Discovery Boronic Acids as Novel and Potent Inhibitors of Fatty Acid Amide Hydrolase

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General methods and materials

Compounds **2**, **11** and **21** were purchased from Boron Molecular Ltd, compounds **1** (purity 97%), **3**, **7**, **8**, and **22** from Sigma-Aldrich, Inc., compounds **4-6**, **9**, **10**, **12**, and **14-19** from Combi-Blocks, Inc., and compounds **13** and **20** from Lancaster Synthesis, and used for testing without further purification. Elemental analyses (CHN) were carried out with a Thermo Quest CE Instrument EA 1110 CHNSO elemental analyzer. The ^{1}H NMR spectra were recorded on a Bruker Avance 500 spectrometer operating at 500.1 MHz. DMSO-d_6 (2.54 ppm) was used as an internal standard of solvent.

Elemental analysis data of compounds 2-23[a,b]

Cmpd	Formula	Calculated	Found
2	$C_9H_{11}BO_4$	C, 55.72; H, 5.72; N, 0	C, 55.47; H, 5.27; N, 0
3[c,d]	$C_7H_6BF_3O_2$	C, 44.27; H, 3.18; N, 0	C, 46.28; H, 2.59; N, 0
4[d,e]	$C_7H_6BNO_2 \cdot 5.1\% H_2O$	C, 54.26; H, 4.48; N, 9.04	C, 53.83; H, 3.95; N, 8.74
5	$C_{12}H_{11}BO_2$	C, 72.78; H, 5.60; N, 0	C, 72.40; H, 5.60; N, 0
6	$C_7H_9BO_3$	C, 55.33; H, 5.97; N, 0	C, 55.52; H, 5.97; N, 0
7[c,d]	$C_6H_6BFO_2 \cdot 12\%H_2O$	C, 45,27; H, 5,15; N, 0	C, 44,82; H, 4,74; N, 0
8	$C_7H_6BF_3O_2$	C, 44.27; H, 3.18; N, 0	C, 43.95; H, 3.60; N, 0
9	$C_6H_6BNO_4$	C, 43.17; H, 3.62; N, 8.39	C, 43.00; H, 3.21; N, 8.44
10	$C_7H_6BNO_2$	C, 57.22; H, 4.12; N, 9.53	C, 56.97; H, 3.68; N, 9.38
11	$C_{12}H_{11}BO_2$	C, 72.78; H, 5.60; N, 0	C, 72.90; H, 5.60; N, 0
12	$C_7H_9BO_3$	C, 55.33; H, 5.97; N, 0	C, 55.28; H, 6.08; N, 0
13	$C_{15}H_{25}BO_2$	C, 72.60; H, 10.15; N, 0	C, 72.38; H, 9.73; N, 0
14[d,f]	$C_6H_6BFO_2 \cdot 2.5\% H_2O$	C, 50.17; H, 4.50; N, 0	C, 49.76; H, 4.07; N, 0
15[d,g]	$C_7H_6BF_3O_2 \cdot 0.9\% H_2O$	C, 43.89; H, 3.25; N, 0	C, 43.44; H, 2.80; N, 0
16[d,h]	$C_{12}H_{11}BO_2 \cdot 1.8\% H_2O$	C, 71.48; H, 5.70; N, 0	C, 71.65; H, 5.71; N, 0
17	$C_7H_9BO_3$	C, 55.33; H, 5.97; N, 0	C, 55.28; H, 6.05; N, 0
18[d,i]	$C_8H_7BO_2S \cdot 4.5\% H_2O$	C, 51.53; H, 4.29; N, 0	C, 51.11; H, 3.84; N, 0

19[d,j]	$C_8H_7BO_3 \cdot 0.5\% H_2O$	C, 59.00; H, 4.39; N, 0	C, 58.65; H, 3.97; N, 0
20[c]	$C_5H_5BFNO_2 \cdot 1.8\% H_2O$	C, 41.82; H, 3.72; N, 9.75	C, 41.66; H, 3.72; N, 9.67
21[d,k]	$C_8H_{11}BO_2 \cdot 4.7\% H_2O$	C, 61.06; H, 7.57; N, 0	C, 60.61; H, 6.91; N, 0
22	$C_{14}H_{13}BO_2$	C, 75.05; 5.85; N, 0	C, 75.36; H, 6.02; N, 0

a. Owing to a possible anhydride formation under high vacuum,[1] compounds were not dried prior to elemental analysis. May contain small amounts of water (for the water containing compounds certificates of analyses and/or ^{1}H NMR spectrums are provided).

b. The structures of all the biologically evaluated compounds (**1**-**22**) were confirmed by ^{1}H NMR spectroscopic methods.

c. Purity >98.0% establish by ^{1}H NMR spectroscopic methods; ^{1}H NMR spectrum provided (page S15-S17).

d. Certificate of analysis provided.

e. Purity 98.1% (HPLC) confirmed by manufacturer (see certificate of analysis).

f. Purity 98.8% (HPLC) confirmed by manufacturer (see certificate of analysis).

g. Purity 99.0% (HPLC) confirmed by manufacturer (see certificate of analysis).

h. Purity 99.5% (HPLC) confirmed by manufacturer (see certificate of analysis).

i. Purity 98.3% (HPLC) confirmed by manufacturer (see certificate of analysis).

j. Purity 100% (HPLC) confirmed by manufacturer (see certificate of analysis).

k. Purity >99.0% (GC, NMR) confirmed by manufacturer (see certificate of analysis).

Enzyme inhibition studies

Animals and preparation of rat brain homogenate for FAAH assay. Eight-week-old male Wistar rats were used in these studies. All animal experiments were approved by the local ethics committee. The animals lived in a 12-h light/12-h dark cycle (lights on at 0700 h) with water and food available *ad libitum*.

The rats were decapitated, forebrains were dissected and homogenized in one volume (v/w) of ice-cold 50 mM Tris-HCl, pH 7.4; 1 mM EDTA with a Potter-Elvehjem homogenizer (Heidolph). The homogenate was centrifuged at 10,000 *g* for 20 min (at 4 °C). The protein concentration of the supernatant was determined by the method of Bradford with BSA as a standard.[2] Aliquots of the supernatant were stored at -80 °C until use.

FAAH assay procedure. The assay for FAAH has been described previously.[3] The endpoint enzymatic assay was developed to quantify FAAH activity with tritium labelled arachidonoylethanolamide [ethanolamine 1-^{3}H]. The assay buffer was 50 mM Tris-HCl (pH 7.4); 1 mM EDTA and test compounds were dissolved in DMSO (the final DMSO concentration was not more than 5% v/v). The incubations were performed in the presence of 0.5% (w/v) BSA (essentially fatty acid free). Test compounds were preincubated with rat brain homogenate protein (18 µg) for 10 min at 37 °C (60 µl). At the 10 min time point, *N*-arachidonoylethanolamide was added to achieve the final concentration of 2 µM (containing 50 x 10^{-3} µCi of 60 Ci/mmol [^{3}H]AEA) with the final incubation volume of 100 µl. The incubations proceeded for 10 min at 37 °C. Ethyl acetate (400 µl) was added at the 20 min time point to stop the enzymatic reaction. Additionally, 100 µl of buffer (50 mM Tris-HCl, pH 7.4; 1 mM EDTA) was added. Samples were centrifuged at 16,000 *g* for 4 min at RT, and aliquots (100 µl) were taken from the aqueous phase, which contained ethanolamine 1-^{3}H, and measured for radioactivity by liquid scintillation counting (Wallac 1450 MicroBeta; Wallac Oy, Finland).

MGL assay procedure. The endpoint enzymatic assay was developed to quantify human recombinant MGL (Cayman Chemical, cat# 10008354) activity with 2-arachidonoylglycerol (2-AG). The assay buffer was 50 mM Tris-HCl, pH 7.4; 1 mM EDTA and test compounds were dissolved in DMSO (the final DMSO concentration was not more than 5% v/v). The incubations were performed in the presence of 0.5% (w/v) BSA (essentially fatty acid free). hrMGL was preincubated with test compounds for 10 min at 37 °C (60 µl). At the 10 min time point, 2-AG was added to achieve the final concentration of 50 µM with the final incubation volume of 100 µl. The incubations proceeded for 10 min at 37 °C. To stop the enzymatic reaction against 2-AG, acetonitrile (200 µl) was added at the 20 min time point and the pH of the samples was simultaneously decreased to 3.0 with phosphoric acid (added to acetonitrile) to stabilize 2-AG against acyl migration to 1-AG. All samples were centrifuged at 16,000 *g* for 4 min at RT. The formation of arachidonic acid and depletion of 2-AG (and 1-AG) was measured by HPLC.

HPLC method. The analytical HPLC was performed as previously described.[4] Briefly, the analytical HPLC system consisted of a Merck Hitachi (Hitachi Ltd., Tokyo, Japan) L-7100 pump, D-7000 interface module, L-7455 diode-array UV detector (190 – 800 nm, set at 211 nm) and L-7250 programmable autosampler, or of a Agilent (Agilent Technologies, Santa Clara, California, U. S. A.) 1100 binary pump, 1100 autosampler, 1100 vacuum degasser, 1100 thermostatted column compartment, 35900E A/D interface module and HP (Hewlett-Packard Company, Palo Alto, California, U. S. A.) 1050 variable wavelength detector. The separations were accomplished on a endcapped Phenomenex C18 SecurityGuard Cartridge (4 x 3.0 mm) (Phenomenex, U.S.A) and Zorbax SB-C18 column (4.6 x 150 mm, 5 µm) (Agilent, U.S.A). The injection volume was 50 µl. A mobile phase mixture of 28% phosphate buffer (30 mM, pH 3.0) in acetonitrile was used at a flow rate of 2.0 ml min^{-1}. Retention times were 5.0 min for 2-AG, 5.3 min for 1-AG and 8.4 min for arachidonic acid. The relative concentrations of 2-AG, 1-AG and arachidonic acid were determined by the corresponding peak areas.

Data analyses.

The results from the enzyme inhibition experiments are presented as mean ± 95% confidence intervals of at least three independent experiments performed in duplicate. Data analyses for the concentration-response curves were calculated as non-linear regressions using a built-in equation "sigmoidal dose-responce curve, variable Hill slope" GraphPad Prism 4.0 for Windows.

Calculation of pK_a values.

The calculated pK_a values were obtained by using the pK_a software (version 4.0) from ACDLabs (Toronto, Canada).

Molecular modelling studies

Structure construction

All compound structures were constructed using Sketch module of SYBYL 8.0.[5] As SYBYL package is lacking parameters for boron-containing compounds by default, we applied similar strategy reported earlier in the literature, that is, mimicking boron atom with sp^3-hybridised carbon (SYBYL atom type 'C.3').[6,7] The method described by Johnsamuel et al.[7] could not be fully implemented as the docking program of our choice (due to availability and earlier positive experiences with FAAH) employs a force field during the posing process. Thus, structure optimization and point charge calculations of the compounds in semi-empirical or *ab initio* level prior to docking would have been groundless. Again, as SYBYL lacks the parameters for boron, and as the docking program was accessed via SYBYL, the sp^3 carbon had to be used.

After the construction, the compounds were minimized employing the Merck molecular force field (MMFF94)[8] with Powell conjugate-gradient minimizer (as implemented in SYBYL) to an energy gradient of 0.005 kcal/(mol Å). The X-ray crystal structure of

murine FAAH complexed with methyl arachidonyl fluorophosphonate (MAFP) (Protein Data Bank code 1MT5)[9] was used as the protein structure for docking calculations. Monomeric enzyme (chain A) was extracted from the crystal data, missing side chain atoms (none at the active site) were added with SYBYL Biopolymer module using suitable conformations with minimal structural violations from the Lovell rotamer library.[10] The side chain amides of Gln48, Gln60, Gln124, Asn159, Gln189, Gln519 and Gln570 were reversed to maximize internal hydrogen bonding, the MAFP atoms were removed, missing hydrogen atoms were added, and protein side chain atoms were energy-minimized with Amber FF99[11] as implemented in Sybyl 8.0 (steepest descent, 300 iterations). All modeling and visualization were done using a dual-processor, dual-core Intel Xeon 3.0 GHz Linux PC workstation.

Molecular docking

Surflex-Dock 2.1[12], as implemented in SYBYL 8.0, was used as the docking tool in this study. Surflex-Dock utilizes 'protomol', a representation of the active site with steric and hydrogen bonding probes, to direct the initial placements of the ligands during the posing phase of the docking process. In this study, FAAH residues within 1.5 Å radius from the MAFP were chosen for the protomol generation. In order to expand the protomol from the ligand-based coordinates, settings for 'threshold' and 'bloat' were modified to 0.01 and 10 Å, respectively. Also, it should be noted that ligand posing during the docking process is done with the full protein structure, and it is not constrained to protomol region only.

For the actual docking process, we used settings aimed at thorough sampling. Prior to docking, Surflex-Dock was allowed to pre-minimize the ligands with the implemented BFGS method employing DREIDING force field.[13] To enhance the sampling during the ligand posing, the number of additional conformations per molecule and the maximum number of conformations per fragment were set to 140 and 200, respectively. Surflex-Dock was set to treat ring systems flexibly. Additionally, ligands were relaxed in the active site after the docking using the aforementioned DREIDING force field method as

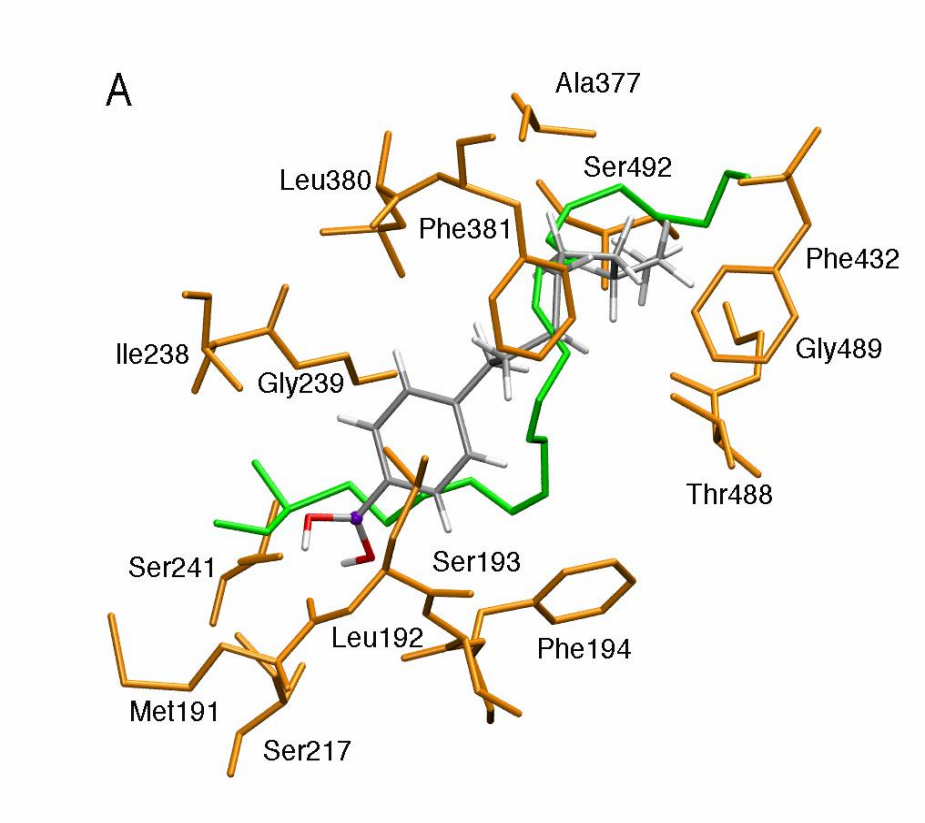
implemented in Surflex-Dock. 25 of the best-ranked conformations for each ligand were retained. Docked poses were visualized with Sybyl 8.0[5] and MOE 2007.0902.[14]

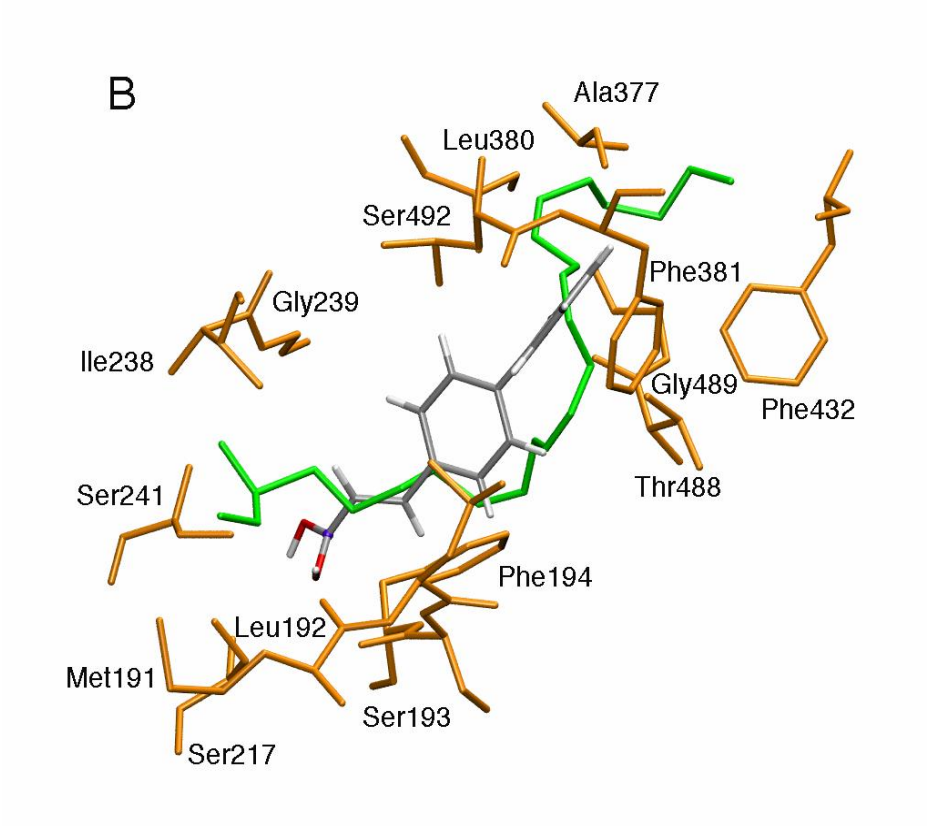
Results and discussion – Molecular Docking

The trade-offs in the modeling of boron atom (see Structure construction), and the fact that the formation of the transition state analog of boronic acid upon covalent binding[15] is not possible to model with docking gave us reason to be cautious when analyzing the docking results. With docking, we can gain information of the initial recognition phase of FAAH-ligand interaction, and the sterical and electrostatical complementarity thereof. Also, it should be noted that by using classical static molecular mechanistic methods, the function of the Ser217-Ser241-Lys142 catalytic triad[9,16] is not modeled correctly. Consequently, the energy surface of binding in the catalytic region is ill-defined and the interaction with Ser241 and ligand is less favorable than in reality. Taking these sources of error into account, we concentrate only on the five most potent compounds with FAAH inhibition IC_{50} values in the nM level (IC_{50}; 9.1 – 80 nM).

All of the 25 top-ranked conformations for each ligand were visualized in the active site of FAAH. As the scores of the poses were deviating only a little within the poses of each ligand (data not shown), the best pose for each ligand could not be judged solely on the basis of the Surflex-Dock scoring function. After the visual inspection, the "best" poses were selected manually. These are shown in Figure S1. As can be seen, apart from **8**, the boronic acid end of the compounds is in close contact with Ser217 and Ser241, and the lipophilic alkyl, aryl or CF_3 substituents are pointing towards the lipophilic acyl chain binding (ACB) channel, similar to MAFP and PF-750 in the published FAAH X-ray crystal structures.[9,17] The binding of **8** in further of ACB, closer to the surface of FAAH, is most likely not due to strong interactions in that particular region (hydroxyl group of boronic acid is forming one hydrogen bonding interaction with the backbone carbonyl of Arg428) but the aforementioned problems in the description of the boronic acid/catalytic triad functionality. At this point, when the exact binding geometry/mechanism of the FAAH inhibition by boronic acids is not clear, we do not believe it to be fruitful to

analyze our dockings in residue-by-residue level. More elaborate modeling and biological studies are certainly needed in order to gain full insight into binding of boronic acids, and to further guide the design of novel boron-based FAAH inhibitors.

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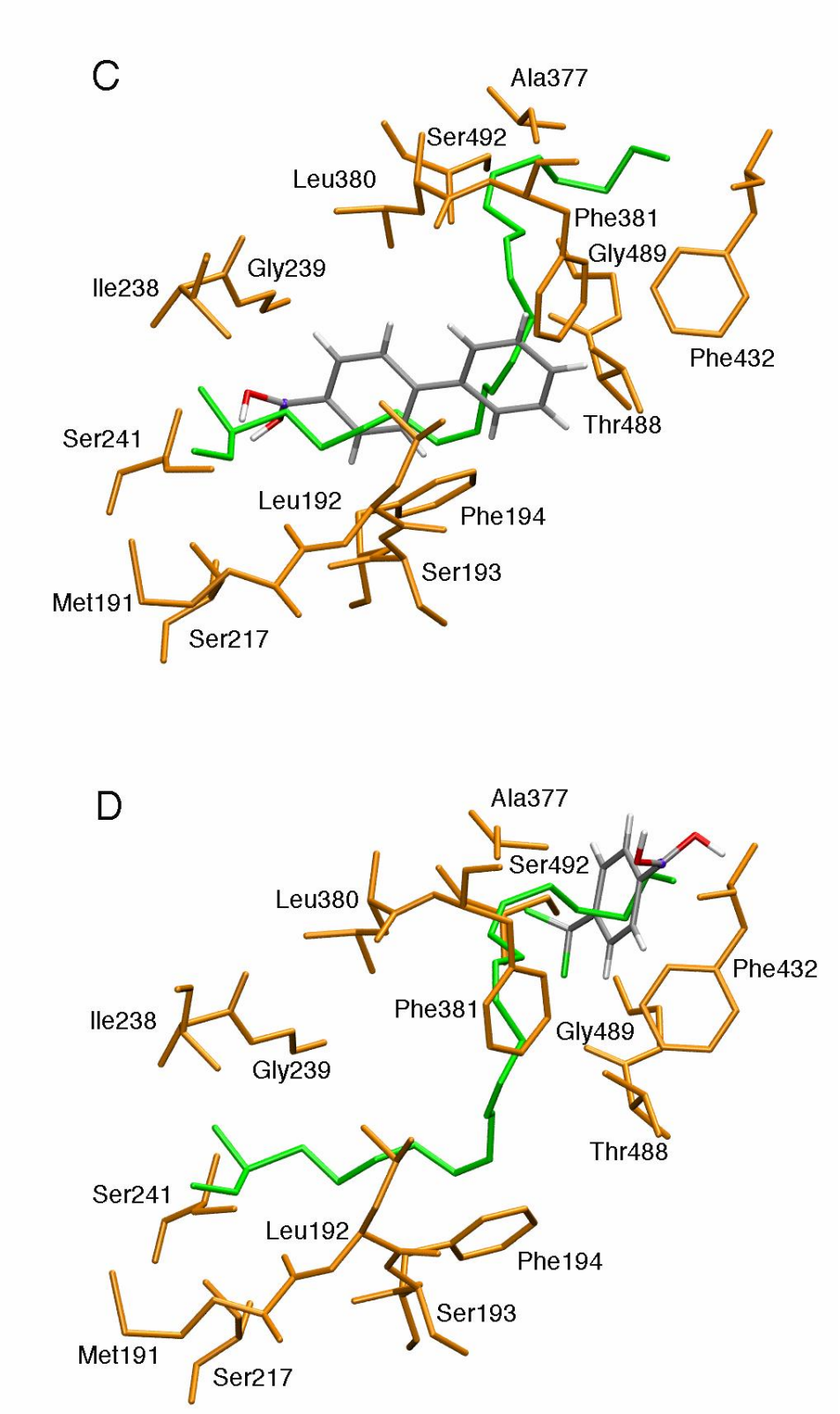
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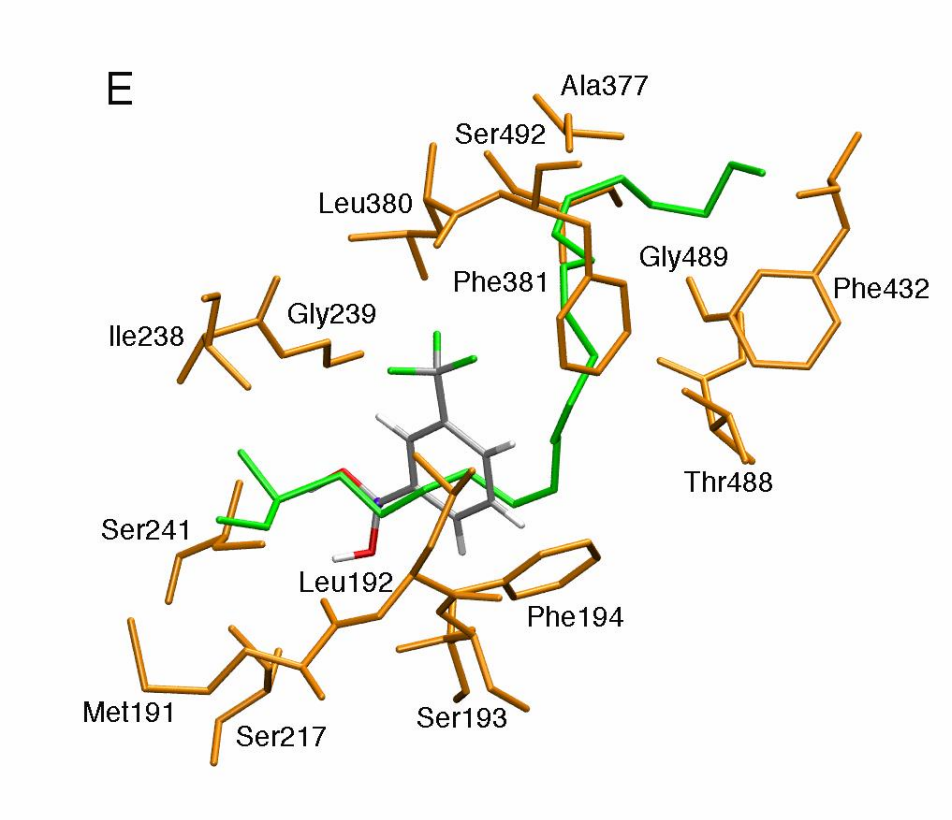

Figure S1. Possible FAAH (protein-ligand recognition phase) binding modes of the boronic acids with nanomolar IC_{50} values. (A) **13** (B) **22** (C) **11** (D) **8** (E) **3**. The crystallized inhibitor (MAFP) from 1MT5 is shown in green.[9] Amino acids are shown in orange. For the sake of clarity, hydrogen atoms of amino acids and MAFP are omitted. Figure rendered with VMD 1.8.6.[18]

References

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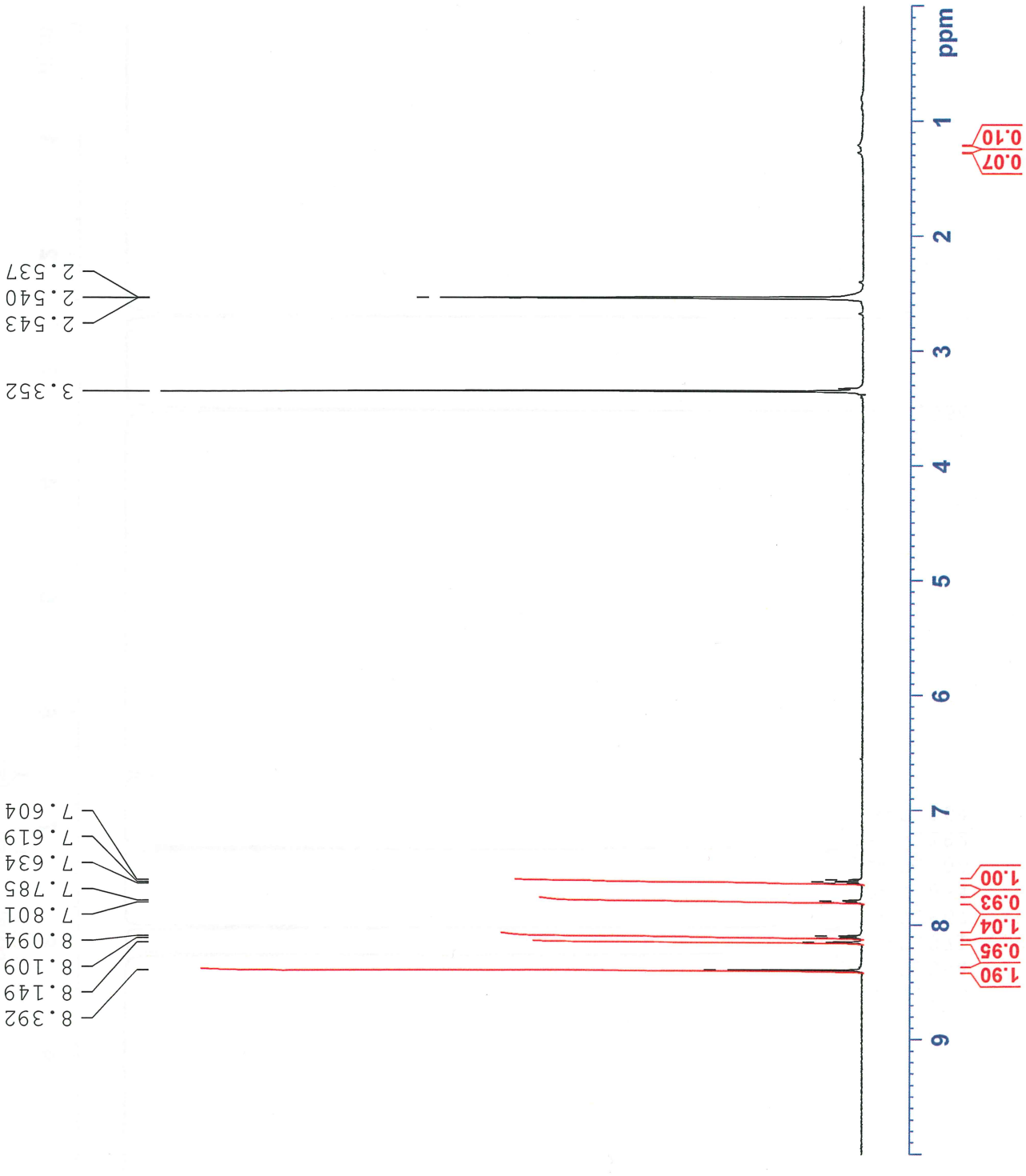
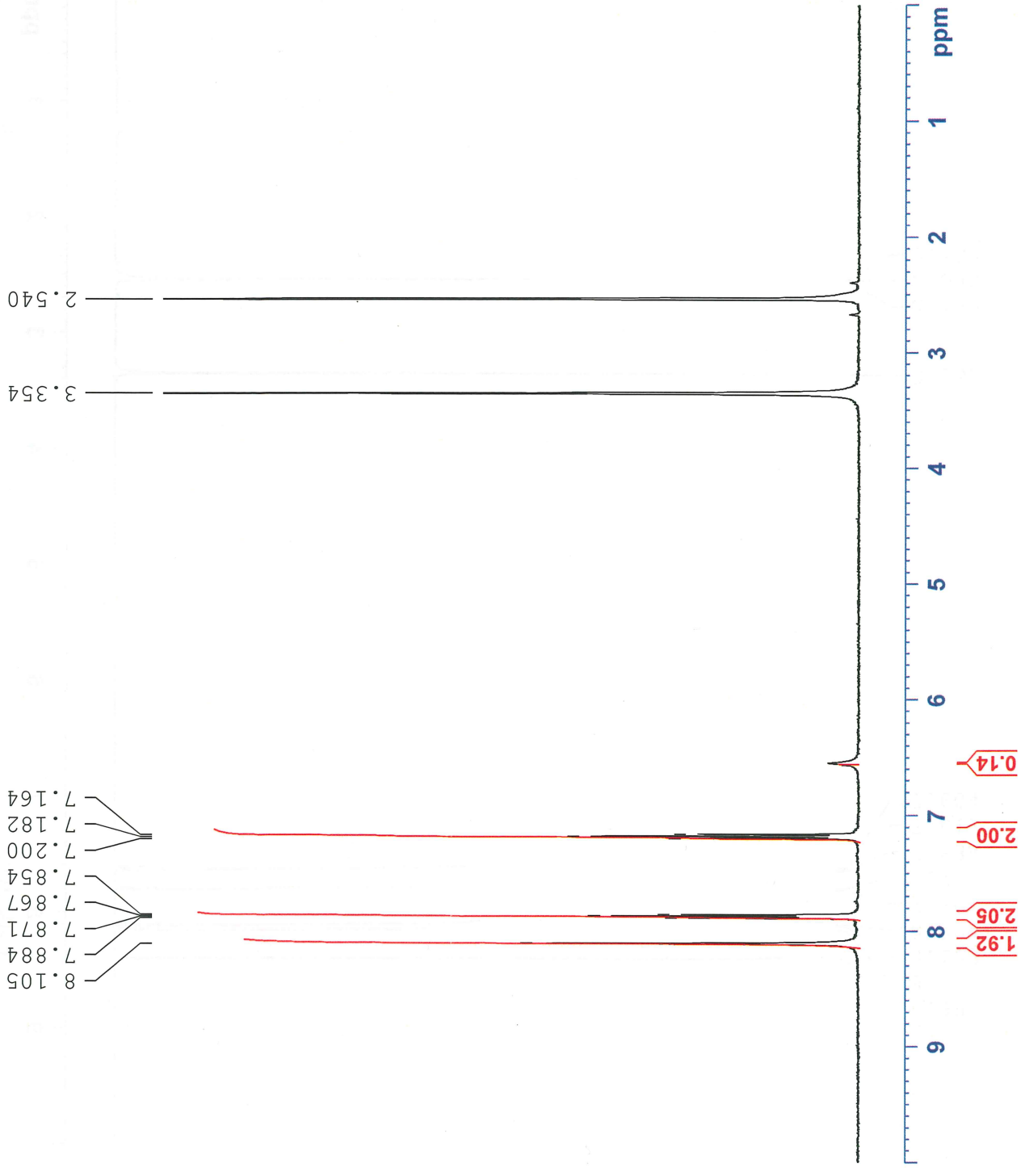
3-(Trifluoromethyl)phenylboronic acid (3), 1H, DMSO



4-Fluorophenylboronic acid (7), 1H, DMSO



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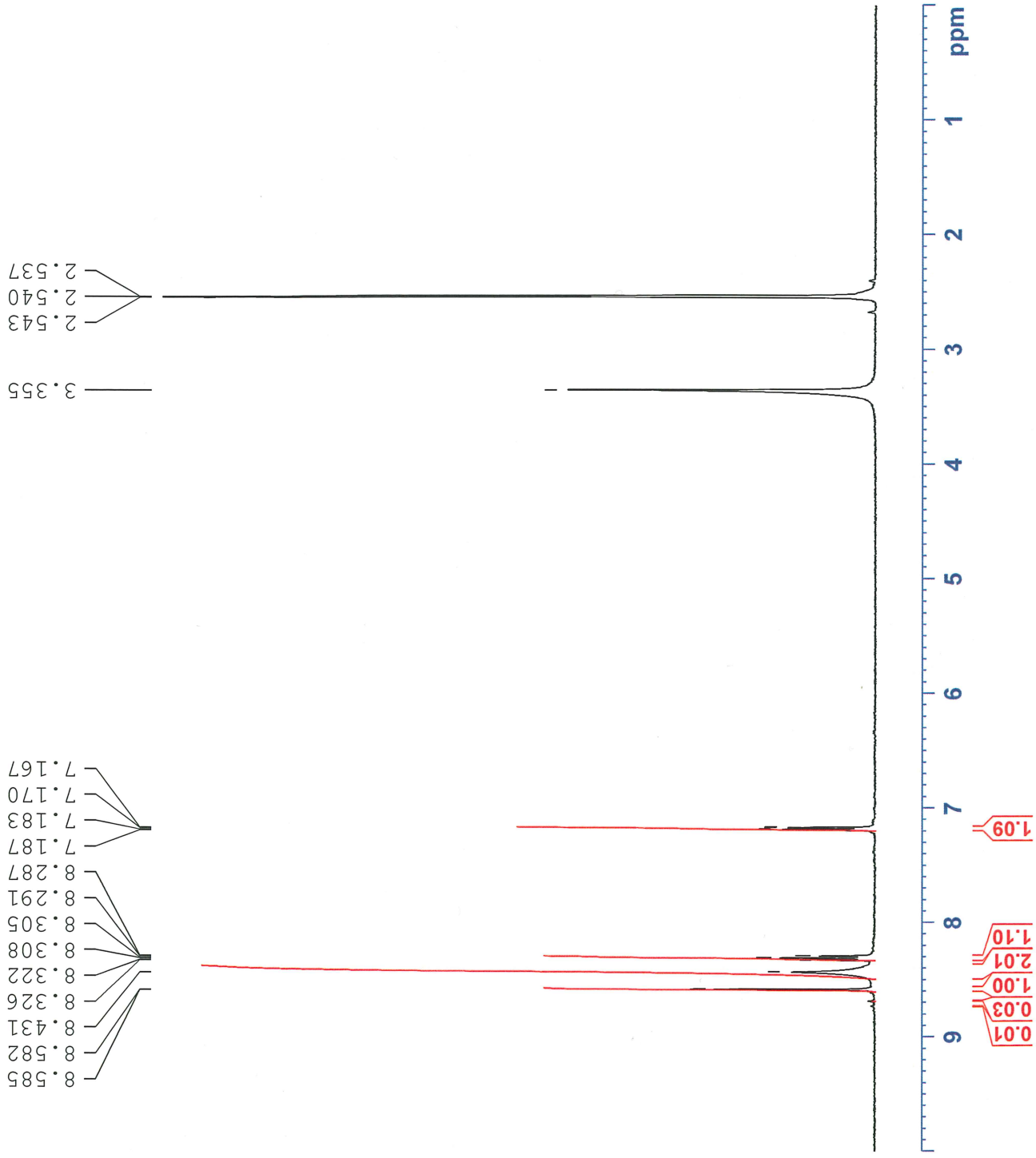

SIGMA-ALDRICH

Certificate of Analysis

Product Name	3-(Trifluoromethyl)phenylboronic acid
Product Number	432032
Product Brand	Aldrich
CAS Number	1423-26-3
Molecular Formula	$CF_3C_6H_4B(OH)_2$
Molecular Weight	189.93

TEST	SPECIFICATION	LOT 02205CO RESULTS
APPEARANCE	WHITE TO LIGHT YELLOW POWDER, CRYSTALS, OR	LIGHT YELLOW CRYSTALLINE POWDER
MELTING POINT		166-169 DEGREES CELSIUS.
INFRARED SPECTRUM	CONFORMS TO STRUCTURE.	CONFORMS TO STRUCTURE.
PROTON NMR SPECTRUM	CONFORMS TO STRUCTURE.	CONFORMS TO STRUCTURE.
TITRATION	95.0% (MINIMUM)	95.4 % (WITH NAOH)
QUALITY CONTROL ACCEPTANCE DATE		MARCH; 2001

Barbara Rajzer, Supervisor
Quality Control
Milwaukee, Wisconsin USA

Combi-Blocks, Inc
7949 Silverton Ave, Suite 915
San Diego, CA 92126, USA

Toll free: 1-877-5-BLOCKS
International: 1-858-635-8950
Fax: 1-858-635-8991
Email: sales@combi-blocks.com
Web Site: www.combi-blocks.com

CERTIFICATE OF ANALYSIS

Product Number	**BB-2455**
Product Name	(3-Cyanophenyl)boronic acid
CAS Number	[150255-96-2]
Molecular Formula	$C_7H_6BNO_2$
Molecular Weight	146.9

TEST RESULTS

BATCH NUMBER	**L15445**
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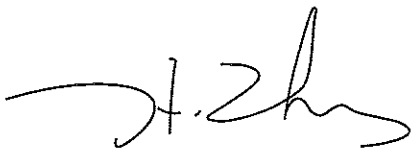
BOILING POINT	No Data
MELTING POINT	298-310° C
NMR	98%, conform with structure
HPLC	98.1%
TLC	98%



Howard Zhang, Ph.D.
CEO

08/25/05

Acceptence Date

SIGMA-ALDRICH

Certificate of Analysis

Product Name	4-Fluorophenylboronic acid
Product Number	417556
Product Brand	Aldrich
CAS Number	1765-93-1
Molecular Formula	$FC_6H_4B(OH)_2$
Molecular Weight	139.92

TEST	SPECIFICATION	LOT 15926DD RESULTS
APPEARANCE	WHITE TO BROWN POWDER	WHITE POWDER
INFRARED SPECTRUM		CONFORMS TO STRUCTURE.
PROTON NMR SPECTRUM	CONFORMS TO STRUCTURE.	CONFORMS TO STRUCTURE.
TITRATION	95.0% (MINIMUM) (WITH NAOH)	102.6% (WITH NAOH)
QUALITY CONTROL ACCEPTANCE DATE		APRIL 2005

Barbara Rajzer, Supervisor
Quality Control
Milwaukee, Wisconsin USA

Combi-Blocks, Inc

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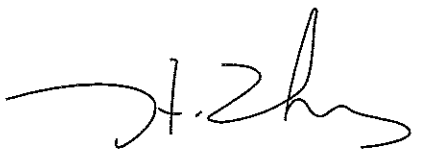
Email: sales@combi-blocks.com
Web Site: www.combi-blocks.com

CERTIFICATE OF ANALYSIS

Product Number	**BB-2658**
Product Name	(2-Fluorophenyl)boronic acid
CAS Number	[1993-03-9]
Molecular Formula	$C_6H_6BFO_2$
Molecular Weight	139.9

TEST RESULTS

BATCH NUMBER	**L16984**
APPARENCE	White solid
BOILING POINT	No Data
MELTING POINT	90-94°C
NMR	97%, conform with structure
HPLC	98.8%
TLC	97%



Howard Zhang, Ph.D.
CEO

07/01/2004

Acceptence Date

Combi-Blocks, Inc
7949 Silverton Ave, Suite 915
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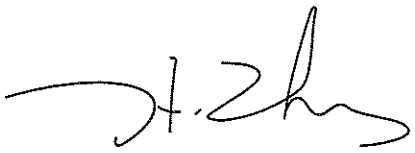
Fax: 1-858-635-8991
Email: sales@combi-blocks.com
Web Site: www.combi-blocks.com

CERTIFICATE OF ANALYSIS

Product Number	**BB-2625**
Product Name	(2-Trifluoromethylphenyl)boronic acid
CAS Number	[1423-27-4]
Molecular Formula	$C_7H_6BF_3O_2$
Molecular Weight	189.9

TEST RESULTS

BATCH NUMBER	**L18162**
APPARENCE	White solid
BOILING POINT	No Data
MELTING POINT	90-94°C
NMR	98%, conform with structure
HPLC	99.0%
TLC	98%



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12/16/2004

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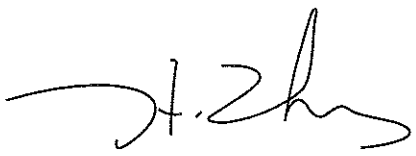
CERTIFICATE OF ANALYSIS

Product Number	**BB-2234**
Product Name	(2-Biphenyl)boronic acid
CAS Number	[4688-76-0]
Molecular Formula	$C_{12}H_{11}BO_2$
Molecular Weight	198.0

TEST RESULTS

BATCH NUMBER	**L17678**
APPARENCE	White solid
BOILING POINT	No Data
MELTING POINT	191.4 $\pm$ 0.2°C (by Mettler-Toledo FP-62)
NMR	98%, conform with structure
HPLC	99.5%
TLC	98%



Howard Zhang, Ph.D.
CEO

07/15/2004

Acceptence Date

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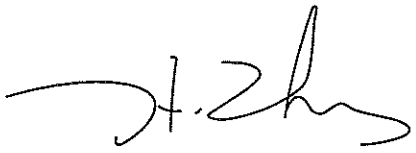
CERTIFICATE OF ANALYSIS

Product Number	**BB-2653**
Product Name	Benzofuran-2-boronic acid
CAS Number	[98437-24-2]
Molecular Formula	$C_8H_7BO_3$
Molecular Weight	162.0

TEST RESULTS

BATCH NUMBER	**L18436**
APPARENCE	Off White solid
BOILING POINT	No Data
MELTING POINT	114-116°C
NMR	98%, conform with structure
HPLC	98.3%
TLC	98%



Howard Zhang, Ph.D.
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02/25/2005

Acceptence Date

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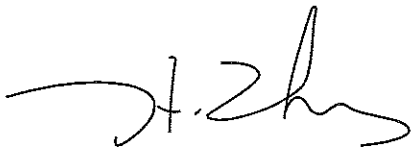
CERTIFICATE OF ANALYSIS

Product Number	**BB-2027**
Product Name	Benzo(b)thiophene-2-boronic acid
CAS Number	[98437-23-1]
Molecular Formula	$C_8H_7BO_2S$
Molecular Weight	178.0

TEST RESULTS

BATCH NUMBER	**L21369**
APPARENCE	White solid
BOILING POINT	No Data
MELTING POINT	268-270° C
NMR	98%, conform with structure
HPLC	100%
TLC	98%



Howard Zhang, Ph.D.
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12/18/2006

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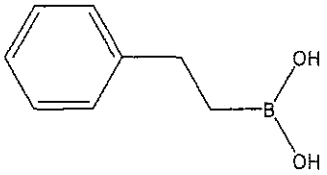
Certificate of Analysis

Structure:



Product Number	BM536
Batch Number	B5360701
Product Name	Phenethylboronic acid
CAS Number	34420-17-2
Molecular Formula	C8H11BO2
Molecular Weight	149.984 g/mol

ANALYSIS	SPECIFICATIONS	RESULTS
Appearance	*white to off-white solid*	*colourless solid*
MPt (°C)	-	-
BPt (°C)	-	-
GC -	*>97%*	*>99%*
NMR - DMSO/D2O	*>97%*	*>99%*
Other	-	-

NOTES: *May contain varying amounts of Anhydrides*

Prepared by [signature] Nelson Shen 30/4/2007
Signature *Print Name* *Date*

Checked by [signature] S. Curtis 30/4/07
Signature *Print Name* *Date*