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Inhibitors of α-chymotrypsin derived from cephalosporins: structure-activity relationships

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Cephalothin and certain other cephalosporins are irreversible inhibitors of α-chymotrypsin in near neutral media. The active inhibiting species is slowly generated in small yield from cephalothin in aqueous solution. Structure-activity studies for 37 other cephalosporins and related compounds establish that inhibitors of the enzyme require in the parent cephalosporin molecule a 7-acyl substituent containing a flat area in the form of an aromatic or heterocyclic ring together with a 3-acetoxymethyl or other substituent which is labile to water. The inhibitory species is considered to be the oxazolinone derived from the corresponding cephalosporoic acid.

After an initial finding that cephalothin irreversibly inhibited α-chymotrypsin, we have examined a wide range of cephalosporins to determine the structure-activity relationships within this group. Interpretation of these relationships in terms of the known topography of the enzyme (Steitz et al 1969; Birktoft et al 1970; Birktoft & Blow 1972; Segal et al 1972) could allow rational design of inhibitors incorporating certain essential features of the cephalosporin molecule for other proteases such as the PMN elastase-like neutral protease which is considered responsible for the lung disease emphysema (Janoff 1972 a,b).

MATERIALS AND METHODS

α-Chymotrypsin (BDH) from bovine pancreas which had been recrystallized four times was used. The cephalosporin analogues of cephalothin were free acids unless otherwise stated and were either obtained commercially or were research samples supplied by Glaxo Research Laboratories which have been described elsewhere and which were not further purified.

Different 7-acyl substituent: (1) Phenylacetamido-, (2) napthylcarboxamido-, (3) 3-*o*-chlorophenyl-5-methylisoxazole-5-carboxamido-, (4) 2-hydroxy-2-phenylacetamido-, (5) 2-methoximinofur-2-yl acetamido- (anti isomer), (6) pyridyl-2-acetamido, (7) benzylthioacetamido-, (Na salt), (8) 2-amino-2-phenylacetamido-, (cephaloglycin), (9) tetrazol-2-ylacetamido-, (10) tetrazol-l-ylacetamido-, (11) glutarimido-, (12) 2-hydroxyiminothienyl-2-acetamido-, (13) 2:2:3-trimethylcyclopent-3-enylacetamido-, (14) cyanoacetamido-(cephacetrile).

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Different 3-substituent: (15) cyclopropylcarbonyloxymethyl-, (16) 2:4-dinitrostyryl-, (17) morpholinocarbamoyloxymethyl-, (18) l-methyl tetrazol-5-yl thiomethyl-, (19) 5-methyl-1,3,4 thiadiazole-2-yl thiomethyl-, (20) l-oxo-pyrid-2-yl thiomethyl-, (21) hydroxymethyl-, (22) pyridinium-l-yl methyl-, (cephaloridine), (23) dimethyl thiocarbamoylthiomethyl-, (dicyclohexylamine salt) (24) carbamoyloxymethyl-.

Different 7-acyl and 3-substituents: (25) 7-oxamido-(dimer)-, 3(1-methyl tetrazol-5-yl thiomethyl)-, (26) 7-phenylacetamido-, 3-(azidomethyl)-, (27) 7(2-amino-2-phenylacetamido)-, 3-methyl, (cephalexin), (28) 7-benzyl-thioacetamido-, 3(dimethyl thiocarbamoylthiomethyl)-, (29) 7(2-hydroxy-2-phenylacetamido)-, 3(1-methyltetrazol-5-yl-thiomethyl)-, (cefamandole), (30) 7(2-amino-2-cyclohex-1′, 4′-dienylacetamido)-, 3-methyl-, (cepharidine), (31) 7-tetrazol-1-yl acetamido-, 3(5-methyl-1,3,4-thiadiazol-2-ylthiomethyl)-, (cefazoline Na), (32) 7-phenylacetamido-, 3(azidomethyl)-.

Miscellaneous compounds (33) 7-methoxycephalothin, (34) benzylpenicillin (Na) (35) 7-aminocephalosporanic acid, (36) cephalothin lactone, (37) benzyl penicillamine.

Assay procedure. The enzyme activity was assayed in a pH stat (Radiometer, Copenhagen) by the continuous titration method (Schwert et al 1948) using the substrate *N*-acetyl tyrosine ethyl ester at pH 7·4 and 25 °C, as previously described (Al Shabibi & Smith 1974). The sample (2 ml) from the inhibition reaction mixture was added to the jacketed assay vessel.

General inhibition procedure. A fresh solution of the cephalosporin (15 mg) in potassium dihydrogen phosphate buffer (5 ml, 0·002M) at pH 6·5 was mixed with a solution of α-chymotrypsin (5 ml, 8 × 10^{-6}M) The mixture was maintained at 25°C and initially and at suitable intervals of time, an aliquot (1 ml) was diluted to 10 ml with water and an aliquot (2 ml) of the resulting mixture was assayed for enzyme activity.

There was a progressive decrease in activity of the enzyme indicative of irreversible inhibition (Fig 1). No residual enzyme activity was observed after 24 h.

Dialysis of a solution of the inhibited enzyme against water (pH 65·) at 4°C gave an inactive enzyme after 72 h dialysis. Native enzyme under these conditions retained 57% of its activity.

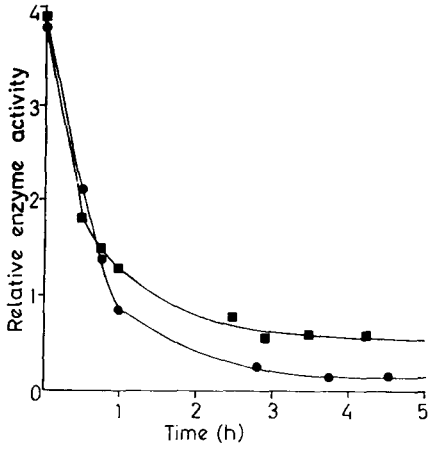


FIG. 1. Inhibition of α-chymotrypsin by cephalothin expt (a)—(●); expt (b)—(■).

Formation of inhibiting species from cephalothin. A solution of cephalothin (9·5 mg) in potassium dihydrogen phosphate buffer (25 ml, 0·002 M) pH 6·5, was incubated at pH 6·5 and 25°C and initially and at suitable intervals of time, an aliquot (2·5 ml) was taken and mixed with a solution of chymotrypsin (0·25 ml, 8 × 10^{-6} M) and buffer (2·25 ml) for 10 min at 25°C and then an aliquot was assayed in the usual manner. The inhibiting species was progressively formed from cephalothin in aqueous solution (Fig. 1).

Spectrophotometric titration of α-chymotrypsin with the inhibitory species. A solution of cephalothin (0·6 M) in phosphate buffer (0·02 M), pH 6·5 was stored overnight (20°C) and then an aliquot (1 ml) diluted with phosphate buffer (99 ml) pH 7·4 and the mixture left for 1·5 h to re-equilibrate. An aliquot (2·8 ml) was placed in a cuvette (1 cm) and its absorbance measured at λ 345 nm on a recording spectrophotometer with chart-speed 2 cm min^{-1}. A fresh solution (0·2 ml) of α-chymotrypsin (1·6 × 10^{-3} M) was then added to the cuvette. The immediate decrease in absorbance observed, ΔA, was calculated from the relationship, ΔA = 0·93A′-A″, where 0·93 is the dilution factor and A′ and A″ are the initial and final absorbances respectively. The ΔA values observed with different enzyme concentrations increased in a linear manner to a limiting value (A) (end point) where the enzyme was in excess (Fig. 2).

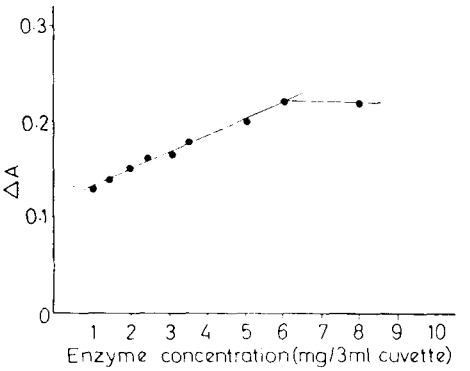


FIG. 2. Titration of inhibitory species in a stored solution of cephalothin by α-chymotrypsin.

Aliquots from a stored solution of cephalothin were taken immediately and at predetermined intervals and titrated with excess α-chymotrypsin solution. The inhibitory species concentration was maximally developed in the solution within 24 h and then slowly decreased in accord with deterioration of the cephalothin (vide infra).

Mass spectra of stored cephalothin solution. An aliquot (A) of a stored solution of cephalothin (1·8 × 10^{-3} M) in ammonium acetate buffer (0·001 M), was lypophilized whilst another aliquot (B) was reacted with excess α-chymotrypsin to remove the inhibitory species and then lyophilized. Both samples in a methanol–water mixture, were examined by mass spectrometry (field desorption) on a Varian 311A machine. At pyrolysis temperature of the emitter (wire current 25 mA), (A) gave a base peak ion at *m/e* 359 (M + 1) with a minor ion (30%) at *m/e* 360. These ions were absent in (B) under these conditions.

Analogues of cephalothin. A wide range of cephalosporins which differed from cephalothin either in the structure of the 7-acyl group and/or the 3-substituent

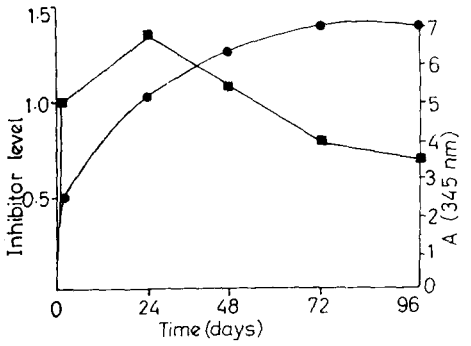


FIG. 3. Development of yellow colour (λ 345 nm) (●) and level of inhibitory species (■) in a stored cephalothin (M) solution. The inhibitor level is expressed as chymotrypsin (mg/3 ml cuvette) required for spectrophotometric titration.

were screened as inhibitors of α-chymotrypsin using the general inhibition procedure. The cephalosporins showing inhibitory activity (see Table 1) were examined in greater detail for, (a) their ability to inhibit the enzyme when fresh solutions were mixed with the enzyme and stored, and (b) the rate of formation of the inhibitory species on storage of the cephalosporin solution. Compounds (7), and (24) showed high inhibitory activity in the screening test but were not further examined. Compounds (16), (17) and (18) had low solubility and were studied in (a) as saturated solutions of known concentrations.

Table 1. Activity of cephalosporins relative to cephalothin as inhibitors of α-chymotrypsin.

Cephalosporin	Expt (a)*	Expt (b)*	(a)/(b)
1	1·25	0·38	3·3
2	1·31	1·2	1·09
3	0·83	Nil	—
4	0·62	0·48	1·29
5	0·52	Nil	—
6	0·19	Nil	—
15	0·96	—	—
16	1·36	0·048	28·4
17	0·95	0·74	1·29
18	0·55	0·38	1·45

* See text for details.

The results for (a) and (b) were expressed as the ratio of the amount of enzyme preparation inhibited by the cephalosporin to that inhibited by cephalothin during a 4 h period (calculated from graph).

The ratio of the values obtained in experiments (a) and (b) for each cephalosporin are shown after allowance for the different cephalosporin concentrations used. The ratio for cephalothin was 1·1 : 1 and was shown to be independent of the difference in concentration used in (a) and (b). Fig. 1 shows the almost equal extent of the inhibition in both experiments where the cephalothin concentration was the same.

DISCUSSION

A fresh solution of cephalothin when mixed with α-chymotrypsin slowly inhibited the enzyme in an irreversible manner to give an inactive modified enzyme. Storage of a solution of cephalothin alone for several hours in buffer pH 6·5 at 25°C gave a solution which was capable of very rapidly achieving a high degree of inhibition of the enzyme on admixture, indicating that an inhibitory species was produced from cephalothin in aqueous solution. Storage of a cephalothin solution with periodic titration of the level of the inhibitor using α-chymotrypsin showed that the level of the species increased with time (Fig. 1). The amount of enzyme inhibited by cephalothin when the enzyme and cephalothin were left together for 4 h and the amount of enzyme titrated by a solution of cephalothin stored for the same period, were similar. These results showed that the inhibition reaction could be mainly accounted for by the action of the species generated from cephalothin and that this species was fairly stable in aqueous solution. Thus, the apparent time-course for the inhibition reaction occurring between the enzyme and a freshly prepared solution of cephalothin was actually the time-course for the production of the inhibiting species from cephalothin.

An alternative means of detecting the inhibitory species was by spectrophotometric observation of its reaction with enzyme. Storage of a solution of cephalothin was accompanied by a gradual increase in absorption at λ 345 nm associated with development of a yellow colour in the solution. Reaction between excess enzyme and the inhibitor was accompanied by an immediate decrease in this absorption to a value which was constant for over an hour. The decrease in absorption was linearly related to the enzyme concentration and reached a limiting value (end point) when excess enzyme was present (Fig. 2). This permitted an approximate calculation of the inhibitor concentration on the assumption that the reaction occurred in the stoichiometric ratio 1:1. The inhibitory species in a stored buffered solution was shown using this method to increase to a maximum level of about 1·3% of the original cephalothin titre after 24 h, and then decrease to about half this level after 4 days, as the cephalothin titre was depleted due to hydrolysis to other products.

(Hamilton-Miller et al 1970; Newton et al (1968). These results are in accord with the inhibitory species being a break-down product of the cephalosporin and having a limited existance.

Many compounds are known which are active site titrants (or potential titrants) of α- (or δ-) chymotrypsin: cinnamoyl imidazole (Schonbaum et al 1961), diphenylcarbamoyl chloride (Erlanger et al 1966), *N*-acetylbenzotriazole (Reboud-Ravausc 1976), 2-hydroxy-5-nitro-4-toluenesulphonic acid sultone (Heidema & Kaiser 1968), esters of *N*-acetyl-*N*-benzylcarbazate (Elmore & Smyth 1968; Barker et al 1974) 2-pheny-4,4-dimethyl-oxazolin-5-one (PDMO) (de Jersey et al 1966), and 4-cis-benzylidene-2-phenyl-Δ^2-oxazolin-5-one (Brocklehurst & Williamson 1974), since they rapidly and quantitatively form a relatively stable acyl-enzyme with a change in absorption in the region λ 300–350 nm. In this work, the spectrophotometric changes were also interpretable in terms of the formation of a stable acyl-enzyme.

Structure-activity relationships for the inhibition reaction

Thirty two other cephalosporins, as well as 7-aminocephalosporanic acid, cephalothin lactone, 7-α-methoxycephalothin, benzylpenicillin and benzyl penicillamine were also studied as inhibitors of the enzyme. Twelve of the cephalosporins studied were inhibitors of the enzyme.

Replacement of the 7-acyl function of cephalothin with a variety of other side chains, led to loss of inhibitory potency in 7 of the 14 compounds tested when fresh solutions of the cephalosporin were mixed and stored with α-chymotrypsin. The inhibitory potency of these compounds is shown in Table 1 (a) relative to cephalothin (= 1). The active inhibitors all possessed a 7-acyl substituent which contained a large flat area in the form of an aryl and/or heterocyclic ring structure.

Buffered solutions of the active inhibitors were stored alone and the level of the active species determined over several hours by titration with the enzyme. This level was compared with the level of the inhibitory species obtained for cephalothin (= 1) (Table 1(b)). Comparison of the ratio of the values for (a) and (b) shows that the more active inhibitors fall into two groups: cephalothin, (2) and (4) where the inhibitory action can be mainly attributed to the production of an active intermediate and (1), (3), (5) and (6) where their mode of action is not clear.

The inhibition noted in (a) for the compounds which do not appear to produce an inhibitory species, does not occur through the intact cephalosporin molecule, since the amount of enzyme preparation inhibited by compound (3) during a 4 h incubation period was unaffected by a ten-fold increase in the enzyme concentration (c.f. Beddoe & Smith 1971; Powers & Tuhy 1973). A likely explanation is that an active intermediate related in structure to that produced from cephalothin is produced in aqueous solution from these compounds but is unstable; the inhibition noted in (a) being due to 'scavenging' by the enzyme which reacts rapidly with the inhibitor before it is destroyed.

Ten cephalosporins were studied where the 3-acetoxymethyl substituent of the 3-cephem-4-carboxylic acid nucleus of cephalothin was replaced by other substituents. The cephalosporins (15), (16), (17), were comparable in inhibitory potency to cephalothin whereas (18) was less potent, (Table 1 (a)). The level of the inhibitory species formed in a stored solution of cephalothin, (17) and (18) mainly accounted for the inhibition noted when mixtures of fresh solutions of the cephalosporins and chymotrypsin were stored.

The cephalosporins, with the exception of (16), which were found to be inhibitors in this work possess a potentially water-labile 3-substituent, either an ester, urethane or suitable substituted tetrazole (isostere of acetoxy) group. It would seem that the presence of this function is a pre-requisite for decomposition of the cephalosporin to produce an inhibitory species. (16) which contains the unique 3-(2,4-dinitrostyryl) substituent, which is not a water-labile function, does not conform to the above pattern although it was shown that it did not react as the intact molecule.

Proposed structure for the inhibitory species from cephalothin

Any structure proposed for the active species formed from cephalothin should be capable of being formed in aqueous media by an acceptable mechanistic pathway and contain an electrophilic carbon centre to form a covalent bond with the enzyme. Suggested structures for the inhibitory species described in this work are the unsaturated oxazolinone (39) or its isomer (40). The alternative thiazine structures (41) and (42) are considered unlikely (see later).

Oxazolinones have been implicated from kinetic measurements as degradation products of cephalosporins formed in near neutral media (Yamana & Tsuji 1976) and the oxazolinones, 2-phenyl-oxazolin-5-one (PO), PDMO, and 4-*cis*-and *trans*-benzylidene-2-

phenyl-Δ^2-oxazolin-5-one are known to be poor substrates of the enzyme and form acyl-enzymes with different degrees of stability (de Jersey et al 1966; Brocklehurst & Williamson 1974).

A mass spectrum derived by the field desorption technique on a lyophilized stored cephalothin solution showed a base peak at *m/e* 359 and a minor peak (30%) at *m/e* 360. Both peaks were absent from the scan of material where the inhibitor had previously been removed by spectrophotometric titration with α-chymotrypsin. The base peak could correspond to the (M + 1) ion of the oxazolinone (39) or (40) but the ion at *m/e* 360 is more difficult to explain.

The minimum E_{max} value calculated for the inhibitor is 4×10^3, assuming that the acyl-enzyme or products do not absorb and the stoichiometry of the reaction is 1:1. This minimum value supports either (39) or (40) since unsaturated oxazolinones show strong absorption in this region (e.g. methyl penicillenate λ_{max} 317nm (ε,20,600): (Cornforth 1949).

Several oxazolinones (loc. cit.) form an acyl enzyme with α-chymotrypsin by reaction between Ser-195 at the active site and the ring carbonyl function. Unsaturated oxazolinones, but not penicillenic acid (43), react with nucleophiles at the β-carbon of the α, β-unsaturated carbonyl moiety, either by addition across the α, β-bond or by substitution at the β-carbon with loss of a substituent (Longridge & Timms 1971a). An alternative mechanism for reaction of (39) or (40) with the enzyme would be reaction at the β-carbon by a nucleophile at the active site of the enzyme. This reaction is considered unlikely since there would be a high energy barrier to either an addition or substitution reaction (with loss of the nitrogen-containing side chain) due to the accompanying decrease in the resonance energy in the extended conjugated system present in (39) or (40). A similar decrease does not occur on formation of an acyl enzyme and this reaction could be expected to be the more facile.

The structure-activity relationships existing within the cephalosporins studied as inhibitors of α-chymotrypsin are also explicable in terms of an oxazolinone structure.

The large flat aromatic ring present in the 7-acyl substituent of the parent cephalosporin molecule could fit into the hydrophobic binding cavity occupied by the aromatic ring of the natural substrates (loc. cit).

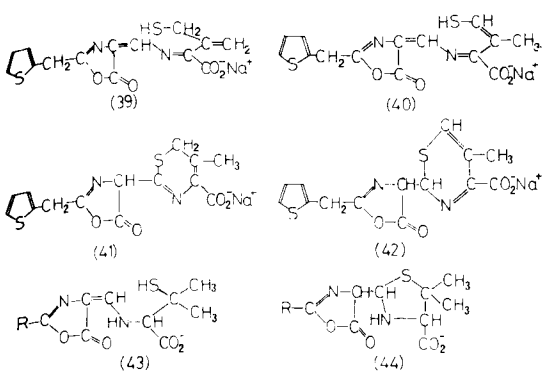
Cephalothin and other 3-acetoxymethyl cephalosporins are slowly hydrolysed in near neutral solution with opening of the β-lactam ring by direct attack by water, and also by spontaneous degradation caused by intramolecular participation of the neighbouring side chain amido group with formation of kinetically-observable oxazolinones (Yamana & Tsuji 1976). Ring opening is associated with concommitant expulsion of the 3-acetoxy or other leaving group and formation of a derivative of 3,10-dehydrodeacetoxy-Δ^4-cephalosporate which rapidly degrades (Hamilton-Miller et al 1970).

The oxazolinones derived from cephalosporins are analogous to the penicillenic acids formed from penicillins and would be expected to be formed by a similar mechanistic pathway so that an electron-withdrawing group would decrease oxazolinone formation and an electron donating group would enhance it (Nayler 1973; Doyle & Nayler 1964). The cephalosporins with electron-withdrawing side chains, (3), (4), (5) and (6), give a lower yield of the inhibitory species in accord with decreased oxazolinone formation. However, the differences in stability of the inhibitory species formed from the different cephalosporins (see Table 1(b)) is difficult to explain in terms of the stability of the corresponding oxazolinone structure.

The carbonium ion formed from the 3-acetoxymethyl side chain (Taylor 1965) would be expected to enhance the activity of the β-lactam carbonyl function in near neutral media and facilitate oxazolinone formation much more effectively than other 3-substituents (c.f. Indelicato et al 1974). Other cephalosporins with a suitable 7-acyl substituent also require for production of the oxazolinone inhibitor a water-labile 3-substituent to generate a carbonium ion for this purpose.

7-Methoxycephalothin (33) is not an inhibitor of α-chymotrypsin. The proposed intermediate (39) or (40) could not be formed from this cephalosporin which may account for its inactivity provided the 7-methoxy function does not affect the steric or electronic environment of the β-lactam carbonyl function so modifying oxazolinone formation. Using the converse argument, structures (41) and (42) are not favoured due to the inactivity exhibited by (33).

The stability of the intermediate in aqueous solution requires some comment since penicillenic acid (43), although an unsaturated oxazolinone, is hydrolysed at pH 7 to penicilloic acid in a matter of minutes (Longridge & Timms 1971b). This behaviour is attributed to the free thiol group in the side chain which can bring about an intramolecular nucleophilic attack to give (44), thus removing the conjugated double bond and reducing the stability of the ring which can then be attacked by the nucleophile. The thiazines (41) and (42) are also saturated oxa-



zolinones and would also be expected to be rapidly hydrolysed in aqueous media.

The proposed structure (39) or its tautomer (40) would be less likely than penicillenic acid to form an unstable saturated oxazolinone by ring closure to the corresponding thiazine since, (1) the complex stereochemistry of the unsaturated side chain places severe restrictions on the positioning of the thiol group with respect to the β-carbon of the unsaturated bond, (2) the reaction would be less facile due to the greater decrease in resonance energy in the transition state and products due to loss of the extended conjugation present in (39) or (40).

Attempts to isolate the inhibitory species derived from cephalothin have been unsuccessful as also have preliminary attempts to isolate the oxazolinone (39) from the product obtained by reaction of the corresponding cephalosporoic acid with acetic anhydride.

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