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Discovery of benzothiazole guanidines as novel inhibitors of thrombin and trypsin IV

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ABSTRACT

In a project to find novel neutral P1 fragments for the synthesis of thrombin inhibitors with improved pharmacokinetic properties, fragments containing a benzothiazole guanidine scaffold were identified as weak thrombin inhibitors. WaterLOGSY (Water-Ligand Observed via Gradient SpectroscopY) NMR was used to detect fragments binding to thrombin and these fragments were followed up by Biacore A100 affinity measurements and enzyme assays. A crystal structure of the most potent compound with thrombin was obtained and revealed an unexpected binding mode as well as the key interactions of the fragment with the protein. Based on these results, the structure-based design and synthesis of a small series of optimized novel substituted benzothiazole guanidines with comparatively low pK_a values was accomplished. Testing of these compounds against human trypsin I and human trypsin IV revealed unexpected inhibitory activity and selectivity of some of the compounds, making them attractive starting points for selective trypsin inhibitors.

The control of the blood coagulation process presents one of the major issues in the treatment of thrombosis (i.e., the formation of a blood clot in a blood vessel) and the prevention of stroke.[1] Warfarin and heparin are still widely used as anticoagulants despite severe side effects and complicated handling. Patients who are prescribed warfarin have to be monitored very closely during the whole treatment. Both substances inhibit a variety of factors which are involved in the blood coagulation cascade. Thrombin as one of these factors has a key function in platelet aggregation and polymerization of fibrin. It is therefore an attractive target for direct inhibition. Recent examples of direct thrombin inhibitors are hirudin, argatroban,[2] melagatran[3] and dabigatran.[4] Early work focused on inhibitors derived from the tripeptide D-Phe-Pro-Arg which binds into the thrombin active site with proline fitting into the S2 pocket and arginine residing in the S1 pocket.[5] Typically, direct thrombin inhibitors possess a P1 group, filling the specific S1 pocket. Very often such P1 groups are considerably basic like arginine in D-Phe-Pro-Arg, since one of the main interactions can be ascribed to an interaction of the basic functional group with the carboxylic acid of the aspartate residue Asp189 sitting deep into the S1 pocket. Such P1 groups are generally associated with unfavourable pharmacokinetic properties and the issues related to these P1 groups cause major problems in inhibitor development so far. Benzamidine is quite often used as a strong binder in the S1 pocket and thus as P1 group in thrombin inhibitors. The basic amidine function forms here a strong bidentate salt bridge with the Asp189 carboxylate which is the major reason for the affinity towards thrombin. Benzamidine itself has a pK_a of 11.6 and thrombin inhibitors with such a basic P1 group will be mainly protonated under physiological conditions which makes it unlikely for these compounds to be absorbed from the GI tract. These substances suffer therefore from low bioavailability. In case of the direct thrombin inhibitors melagatran and dabigatran, the amidine function is masked and the drugs are administered in form of the prodrugs ximelagatran and dabigatran etexilate which are readily orally absorbed. Hence, the discovery of novel neutral P1 groups, which provide adequate potency and selectivity, is central to the synthesis of new direct thrombin inhibitors. Proteases belonging to the human trypsinogen family show a substantial overlap in substrate specificity and susceptibility to inhibitors, for example, melagatran, with thrombin.[6] A longstanding research interest of ours is trypsin IV, a serine protease belonging to the human trypsinogen family, that can cleave protease-activated receptors 1 and 2 to induce inflammation, hyperalgesia and pain.[6,7] However, so far lack of trypsin IV specific small molecular inhibitory compounds hindered evaluation of this potential interesting target in inflammation and pain. Therefore developing starting points for specific inhibitors of trypsin IV is of increasing interest, especially those that would discriminate between trypsin IV and other trypsin-like proteases. In this work, NMR-based fragment screening[8–11] against thrombin identified benzothiazole guanidine as a lead scaffold from a library of modified potential P1 fragments. Since the aim of this project was not the generation of full inhibitors but the

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focus on neutral P1-groups we decided to further examine the potential of this scaffold for fragment generation. A crystal structure of thrombin with the most potent inhibitor of this set assisted the structure based design and synthesis of a second set of compounds containing the benzothiazole guanidine motif, aiming for fragments with lower molecular complexity and lower pK_a. Additional, screening of these compounds against human trypsin I and human trypsin IV revealed inhibitory activity and selectivity of some of the compounds, making them attractive starting points for selective trypsin inhibitors. The pK_a of guanidine is very sensitive to substitution. For Oxyguanidines a comparatively low pK_a of 7.0–7.5 was measured regarding the guanidine pK_a of 13–14. Thrombin inhibitors with this rather neutral P1 group showed an acceptable permeability coefficient in the human Caco-2 monolayer assay.[12] In the case of benzothiazole guanidine, an even lower pK_a of 5.7 has been determined experimentally.[13] The absorption potential of substances with such a P1 group can also be estimated acceptable regarding the pH range 5.5–7 of the lower GI tract resulting in a higher proportion of uncharged species. Therefore, incorporation of the benzothiazole guanidine motif into thrombin and trypsin inhibitor design may lead to inhibitors with improved pharmacokinetic properties and unique specificity.

An interesting approach in the search for less basic or neutral P1 fragments for thrombin inhibitor design is illustrated by the incorporation of a *p*-chlorophenoxy acetamide P1 substituent to afford potent thrombin inhibitors.[14,15] Lacking a basic functionality, there is no longer a direct interaction between Asp189 and the P1 group. Instead, H-bonding of the acetamide side chain to Gly219 in the S1 pocket as well as a hydrophobic interaction of the chloro group with the aromatic system of Tyr 228 located at the bottom of the S1 pocket seems to be responsible for the potency of such a fragment. Thus, it was hypothesized that attachment of suitable side chains to fragments fitting into the thrombin S1 pocket could lead to P1 fragments with increased affinity or selectivity towards thrombin. Based on this, a set of 14 neutral and weakly basic potential P1 fragments was chosen for the synthesis of a library of modified fragments. The fragments were alkylated with in total 12 different side chains. After purification, a set of 114 modified fragments was obtained and the whole library, including the unmodified fragments, was submitted for WaterLOGSY NMR fragment screening.[16,17] WaterLOGSY (Water-Ligand Observed via Gradient SpectroscopY) represents a powerful NMR technique to detect ligands binding to macromolecules. Bulk water magnetisation is transferred via bound water molecules in the protein binding site to the ligand. The method proved to be fast and sensitive requiring only a small amount of non-deuterated protein and ligand. Competition experiments with melagatran and benzamidine were performed with binding fragments to confirm binding in the thrombin binding site and the S1 pocket. From our library, four compounds were identified showing affinity towards thrombin, whereof three compounds featured the benzothiazole guanidine scaffold (data not shown). Among those fragments, **1**, **2** and **3** with benzothiazole guanidine as core scaffold (Fig. 1) showed promising activity and attractive possibilities for the development of further lead structures. Compared to the unsubstituted benzothiazole guanidine **1**, which showed only weak binding to thrombin in the WaterLOGSY experiment and a 13% inhibition of thrombin enzyme activity at 5.3 mM compound concentration, attachment of an ethyl acetate side chain led to a significantly increased potency. Fragment **3** is slightly less active than **2**, nevertheless **3** was shown to be more potent than the unsubstituted derivative (Table 1). The primary screening against thrombin using WaterLOGSY NMR proved to be a fast and robust method to determine binding to the protein and to confirm binding to the active site as well as to provide the possibility to rank the compounds according to their affinity. Nevertheless, K_D values calculated from data collected by WaterLOGSY NMR fragment screening showed a large variation and this technique was not suitable to obtain quantitative binding data. Also compound **1** only showed minor inhibition at high concentration in a thrombin activity assay. We therefore tested plasmon surface resonance technique to determine direct binding data for all other compounds, which proved to be a much more reliable method in our case. For **2** and **3**, K_D values of 95 and 145 μM, respectively, were measured using a Biacore A100 and human thrombin (Table 1). When these measurements were followed up with enzymatic assays for thrombin and additionally for trypsins, compound **2** inhibited trypsin I and trypsin IV more than thrombin, while compound **3** showed certain selectivity for trypsin IV.

The X-ray structure of **2** with thrombin revealed an unexpected binding mode and indicates the principal interactions of the fragment with the protein (Fig. 2). The structure has been deposited with PDB (code 3PO1). Preliminary docking studies on compound **1** predicted the guanidine part to be oriented deep into the S1 pocket, developing the expected interaction with Asp189. Instead, the whole molecule is flipped 180° with the guanidine part directed towards the carboxyl group of Glu192. The hydrogen bond distance here is 2.6 Å and compared to the thrombin crystal structure with melagatran, the carboxyl function of Glu192 is shifted about 2 Å towards the guanidine function, which reflects a significant interaction with the ligand. The guanidine group is placed in-between Glu192 and Gly216, demonstrating also a weak hydrogen bond to Gly216. With the phenol directed down towards the S1 pocket, the hydroxy function can interact with Asp189 (distance 2.7 Å). A similar interaction has been reported for hydroxy benzothiophene based thrombin inhibitors.[18] Further weaker interactions can be seen both between the ester carbonyl and Gly216 and between the ethoxy oxygen and the imidazole ring in His57.

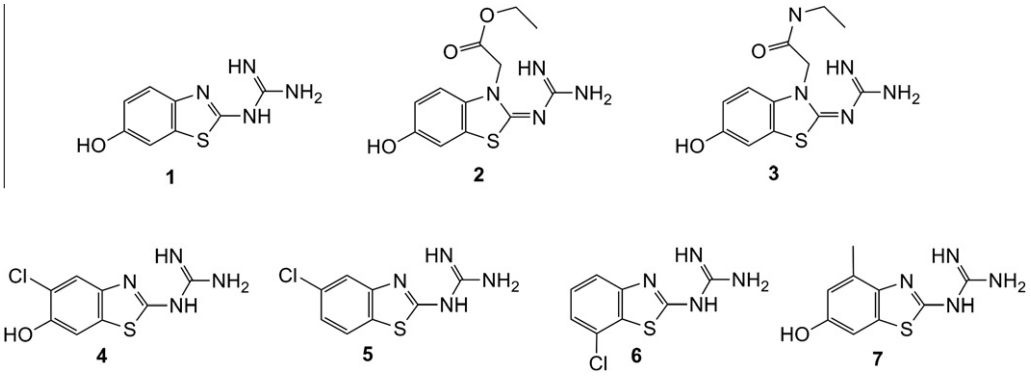


Figure 1. Benzothiazole guanidine fragments **1**–**7**.

Table 1
Affinity constants and inhibition constants

Compound	Thrombin (Biacore) K_D (μM)	Thrombin (enzyme assay) K_{ic} (μM)	Trypsin I (enzyme assay) K_{ic} (μM)	Trypsin IV (enzyme assay) K_{ic} (μM)	pK_a
1	n.d.	See footnote[c]	No effect[a]	No effect[a]	9.6[d]
2	95 ± 4 (n = 4)	481 ± 58	198 ± 47	168 ± 16	5.1[d]
3	145 ± 46 (n = 4)	2731 ± 2314	No effect	1094 ± 390	9.0
4	No effect	No effect	647 ± 51	476 ± 49	5.7
5	No effect	No effect	No effect	No effect	5.5
6	No effect	No effect[a]	No effect[a]	No effect[a]	5.4
7	No effect	No effect	No effect	794 ± 107	6.0
4-Aminobenzamidine dihydrochloride	78 ± 11 (n = 3)	95 ± 25	8.9[b]	13.1[b]	13.1[d]

The affinity constants for thrombin measured with the Biacore A100 are given as K_D ± SD. The number of measurements is given in brackets. K_{ic} and standard errors were calculated from IC_{50} values and their standard errors. The standard errors were reported by the fitting software Sigma Plot 8 from the global IC_{50} fit to all data.
n.d.—not done.
No effect—no K_D or IC_{50} could be determined.
[a] Precipitates at concentrations >1 mM.
[b] Data from [5].
[c] Compound **1** at 5.3 mM did inhibit 13% of thrombin activity using an alternative less affine thrombin substrate (pyroGlu-Pro-Arg-pNA HCL at 300 μM) instead of 50 μM S2238.
[d] Calculated from ACD/p*K*a DB.

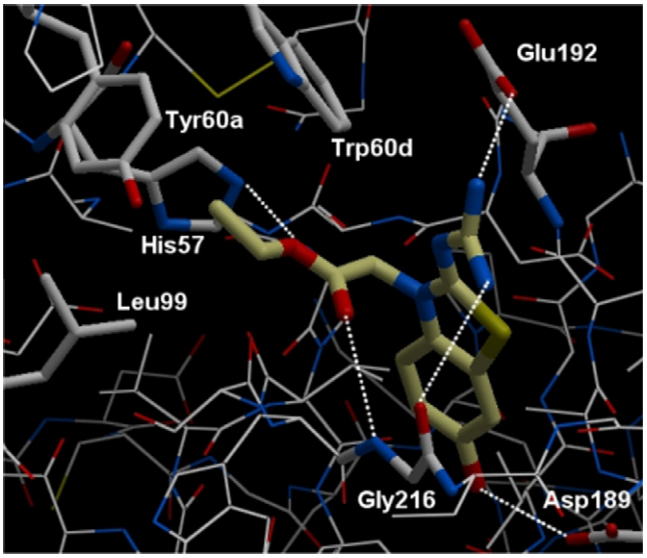


Figure 2. X-ray structure of **2** in thrombin. Hydrogen bonds are represented as white dotted lines.

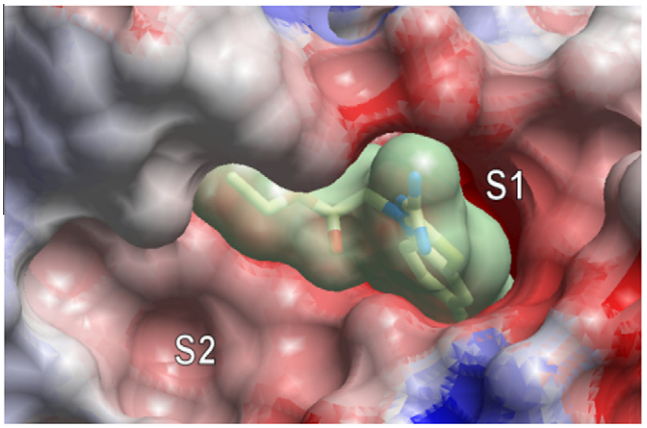
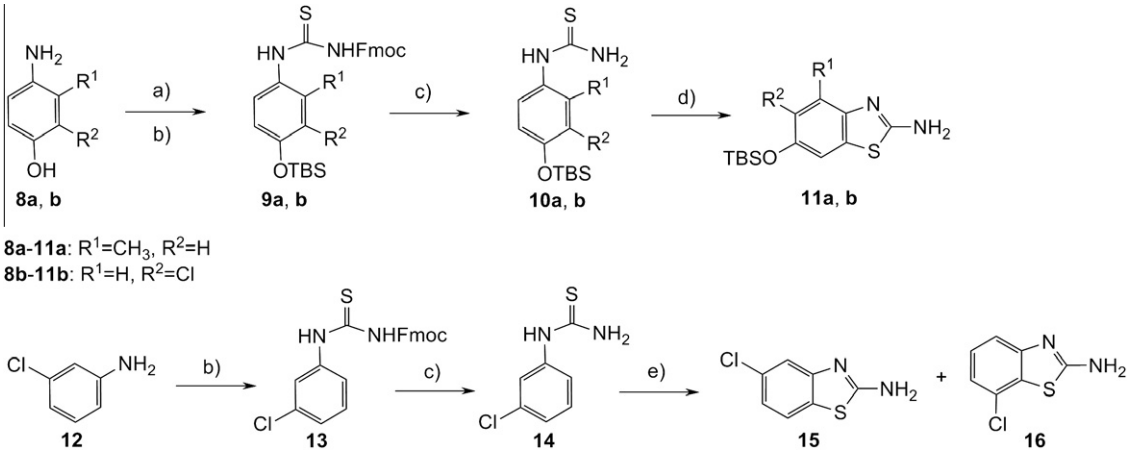


Figure 3. X-ray structure of **2** in thrombin, electrostatic surface of the protein. Orientation of the ethyl acetyl side chain in the S2 subpocket.

It is hypothesized that hydrophobic interaction also plays a crucial role in the increased affinity for **2** (Fig. 3). The ethyl ester group is situated in a non-polar S2 subpocket formed by Trp60d, Tyr60a, Leu99 and His57. The branching point of the side chain can be responsible for the unexpected orientation flip with the guanidine part interacting with Glu192 instead of Asp189. Other derivatives of **1**, N-alkylated with different larger alkyl acetamides were also synthesized. Of these, only the ethyl acetamide substituted benzothiazole guanidine **3** showed activity (data not shown), which demonstrates the spatial limitations of the subpocket. The weaker affinity of **3** compared to **2** can be ascribed to an increased rigidity changing the ester bond with the amide bond resulting in a slightly disfavoured position of the ethyl acetamide part in the subpocket. Also the differences in lipophilicity of the side chain may be crucial regarding the rather hydrophobic character of the subpocket. In the search of further active fragments, the obtained crystal structure was used for the structure based design of a second set of fragments based on the benzothiazole guanidine scaffold. Identifying a potent thrombin inhibitor which contains a P1 fragment based on the benzothiazole guanidine scaffold involves finding an optimal way to link this fragment with the rest of the inhibitor. Potent P2 fragments which we used as proline replacement in our project possessed a methyl group pointing also into the above mentioned hydrophobic subpocket and were unsuitable to be connected with **2** due to an obvious overlap of both molecules. It was therefore desirable to find novel P1 fragments which allow the P2 fragment to reach into the subpocket. Introduction of other functional groups utilizing further interaction alternatives of the ligand with the S1 pocket was supposed to increase potency or to at least compensate potency loss if the side chain is no longer present. Regarding possible toxic effects of some phenols[19] it was also preferable to avoid the alcohol function. Electron withdrawing substituents at the benzene ring should lower the pK_a. Thus, benzothiazole guanidines **4**, **5** and **6** were designed and synthesized (Fig. 1). To address the question whether a substitution at the 4-position is tolerated, the 4-methyl derivative **7** was also synthesized. Besides attachment at the ring nitrogen, the 4-position is, according to docking studies, also regarded to be favourable to link P2 groups. The introduction of the chloro group should examine the possibility to establish a further hydrophobic interaction, for instance with the aromatic system of Tyr228. As expected, the measured pK_a values for the guanidine group of **4–7** were considerably lower compared to **3** (pK_a 9.0), ranging from 5.4 to 6.0. We have to mention that those smaller fragments could adopt both possible

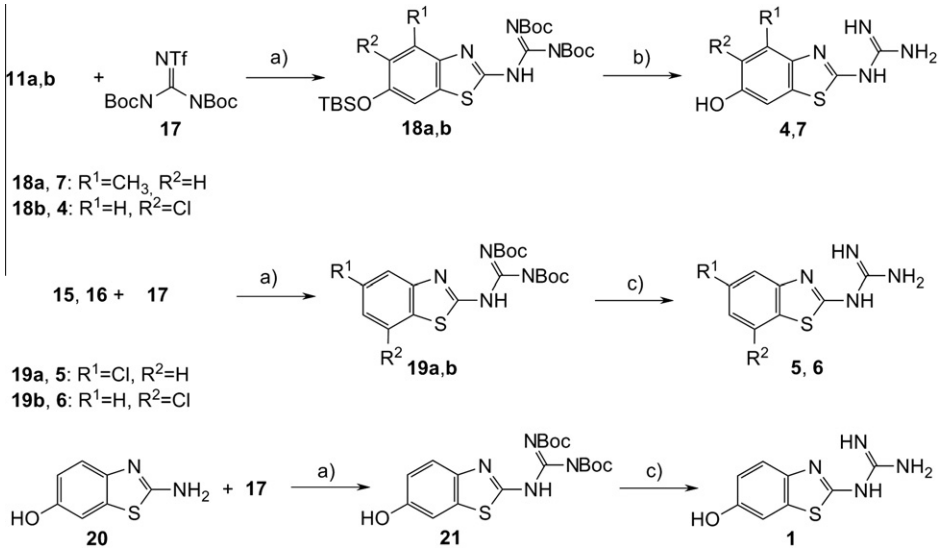


Scheme 1. Reaction conditions: (a) TBDMSCl, Imidazole, DCM, rt; (b) FmocSCN, DCM, rt; (c) morpholine, DCM, rt; (d) $ANMe_3Br_3$ (A = Ph or $PhCH_2$), DCM; (e) $PhCH_2NMe_3Br_3$, DCM, **15**:**16** = 5:1.

orientations in the S1 pocket, depending on a future connection to P2 fragments. The second set of substituted benzothiazole guanidines was analyzed by WaterLOGSY NMR fragment screening against thrombin and affinity measurements using a Biacore A100. Though WaterLOGSY NMR indicated very weak binding of these fragments to thrombin, we could not show binding of these compounds to thrombin nor inhibition of thrombin in the enzymatic assay (Table 1). However, compound **4** inhibited trypsin I and IV while compound **7** showed a selective inhibition of trypsin IV. Thus, based on these fragments, novel lead structures as trypsin inhibitors can be developed with an additional opportunity to increase selectivity towards trypsin IV.

Benzothiazole guanidines **2** and **3** were part of a library of functionalised 2-guanidino-6-hydroxy benzothiazole **1**. Alkylation of the benzothiazole guanidine scaffold was achieved by Mitsunobu reaction of the di-Boc protected precursor of **1** with suitable alcohols and subsequent Boc-deprotection. Benzothiazole guanidines **4**–**7** were prepared as outlined in Scheme 1 and Scheme 2. A key step in this synthesis was the preparation of substituted 2-aminobenzothiazoles **11a**, **11b**, **15** and **16** (Scheme 1). Starting from anilines **8a**, **8b** and **12**, condensation with Fmoc-isothiocyanate in dichloromethane (DCM) at rt provided the Fmoc-protected thiourea. Subsequent Fmoc-deprotection with morpholine gave thioureas **10a**, **10b** and **14**. Cyclisation of the thioureas using benzyltrimethylammonium tribromide provided 4-, 5-, 6- and 7-substituted 2-aminobenzothiazoles **11a**, **11b**, **15** and **16**. Di-Boc protected 2-guanidinobenzothiazoles **18a**, **18b**, **19a**, **19b** and **21** were obtained by treatment of the corresponding 2-aminobenzothiazoles with *N*,*N*′-di-Boc-*N*′-triflylguanidine **17** as guanidinylation reagent[20,21] (Scheme 2). Deprotection of the Boc-group with trifluoroacetic acid (TFA) in DCM yielded target compounds **1**, **5** and **6**. Protected guanidines **18a** and **18b** were deprotected using a mixture of TFA and HF in DCM to give **4** and **7**.

Fragment screening techniques are powerful tools to find new weak thrombin inhibitors with comparatively low molecular weight. In this work, WaterLOGSY fragment screening proved to be successful to identify binding fragments. Binding data could then be obtained using the plasmon surface resonance technology and enzymatic assays. To design potent thrombin inhibitors starting from such fragments with comparatively low affinity, it is essential to know the binding mode of these fragments. The crystal structure of the most potent compound with thrombin revealed a rather unexpected binding mode. So far, the key interaction of strong basic P1 fragments containing the amidine motif is



Scheme 2. Reaction conditions: (a) Dioxane, NEt_3, microwave heating 110 °C; (b) TFA, HF, DCM, rt; (c) TFA, DCM, rt.

interaction of the amidine with Asp189. In our case we could demonstrate that the guanidine group in benzothiazole guanidines does not necessarily behave in the same way. Instead, the guanidine group can also interact with Glu192 and the molecule can adopt a binding mode where the molecule is flipped 180° compared to the expected binding mode. This behaviour seems to be triggered by attachment of suitable side chains. Based on this, our work resulted in the synthesis of further weak thrombin inhibitors, with a stronger focus on lower molecular complexity and lower pK_a which is considered to be favourable for increased bioavailability. However, this has to be proved for full inhibitors employing our fragments as P1-group. This approach led to a significant drop in potency against thrombin. However testing of the compounds against another emerging target protein, trypsin IV revealed activity and selectivity of some of the compounds making them attractive starting points for further work. Therefore, finding an optimal way to incorporate these novel fragments into suitable scaffolds can contribute to the future synthesis of neutral thrombin and also trypsin IV inhibitors.

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Supplementary data

Supplementary data (experimental procedures for the synthesis of **1**–**7**, Biacore and enzymatic assay protocols and crystallographic data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.05.046.

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