Final Report

PROTEASE INHIBITORS AS ANTIVESICANTS

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8/30/91

James C. Powers DATE

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Keywords: Antivesicants, Protease Inhibitors, Serine Protease, Sulfur Mustard

ABSTRACT

Bis-(2-chloroethyl)sulfide (HD, sulfur mustard) is a potent vesicant which was used in the Iran-Iraq conflict. Sulfur mustard penetrates the skin rapidly and after a latent period of several hours under temperate climatic conditions, causes extensive blistering of the skin. The mustard is thought to alkylate DNA which eventually results in the release of potent proteolytic (protein degrading) enzymes, particularly serine proteases. These serine proteases are capable of destroying a number of connective tissue proteins and several of the enzymes seem to be able to specifically cleave proteins at the junction of the dermis and epidermis (outer non-vascular layer of skin). The destruction of these basement membrane and connective tissue proteins results in an epidermal-dermal separation, fluid accumulation, and formation of a blister.

A total of 45 serine protease inhibitors have been submitted in 3-5 g quantities for animal testing. The structural classes include 9 isocoumarins, 5 saccharins, 6 peptide phosphonates, 3 benzoxazinones, 11 benzamidines, 5 derivative of *p*-guanidino benzoic acid and 6 miscellaneous compounds. The inhibitors have been surveyed for inhibitory potency against human neutrophil elastase, human neutrophil cathepsin G, rat skin tryptase, human skin tryptase, human skin chymase, human plasminogen activator, porcine pancreatic elastase, rat mast cell protease II, dog skin chymase, human lung tryptase and bovine chymotrypsin.

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BACKGROUND

Sulfur Mustard. Bis-(2-chloroethyl)sulfide (HD, sulfur mustard) is a potent vesicant which was used in the Iran-Iraq conflict. Sulfur mustard penetrates the skin rapidly and after a latent period of several hours under temperate climatic conditions, causes extensive blistering of the skin. Antimustard ointments which attempt to inactivate the free mustard are ineffective since the mustard quickly reacts with components in the skin and other tissues. Therefore, an effective therapy for sulfur mustard exposure must be based on reversing the physiological processes that result upon contact with this potent vesicant (Cullumbine, 1947).

Bis-(2-chloroethyl)sulfide is a potent alkylating agent which can react with the heterocyclic bases in nucleic acids (Ludlum et al., 1984) and with a wide variety of the side chain functional groups (thiols, thioethers, carboxyl groups, amino groups, imidazole rings, etc.) found in proteins. The majority of the sulfur mustard is secreted in the form of various metabolites such as thiodiglycol, but some is carried by the circulation to other organs, and a significant portion is stored in skin reservoirs (Klain and Bonner, 1987). While the most significant mustard induced injury occurs in the skin (vesication and inflammation), significant numbers of mustard casualties have ocular injuries and cornea impairment for 2-4 months. In cases of severe exposure, there is serious lung and bone marrow damage which results in death.

Mechanism of Sulfur Mustard Induced Blistering. The molecular mechanisms by which sulfur mustard causes toxicity are unknown but mustard is a powerful alkylating agent of DNA and RNA. Papirmeister has suggested that the alkylated purine bases in DNA are unstable and undergo both spontaneous and enzymatic depurination (Papirmeister et al., 1985). This results in DNA strand breaks, and activation of nucleases and other DNA repair mechanisms. As a result, poly(ADP-ribose)polymerase is activated, NAD^+ is depleted, glycolysis is inhibited, and the hexose monophosphate shunt is stimulated (Meier et al., 1987). This causes the release of potent proteolytic enzymes which produces the observed pathology of basal cell necrosis and vesication.

Evidence for the Papirmeister hypothesis includes the isolation and structural characterization of several DNA alkylation products upon treatment of DNA with sulfur mustard (Benschop et al., 1989) and the demonstration of single strand breaks in the DNA after exposure of keratinocyte cultures to low levels of sulfur mustard (Bernstein et al., 1989). In addition, other agents which result in DNA damage such as UV light and radiation have been shown to stimulate the synthesis or release of proteases in fibroblast cultures (Miskin and Reich, 1980).

Proteases are normally controlled by natural plasma protein protease inhibitors such as α_1-protease inhibitor, α_1-

antichymotrypsin, and α_2-macroglobulin. If this antiprotease screen is destroyed, tissue destruction results. Several of the plasma serpins (serine protease inhibitors) including α_1-protease inhibitor and α_1-antichymotrypsin have essential methionine residues and are susceptible to inactivation by oxidizing agents or alkylating agents. A single dose of sulfur mustard in the mouse brain has recently been shown to cause a burst of oxidants (Elsayed et al., 1989). The serpin screen could then be removed directly by sulfur mustard alkylation or indirectly by oxidation as a result of this oxidative burst. Thus, sulfur mustard exposure probably results both in the release of powerful proteolytic enzymes and in the partial destruction of the protease inhibitor screen which would normally protect the organism from proteolysis.

Other Blistering Disease States Involve Proteases. Blistering disease states which have been described include dermatitis herpetiformis (DH), bullous pemphigoid (BP), chronic bullous disease of childhood, and pemphigus vulgaris. These diseases are characterized by destruction of various connective tissue components of the epidermis or dermis followed by tissue separation and the formation of fluid-filled blisters. Blister fluids from patients with all of these diseases have been shown to contain proteases including elastase and collagenase (Oikarinen et al., 1983). Human polymorphonuclear leukocyte elastase is the major enzyme in DH fluid, while BP fluid predominantly contains the metalloprotease collagenase. A trypsin-like enzyme and a thiol protease have also been implicated in blister formation respectively in recessive dystrophic epidermolysis bullosa and epidermolysis bullosa simplex (Takamori et al., 1985). Incubation of normal human skin with the blister fluid from patients with epidermolysis bullosa letalis, a severe and usually fatal congenital blister disease, results in dermal-epidermal separation. A number of common serine protease inhibitors prevented the separation (Matsumoto and Hashimoto, 1986).

Proteases are Associated with Inflammation. Proteases are important mediators and modulators of inflammation and have been demonstrated in non-blistering inflammatory disease states such as psoriasis and arthritis. The most abundant enzymes are the serine proteases elastase and cathepsin G (a chymotrypsin-like enzyme) from leukocytes; chymases (chymotrypsin-like enzymes), and tryptases (trypsin-like) enzymes from mast cells; plasminogen activator; and the metalloprotease collagenase from leukocytes. These enzymes are capable of cleaving a variety of connective tissue proteins including elastin, collagen, proteoglycans, and other basement membrane components.

Sulfur Mustard Induced Inflammatory Lesions Contain Proteases and Protease-Inhibitor Complexes. The proteolytic enzymes released upon exposure to sulfur mustard have not yet been isolated or characterized, but likely candidates include chymases and tryptases from mast cells, elastase and cathepsin G from leukocytes, plasminogen activator, and collagenase. Culture

fluids from mustard-induced inflammatory lesions in rabbit skin show 3 to 6 fold increased levels of proteases both in developing and healing lesions (Higuchi et al., 1987). These fluids will hydrolyze two synthetic peptide substrates, Boc-Leu-Gly-Arg-AFC (Boc = *t*-butyloxycarbonyl, AFC = 7-amino-4-trifluoromethyl coumarin) and Bz-Phe-β-naphthyl ester (Bz = benzoyl). The first peptide is a substrate for trypsin, tryptases, plasmin, plasminogen activator and other trypsin-like enzymes, while the latter is a substrate for chymotrypsin-like enzymes including chymases and cathepsin G. The rabbit skin culture fluids did not consistently hydrolyze four other synthetic peptide substrates (two for elastase and two for cathepsin G) or the protein elastin (elastase's natural substrate). Exposure of human skin in culture to sulfur mustard results in a 41 % increase in plasminogen activator activity (Dannenberg et al., 1989), an enzyme which is known to be associated with blister formation (Hashimoto et al., 1983). The enzymatic activity of chymases, tryptases, and angiotensin converting enzyme toward small synthetic substrates were not elevated.

The proteases found in the culture fluids from mustard-induced inflammatory lesions in rabbit skin are not present as free active enzymes, but are found as inactive complexes with their natural plasma protease inhibitors α_1-protease inhibitor and α_2-macroglobulin (Harada et al., 1987; Dannenberg et al., 1987; Higuchi et al., 1987). These complexes are formed as natural protein protease inhibitors from the plasma react with the proteases being released at the site of inflammation. The protease-inhibitor complexes are incapable of hydrolyzing protein substrates and complex formation thus protects the organism from further damage. The natural plasma protease inhibitors are probably not completely destroyed by exposure to low levels of sulfur mustard and are still available to react with some of the proteases released in the blister.

Protease-inhibitor complex formation hinders the identification of the proteases present in sulfur mustard induced culture fluid. Complexes of elastase and cathepsin G respectively with α_1-protease inhibitor and α_1-antichymotrypsin are inactive toward both synthetic peptide substrates and natural protein substrates. Thus the lack of hydrolysis of elastin or elastase substrates does not preclude the presence of inactivated elastase in the culture fluids. Similarly the lack of hydrolysis of the protein fibrin by the culture fluids doesn't exclude the presence of plasmin or plasminogen activator. In summary, it is now clear that there is a chymotrypsin-like enzyme (chymase), a trypsin-like enzyme (tryptase), and plasminogen activator in the sulfur mustard induced inflammatory lesions, but the presence of other enzymes has not been excluded.

Skin Serine Proteases Have Been Isolated and Characterized. The dermis of human skin is a rich source of mast cells and salt extraction of human skin has yielded two serine proteases, a chymase and a tryptase. These serine

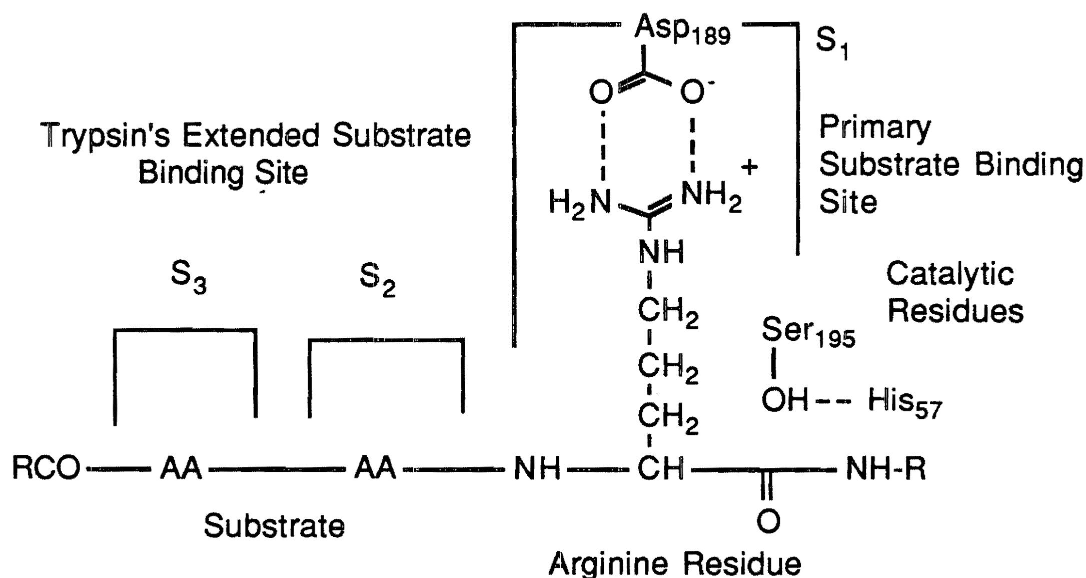
proteases are localized in the granule fraction of mast cells, a cell type which is located predominantly in connective tissue. The chymase has been demonstrated immunocytochemically to bind to the dermo-epidermal junction in skin (Sayama et al., 1987). Both the mast cell chymase and tryptase are able to specifically cleave proteins found in the dermal-epidermal boundary and cause vesication. The chymase is incompletely inhibited by plasma due to a 650 fold slower rate of reaction with the serpins α_1-protease inhibitor and α_1-antichymotrypsin (Schechter et al., 1989), while the tryptase appears not to be inhibited by most protein protease inhibitors (Schechter et al., 1983). This may explain the ready detection of chymase and tryptase activity in culture fluids from mustard-induced lesions in rabbit skin.

Rat mast cells contain two chymases (RMCP I and RMCP II) which have been more extensively characterized than the chymase from human skin. Both of these serine proteases are highly homologous to human mast chymase and human leukocyte cathepsin G. RMCP I and RMCP II have both been sequenced and the x-ray crystal structure of RMCP II has been determined, while the x-ray structure of RMCP I is underway (Woodbury and Neurath, 1980; Remington et al., 1988). The sequence of dog mast cell tryptase and a related dog mast cell protease have been determined by gene sequencing techniques (Vanderslice et al., 1989) and it is likely that the sequences for human skin tryptase and chymase will be available in the next few years.

The substrate specificity and inhibition profile of human skin chymase, human skin tryptase, RMCP I, RMCP II, and related enzymes have been studied in the laboratory of the principle investigator (Powers et al., 1985). Human skin chymase hydrolyzes peptide substrates containing aromatic amino acid residues and prefers Phe-AA and Tyr-AA bonds over Trp-AA bonds (AA = any amino acid residue) in contrast to chymotrypsin which prefers Trp over Phe and Tyr. One of the best peptide substrates is Suc-Phe-Val-Pro-Phe-NA (Suc = succinyl, NA = 4-nitroanilide). Human skin tryptase is a trypsin-like enzyme, but seems to prefer double basic residues in its substrates (Tanaka et al., 1983). For example, the thioester substrate Z-Lys-Arg-SBu-i (Z = benzyloxycarbonyl, SBu-i = thioisobutyl ester) is hydrolyzed by human skin tryptase with a k_{cat}/K_M = 59,000,000 $M^{-1}s^{-1}$, a second order rate constant which is close to the diffusion controlled rate.

Serine Protease Specificity. The specificity of serine proteases toward natural peptide substrates or synthetic inhibitors is determined by the nature of the primary substrate specificity pocket (S_1) and secondary subsites (S_2, S_3, etc.) on the surface of each individual enzyme. Trypsin's primary specificity site contains an Asp residue in the back of the S_1 pocket so that trypsin will only bind to and hydrolyze peptide substrates containing lysine or arginine residues (a schematic model of trypsin with a bound substrate is shown below). The

three-dimensional structure of chymotrypsin is quite similar except that the Asp-189 in trypsin is replaced by Gly-189 in chymotrypsin. As a result the S_1 pocket of chymotrypsin in very hydrophobic and chymotrypsin prefers substrates containing aromatic amino acid residues such as Trp, Tyr, and Phe. With many serine proteases, interactions of inhibitors with the extended substrate binding site (S_2, S_3, etc.) are important to increase the specificity and reactivity of the inhibitor. This is clearly the case with human skin chymase and tryptase. For example, interaction of the Lys in the substrate Z-Lys-Arg-SBu-i with the S_2 subsite of human tryptase results in an accelerated rate of hydrolysis, while little change in hydrolysis rate is observed with trypsin.



HYPOTHESIS

It is clear—no matter the exact mechanism of their release or their source—that proteases are major factors in the tissue destruction that accompanies mustard induced vesication. We propose that protease inhibitors will be effective antivesicants and should be useful both in preventing blistering and in the treatment of blisters. Appropriate target proteases are the mast cell chymase and tryptase, serine proteases which are localized in the skin and have the ability to cleave proteins at the dermal-epidermal junction. However other serine protease such as elastase and cathepsin G from leukocytes, and plasminogen activator may also be involved. Evidence for the involvement of other classes of proteases such as the metalloprotease collagenase or the thiolprotease cathepsin B is incomplete or lacking at present, although the mast cell tryptase is able to activate latent collagenase (Gruber et al., 1989).

Research Strategy. Since the exact target enzyme (or enzymes) is not known with certainty, we decided to synthesize general serine protease inhibitors, specific chymase inhibitors, specific tryptase inhibitors, specific plasminogen activator inhibitors, and specific inhibitors for other important serine proteases. During the last portion of this contract, we focused on inhibitors for trypsin-like enzymes since Smith et al. (1991) have recently reported increased hydrolysis rates for substrates of trypsin-like enzymes by mustard stimulated lymphocytes. Thus, these enzymes may be significant proteases involved in blistering.

PROGRESS REPORT

Research Goals

1. Prepare and submit for animal testing 3-5 inhibitors of serine proteases such as 3,4-dichloroisocoumarin and saccharins each year.

2. Prepare and submit for testing 3-5 inhibitors of human skin chymase each year.

3. Prepare and submit for testing 3-5 inhibitors of human skin tryptase each year.

4. Prepare and submit for testing each year 3-5 inhibitors for other human serine proteases-such as human leukocyte elastase, cathepsin G, and plasminogen activator-which may have a role in vesication.

5. Assay all inhibitors with human skin serine proteases and related enzymes for *in vitro* effectiveness.

Research Progress-Summary

1. A total of 45 serine protease inhibitors have been submitted in 3-5 g quantities for animal testing during the first two years of this contract. The structural classes include 9 isocoumarins, 5 saccharins, 6 peptide phosphonates, 3 benzoxazinones, 11 benzamidines, 5 derivative of *p*-guanidino benzoic acid and 6 miscellaneous compounds. All the inhibitors which were submitted are listed in the following table along with their sample numbers.

2. Various inhibitors have been surveyed for inhibitory potency against human neutrophil elastase, human neutrophil cathepsin G, rat skin tryptase, human skin tryptase, human skin chymase, human plasminogen activator, porcine pancreatic elastase, rat mast cell protease II, dog skin chymase, human lung tryptase and bovine chymotrypsin.

SAMPLES SUBMITTED: DMAD17-89-C-9008

BOT-NUM	WR-NUM	SUB-ID	RCHD	COMPOUND
ISOCOUMARINS (9 submitted)				
BL58572	268195	II-148	89/05/15	3,4-dichloroisocoumarin
BL57637	268119	II-134	89/03/20	3-chloroisocoumarin
BL57413	259666	II-137	89/03/13	4-chloro-3(2-phenylethoxy)isocoumarin
BM00482	268387	II-151	89/08/08	4-chloro-3-benzyloxyisocoumarin
BM04319	268693	MA-134	90/04/17	7-amino-4-chloro-3-cyclohexylmethoxyisocoumarin
BM04668	268715	GP1	90/05/17	4-chloro-3-methylisocoumarin
BM01096	268440	JO-138	89/09/12	4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxy isocoumarin
BM00642	268397	II-152	89/08/21	4-chloro-3-(3-S-isothiureidopropoxy)isocoumarin
BM06288		GP2	90/09/05	4-chloro-3-methoxyisocoumarin
SACCHARINS (5 submitted)				
BL57977	268145	MA-84	89/04/19	N-benzoyl saccharin
BL57995	268147	MA-89	89/04/19	N-phenylacetyl saccharin
BL57986	268146	MA-87	89/04/19	N-diphenylacetyl saccharin
BL57931	268141	MA-86	89/04/19	N-furoyl saccharin
BM00464	268385	MA-96	89/08/01	N-cyanomethyl saccharin
PHOSPHONATES (6 submitted)				
BL57959	268143	II-22	89/04/19	$\text{Z-Met}^{P}\text{(OPh)}_2$
BL57968	268144	II-138A	89/04/19	$\text{Z-Val}^{P}\text{(OPh)}_2$
BL57422	259858	II-137A	89/03/13	$\text{Z-Phe}^{P}\text{(OPh)}_2$
BL57842	268132	II-139	89/04/10	$\text{Z-Phe-Phe}^{P}\text{(OPh)}_2$
BL59382	268241	II-147	89/06/14	$\text{Suc-Val-Pro-Phe}^{P}\text{(OPh)}_2$
BM04328	268694	III-3	90/04/17	$\text{Z-NHCH(AmPh)PO(OPh)}_2$

p-GUANIDINOBENZOIC ACID DERIVATIVES (5 submitted)

BM01363	268476	MA101	89/10/06	*p*-guanidino benzoic acid
BM01185	184335	MA-111	89/09/19	ethyl-*p*-guanidinobenzoate
BM02655	268570	MA-121	89/12/13	N(*p*-guanidinobenzoyl)valine amide
BM03143	268596	MA-115	90/01/23	O(*p*-guanidinobenzoyl)glycol amide
BM06304		MA-148	90/09/11	4-cyanophenyl *p*-guanidinobenzoate

BENZOXAZINONES (3 submitted)

BM00651	268398	JO-12	89/08/21	2-ethoxybenzoxazinone
BM05441		GP3	90/06/29	2-phenylamino-4H-3,1-benzoxazin-4-one
BM05807		GP4	90/07/24	2-benzylamino-4H-3,1-benzoxazin-4-one

BENZAMIDINES (11 submitted)

BM06804		GP5	90/11/02	1-(4-amidinophenyl)-3-phenylurea
BM06840		GP6	90/11/14	1-(4-amidinophenyl)-3-(4-chlorophenyl)urea
BM07481		GP7	90/12/18	1-(4-amidinophenyl)-3-benzylurea
BM07829		GP8	91/01/31	1-(4-amidinophenyl)-3-(4-phenoxyphenyl)urea
BM08004		GP9	91/02/19	(4-amidinobenzyl)benzyl ether
BM08184		GP10	91/03/08	bis(4-amidinophenyl)urea
BM08308		GP11	91/04/02	1-(3-amidinophenyl)-3-phenylurea
BM08595		GP12	91/04/23	(4-amidinobenzyl)phenylethyl ether
BM08764		GP13	91/05/05	1-(3-amidinophenyl)-3-(4-phenyoxyphenyl)urea
BM09903		GP14	91/08/05	(4-amidinobenzyl)-3-phenylpropyl ether
BM09912		GP15	91/08/05	(4-amidinobenzyl)-3-phenoxybenzyl ether

MISC. (6 submitted)

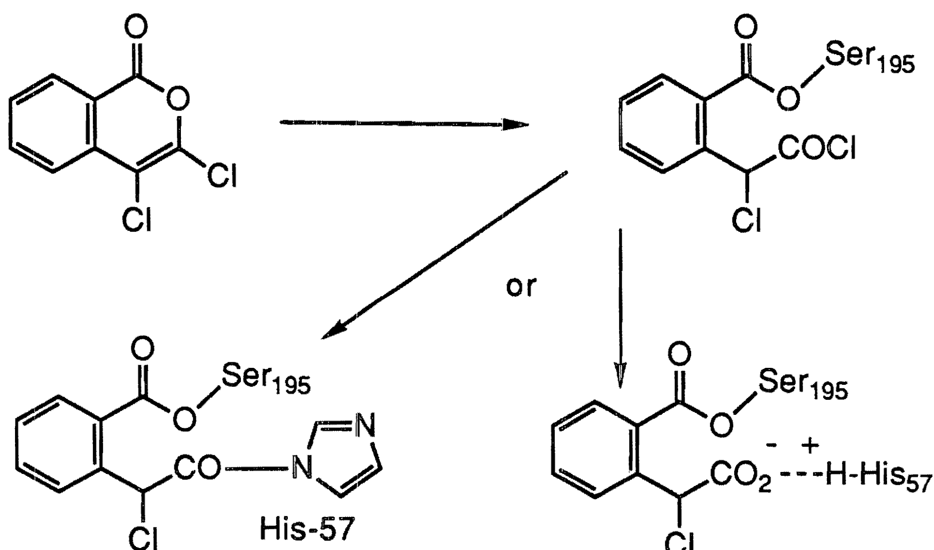
BL57646	015392	II-137B	89/03/20	isatoic anhydride
BL57940	268142	II-145	89/04/19	di(4-isovaleroylphenyl)sulfide
BM00491	099874	II	89/08/08	5-nitro-3H-1,2-benzoxathiole-2,2-dioxide
BM07490		MA-60	90/12/18	1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole
BM07838		MA-76	91/01/31	1-benzoyloxy-1,2,3-benzotriazole
BM07132		MA-68	90/11/27	N-(2-isothiureidoethyl)pthalimide

TOTAL SUBMITTED (8/30/91): 45

Inhibitors Submitted.

Isocoumarins-General Inhibitors. Dichloroisocoumarin (WR268195) is an excellent general inhibitor of serine proteases and was discovered in the laboratory of the principal investigator (Harper et al., 1985). With the exception of the bacterial enzyme subtilisin, 3,4-dichloroisocoumarin is an inactivator of all serine proteases which have been tested, including human leukocyte elastase, human skin chymase, dog skin chymase, rat mast cell protease I, and rat mast cell protease II.

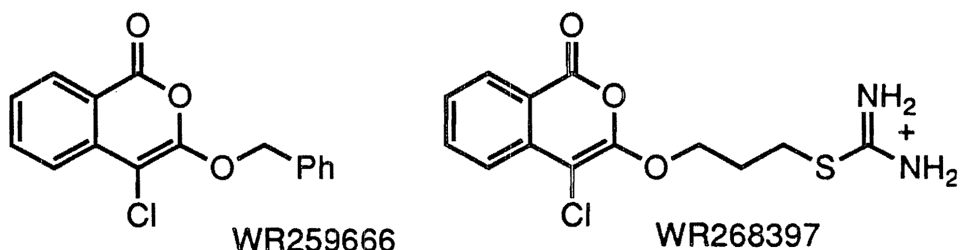
The dichloroisocoumarin ring system contains a masked acid chloride (or ketene) functional group which is exposed when an acyl enzyme is formed upon reaction with the active site serine of a serine protease (Harper et al., 1985). The acyl enzyme (top right of figure) which is formed initially can react further by acylating the active site histidine to form a doubly acylated enzyme derivative (bottom left) or can hydrolyze to form an acyl enzyme stabilized by a salt link between the protonated histidine and the inhibitor carboxyl group (bottom right). The monochloro derivative, 3-chloroisocoumarin (WR268119), inhibits chymotrypsin-like enzymes at slower rates than 3,4-dichloroisocoumarin and does not touch trypsin. The acyl enzymes formed upon reaction with dichloroisocoumarin have variable stabilities, but in general the half-lives for reactivation (deacylation) are greater than 8 hrs at pH 7.5.



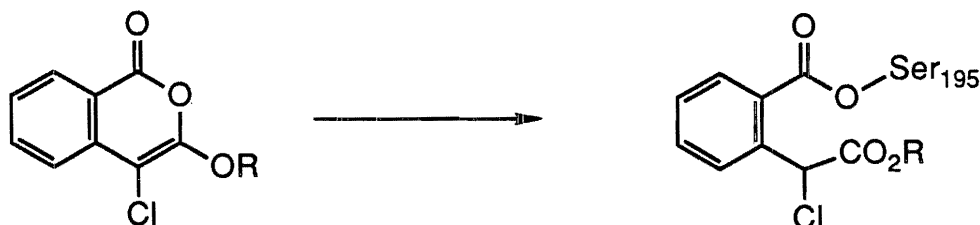
Dichloroisocoumarin and 3-chloroisocoumarin are formed by reaction of homophthalic acid with PCl_5.

Isocoumarins-Specific Inhibitors. We have also synthesized a number of isocoumarin inhibitors which are more specific for the active sites of chymases or tryptases.

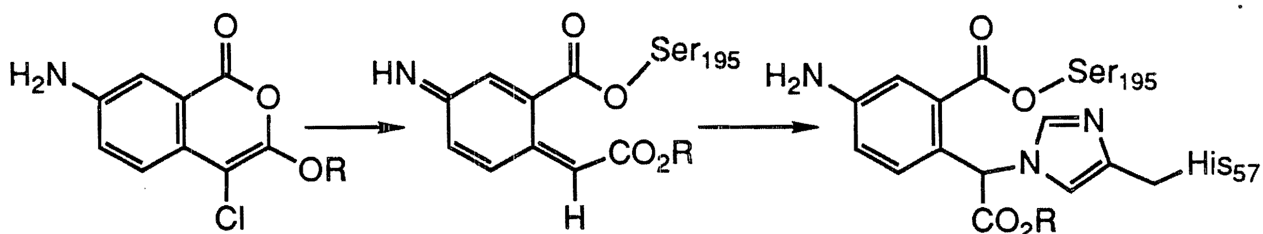
Inhibitors targeted for chymase should contain an aromatic side chain which resembles the side chain of Phe, Tyr or Trp, while those inhibitors targeted for tryptase should containing a charged group which resembles the side chain of Arg or Lys. Several of the more specific isocoumarin inhibitors are shown below. Inhibitors with the benzyloxy (such as WR268387) or phenylethoxy groups (WR259666) were targeted at the chymases, while those with basic side chains (such as WR268397) were targeted at the tryptases.



The mechanism of inhibition of serine proteases by 3-alkoxy-4-chloroisocoumarins involves acylation of the active site serine-195 to form acyl enzymes with varying stabilities ($t_{1/2}$ = hrs to days) depending on the nature of the alkoxy group (Harper and Powers, 1985).

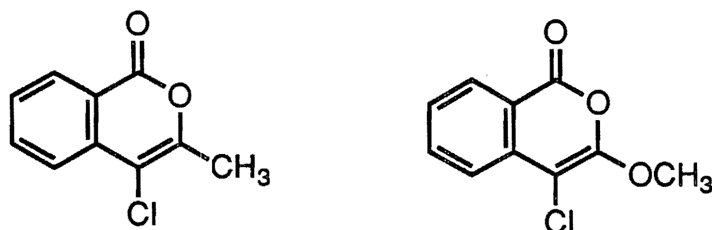


Analogous isocoumarins with electron donating substituents in the 7-position such as 3-alkoxy-7-amino-4-chloroisocoumarins (below) are mechanism-based or suicide inhibitors of serine proteases (Powers et al., 1989). These inhibitors also acylate serine proteases, but form stable acyl enzymes which are not reactivated upon long standing or upon treatment with hydroxylamine. The inhibition mechanism involves formation of an acyl enzyme which can then eliminate chloride to form a quinone imine methide (center). This intermediate then irreversibly alkylates His-57 with the formation of a stable covalent bond between enzyme and inhibitor. This mechanism is supported by x-ray crystallographic studies of complexes of isocoumarin inhibitors bound to the active site of porcine pancreatic elastase (Bode et al., 1989). Thus far, five separate isocoumarins have been studied crystallographically, two give simple acyl enzyme structures (above), two give acyl enzyme structures where His-57 has been alkylated, and one gives a mixture of both structures.



Isocoumarins which have been submitted and should inhibit serine proteases by the above mechanism include 7-amino-4-chloro-3-(cyclohexylmethoxy)isocoumarin (WR268693) and 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin (WR268440).

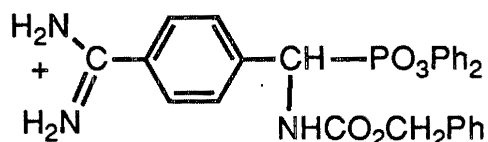
Two analogs of 3,4-dichloroisocoumarin are shown below. These two compounds were synthesized with the expectation that any isocoumarins with two small electronegative functional groups would be effective general serine protease inhibitors.



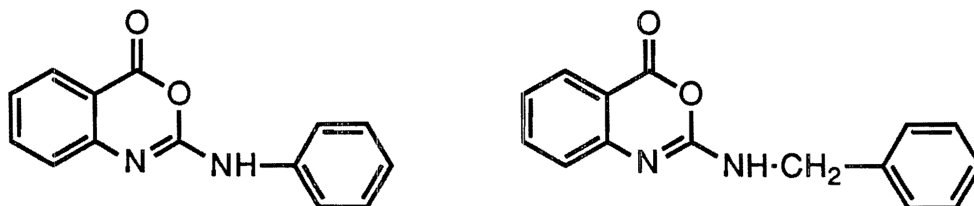
α-Aminoalkylphosphonates Diphenyl Esters. Peptidyl derivatives of α-aminoalkylphosphonate diphenyl ester are effective and specific inhibitors of serine proteases at low concentrations (Oleksyszyn and Powers, 1989; 1991). These peptide derivatives phosphonylate the active site serine to form stable phosphonyl derivatives. Good interactions with the S_1 pocket of the target serine protease are necessary before nucleophilic substitution on phosphorus atom occurs to give a stable phosphonyl derivative.

Phosphonate diphenyl ester inhibitors are chemically stable, relatively easy to synthesize, do not react with acetylcholinesterase, form very stable derivatives possibly due to their resemblance to the tetrahedral intermediate involved in peptide bond hydrolysis, and have considerable potential utility as therapeutic agents.

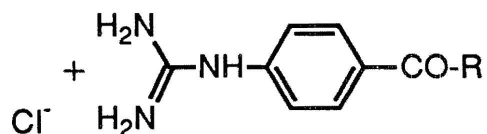
We have submitted a number of simple amino acid and peptide derivatives of phosphonate diphenyl esters including Z-$Met^P(OPh)_2$ (WR268143), Z-$Val^P(OPh)_2$ (WR268144), Z-$Phe^P(OPh)_2$ (WR259858), Z-Phe-$Phe^P(OPh)_2$ (WR268132), and Suc-Val-Pro-$Phe^P(OPh)_2$ (WR268241). More recently, we have accomplished the synthesis of the amidinophenyl phosphonate derivative (WR268694) shown below. The benzyloxycarbonyl (Z) derivative has been submitted recently for testing. We have also synthesized a few peptide derivatives of the amidinophenyl phosphonate, but not in sufficient quantities for submission.



Benzoxazin-4-ones. Substituted benzoxazin-4-ones were discovered to be potent inhibitors of human leukocyte (HL) elastase, porcine pancreatic (PP) elastase, cathepsin G, and chymotrypsin by the PI (Teshima et al., 1982). Mechanistic studies by Abeles showed that these compounds were forming stable acyl enzyme derivatives (shown below) with chymotrypsin (Hedstrom et al., 1984) and this has been confirmed by x-ray crystallographic studies with two benzoxazinones bound to PP elastase (Radhakrishnan et al., 1987). Due to the potential of benzoxazinone inhibitors of HL elastase for treatment of emphysema, a group at Syntex Canada has synthesized over 100 new benzoxazinones, carried out a structure-function study as substituents were varied on the ring system, and studied the plasma stability of these compounds (Spencer et al., 1986; Krantz et al., 1987; Krantz et al., 1990). We have submitted two benzoxazinones (BM05441 and BM05807) as elastase inhibitors.

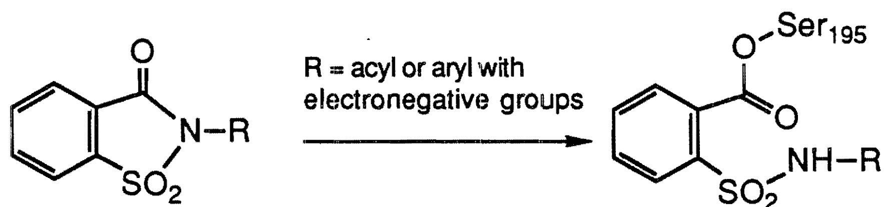


Guanidinobenzoic Acid Inhibitors. Esters of *p*-guanidinobenzoic acid have been reported to be potent inhibitors for various trypsin-like enzymes (Okutome et al., 1984; Fujii et al., 1977) and *p'*-nitrophenyl-*p*-guanidinobenzoate is widely used as an active-site titrant for these enzymes (Chase and Shaw, 1970). An active-site titrant for trypsin-like enzymes developed in the laboratories of the PI is benzyl *p*-guanidinothiobenzoate (Cook and Powers, 1983). We have submitted samples of five *p*-guanidinobenzoic acid derivatives for testing.

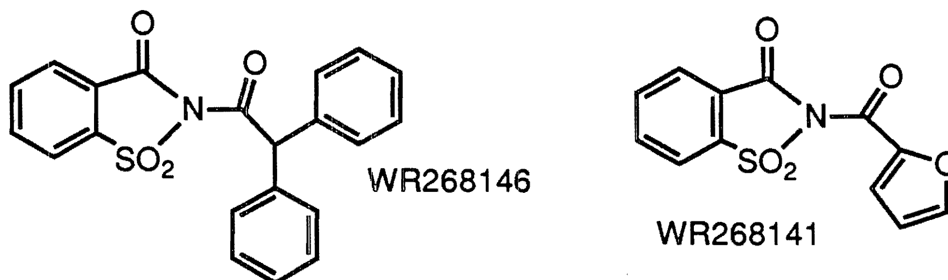


R = OH (WR268476)
OEt (WR184335)
NH-Val-NH_2 (WR268570)
OCH_2CONH_2 (WR268596)
C_6H_4-CN (BM06304)

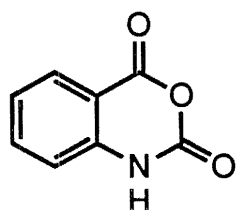
Saccharin Inhibitors. N-Acyl and N-aryl saccharins are potent acylating agents of HL elastase, cathepsin G, and chymotrypsin (Zimmerman et al., 1980; Ashe et al., 1981). A few of the N-acyl derivatives such as N-furoyl, N-thienoyl, and N-benzoylsaccharin inhibit trypsin with IC_{50} values of 0.7-2.4 µM. These structures were initially designed as acyl transfer reagents, but studies using ^{35}S-labeled N-furoylsaccharin indicated that the saccharin portion of the inhibitor becomes covalently and stoichiometrically bound to both HL elastase and pancreatic elastase upon acylation.



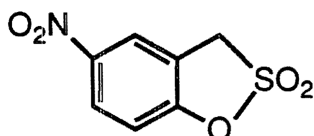
Two of the saccharins which we have submitted are shown below.



Miscellaneous Inhibitors. Two heterocyclic general serine protease inhibitors have been submitted. They are isatoic anhydride which has been shown to acylate the active site of chymotrypsin and form a stable acyl enzyme (Moorman and Abeles, 1982) and 5-nitro-3H-1,2-benzoxathiole-2,2-dioxide which forms a stable sulfonyl derivative also with chymotrypsin.

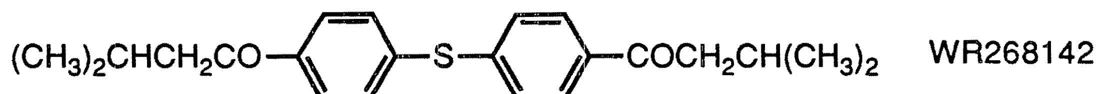


Isatoic Anhydride
WR015392

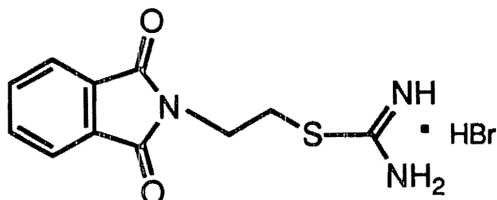


5-Nitro-3H-1,2-benzoxathiole-2,2-dioxide
WR099874

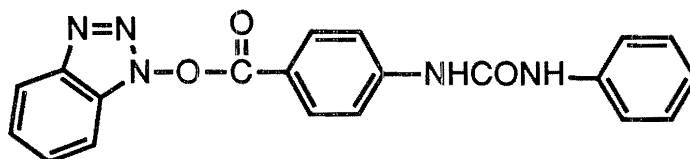
The sulfide shown below is an effective inhibitor of elastase which was discovered in the laboratory of the PI.

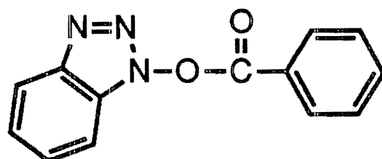


The following phthalimide derivative was designed as a trypsin inhibitor.

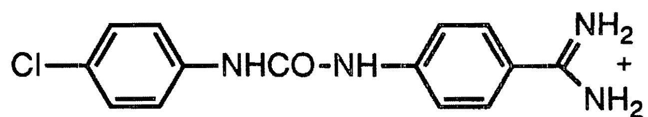


The following acyl derivatives of N-hydroxybenzotriazole inhibit trypsin.

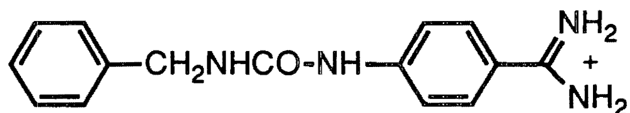




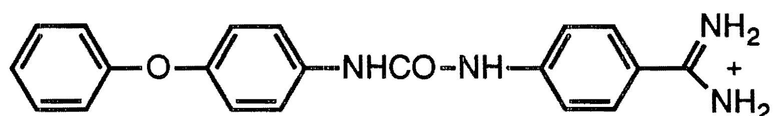
Benzamidine Inhibitors. Eleven benzamidine inhibitors have been submitted as reversible inhibitors for trypsin-like enzymes. These compounds interact with the S_1 pocket as well as other portions of the active site. Some representative examples are shown on the next page.



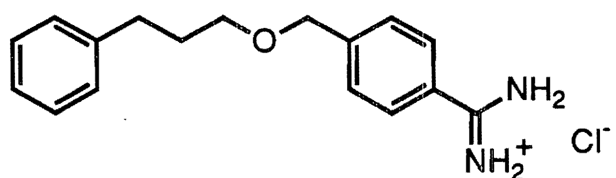
BM06840



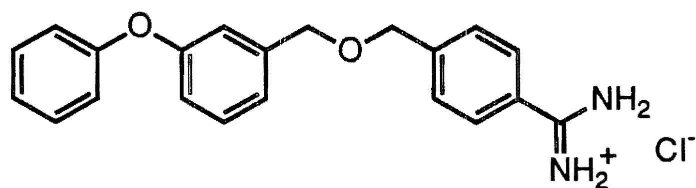
BM07481



BM07829



BM09903



BM09912

Biological Test Data.

All the inhibitors which we have synthesized have been tested for inhibitory potency against a variety of serine proteases. Kinetic data obtained with the various inhibitors are shown in Tables I-III. Most of the inhibitors are irreversible or slowly reversible inhibitors and we report the second order inhibition rate constants $k_{obs}/[I]$. Several of the inhibitors reported in the tables have $k_{obs}/[I]$ values of 100,000 $M^{-1}s^{-1}$ or greater. A second order inhibition rate constant of this magnitude indicates that the reaction between equimolar concentrations of enzyme and the inhibitor is over in less than 0.2 min. (the time required for mixing the enzyme and inhibitor in the assays). The half-life of the inhibition reaction can be calculated from the equation $t_{1/2} = 0.693/\{[I]*k_{obs}/[I]\}$. Thus, an inhibitor with a $k_{obs}/[I]$ value of 100,000 $M^{-1}s^{-1}$ would have an inhibition half-life of 6.93 sec. at an inhibitor concentration of 1 μM, while an inhibitor with $k_{obs}/[I]$ = 10,000 would have a half-life of 69 sec. For the few reversible inhibitors investigated, K_I values (dissociation constant of the enzyme-inhibitor complex) or IC_{50} values are given.

The data with bovine chymotrypsin, cathepsin G, rat mast cell protease II, human skin chymase, and dog skin chymase is given in Table I. The best isocoumarin inhibitor in this table is 3-benzyloxy-4-chloroisocoumarin (WR268387) with a $k_{obsd}/[I]$ = 12,000 $M^{-1}s^{-1}$ for the human skin chymase. Increasing the length of the side chain at position 3 by one methylene group [4-chloro-3-(2-phenylethoxy)isocoumarin, WR259666] reduces the activity by a factor of 35.

The best phosphonate inhibitor for chymotrypsin-like enzymes is Suc-Val-Pro-Phe^P(OPh)$_2$, which corresponds to the sequence of an excellent 4-nitroanilide substrate for these enzymes. NMR studies with chymotrypsin indicate that only one of the two stereoisomers reacts with the enzyme ($k_{obsd}/[I]$ = 146,000 $M^{-1}s^{-1}$ calculated for the single isomer, the value in the table is for the DL mixture). The ^{31}P NMR of chymotrypsin inhibited by this peptide phosphonate shows one broad signal at 25.98 ppm corresponding to the Ser-195 phosphonylated enzyme derivative (Oleksyszyn and Powers, 1991). The tripeptide phosphonate Suc-Val-Pro-Phe^P(OPh)$_2$ makes better interactions with the extended substrate binding site of the enzyme than is possible with the shorter dipeptide or amino acid phosphonate derivatives.

All of the saccharins submitted so far have high $k_{obsd}/[I]$ values with the various chymotrypsin-like enzymes and low IC_{50} values with the elastases tested. One of the better inhibitors in this family is N-furoylsaccharin. The acyl enzymes formed upon acylation of serine proteases by acyl saccharins have variable stabilities. Furoyl saccharin and benzoyl saccharin form inhibited elastase derivatives which are very stable and have half-lives for deacylation of 80-160 hrs. In contrast the

chymotrypsin derivatives have much shorter half-lives in the range of 1.9 hrs. One disadvantage of some acyl saccharins is their fairly rapid hydrolysis at neutral pH values.

The data with porcine pancreatic elastase (PPE) and human leukocyte elastase (HLE) is given in Table II. All the isocoumarin derivatives reported in this table are excellent inhibitors of HLE, the best one being the 7-ureido derivative WR268440 with a $k_{obsd}/[I] = 140,000\ M^{-1}s^{-1}$ for HLE. It is also highly selective compared with 3,4-dichloroisocoumarin and 3-chloroisocoumarin which is a logical consequence of the improvement in binding resulting from substitution at the 7-amino group. We have also submitted an excellent benzoxazinone inhibitor for HLE (WR268398). Two analogs of this compound were poor inhibitors of the elastases, and were only moderate inhibitors of chymotrypsin-like enzymes.

The phosphonate derivatives which we have submitted thus far are not good inhibitors for PPE or HLE. This is not surprising since the sequence of the tripeptide was chosen for chymase inhibition and this sequence is very specific for the chymases as discussed earlier. An excellent phosphonate inhibitor for the elastases has been synthesized only in a small scale. This inhibitor, Boc-Val-Pro-Val^{P}(OPh)$_2$, has $k_{obsd}/[I]$ value of 27,000 $M^{-1}s^{-1}$ for human leukocyte elastase (HLE) and 11,000 $M^{-1}s^{-1}$ for porcine pancreatic elastase (PPE). Again this sequence corresponds to a good HLE substrate sequence and at low concentrations this peptide did not react with chymotrypsin.

The inhibition data obtained with bovine trypsin, human lung tryptase, rat skin tryptase, human skin tryptase, and human recombinant tissue plasminogen activator is given in Table III. The best tryptase inhibitor submitted is the 3-(isothioureidopropoxy)isocoumarin WR268397 with a $k_{obs}/[I] = 650,000\ M^{-1}s^{-1}$ for the rat skin tryptase, an extremely rapid inhibition rate. However, the acyl-enzyme formed with this inhibitor is unstable and the enzyme regains its activity within 5 min.

Several *p*-guanidinobenzoic acid derivatives have been tested as inhibitors for the various tryptases. One of these derivatives, O-(*p*-guanidinobenzoyl)glycolamide is an excellent inhibitor of the rat skin tryptase, but is a much poorer inhibitor of human skin tryptase. In contrast to the isocoumarin WR268397, the inhibited derivative did not regain enzyme activity upon standing. We pursued this lead and synthesized additional derivatives. The 4-cyanophenyl *p*-guanidinobenzoate was also a very potent inhibitor of trypsin and human lung tryptase. However it is probably not very stable in solution.

We then shifted our emphasis away from guanidino compounds to amidines when we learned that amidines are likely to be less toxic than guanidino derivatives. In the last few months of this contract we have been concentrating only on inhibitors for

tryptases since this may be the more important enzyme in blister formation. Aromatic benzamidine derivatives are reversible inhibitors for trypsin-like enzymes and we have been preparing a series of derivatives to see if we can increase their inhibitory potency for tryptases. The most potent inhibitor thus far for both trypsin and the human lung tryptase is 1-(amidinophenyl)-3-(4-phenoxyphenyl)urea (GP8, BM07829) which has a K_I value of 1.6 μM with bovine trypsin and inhibits 71% of the activity of human lung tryptase at 226 μM.

Table I. Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
WR Compound No.	Inhibitor	ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
		Isocoumarin Inhibitors				
268195	3,4-dichloroIC[g]	570	28	580	27	82
268119	3-chloroIC[g]	330	24	85		
259666	4-chloro-3-(2-phenylethoxy)IC	3800	100	200	340	
268387	4-chloro-3-benzyloxyIC	32,000	220	3200	12,000	39,000
268693	7-amino-4-chloro-3-cyclohexylmethoxyIC				25	
268715	4-chloro-3-methyl IC	66	8			
BM06288	4-chloro-3-methoxy IC	206	25			
		Benzoxazinone Inhibitors				
BM05441	2-phenylamino-4H-3,1-benzoxazin-4-one	77	reactivates			

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ $(M^{-1}s^{-1})$				
		ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
BM05807	2-benzylamino-4H-3,1-benzoxazin-4-one	NI	NI			
	Miscellaneous Inhibitors					
015392	isatoic anhydride	580	114	250		
268142	di(4-isovaleroylphenyl)sulfide		NI[h]	25%[i]		
	Phosphonate Inhibitors					
268143	Z-Met^{P}(OPh)$_2$	1.6	1.1	24		
268144	Z-Val^{P}(OPh)$_2$	0.4	NI[h]	NI[h]		
259858	Z-Phe^{P}(OPh)$_2$	260	76	89		
268132	Z-Phe-Phe^{P}(OPh)$_2$	7.5	NI[h]	18		
268241	Suc-Val-Pro-Phe^{P}(OPh)$_2$	41,000	36,000	15,000	190,000	

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ $(M^{-1}s^{-1})$				
		ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
		Saccharin Inhibitors				
268145	N-benzoylsaccharin	15,000	45,000	16,000		
268147	N-phenylacetylsaccharin	11,000	31,000	1,300		
268146	N-diphenylacetylsaccharin	10,000	14,000	9,800		
268141	N-furoylsaccharin	22,000	39,000	20,000		
268385	N-cyanomethylsaccharin	NI[h]				

[a]Inactivation rates were measured by an incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO at 25 °C. Enzyme concentrations were: chymotrypsin, 1.6 μM; cathepsin G, 0.8-1.6 μM; RMCP II, 38 nM; human skin chymase, 0.07 μM. Chymotrypsin and cathepsin G were assayed with Suc-Val-Pro-Phe-NA (0.5 μM), human skin chymase and RMCP II were assayed with Suc-Ala-Ala-Pro-Phe-SBzl (88 μM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

[b]Inhibitor concentrations were in the range 5.2 μM-1000 μM. N-Cyanomethyl saccharin was inactive at 1 mM.

[c]Inhibitor concentrations were in the range: 4.8 μM-1000 μM.

[d]Inhibitor concentrations were in the range: 3-148 μM.

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

[e]Inhibitor concentrations were as follows: 4-chloro-3-(2-phenylethoxy)IC, 45 μM; 7-amino-4-chloro-3-cyclohexylmethoxyIC, 0.44 mM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 0.54 μM.

[f]Inhibitor concentrations were as follows: 3,4-dichloroisocoumarin, 540 μM; 3-benzyloxy-4-chloroIC, 1.0 μM.

[g]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) Biochemistry **24**, 1831-1841.

[h]No inhibition.

[i]Inhibition was not time dependent, and the % inhibition was measured at 92 μM.

Table II. Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)	
		PPE[b]	HLE[c]
	Isocoumarin Inhibitors		
268195	3,4-dichloroIC[d]	2,500	9,000
268119	3-chloroIC[d]	510	3,900
259666	4-chloro-3-(2-phenylethoxy)IC		
268440	4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyIC	59	140,000
268715	4-chloro-3-methyl IC	NI[e]	72%[f]
BM06288	4-chloro-3-methoxy IC	601	87
	Miscellaneous Inhibitors		
015392	isatoic anhydride		
268142	di(4-isovaleroylphenyl)sulfide		2 μM[g]
099874	5-nitro-3H-1,2-benzoxathiole-2,2-dioxide		50
	Benzoxazinones		
268398	2-ethoxy-4H-3,1-benzoxazin-4-one		110,000
BM05441	2-phenylamino-4H-3,1-benzoxazin-4-one	216	NI
BM05807	2-benzylamino-4H-3,1-benzoxazin-4-one	NI	NI

Table II (Continued). Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)	
		PPE[b]	HLE[c]
	Saccharin Inhibitors		
268145	N-benzoylsaccharin	5.2 μM[h]	2.4 μM[h]
268147	N-phenylacetylsaccharin		
268146	N-diphenylacetylsaccharin		
268141	N-furoylsaccharin	0.58 μM[h]	0.36 μM[h]
268385	N-cyanomethylsaccharin		
	Phosphonate Inhibitors		
268143	Z-Met^P(OPh)$_2$	NI[e]	0.8
268144	Z-Val^P(OPh)$_2$	2.5	90
259858	Z-Phe^P(OPh)$_2$	NI	6
268132	Z-Phe-Phe^P(OPh)$_2$	NI	NI
268241	Suc-Val-Pro-Phe^P(OPh)$_2$	NI	NI

[a]Inactivation rates were measured by an incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 8-12 % Me_2SO at 25 °C. Enzyme concentrations were: PPE, 1.6 μM; HLE, 0.3 μM. PPE was assayed with Suc-Ala-Ala-Ala-NA (0.75 mM), and HLE was assayed with MeO-Suc-Ala-Ala-Pro-Val-NA (0.5 mM).

[b]Inhibitor concentrations were in the range: 3-1000 μM.

[c]Inhibitor concentrations were in the range: 1.2-1000 μM.

[d]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) Biochemistry **24**, 1831-1841.

Table II (Continued). Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

[e]No inhibition.

[f]Inhibition was not time dependent.

[g]Inhibition was not time dependent and the IC_{50} was obtained.

[h]IC_{50} values obtained from Zimmerman, M., Morman, H., Mulvey, D., Jones, H, Frankshun, R. and Ashe, B. M. (1980) J. Biol. Chem. **255**, 9848-9851.

Table III. Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Phosphonate Inhibitors, Benzamidines, and Miscellaneous Inhibitors.

		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
WR Compound No.	Inhibitor	Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
	Isocoumarin Derivatives					
268195	3,4-dichloroisocoumarin	200	190	610		70
268397	4-chloro-3-(3-isothioureido-propoxy)isocoumarin	46,000	260,000	650,000	83,000	13,000
	Guanidinobenzoic Acid Derivatives					
268476	*p*-guanidinobenzoic acid	NI[g]				
184335	ethyl *p*-guanidinobenzoate	4.3	1.7	0.7		NI[g]
268570	N-(*p*-guanidinobenzoyl) valine amide	4.4	4.7	1.3	2,000	
268596	O-(*p*-guanidinobenzoyl) glycolamide	100	19%[h]	130,000	5.8	NI[g]

Table III (Continued). Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Phosphonate Inhibitors, Benzamidines, and Miscellaneous Inhibitors.

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
		Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
BM06304	4-cyanophenyl *p*-guanidinobenzoate	150,000	91,000			
	Phosphonate Inhibitors					
268694	Z-NHCH(AmPh)PO_3Ph_2[i]	2,000		16		
	Benzamidine Derivatives	K_I (μM)	% Inhibition[j]			
BM06804	1-(4-amidinophenyl)-3-phenylurea	23	41			
BM06840	1-(4-amidinophenyl)-3-(4-chlorophenyl)urea	18	51			
BM07481	1-(4-amidinophenyl)-3-benzylurea	19	49			
BM07829	1-(4-amidinophenyl)-3-(4-phenoxyphenyl)urea	1.6	71			
BM08004	(4-amidinobenzyl)benzyl ether	47	31			
BM08184	bis(4-amidinophenyl)urea	8				

Table III (Continued). Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Phosphonate Inhibitors, Benzamidines, and Miscellaneous Inhibitors.

		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
WR Compound No.	Inhibitor	Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
BM08308	1-(3-amidinophenyl)-3-phenylurea	50				
BM08595	(4-amidinobenzyl)phenylethyl ether	14				
BM08764	1-(3-amidinophenyl)-3-(4-phenoxyphenyl)urea	10				
BM09903	(4-amidinobenzyl)-3-phenylpropyl ether	16				
BM09912	(4-amidinobenzyl) 3-phenoxybenzyl ether	20				

Table III (Continued). Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Phosphonate Inhibitors, Benzamidines, and Miscellaneous Inhibitors.

		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
WR Compound No.	Inhibitor	Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
	Miscellaneous Inhibitors					
BM07490	1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole	14,000	600			
MA-76	1-benzoyloxy-1,2,3-benzotriazole	1,100				
BM07132	N-(2-isothiureidoethyl)pthalimide	NI				

[a]Inactivation rates were measured by an incubation method. Enzyme concentrations were: trypsin, 0.12 μM; rat skin tryptase, 0.015 μM; human skin tryptase, 0.12 μM and 0.02 μM; human r-*t*-PA, 0.017 μM Bovine trypsin was assayed with Z-Phe-Gly-Arg-NA·HCl (0.07 mM). Human lung tryptase, human skin tryptase and rat skin tryptase were assayed with Z-Arg-SBzl·HCl (0.07 mM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

[b]Conditions were as follows: 0.01 M Hepes, 0.01 M $CaCl_2$, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 1μM; *p*-guanidinobenzoic acid, 0.44 mM; ethyl *p*-guanidinobenzoate, 0.43 mM; *p*-guanidinobenzoyl valine amide,0.44 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.44 mM; 4-cyanophenyl *p*-

Table III (Continued).

guanidinobenzoate, 0.46 µM; 1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole, 1.22 µM; 1-benzoyloxy-1,2,3-benzotriazole, 22 µM.

[c]Conditions were as follows: 0.1M Hepes, 0.5M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureido-propoxy)isocoumarin, 0.42 µM; ethyl *p*-guanidinobenzoate, 0.42 mM; *p*-guanidinobenzoyl valine amide, 0.42 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.42 mM; 4-cyanophenyl *p*-guanidinobenzoate, 0.22 µM; 1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole, 11.6 µM.

[d]Conditions were as follows: 25 mM phosphate, 0.5M NaCl, 1mM EDTA, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 0.44 µM; ethyl *p*-guanidinobenzoate, 0.45 mM ; *p*-guanidinobenzoyl valine amide, 0.42 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.44 µM.

[e]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 0.35 µM; *p*-guanidinobenzoyl valine amide, 3.5 µM; O-(*p*-guanidinobenzoyl)glycolamide, 1.7 mM.

[f]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 4.3 µM; ethyl *p*-guanidinobenzoate, 0.44 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.43 mM.

[g]No inhibition.

[h]Inhibition was not time dependent and the % inhibition was measured at 0.42 mM.

[i] AmPh = 4-amidinophenyl

[j]Inhbitior concentrations were 226 µM.

Animal Testing Priorities

The various compounds which we have submitted have been prioritized for animal testing and the following table lists the priority which we have assigned to each compound and the reasons for that priority.

High-1 WR268195 3,4-dichloroisocoumarin
This isocoumarin is a general serine protease inhibitor and effectively inhibits most of the enzymes tested.

High-2 GP8 1-(amidinophenyl)-3-(4-phenoxyphenyl)urea
This benzamidine should be very stable in solution and is an excellant competitive inhibitor of trypsin-like enzymes.

High-3 WR268241 Suc-Val-Pro-Phe^P(OPh)$_2$
This peptide phosphonate is a reactive and specific inhibitor for chymotrypsin-like enzymes including chymases.

High-4 WR268397 4-chloro-3-(3-isothioureidopropoxy)isocoumarin
This isocoumarin is a very reactive inhibitor for the rat skin tryptase.

High-5 WR268398 2-ethoxy-4H-3,1-benzoxazin-4-one
This benzoxazinone inhibitor is an effective inhibitor for elastase.

High-6 WR268141 N-furoylsaccharin
This saccharin is a general protease inhibitor and inhibits elastases and chymotrypsin-like enzyme quite effectively and is probably a moderate inhibitor for trypsin-like enzymes.

High-7 BM06304 4-cyanophenyl *p*-guanidinobenzoate
This guanidinium substituted derivative is an effective inhibitor for trypsin-like enzymes

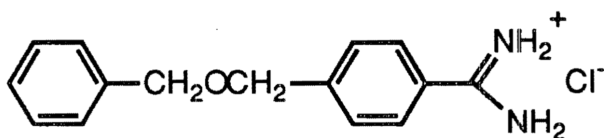
High-8 WR268440 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin
This isocoumarin is an effective elastase inhibitor.

High-9 WR268142 di(4-isovaleroylphenyl)sulfide
This aromatic derivative is an effective elastase inhibitor.

High-10 WR268387 4-chloro-3-benzyloxyisocoumarin
This isocoumarin is an effective inhibitor for chymotrypsin-like enzymes.

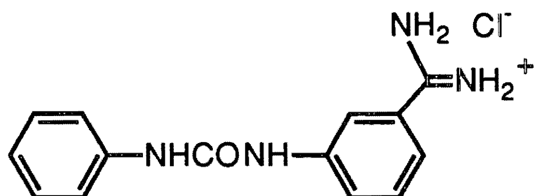
SYNTHESIS

Benzamidines. Six new benzamidines were synthesized in the final six months of the project We successfully overcame the initial difficulties in the synthesis of these class of compounds and submitted a total of eleven benzamidines. The last compounds submitted are (4-amidinobenzyl) benzyl ether hydrochloride (**GP 9**), bis(4-amidinophenyl)urea dihydrochloride (**GP 10**), 1-(3-amidinophenyl)-3-phenylurea hydrochloride (**GP 11**), (4-amidinobenzyl)phenylethyl ether hydrochloride (**GP 12**), 1-(3-amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (**GP 13**), (4-amidinobenzyl)-3-phenylpropyl ether hydrochloride (**GP 14**) and (4-amidinobenzyl)3-phenoxybenzyl ether hydrochloride (**GP 15**). The structures are shown below.

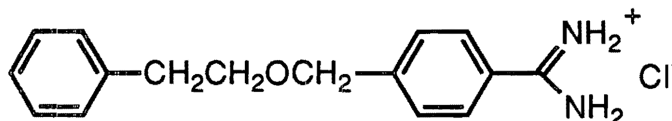


GP 9, BM08004

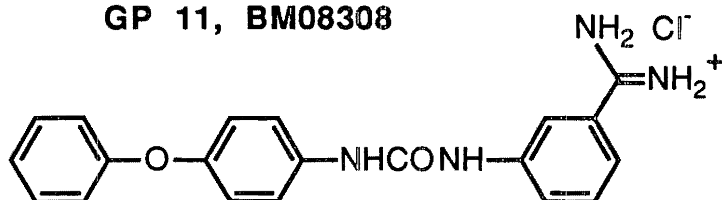
GP 10, BM08184



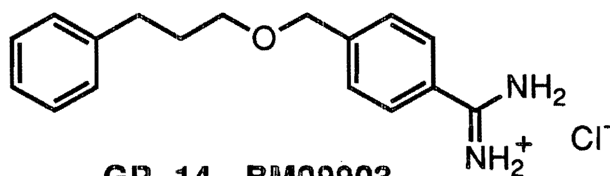
GP 11, BM08308



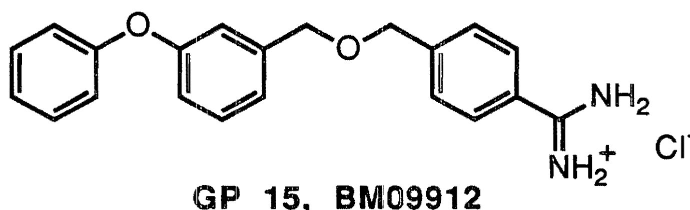
GP 12, BM08595



GP 13, BM08764



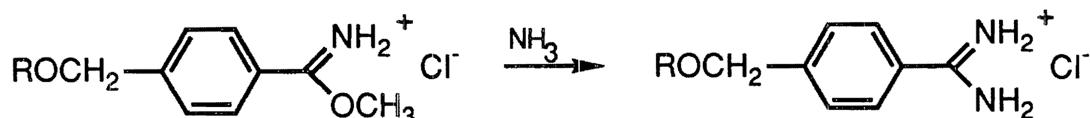
GP 14, BM09903



GP 15, BM09912

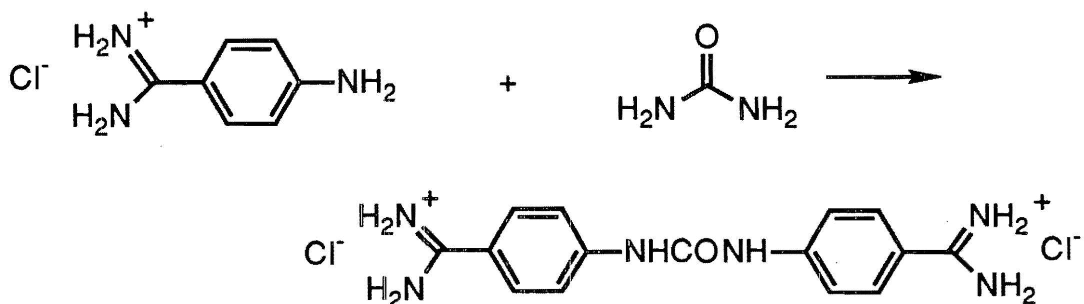
Synthetic Schemes. (4-Amidinobenzyl)benzyl ether (**GP 9**) was synthesized from the cyano ether obtained by the reaction of sodium benzyloxide and α-bromo-*p*-tolunitrile. The cyano ether was converted to the imidate ester by treating it with dry methanol in the presence of HCl. The imidate ester on reacting with ammonia gave the desired amidino compound.

(4-Amidinobenzyl)phenylethyl ether hydrochloride (**GP 12**) was prepared by replacing benzyl alcohol with phenylethyl alcohol and keeping the rest of the procedure as in **GP 9**.



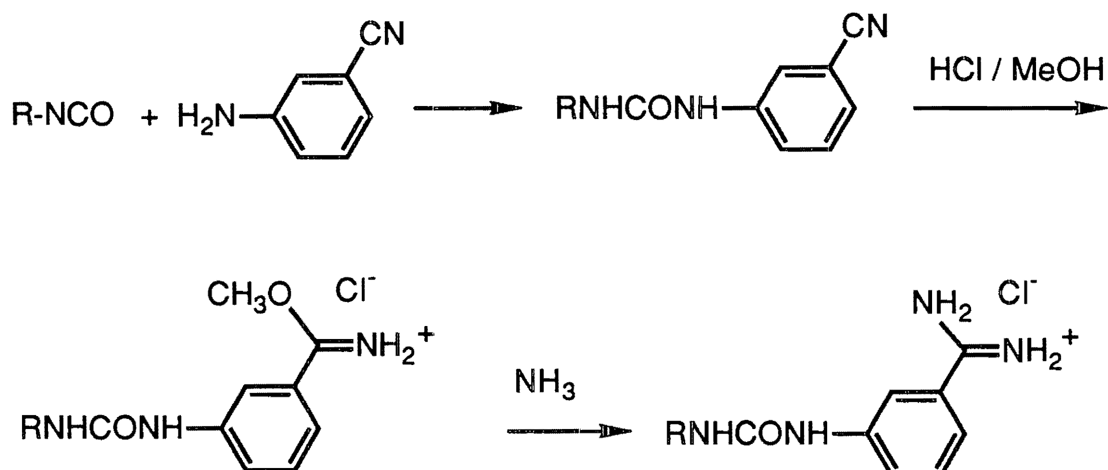
(4-Amidinobenzyl)phenylpropyl ether hydrochloride (**GP 14**) and (4-amidinobenzyl)3-phenoxybenzyl ether hydrochloride (**GP 15**) were also synthesized using the same method with ROH being phenylpropyl alcohol and 3-phenoxybenzyl alcohol respectively.

Bis(4-amidinophenyl)urea dihydrochloride (**GP 10**) was made by condensing 4-aminobenzamidine dihydrochloride with urea.



1-(3-Amidinophenyl)-3-phenylurea hydrochloride (**GP 11**) was prepared starting from 3-aminobenzonitrile. Phenyl isocyanate and 3-aminobenzonitrile were condensed in refluxing benzene and the resulting urea was converted to the imidate ester by the treatment with dry methanol in presence of dry HCl. Refluxing the imidate ester in dry isopropanol saturated with ammonia afforded the desired amidino compound.

1-(3-Amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (**GP 13**) was prepared using the procedure described above except that phenyl isocyanate was replaced with 4-phenoxyphenyl isocyanate.



EXPERIMENTAL SECTION

(4-Amidinobenzyl)benzyl ether hydrochloride (GP 9). A solution of benzyl alcohol (1.08 g, 0.01 mol) in dry THF (10 mL) was added slowly to sodium hydride (2.4 g, 0.10 mol) and the suspension stirred at room temperature for 15 min. To this stirred suspension was then added dropwise a solution of α-bromo-*p*-tolunitrile (1.96 g, 0.01 mol) in dry THF (10 mL) and the reaction stirred at room temperature for 24 h. Excess sodium hydride was destroyed by careful addition of water and the reaction taken up in ethyl acetate. The organic layer was washed successively with dil. HCl, water and brine, dried over anhydrous magnesium sulfate and solvent evaporated to get the cyano ether as a liquid.

Dry HCl was passed through a cooled solution of the cyano ether (1.00 g, 0.004 mol) and dry methanol (0.67 g, 0.020 mol) in dry dimethoxyethane (30 mL) for 1 h and the reaction stored in a refrigerator for 16 h. Solvent was then evaporated under reduced pressure and the imidate ester, obtained as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (1 g) was dissolved in dry isopropanol saturated with ammonia (50 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white crystals of the title compound (0.41 g, 44 %); mp 102-104 °C. ^{1}H NMR (DMSO-d_6) δ: 4.58 (s, 2H); 4.65 (s, 2H); 7.25-7.40 (m, 5H); 7.59 (d, 2H); 7.84 (d, 2H); 9.23 (bs, 2H); 9.41 (bs, 2H). Anal. Calcd. for $C_{15}H_{17}Cl_1N_2O$: C, 65.10; H, 6.19 Cl, 12.81; N, 10.12. Found: C, 64.98; H, 6.20; Cl, 12.73; N, 10.02.

Bis(4-amidinophenyl)urea dihydrochloride (GP 10). A suspension of urea (0.60 g, 0.01 mol) and 4-aminobenzamidine (4.16 g, 0.02 mol) in water (5 mL) was heated under reflux for 24 h. The condenser was then removed and the heating further continued for 24 h. The reaction was then trichurated with water (25 mL). The solid was filtered out dissolved in water and the clear solution acidified with HCl to get the product as a white solid

(3.1 g, 84 %); mp >250 °C. ^1H NMR (DMSO-d_6) δ: 7.68 (d, 4H); 7.84 (d, 4H); 9.00 (s, 4H); 9.23 (d, 4H); 10.45 (s, 2H). Anal. Calcd. for $C_{15}H_{18}Cl_2N_6O \cdot 0.3\ H_2O$: C, 48.07; H, 4.96; Cl, 18.96; N, 22.43. Found: C, 48.06; H, 5.05; Cl, 19.03; N, 22.55.

1-(3-Amidinophenyl)-3-phenylurea hydrochloride (GP 11). To a stirred solution of 3-aminobenzonitrile (4.72 g, 0.04 mol) in benzene (100 mL) was added phenyl isocyanate (5.00 g, 0.042 mol) and the reaction refluxed for 5 h and further stirred at room temperature overnight. The separated white solid was filtered out and recrystallised from methanol (8.2 g, 84 %).

Dry HCl was passed through a cooled solution of the cyano urea (4.00 g, 0.016 mol) and dry methanol (2.7 g, 0.08 mol) in dry dimethoxyethane (100 mL) for 2 h and the reaction stored in a refrigerator for 14 h. Solvent was then evaporated under reduced pressure and the imidate ester obtained, as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (4 g) was dissolved in dry isopropanol saturated with ammonia (200 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white solid of the title compound (2.1 g, 55 %); mp 252-254 °C. ^1H NMR (DMSO-d_6) δ: 6.98 (t, 1H); 7.20-7.35 (m, 3H); 7.40-7.55 (m, 3H); 7.70 (d, 1H); 7.95 (s, 1H); 9.1 (bs, 2H); 9.32 (bs, 2H); 9.45 (bs, 1H); 9.18 (bs, 1H). Anal. Calcd. for $C_{14}H_{15}Cl_1N_4O$: C, 57.83; H, 5.20 Cl, 12.19; N, 19.27. Found: C, 57.74; H, 5.20; Cl, 12.16; N, 19.21.

(4-Amidinobenzyl)phenylethyl ether hydrochloride (GP 12). This compound was prepared using the procedure described for compound **GP 9** and replacing benzyl alcohol with phenyl ethyl alcohol. ^1H NMR (DMSO-d_6) δ: 2.88 (t, 2H); 3.69 (t, 2H); 4.60 (s, 2H); 7.15-7.30 (m, 5H); 7.49 (d, 2H); 7.80 (d, 2H); 9.17 (bs, 2H); 9.37 (bs, 2H). Anal. Calcd. for $C_{14}H_{14}Cl_2N_4O$: C, 66.09; H, 6.59; Cl, 12.19; N, 9.63. Found: C, 66.18; H, 6.61; Cl, 12.09; N, 9.56.

1-(3-Amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (GP 13). This compound was prepared using the

procedure described for compound **GP 11** and substituting 4-phenoxyphenyl isocyanate for phenyl isocyanate. ^1H NMR (DMSO-d_6) δ: 6.91-7.20 (m, 4H); 7.10 (t, 1H); 7.30-7.40 (m, 3H); 7.45-7.58 (m, 3H); 7.70(d, 1H); 7.95 (s, 1H); 9.00 (s, 2H); 9.34 (s, 1H); 9.36 (s, 2H); 9.55 (s, 1H). Anal. Calcd. for $C_{20}H_{19}Cl_1N_4O_2 \cdot 0.75 H_2O$: C, 60.60; H, 5.17; Cl, 8.96; N, 14.14. Found: C, 60.62; H, 5.19; Cl, 8.96; N, 14.05.

(4-Amidinobenzyl)-3-phenylpropyl ether hydrochloride (GP 14). A solution of 3-phenylpropyl alcohol (2.72 g, 0.02 mol) in dry THF (20 mL) was added slowly to sodium hydride (2.4 g, 0.10 mol) and the suspension stirred at room temperature for 15 min. To this stirred suspension was then added dropwise a solution of α-bromo-*p*-tolunitrile (3.92 g, 0.02 mol) in dry THF (20 mL) and the reaction stirred at room temperature for 24 h. Excess sodium hydride was destroyed by careful addition of water and the reaction taken up in ethyl acetate. The organic layer was washed successively with dil. HCl, water and brine, dried over anhydrous magnesium sulfate and solvent evaporated to get the cyano ether as a liquid.

Dry HCl was passed through a cooled solution of the cyano ether (1.00 g, 0.004 mol) and dry methanol (0.67 g, 0.020 mol) in dry dimethoxyethane (30 mL) for 1 h and the reaction stored in a refrigerator for 16 h. Solvent was then evaporated under reduced pressure and the imidate ester, obtained as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (1 g) was dissolved in dry isopropanol saturated with ammonia (50 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. The solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white crystals of the title compound (0.295 g, 31 %); mp 76-78 °C. ^1H NMR (DMSO-d_6) δ: 1.84 (m, 2H); 2.62 (t, 2H); 3.45 (t, 2H); 4.55 (s, 2H); 7.10-7.90 (aromatic, 9H); 9.20 (bs, 2H); 9.45 (bs, 2H). Anal. Calcd. for $C_{17}H_{21}Cl_1N_2O$: C, 66.99; H, 6.94 Cl, 11.63; N, 9.19. Found: C, 67.05; H, 6.95; Cl, 11.54; N, 9.20.

(4-Amidinobenzyl)-3-phenoxylbenzyl ether hydrochloride (GP 15). This compound was prepared using the procedure described for compound **GP 14** and replacing 3-phenylpropyl alcohol with 3-phenoxylbenzyl alcohol. ^1H NMR (DMSO-d_6) δ: 4.55 (s, 2H); 4.63 (s, 2H); 6.95-7.80 (aromatic, 13H); 9.16 (bs, 2H); 9.40 (bs, 2H). Anal. Calcd. for $C_{21}H_{21}Cl_1N_2O_2$: C, 68.38; H, 5.74; Cl, 9.61; N, 7.59. Found: C, 68.20; H, 5.78; Cl, 9.48; N, 7.44.

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