Supporting Information

for the article

Reemerging aspartic protease targets: Examining *C. neoformans* Major aspartyl peptidase 1 as a target for antifungal drug discovery

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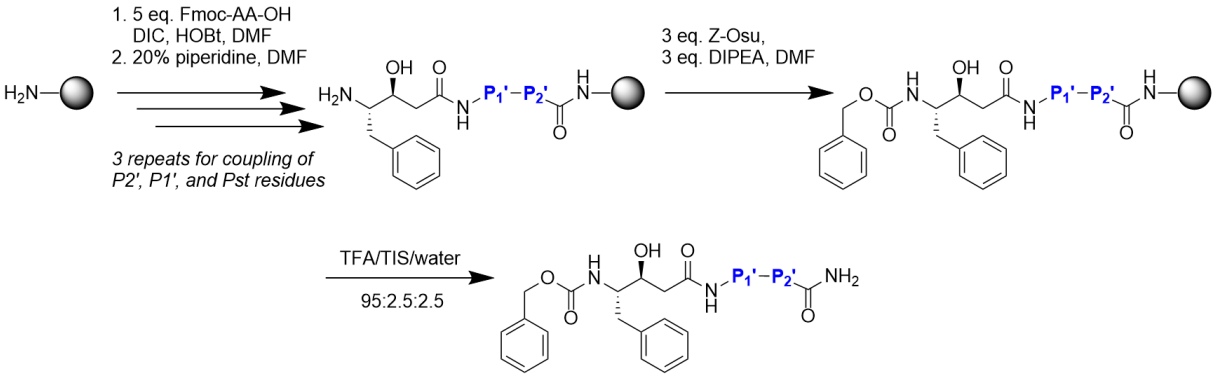
Chemical syntheses



	cap		TSA		P1'		P2'
1a	Z	-	Pst	-	L-Glu	-	L-Abu - NH_2
2a	Z	-	Pst	-	L-Glu	-	L-Nva - NH_2
3a	Z	-	Pst	-	L-Glu	-	L-Val - NH_2
4a	Z	-	Pst	-	L-Glu	-	L-Nle - NH_2
5a	Z	-	Pst	-	L-Glu	-	L-Leu - NH_2
6a	Z	-	Pst	-	L-Glu	-	L-Ile - NH_2
7a	Z	-	Pst	-	L-Glu	-	L-Thr - NH_2
8a	Z	-	Pst	-	L-Glu	-	L-Met - NH_2
9a	Z	-	Pst	-	L-Glu	-	L-Trp - NH_2
10a	Z	-	Pst	-	L-Glu	-	L-Cha - NH_2
11a	Z	-	Pst	-	L-Glu	-	L-Phg - NH_2
12a	Z	-	Pst	-	L-Glu	-	L-Phe - NH_2
13a	Z	-	Pst	-	L-Glu	-	L-Hph - NH_2

	cap		TSA		P1'		P2'
1b	Z	-	Pst	-	L-Gln	-	L-Abu - NH_2
2b	Z	-	Pst	-	L-Gln	-	L-Nva - NH_2
3b	Z	-	Pst	-	L-Gln	-	L-Val - NH_2
4b	Z	-	Pst	-	L-Gln	-	L-Nle - NH_2
6b	Z	-	Pst	-	L-Gln	-	L-Ile - NH_2
7b	Z	-	Pst	-	L-Gln	-	L-Thr - NH_2
8b	Z	-	Pst	-	L-Gln	-	L-Met - NH_2
10b	Z	-	Pst	-	L-Gln	-	L-Cha - NH_2
11b	Z	-	Pst	-	L-Gln	-	L-Phg - NH_2
12b	Z	-	Pst	-	L-Gln	-	L-Phe - NH_2
13b	Z	-	Pst	-	L-Gln	-	L-Hph - NH_2

Fmoc-protected amino acids, Fmoc-(3S,4S)-AHPPA-OH (phenylstatine, Pst) transition state analogue (TSA) building block, and Rink amide MBHA resin were purchased from Iris Biotech (Marktredwitz, Germany) and Bachem (Bubendorf, Switzerland). N-(benzyloxycarbonyl)succinimide (Z-Osu) from Merck (Kenilworth, USA). Compounds were synthesized by standard Fmoc-chemistry solid phase peptide synthetic protocols on Rink amide MBHA resin support (subst. 0.69 mmol/g) in N,N-dimethylformamide (DMF) as a solvent. Syntheses were performed in 0.1 mmol scale, couplings with 5 equivalent amino acid excess to resin and 1-hydroxybenzotriazol (HOBt, 1.5 eq. to amino acid) and N,N'-diisopropylcarbodiimide (DIC, 1.5 eq. to amino acid) activation. Fmoc groups were removed with 20% piperidine. Coupling and deprotection as repeated to incorporate 3 residues for each compound. The N-(benzyloxycarbonyl) capping group was introduced with N-(benzyloxycarbonyl) succinimide (Z-Osu, 3 eq. to resin) in presence of N,N-diisopropylethylamine (DIPEA, 3 eq. to resin) in DMF as a solvent. Resin was then washed with dichloromethane and dried in vacuo. Compounds were deprotected and cleaved off the resin by incubation with a mixture of TFA/triisopropylsilane/water (95:2.5:2.5) for an hour.

Table S1. Data collection and refinement statistics.

	Apo-MayI	MayI-pepstatin A
Crystal data		
Space group	$C222_1$	$C222_1$
a, b, c (Å)	97.42, 112.06, 91.21	97.36 112.64 91.03
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Molecules per asymmetric unit	1	1
Matthews coefficient ($Å^3$ Da^{-1})	3.38	3.33
Solvent content (%)	63.62	63.06
Max. resolution (Å)	1.75	1.80
Data collection and processing		
Wavelength (Å)	0.918	1.542
Resolution limits (Å)	48.71-1.75 (1.86-1.75)	50.0-1.8 (1.91-1.80)
No. of observed reflections	264,390 (23,308)	138,736 (2,791)
No. of unique reflections	48515 (6,330)	39317 (2,229)
Multiplicity	5.4 (3.7)	3.5 (1.3)
R_{merge}[a]	0.135 (1.356)	0.087 (0.565)
$CC_{1/2}$[b]	0.997 (0.482)	0.997 (0.590)
Completeness (%)	95.8 (78.2)	84.3 (29.8)
$\langle I/\sigma I \rangle$	10.3 (1.0)	11.7 (0.99)
Refinement statistics		
Resolution (Å)	48.7-1.75 (1.80-1.75)	73.7-1.8 (1.85-1.80)
No. of reflections in working set	46,413 (2,589)	39,317 (2,229)
No. of reflections in test set	2101 (118)	1710 (30)
R_{work}[c] (%)	18.0 (39.5)	17.7 (44.5)
R_{free}[d] (%)	22.3 (39.3)	20.7 (42.9)
Average B-factor ($Å^2$)	29.6	24.6
RMSD bond length (Å)	0.013	0.012
RMSD angle (°)	1.6	1.6
Number of atoms in AU (protein/inhibitor/water molecules)	2642/0/346	2617/60/314
Ramachandran plot		
Most favored regions[e] (%)	97.99	97.65
Additional allowed regions[e] (%)	2.01	2.08
Disallowed regions[e] (%)	0.0	0.27
PDB code	6R5H	6R6A

Values in parentheses report the values in the highest resolution shell.

[a] $R_{merge} = \Sigma_{hkl} \Sigma_i |I_i(hkl)_i - \langle I(hkl) \rangle| / \Sigma_{hkl} \Sigma_i I_i (hkl)_i$.

[b] $CC_{(1/2)}$ is the correlation coefficient between random half data sets and from its value the Pearson correlation coefficient of the true level of signal can be calculated: $CC^* = \sqrt{2\frac{CC_{\frac{1}{2}}}{1} + CC_{\frac{1}{2}}}$ [S1].

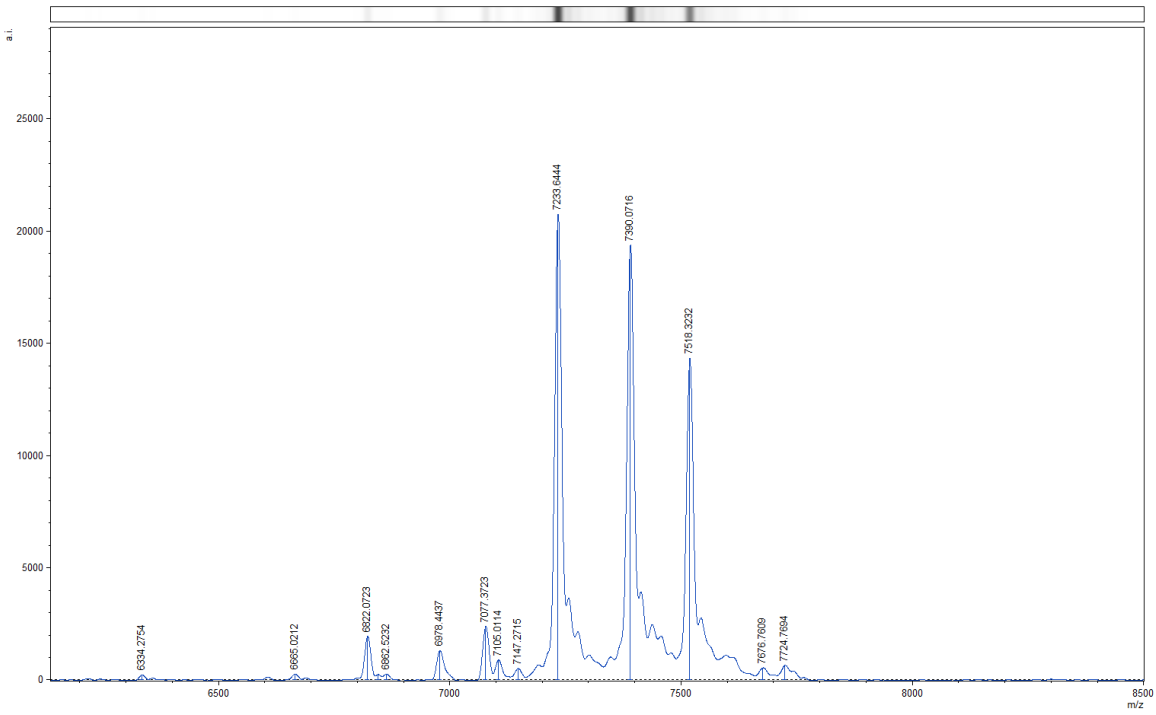
[c] R-value = $||F_o| - |F_c|| / |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

[d] R_{free} is equivalent to the R-value but is calculated for 5% of the reflections chosen at random and omitted from the refinement process [S2].

[e] As determined by MolProbity [S3].

Table S2. Cytotoxicity of Z-Pst-*L*-Glu-Hph-NH_2 toward various human cell lines. Data were obtained through measurements of cell viability by luminometric CellTiter-Glo® 2.0 Cell Viability Assay (Promega).

	HeLa	CCRF-CEM	HL-60	MCF-7	HepG2
CC_{50}	> 80 μM	> 80 μM	> 80 μM	> 80 μM	> 80 μM



	m/z	
Δ	calcd.	found
-0,5	7077,87	7077,37
-0,41	7234,05	7233,64
-0,17	7390,24	7390,07
-0,09	7518,41	7518,32

RSAPSNSVPLTPLNSRQYSEDLAERQSWLLDQAKGLRSKYAPHLGERGQELRRRDIIDEGIT
RSAPSNSVPLTPLNSRQYSEDLAERQSWLLDQAKGLRSKYAPHLGERGQELRRRDIIDEGITR
RSAPSNSVPLTPLNSRQYSEDLAERQSWLLDQAKGLRSKYAPHLGERGQELRRRDIIDEGITRR
RSAPSNSVPLTPLNSRQYSEDLAERQSWLLDQAKGLRSKYAPHLGERGQELRRRDIIDEGITRRK

1 10 20 30 40 50 60 70 80

CNAG_05872 MHYLAVALPLLTLALAAPSNSVPLTPLNSRQYSEDLAERQSWLLDQAKGLRSKYAPHLGERGQELRRRDIIDEGITRRKRAN

Figure S1a. MALDI mass spectrometry analysis of May1 prodomain fragments. The mismatching N-terminal Arg-Ser dipeptide in the fragments is a remnant of the *Bgl*II restriction site product.

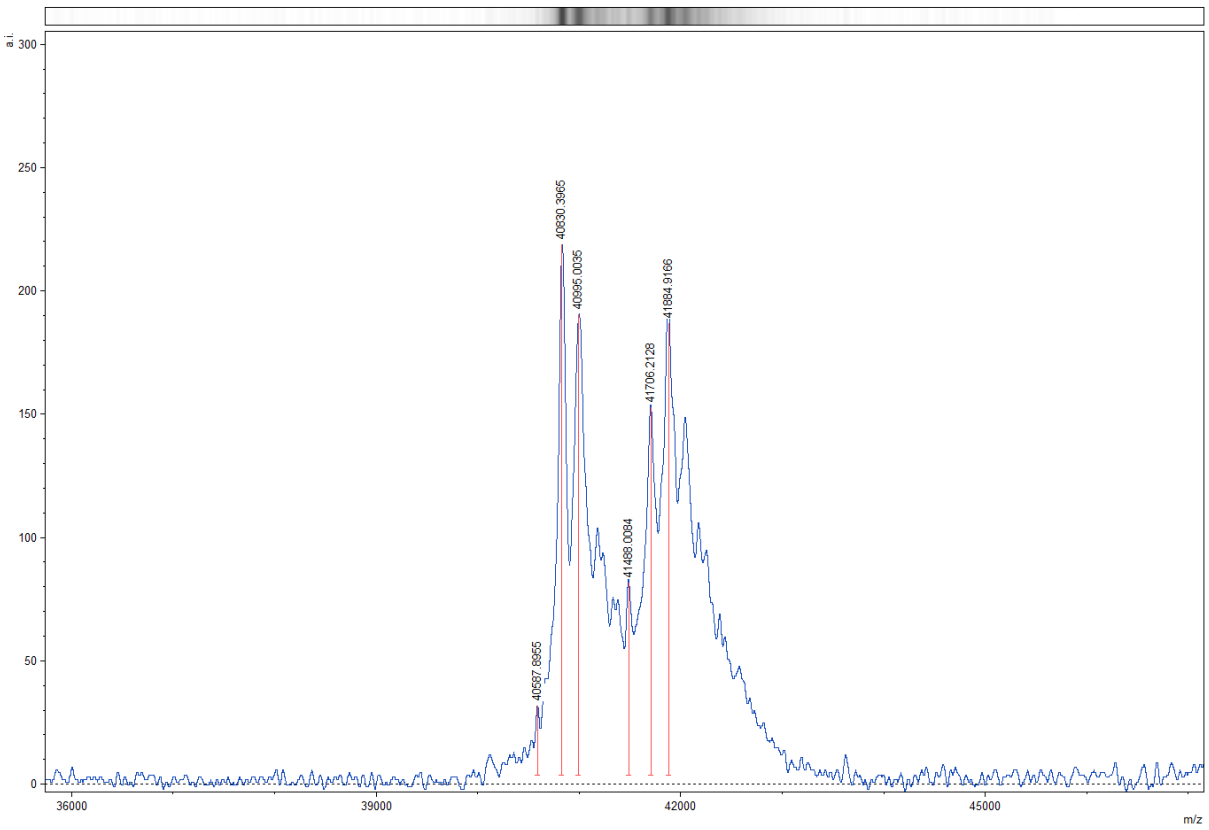


Figure S1b. (continued) MALDI mass spectrometry analysis of active May1 with its prodomain cleaved off. Largest possible average molecular weight of MayI fragment 77-434, including C-terminal tag and its biotinylation, is 40145.35 Da – this corresponds to the loss of the shortest detected prodomain fragment 15-76 (first entry in the table above).

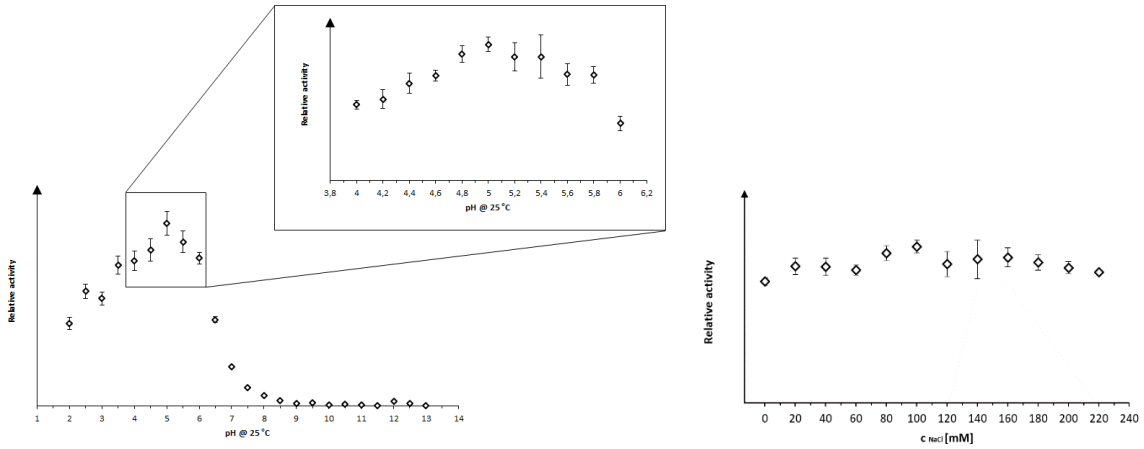


Figure S2. Activity profiles of May1 detected by cleavage of IQ-2 fluorescent substrate. Left side: Activity profile across pH 2.0 to 13.0 with steps of 0.5 in a Britton-Robinson buffer system. The activity maximum was further resolved with a series of buffers of pH 4.0 to 6.0 with steps of 0.2. Right side: Activity profile with varying salt concentration at pH 5.0.

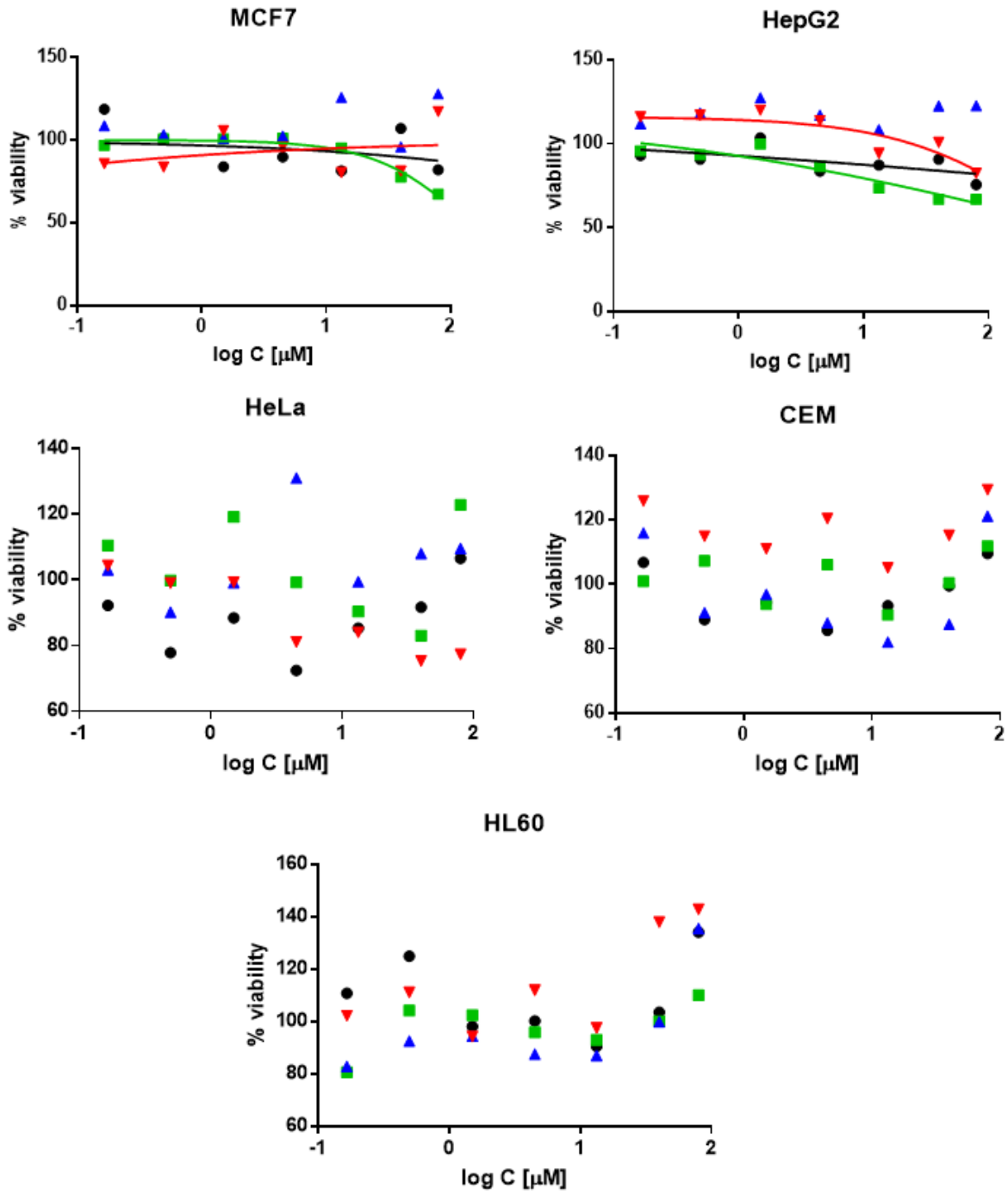


Figure S3. Cytotoxicity curves for five cell lines. Data were obtained through measurements of cell viability by luminometric CellTiter-Glo® 2.0 Cell Viability Assay (Promega).

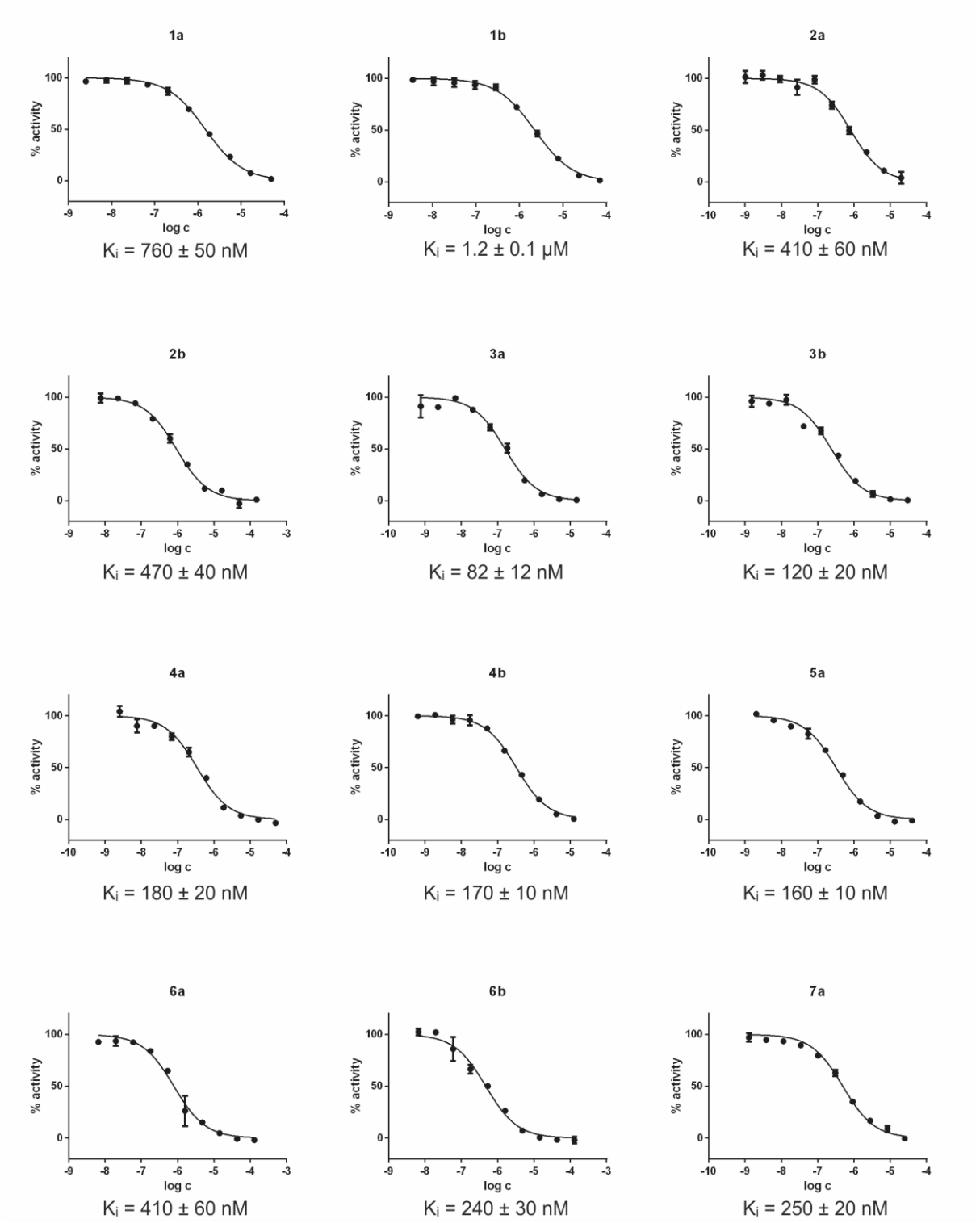


Figure S4a. May1 inhibition curves for compounds 1 – 13a and 1 – 13b with fitted K_i values and 95% CI. Figure continues on the next page.

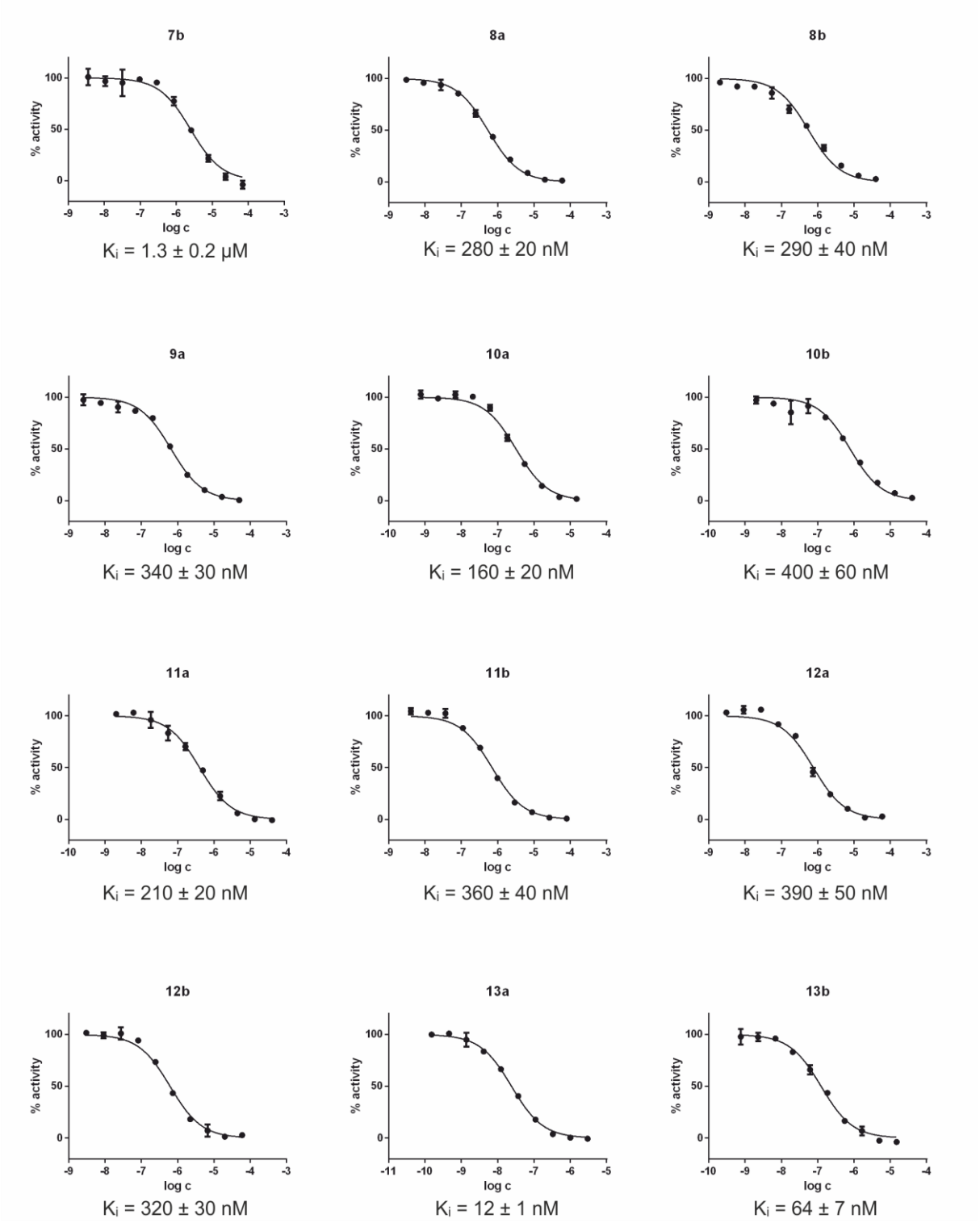
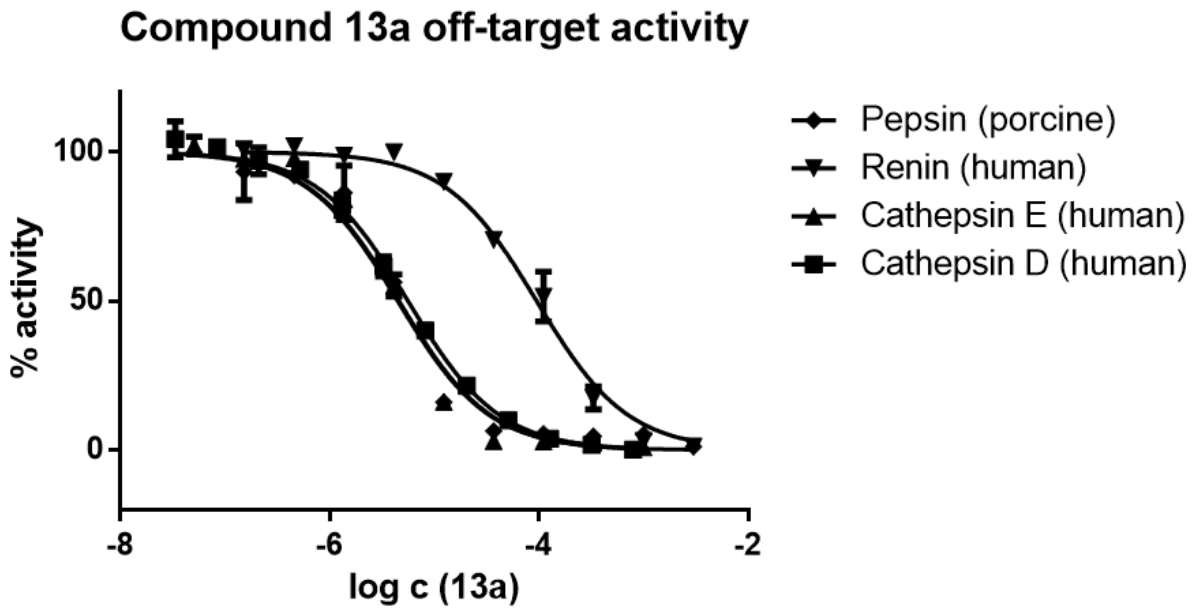


Figure S4b. (continued) May1 inhibition curves for compounds 1 – 13a and 1 – 13b with fitted K_i values and 95% CI.



enzyme	substrate K_m	substrate concentration	compound 13a K_i ± 95% CI
pepsin (porcine)	2.5 µM	10 µM	1.6 ± 0.3 µM
renin (human)	80 µM	150 µM	19 ± 2 µM
cathepsin E (human)	3.3 µM	30 µM	440 ± 70 nM
cathepsin D (human)	3.7 µM	30 µM	620 ± 50 nM

Figure S5. Inhibition curves for compound 13a against a panel of aspartic protease off-targets. Bottom table details the assay conditions used to calculate the fitted K_i values and 95% CI.

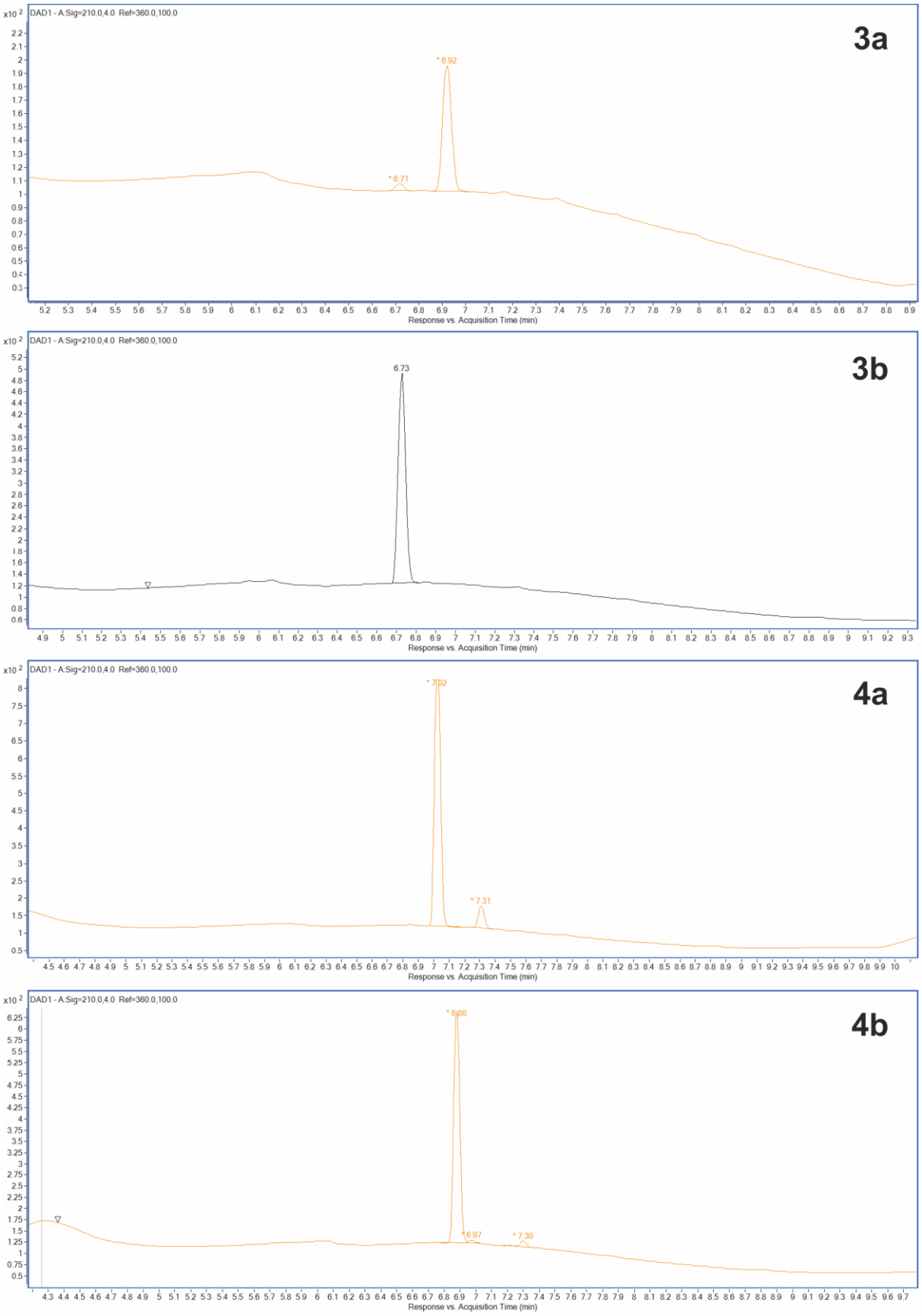


Figure S6a. LC traces of compounds 3a, 3b, 4a and 4b. Figure continues on the next page.

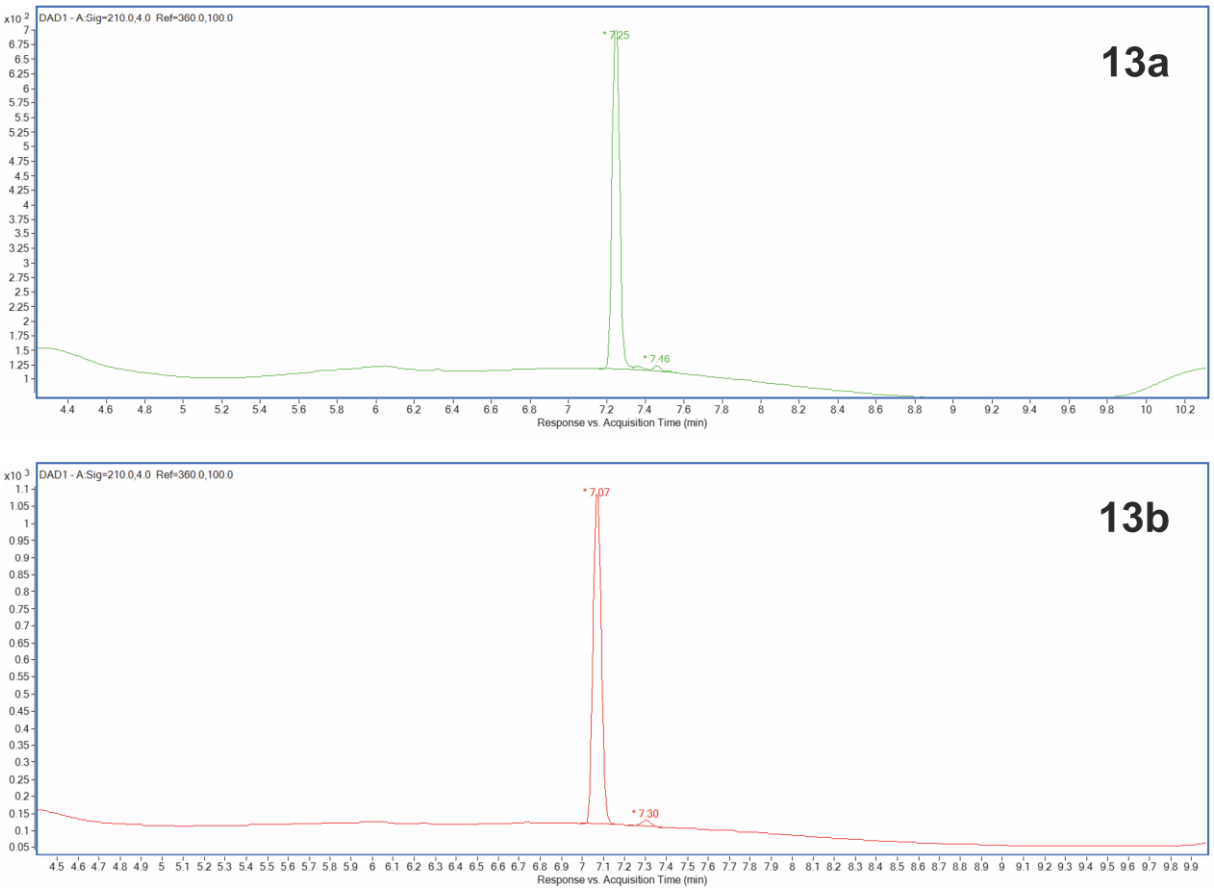


Figure S6b. (continued) LC traces of compounds 13a and 13b.

Supplementary references:

S1 Karplus, P. A. & Diederichs, K. Linking crystallographic model and data quality. *Science* **336**, 1030-1033 (2012).

S2 Brunger, A. T. Free R value: a novel statistical quantity for assessing the accuracy of crystal structures. *Nature* **355**, 472-475 (1992).

S3 Chen, V. B. *et al.* MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Cryst D* **66**, 12-21, (2010).