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# BENZENESULFONAMIDE DERIVATIVES OF 2-SUBSTITUTED 4H-3,1-BENZOXAZIN-4-ONES AND BENZTHIAZIN-4-ONES AS INHIBITORS OF COMPLEMENT C1r PROTEASE

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**Abstract:** A series of 2-sulfonyl-4*H*-3,1-benzoxazinones was prepared that inhibit C1r protease in vitro. Several compounds were found to be selective for C1r verses the related serine protease trypsin. Selected compounds demonstrated functional activity in a hemolysis assay. 

## Introduction

The complement system is both an effector and regulator of inflammation injury via the invocation of two distinct biochemical cascades, namely the classical and alternative pathway. When functioning normally the complement system is activated upon exposure to bacteria, and other antigens, resulting in cell destruction by opsonization and lysis.[1] Immunocytochemical studies of the brains from Alzheimer's disease (AD) patients have revealed that the pathological hallmark of AD, β-amyloid plaques, are associated with several components of the complement system. [2]

From an in vitro perspective it has previously been demonstrated that β-amyloid(Aβ) can initiate the classical complement pathway by binding to the C1q subcomponent of C1,[3] resulting in the activation of serine proteases C1r and C1s. This activation leads to the opsonization of Aβ and the formation of proinflammatory anaphylatoxin peptides.[4] These in vitro inflammatory events induced by Aβ can be prevented by inhibiting complement activation.[5] Thus, inhibition of the complement system in the brain may therefore slow the destruction of neurons in the brains of AD patients. While there are clearly several potential targets within the complement cascade to prevent its activation by amyloid plaques, one pharmacological approach would be to inhibit C1r, the first protease activated in the cascade.

The catalytic domain of human C1r protein, possesses significant sequence similarity to the serine protease trypsin. C1r, like trypsin, cleaves at an arginine residue. In a model of the C1r catalytic domain (Figure 1) that we have developed, using standard homology-based modeling techiniques, there is an aspartic acid at the bottom of the S1 pocket. As in trypsin this Asp residue is likely to be the functionality that interacts favorably with the arginine side chain of the substrate. This is consistent with literature reports on inhibitors of

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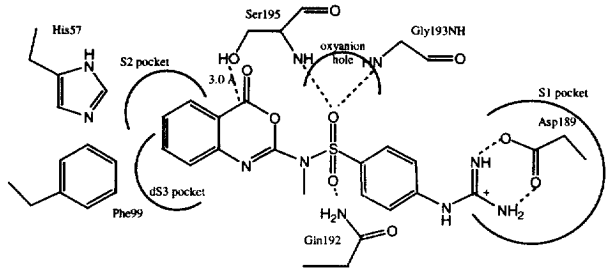
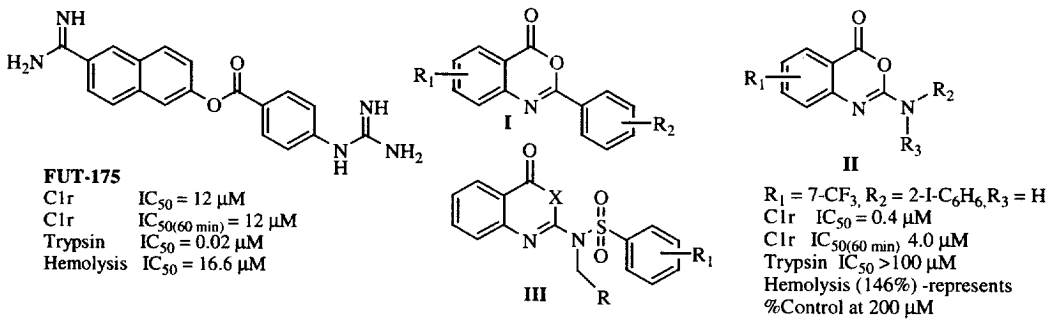
C1r also being potent inhibitors of trypsin. For example, FUT-175[6] while moderately inhibiting C1r, is a potent inhibitor of trypsin (Figure 2). Previously, our discovery efforts directed toward inhibitors of C1r have reported

**Figure 1** Model of **16** bound in C1r catalytic site




benzoxazinones of structures **I**[7] and **II**[8] to be inhibitors C1r. An example of a potent inhibitor characterized by structure **II** is shown in Figure 2. The current report describes further our synthetic efforts in this area to obtain more potent and selective C1r inhibitors with activity in the functional hemolysis assay. The synthesis and biological data relating to benzenesulfonamide derivatives of 2-substituted 4*H*-3,1-benzoxazin-4-ones **III** will be presented here.

**Figure 2**

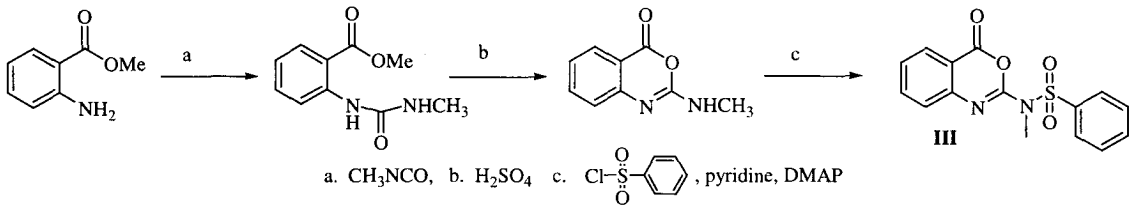



## Chemistry

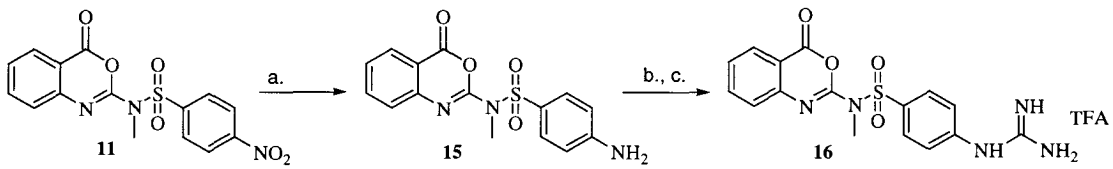
The synthesis of the type III benzoxazin-4-ones will be illustrated by one representative example, which is a minor modification of a literature procedure.[9] Treatment of methyl anthrinilate with methyl isocyanate or alky isothiocyanates provides the alkyl urea (Scheme 1), which is cyclized with concentrated sulfuric acid to afford 2-methylamino-benzo[3,1-d]-oxazin-4-one. This benzoxazinone reacted with benzene sulfonyl chloride in the presence of pyridine and dimethylaminopyridine (DMAP) to give the inhibitor **1** 64% overall yield.

Preparation of **16** (Scheme 2) required reduction of compound **11** using transfer hydrogenation conditions with 10% Pd/C and 1,4-cyclohexadiene in refluxing benzene (67%). The aniline **15** was treated with *N,N'*-bis(*t*-butyloxycarbonyl)guanidine in THF to yield the protected guanidine compound in 46% yield. Final deprotection was accomplished with TFA:$CH_2Cl_2$ (1:1) to yield **16** in 74% yield.

**Scheme 1**




**Scheme 2**




a. 10% Pd/C, 1,4-cyclohexadiene; b. N,N'-bis(*t*-butyloxycarbonyl)guanidine, THF; c. TFA:$CH_2Cl_2$(1:1)

**Biological Testing**

The in vitro enzyme assays were performed according to protocols previously described.[8] 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 less than or equal to 10% of the mean value.

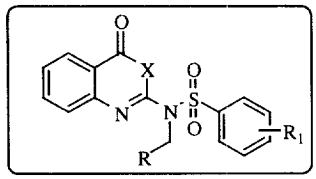
The compounds were tested for inhibitory activity against purified C1r in two assays. In the first assay (initial), the enzyme C1r was incubated with the inhibitor and peptide substrate (Z-Gly-Arg-S-Bzl). In this assay, the inhibitor competes with the peptide substrate for the enzyme. The second assay (60 min) involves incubation of the inhibitor in the assay buffer for 60 min and then the enzyme assay is performed. This value reflects the compounds' stability in aqueous buffer.

A functional hemolysis assay was performed on active compounds utilizing a DialMedix EZ Complemment 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 is incubated for 60 min at room temperature, then centrifuged. The absorbance of the supernate is read at 405 nm. The results are expressed as % of inhibition at 200 μM or given as an $IC_{50}$.

**Results and Discussion**

Benzoxazinones of the general structure **I** (Figure 1) were found to be inhibitors of C1r. Structure activity relationships around this series led to 2-aryl subsituted 4*H*-3,1-benzoxazin-4-ones, which inhibited C1r; however, these compounds were hydrolytically labile and were not selective over the serine protease tyrpsin.[7] An extension of this work led to a series of 2-amino-4*H*-3,1-benzoxazin-4-ones, **II**.[8] Several compounds in this

**Table 1.** In Vitro $IC_{50}$[a] Values (μM) of compounds **1-18**[b] against purififed C1r enzyme [c] and trypsin[d]



| | | | | Inhibition of C1r ($IC_{50}$ μM) | | Inhibition of trypsin | Hemolysis |
|---|---|---|---|---|---|---|---|
| Compounmd | X | R | $R_1$ | initial | 60 min | ($IC_{50}$ μM) | ($IC_{50}$ μM) |
| 1 | O | H | H | 4 | 6 | | (162)[g] |
| 2 | O | H | 4-Cl | 40%@31.2 | >62 | 1.3 | |
| 3 | O | H | 4-tBu | >60 | >60 | 5.83 | |
| 4 | O | $CH_3$ | H | 3.2 | 8.3 | 1.5 | 86[f] |
| 5 | O | $CH_3$ | 4-Cl | 30 | 62 | 0.93 | |
| 6 | O | H | 4-$CH_3$ | 7.8 | 40%@62 | 4.5 | |
| 7 | O | H | 2-Br | 2.8 | 25 | >100 | |
| 8 | O | H | 2,6-Cl | 7.5 | >62 | 10.1 | |
| 9 | O | H | 4-OMe | 0.7 | 6 | >100 | 80f |
| 10 | O | H | 3,4-Cl | 20%@15 | >62.5 | 8.3 | |
| 11 | O | H | 4-$NO_2$ | 31 | 15%@62.5 | >100 | |
| 12 | O | H | 2,3-$C_4H_4$ | 6 | 10 | 4.3 | 56[f] |
| 13 | O | H | 4-CN | 20 | 30%@62 | >100 | |
| 14 | O | H | 3-Cl | 24 | 58 | 4.2 | 49[f] |
| 15 | O | H | 4-$NH_2$ | 7 | 62 | 2.2 | 8 |
| 16 | O | H | 4-NHC(NH)$NH_2$ | 1.5 | 3 | NT[e] | 18 |
| 17 | S | H | H | >62.5 | NT[e] | NT[e] | (162)[g] |
| 18 | S | H | 4-OMe | >62.5 | NT[e] | >200 | |
| FUT-175 | x | x | x | 12 | 12 | 0.017 | 16.6 |

a Concentration (μM)of **1-18** necessary to inhibit enzymatic cleavage of the substrate described in ref. 6 by 50%.
b. All new compounds were characterized by $^1$H-NMR, mass spectroscopy and CHN. c. human C1r. d. Bovine trypsin.
e. not tested f. represents %inhibition @200 μM g. represents %control @200μM

series proved to be potent C1r inhibitors with selectivity over trypsin and were stable in the aqueous buffer medium. Further evaluation of this series led to compounds containing a sulfonamide group at the 2-position, **III**. This report describes the Topliss tree analysis[10] of these novel inhibitors.

The parent compound of this series (**1**) showed improved activity toward the serine protease C1r ($IC_{50}$ = 4 μM) relative to the reference compound FUT-175 ($IC_{50}$= 12 μM). We sought to improve enzyme inhibition by modifying the aryl sulphonamide substituent. We decided to apply the Topliss decision tree, or more formally the Hansch substituent effect, to select compounds that encompassed a range of pi and sigma values. The initial set of compounds selected incorporated a 4-Cl, 4-$CH_3$, 4-OMe, and 3,4-diCl (**2**, **6**, **9** and **10**) as dictated by the initial levels of the Topliss tree. Gratifyingly a significant improvement in potency for the 4-OMe substituent was apparent in the "initial" C1r assay, which further translated to observable activity in the hemolysis secondary functional assay.

In an effort to further verify the Topliss analysis we prepared compounds from the second tier of the decision tree **3**, **10**, **11**, and **14**. The steric effect of the 4-*t*-Bu derivative **3** ($IC_{50}$ > 60 μM) led to an inactive compound. The 3-Cl substituent of compound **14** relieves the steric hinderence at the 4-position however this substituent led to a compound much less potent than **1** (**14**, $IC_{50}$ = 24 μM). Increasing sigma values as demonstrated by **11** ($IC_{50}$ = 31 μM) and **10** led to decreases in activity. Additional compounds such as **7**, **8**, **12**, and **13** further explored the SAR. Ortho substitution led to compounds **7** and **8** with $IC_{50}$'s 2.8 and 7.5 μM respectively. Interestingly, **7** did not inhibit trypsin[8] whereas the disubstituted **8** showed similar inhibition towards C1r and trypsin. The 1-napthyl substituent **12** ($IC_{50}$ = 6 μM) showed good activity and was hydrolytically stable.

Given the improvement in activity seen for the 4-OMe substituent, **9**, further analysis of the Topliss decision tree revealed that an amino substituent might also confer favorable activity. This was supported by our model of C1r and how the benzoxazinones might fit the active site. From inspection of this model it appeared that favorable hydrogen bonding interactions might be possible, between the aspartic acid at the base of the S1 pocket and a basic residue added to the inhibitor. Indeed preparation of the 4-$NH_2$ analog **15**, and the 4-guanidino analog **16**, resulted in compounds that displayed good potency toward the isolated enzyme C1r though not more potent than **9**.

The functional assay of hemolysis showed our most potent compounds to have good activity. Compound **9**, the most potent inhibitor and a selective inhibitor, showed 80% inhibition of hemolysis at 200 μM in the functional assay, unfortunately this compound was insoluble at higher concentrations and an $IC_{50}$ could not be determined. Placing an amino or guanidino group in the para position as suggested by the homology model aided in the solubility of these compounds in this functional assay. Compound **15**, which contains the 4-amino

substituent, had an $IC_{50}$ in the functional assay of 8 μM, which is twice as potent at the literature reference FUT-175 ($IC_{50}$ 16.6 μM). Compound **16**, which contains the 4-guanidino substituent, had an $IC_{50}$ = 18 μM similar to the reference compound FUT-175.

It was evident from the in vitro incubation studies with the serine protease C1r that a noticeable decrease in activity was evident after incubating the potential inhibitor in buffer for 60 min. Four benzoxazinones **1**, **4**, **12**, and **16** showed modest stability in the assay buffer, losing only one half their enzymatic activity after incubation for 60 min in buffer. Since there have been several reports that benzthiazones were hydrolytically more stable[11] than benzoxazinone we prepared compounds **17** and **18**. These compounds were indeed hydrolytically stable but unfortunately were inactive against C1r.

**Conclusion**

Application of the Topliss decision tree approach in combination with homology molecular modeling has led to improved activity of the initial compound **1**. Compound **9** was an order of magnitude more potent than the reference compound FUT-175 with improved selectivity for C1r over trypsin. Compounds **9**, **15**, and **16** are particularly noteworthy in that they demonstrate improved functional activity in the hemolysis assay. While these compounds are generally quite hydrolytically labile, the analogs prepared have served to define critical pharmacophores that may be useful in the subsequent design and synthesis of further inhibitors of C1r.

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