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SYNTHESIS AND EVALUATION OF 2-ARYL-4H-3,1-BENZOXAZIN-4-ONES AS C1r SERINE PROTEASE INHIBITORS

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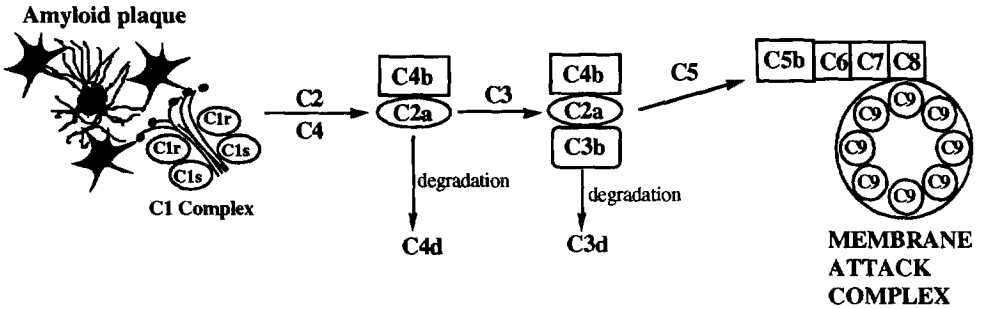
Abstract: A series of 2-aryl-4H-3,1-benzoxazin-4-ones have been synthesized and tested for inhibitory activity against C1r serine protease. Compounds were found that were equipotent and more selective than the reference compound FUT-175.

The complement system is a part of the host's immune response which provides a critical and multifaceted defense system against infection.[1] The classical complement pathway involves the sequential activation of nine major proteins or protein complexes designated as C1 through C9 (Figure 1). This activation is specific, requiring the interaction of the C1q subunit of C1 with an antigen-antibody complex. Once this pathway is activated, a highly regulated cascade of events occurs in which discrete interactions of soluble proteins catalyze subsequent steps. Complement activation culminates in the formation of a membrane attack complex which causes lethal and sublethal damage to cells.

Under normal conditions, the complement system is highly controlled by regulatory proteins against self attack. However, fibrillar ß-amyloid deposits have recently shown the ability to activate the classical complement system (Figure 1).[2] This activation by ß-amyloid may represent a major contributing event to the neuropathology of Alzheimer's disease(AD).[3] Inhibition of the complement system represents one approach to the modulation of the progression of AD.[2b] However, chronic blockage of the complement system may enhance susceptability to bacterial infection unless the alternative pathway can compensate.[4] While there are many potential targets for

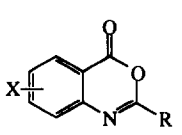
Figure 1. Activation of the Classical Complement Pathway by β-Amyloid



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 complement system. By selecting a target that is early in the cascade, it may be possible to prevent many of the amplification events that occur when the cascade is fully activated.

Chemistry

A series of 2-aryl-4H-3,1-benzoxazin-4-ones(**A**) have been synthesized and evaluated for C1r inhibitory activity.[5] These compounds were compared to the reference compound 6-amidino-2-naphthyl 4-guanidinobenzoate (FUT-175).[6] The synthesis of the 2-aryl-4H-3,1-benzoxazin-4-ones **2 - 24** was carried out



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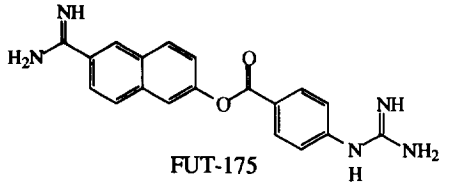
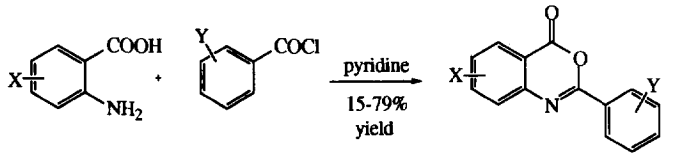


FUT-175

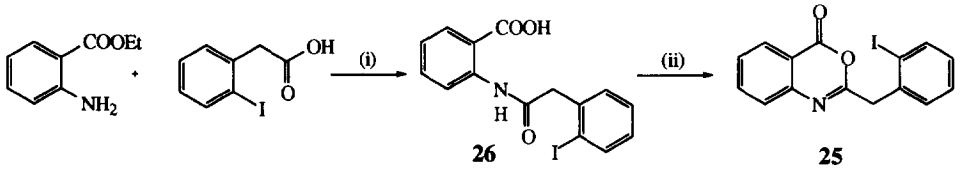
according to Scheme 1.[7] Treatment of appropriately substituted anthranilic acids with 2.2 equivalents of the desired benzoyl chloride in pyridine yielded the 2-aryl-4H-3,1-benzoxazinones in moderate yields.

Scheme 1



Compound **25** was made in a similar, but stepwise manner (Scheme 2). Ethyl anthranilate was coupled with *o*-iodophenylacetic acid in CH_2Cl_2 using dicylohexylcarbodiimde (DCC) followed by hydrolysis of the ethyl ester to give intermediate **26**. This compound was then cyclized with benzoyl chloride in pyridine to give compound **25**.

Scheme 2



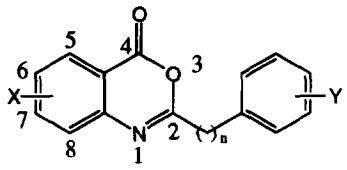
26 **25**

(i) 1. DCC CH_2Cl_2, 41%; 2. NaOH, EtOH, H_2O, Δ, 84%. (ii) PhCOCl, pyridine, 51%.

Enzyme Inhibition Assay

Compounds **1 - 25** were tested for inhibitory activity against purified human C1r enzyme using standard protocols.[8] The compounds were tested in three assays. In assay 1, the inhibitor was preincubated with the C1r enzyme for 10 minutes prior to the addition of substrate (Z-Gly-Arg-SBzl); enzyme activity was monitored by the production of $PHCH_2SH$. To more accurately assess the ability of the inhibitor to compete with substrate for the active site, assay 2 was utilized. In this assay, C1r was added to a solution of inhibitor and substrate, then enzyme activity was monitored as above. Active compounds were found to cause a time-dependent inhibition of C1r which is reflected in the lower IC_{50} values observed in assay 1. The final assay was used to determine the chemical stability of the compounds in aqueous media (Assay 2; 60 min). The compounds were allowed to incubate in aqueous buffer for 60 minutes before the substrate and C1r enzyme were added. Enzyme activity was then determined as above. Results are expressed as IC_{50} values or % inhibition at the highest concentration tested and are shown in Table 1.

Table 1. Inhibition of C1r by of 2-Aryl-4H-3,1-benzoxazin-4-ones



Cmpd #	X	Y	n	Inhibition of C1r IC_{50} (μM)[9]		
				Assay 1	Assay 2	Assay 2 (t = 60 min)
1		FUT-175		1.0	12	12
2	H	*o*-I	0	1.4	16.7	50
3	H	*o*-Br	0	9.5	32	46
4	H	*o*-Cl	0	17.3	45	>62.5
5	H	*o*-F	0	12.1	>62.5	>62.5
6	H	*m*-I	0	0%[a]	>62.5	>62.5
7	H	*p*-I	0	0%[a]	>62.5	>62.5
8	H	*o*-OH	0	20%[a]	>62.5	>62.5
9	H	*o*-OMe	0	17.7	>62.5	>62.5
10	H	*o*-COOH	0	25%[a]	>62.5	>62.5
11	H	*o*-NO_2	0	36.3	15	>62.5
12	H	*o*-CF_3	0	4.2	35.4	>62.5
13	5-Cl	*o*-I	0	3.0	13.5	57
14	6-Cl	*o*-I	0	3.0	7.8	27.3
15	7-Cl	*o*-I	0	0%[a]	>62.5	>62.5
16	6,7-diCl	*o*-I	0	1.7	>62.5	>62.5
17	5-Me	*o*-I	0	7.5	>62.5	>62.5
18	6-Me	*o*-I	0	10	51	>62.5
19	7-Me	*o*-I	0	46%[a]	>62.5	>62.5
20	6-OMe	*o*-I	0	5.0	>62.5	>62.5
21	7-OMe	*o*-I	0	27%[a]	>62.5	>62.5
22	6,7-OMe	*o*-I	0	18%[a]	>62.5	>62.5
23	7-NO_2	*o*-I	0	0%[a]	22	>62.5
24	b	*o*-I	0	0.7	>62.5	>62.5
25	H	*o*-I	1	6.0	26	40

(a) at 10μM, (b) benzene ring of benzoxazinone replaced with 2,3-naphthyl ring.

Results and Discussion

The most potent 2-aryl-4H-3,1-benzoxazin-4-ones were compounds **2** and **13** which were found to be equipotent with the reference compound FUT-175. In general, the 2-aryl-benzoxazinones were found to be unstable in aqueous buffer as shown by loss the of activity in assay 2 after 60 minutes in aqueous buffer; by comparison, FUT-175 exhibited no loss of activity. The optimal substitution for the aryl group (substituent Y) was found to be an ortho iodo group. All other substitutions resulted in loss of activity, as shown by entries **3 - 12.** Substitution of the phenyl ring on the benzoxazinone core was also explored, as shown by entries **13 - 24.** Compounds **16** and **24** exhibited IC_{50}'s equipotent to that of FUT-175 in assay 1 but were found to be inactive in the other assays. Entry **13**, the 5-chloro compound, was the only 2-arylbenzoxazinone that exhibited inhibitory potency equivalent to compound **2** in all three assays. FUT-175 and compound **2** were also tested against other serine proteases to determine their selectivities. As shown in Table 2, compound **2** is more selective against C1r than FUT-175.

Table 2. Inhibition of Serine Proteases by Compound **2** and FUT-175

	Inhibition IC_{50} (μM)[9]	
Protease	**2**	FUT-175
C1r	1.37	1.04
C1s	1.25	0.14
Trypsin	15.3	0.02
Plasmin	6.68	0.24
Thrombin	0.51	0.29
Kallikrein	9.89	0.65

In summary, benzoxazinones **2** and **13** have been found to be potent inhibitors of C1r with analog **2** showing greater selectivity for C1r versus related serine proteases than the reference agent FUT-175. Studies are now in progress to find derivatives with greater aqueous stability and increased potency and selectivity for the target enzyme.

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5. Compound **2** was identified as an inhibitor of C1r from a mass screening assay.
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9. All assays were done in triplicate with the mean and standard deviation of the IC_{50} value calculated for each determination. The IC_{50} value was determined by plotting % of control activity versus the inhibitor concentration. In all cases, the standard deviation of each calculated mean was less than 10% of the mean of all compounds assayed.

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