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A simple, general approach of allosteric coagulation enzyme inhibition through monosulfated hydrophobic scaffolds

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ABSTRACT

Allosteric inhibition of coagulation enzymes offers the advantage of controlled inhibition. In this study, a small library of mono sulfated indole and benzothiazole based molecules was synthesized and screened against the panel of coagulation proteases. The results reveal that selected molecules inhibit the thrombin, factor Xa and factor XIa with moderate potency. Compound **6a** was found to have an allosteric mode of inhibition against thrombin. Plasma clotting assays suggest that selected inhibitors **14b**, **14c** and **14d** prolong both prothrombin and activated partial thromboplastin time. Overall, this work presents the newer class of allosteric inhibitors of thrombin and factor XIa with improved aqueous solubility profile.
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We recently designed the first small molecules that act as allosteric inhibitors of thrombin.[1–5] A library of monosulfated benzofuran derivatives exhibited moderate to potent inhibition of human α-thrombin under physiologically relevant conditions. Michaelis–Menten kinetic studies suggested a noncompetitive mode of inhibition and site-directed mutagenesis studies in combination with computational studies suggested two different sites of binding based on size of molecule. Whereas monosulfated benzofuran dimers preferred to bind in the hydrophobic pocket close to Arg173,[3] the trimer derivatives appears to bind in the hydrophobic pocket near Arg233.[4] Both of these sites on thrombin are part of exosite 2, a fairly large electropositive domain that is known to bind natural glycosaminoglycans, especially heparin and chondroitin sulfate,[6–10] and modulate enzymatic function. In addition, Arg173 is also involved in recognition of fibrinogen and thereby plays a key role in propagation of the natural hemostatic signal.

To further the design of allosteric inhibitors of coagulation enzymes, especially thrombin, we sought to explore hydrophobic scaffolds related to benzofuran that might provide added benefits, such as ease of synthesis, and enhance the structural search space. A key requirement was the presence of one sulfate group on the scaffold, which was earlier deduced to be essential for inducing allosteric inhibition.[2,3] As described in our earlier work, monosulfated benzofuran dimers and trimers required nearly 7 to 9 steps, of which the first step of oxidative cyclization was a particularly challenging, moderate yielding step. Thus, discovery of an equivalent, or more potent, scaffold would enable advanced structure–activity studies in the nascent area of allosteric inhibitors of coagulation enzymes.

We selected nearly 50 different heterocyclic core scaffolds and designed an in silico library of molecules mimicking the monosulfated benzofuran dimers and trimers. Using GOLD-based docking strategy,[5] the in silico library was screened for interaction with thrombin and two closely related scaffold, the indole and benzothiazole scaffolds, were selected based on their high docking scores and consistency of binding. We present a1 to 5-step synthesis of a small library of monosulfated indole and benzothiazole monomers, dimers and trimers, and their ability to inhibit thrombin and other enzymes of the coagulation cascade. Four molecules were identified to inhibit thrombin with moderate potency. The inhibitors present a growing body of evidence that allosteric inhibition of coagulation enzymes could be a fairly general strategy of achieving anticoagulation.

Chemistry: Scheme 1 and Scheme 2 describe the synthesis of monosulfated molecules **6a**–**6d**, **7**, **14a**–**14d**, and **15** starting from commercially available benzothiazole (2-methylbenzo[d]thiazol-6-ol) (**1**) and indole core structures (ethyl 5-hydroxy-2-methyl-1H-indole-3-carboxylate) (**8**).The intermediates **A**, **B** and **C** used in their synthesis were obtained as described earlier in our work.[1,2]

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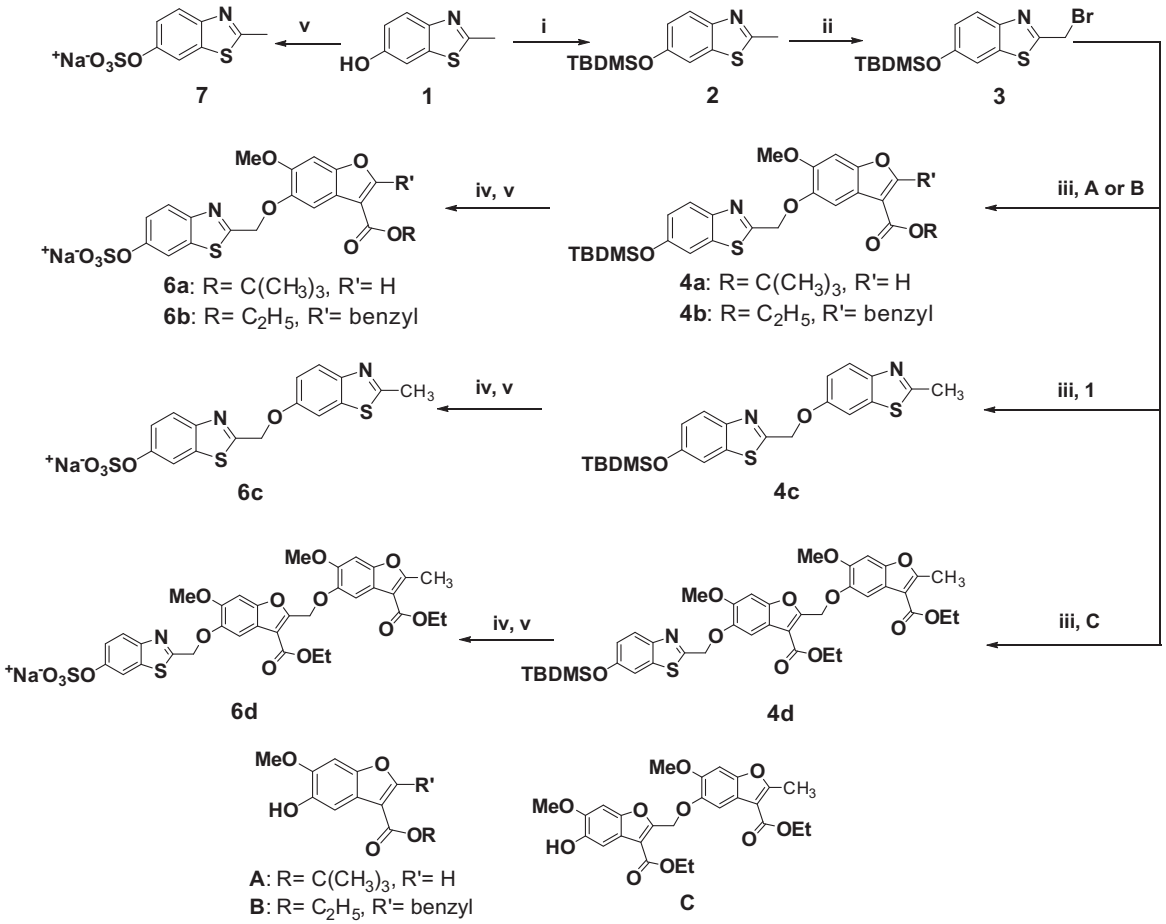

Scheme 1. Synthesis of potential thrombin inhibitors based on the benzothiazole scaffold. (i) TBDMSCl, imidazole, DMF (ii) NBS, CCl_4 (iii) Cs_2CO_3, DMF, MW, 100 °C (iv) KF, CH_3COOH, DMF (v) $Me_3N.SO_3$, Et_3N, MW, 100 °C.

Briefly, protection of the 5-hydroxy group of benzothiazole**1**by *t*-butyltrimethylsilyl chloride in presence of imidazole led to formation of intermediate **2** (Scheme 1). Likewise, intermediate **9** was synthesized by *t*-butyltrimethylsilyl protection of the 5-hydroxyl group of **8**and BOC protection of the indole's NH group using di-*t*-butyl dicarbonate (Scheme 2). Bromination of the allylic position of **2** and **9** by *N*-bromo succinimide led to reactive handles **3** and **10**, which were used in the synthesis of **4a**–**4d** and **12a**–**12d** through nucleophilic displacement of allylic bromide by 5-phenolate ion of either **1**, **A**, **B**, **C** or **8**. The coupling of two aromatic rings was typically low yielding (<50%) due to steric interference, however under microwave conditions good yields were realized in shorter amount of time (see Supplementary information). The targeted compounds **6a**–**6d**, **7**, **14a**–**14d** and **15** were prepared by deprotection followed by sulfation of intermediates **1**, **4a**–**4d**, **8** and **12a**–**12d** using microwave- based chemical sulfation developed earlier in our laboratory.[11] The exchange of triethylammonium ion by Na^+ was performed by using weak cation exchange resin and the monosodium benzothiazole or indole derivative was purified by using flash chromatography. The overall yield of potential inhibitors was 40–60%, which was higher in comparison to the monosulfated benzofuran dimers and trimers developed earlier.[2,4] Detailed description of reactions and characterization data is provided as Supplementary information.

Screening against a panel of serine proteases: The library of 10 monosulfated potential inhibitors was screened against a panel of related coagulation proteases consisting of factors IIa (thrombin), VIIa, IXa, Xa, XIa and XIIa to better understand their inhibition properties. Initial screening was performed at 500 μM concentration of inhibitors using a single-point substrate hydrolysis assay, as described in the Experimental Section (see Supplementary material).The fractional residual activity of each enzyme was calculated from ratio of the initial rate of substrate hydrolysis in the presence of the inhibitor to that in its absence. The screening results indicated that none of the monosulfated molecule inhibited factors VIIa, IXa and XIIa by more than 20% (Table 1, Fig. 1). But thrombin, factor Xa and factor XIa were inhibited well (more than 50%) by several molecules including **6a**, **14b**, **14c** and **14d**. Considering that these enzymes are most related to each other, the results indicated a high level of consistency. Yet, the results also suggested that inhibitor fine structure was likely to govern inhibition potential.

Enzyme inhibition potential: The inhibitors that showed significant promise in the initial screen were evaluated for quantitative inhibition potency using small peptide substrate hydrolysis assay in the presence of inhibitor concentrations over several log units. The sigmoidal fractional decrease (on a semi-log plot) in the initial rate of substrate hydrolysis was fitted by the traditional dose–response equation (see Supplementary material) to calculate the IC_{50} of each potential inhibitor. Figure 2 shows the semi-log curves for thrombin, factor Xa and factor Xia inhibition at pH 7.4 and Table 2 lists the IC_{50} values. The inhibitors studied in detail exhibited a potency range of 146–425 μM against thrombin, 87–220 μM against factor XIa and 195–352 μM against factor Xa. These potency ranges indicates that benzothiazole and indole scaffolds are moderately active against the three enzymes. The previously

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