SPECIFIC IRREVERSIBLE ENZYME INHIBITORS

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Introduction

The study of enzyme inhibition has its roots in two lines of scientific pursuit. The first was the study of enzyme kinetics and was not directly concerned with pharmacology; the second was the study of the mechanism of action of pharmacologically active compounds at the molecular level of the enzyme. As the knowledge of molecular pharmacology and of enzyme systems widened, it became feasible to design, synthesize, and evaluate enzyme inhibitors with isolated enzyme systems.

Reversible versus irreversible inhibitors.—The concepts of reversible enzyme inhibition need no explanation here; substrate and inhibitor compete for the enzyme and the enzyme reaction is slowed an amount dependent upon the relative concentrations of substrate and inhibitor and their dissociation constants (K_m, K_i) when complexed to the enzyme.

An irreversible inhibitor no longer dissociates from the enzyme and the enzyme reaction is slowed an amount dependent upon only enzyme and inhibitor concentrations, but is independent of substrate concentration or K_m; thus one inhibitor molecule could theoretically slow to zero the action of one enzyme molecule. There are two types of irreversible inhibitors. The first type is so strongly complexed to the enzyme that it fails to dissociate from the enzyme under physiological conditions (1), but can be dissociated by dialysis or by chromatography; an example of such a "pseudo-irreversible" inhibitor is amethopterin (methotrexate) which "titrates" dihydrofolic reductase (1–3).

The second type of irreversible inhibition is due to the formation of a covalent bond between the enzyme and the inhibitor; if the new covalent bond stops conversion of substrate to product the enzyme has been inactivated irreversibly. There are two main types of these irreversible inhibitors that operate by formation of a covalent bond. The first type reacts with an essential functional group on the enzyme by a bimolecular process (Eq. 1); such a process has little specificity

$$E + I\text{–}X \xrightarrow{k_b} E\text{–}I + X^{\ominus} \qquad 1.$$

since all groups on the surface of all enzymes with the nucleophilic capacity to do so will react at varying rates depending on their rate constant, k_b (1, 4).

The second type of irreversible inhibition by covalent bond formation is expressed by Equation 2. The enzyme forms a reversible complex

$$E + I\text{–}X \underset{K_i}{\rightleftharpoons} E \cdots I\text{–}X \xrightarrow{k_d} E \cdots I + X^{\ominus} \qquad 2.$$

with the inhibitor which bears a leaving group, X. If a nucleophilic group on the enzyme of the leaving group, X, are closely juxtaposed within the reversible enzyme-inhibitor complex, then a rapid neighboring group reaction can occur within the complex; such formation of a covalent bond can be highly specific since properly positioned neighboring groups can react 10^3 to 10^4 more rapidly than the identical bimolecular reaction, that is, $k_d > 10^3 k_b$. This highly specific reaction within an enzyme-inhibitor reversible complex is called "active-site directed irreversible inhibition (1, 5)" or "affinity labeling (6, 7)". The concept of active-site directed irreversible inhibition has been stated as follows (1, 8, 9):

> The macromolecular enzyme has functional groups on its surface which logically could be attacked selectively in the tremendously accelerated neighboring group reactions capable of taking place within the reversible complex formed between the enzyme and an inhibitor substituted with a properly placed neighboring group.

Furthermore, active-site directed irreversible inhibitors have an extra dimension of specificity dependent on k_d that does not exist with reversible inhibitors (5, 10, 11); this is called the bridge principle of specificity.

There are two classes of active-site directed irreversible enzyme inhibitors. The first class operates by formation of a covalent bond within the active-site (*endo* mechanism) (1, 5); these *endo* types are sought primarily by protein-structure chemists who wish to label the active-site, but are quite limited for drug design. The second class form a covalent bond outside the active-site (*exo* mechanism) and have a much broader utility for drug design (1, 5).

The first example of an active-site directed irreversible inhibitor operating by the *endo* mechanism was diisopropyl fluorophosphate (DFP) (12), although the mechanism was not established until almost 20 years later (13). An early example of an irreversible antimetabolite operating by the *endo* mechanism was the L-glutamine antagonist, L-azaserine (14); the latter specifically alkylates a single cysteine in the active-site of the enzyme that converts formylglycineamide ribotide to its amidine (15). The first example of an irreversible inhibitor operating by the *exo* mechanism was the inactivation of glutamate dehydrogenase by 4-(iodoacetamido)salicylic acid (10, 16).

Dihydrofolate Reductase

More progress towards specific inhibition of this enzyme from diverse species and different tissues has been made than with any other enzyme sys-

tem; therefore inhibition of this enzyme will be considered first. The enzyme reduces dihydrofolate (*2*) to the pre-cofactor form tetrahydrofolate (*3*) using TPNH as the reducing agent. Some, but not all, dihydrofolate reductases can reduce folate (*1*) to tetrahydrofolate (*3*). The enzymes from species that synthesize dihydrofolate *de novo* apparently cannot use folate (*1*) as a substrate; in contrast mammals do not make dihydrofolate (*2*) *de novo* and therefore depend on an exogenous source of folate (*1*) which can be reduced to tetrahydrofolate (*3*) by their dihydrofolate reductases.

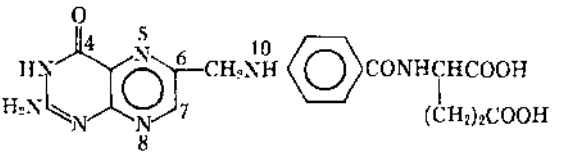
Types of inhibitors.—There are two major classes of inhibitors of dihydrofolate reductase (17–19). The first class are isosteric analogues of folic acid (*1*) where the 4-oxo group has been replaced by amino such as aminopterin (*4*) and amethopterin (methotrexate, 5); these isosteres are "pseudo-irreversible" inhibitors (1–3) of dihydrofolate reductase that show little specificity in inhibition of the enzyme from different species or different tissues (17, 19). Nevertheless, *5* is effective in treatment of about 20 per cent of childhood leukemias; the specificity of action resides in a more efficient uptake of *5* into susceptible leukemic cells (20, 21), presumably by the active transport system used for folic acid (*1*) (22).

The second major class of inhibitors of dihydrofolate reductase do not contain the *p*-aminobenzoyl-L-glutamate moiety of folic acid (*1*), but have instead a hydrocarbon-like moiety; examples are the antimalarials, pyrimethamine (Daraprim, *6*) and cycloguanyl (*7*), and the antibacterial agent, trimethoprim (*8*). The chlorophenyl moiety of *6* and *7* is complexed to a hydrophobic bonding region on the enzyme (17, 23, 24); this hydrophobic region is most probably not part of the active-site, but is adjacent to the area where the 4-oxo or 8-N of folate (*1*) resides on the enzyme (17, 25, 26). This type of inhibitor (*6* to *8*) without the ionized *p*-aminobenzoyl-L-glutamate moiety penetrates a cell wall by passive diffusion (17, 19, 22, 27).

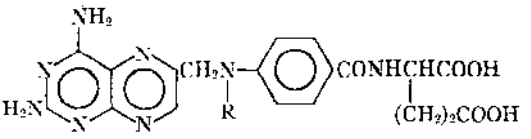
Trimethoprim (*8*) has a selective action towards bacteria, since the bacterial dihydrofolate reductases are complexed 50,000-fold better by *8* than are vertebrate enzymes (19, 28, 29); in fact *8* is complexed so tightly to bacterial enzymes that *8* is a "pseudo-irreversible" inhibitor with $K_i < 10^{-10}$. Similarly, pyrimethamine (*6*) binds 2000-fold better to a susceptible malarial dihydrofolate reductase than to a mammalian enzyme (30); again, *6* is a "pseudo-irreversible" inhibitor of the parasitic enzyme.

Hydrophobic bonding.—The evidence available supports the concept that the hydrophobic bonding region on dihydrofolate reductase is not part of the active-site, but is just adjacent to the active site; therefore one can anticipate that evolutionary changes should have occurred in this nonessential region of the protein structure (29, 31). Linear sequence studies on cytochrome *c* from various sources gave direct support to the theory that 70 per cent of a protein could have undergone evolutionary changes between species (32, 33) and still be functional.

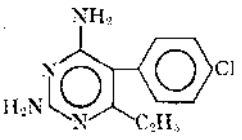
The 10^3 difference in binding of a *p*-chlorophenyl group of pyrimethamine (*6*) to dihydrofolate reductases from malaria and vertebrates are readily rationalized (29, 31) on this basis; similarly the 5×10^4 difference in binding of



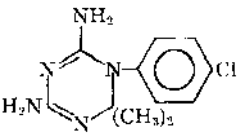
1, Folate
2, 7,8-H_2 = dihydrofolate
3, 5,6,7,8-H_4 = tetrahydrofolate



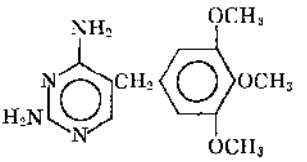
4, R = H, aminopterin
5, R = CH_3, amethopterin (methotrexate)



6, pyrimethamine

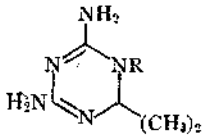


7, cycloguanyl

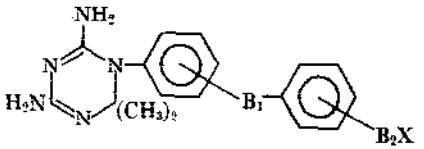


8, trimethoprim

trimethoprim (*8*) to bacterial and vertebrate enzymes could also be rationalized (29), providing some polar binding of a CH_3O group adjacent to the active site is also considered. Based on these hypotheses, Baker (29) compared this binding adjacent to the active-site of dihydrofolate reductase from *E. coli* B and the enzyme coded by T_2-phage when it infects *E. coli* (34, 35) by inhibition with 49 selected 5-substituted-2, 4-diaminopyrimidines and 1-substituted-4, 6-diamino-1, 2-dihydro-*s*-triazines. The parent 1-methyl-1, 2-dihydro-*s*-triazine (*9*) showed no difference in binding between the *E. coli* B enzyme and the T_2-phage coded enzyme (29); such a result would be expected if only complexing to the active-site had occurred. In contrast, trimethoprim (*8*) complexed 2300-fold stronger to the *E. coli* B enzyme than the T_2-phage coded enzyme, but only a 20-fold difference between the pigeon liver enzyme and the T_2-phage coded enzyme was observed (29); furthermore, the "inhibition profile" (19) with the 49 compounds was distinctly different between the three sources of enzyme—the paleontological consequences of which have been discussed (29).



9, R = CH_3
10, R = $(CH_2)_4C_6H_5$



11, B = bridge, X = leaving group

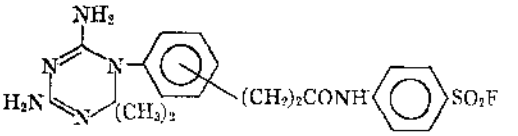
The T_2-phage coded enzyme can be considered as a model for a tumorigenic virus such as SV40 or polyoma virus which infect mammalian cells (36). Therefore a comparison was made of the hydrophobic bonding region of dihydrofolate reductase of the Walker 256 rat tumor compared to the enzyme from rat liver (37, 38)—even though it is not known whether this tumor was originally of viral origin. In a number of cases, 3- to 20-fold differences were observed, the largest difference being 100-fold with the 1-phenylbutyl-1, 2-dihydro-*s*-triazine (*10*); the 1-methyl derivative (*9*) again showed no difference. These differences are insufficient to enable us to expect chemotherapeutic advantages on the Walker 256 tumor, particularly when 10^3 to 10^4 differences are noted with *6* and *7* on infectious diseases. Therefore another parameter was introduced to achieve tumor specificity, as described in the next section.

An "inhibition profile" study with 63 diaminoheterocycles has also been done on the dihydrofolate reductase from *Trypanosoma equiperdum* (39); this profile was distinct from the profiles of the enzyme from rat liver, chicken *E. coli*, or *P. berghei*.

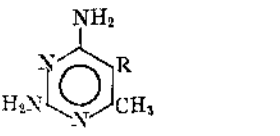
Active-site directed irreversible inhibitors.—It was predicted (40) that the greatest difference in irreversible inhibition of dihydrofolic reductases from normal and tumor tissue should occur if the group on the inhibitor that forms a covalent bond would be attached past the point where the inhibitor is com-

plexed to a hydrophobic region; in this way, any small differences in hydrophobic bonding would be greatly magnified by their influence on the juxtapositioning of the inhibitor's leaving group to a nucleophilic group on the enzyme surface. This premise was based on the bridge principle of specificity (11, 41).

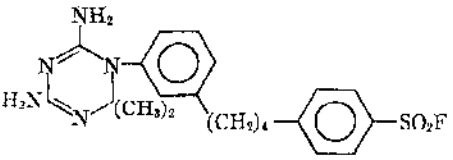
To use this modification of the bridge principle of specificity, it would be necessary to know where the hydrophobic bonding region on dihydrofolate reductase ends by emerging into a polar region. The answer to this question was achieved by synthesis and evaluation of inhibitors of type *11* (40, 42, 43); the hydrophobic bonding region ended at a B_1 bridge of 2 to 3 carbons, which then placed the benzene ring bearing B_2-X on a polar region of the



12, *meta* isomer
13, *para* isomer



14, R = $(CH_2)_3O$–NHCO–
15, R = $(CH_2)_3OC_6H_4NH_2$–*p*
16, R = $C_6H_3Cl_2$–3,4
17, R = $-(CH_2)_4C_6H_5$



17b

enzyme. The best B_2-X group found was SO_2-F as shown in *12* and *13*; *12* was an excellent reversible inhibitor of dihydrofolic reductases from pigeon liver, rat liver, Walker 256 rat tumor, and L1210 mouse leukemia with $K_i = 2-17 \times 10^{-9}$ M (44). However, of these four sources of enzyme, only the pigeon liver enzyme was irreversibly inhibited—the half-life being 8 min with *12* at 2×10^{-7} M.

When the side chain of *12* was moved to *para*-position, the resulting *13* showed little change in reversible inhibition; however, the irreversible inhibition was dramatically altered in that all vertebrate dihydrofolic reductases so far tested were inactivated with 0.05 to 0.10 μM of *13* with half-lives of less than 2 min; this included L1210 mouse leukemia, normal mouse tissues (liver, spleen, intestine), rat liver, Walker 256 rat tumor, and pigeon liver (44, 45).

Extensive synthetic and enzymatic studies on three classes of 2, 4-diaminoheterocyclics led to several compounds with the ability to inactivate L1210 mouse leukemia dihydrofolate reductase with no significant inactiva-

tion of the enzyme from mouse liver, spleen, or intestine (45); one such compound was *14* which had $K_i = 3 \times 10^{-9} M$, which at $5 \times 10^{-8} M$ gave complete inactivation of the L1210 enzyme with a half-life of 2 min (46).

Cell wall transport.—Although *13* and *14* showed similar inactivation of the L1210 enzyme, and in addition *14* showed no inactivation of the enzyme from normal tissues, only *13* showed *in vivo* activity giving a 40 to 70 per cent life extension of mice bearing L1210 (45). The failure of *14* to be effective *in vivo* was traced to slow penetration of the L1210 cell wall as estimated by kill of L1210 cell culture (47); *14* was 10^5 less effective than the 3, 4-dichlorophenylpyrimidine standard (*16*) in cell culture and *15* was 10^6 less effective than the standard (*16*). Similarly, the 5-phenylbutylpyrimidine (*17*) was 6000-fold less effective than the standard *16*; in contrast, the 1-phenylbutyl-1, 2-dihydro-*s*-triazine (*10*) was just as effective as the standard (*16*) in cell culture.

Whether or not the dihydro-*s*-triazine analogue of *14* will still show good transport and good selectivity awaits completion of its synthesis. Another structural type that gives good L1210 cell kill, but poor selectivity of inactivation is *17b* (48); whether *17b* can be modified for selectivity and still maintain good transport is under active investigation.

In vivo *activity.*—Introduction of a methyl group ortho to the SO_2F function of *13* gave *13a* which showed increased irreversible specificity towards the L1210 enzyme (102). Furthermore, *13a* showed good irreversible specificity in the rat towards the Walker 256 rat tumor enzyme. At 0.065μM, *13a* rapidly gave 100 per cent inactivation of the Walker 256 enzyme; under the same conditions *13a* gave 28, 27, and 7 per cent inactivation of the enzyme from normal rat liver, spleen, and kidney, respectively. When rats were inoculated with 10^6 Walker 256 ascites cells, death occurred in 8 days; treatment with 50 or 25 mg/kg per day of *13a* for 9 days gave 6/6 30 day survivors. When treatment with 25 mg/kg per day *13a* was started 5 days after inoculation and 2 days before death of the control group, 4/6 rats still survived >30 days (103).

In contrast, with the optimum dose of a potent, nonselective reversible inhibitor of dihydrofolate reductase (the 3′-chloro derivative of *7*) only 1/6 rats survived 30 days when treatment was started on day 1 (103); this supports the concept that selective irreversible inhibitors will be more effective than nonselective reversible inhibitors for treatment of tumors.

Treatment of L1210 mouse leukemia with *13a* was much less effective; only a 50 per cent life extension at optimum dosage was observed. This difference between rat and mouse has been traced to a macromolecule in mouse serum that rapidly destroys the SO_2F function of *13a* by covalent bond formation; this serum factor was absent in rat, dog, calf, horse, and man (104).

Anabolic and Catabolic Enzymes for Nucleosides

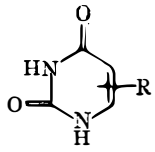
The selective anticancer action of a number of nucleosides, pyrimidines, and purines such as 6-mercaptopurine (MP) 5-fluorouracil (FU), FUDR, MPR, thioguanine, and cytosine-β-arabinoside is primarily the result of a

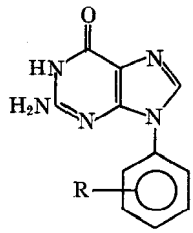
favorable balance of anabolism and catabolism of these compounds in malignant versus normal cells (5); only a few of these many enzymes have been studied from the standpoint of design of active-site directed irreversible inhibitors that also utilize regions on the enzyme adjacent to the active-site.

FUDR phosphorylase.—5-Fluorouracil (FU) and its deoxyriboside (FUDR) are interconverted in the cell by the enzymes that normally utilize thymine or uracil. If such an enzyme could be selectively blocked in a tumor cell with no inhibition of the enzyme in normal cells, then useful adjuncts to FUDR therapy could emerge (49, 50). Initial studies on inhibition were conducted with the *E. coli* B enzyme where 400 μM FUDR was employed as the substrate (49).

The base-line compound for such studies was uracil (*18*) which had $I_{50} = 1600\ \mu M$ for the *E. coli* B enzyme (50). Binding was enhanced 17-fold by introduction of a 6-benzyl group (*19*) (50) and 4000-fold by introduction of a 6-(2, 3-dichloroanilino) group (*20*) (51); that is, *20* had $I_{50} = 0.35\ \mu M$ which is 1100-fold better binding than the substrate, 400 μM FUDR. This interaction of the 6-substituent is believed to be a hydrophobic one adjacent to the active-site (50). As might be anticipated from the "inhibition profiles" with the dihydrofolate reductases from *E. coli* B and a mammalian source, *19* and *20* were considerably less effective on the FUDR phosphorylase from rat liver or Walker 256 rat tumor; *19* showed no increment in binding and *20* showed only a 7-fold increment in binding over the parent uracil, even though the I_{50} for uracil was about the same for the three sources of enzyme (52).

Good hydrophobic bonding to the rat enzymes was seen with a 5-benzyl substituent (*21*) ($I_{50} = 5\ \mu$M) which gave a 600-fold increment over the parent uracil (*18*). A cross-over specificity was noted with the *E. coli* B enzyme; that is, no increment in binding by the 5-benzyl substituent of *21* was ob-





18, R = H
19, R = 6-$CH_2C_6H_5$
20, R = 6-$NHC_6H_3Cl_2$-2,3
21, *R* = 5-$CH_2C_6H_5$

22, R = H
23, *R* = *m*-$NHCOC_6H_5$
24, *R* = m-$NHCOC_6H_4$-*m*-SO_2F
25, *R* = *m*-$NHSO_2C_6H_4SO_2F$-*m*
26, R = *p*-$NHCOC_6H_4$-SO_2F-*m*

served. Stated another way *20* was 110 times more effective on the *E. coli* enzyme than the Walker 256 enzyme, but *21* was 300 times more effective on the Walker 256 enzyme than the *E. coli* B enzyme. Whether appropriate active-site directed irreversible inhibitors of the Walker 256 enzyme can be found that will not inactivate the rat liver enzyme is under investigation (52).

Guanine deaminase.—This catabolic enzyme normally converts guanine to xanthine, but also converts thioguanine to 6-thioxanthine which is then oxidized to the nontoxic 6-thiouric acid by xanthine oxidase (53, 54). The enzyme from rabbit liver is inhibited by 9-phenylguanine (*22*); the 9-phenyl group gives a 28-fold increment in binding compared to 9-methyl which is caused by a hydrophobic interaction adjacent to the active-site (55). Similar results were obtained with the enzyme from Walker 256 rat tumor. Binding was further enhanced 200-fold by a ***m***-benzamido group, *23* being complexed 270-fold better than the substrate, guanine (56). An excellent irreversible inhibitor emerged when *23* was further substituted by an SO_2F group on the terminal phenyl to give *24*; *24* was complexed reversibly to the enzyme 120-fold better than guanine and rapidly inactivated the enzyme from both rat liver and Walker 256 rat tumor by active-site directed irreversible inhibition (57). Walker 256 enzyme could be partially inactivated by *25* with no inactivation of the rat liver enzyme (57); however *25* was not nearly as effective as *24* and further studies are needed to combine the better features of *24* and *25*, hopefully to give an efficient, highly selective irreversible inhibitor of Walker 256 guanine deaminase.

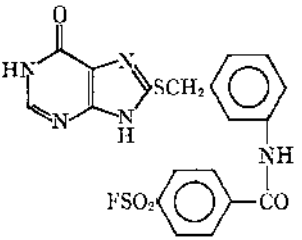
Xanthine oxidase.—Milk xanthine oxidase is also inhibited by 9-phenylguanine (*22*), the 9-phenyl group giving a 140-fold increment in binding by hydrophobic interaction adjacent to the active-site (55). Although there were some differences, the binding of substituted 9-phenylguanines to guanine deaminase and xanthine oxidase were similar enough to suggest these two enzymes may have had a common ancestral precursor enzyme. In spite of these similarities, the two enzymes are readily differentiated by irreversible inhibitors; for example, *24* failed to inactivate xanthine oxidase, but *26* showed a cross-over specificity by activating xanthine oxidase (58) and not rabbit liver guanine deaminase (57).

Other rapid active-site directed irreversible inhibitors of milk xanthine oxidase are *27*, *28* (59), and *29* (60). Further studies for specific inactivation of a tumor enzyme with no inactivation of enzyme from normal tissue is continuing in these four series.

Adenosine deaminase.—This enzyme can be inhibited by 9-alkyl and 9-aralkyladenines where the hydrocarbon moiety interacts with the enzyme by hydrophobic bonding (61–63); an example is 9-benzyladenine (*30*). A bromoacetamido group on the ***p***-position (*31*) of *30* gave an active-site directed irreversible inhibitor with $K_i = 1.4 \times 10^{-5} M$ and $k_d = 11 \times 10^{-3}$ min.$^{-1}$ (64); the ortho isomer (*32*) had a poorer $K_i = 5 \times 10^{-4}$M, but a better inactivation rate, $k_d = 70 \times 10^{-3}$ min^{-1}. Further work on appropriate analogues that might give a species or tissue-specific blockade of adenosine deaminase—to be used in conjunction with cytotoxic nucleosides—will be awaited with interest.

Serine-type Proteases

At least fifteen proteases have been isolated from mammalian sources. These can be divided into three major classes: (*a*) digestive enzymes such as trypsin, chymotrypsin, and pepsin, (*b*) enzymes involved in blood clot forma-



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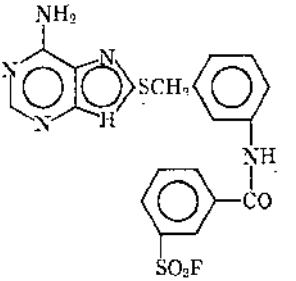

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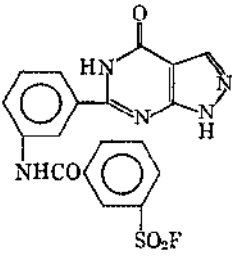

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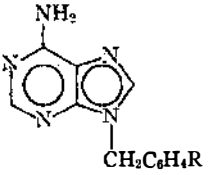


30, R = H
31, R = *p*-NHCOCH$_2$Br
32, R = *o*-NHCOCH$_2$Br

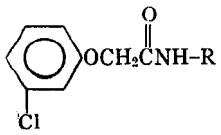
tion and solution such as thrombin and plasmin, and (*c*) the complement and related systems involved in rejection of foreign cells, antigen-induced histamine release, and certain arthritic conditions. These enzyme systems are complicated by zymogen forms that must be activated and by other enzymes that destroy the activated enzyme.

Since all of these enzymes hydrolyze peptide linkages, they must be closely related mechanistically; a large part of the relative specificity resides in the type of acylated amino acid amide preferred for complexing in the active site. The design of a specific inhibitor of one of this myriad of closely related enzymes is not simple. Such specificity should be achievable by utilization of binding areas on the enzyme adjacent to the active-site and by use of active-site directed irreversible inhibitors; these could be useful for a number of disease states such an organ transplantation, cardiovascular disease, arthritis, and viral infection of mammalian cells. The current status on selective inhibition of these enzymes will be reviewed.

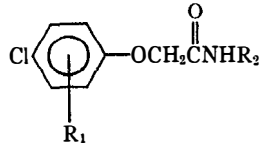
α-Chymotrypsin.—α-Chymotrypsin prefers to hydrolyze the carboxamide function of the acyl peptides Phe, Tyr, and Try. Good substrates are N-benzoyl-L-tyrosine ethyl ester (BTEE) (65) and N-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA) (66); we consider BTEE to be the superior substrate of the two since only about 1 per cent as much enzyme is required for the same rate of optical density change—an important factor with irreversible inhibitors with $K_i < 70\ \mu M$. BTEE has $K_m = 4$ mM and GPNA has $K_m =$ mM (65, 66).

A typical classical inhibitor is N-benzoyl-L-tyrosine methylamide with $K_i = 6$ mM (67). By utilization of a binding area adjacent to the amide linkage, much better reversible inhibitors can be obtained; examples with better K_i's are *33* = 36 μM (69), *34* = 81 μM (68), *35* = 7 μM (68), and *36* = 61 μM (68).

Active-site directed irreversible inhibitors of α-chymotrypsin that operate by the *endo* mechanism (67) are diisopropyl fluorophosphate (DFP) (70), α-tolylsulfonyl fluoride (71), and 1-chloro-4-phenyl-3-tosylamido-2-butanone (TPCK) (72). DFP apparently attacks the active-site serine of all serine proteases and esterases such as acetylcholinesterase (70), trypsin (70), chymotrypsin (70), C'1a component of complement (73), and others (70); similarly, α-tolylsulfonyl fluoride attacks the active-site serine of these enzymes and the active-site cysteine of papain (74), but at different relative rates (71).



33, R = $C_6H_4SO_2F$-*m*



34, R_1 = 2-COOH, R_2 = *m*-$CH_2C_6H_4NHCONHC_6H_4SO_2F$-*m*

35, R_1 = 3-Cl, R_2 = -CH_2(pyridinium)-N-$CH_2C_6H_4SO_2F$-*p*

36, R_1 = 3-Cl, R_2 = -CH_2(pyridinium)-N-$CH_2C_6H_4SO_2F$-*m*

TPCK is more specific but is broad in the number of enzymes with which it will interact; for example, TPCK also reacts with the SH in the active-site proteases such as papain (74) and ficin (75). However, TPCK does not react with tryptic-type enzymes such as trypsin or thrombin (72). Thus these three *endo* type irreversible inhibitors are most useful for specific labeling of the active site of pure enzymes, but are probably not useful for specific pharmarcological action.

Four newer types of active-site directed irreversible inhibitors that were designed to operate by the *exo* mechanism by covalently linking serine (44) are *33* to *36* (68, 69). Covalent linkage of a serine of chymotrypsin by *33*, *34*, and *36*, has been shown (76); the serine covalently linked by *36* was shown definitely not to be the active-site serine-195, but was probably serine-217 outside the active-site (76). Inactivation by K_i concentrations of *34* to *36* occurs with a half-life of 2 min or less (69); a K_i concentration of *33* inactivates with a half-life of 13 min (68).

Guinea pig complement is inhibited by *34* to *36* (77); *35* is an irreversible inhibitor of the C'1a component, but *34* is not (78). Whether or not com-

pounds of type *33* to *36* also inactivate SH-proteases such as ficin or papain has not yet been determined.

Tryptic enzymes.—These are enzymes that preferentially hydrolyze the carboxamide of lysine and arginine peptides; examples are trypsin, thrombin, plasmin, and complement. Trypsin is readily assayed spectrophotometrically using as substrates N-benzoyl-DL-arginine p-nitroanilide (BANA) or N-tosyl-L-arginine methyl ester (TAME) (79); the latter is preferred with irreversible inhibition since the enzyme concentration is lower (80).

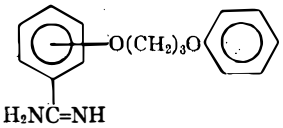
The best inhibitors of trypsin are derived from benzamidine. The latter has $K_i = 20\ \mu M$ (81), but its potency can be enhanced twofold by placing a phenoxypropyloxy group on either the *p*-position (*37*) or fourfold if on the *m*-position (*38*) (82).

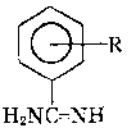
DFP (70), α-tolylsulfonyl fluoride (71), 7-amino-1-chloro-3-tosylamido-2-heptanone (TLCK) (83), ethyl *p*-guanidinobenzoate (84), and a series of O-*p*-nitrophenyl-O-ethylphosphonates (85) are active-site directed irreversible inhibitors that operate by the *endo*-mechanism. These *endo* type irreversible inhibitors lacked specificity and attacked other related enzymes. For example, TLCK also inactivated thrombin as well as the SH-proteases, papain (74) and ficin (75).

In contrast, a greater degree of specificity could be obtained by active-site directed irreversible inhibitors operating by the *exo*-mechanism. For example, the sulfonyl fluoride (*39*) had a $K_i = 3\ \mu M$ and at a K_i concentration inactivated trypsin with a half-life of 7 min; in contrast, *39* at 30 μM failed to show irreversible inhibition of thrombin (80).

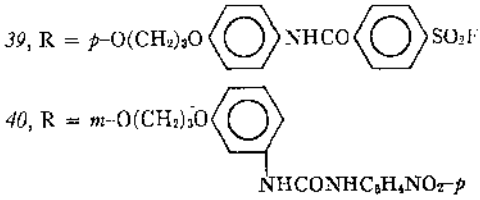
Complement.—Complement consists of nine components which are derived from eleven distinct proteins—all of which are needed in the lysis of a foreign cell by a mammal (73). Although the system is complex it is readily assayed by inhibition of lysis of sheep red blood cells caused by guinea pig complement and antibody (hemolysin) (86). The complement system has both "tryptic" and "chymotryptic" properties and therefore could be expected to be inhibited by some of the inhibitors effective on trypsin and chymotrypsin; most of the published information on inhibition of complement have come from the laboratories of Becker (85, 87) and Baker (77, 78, 86, 88, 89). The most effective compound reported from Becker's laboratory is maleopimaric acid (*41*) (87) which at 0.5 mM shows 50 per cent inhibition of complement and 81 per cent at 1 m*M*.

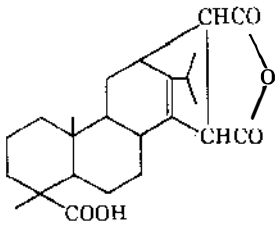
Benzamidine is a weak inhibitor of complement. Potency is increased with a *m*-phenoxypropoxy side chain (*38*), 54 per cent inhibition being seen at 0.5 mM (86, 87); thus *38* is as active as maleopimaric acid. It is noteworthy that *p*-phenoxypropoxybenzamidine (*37*) is not an inhibitor of complement. Activity was considerably enhanced with the *p*-nitrophenyl-ureido substitutent (*40*) on *38*; *40* at 0.062 and 0.016 mM showed 95 per cent and 60 per cent inhibition, respectively, of complement; thus *40* is the most potent inhibitor of complement yet reported (89), being 400 times more potent than benzamidine and 1300 times more potent than N-tosyl-L-arginine methyl ester (77). The α-chymotrypsin inhibitors such as *34* to *36* were also





37, *para*
38, *meta*





41, maleopimaric acid

potent inhibitors of complement (77, 78). Whether irreversible inhibitors related to *38* and *40* will give further enhancement of activity is under investigation in Baker's laboratory.

Other Enzymes

Active-site directed irreversible inhibitors of a number of other enzyme systems have been evaluated; since specificity towards other enzymes or other sources of the enzyme were not studied in most cases, these inhibitors are merely listed:

3-(Bromoacetamidopropyl) guanidine slowly attacks the active-site serine of trypsin and thrombin (90).

5-Diazo-4-oxo-L-norvaline is an asparagine antagonist that inactivates asparaginase (91).

2-Amino-4-oxo-5-chloropentanoic acid is a glutamine antagonist that rapidly inactivates carbamyl phosphate synthetase (92).

4-Iodoacetamidosalicylic acid (10) inactivates lactate dehydrogenase from *Schistosoma mansoni* and rabbit skeletal muscle, but has little or no effect on the enzyme from ox heart, mouse liver, or human serum (93); however, the lactate dehydrogenase from pig heart is inactivated tenfold more rapidly by 3-bromoacetylpyridine than α-bromoacetophenone (94).

N^1-(2-Bromoacetamidoethyl) nicotinamide chloride inactivated yeast

alcohol dehydrogenase, but not horse liver alcohol dehydrogenase, pigheart lactate dehydrogenase, or beef liver glutamate dehydrogenase (95).

Bromomesaconate is an active-site directed irreversible inhibitor of fumarase (96).

p-(Trimethylammonium)benzenediazonium fluoroborate attacks the acetylcholine site in the electropax (97) as well as inactivating acetylcholinesterase (98, 99); N-(*p*-trimethylammoniumphenyl)maleimide can also be used (100).

Membrane bound Na-K ATPase, the enzyme involved in Na and K membrane transport, can be specifically labeled with helebrigenin 3-iodoacetate (101).

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