Boron Chemistry

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Dynamic Combinatorial Chemistry Employing Boronic Acids/Boronate Esters Leads to Potent Oxygenase Inhibitors**

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The application of dynamic reactions is a promising approach for the discovery of small-molecule ligands for proteins. To date, however, this method is limited by the few appropriate reactions and the techniques used for the analysis of protein–ligand complexes.[1] "Dynamic" functional group interconvertions that have been employed include the conversion of thiols to disulfides, the aldol reaction, and the addition of nucleophiles to ketones and aldehydes.[2] The reaction of boronic acids with diols to form boronate esters is attractive for dynamic-library formation, because it is reversible in aqueous solution in a pH-dependent manner.[3] The dynamic boronic acid/boronate ester system has been used to form supramolecular switches, some of which have been used for sugar detection.[4,5] However, this system has not been used for the identification of protein ligands. Proof of principle work with proteases, which react reversibly with boronic acids, suggests that boronic acid/boronate ester systems might be useful for the identification of enzyme inhibitors.[6]

One issue with the application of reversible reactions for ligand identification is the need to analyze labile complexes that are derived from mixtures. High-resolution techniques, such as NMR spectroscopy and X-ray crystallography, are applicable, but these are time-consuming.[7] Our research group and that of Poulsen, have used non-denaturing protein mass spectrometry to identify protein–ligand complexes formed from equilibrating mixtures of thiols/disulfides[8–11] and aldehydes/hydrazones.[7] The dynamic-combinatorial mass spectrometry (DCMS) technique has the advantages of being efficient and providing information on mass shifts, which can be used for assigning structures to the ligands that bind preferentially.

Herein we demonstrate that boronic acid/boronate ester dynamic systems coupled with protein mass spectrometry analysis are useful for the identification of protein inhibitors (Scheme 1). Our target model enzyme was prolyl hydroxylase domain isoform 2 (PHD2), which is a Fe^{II} and 2-oxoglutarate (2OG) oxygenase that regulates the human hypoxic response. PHD2 inhibition is of therapeutic interest for the treatment of anemia and ischemia-related diseases.[12]

DCMS experiments were carried out using "support ligands" **2** and **3** (Scheme 2), which were designed to participate in Fe^{II} chelation in the active site and, through the incorporation of a boronic acid moiety, participate in boronate ester exchange. We selected the 2-(picolinamido)-acetic acid scaffold because, based on crystal structures of PHD2,[13] it is predicted to fit into the active site through its chelation with Fe^{II}. The low potency of 2-(picolinamido)acetic acid ($IC_{50} > 1$ mM) enabled the effect of boronate ester substitution to be monitored.

Modeling studies suggested that whereas the boronic acid group in support ligand **2** would fit into the active-site subpocket, that of **3** would clash with the active-site wall.[14] Hence, it was envisaged that the reactivity of **3** might serve as a control to investigate possible non-specific binding. The analysis of mixtures of **2** or **3** with PHD2·Fe^{II} through the use of non-denaturing ESI-MS led to the observation of a new peak at 27887 Da (187 ± 2 Da shift), corresponding to a small molecule/protein adduct, in which the OH groups of the boronic acids moiety are cleaved. We have previously observed, through the use of non-denaturing ESI-MS, analogous apparent fragmentation of boronic acids complexed with other enzymes.[15] Notably, the mixture of boronate ester **4** and PHD2·Fe^{II} gave the same mass shift (187 ± 2 Da) as that observed with **2** and **3** at a cone voltage of 80 V.[14] However, when a lower cone voltage was used (30 V), the mass shift corresponding to an adduct of **4** with the protein, without fragmentation, was apparent (358 ± 2 Da), demonstrating that boronate ester formation can be observed when sufficiently mild ionization is used.

Both **2** and **3** compete with the 2OG analogue *N*-oxalylglycine (NOG) for the 2OG binding site of PHD2.[14] To ensure that boronate ester formation involving **2** and **3** was favorable under the conditions used (NH_4OAc

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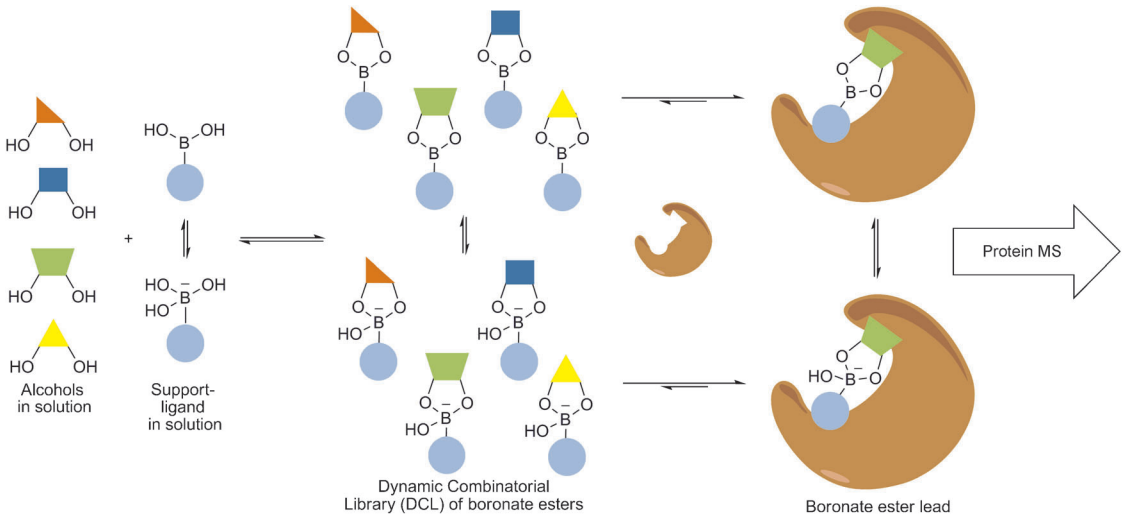
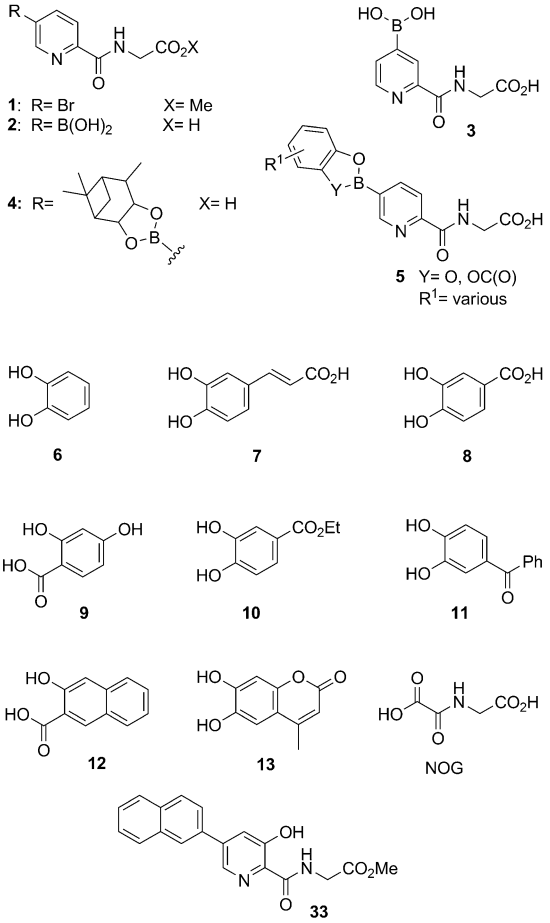
Supporting information, including syntheses and characterizations, MS methods, inhibition assays, protein purification, crystallization, and structure solution methods for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201202000.



Scheme 1. Schematic representation of the dynamic combinatorial mass spectrometry (DCMS) method that uses the conversion between boronic acids and boronate esters as the reversible reaction.



Scheme 2. Structures of selected compounds used in this work.

buffer, pH 7.5), the pK_as of the boronic acids were measured by NMR spectroscopy. The pK_a values of **2** (approximately 7.4) and **3** (approximately 6.9) [14] are appropriate for boronate ester formation under the incubation conditions.[14]

We then investigated whether boronate esters could be used in DCMS. Forty diols were separated into four sets (i–iv) of ten diols[14] according to molecular weight (see the Supporting Information for details). Each set was treated with either support ligands, **2** or **3**, in the presence of PHD2·Fe^{II}. ESI-MS was then used to determine which of the in situ formed boronate esters bind preferentially to PHD2·Fe^{II} (Figure 1).

The reaction of support ligand **2** with the diol sets i–iv resulted in the observation of peaks with mass shifts corresponding to complexes of PHD2 with boronate esters of **2** (**5**, Scheme 2), which were derived from 7 diols (**7**–**13**, Scheme 2). ESI-MS analysis of mixtures of **2** with the individual diols validated the above DCMS results.[14] In some cases, diols were observed to bind to PHD2·Fe^{II} in the absence of **2**, presumably through Fe chelation (Figure 1).[14]

In contrast to the results with **2**, when **3** was employed, no binding of boronate esters was observed in the corresponding assay. Direct binding of some diols to PHD2·Fe^{II} was again observed (Figure 1). The results imply that the mass shifts observed with **2** represent protein adducts involving boronate esters derived from support ligand **2** rather than adducts derived from simultaneous binding of **2** and diols. In the case of diol **8**, which was observed to bind to PHD2·Fe^{II} both in the absence of a support ligand (**2** and **3**) and in the form of the boronate ester derived from **2**, addition of the 2OG competitor NOG to the PHD2/Fe^{II}/**8** mixture resulted in separate peaks representing the adducts derived from the binding of PHD2·Fe^{II} with both **8** and NOG; no peak derived from simultaneous binding of **8** and NOG was observed. These observations support the hypothesis that boronate esters derived from **2** are formed and bind to the protein and that, in the absence of **2**, diol **8** and also the diols **11**, **13**, and **34**–**38** (see the Supporting Information for details) bind to PHD2 through chelation with Fe^{II}.[14]

The results of ESI-MS analyses are not always representative of what exists in solution,[16] therefore the DCMS results were validated by solution studies. NMR-based water

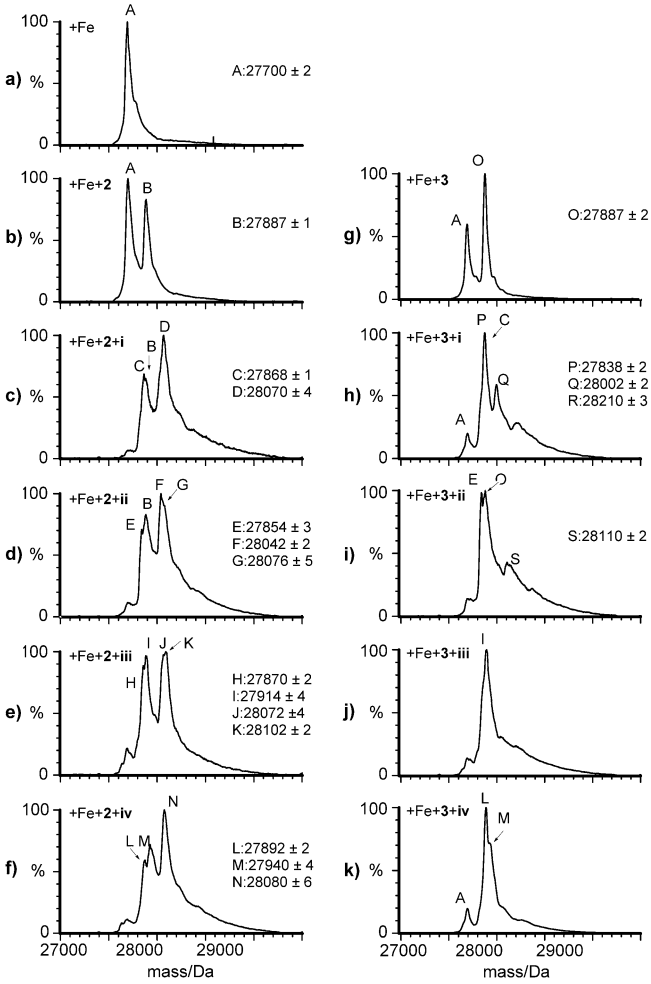


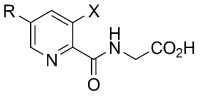
Figure 1. DCMS analyses of PHD2 with boronic acids **2**/**3** and diol sets i–iv. Spectra of deconvoluted non-denaturing ESI-MS (cone voltage 30 V) showing: a) PHD2·Fe^{II}; b) PHD2·Fe^{II} with **2** (1:1); c–f) PHD2·Fe^{II} with **2** and i–iv (1:1:1), respectively; g) PHD2·Fe^{II} with **3** (1:1), h–k) PHD2·Fe^{II} with **3** and diols i–iv (1:1:1), respectively. Assignment of labeled peaks: PHD2·Fe·**2** (peak B); PHD2·Fe·**3** (peak O); PHD2·Fe··diol (peaks C, E, H, I, L, M, P, and Q); PHD2·Fe·**5** (peaks D, F, G, J, K, and N).

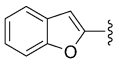
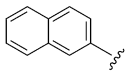
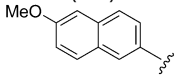
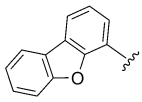
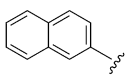
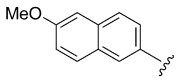
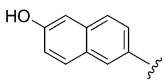
relaxation experiments [17,18] were used for the measurement of the apparent binding constants ($K_{D,app}$) of **2** with PHD2, in the presence of different diols. In this method, paramagnetic Mn^{II} was used as a substitute for the diamagnetic Fe^{II}; $K_{D,app}$ values were determined by monitoring the bulk water relaxation rate, which decreases when water access to the paramagnetic metal center is hindered through binding of compounds to the metal. The support ligand **3** displayed no significant PHD2 binding, as determined by NMR spectroscopy.[14] When the NMR experiments were carried out using **2** in the absence or in the presence of diols (**6**–**13**), it was found that its apparent affinity for PHD2 ($K_{D,app} = 24.8$ μM) was enhanced in the presence of the diols that had been identified by DCMS as being able to form boronate esters (e.g. **2**/**12**: $K_{D,app} = 3.0$ μM, **2**/**10**: $K_{D,app} = 1.3$ μM, **2**/**11**: $K_{D,app} = 0.6$ μM). The presence of diols that were not observed to form boronate esters in the DCMS assays caused a much weaker increase in the apparent binding affinity of **2** (**2**/**6**: $K_{D,app} =$ 13.2 μM, **2**/**39**: $K_{D,app} = 18.2$ μM).[14] Hence there is qualitative agreement between the gas-phase and solution studies.

To verify the potential of the boronic acid/boronate ester-based DCMS for identification of PHD2 inhibitors, the synthesis of stable analogues that mimic the structure of the identified hits (boronate esters derived from **2** and diols **7**–**13**) from DCMS experiments was attempted. To prepare stable analogues of the boronate esters, we made use of the boronic acid functional group, which was also employed in the DCMS chemistry, in Suzuki cross-coupling reactions.[14] Benzofuran- and naphthalene-based analogues, **16** and **18**, respectively, were prepared; the benzofuran and naphthalene moieties function as 6/5- and 6/6-fused bicyclic mimics of the catechol boronate ester moiety in the boronate ester derived from **2** and catechol (**6**). The binding constants of **16** and **18** were similar (**16**: $K_D = 9.5$ μM, **18**: $K_D = 7.0$ μM) suggesting that both ring systems are accommodated in the protein binding pocket. The 5-methoxynaphthalene derivative **20** was used as a mimic of the boronate esters derived from 4-substituted catechols (**7**–**11**) and, notably, **20** ($IC_{50} = 107$ μM, $K_D = 1.6$ μM) showed greater inhibition and binding affinity than **16** and **18**; these results are consistent with the DCMS results. NMR water relaxation measurements showed that all the stable analogues bind significantly stronger to PHD2 than the parent compound **2** (Table 1); however they were generally poorer binders than the boronate esters identified by DCMS.[14]

Analysis of PHD2 structures[14] suggests that hydrogen-bonding interactions between the inhibitor and the side chain

Table 1: Binding constants (K_D) as measured by NMR spectroscopy and IC_{50} values for selected inhibitors.[14]



Compound	R	X	K_D [μM]	IC_{50} [μM]
14	H	H	N/A	>1000
16		H	9.5	>500
18		H	7.0	>500
2	$B(OH)_2$	H	24.8	126
20		H	1.6	107
22		H	8.7	>100
23	H	OH	3.5	409
30		OH	0.5	0.017
28		OH	0.8	0.013
32		OH	0.9	0.004

hydroxy group of Tyr303 might promote inhibitor binding.[13] Thus, a derivative of inhibitor **20** containing a hydroxy group at the C3 position, **28**, was prepared.[14] Compound **28** showed significantly higher potency and affinity ($IC_{50} = 13$ nM, $K_D = 0.8$ μM) than **20**. As indicated by DCMS, the aryl substituent at the 5-position is important: **28** showed higher potency than **23** (Table 1).[11] In the presence of the C3 hydroxy group, the methoxy group on the naphthalene ring, which improved the activity of the initial inhibitors, is less important, as shown by the similar potency of **28** and **30** (Table 1). However, substitution at the C5 position of the naphthalene moiety remains important as indicated by the higher activity of **32** relative to **30**.

To investigate the mode of binding of **28**, it was crystalized in complex with the PHD2 catalytic domain incorporating Mn^{II}, which substitutes for Fe^{II}.

The crystal structure reveals that **28** complexes to the metal in a bidentate manner, with the C3 hydroxy group interacting with Tyr303 through a hydrogen bond. The naphthalene group of **28** is twisted relative to the pyridinyl ring (torsion angle 52.8°), thus allowing the naphthalene moiety to occupy the substrate-binding site (Figure 2).

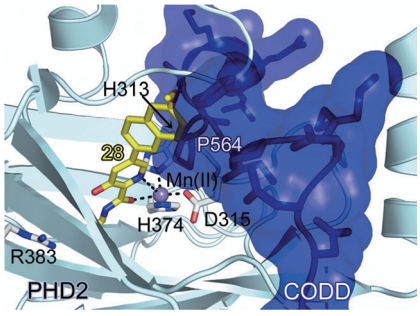


Figure 2. View from a crystal structure of PHD2·Mn^{II} (light blue) complexed with **28** (yellow) superimposed with a structure of PHD2·Mn^{II} in complex with HIF-1α residues 558-574 ("CODD": blue surface; PDB ID 3HQR). The image shows the proximity of the C5 substituent of **28** (5-methoxynaphthalene) to the substrate binding site. The active-site metal is in purple and Pro564 of HIF-1α is labeled.

Finally, to investigate the efficacy of the C5 pyridyl compounds in cells, we tested the methyl ester derivative of **30** (**33**, Scheme 2) owing to its better cell penetration. **33** was shown to upregulate HIF1α in cells by selectively inhibiting PHDs but not Factor Inhibiting Hypoxia (FIH).[14]

Overall, we have demonstrated the potential of boronic acids/boronate esters for inhibitor discovery using a biologically relevant human hydroxylase. We envision that similar approaches that use this boron chemistry will be applicable to other proteins. Suitably protected boronic acids were also used for the synthesis of stable analogues through organometallic chemistry. We have found that there are potential limitations of the DCMS approach, for example, boronic acids can sometimes fragment under the conditions of MS. However, we foresee that improved MS screening methods that give results that are more representative of the composition of the analyte in solution will become available as the technology develops. We have also shown that NMR-based screening methods, and other types of analysis, can also be employed in the evaluation of boronate ester binding.

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