Forschung, Wissenschaft

Recent Developments in the Field of Enzyme Inhibitors

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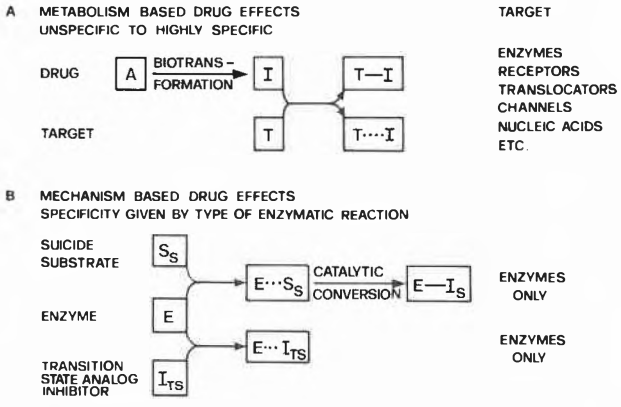
Summary

The present article summarizes the recent developments in the design and use of enzyme inactivators as put forward on the occasion of the joint meeting on "Enzyme Inhibitors" held under the auspices of the Swiss Chemical Society and the Division of Medicinal Chemistry of the German Chemical Society at Sandoz headquarters in Basel, March 1980. In total 25 papers were presented by investigators from industrial and university laboratories. The two day meeting once again showed that the bridge in science and technology between industry and university is important and of mutual benefit. It finely demonstrated how scientists of both groups can profit from each other. Those carrying out applied research were confronted with the latest ideas and developments pertaining the design of highly specific enzyme inhibitors; those doing basic research again became aware of the long road a bioactive compound has to travel if at all it becomes marketable.

New drugs may be found and developed in a variety of different ways, the two extremes being serendipity and the "on purpose design" of novel structures. Within the general frame of the "biospecific drug design" the papers presented mainly dealt with theory, proposition of structures, synthesis of novel inhibitors and their effects on isolated enzymes as well as on the living organism. The present article gives a summary only as a full account will be published [1].

The trends outlined during this meeting point towards the "biospecific drug design" i.e. to find molecules that are able to interact specifically with one target molecule. Biospecificity may be achieved either by a metabolism specific to a target organ or by an intrinsically high affinity for a macromolecular target (Scheme 1). Molecules that *per se* do not posess therapeutic activity may be transformed to an active drug by metabolic conversion, a process that leads to a metabolism based biospecificity. Alternatively molecules may directly interact with a particular enzyme either owing to an intrinsically high affinity alone or by additional enzymatic conversion which produces the enzyme's own inhibitor. Such inhibition is the result of a "mechanism based biospecificity". Within the group of mechanism based enzyme inactivators three different kinds can be distinguished: a) suicide substrates, or alternatively termed k_{cat}-inhibitors, b) molecules that act by paracatalytic modification of an enzyme's active site and c) transition state analog inhibitors (Table 1). Apart from these classes other lines of enzyme inhibitors extend to the so called "bi-substrate" or "bi-product" inhibitors, coenzyme-analogs, molecules with chemically or photo reactive groups etc. Presently these latter types of enzyme inhibitors will be mentioned only in connection with specific contributions to the meeting on "Enzyme Inhibitors".



Scheme 1: Biospecific drug design

Table 1: Mechanism based Enzyme Inactivation

Suicide Substrates	*R. H. Abeles* [2]
k_{cat}-Inhibitors	*R. R. Rando* [3]
Paracatalytic Modification	*P. Christen* [4]
Transition-state-analog-inhibitors	*L. Pauling* [5]
	G. E. Lienhard [6,7]
	R. Wolfenden [8,9]

Numbers in parenthesis refer to references listed at the end of this article

Suicide Substrates

Since the initial observation by *Bloch* [10], that 3-decynoyl-CoA is an irreversible inactivator of bacterial-β-hydroxydecanoyl thiolester dehydrase by virtue of enzymic processing of the electrophilic conjugated allene, a number of groups have designed and tested a variety of molecules as possible suicide substrates of enzymes. From this work a catalog of latently reactive functional groups emerged that have to be introduced into a substrate analog molecule for it to become a

Table 2: Recent developments in suicide substrates

Suicide substrates / Reaction occuring	Target Enzymes
Acetylenic substrate analogs / rearrangement into conjugated allenes	Flavin dependent oxidoreductases Pyridoxal-phosphate dependent enzymes; isomerases
Olefinic substrate analogs	Flavin dependent oxidoreductases: sarcosine oxidase, monoaminooxidase; alcoholdehydrogenase Pyridoxal-phosphate dependent enzymes Lyases: β-cystathionase, 5-deoxyribosephosphate aldolase
Nitrile group containing analogs / conversion into ketones or ketimines	Flavine dependent oxidoreductases: lysine oxidase, monoaminooxidase Tryptophan synthetase
β-Substituted aminoacids and amines / loss of HX	Monoaminooxidase Pyridoxal-phosphate dependent enzymes
Allylsulfoxide containing substrate analogs / 2,3-sigmatropic rearrangement to allylsulfenate esters	Cystathion-γ-synthetase Methionine-γ-lyase

compiled from: *Ch. Walsh,* Horizons Biochem. Biophys. *3* (1977) 36–81 and *Ch. Walsh,* in reference [1], p. 1.

suicide enzyme inactivator (Table 2). These include acetylenic and olefinic substrate analogs, nitrile group containing molecules and β-substituted amino acids. Most recently Ch. Walsh introduced allylsulfoxide containing substrate analogs which are converted to an allylsulfenate ester by a 2,3-sigmatropic rearrangement (see table 2 for reference). The allylsulfoxide is unreactive whereas the rearranged ester is reactive to nucleophilic capture at sulfur. From the list of enzymes that are affected by suicide substrates, it is seen that mainly flavin dependent oxidoreductases and pyridoxal-phosphate dependent enzymes are prominent candidates for inactivation.

The former subclass of enzymes has been thoroughly reviewed by *S. Ghisla* (cf. reference [1]). Those flavine dependent enzymes which proceed by the formation of a carbanion intermediate appear to be susceptible to suicide inactivation by compounds bearing acetylenic, allenic or methylenecyclopropane moieties.

The inhibition of monoamino oxidase by suicide substrates is of clinical importance as this enzyme plays a central role in the metabolism of biogenic amines. Compounds such as propargylamines (fig. 1), allenic amines, cyclopropyl amines and phenylhydrazines inactivate monoamino oxidase in suicidal fashion.

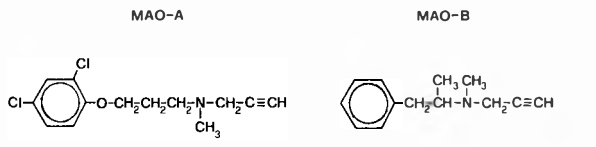


Fig. 1: Suicide substrates of monoaminooxidase (MAO)

γ-Aminobutyric acid: 2-oxoglutarate aminotransferase (GABA-T) is another enzyme involved in the metabolism of a specific neurotransmitter, γ-aminobutyric acid (GABA). This pyridoxal-phosphate dependent enzyme is irreversibly inhibited by compounds such as γ-acetylenic-, and γ-vinyl-GABA or gabaculine (fig. 2). The design, biochemistry and pharmacology of this type of GABA-T inhibitors has been reviewed by *M. J. Jung*. His contribution gave an illustrative example of how a suicide substrate has been developed to the state of clinical trials (cf. reference [1]).

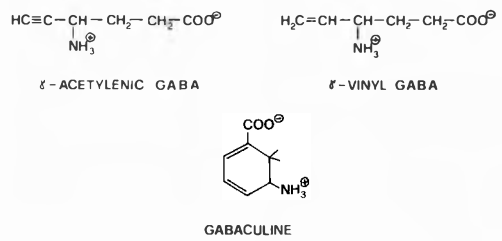


Fig. 2: Structures of suicide substrates of GABA-transaminase

The inhibition of β-lactamases is also of clinical interest. This subclass of enzymes is produced in lactam-resistant bacteria and brings about a rapid hydrolysis of β-lactam antibiotics. As reviewed by *J. R. Knowles*, the inhibition of this enzyme by suicide substrates is but one approach to overcome the defensive production of β-lactamase. Clavulanic- and olivanic acid (fig. 3) give early examples of β-lactamase inhibitors.

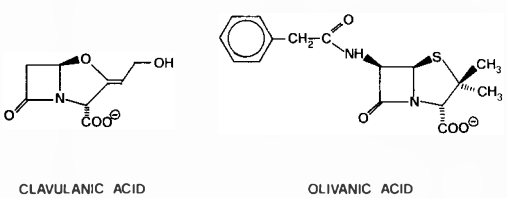


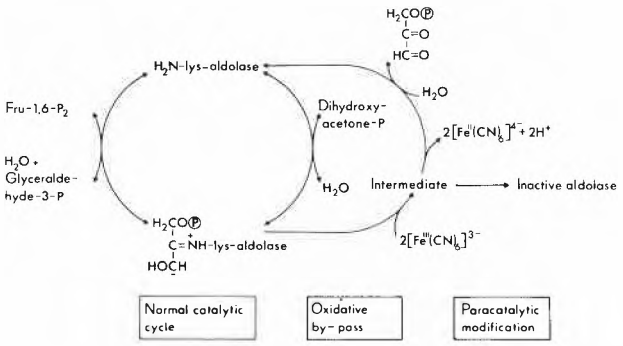
Fig. 3: Suicide substrates of β-lactamase

According to Knowles, effective inactivators of β-lactamase must fulfill three criteria: a) the inactivator must contain a β-lactam ring which allows the formation of an acylenzyme intermediate. b) the inhibitor must posses a reactive proton which upon β-elimina-

tion yields an α,β-unsaturated ester and c) a good leaving group must fascilitate the β-elimination. Based on these criteria Knowles argued that those β-lactames which produce a long-lived acyl-enzyme intermediate i.e. poor substrates, may survive long enough for β-elimination to occur, a prerequisit for suicide inactivation of β-lactamases. Three representative compounds, the sulfones of cloxacillin, methicillin and quinacillin fulfill these requirements and as inhibitors excel penicillanic acid sulfone (cf. reference [1]).

Inhibition by paracatalytic selfinactivation

The concept of enzyme inhibition by paracatalytic selfinactivation was originally put forward by *P. Christen* [4]. It states that a substrate in its transition state may not only be converted to products but owing to its intrinsically high reactivity may also undergo side reactions with agents not belonging to the ordinary catalytic cycle. Such side reactions often lead to a covalent modification of the active site and as a consequence to irreversible enzyme inactivation. Especially for enzymes producing carbanion intermediates, the combination of normal substrates with a suitable electron acceptor constitutes a highly specific binary system for active site directed paracatalytic enzyme inactivation. In her lecture, *M. Cogoli,* a collaborator of *P. Christen* elaborated on fructose-1,6-bisphosphate aldolase as the most thoroughly studied example of those enzymes undergoing paracatalytic reactions (scheme 2). It is of interest to note that paracatalytic enzyme modification has implications for isolated enzymes as it can provide an effective means to label the active site of certain enzymes with high specificity.



Scheme 2: Mechanism of aldol cleavage/condensation reaction, paracatalytic oxidation and paracatalytic modification of Schiff base forming fructose-1,6-bisphosphate aldolase
According to *Cogoli, M., Lubini, D.* and *Christen, P.* ref. [1], pp. 27–42

According to Christen, paracatalytic reactions are unspecific with respect of the oxidants. This raises the possibility that they might also occur with intracellular oxidants such as hydrogen peroxides which might contribute to processes of aging and turnover of enzymes in the cells [1, 4].

Transition State Analog Inhibitors

Another group of mechanism based enzyme inactivators comprises the transition state analog inhibitors. The design of such molecules is based on Pauling's hypothesis [5] that molecules which resemble the substrate in its transition state geometry have a much higher affinity for an enzyme than the substrate itself. The postulate of *Pauling* was later taken up by *G. E. Lienhard* [6, 7] and *R. Wolfenden* [8, 9] who created the concept of enzyme inhibition by transition state analog compounds. The design of such inhibitors requires some knowledge of the mechanism of the enzymatic reaction, i.e. the conformation of the substrate in its activated form should be known well enough to allow the design a stable analog.
Of a total of approximately 2000 known enzymes, so far only about 70 are recognized targets for inhibition by transition state analogs. Table 3 shows that this type of inhibitors affects enzymes of all 6 classes and a list of a representative example in each class is given in table 4. The majority of transition state analog compounds tested so far refer to hydrolases and to a lesser extent oxidoreductases and transferases (Table 3).

Table 3: Number of Examples of Transition State Analog Inhibitors

Enzyme-Class	A	B
1 Oxidoreductases	464	10
2 Transferases	561	13
3 Hydrolases	577	30
4 Lyases	241	7
5 Isomerases	95	4
6 Ligases	88	8

A: Number of enzymes known in each class (from Enzyme Nomenclature, Recommendations 1978 of the IUB)
B: Number of enzymes for which transition state analog inhibitors have been designed (status March 1980)

In general hydrolytic cleavage of a substrate and group transfer reactions proceed by a change in bond order, i.e. the substrates undergo trigonal-tetrahedral or tetrahedral-trigonalbipyramidal transformations between ground- and transition state. Enzymes which follow this type of reaction mechanism appear to be prominent targets for inhibition by transition state analogs. The literature published since the compilation of *Wolfenden* in 1976 (for reference see table 4) corroborates this finding as the majority of newer publications refer to hydrolases of all kind (see reference 1 for an updated list). Of the recent developments 3 examples are cited presently: the on "purpose design" of transition state analog inhibitors of acetylcholinesterase and carboxypeptidase and a naturally occuring inhibitor of glycosidases that presumably acts as transition state analog.
Acetylcholinesterase is a typical example for a serine-hydrolase that functions by a change in bond order. It catalyzes its own acylation and deacylation in a double

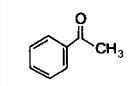
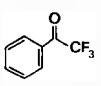
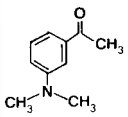
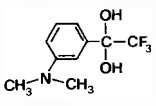
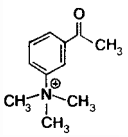
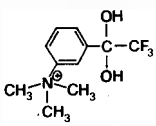
Table 4: Selected examples of transition state analog inhibitors

	Enzyme Class	Enzyme	Substrate	Transition-State-Analog Inhibitor
1	Oxidoreductases	Dihydrofolate reductase	Folate $K_s = 1.4 \cdot 10^{-5}$ M	Methotrexate $K_i = 3 \cdot 10^{-11}$ M
2	Transferases	Aspartate transcarbamoylase	Carbamylphosphate $K_m = 2.7 \cdot 10^{-5}$ M Aspartate $K_m \approx 2 \cdot 10^{-2}$ M	N-Phosphonoacetyl-L-Aspartate $K_i = 2.7 \cdot 10^{-8}$ M
3	Hydrolases	Adenosine deaminase	Adenosine $K_m = 3.1 \cdot 10^{-5}$ M	Coformycin $K_i \approx 10^{-10}$ M
4	Lyases	Acetoacetate Decarboxylase	Acetoacetate $K_m = 9 \cdot 10^{-3}$ M	Acetopyruvate $K_i = 2 \cdot 10^{-7}$ M
5	Isomerases	Proline racemase	L-Proline $K_m = 2.3 \cdot 10^{-3}$ M	Pyrrole-2-Carboxylate $K_i \approx 10^{-5}$ M
6	Ligases	t-RNA Ligases	L-Alanine $K_m = 7.2 \cdot 10^{-4}$ M	DL-Alaninol-AMP-ester $K_i = 9 \cdot 10^{-6}$ M

Ref.: *Wolfenden, R.:* Ann. Rev. Biophys. Bioeng. (1976) *5*, 271–306.

displacement type mechanism. Compounds that facilitate the nucleophilic attack by the reactive hydroxyl moiety of the active center serine residue are prominent transition state analogs. Recently we have shown that trifluoromethylketones are effective inhibitors of acetylcholinesterase (Table 5). The most prominent compound tested so far is N,N,N-trimethyl-ammonium trifluoracetophenone that owing to the formation of a hemiketal linkage and thus tight binding to the active site leads to an essentially irreversible inactivation of acetylcholinesterase.

Table 5: Inhibition of acetylcholinesterase by ketones and trifluoromethylketones

Parent Compounds		Fluorinated Compounds	
	K_i	K_i	
	$1.6 \cdot 10^{-3}$ M	$1.8 \cdot 10^{-5}$ M	
	$9.0 \cdot 10^{-6}$ M	I_{50} $1.0 \cdot 10^{-7}$ M $(2.3 \cdot 10^5)$	
	$5.0 \cdot 10^{-7}$ M	$1.3 \cdot 10^{-8}$ M $(1.8 \cdot 10^8)$	
		(Second Order Rate Constant)	

According to *U. Brodbeck* and *M. Rottenberg*, reference [1], p. 12

Another type of hydrolases comprises the metallo-enzymes as they contain a functional heavy metal cation in their active center. Carboxypeptidase is a typical example of such a metallo-enzyme and the hydrolytic cleavage of the peptide bond proceeds too via a change in bond order.

In his contribution *M. Rottenberg* elaborated on the design of a potent transition state analog inhibitor of carboxy-peptidase. Starting from a detailed knowledge about the enzyme (i.e. its mechanism of action, its active site and even its three dimensional structure) and applying analog thinking *Rottenberg* found a structure, that as a transition state analog proved to be more effective than all hitherto known inhibitors of carboxypeptidase.

The novel inhibitor is L-2-phosphoryloxy-3-phenyl-propionic acid ($K_i = 1.4 \cdot 10^{-7}$ M) and this compound excels, by a factor of 4, L-benzylsuccinic acid ($K_i = 6 \cdot 10^{-7}$ M) the bi-product inhibitor designed by Byers and Wolfenden.

The last example refers to Acarbose (Bay g 5421). This compound is a tetrasaccharide derived from a natural poly-saccharide precursor isolated from Actino-planaceae (Fig. 4). In a series of papers scientists from

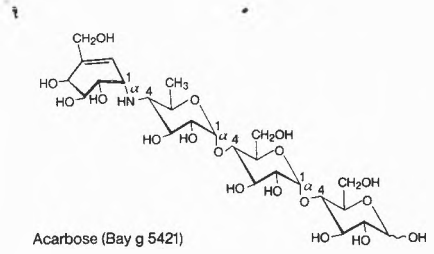


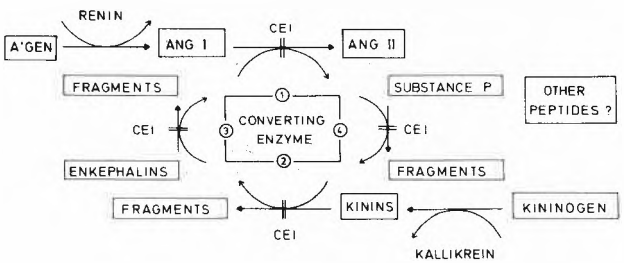
Fig. 4: Structure of Acarbose (Bay g 5421)

Bayer Pharmaceuticals discussed the isolation, mechanism of action and possible clinical usefulness of this compound. Acarbose inhibits a number of α-glucosidases such as pancreatic α-amylase and intestinal glucoamylase, sucrase and maltase. It gives an example of a naturally occuring compound that presumably acts as a transition state analog inhibitor of these enzymes. In vivo Acarbose not only delays the diges-

tion of sucrose but is also a potent inhibitor of starch degradation. Owing to a retarded carbohydrate digestion, the postprandial increment of blood glucose and serum insulin in animals and man is dose dependently reduced by Acarbose. As suggested by *L. Müller* and collaborators, the principle of enzyme inhibition should be an appropriate way to regulate carbohydrate digestion, to influence carbohydrate absorption kinetics and to finally control intermediary carbohydrate metabolism. It is envisioned that penultimately Acarbose may become of clinical usefulness in treating diseases associated with carbohydrate (and lipid) metabolism such as diabetes, adipositas and hyperlipemia.

Substrate- and Coenzyme Analog Inhibitors

Besides the mechanism based enzyme inactivators a number of additional inhibitors affecting a variety of different enzymes were presented during the meeting. *T. Unger* reviewed those molecules - that specifically inhibit dipeptidyldipeptide carboxyhydrolase (converting enzyme). This enzyme is involved in blood pressure regulation; it catalyzes the conversion of angiotensin I to angiotensin II which constitutes one of the most powerful vasoconstricting, salt retaining and thus blood pressure increasing agent. In addition the converting enzyme inactivates the vasodilatory and natriuretic nonapeptide bradykinin; converting enzyme is identical with kininase II. Converting enzyme thus increases blood pressure by a dual action. Since it is known that the renin-angiotensin system plays an important role in the pathogenesis of human high blood pressure disease it is expected that inhibitors of converting enzyme would become useful antihypertensive agents (Scheme 3).



Scheme 3: Mechanism by which converting enzyme inhibitors can interfere with peptidergic systems
①, ② established mechanisms; ③, ④ hypothesized mechanisms; A'GEN: angiotensinogen; ANG: angiotensin; ACE: angiotensin converting enzyme; CEI: converting enzyme inhibitors
According to *T. Unger* et al. (reference [1], pp. 238)

Starting from a hypothetical model of the active site of converting enzyme (fig. 5), *Unger* presented arguments that lead to the development of succinyl-1-proline derivatives which are specific, orally active converting enzyme inhibitors. In this series, 2-D-methyl-3-mercapto-propranoyl-1-proline (Captopril, SQ 14225) proved to be most promising as blood pressure lowering drug in experimental hypertension in animals and in human hypertension.

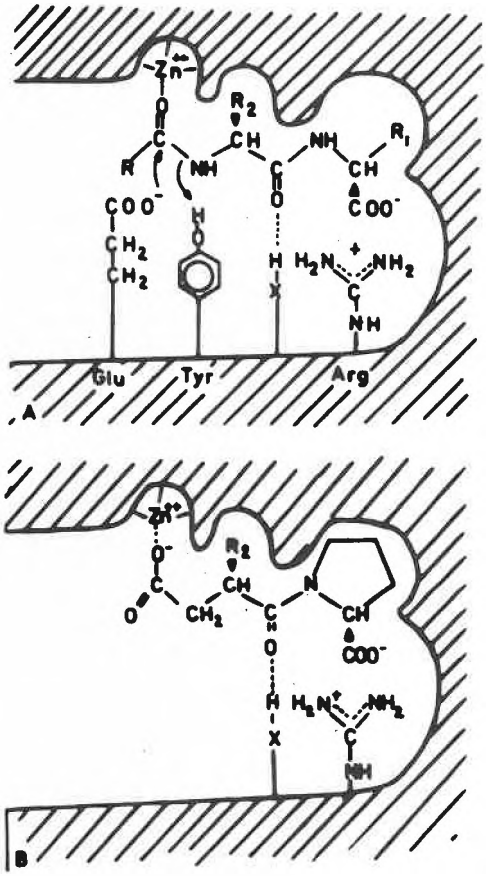
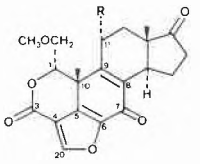


Fig. 5: Hypothetical model of the active site of converting enzyme. A: proposed binding and hydrolytic cleavage of a peptide substrate; B: proposed binding of succinyl-1-proline derivative inhibitors.
According to: *T. Unger* et al., Reference [1], pp. 223–241.

Other contributions pertained to the inhibition of prostaglandin biosynthesis thereby affecting vasodilation, platelet aggregation as well as inflammation. *S. Moncada* reviewed the biochemistry of prostacyclin (PGI_2) and thromboxane A_2 formation, two potent substances with opposing biological effects. The balance between these two substances is important for an equilibrated interaction of platelets with the wall of a blood vessel. *Moncada* delineated the different ways of interfering with this balance and its impact in the development of thrombosis and artheriosclerosis. As shown by *Haefliger* and coworkers, a metabolic product from the fungus Pennicillium funiculosum. desacetoxywortmannin (fig. 6) has a potent effect in various inflammation models. This compound does appear not to inhibit prostaglandin synthesis and its

mechanism of action as an anti-inflammatory agent is subject to further investigations.



R = H Desacetoxywortmannin
R = OAc Wortmannin

1. W. Haefliger und D. Hauser, Helv. 56, 2901 (1973)
2. D. Wiesinger, H.U. Gubler, W. Haefliger und D. Hauser, Experientia 30, 135 (1974)

Fig. 6: Structures of wortmannin and desacetoxywortmannin

C. H. Hassal discussed small molecular weight inhibitors of elastase which might find uses in combatting degenerative tissue degrading processes found in diseases such as pancreatitis, arthritis and emphysema. Using X-ray crystallographic data on porcine pancreatic elastase *Hassal* defined those factors contributing to the binding of oligopeptides to the active site of the enzyme. As a result elastase inhibitors such as propionyl-ala-pro-cyclohexylamide have been designed which were shown to have activity in vivo as they inhibit elastase induced rat paw oedema.

The enzymatic methylation of macromolecules has gained considerable interest since the discovery of abnormal methylation patterns of t-RNA in a variety of tumors as well as in cells transformed by oncogenic viruses. In her lecture *M. Robert-Géro* discussed synthetic analogs of S-adenosyl-homocysteine which were shown to be inhibitors of oncogenic transformation of chick embryo fibroblasts infected by Rous sarcoma virus. They also inhibit other oncogenic RNA and DNA viruses and are particularly toxic for cells transformed by chemical carcinogens. Analogous activities have been found for two antifungal antibiotics, sinefungin and dehydrosinefungin, the former being especially active against Plasmodium falciparum in human erythrocytes. In vitro these inhibitors effect enzymatic methylation of t-RNA, m-RNA, protein and phospholipid. Furthermore they inhibit various enzymes involved in the metabolism of S-adenosylhomocysteine.

Typical and well known examples of a coenzyme-analog inhibitors are the folic acid antagonists which inhibit bacterial dihydrofolic acid reductase. Novel structures and new derivatives of known compounds were presented by H. Vergin and R. Kompis. These contributors stressed once again the usefulness of this type of enzyme inhibitors in chemotherapy.

Current and Future uses of Enzyme Inhibitors

The lectures presented during the meeting on "enzyme inhibitors" gave a wealth of valuable information to those investigators seeking novel structures in the design of biospecific enzyme inactivators. In a number of cases the possible or probable use of such compounds in therapy of diseases was confirmed or suggested. Although limited in number the examples as summarized in table 6 clearly show that the concept of biospecific drug design is on the verge of reaching adolescence. As novel target enzymes are being defined, new mechanism based inhibitors will be designed and tested for their clinical usefulness. In addition the concept of mechanism based inhibitors will lead to novel effector molecules that will find applications in agricultural chemistry and applied as well in basic enzymology (Table 7). One of the unresolved problems in analytical enzymology pertains to the determination of the absolute amount of an enzyme present in a given solution or in a biological fluid. Novel inhibitors, highly specific for a particular enzyme, will allow the determination of its functional molarity, a procedure that up to now has found only limited application. Furthermore highly specific inhibitors are and will be

Table 6: Suggested applications of mechanism based enzyme inhibitors in therapy of diseases

Disease	Inhibitor(s)	Affected enzyme/reaction *
Adipositas, Diabetes, Hyperlipemia	Acarbose (Bay g 5421)	Glycosidases (110)
Arthritis, Emphysema, Pancreatitis	Derivatives of peptides	Elastase (259)
Cancer	N-(Phosphonoacetyl)-L-aspartate	Aspartate transcarbamoylase (8)
	Maltol, squaric acid	Glyoxylase (13)
	Derivatives of S-adenosylmethionine	Methyltransferases (62)
Huntington's disease	Derivatives of GABA	GABA-amimotransferase (92)
Hypertension	Derivatives of succinyl-proline	Converting enzyme (224)
Infections: bacterial	β-Lactam antibiotics	β-Lactamase (163)
bacterial, viral	2,4-Diamino-5-benzyl-pyrimidines	Dihydrofolate reductase (177, 191)
fungal	Sinefungin	Methyltransferases (61)
parasitic	Derivatives of S-adenosyl methionine	Methyltransferases (61)
Inflammation	Wortmannin and derivatives	Prostaglandin biosynthesis (245)
	Peptide-chlorobenzyl esters	C3-Convertase (261)

Compiled from reference [1];
* Numbers in parenthesis refer to page numbers of reference [1]

Table 7: Further use of mechanism based enzyme inhibitors

Agricultural chemistry –	Inhibition of a metabolic pathway unique to a particular living organism
Industrial enzymology –	Large scale enzyme purification by biospecific adsorption/desorption
Clinical enzymology –	enzyme standardization by determination of functional molarity
Basic enzymology –	delineation of enzyme mechanism

used in the purification of enzymes on a technical scale. They certainly prove to be useful tools in the delineation of enzyme mechanism.

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