford) software programme in conjunction with a VAX computer was used for the graphics display and calculations. Computer generated molecular models of the compounds considered here were obtained from a search of the Cambridge Crystallographic Data Base (via the Daresbury Chemical Data Bank Service) for the X-ray co-ordinates of suitable starting fragments. Final models were generated by modification of the starting fragments followed by the geometry optimisation and energy minimisation procedures available within the Chem-X Molecular Mechanics Suite. The minimisation procedure involved setting the 6-acyl amino substituent in the trans position with rotation of all other bonds by the programme to obtain a global energy minimum. This procedure was followed several times from different rotational positions to ensure that a local minimum was not obtained.

RESULTS AND DISCUSSION

A range of penicillins (see Table I for formulae) together with related compounds have been examined as inhibitors of enkephalinase (see Table II). Carfecillin ($K_i = 0.18\ \mu M$) was found to be the most potent compound in the series examined whereas cloxacillin (27.5 μM), ampicillin (41.0 μM), nafcillin (58.7 μM) and carbenicillin (158 μM) were of medium potency and benzyl penicillin (885 μM),

TABLE I
Structures of penicillins used in this study

GENERAL FORMULA



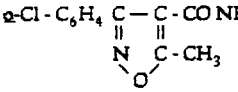
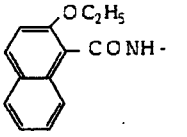
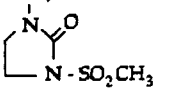
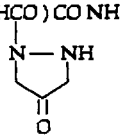
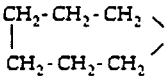
R	Compound
C_6H_5-CH(COOC_6H_5)CONH-	Carfecillin
o-Cl-C_6H_4 	Cloxacillin
C_6H_5-CH(NH_2)-CONH-	Ampicillin
	Nafcillin
C_6H_5CH(COONa)CONH-	Carbenicillin
C_6H_5-CH(NHCO)CONH- 	Mezlocillin
C_6H_5CH(NHCO)CONH- 	Azlocillin
$C_6H_5CH_2CONH$-	Benzyl Penicillin
C_6H_5-C(=N-C(=O)-O-)-CONH-	Oxacillin
HS-CH_2-CH($CH_2C_6H_5$)CONHCH_2COOH	Thiorphan
	Mecillinam

TABLE II
K_i and log P values for some penicillins and related compounds as enkephalinase inhibitors

Compound	K_i (μM)	Log P	
		PGDP***	Octanol*
Carfecillin	0.180 ± 0.021	+1.5	+3.1 (+2.96)
Cloxacillin	27.5 ± 2.4	+1.2	+2.0 (+2.43)
Ampicillin	41.0 ± 5.1	−2.5	−1.3
Nafcillin	58.7 ± 17	+1.8	+3.5
Carbenicillin	158.0**	−1.0	+1.6 (+1.13)
Mezlocillin	473.0 ± 14.5	very hydrophilic	N/D
Azlocillin	566.0 ± 44	very hydrophilic	N/D
Benzyl penicillin	885.0 ± 66	0	+1.7 (+1.83)
Oxacillin	ϕ	+0.2	+1.3 (+2.38)
Thiorphan	0.0048 ± 0.0012		+1.5
Mecillinam	ϕ		N/D
Penicilloic acid (ampicillin)**	inactive		−4.8
6-Aminopenicillanic acid	ϕ		−0.3

ϕ = Less than 50% inhibition at 1 mM concentration. * = Calculated using MEDCHEM programme (experimentally determined values given by programme are shown in parenthesis). ** = Data from ref. 16. N/D = not determined. *** = Calculated for R fragment from Leahy *et al.*[22]

mezlocillin (473 μM) and azlocillin (556 μM) were weak inhibitors. Oxacillin, mecillinam and 6-aminopenicilloic acid were practically inactive. The penicilloic acid of ampicillin has previously been found to be inactive.[16]

We have examined the enkephalinase inhibitory potency of the different penicillins with the different structural features present in the 6-acylamino side chains utilising molecular graphics and calculated log P values.

Molecular Graphics

Representative compounds were examined using the VAX/Chem-X software system.

Thiorphan is a potent inhibitor of enkephalinase and is considered to bind at the active site through a three point attachment:[23] (i) The terminal ionized carboxyl group forms an electrostatic link with a protonated amino (guanidine) group on the protein, (ii) the amide carbonyl oxygen atom forms a hydrogen bond with a hydrogen donor group on the protein and (iii) the thiol group ligands to the zinc ion. Using their minimum energy conformations, benzyl penicillin and certain other penicillins have been fitted to thiorphan through the carboxyl groups and amide carbonyl groups (using the β-lactam carbonyl group of the penicillins). Using this procedure it was found that the thiol group of thiorphan and the 6-acyl amino carbonyl oxygen atom of the penicillin were closely positioned. The molecular graphics programme was then run on a 'best-fit' basis for these matching functions in the two compounds. The distances between the matched pairs of atoms are shown in the bar graph (Figure 1).

The graph shows that there is an excellent fit between thiorphan and benzylpenicillin which is much superior to that seen for the other four penicillins where the overall fit decreases in the order ampicillin = carbenicillin > carfecillin > cloxacillin. This is not unexpected since substitution of additional groups, especially the bulky groups

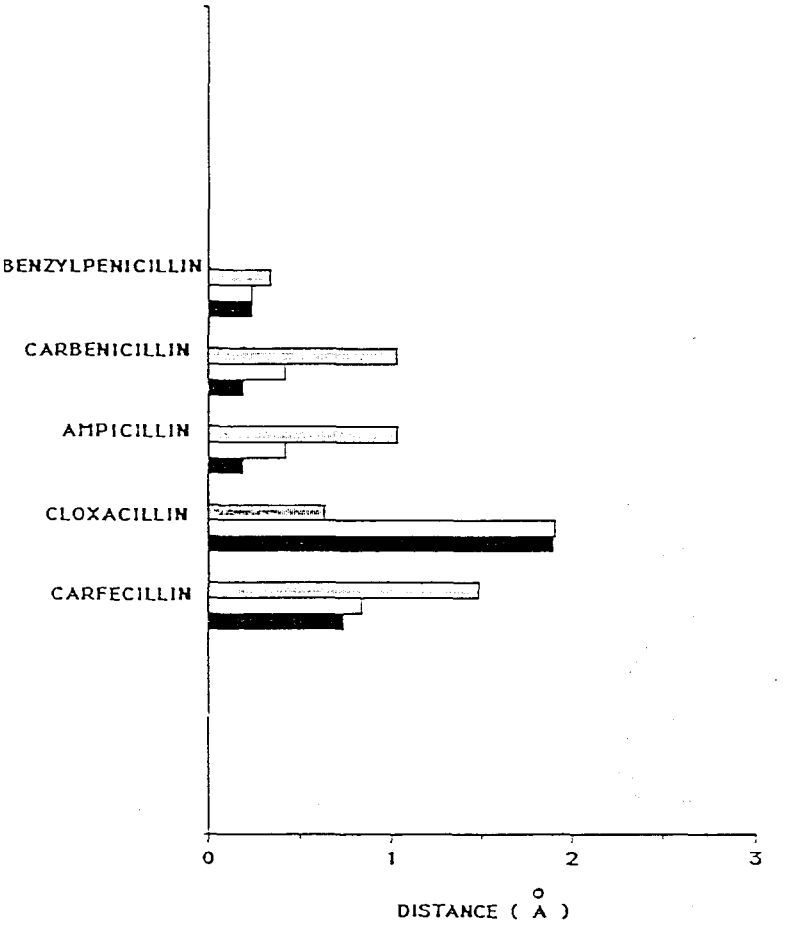


FIGURE 1 Distance (Å) between specific atoms in penicillins and thiorphan fitted in minimum energy conformations: ■—thiol sulphur atom of thiorphan and 6-acylamino oxygen atom of penicillin; □—amide carbonyl oxygen atom of thiorphan and β-lactam carbonyl oxygen atom of penicillin; ▨—average for terminal carboxylate oxygen atoms in thiorphan and penicillin.

present in carfecillin and cloxacillin will alter the conformation of the side chain sufficiently to affect the 6-acyl amino carbonyl oxygen-thiorphan sulphur distance. This resulting change will also increase all the other distances when an equal weighting is placed on the 'best-fit' calculation as the programme attempts to minimise all three distances under study.

In conclusion, the minimum energy conformation of benzyl penicillin, fits the enzyme active site better than the other penicillins studied with regard to the postulated three point attachment. However the other three compounds may fit the receptor equally well at the higher energy level since such an increase in energy could be offset by an overall decrease in energy on binding brought about by additional groupings present in the 6-acyl side chain binding to sites not available to benzyl penicillin.

Partition Coefficients

The partition in octanol-water of the penicillins under study was calculated. The penicillins contain a carboxylic acid and are ionised nearly completely at pH 7.4 where the inhibition studies were conducted. The log P however is calculated for the unionised molecule and whereas it does not reflect log P at physiological pH it provides a comparative parameter within the series and with thiorphan which is also ionised at physiological pH. The additional –COOH function present in carbenicillin and penicilloic acid will be similarly ionised. The amino group present in ampicillin (pKa, 7.2) and 6-aminopenicillanic acid (pKa, 4.9) will be present[24] in the base form to the extent of 61.3% and 99.7% respectively at pH 7.4.

The log P values in octanol-water of the penicillins and the 6-acyl group in PGDP are compared with the inhibitory potency of the compounds in Table II. Examination of the Table shows that there is not a clear relationship between K_i and log P in either the octanol or the PGDP system. The PGDP system is considered to resemble a hydrocarbon-water environment[22] more closely since proton acceptor groups such as esters, amides will be markedly more hydrophilic than in the octanol system. Although additional hydrophobic binding of the 6-acyl group in carfecillin, cloxacillin and nafcillin could account for their greater potency than benzyl penicillin, the potency of the more hydrophilic residues in ampicillin, carbenicillin, mezlocillin and azlocillin is unaccounted for.

It was considered as an alternative possibility that the greater potencies of ampicillin and carbenicillin over that of benzyl penicillin could be due to stronger binding to the zinc through the α-amino and α-carboxylate groups (present only in these penicillins within the series examined) rather than to weak binding through the carbonyl-oxygen atom. Matching of thiorphan with ampicillin and carbenicillin using these alternative binding sites gives the 'best fit' for all 3 binding groups involved as shown in the bar graph (Figure 2). The bar graph (Figure 2) shows that whereas the overall fit obtained for binding through the α-function is decreased compared with the original fit through the carbonyl oxygen atom it is still reasonable within the series and the discrepancy could be offset by stronger liganding to the zinc.

However, this argument cannot be applied to the strongly hydrophilic side chains in mezlocillin and azlocillin and an alternative explanation was sought. We consider that it is possible that these four hydrophilic 6-acyl side chains provide additional binding to the enzyme through their proton acceptor groups ($HN-\overset{\overset{O}{\|}}{C}-$, $-NH_2$, COO^-) with a common proton donor group on the enzyme; the potency of carfecillin ($-\overset{\overset{O}{\|}}{C}-O-$) could also be increased in this manner.

In summary, a good spacial match between the penicillins and the potent enkephalinase inhibitor thiorphan with regard to the functions predicted to bind at the active site of enkephalinase *viz.* COO^- (to HN^+), β-lactam C=O (to H-bond donor) and $-\overset{\overset{O}{\|}}{C}-NH-$ (to Zn^{++}) is insufficient to give a potent inhibitor. Potency is additionally determined by the hydrophobic nature of the 6-acyl amino side chain but introduction in the 6-acyl amino side chain of a suitably positioned proton acceptor group in hydrophilic penicillins can offset loss of hydrophobic character and increase potency.

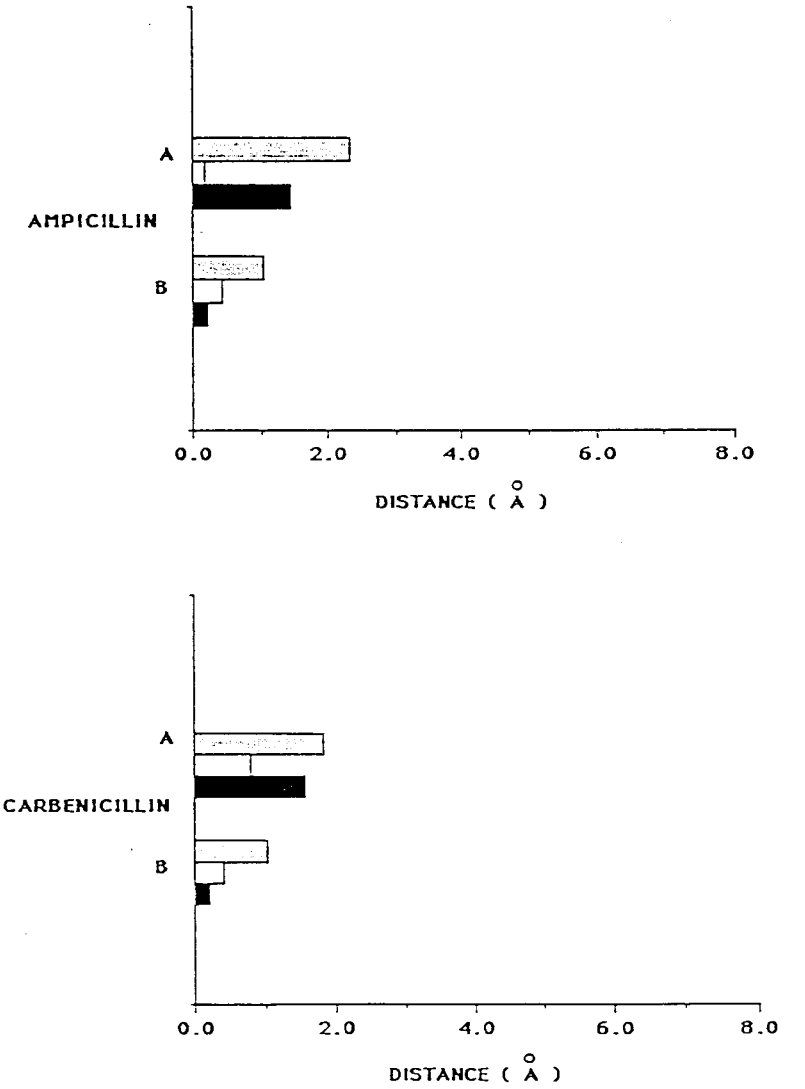


FIGURE 2 (A) As for Figure 1 except that: ■—thiol sulphur atom of thiorphan and α-amino (nitrogen) or α-carboxylate (average distance for oxygens). (B) Data from Figure 1 shown for comparison.

References

1. Meek, J.L., Yang, H.-Y.-T. and Costa, E. (1979) *Neuropharmacol.*, **16**, 151–154.
2. Stime, S.M., Yang, H.-Y.-T. and Costa, E. (1980) *Brain Res.*, **188**, 295–299.
3. Malfroy, B., Swerts, J. P., Guyon, A., Roques, B.P. and Schwartz, J.C. (1978) *Nature* (*Lond.*), **276**, 523–526.
4. De La Baume, S., Patey, G. and Schwartz, J.-C. (1981) *Neurosci.*, **6**, 315–321.
5. Malfroy, B., Swerts, J.P., Roques, B.P. and Schwartz, J.-C. (1978) *Nature* (*Lond.*), **276**, 523–526.

6. Schwartz, J.-C., Malfroy, B. and De La Baume, S. (1981) *Life Sci.*, **29**, 1715–1740.
7. Llorens, C., Malfroy, B., Schwartz, J.-C., Gacel, G., Roques, B.P., Roy, J., Morgat, J.L., Javoy-Agid, F. and Agid, Y. (1982) *J. Neurochem.*, **39**, 1081–1089.
8. Waksman, G., Hamel, E., Bouboutou, R., Besselievre, R., Fournie-Zaluski, M.-C. and Roques, B.P. (1984) *C.R. Acad. Sci. (Paris)*, **299**, 613–615.
9. Roques, B.P., Fournie-Zaluski, M.C., Soroca, E., Lecomte, J.M., Malfroy, B., LLorens, C. and Schwartz, J.-C. (1980) *Nature (Lond.)*, **288**, 286–288.
10. Lecomte, J.-M., Costentin, J., Vlaiculescu, A., Chaillet, P., Marcais-Collado, H., Llorens-Cortes, C., Leboyer, M. and Schwartz, J.-C. (1986) *J. Pharmacol. Exp. Ther.*, **237**, 937–944.
11. Fournie-Zaluski, M.C., Coulaud, A., Bouboutou, R., Chaillet, P., Devin, J., Waksman, G., Costentin, J. and Roques, B. (1985) *J. Med. Chem.*, **28**, 1158–1169.
12. Chipkin, R.E., Berger, J.G., Billard, W., Iorio, L.C., Chapman, R. and Barnett, A. (1988) *J. Pharmacol. Exp. Therap.*, **245**, 829–838.
13. Haslanger, M.F., Sybertz, E.J., Neustadt, B.R., Smith, E.M., Nechuta, T.L. and Berger, J. (1989) *J. Med. Chem.*, **32**, 739–742.
14. Stephenson, S.L. and Kenny, A.J. (1987) *Biochem. J.*, **243**, 183.
15. Kenny, A.J. and Stephenson, S.L. (1988) *FEBS Lett.*, **232**, 1.
16. Williams, P.S., Sewell, R.D.E., Smith, H.J. and Gonzalez, J.P. (1989) *J. Enz. Inhib.*, **3**, 91–101.
17. Hudgin, R.L., Charleson, S.E., Zimmerman, M., Mumford, R. and Wood, P.L. (1981) *Life Sci.*, **29**, 2593–2601.
18. Lineweaver, H. and Burke, D. (1934) *J. Amer. Chem. Soc.*, **56**, 658–666.
19. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.*, **193**, 265–275.
20. Cheng, Y.C. and Prusoff, W.H. (1973) *Biochem. Pharmacol.*, **22**, 3099–3108.
21. Yu, Z., Williams, P.S., Smith, H.J., Sewell, R.D.E., Patel, A. and Gonzalez, J.P. (1989) *J. Enz. Inhib.*, **3**, 103–117.
22. Leahy, D.E., Taylor, P.J. and Wait, A.R. (1989) *Quant. Struct.-Act. Relat.*, **8**, 17–31.
23. Roques, B.P., Fournie-Zaluski, M.C., Soraca, M.C., Lecomte, E., Malfroy, J.M., Llorens, B. and Schwartz, J.-C. (1980) *Nature (Lond.)*, **288**, 286–288.
24. Rapson, H.D.C. and Bird, A.E. (1963) *J. Pharm. Pharmacol.*, **15**, 222T.