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STEREOSPECIFICITY OF HYDROLYTIC ENZYMES IN THEIR REACTION WITH OPTICALLY ACTIVE ORGANOPHOSPHORUS COMPOUNDS—II

THE INHIBITION OF ALIESTERASE, ACETYLESTERASE, CHYMOTRYPSIN AND TRYPSIN BY S-ALKYL *p*-NITROPHENYL METHYLPHOSPHONOTHIOLATES

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Abstract—Rate constants of the irreversible inhibition of aliesterase, acetylesterase, chymotrypsin and trypsin by racemic and enantiomeric forms of some S-alkyl *p*-nitrophenyl methylphosphonothiolates were measured. Of the esterases investigated acetylesterase showed the most pronounced stereospecificity. The stereospecificity pattern corresponded with that reported previously for acetylcholinesterase. In the case of chymotrypsin an inversion of the stereospecificity throughout the enantiomeric series of inhibitors was observed.

IN A PREVIOUS paper[1] we described the stereospecificity exhibited by cholinesterases in their reaction with optically active S-alkyl *p*-nitrophenyl methylphosphonothiolates. We now wish to report the results obtained with other hydrolytic enzymes viz. horse liver aliesterase (carboxylic ester hydrolase, E.C.3.1.1.1), citrus acetylesterase (acetic ester acetyl-hydrolase, E.C.3.1.1.6), chymotrypsin (E.C.3.4.4.5) and trypsin (E.C.3.4.4.4).

Little is known about the stereospecificities of these enzymes for organophosphorus inhibitors. Michel[2] observed a biphasic reaction when chymotrypsin was inhibited with sarin (isopropyl methylphosphonofluoridate). Since the sarin molecule is asymmetric, he held the existence of enantiomeric forms responsible for the biphasic behaviour of the inhibition reaction. Repeating this experiment, using equimolecular concentrations of enzyme and inhibitor, Ooms and van Dijk measured a rate constant $2{\cdot}3 \times 10^4$ *l* mole^{-1} min^{-1} (pH 7·7, 25°) for the reaction with the more active enantiomer. The ratio of the rate constants of the optical isomers was calculated to be 7·7. Data concerning the stereospecific properties of chymotrypsin when inhibited with other phosphonofluoridates were given.[3]

The use of an inhibitor in its racemic form generally gives limited information about the stereospecific properties of the enzyme under consideration. When for instance the ratio of activity of the optical isomers is great the enzyme will be inhibited almost exclusively by the more active enantiomer. In that case the fore-mentioned calculation is not applicable. Therefore the investigation represented here was carried

out with enantiomeric forms of S-alkyl *p*-nitrophenyl methylphosphonothiolates. For comparison inhibition experiments with racemic mixtures were included whenever possible.

EXPERIMENTAL

Enzymes, assay methods and measurements of inhibition rates

Detailed descriptions of the aliesterase, acetylesterase, chymotrypsin and trypsin preparations, assay methods and measurements of the rates of inhibition have been published elsewhere.[4, 5, 3]

S-alkyl p-*nitrophenyl methylphosphonothiolates*

The same preparations as described previously[1] were used.

RESULTS AND DISCUSSION

Rate constants of the irreversible inhibition of the different enzymes by racemic and enantiomeric forms of S-alkyl *p*-nitrophenyl methylphosphonothiolates are collected in Table 1. The ratio of activity (r_a) of the enantiomers with Lp- and Dp-configuration respectively (compare[1]) serves as a measure for the exhibited stereospecificity.

Acetylesterase. As is shown in Table 1 acetylesterase shows the most pronounced stereospecificity of the enzymes investigated. The Lp-series contains the more active inhibitors; the r_a value given rises with increasing rate constant of the members of this series. In view of the relatively high rate constants the Lp-series rather than its enantiomeric series represents the complementarity of this type of organophosphorus inhibitors to the active center of the enzyme. The above-mentioned relationship between stereospecificity and specificity is visualized in Fig. 1 where the r_a value is plotted in logarithmic units versus the rate constant for the members of the Lp-series. Going down from the *n*-pentyl compound a steep decrease in log r_a value at the lower homologues of the series is observed. As is represented by the broken line it is to be expected that for a possibly existing homologous phosphonothiolate of which the Lp-isomer fits badly on the active center (and consequently shows a low rate constant) the log r_a value will approach zero. This means that the enzyme will not show any preference for one of the two enantiomorphs during inhibition.

In the inhibition experiments the enzyme was used in $5{\cdot}6 \times 10^{-10}$M concentration. Depending on their reactivities the racemic inhibitors were applied in concentrations varying from $10^{-6} - 10^{-7}$M. As a consequence of the high r_a values encountered rate constants for the racemic inhibitors were calculated substituting half of the molar concentration in the pseudo-first order kinetic formula.[4] As appears from Table 1 these rate constants almost equal the rate constants obtained for the corresponding faster reacting isomers. This is in agreement with the relation derived in the Appendix.

The equality of the rate constants for the racemic inhibitors and those of the corresponding Lp-isomers indicates that (a) the difference in activity between the enantiomers is not due to traces of a more active impurity present in the Lp-isomers (b) the less active isomer does not interfere in the inhibition by the more active enantiomer when the racemic mixture is administered to the enzyme.

Chymotrypsin. This enzyme behaves peculiar. For the smaller alkyl groups (R = Me and Et) the compounds with Dp-configuration turned out to be more active; with

TABLE 1. RATE CONSTANTS (l MOLE^{-1} MIN^{-1}) OF THE INHIBITION REACTION OF SOME ESTERASES BY RACEMIC AND ENANTIOMERIC FORMS OF S-ALKYL *p*-NITROPHENYL METHYLPHOSPHONOTHIOLATES, (RS)MeP(O)$OC_6H_4NO_2$, AT pH 7·7 AND 25°. RATIOS OF ACTIVITY (r_a) OF THE ENANTIOMERS ARE INCLUDED

R	Isomer	Aliesterase	r_a	Acetylesterase	r_a	Chymotrypsin	r_a	Trypsin	r_a
Me	(±)	$1·5 \times 10^6$	2·2	$2·6 \times 10^4$	8·9	$1·3 \times 10^4$	0·07	$8·3 \times 10^1$	0·66
	Dp(−)	$6·0 \times 10^5$		$2·8 \times 10^3$		$1·1 \times 10^4$		$7·3 \times 10^1$	
	Lp(+)	$1·3 \times 10^6$		$2·5 \times 10^4$		$7·9 \times 10^2$		$4·8 \times 10^1$	
Et	(±)	$1·4 \times 10^6$	0·15	$4·6 \times 10^4$	49	$4·8 \times 10^3$	0·29	$1·9 \times 10^2$	1·5
	Dp(+)	$1·7 \times 10^6$		$8·5 \times 10^2$		$1·0 \times 10^4$		$1·3 \times 10^2$	
	Lp(−)	$2·5 \times 10^5$		$4·2 \times 10^4$		$2·9 \times 10^3$		$2·0 \times 10^2$	
n-Pr	(±)	$1·2 \times 10^6$	0·52	$4·2 \times 10^4$	42	$2·0 \times 10^4$	2·1	$5·7 \times 10^2$	3·0
	Dp(+)	$2·1 \times 10^6$		$9·0 \times 10^2$		$1·5 \times 10^4$		$2·6 \times 10^2$	
	Lp(−)	$1·1 \times 10^6$		$3·8 \times 10^4$		$3·1 \times 10^4$		$7·8 \times 10^2$	
n-Bu	(±)	$1·5 \times 10^6$	0·72	$3·2 \times 10^5$	125	$5·9 \times 10^4$	2·8	$5·6 \times 10^2$	1·4
	Dp(+)	$1·8 \times 10^6$		$2·4 \times 10^3$		$4·0 \times 10^4$		$5·6 \times 10^2$	
	Lp(−)	$1·3 \times 10^6$		$3·0 \times 10^5$		$1·1 \times 10^5$		$7·8 \times 10^2$	
n-Pe	(±)	$2·2 \times 10^6$	0·21	$5·8 \times 10^5$	540	—	6·9	$1·1 \times 10^3$	2·7
	Dp(+)	$3·1 \times 10^6$		1.1×10^3		$5·5 \times 10^4$		$5·1 \times 10^2$	
	Lp(−)	$6·5 \times 10^5$		$5·9 \times 10^5$		$3·8 \times 10^5$		$1·4 \times 10^3$	

larger groups (R = *n*-Pr, *n*-Bu and *n*-Pe) the Lp-series contained the more potent inhibitors. This crossing of reactivities is illustrated in Fig. 2. A reasonable explanation of this phenomenon lies in all probability in the similar approach of all the compounds to the enzyme active center. When R is small it is possible that the radical RS fits better on a particular site of the enzyme surface than the relatively small methyl group directly bound to the phosphorus atom. When the size of R increases this site might show a preference for the smaller methyl radical.

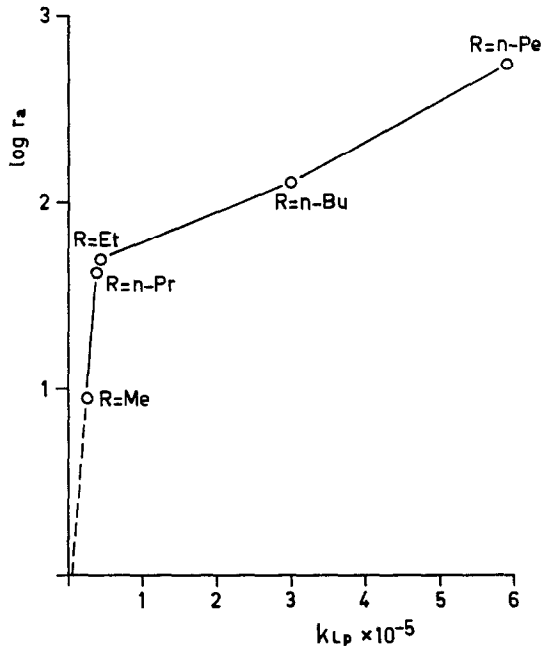

FIG. 1. Plot of the ratio of the rate constants of the enantiomers versus the rate constant obtained for the faster reacting isomer(l mole^{-1} min^{-1}) of some S-alkyl *p*-nitrophenyl methylphosphonothiolates, (RS)MeP(O)$OC_6H_4NO_2$, inhibiting acetylesterase at pH 7·7 and 25°.

Concerning the rate constants obtained for the racemic inhibitors (see Table 1) that of the methyl homologue was calculated using half of the inhibitor concentration because of the great difference in activity of the enantiomers. It corresponds fairly well with the rate constant obtained for the faster reacting isomer. The rate constant for the racemic mixture of the *n*-pentyl homologue could not be measured as a biphasic reaction was observed. This seems to be a consequence of the equality of enzyme and inhibitor concentration (6×10^{-6} M) used in addition to the difference in activity of the enantiomorphs (see Appendix). In the other cases where relatively small differences in activity between the enantiomers were observed the arithmic mean of the rate constants for the isomers was compared with the rate constant obtained for the racemic mixture. Only for the *n*-propyl homologue a satisfactory agreement was found.

Trypsin and aliesterase. Trypsin does not show a pronounced stereospecificity and aliesterase only slightly more as is represented in Table 1. The exhibited stereospecificities are opposite for these two enzymes. The results obtained for aliesterase show the

same crossing effect as was found for chymotrypsin but to a much smaller extent. In about half of the cases a satisfactory agreement between the arithmic mean of the rate constants of the enantiomers and those obtained for the racemic mixtures was found.

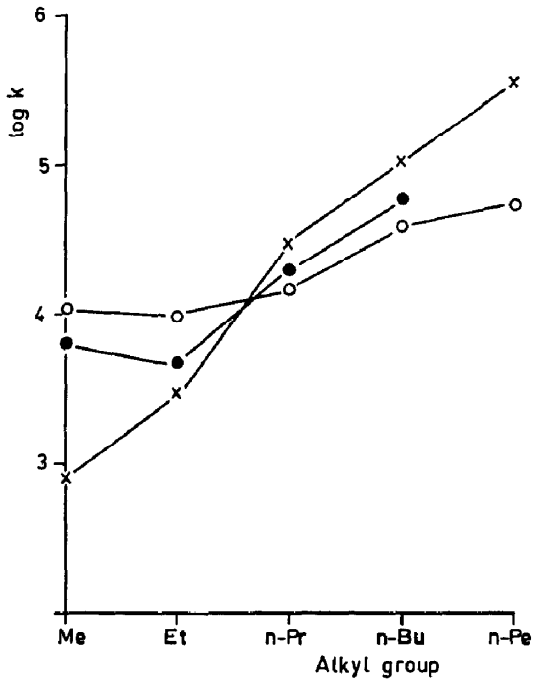

FIG. 2. The inhibition of chymotrypsin by racemic and enantiomeric forms of S-alkyl *p*-nitrophenyl methylphosphonothiolates at pH 7·7 and 25°. ●—Rac. series; ○—Dp-series; ×—Lp-series.

APPENDIX

If an enzyme is inhibited irreversibly by a racemic inhibitor, the following reactions occur simultaneously

$$E + I_1 \xrightarrow{k_1} EI_1$$

$$E + I_2 \xrightarrow{k_2} EI_2$$

where I_1 and I_2 represent the enantiomeric forms of the inhibitor present with equal initial concentrations and symbols EI_1 and EI_2 stand for the enzyme inhibited by the optical isomers. During the inhibition experiment the rate of disappearance of E is measured. The over-all reaction may be expressed by combination of the rates of inhibition by the isomers[3]

$$\frac{dx}{dt} = k_1 (c - x) \{(a - x_1) + r_a(a - x_2)\} \quad (1)$$

where c and a represent the initial concentrations of the enzyme and the optical isomers respectively, x_1 and x_2 the concentrations of the isomers that has reacted in time t and x the enzyme concentration inhibited in the same time. The ratio of activity (r_a) of the enantiomers is defined as k_2/k_1. The following possibilities may be considered:
(1) $r_a = 1$ in that case we have: $x_1 = x_2 = 1/2x$. Substitution in (1) gives

$$\frac{dx}{dt} = k_1(c - x)\,(2a - x)$$

or

$$k_r = k_1 = k_2$$

where k_r is the rate constant obtained for the racemic inhibitor.
(2) $r_a \gg 1$ This implies: $x_1 = 0$ and $x_2 = x$. Insertion in (1) results in

$$\frac{dx}{dt} = k_1\,(c - x)\,\{a(r_a + 1) - r_a.x\}. \tag{2}$$

Since r_a is great, $(r_a + 1)$ approximately equals r_a and (2) simplifies to

$$\frac{dx}{dt} = r_a.k_1\,(c - x)\,(a - x) \tag{3}$$

or

$$k_r = r_a.k_1 = k_2.$$

Equation (3) shows that in this case the initial concentration of the racemic inhibitor is given by a (and not $2a$) since the less active inhibitor does not play a role in the inhibition process.

(3) $r_a \sim 1$ in this case two possibilities must be considered:
(*a*) The racemic inhibitor is present in large excess. This implies: x_1 and $x_2 \ll a$. Equation (1) may be written as

$$\frac{dx}{dt} = k_1(c - x)\,\{a(r_a + 1)\} = 1/2(k_1 + k_2)\,(c - x).2a$$

or

$$k_r = 1/2\,(k_1 + k_2).$$

(b) The racemic inhibitor and the enzyme are present in comparable concentrations. In this case the following relation may be derived[3]

$$k_r = k_1.r_a + k_1\,(1 - r_a)\frac{a - x_1}{2a - x}. \tag{4}$$

Equation (4) indicates that no constant value for the rate constant of the racemic inhibitor will be obtained as it depends on the variable quantities x_1 and x.

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