J. Med. Chem. **1998,** *41,* 1060−1067

2-Amino-4*H*-3,1-benzoxazin-4-ones as Inhibitors of C1r Serine Protease

Sheryl J. Hays,*,‡ Bradley W. Caprathe,‡ John L. Gilmore,‡ Nilam Amin,† Mark R. Emmerling,† Walter Michael,† Ravi Nadimpalli,† Rathna Nath,† Kadee J. Raser,† Daniel Stafford,† Desiree Watson,† Kevin Wang,† and Juan C. Jaen‡

Departments of Chemistry and Neuroscience Therapeutics, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, Michigan 48105

Received June 13, 1997

A series of 2-amino-4*H*-3,1-benzoxazin-4-ones have been synthesized and evaluated as inhibitors of the complement enzyme C1r. C1r is a serine protease at the beginning of the complement cascade, and complement activation by β-amyloid may represent a major contributing pathway to the neuropathology of Alzheimer's disease. Compounds such as 7-chloro-2-[(2-iodophenyl)-amino]benz[*d*][1,3]oxazin-4-one (**32**) and 7-methyl-2-[(2-iodophenyl)amino]benz[*d*][1,3]oxazin-4-one (**37**) show improved potency compared to the reference compound FUT-175. Many of these active compounds also possess increased selectivity for C1r compared to trypsin and enhanced hydrolytic stability relative to 2-(2-iodophenyl)-4*H*-3,1-benzoxazin-4-one (**1**).

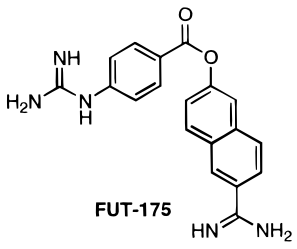
Introduction

The complement system is an important part of the host's immune response to foreign antigens.[1] The classical complement pathway involves the sequential activation of nine major proteins or protein complexes designated as C1−C9 (Figure 1). This activation requires the interaction of the C1q subunit of C1 with an antigen−antibody complex. Following activation, a highly regulated cascade of events occurs in which discrete interactions of soluble proteins catalyze subsequent steps. The final step in the pathway is the formation of a membrane attack complex which creates a pore in any accessible cellular membrane causing lethal and sublethal damage. The complement cascade can additionally increase vascular permeability and promote phagocytic recognition by chemotaxis.

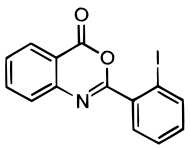
Alzheimer's disease (AD) is the most common degenerative dementia affecting primarily the elderly population. The disease is characterized by the decline of multiple cognitive functions and a progressive loss of neurons in the central nervous system. Deposition of β-amyloid peptide in the form of plaques is a classical feature of the disease, and these deposits have recently been shown to activate the classical complement system (Figure 1).[2,3] This activation by β-amyloid may represent a major contributing event to the neuropathology of AD.[4−6] These initial observations which suggest the existence of an inflammatory component in the neurodegeneration observed in AD have been extended to the clinic. A small clinical study using the nonsteroidal antiinflammatory drug indomethacin indicated that indomethacin significantly slowed the progression of the disease.[7] Additional studies with various antiinflammatory drugs have confirmed this initial observation.[8] Specific inhibition of the complement system may represent another approach to modulate the neuroinflammatory component of AD. While there are many potential targets for the prevention of complement activation, we have chosen to explore C1r inhibition.

C1r is a trypsin-like serine protease that is part of the C1 complex (one C1q, two C1r, and two C1s molecules) and is located at the beginning of the classical complement cascade. C1r is responsible for the proteolytic cleavage of C1s, the next protein in the pathway.[9] By selecting a target that is early in the cascade, it should be possible to prevent many of the amplification events that occur once the cascade is fully activated.

6-Amidino-2-naphthyl-4-guanidinobenzoate (FUT-175)[10] and 2-(2-iodophenyl)-4*H*-3,1-benzoxazin-4-one (**1**)[11] have been shown to act as inhibitors of C1r. FUT-175 has demonstrated little selectivity for C1r over other serine proteases, while compound **1** suffers from a lack of chemical stability. A series of 2-amino-benzo[*d*][3,1]-oxazin-4-ones have been targeted to overcome these difficulties. The synthesis and C1r inhibition for this series of compounds are reported here.



FUT-175



1

Chemistry

2-Aminobenz[*d*][1,3]oxazin-4-one (**2**) was synthesized from anthranilic acid and cyanogen bromide as described previously (see Scheme 1).[12] Treatment of **2** with sodium hydride and trifluoroethyl iodide afforded the alkylated product **3**.

The syntheses of the majority of the benzoxazinones are shown in Scheme 2. An appropriately substituted methyl anthranilate **I** was treated with an isocyanate to produce the urea **III** in moderate to good yield. Alternatively, the methyl anthranilate isocyanate **II** (purchased or prepared from the methyl anthranilate **I** and phosgene) was reacted with an amine to form the

‡ Department of Chemistry.
† Department of Neuroscience Therapeutics.

S0022-2623(97)00394-4 CCC: $15.00
Published on Web 03/05/1998

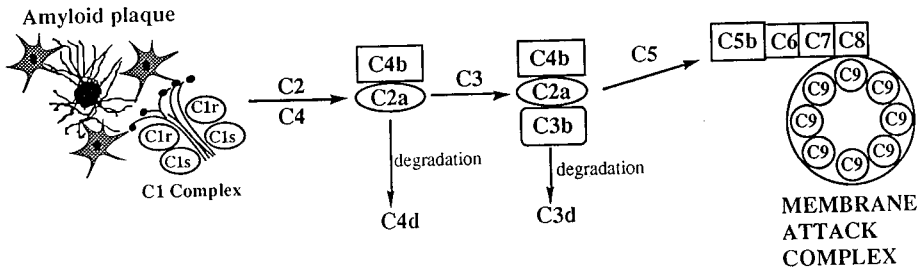
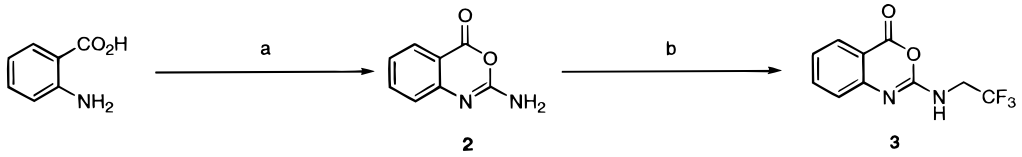


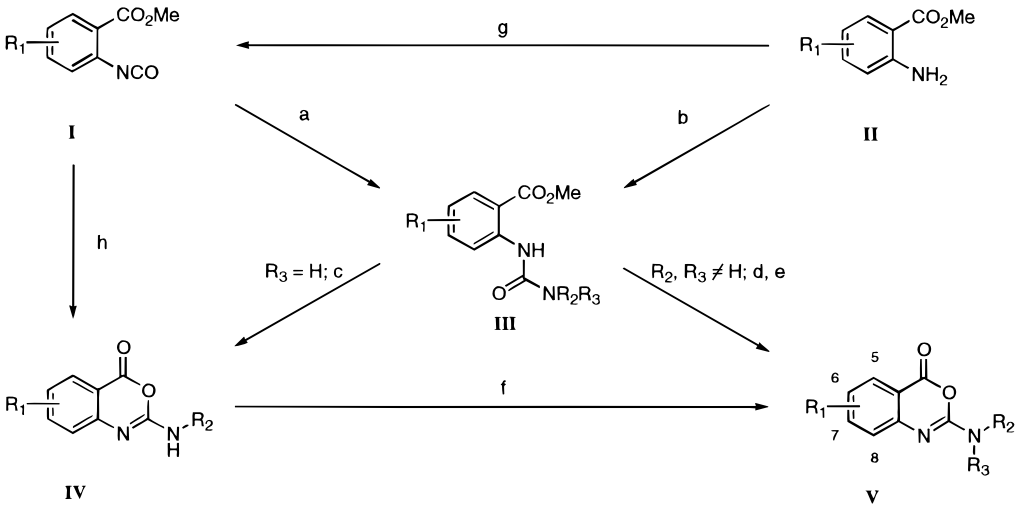
Figure 1. Activation of the classical complement pathway by β-amyloid.

Scheme 1[a]



[a] (a) CNBr, NaOH, H_2O; (b) CF_3CH_2I, NaH, DMF, 150 °C.

Scheme 2[a]



[a] (a) HNR_2R_3, pyridine or THF or toluene; (b) R_3 = H, R_2-N=C=O, pyridine or THF or toluene; (c) H_2SO_4, rt, N_2; (d) aq NaOH, EtOH, rt; (e) EDCI·HCl, CH_2Cl_2, rt; (f) NaH, R_3I, THF, 60 °C; (g) phosgene, HCl, toluene, EtOAc, reflux; (h) H_2NR_2, NaH, THF or DMF, 50 °C. Method A: steps a, c. Method B: steps b, c. Method C: step h.

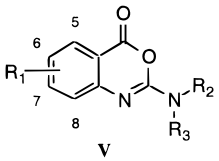
intermediate urea **III**. When R_3 was hydrogen, the urea **III** was cyclized with concentrated sulfuric acid to produce the requisite benzoxazinones **IV**. This product **IV** could be further reacted by treatment with sodium hydride and methyl or ethyl iodide to obtain the alkylated derivative **V**. The urea **III** could also be cyclized to the final heterocyclic target by a two-step procedure using sodium hydroxide to hydrolyze the ester, followed by ring closure with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) to produce the desired benzoxazinone **V**. Physical data and method of synthesis for the benzoxazinones and intermediate ureas are summarized in Table 1.

Biological Testing

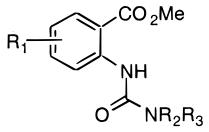
Benzoxazinones were assayed for inhibition of purified human C1r using CbzGly-Arg-S-Bzl as a substrate. This substrate was chosen as a result of the more comprehensive substrate specificity study using human C1r described previously by McRae and co-workers.[13] The compounds were dissolved in DMSO, diluted to concentrations ranging from 1 μM to 0.01 mM with Tris buffer, and assayed immediately. Enzyme activity was monitored by the production of benzyl mercaptan using standard protocols.[13] All assays were performed in triplicate at each inhibitor concentration, and the results are expressed as the mean IC_{50}. In all cases, standard errors are ≤10% of the mean value. To determine a compound's stability in aqueous buffer, the benzoxazinone was allowed to incubate in assay buffer for 60 min, and the enzyme assay was repeated. The loss of enzyme inhibition from the "initial" value to the "60-min" value is assumed to reflect the aqueous hydrolysis of the benzoxazinone ring to its inactive acyclic form. The results of the enzyme assays can be seen in Table 2.

To explore the benzoxazinone's selectivity for C1r versus other serine proteases, trypsin inhibition was examined for the most potent of the C1r inhibitors. A functional test for complement inhibition was performed on selected inhibitors of C1r. Complement hemolytic activity was measured by the hemolysis of sheep erythrocytes that have been sensitized by specific antibodies against the erythrocytes. The degree of hemolysis is directly correlated to the total complement activity. The results of the trypsin and complement inhibition studies are shown in Table 3.

Table 1. Synthetic Data



V



III

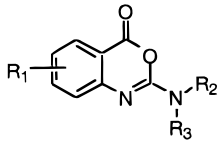
2-amino-4*H*-3,1-benzoxazin-4-one product (**V**)						corresponding urea starting material (**III**)	
no.	R_1	R_2	R_3	method (yield)	mp, °C	no.	mp, °C (yield)
2	H	H	H	*a* (92%)	200−202		
3	H	CF_3CH_2	H	*b* (11%)	244−245		
4	H	Me	H	A (80%)	202−203	**54**	120−121 (63%)
5	5-Me	$(CH_3)_2CH$	H	B[a] (35%)	194−197	**55**	not taken (52%)
6	H	C_6H_5	H	*c*	190−191	**56**	141−142 (85%)
7	H	2-I-C_6H_4	H	B[b,d] (86%)	140−143	**57**	162−164 (78%)
8	H	3-I-C_6H_4	H	A (81%)	237−239	**58**	167−168 (60%)
9	H	4-I-C_6H_4	H	A (67%)	190−224	**59**	190−192 (71%)
10	H	2-I-C_6H_4	Me	A[b]	104−108	**60**	144−148 (80%)
11	H	2-I-C_6H_4	Et	*b* (24%)	129−130		
12	H	2-I-$C_6H_4CH_2$	H	A (92%)	202−203	**61**	169−170 (60%)
13	H	2-I-$C_6H_4CH_2CH_2$	H	A[b] (94%)	151−153	**62**	132−135 (86%)
14	H	2-F-C_6H_4	H	A (99%)	152−155	**63**	178−179 (56%)
15	H	2-Cl-C_6H_4	H	A (79%)	146−149	**64**	179−180 (25%)
16	H	2-Br-C_6H_4	H	A (91%)	153−155	**65**	not taken (27%)
17	H	2-Me-C_6H_4	H	*c* (34%)	157−159	**66**	201−202 (92%)
18	H	2-(OCF_3)-C_6H_4	H	A (60%)	168−169	**67**	140−142 (75%)
19	H	2-(SMe)-C_6H_4	H	C (11%)	256−258		
20	H	2,6-diCl-C_6H_3	H	A (55%)	261−263	**68**	205−206 (33%)
21	H	2,5-diCl-C_6H_3	H	*c* (62%)	176−179	**69**	219−222 (72%)
22	H	2,6-diMe-C_6H_3	H	B (84%)	198.5−200	**70**	237−239 (94%)
23	H	3-thiophenylmethyl	H	C (57%)	251−254		
24	H	2-(1,3,4-thiadiazolyl)	H	A (31%)	276−277	**71**	not taken (95%)
25	H	2-(1,3,4-triazolyl)	H	C (15%)	326−329 dec		
26	H	2-thiazolidinyl	H	C (24%)	288 dec		
27	H	2-thiazolyl	H	C (18%)	290−293		
28	H	3-tetrahydrothiophenyl	H	C (71%)	287−289		
29	7-NMe_2	2-I-C_6H_4	H	B (46%)	231−232	**72**	not taken (70%)
30	5-Cl	2-I-C_6H_4	H	B (51%)	187−189	**73**	183−185 (80%)
31	6-Cl	2-I-C_6H_4	H	B (63%)	188−191	**74**	226−227 (53%)
32	7-Cl	2-I-C_6H_4	H	B (77%)	225−227	**75**	187−188 (25%)
33	6,7-diCl	2-I-C_6H_4	H	B (34%)	179−180	**76**	212−214 (33%)
34	5,7-diCl	2-I-C_6H_4	H	B (55%)	235−236	**77**	220−221 (30%)
35	5-Me	2-I-C_6H_4	H	B (56%)	203−204	**78**	185−187 (53%)
36	6-Me	2-I-C_6H_4	H	C (4%)	275−277		
37	7-Me	2-I-C_6H_4	H	B (79%)	174−176	**79**	189−190 (64%)
38	6-OMe	2-I-C_6H_4	H	B (40%)	156−157	**80**	not taken (46%)
39	7-OMe	2-I-C_6H_4	H	C (15%)	214−218		
40	6,7-diOMe	2-I-C_6H_4	H	B (59%)	214−216	**81**	178−180 (70%)
41	7-NO_2	2-I-C_6H_4	H	B[d] (44%)	237−239	**82**	178−181 (87%)
42	7-F	2-I-C_6H_4	H	B (72%)	186−189	**83**	186−187 (29%)
43	6,7-diF	2-I-C_6H_4	H	B (58%)	173−174	**84**	241−243 (19%)
44	7-CF_3	2-I-C_6H_4	H	B (37%)	177−179	**85**	177−178 (30%)
45	7-$(CH_3)_2CH$	2-I-C_6H_4	H	B (71%)	121−123	**86**	93−95 (84%)
46	7-cyclohexyl	2-I-C_6H_4	H	B (63%)	162−164	**87**	194−198 (79%)
47	6,7-CH=CH−CH=CH−	2-I-C_6H_4	H	B[d] (64%)	190−194	**88**	210−211 (36%)
48	7-Cl	2-Cl-C_6H_4	H	B (43%)	209.5−211	**89**	207−209 (27%)
49	6,7-diCl	2-Cl-C_6H_4	H	B (47%)	237−238	**90**	198−200 (82%)
50	7-Cl	2,6-diCl-C_6H_3	H	B[d] (53%)	225−229	**91**	180−183 (40%)
51	7-Me	2-Cl-C_6H_4	H	B (69%)	153−156	**92**	183−187 (43%)
52	7-NO_2	2-Cl-C_6H_4	H	B (56%)	243−246	**93**	186−188 (72%)
53	7-CF_3	2,6-diCl-C_6H_3	H	B (15%)	208−211	**94**	160−162 (74%)

[a] Reference 12. [b] Procedure is described in the Experimental Section. [c] Reference 14. [d] Ethyl ester of the anthranilic acid.

Results and Discussion

Active benzoxazinones have been shown to exhibit a time-dependent inhibition of C1r. This is believed to occur via a nucleophilic attack of the active site serine of the enzyme on the carbonyl of the heterocycle to produce acyl enzyme intermediates. Substitution at the 2-position of the benzoxazinone is a sensitive parameter that affects enzyme activity as well as chemical stability. Directly bonded aryl derivatives such as compound **1** (IC_{50} = 16.7 μM) are potent inhibitors of C1r as described previously but are hydrolytically labile.[11] Replacement of the aryl substituent by primary or aliphatic amines has been used as a successful strategy to inhibit the serine protease human elastase and improve benzoxazinone stability[12] but was unsuccessful for inhibiting C1r (compounds **2**−**6**, IC_{50} > 62.5 μM). Additionally, benzyl- and phenethylamine derivatives **12** and **13** were also inactive.

Table 2. C1r Inhibition by 2-Amino-4*H*-3,1-benzoxazinones



				C1r inhibition (IC_{50}, μM)	
no.	R_1	R_2	R_3	initial	60 min
FUT-175				12	12
1				16.7	50
2	H	H	H	>62.5	
3	H	CF_3CH_2	H	>62.5	
4	H	Me	H	>62.5	
5	5-Me	$(CH_3)_2CH$	H	>62.5	
6	H	C_6H_5	H	>62.5	
7	H	2-I-C_6H_4	H	3.5	14.7
8	H	3-I-C_6H_4	H	>62.5	
9	H	4-I-C_6H_4	H	>62.5	
10	H	2-I-C_6H_4	Me	1.4	16.6
11	H	2-I-C_6H_4	Et	>62.5	
12	H	2-I-$C_6H_4CH_2$	H	>62.6	
13	H	2-I-$C_6H_4CH_2CH_2$	H	>62.5	
14	H	2-F-C_6H_4	H	50	>62.5
15	H	2-Cl-C_6H_4	H	6.5	8.0
16	H	2-Br-C_6H_4	H	5.1	15.2
17	H	2-Me-C_6H_4	H	31	38
18	H	2-OCF_3-C_6H_4	H	4.0	>62.5
19	H	2-SMe-C_6H_4	H	>62.5	
20	H	2,6-diCl-C_6H_3	H	0.9	0.9
21	H	2,5-diCl-C_6H_3	H	2.0	2.0
22	H	2,6-diMe-C_6H_3	H	>62.5	
23	H	3-thiophenylmethyl	H	>62.5	
24	H	2-(1,3,4-thiadiazolyl)	H	>62.5	
25	H	2-(1,3,4-triazolyl)	H	>62.5	
26	H	2-thiazolidinyl	H	>62.5	
27	H	2-thiazolyl	H	>62.5	
28	H	3-tetrahydrothiophenyl		>62.5	
29	7-$N(CH_3)_2$	2-I-C_6H_4	H	>62.5	
30	5-Cl	2-I-C_6H_4	H	14.5	>62.5
31	6-Cl	2-I-C_6H_4	H	4.0	>62.5
32	7-Cl	2-I-C_6H_4	H	0.7	0.9
33	6,7-diCl	2-I-C_6H_4	H	0.5	>62.5
34	5,7-diCl	2-I-C_6H_4	H	>62.5	
35	5-Me	2-I-C_6H_4	H	>62.5	
36	6-Me	2-I-C_6H_4	H	>62.5	
37	7-Me	2-I-C_6H_4	H	0.8	1.5
38	6-OMe	2-I-C_6H_4	H	55	>62.5
39	7-OMe	2-I-C_6H_4	H	>62.5	
40	6,7-diOMe	2-I-C_6H_4	H	47	47
41	7-NO_2	2-I-C_6H_4	H	0.8	7.8
42	7-F	2-I-C_6H_4	H	1.7	2.8
43	6,7-diF	2-I-C_6H_4	H	7.0	>62.5
44	7-CF_3	2-I-C_6H_4	H	0.4	4.0
45	7-$(CH_3)_2CH$	2-I-C_6H_4	H	>62.5	
46	7-cyclohexyl	2-I-C_6H_4	H	>62.5	
47	6,7-CH=CH−CH=CH−	2-I-C_6H_4	H	0.2	16.6
48	7-Cl	2-Cl-C_6H_4	H	0.8	0.8
49	6,7-diCl	2-Cl-C_6H_4	H	2.0	>62.5
50	7-Cl	2,6-diCl-C_6H_4	H	0.4	0.9
51	7-Me	2-Cl-C_6H_4	H	1.4	0.9
52	7-NO_2	2-Cl-C_6H_4	H	1.4	>62.5
53	7-CF_3	2,6-diCl-C_6H_4	H	1.5	4.2

Appropriately substituted 2-anilinobenzoxazinones have demonstrated good activity as inhibitors of C1r. While no inhibition was observed for the unsubstituted aniline **6**, derivatives which contain at least one ortho substituent (compounds **7**, **15**, **16**, **18**, **20**, and **21**) have demonstrated improved potency relative to the reference agent FUT-175 (IC_{50} = 12 μM).[11] The best anilino substituents included the *o*-iodo (**7**, IC_{50} = 1.4 μM) and 2,6-dichloro (**20**, IC_{50} = 0.9 μM) analogues, while the *m*- and *p*-iodoanilino derivatives (compounds **8** and **9**) were inactive. Several ortho substituents which were small such as fluoro or electronically more neutral such as methyl and thiomethyl analogues (compounds **14**, **17**, and **19**, respectively) produced poor enzyme inhibition. Derivatives which replaced the ortho-substituted aniline group with a heterocycle (compounds **23**−**28**) demonstrated no enzyme inhibition. Changing R_3 from hydrogen to methyl produced an equipotent derivative (**10**, IC_{50} = 1.4 μM), while the slightly larger ethyl replacement abolished activity (**11**, IC_{50} > 62.5 μM).

Table 3. Biological Activity of Benzoxazinones

	IC_{50}, μM		
no.	initial C1r	trypsin	hemolysis
FUT-175	12	0.017	16.6
1	16.7	15.3	(61%)[a]
10	1.4	4.5	(72%)[a]
16	5.1	>100	>200
21	2.0	>100	(131)[a]
37	0.8	>100	(114)[a]
41	0.75	>100	(126%)[a]
42	1.7	>100	ND[b]
44	0.4	>100	(146%)[a]
48	0.8	>100	(138%)[a]
53	1.5	87.1	(120%)[a]

[a] Represents percent of control at 200 μM. [b] ND, not determined.

Substitution of the benzoxazinone ring also greatly affects enzyme activity. Chloro or methyl substitution of the 5-position (**30**, 5-chloro, $IC_{50} = 14.5$ μM; **34**, 5,7-dichloro, $IC_{50} > 62.5$ μM; **35**, 5-methyl, $IC_{50} > 62.5$ μM) reduced or abolished enzyme activity. This result was in contrast with the 5-substituted benzoxazinones prepared as human elastase inhibitors where 5-alkyl substitution was highly advantageous for enzyme inhibition.[12] Compounds that are substituted in the 6-position with a neutral or electron-donating group (**36**, 6-methyl, $IC_{50} > 62.5$ μM; **38**, 6-methoxy, $IC_{50} = 55$ μM; **40**, 6,7-dimethoxy, $IC_{50} = 47$ μM) showed little or no activity, while electron-withdrawing groups in this position (**31**, 6-chloro, $IC_{50} = 4.0$ μM; **33**, 6,7-dichloro, $IC_{50} = 0.5$ μM; **43**, 6,7-difluoro, $IC_{50} = 7.0$ μM) maintained or improved initial activity. Electron-donating groups in the 7-position of the benzoxazinone ring showed no activity (**29**, 7-dimethylamino; **39**, 7-methoxy), while neutral (**37**, 7-methyl, $IC_{50} = 0.8$ μM) or electron-withdrawing (**32**, 7-chloro, $IC_{50} = 0.7$ μM; **41**, 7-nitro, $IC_{50} = 0.8$ μM; **42**, 7-fluoro, $IC_{50} = 1.7$ μM; **44**, 7-trifluoromethyl, $IC_{50} = 0.4$ μM) groups demonstrated improved activity relative to the unsubstituted parent **7**. Neutral substituents in the 7-position that are relatively bulky compared to the methyl group (**45**, 7-isopropyl; **46**, 7-cyclohexyl) demonstrated no appreciable enzyme inhibition.

Stability of the compounds was assessed using the enzyme assay as described above. The advantage of this technique is that it provides a fast and easy method to determine a derivative's aqueous stability with the disadvantage that a compound must be biologically active for a "60-min" value to be obtained. Attempts to determine the half-life of a compound in aqueous buffer using ultraviolet spectroscopy were unsuccessful due to the multiple chromophores in the molecule. Although aliphatic amino substitution has been reported to increase benzoxazinone stability, anilino substitution does not necessarily improve compound stability relative to the 2-iodophenyl derivative **1**. Comparison of **1** with the 2-substituted iodoaniline derivative **7** shows a comparable decrease in enzyme inhibition after the 60-min incubation. Additionally, methyl substitution of the anilino nitrogen (compound **10**) further destabilizes the benzoxazinone ring structure. While these *o*-iodo derivatives do not improve stability, the *o*-chloro analogues do show increased hydrolytic stability with little or no benzoxazinone breakdown after 60 min (compounds **15**, **20**, and **21**). The smaller chloro group may provide less conformation strain than the larger iodo and bromo substituents, thereby increasing the compounds' aqueous stability.

Phenyl substitution of the benzoxazinone's benzene ring also profoundly influences the compounds' hydrolytic stability. The presence of a chloro, methyl, or fluoro group in the 7-position greatly stabilizes the heterocyclic ring structure to aqueous addition (compounds **32**, **37**, and **42**), while substitution at the 6-position has a deleterious effect (compounds **31**, **33**, **43**, and **47**). It is difficult to determine the influence of substitution at the 5-position since only one analogue exhibited enzymatic inhibition. This result (**30**, $IC_{50} = 14.5$ μM) suggests that this substitution pattern negatively influences hydrolytic stability.

In an effort to maximize C1r inhibition and to increase inhibitor stability, a series of 2-*o*-chloroanilino derivatives with electron-withdrawing groups in the 7-position were synthesized. Several of these compounds substituted with either a 7-chloro (**48**, $IC_{50} = 0.8$ μM; **50**, $IC_{50} = 0.4$ μM), 7-methyl (**51**, $IC_{50} = 1.4$ μM), or 7-trifluoromethyl (**53**, $IC_{50} = 1.5$ μM) are among the most stable and potent benzoxazinones tested for C1r activity. The 6,7-dichloro derivative **49** and 7-nitro analogue **52** are potent C1r inhibitors but are relatively unstable in aqueous media.

The benzoxazinones with the most potent C1r inhibitory activity and hydrolytic stability were examined for trypsin inhibition. This serves as a quick determination of the selectivity of the compound as an inhibitor of C1r versus other serine proteases. The reference compounds FUT-175 and **1** were better or equipotent inhibitors of trypsin compared to C1r, and it was one of the goals of the project to improve the compounds' selectivity. While the N-methylated analogue **10** shows no selectivity, all the other derivatives demonstrated increased inhibition of C1r when compared to trypsin (see Table 3).

These same compounds were also examined in a functional assay using erythrocyte hemolysis as a functional end point of complement inhibition. FUT-175 showed good activity in this model with an IC_{50} of 16.6 μM. The benzoxazinones, however, were marginally active at 200 μM (compound **1** or **10**) or showed no activity in this model. Several possible explanations exist for this lack of functional activity. Although these aminobenzoxazinones showed good aqueous stability, one of the components of this assay is human serum and these compounds may be less stable to enzymatic degradation. Alternatively, the inhibitors may be tightly binding to plasma proteins. In conclusion, while the initial goals of enhanced C1r inhibition, improved serine protease selectivity, and increased aqueous stability over the 2-arylbenzoxazinone series were achieved, the compounds proved to be inactive in the functional hemolysis assay. Additional series of benzoxazinones are under investigation to address this problem.

Experimental Section

Chemistry. All melting points were determined on a MELT-TEMP II capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were determined as KBr pellets on a Mattson Cygnus 100 or a Nicolet MX1 instrument. Proton magnetic resonance (NMR) was recorded on either a Varian XL-300 or a Bruker AM250 spectrometer; chemical shifts are reported in δ units relative to internal tetrameth-

ylsilane. All mass spectra were obtained on a Finnigan 4500 GCMS or a VG analytical 7070 E/F spectrometer. Elemental analyses were performed on a CEC model 240 elemental analyzer and are within 0.4% of the theoretical values unless otherwise indicated. Medium-pressure liquid chromatography utilized E. Merck silica gel, 230−400 mesh. All reactions were run under N_2, unless indicated otherwise. All analytical (C,H,N) and spectroscopic (^{1}H NMR, IR, MS) data are in agreement with the proposed structures.

Method A. 2-[[2-(2-Iodophenyl)ethyl]amino]benz[*d*][1,3]oxazin-4-one (13). Thionyl chloride (13.9 mL, 190.6 mmol) was added dropwise to a stirring solution of 2-iodophenylacetic acid (5.0 g, 19.1 mmol) in 500 mL of methylene chloride. The solution was refluxed for 4 h, cooled, and concentrated to a light-brown oil. Cold concentrated ammonium hydroxide (100 mL) was carefully added dropwise. Solid formed, and the mixture was filtered, washed with water, and vacuum-dried to yield 4.18 g (84%) of 2-(2-iodophenyl)-acetamide as a tan solid: mp 180−182 °C; ^{1}H NMR (DMSO-d_6) δ 3.56 (s, 2H), 6.96−7.00 (m, 2H), 7.30−7.36 (m, 2H), 7,43 (bs, 1H), 7.82 (dd, 1H, J = 0.8, 8.0 Hz). Anal. Calcd for C_8H_8INO: C, 36.81; H, 3.09; N, 5.37. Found: C, 36.90; H, 3.09; N, 5.22.

Boron trifluoride etherate (9.2 mL, 74.8 mmol) was added dropwise to a stirring suspension of 2-(2-iodophenyl)acetamide (3.9 g, 15.0 mmol) and sodium borohydride (2.1 g, 56.0 mmol) in 500 mL of THF maintained at 0 °C. The mixture was allowed to warm to room temperature overnight. The mixture was cooled to 0 °C, carefully quenched by dropwise addition of water (50 mL), concentrated to remove the THF, basified with concentrated ammonium hydroxide to a pH of 9−10, and then extracted into ethyl acetate. The ethyl acetate extract was washed with brine, dried ($MgSO_4$), filtered, and concentrated to a hygroscopic white solid. The HCl salt was made and crystallized from ethanol to give 3.1 g (73%) of 2-(2-iodophenyl)ethylamine hydrochloride as a white solid: mp 251−255 °C; ^{1}H NMR (DMSO-d_6) δ 2.96−3.04 (m, 4H), 7.01−7.05 (m, 1H), 7.34−7.41 (m, 2H), 7.87 (dd, 1H, J = 0.8, 7.8 Hz), 8.24 (bs, 3H). Anal. Calcd for $C_8H_{10}IN\cdot HCl$: C, 33.89; H, 3.91; N, 4.94. Found: C, 34.05; H, 3.83; N, 4.88.

A solution of 2-carbomethoxyphenyl isocyanate (0.55 g, 3.10 mmol), 2-(2-iodophenyl)ethylamine hydrochloride (0.80 g, 2.82 mmol), and *N*,*N*-diisopropylethylamine (0.75 mL, 4.30 mmol) in 100 mL of THF was stirred at room temperature for 24 h. The solution was concentrated and then partitioned between ethyl acetate and 10% HCl. The ethyl acetate extract was washed with brine, dried ($MgSO_4$), and concentrated. The residue was chromatographed (silica gel, 20% ethyl acetate in hexanes) to yield 1.02 g (86%) of methyl 2-[3-[2-(2-iodophenyl)ethyl]ureido]benzoate (**62**) as a white solid: mp 132−135 °C; ^{1}H NMR (DMSO-d_6) δ 2.88 (t, 2H, J = 7.4 Hz), 3.28−3.30 (m, 2H), 3.86 (s, 3H), 6.96−7.01 (m, 2H), 7.31−7.38 (m, 2H), 7.48−7.53 (m, 1H), 7.66 (bs, 1H), 7.84 (dd, 1H, J = 0.8, 7.8 Hz), 7.89 (dd, 1H, J = 1.6, 8.1 Hz), 8.36 (dd, 1H, J = 0.8, 8.6 Hz), 9.75 (bs, 1H). Anal. Calcd for $C_{17}H_{17}IN_2O_3$: C, 48.13; H, 4.04; N, 6.60. Found: C, 48.16; H, 4.09; N, 6.64.

Concentrated sulfuric acid (3.0 mL, 54.3 mmol) was added to **62** (0.60 g, 1.41 mmol), and the solid slowly dissolved. The sample was stirred at room temperature for 1.5 h and then carefully poured into a vigorously stirred mixture of sodium bicarbonate (14.0 g, 166.6 mmol, ca. 3 equiv vs H_2SO_4 used) in water (250 mL) and ethyl acetate (250 mL). The ethyl acetate extract was washed with brine, dried ($MgSO_4$), filtered, and concentrated to afford 0.52 g (94%) of the title compound as a white solid: mp 151−153 °C; FT-IR (KBr) 3298, 1746, 1632, 1476 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.09−3.13 (m, 2H), 3.68−3.73 (m, 2H), 4.97 (bs, 1H), 6.91−6.95 (m, 1H), 7.16−7.24 (m, 1H), 7.24−7.32 (m, 3H), 7.62−7.66 (m, 1H), 7.83 (d, 1H, J = 8.0 Hz), 8.02 (dd, 1H, J = 1.5, 7.8 Hz). Anal. Calcd for $C_{16}H_{13}IN_2O_2$: C, 49.00; H, 3.34; N, 7.14. Found: C, 48.93; H, 3.25; N, 7.07.

Method B. 2-[(2-Iodophenyl)amino]benz[*d*][1,3]oxazin-4-one (7). To a suspension of 2-iodoaniline hydrochloride (prepared from 5.7 g, 26.0 mmol, of 2-iodoaniline and 33 mL of 1 M HCl in ether solution) in 500 mL of toluene was added dropwise a 12.5% solution of phosgene in toluene (26 mL, 32.8 mmol) at room temperature. The mixture was refluxed for 1 h, cooled to room temperature, treated with another portion of phosgene in toluene solution (26 mL), and refluxed for an additional 12 h. The cloudy solution was concentrated, suspended into 250 mL of hexanes, and filtered through a pad of Celite. The filtrate was concentrated and distilled (bp 50−60 °C, 0.50−0.25 mmHg) to give 5.0 g (78%) of 2-iodophenyl isocyanate as a colorless liquid: ^{1}H NMR ($CDCl_3$) δ 6.88−6.92 (m, 1H), 7.13 (dd, 1H, J = 1.4, 8.0 Hz), 7.27−7.31 (m, 1H), 7.79 (dd, 1H, J = 1.4, 8.0 Hz). Anal. Calcd for C_7H_4INO: C, 34.32; H, 1.65; N, 5.72. Found: C, 34.38; H, 1.68; N, 5.67.

A solution of 2-iodophenyl isocyanate (3.36 g, 13.7 mmol) and ethyl 2-aminobenzoate (5.1 mL, 34.5 mmol) in 250 mL of THF was stirred at room temperature for 24 h. The solution was concentrated and then partitioned between ethyl acetate and 10% HCl. The ethyl acetate extract was washed with brine, dried ($MgSO_4$), and concentrated to an oily white solid. The sample was suspended in ether and the insoluble white solid that resulted from filtration yielded 4.38 g (78%) of ethyl 2-[3-(2-iodophenyl)ureido]benzoate (**57**): mp 162−164 °C; ^{1}H NMR ($CDCl_3$) δ 1.41 (t, 3H, J = 7.2 Hz), 4.37 (q, 2H, J = 7.2 Hz), 6.82−6.87 (m, 2H), 7.01−7.05 (m, 1H), 7.34−7.38 (m, 1H), 7.51−7.55 (m, 1H), 7.80 (dd, 1H, J = 1.4, 8.0 Hz), 7.99 (dd, 1H, J = 1.7, 8.2 Hz), 8.03 (dd, 1H, J = 1.7, 8.0 Hz), 8.51 (dd, 1H, J = 1.0, 8.4 Hz), 10.76 (s, 1H). Anal. Calcd for $C_{16}H_{15}IN_2O_3$: C, 46.85; H, 3.69; N, 6.83. Found: C, 46.75; H, 3.62; N, 6.56.

A solution of ethyl 2-[3-(2-iodophenyl)ureido]benzoate (**57**; 0.50 g, 1.22 mmol) in concentrated sulfuric acid (10 mL, 181.0 mmol) was stirred at 50 °C for 1 h. The solution was cooled to room temperature and poured into ice−water. After neutralization with saturated $NaHCO_3$ solution, the product was collected by filtration. The solid was washed with water and vacuum-dried to yield 0.38 g (86%) of **7** as an off-white solid: mp 140−143 °C; FT-IR (KBr) 1763 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.85−6.90 (m, 1H), 7.26−7.32 (m, 2H), 7.41−7.45 (m, 1H), 7.68−7.73 (m, 1H), 7.82 (dd, 1H, J = 1.4, 8.0 Hz), 8.11 (dd, 1H, J = 1.6, 7.8 Hz), 8.44 (dd, 1H, J = 1.6, 8.1 Hz). Anal. Calcd for $C_{14}H_9IN_2O_2$: C, 46.18; H, 2.49; N, 7.69. Found: C, 46.13; H, 2.43; N, 7.51.

2-[*N*-(2-Iodophenyl)-*N*-methylamino]benz[*d*][1,3]oxazin-4-one (10). A solution of 2-iodoaniline (6.0 g, 27.4 mmol) and ethyl formate (11.1 mL, 137.4 mmol) in 200 mL of THF was added dropwise to a stirring suspension of sodium hydride (1.4 g, 34.2 mmol, 60% dispersion in mineral oil washed twice with 50 mL of hexanes) in 250 mL of THF at room temperature. The mixture was stirred for 24 h, cooled in an ice bath, and quenched by dropwise addition of water. The sample was concentrated (to remove the THF) and then partitioned between ethyl acetate and saturated KH_2PO_4 solution. The ethyl acetate extract was washed with brine, dried ($MgSO_4$), filtered, and concentrated to give 6.3 g (93%) of *N*-(2-iodophenyl)formamide as an off-white solid: mp 118−119 °C; ^{1}H NMR (DMSO-d_6) δ 6.93−7.02 (m, 1H), 7.33−7.41 (m, 1H), 7.78 (dd, 1H, J = 1.1, 8.1 Hz), 7.88 (dd, 1H, J = 1.1, 7.8 Hz), 8.34 (s, 1H), 9.54 (bs, 1H). Anal. Calcd for C_7H_6INO: C, 34.03; H, 2.45; N, 5.67. Found: C, 34.16; H, 2.47; N, 5.67.

A suspension of sodium borohydride (3.6 g, 95.7 mmol) and *N*-(2-iodophenyl)formamide (6.3 g, 25.5 mmol) in 500 mL of THF was treated dropwise with boron trifluoride etherate (15.7 mL, 127.6 mmol) at 0 °C. The mixture was allowed to warm to room temperature overnight. The mixture was cooled to 0 °C and carefully quenched by dropwise addition of water (125 mL). The THF was removed under vacuum, and the residue was basified with concentrated ammonium hydroxide to a pH of 9−10 and then extracted with ethyl acetate. The organic extract was washed with brine, dried ($MgSO_4$), filtered, and concentrated to a tan oil. The residue was treated with HCl and crystallized from ethanol to give 5.1 g (74%) of *N*-methyl-2-iodoaniline hydrochloride as a white solid: mp 154−155 °C dec; ^{1}H NMR (DMSO-d_6) δ 2.77 (s, 3H), 6.48−6.52 (m, 1H), 6.70 (d, 1H, J = 8.0 Hz), 7.24−7.28 (m, 1H), 7.66 (dd, 1H, J =

1.4, 7.7 Hz), 7.80 (bs, 2H). Anal. Calcd for $C_7H_8IN \cdot HCl$: C, 31.20; H, 3.37; N, 5.20. Found: C, 30.90; H, 3.20; N, 5.17.

A solution of 2-carbomethoxyphenyl isocyanate (0.93 g, 5.25 mmol), *N*-methyl-2-iodoaniline hydrochloride (1.56 g, 5.79 mmol), and *N*,*N*-diisopropylethylamine (1.1 mL, 6.32 mmol) in 100 mL of toluene was refluxed for 36 h. The solution was concentrated and then partitioned between chloroform and saturated KH_2PO_4 solution. The chloroform extract was washed with brine, dried ($MgSO_4$), and concentrated. The residue was chromatographed (silica gel, CH_2Cl_2) to yield 1.18 g (55%) of methyl 2-[3-(2-iodophenyl)-3-methylureido]benzoate as a light-yellow solid: mp 144−148 °C; ^{1}H NMR ($CDCl_3$) δ 3.29 (s, 3H), 3.67 (s, 3H), 6.91−6.95 (m, 1H), 7.12−7.16 (m, 1H), 7.41 (dd, 1H, J = 1.6, 7.8 Hz), 7.46−7.52 (m, 2H), 7.87 (dd, 1H, J = 1.7, 8.0 Hz), 7.99 (dd, 1H, J = 1.4, 8.0 Hz), 8.60 (dd, 1H, J = 1.0, 8.7 Hz), 9.91 (bs, 1H). Anal. Calcd for $C_{16}H_{15}IN_2O_3$: C, 46.85; H, 3.69; N, 6.83. Found: C, 47.15; H, 3.77; N, 6.86.

A mixture of methyl 2-[3-(2-iodophenyl)-3-methylureido]-benzoate (0.50 g, 1.22 mmol) in 20 mL of ethanol and 0.1 N NaOH (17 mL, 1.70 mmol) was refluxed for 30 min. The solution was concentrated to remove the ethanol, acidified with 10% HCl to pH 1−2, and then extracted into ethyl acetate. The organic extract was washed with brine, dried ($MgSO_4$), filtered, and concentrated to give 2-[3-(2-iodophenyl)-3-methylureido]benzoic acid as a light-yellow solid. The solid was dissolved into 150 mL of chloroform (ethanol-free) and treated with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.26 g, 1.36 mmol). The solution was stirred at room temperature for 2 h and washed with saturated $NaHCO_3$ solution. The organic extract was dried ($MgSO_4$) and concentrated to give a colorless oil, which upon crystallization from ether−hexanes afforded 0.42 g (91%) of 2-[*N*-(2-iodophenyl)-*N*-methylamino]benz[*d*][1,3]oxazin-4-one as a light-yellow solid: mp 104−108 °C dec; FT-IR (KBr) 1749, 1620, 1603 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.44 (s, 3H), 7.08−7.12 (m, 1H), 7.17−7.24 (m, 1H), 7.31−7.46 (m, 3H), 7.62−7.65 (m, 1H), 7.93 (dd, 1H, J = 1.2, 8.0 Hz), 8.02 (dd, 1H, J = 1.2, 8.0 Hz). Anal. Calcd for $C_{15}H_{11}IN_2O_2$: C, 47.64; H, 2.93; N, 7.41. Found: C, 47.50; H, 3.00; N, 7.37.

Method C. 2-[[(3-Thiopheneyl)methyl]amino]benz[*d*]-[1,3]oxazin-4-one (23). 3-Thiophenecarboxaldehyde (10.0 g, 89 mmol), hydroxylamine hydrochloride (18.8 g, 270 mmol), and sodium acetate (22.1 g, 270 mmol) were combined in ethanol (175 mL) and water (175 mL) and refluxed overnight. The reaction mixture was cooled, and most of the ethanol was removed in vacuo. The sample was extracted into ethyl acetate (200 mL). The organic layer was dried ($MgSO_4$), and the volatiles were removed under reduced pressure. Water (400 mL) was added to the residue, and a crystalline product was obtained. The product was filtered and recrystallized from ethyl acetate−hexanes to afford 3.0 g (27%) of the oxime of 3-thiophenecarboxaldehyde. This oxime (1.03 g, 8.1 mmol) was dissolved in methanol saturated with ammonia (100 mL) and hydrogenated with Raney nickel (1.0 g). The reaction mixture was filtered, and the volatiles were removed under reduced pressure. A 1 N solution of HCl in ether (150 mL) was added to the crude residue, and a precipitate formed. The solid was filtered and washed well with ether to afford 0.582 g (49%) of (3-thienyl)methylamine hydrochloride as a solid: ^{1}H NMR (DMSO-d_6) δ 3.95−4.05 (s, 2H), 7.2−7.3 (m, 1H), 7.5−7.7 (m, 2H), 8.3−8.7 (bs, 1H).

To a suspension of (3-thienyl)methylamine hydrochloride (0.57 g, 3.80 mmol) in 10 mL of DMF was added sodium hydride (0.34 g of 60% dispersion in mineral oil, 8.50 mmol) at room temperature. After bubbling ceased, 2-carbomethoxyphenyl isocyanate (0.34 g, 8.4 mmol) was added. The sample was heated at 75 °C for 5 days, cooled to room temperature, and poured into water. A precipitate formed which was recrystallized from methanol−ethyl acetate to give 0.56 g (57%) of 2-[[(3-thiopheneyl)methyl]amino]benz[*d*][1,3]oxazin-4-one (**23**) as a solid: mp 251−254 °C; ^{1}H NMR (DMSO-d_6) δ 5.07 (s, 2H), 7.09−7.11 (dd, 1H, J = 1.2, 4.8 Hz), 7.18−7.23 (m, 2H), 7.37−7.38 (m, 1H), 7.45−7.47 (m, 1H), 7.63−7.68 (m, 1H), 7.93−7.96 (dd, 1H, J = 1.4, 8.4 Hz), 11.5 (s, 1H). Anal. Calcd for $C_{13}H_{10}N_2O_2S$: C, 60.45; H, 3.90; N, 10.85. Found: C, 60.24; H, 3.92; N, 10.72.

2-[(2,2,2-Trifluoroethyl)amino]benz[*d*][1,3]oxazin-4-one (3). 2-Aminobenz[*d*][1,3]oxazin-4-one (**2**; 1.0 g, 6.2 mmol), trifluoroethyl iodide (2.6 g, 12.4 mmol), and sodium hydride (0.32 g of a 60% oil dispersion, 8.0 mmol) were combined with DMF (25 mL) in a rocking autoclave and heated to 150 °C for 4 h. The reaction mixture was cooled and partitioned between CH_2Cl_2 (200 mL) and water (2 × 200 mL). The organic layer was dried ($MgSO_4$), and the volatiles were removed under reduced pressure. The crude residue was chromatographed (silica gel, 25% ethyl acetate in hexanes). The product was then recrystallized from ethyl acetate−hexanes to afford 170 mg (11%) of 2-[(2,2,2-trifluoroethyl)amino]benz[*d*][1,3]oxazin-4-one as a solid: mp 244−245 °C; FT-IR (KBr) 3075, 2946, 1728, 1669, 1453 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.68−4.78 (q, 2H, J = 9.2, 18.4 Hz), 7.20−7.28 (m, 2H), 7.69−7.75 (m, 1H), 7.95−7.98 (d, 1H, J = 7.9 Hz), 11.70 (bs, 1H). Anal. Calcd for $C_{10}H_7F_3N_2O_2$: C, 49.19; H, 2.89; N, 11.47. Found: C, 49.11; H, 3.07; N, 11.50.

2-[*N*-Ethyl-*N*-(2-iodophenyl)amino]benz[*d*][1,3]oxazin-4-one (11). 2-[(2-Iodophenyl)amino]benz[*d*][1,3]oxazin-4-one (**7**; 0.308 g, 0.85 mmol) was dissolved in THF (5 mL), and NaH (0.040 g of a 60% oil dispersion, 1.0 mmol) was added. The reaction mixture was stirred for 30 min, and ethyl iodide (0.312 g, 2.0 mol) was added. The reaction mixture was heated to 60 °C and stirred for 24 h. The reaction mixture was partitioned between water (200 mL) and ethyl acetate (200 mL). The ethyl acetate layer was dried ($MgSO_4$), and the volatiles were removed under reduced pressure. The crude residue was chromatographed (silica gel, 30% ethyl acetate in petroleum ether). This residue was then recrystallized from ether−hexanes to afford 80 mg (25%) of the desired product (**11**) as a white solid: mp 129−130 °C; FT-IR (KBr) 3422, 2980, 1713, 1664, 1609, 1481 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.39−1.43 (t, 3H, J = 7.0 Hz), 4.17−4.35 (m, 2H), 7.14−7.19 (m, 1H), 7.28−7.34 (m, 2H), 7.48−7.53 (m, 1H), 7.72−7.77 (m, 1H), 7.96−7.98 (dd, 1H, J = 1.4, 8.2 Hz), 8.28−8.31 (dd, 1H, J = 1.4, 8.0 Hz). Anal. Calcd for $C_{16}H_{13}IN_2O_2$: C, 49.00; H, 3.34; N, 7.14. Found: C, 49.24; H, 3.52; N, 7.03.

Preparation of Starting Materials. Methyl 2-Amino-4-isopropylbenzoate (Starting Material for 45 and 86). Nitric acid (63 g, 1 mol) was added to a solution of 4-isopropylbromobenzene (50 g, 0.25 mol) dropwise at 0 °C. The reaction mixture was stirred for 3 h, poured onto ice, and extracted with ether (2 × 500 mL). The organic layer was dried ($MgSO_4$), and the volatiles were removed under reduced pressure. The crude residue was distilled (1 mmHg, 105−129 °C) to afford 19.5 g (32%) of 2-nitro-4-isopropylbromobenzene: FT-IR (LF) 2966, 1535, 1358 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.26−1.28 (d, 6H, J = 7.0 Hz), 2.93−3.00 (m, 1H), 7.28−7.31 (dd, 1H, J = 2.2, 8.2 Hz), 7.62−7.64 (d, 1H, J = 8.4 Hz), 7.68−7.69 (d, 1H, J = 2.2 Hz). Anal. Calcd for $C_9H_{10}BrNO_2$: C, 44.29; H, 4.13; N, 5.74. Found: C, 43.91; H, 4.10; N, 5.48.

2-Nitro-4-isopropylbromobenzene (19.5 g, 80 mmol) and copper(I) cyanide (14.3 g, 160 mmol) were refluxed in DMF (250 mL) for 24 h. The reaction mixture was poured into water (500 mL) and extracted with ether (3 × 500 mL). The combined organic layers were filtered through Celite and washed with water (3 × 500 mL). The organic layer was dried ($MgSO_4$), and the volatiles were removed under reduced pressure. The crude residue was chromatographed (silica gel, CH_2Cl_2−petroluem ether, 1:1) to afford 4.3 g (28%) of 2-nitro-4-isopropylbenzonitrile: FT-IR (KBr) 2969, 1539, 1344 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.32−1.34 (d, 6H, J = 7.0 Hz), 3.08−3.13 (m, 1H), 7.65−7.68 (dd, 1H, J = 1.7, 8.0 Hz), 7.82−7.84 (d, 1H, J = 8.0 Hz), 8.18−8.19 (d, 1H, J = 1.7 Hz). Anal. Calcd for $C_{10}H_{10}N_2O_2$: C, 63.15; H, 5.30; N, 14.73. Found: C, 63.13; H, 5.22; N, 14.90.

2-Nitro-4-isopropylbenzonitrile (4.3 g, 22.6 mmol) was refluxed overnight in a mixture of water−acetic acid−H_2SO_4 (2:1:1, 60 mL). The reaction mixture was poured onto ice water (200 mL), basified with 1 N NaOH, and extracted with ether

(250 mL). The aqueous layer was separated, acidifed with concentrated HCl, and extracted with ether (250 mL). The organic layer was dried ($MgSO_4$), and the volatiles were removed under reduced pressure to afford 4.8 g (68%) of 2-nitro-4-isopropylbenzoic acid as a solid: FT-IR (KBr) 2968, 2663, 1703, 1535, 1356 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.22−1.25 (d, 6H, J = 7.0 Hz), 3.02−3.10 (m, 1H), 7.65−7.68 (dd, 1H, J = 1.4, 8.0 Hz), 7.79−7.83 (m, 2H). Anal. Calcd for $C_{10}H_{11}NO_4$: C, 57.41; H, 5.30; N, 6.70. Found: C, 57.78; H, 5.46; N, 6.84.

2-Nitro-4-isopropylbenzoic acid (3.09 g, 14.8 mmol) was hydrogenated with Raney nickel in water (100 mL) and NH_4OH (10 mL). The reaction mixture was filtered and acidified with concentrated HCl. A precipitate formed and was collected by filtration. The solid was heated in MeOH and filtered again to afford 1.2 g (45%) of 2-amino-4-isopropylbenzoic acid hydrochloride, which was carried on without further purification: ^1H NMR (DMSO-d_6) δ 1.05−1.20 (d, 6H, J = 7.0 Hz), 2.6−2.8 (m, 1H), 6.3−6.5 (m, 2H), 7.6−7.7 (m, 3H).

2-Amino-4-isopropylbenzoic acid hydrochloride was refluxed in H_2SO_4 (20 mL) and methanol (80 mL) overnight. The reaction mixture was poured onto ice water (400 mL) and extracted with methylene chloride (200 mL). The organic layer was washed with saturated $NaHCO_3$ (200 mL) and dried ($MgSO_4$), and the volatiles were removed under reduced pressure to afford 0.805 g (62%) of methyl 2-amino-4-isopropylbenzoate as a solid: mp 40−41 °C; FT-IR (KBr) 3472, 3367, 2960, 1691, 1622, 1437, 1307 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.1−1.34 (d, 6H, J = 7.0 Hz), 2.7−2.9 (m, 1H), 3.85 (s, 3H), 6.5−6.6 (m, 2H), 7.7−7.87 (d, 1H). Anal. Calcd for $C_{11}H_{15}NO_2$: C, 72.07; H, 8.21; N, 6.00. Found: C, 71.87; H, 8.04; N, 5.97.

Biological Methods. Inhibition of C1r (Initial): Compounds to be tested were dissolved in DMSO at concentrations ranging from 1 mM to 0.01 mM, and a 10-μL aliquot of each concentration was deposited into a well of a 96-well microtiter plate; 50 μL of Cbz-Gly-Arg-S-Bzl (1.5 mM in DMSO/H_2O, 3:7) and 50 μL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) solution (0.75 mM in 150 mM Tris buffer) were dispensed in the wells of the microtiter plate, followed by the immediate addition of 50 μL of the C1r enzyme solution (20 μg/mL purified human C1r in 10 mM Tris buffer (pH 7.4)). After a 30-s incubation, the hydrolyzed substrate was read in the kinetic mode using a Molecular Devices Thermomax microplate reader set to a wavelength of 405 nm. The assay background was followed by monitoring wells that contain substrate, buffer, and DTNB solutions but no enzyme. All assays were performed in triplicate at each inhibitor concentration tested. The results are expressed in IC_{50} values.

Inhibition of C1r (60 min): A 50-μL aliquot of 150 mM Tris buffer (pH 7.5) was added to wells containing 10 μL of inhibitor in DMSO. The mixture sat at room temperature for 60 min; then 50 μL of substrate solution, 50 μL of DTNB, and 50 μL of enzyme solution were added to the wells to start the reaction. The plate was read as described above.

Inhibition of Trypsin: A microplate caseinolysis assay was carried out as previously described.[15] Briefly, 0.5 mg/mL casein (sodium salt; Sigma Co.), 20 mM dithiothreitol, and 50 mM Tris-HCl (pH 7.4) were mixed in the presence of various concentrations of an inhibitor. After 1.25 μg of trypsin (bovine pancreas; Sigma Co.) was added, the microtiter plate (250 μL) was incubated for 60 min at 25 °C. The samples were processed for colorimetric development and read on a Molecular Devices Thermomax microplate reader at an absorbance of 595 nm. Based on a plot of percent inhibition of trypsin against log [inhibitor], an IC_{50} was generated using Sigma plot. Data are the average of two to four determinations with data ranges within 20% of the average values.

Hemolysis Assay: The hemolysis assay was performed using a DiaMedix EZ Complement kit. Compounds (5 μL) to be tested were added to a test tube containing a standardized suspension of sheep erythrocytes sensitized with antibodies to sheep erythrocytes. The mixture was incubated for 60 min at 25 °C and then centrifuged. The absorbance of the supernate was read at 405 nm. The results are expressed as percent of control at 200 μM.

References

(1) For reviews see: Tomlinson, S. Complement defense mechanisms. *Curr. Opin. Immunol.* **1993**, *5*, 83−89. (b) Benjamini, E.; Leskowitz, S. *Immunology: A Short Course*; Wiley-Liss: New York, 1991; pp 135−150.

(2) Rogers, J.; Cooper, N. R.; Webster, S.; Schultz, J.; McGeer, P. L.; Styren, S. D.; Civin, W. H.; Brachova, L.; Bradt, B.; Ward, P.; Lieberburg, I. Complement activation by β-amyloid in Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 10016−10020.

(3) Jiang, H.; Burdick, D.; Glabe, C. G.; Cotman, C. W.; Tenner, A. β-Amyloid activates complement by binding to a specific region of the collagen-like domain of the C1q A chain. *J. Immunol.* **1994**, *152*, 5050−5059.

(4) Kalaria, R. N. The immunopathology of Alzheimer's disease and some related disorders. *Brain Pathol.* **1993**, *3*, 333−347.

(5) McGeer, P. L.; McGeer, E. G. The inflammatory response system of brain: Implications for therapy of Alzheimer and other neurodegenerative diseases. *Brain Res. Rev.* **1995**, *21*, 195−218.

(6) Eikelenboom, P.; Zhan, S.-S.; van Gool, W. A.; Allsop, D. Inflammatory mechanisms in Alzheimer's disease. *Trends Pharmacol. Sci.* **1994**, *15*, 447−450.

(7) Rogers, J.; Kirby, L. C.; Hempelman, S. R.; Berry, D. L.; McGeer, P. L.; Kaszniak, A. W.; Zalinski, J.; Cofield, M.; Mansukhani, L.; Willson, P.; Kogan, F. Clinical trial of indomethacin in Alzheimer's disease. *Neurology* **1993**, *43*, 1609−1611.

(8) McGeer, P. L.; Schulzer, M.; McGeer, E. G. Arthritis and antiinflammatory agents as possible protective factors for Alzheimer's disease: A review of 17 epidemiologic studies. *Neurology* **1996**, *47*, 425−432.

(9) Fothergill, J.; Kemp, G.; Paton, N.; Carter, P.; Gray, P. The structures of human C1r and C1s and their relationship to other serine proteases. *Behring Inst. Mitt.* **1989**, *84*, 72−79.

(10) Aoyama, T.; Ino, Y.; Ozeki, M.; Oda, M.; Sato, T.; Koshiyama, Y.; Suzuki, S.; Fujita, M. Pharmacological studies of FUT-175, nafamstat mesilate I. Inhibition of protease activity in in vitro and in vivo experiments. *Jpn. J. Pharmacol.* **1984**, *35*, 203−227.

(11) Gilmore, J. L.; Hays, S. J.; Caprathe, B. W.; Lee, C.; Emmerling, M. R.; Michael, W.; Jaen, J. C. Synthesis and evaluation of 2-aryl-4H-3,1-benzoxazin-4-ones as C1r serine protease inhibitors. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 679−682.

(12) Krantz, A.; Spencer, R. W.: Tam, T. F.; Liak, T. J.; Copp, L. J.; Thomas, E. M.; Tafferty, S. P. Design and synthesis of 4H-3,1-benzoxazin-4-ones as potent alternate substrate inhibitors of human leukocyte elastase. *J. Med. Chem.* **1990**, *33*, 464−479.

(13) McRae, B. J.; Lin, T. Y.; Powers, J. C. Mapping the substrate binding site of human C1r and C1s with peptide thioesters. Development of new sensitive substrates. *J. Biol. Chem.* **1981**, *256*, 12362−12366.

(14) Papadopoulos, E. P.; Torres, C. D. Convenient preparation of N-substituted 2- amino-4H-3,1-benzoxazin-4-ones and 3-substituted 2, 4(1H,3H)-quinazoline-diones. *J. Heterocycl. Chem.* **1982**, *19*, 269−272.

(15) Buroker-Kilgore, M.; Wang, K. K. W. A Coomassie brilliant blue G-250-based colorimetric assay for measuring activity of calpain and other proteases. *Anal. Biochem.* **1993**, *208*, 389−392.

JM970394D