Cyclic peptide unguisin A is an anion receptor with high affinity for phosphate and pyrophosphate

A. Daryl Ariawan, James E. A. Webb, Ethan N. W. Howe, Philip A. Gale, Pall Thordarson and Luke Hunter**

SUPPORTING INFORMATION

Contents:

1. Antimicrobial assay data S-2
2. ^{1}H NMR titration information S-5
3. Binding data for compounds **2**–**5** with Cl^{-} S-17
4. Links to online repositories of binding data S-18
5. Transmembrane chloride transport study S-19

1. Antimicrobial assay data

1.1. Sample preparation

Samples were provided by the collaborator as dry material, and were made to 10 mg/mL in DMSO as a stock solution and stored at -20 ^{o}C.

An aliquot of each sample was further diluted to 320 μg/mL in water in 384-well polypropylene plates (PP), and 5 μL was plated in duplicate (n=2) into a 384-well non- binding surface plate (NBS) for each strain assayed against.

1.2. Antimicrobial Assay

Procedure

All bacteria were cultured in CAMHB at 37 °C overnight. A sample of each culture was then diluted 40-fold in fresh broth and incubated at 37 °C for 1.5-3 h. The resultant mid-log phase cultures were diluted to a final concentration of 5×10^5 CFU/mL, then 45 μL was added to each well of the compound containing plates, giving a final concentration of 32 μg/mL for the tested samples and a concentration range of 0.002 to 10 μg/mL for the antibiotic controls. All the plates were covered and incubated at 37 °C for 18 h.

Analysis

Inhibition of bacterial growth was determined using resazurin as a marker for cell viability. Resazurin was added to each well, at 0.001% final concentration, and plates incubated at 37 °C for 2h. Fluorescence intensity is measured, using F (top read), ex 560/10 nm, em 590/10 nm, using a Tecan M1000 Pro monochromator plate reader. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate as references. The significance of the inhibition values was determined by Z-scores, calculated using the average and standard deviation of the sample wells (no controls) on the same plate. Samples were classed as actives with, inhibition value above 80% and Z-Score above 2.5, for either replicate (n=2 on different plates).

1.3. Antifungal Assay

Procedure

Fungi strains were cultured for 3 days on Yeast Extract-Peptone Dextrose (YPD) agar at 30 °C. A yeast suspension of 1 x 10^6 to 5 x 10^6 cells/mL was prepared from five colonies. These stock suspensions were diluted with Yeast Nitrogen Base (YNB) broth to a final concentration of 2.5 $\times 10^3$ CFU/mL. Then, 45µL of the fungi suspension was added to each well of the compound-containing plates, giving a final concentration of 32 µg/mL for the tested samples and a concentration range of 0.01 to 32 µg/mL for the antifungal control, fluconazole. Plates were covered and incubated at 35 °C for 24 h.

Analysis

Growth inhibition of *C. albicans* was determined measuring absorbance at 530 nm (OD530), while the growth inhibition of *C. neoformans* was determined measuring the difference in absorbance between 600 and 570 nm (OD600-570), after the addition of resazurin (0.001% final concentration) and incubation at 35 °C for 2 h. The absorbance was measured using a Biotek Multiflo Synergy HTX plate reader. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate. The significance of the inhibition values was determined by Z-scores, calculated using the average and standard deviation of the sample wells (no controls) on the same plate. Samples were classed as actives with, inhibition value above 80% and Z-Score above 2.5, for either replicate (n=2 on different plates).

1.4. Antibiotic standards preparation and quality control

Colistin and Vancomycin were used as positive bacterial inhibitor standards for Gram- negative and Gram-positive bacteria, respectively. Fluconazole was used as a positive fungal inhibitor standard for *C. albicans and C. neoformans.*

The antibiotics were provided in 4 concentrations, with 2 above and 2 below its MIC value, and plated into the first 8 wells of column 23 of the 384-well NBS plates.

The quality control (QC) of the assays was determined by the antimicrobial controls and the Z'-factor (using positive and negative controls). Each plate was deemed to fulfil the quality criteria (pass QC), if the Z'-factor was above 0.4, and the antimicrobial standards showed full range of activity, with full growth inhibition at least at their highest concentration, and no growth inhibition at least at their lowest concentration.

1.5. Control Results

Species	***Antibiotic***	***Minimum Inhibitory Concentration [µg/mL]***		***Pass/Fail***	
		Rep 1	***Rep 2***	***Rep1***	***Rep 2***
E. coli	Colistin	.0.2	.0.2	Pass	.Pass
K. pneumoniae (MDR)	Colistin	.0.2	.0.2	Pass	.Pass
A. baumannii	Colistin	0.2	0.2	Pass	Pass
P. aeruginosa	Colistin	.0.2	.0.2	Pass	.Pass
S. aureus (MRSA)	.Vancomycin	.1	.1	..Pass	Pass
C. albicans	Fluconazole	0.25	0.25	.Pass	Pass
C. neoformans H99	Fluconazole	≥1	≥1	Pass	Pass

1.6. Antimicrobial screening

Species	***Unguisin antimicrobial activity at 32 µg/mL***
E. coli	Inactive
K. pneumoniae (MDR)	Inactive
A. baumannii	Inactive
P. aeruginosa	Inactive
S. aureus (MRSA)	Inactive
C. albicans	Inactive
C. neoformans H99	Inactive

2. ^{1}H NMR titration information

2.1. Methodology

NMR titrations were conducted on a Bruker Avance III 600 spectrometer with the probe temperature maintained at 298 K. In all cases, NMR titrations were performed maintaining the concentration (usually around 1.0 mM) of the host constant by dissolving the guest in the same host solution, followed by addition of that guest dissolved in the host solution to the NMR sample of the host (+ any previously added guest in host solution or other additives), delivered accurately using 25 μL micro syringes. After each addition, the samples were shaken thoroughly within the air-tight screw-cap NMR sample tubes and then allowed to equilibrate in the NMR probe for 1 min before the spectra were recorded. Titration was performed from 0.2 to 50 equivalent of the guest molecule.

In most cases four proton resonances were recorded providing four sets of data from which the association constants can be determined by fitting to binding models using a custom written *python* program *BindFit* developed and deployed on the web by Prof. Pall Thordarson and based on previously published binding programs. The full set of scripts for BindFit is available at www.supramolecular.org under the source code option (Help→Source code).

In the paper three different binding models are considered (see previous work for full details on the equations and terminology used here).

1:1 Equilibria

Here, we define the NMR resonance for the host as δ_H, the guest as δ_G and the host-guest complex as δ_{HG}. From this, we can also define the change in resonance for the host-guest complexation as $\delta_{\Delta HG} = \delta_{HG} - \delta_H$. If we then define δ_0 = NMR resonance of the host before the guest is added (before the start of titration) we can define the change in in resonance as $\Delta\delta = \delta - \delta_0$. We can now write the NMR version of our simple 1:1 equilibria according to equation (S1) which is derived from the generic quadratic equation used to calculate the concentration of host-guest complex [HG] as previously described.

$$\Delta\delta = \frac{\delta_{\Delta HG}}{[H]_0}\left(\frac{1}{2}\left\{\left([G]_0 + [H]_0 + \frac{1}{K_a}\right) - \sqrt{\left([G]_0 + [H]_0 + \frac{1}{K_a}\right)^2 + 4[H]_0[G]_0}\right\}\right) \quad \text{Eq. (S1)}$$

Full 1:2 model

Here we make the assumption that the binding is cooperative ($K_1 \neq 4K_2$) and that the chemical shift changes are not additive $\delta_{\Delta HG_2} \neq 2\delta_{\Delta HG}$. This yields equation (S2).

$$\Delta\delta = \frac{\delta_{\Delta HG} K_1[G] + \delta_{\Delta HG_2} K_1 K_2 [G]^2}{1 + K_1[G] + K_1 K_2 [G]^2} \qquad \text{Eq. (S2)}$$

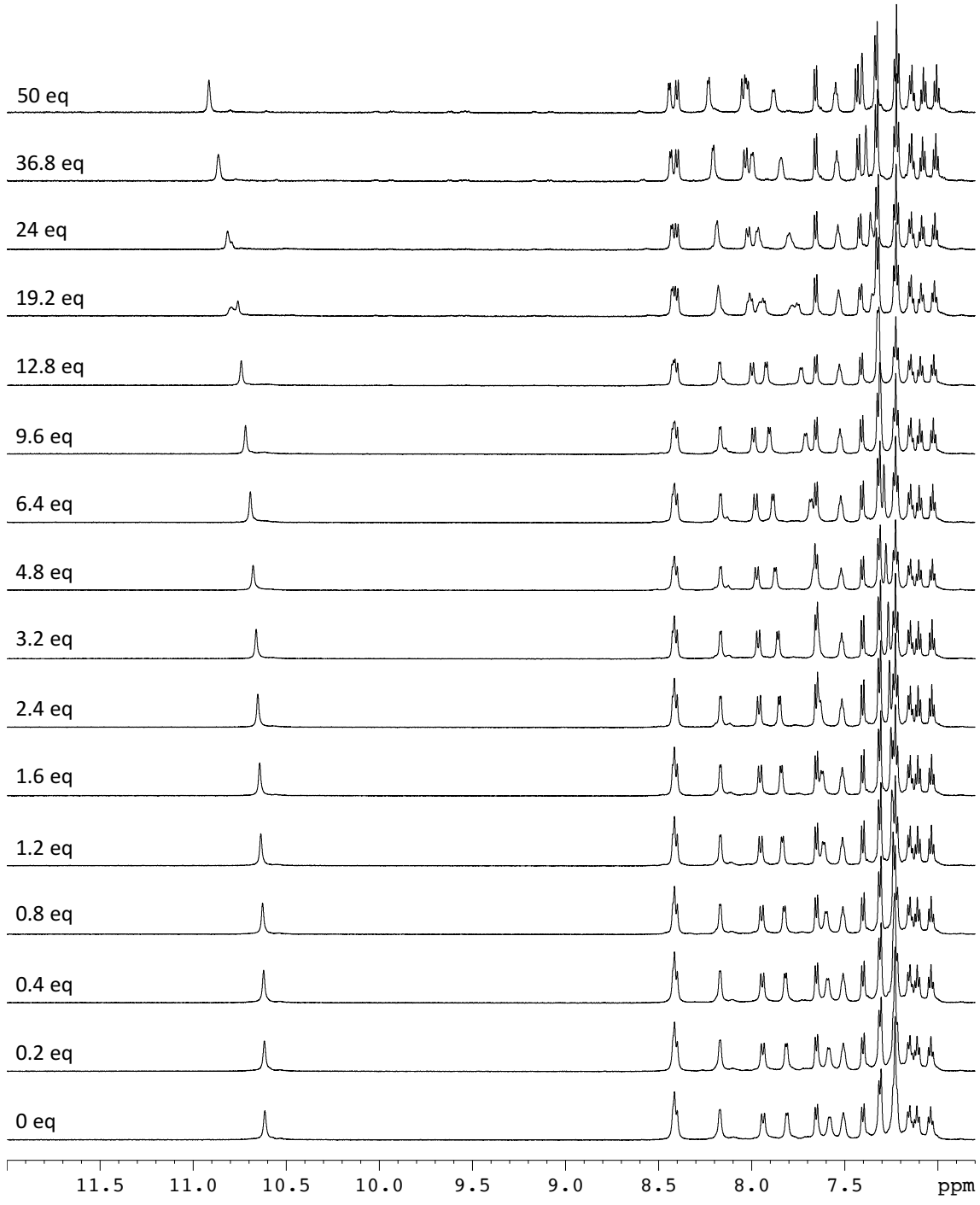
Full 2:1 model.

This model effectively mirrors the 1:2 binding model (Eq. S3) except that it assumes a 2:1 stoichiometry of the measured host and the guest. This yields equation (S3).

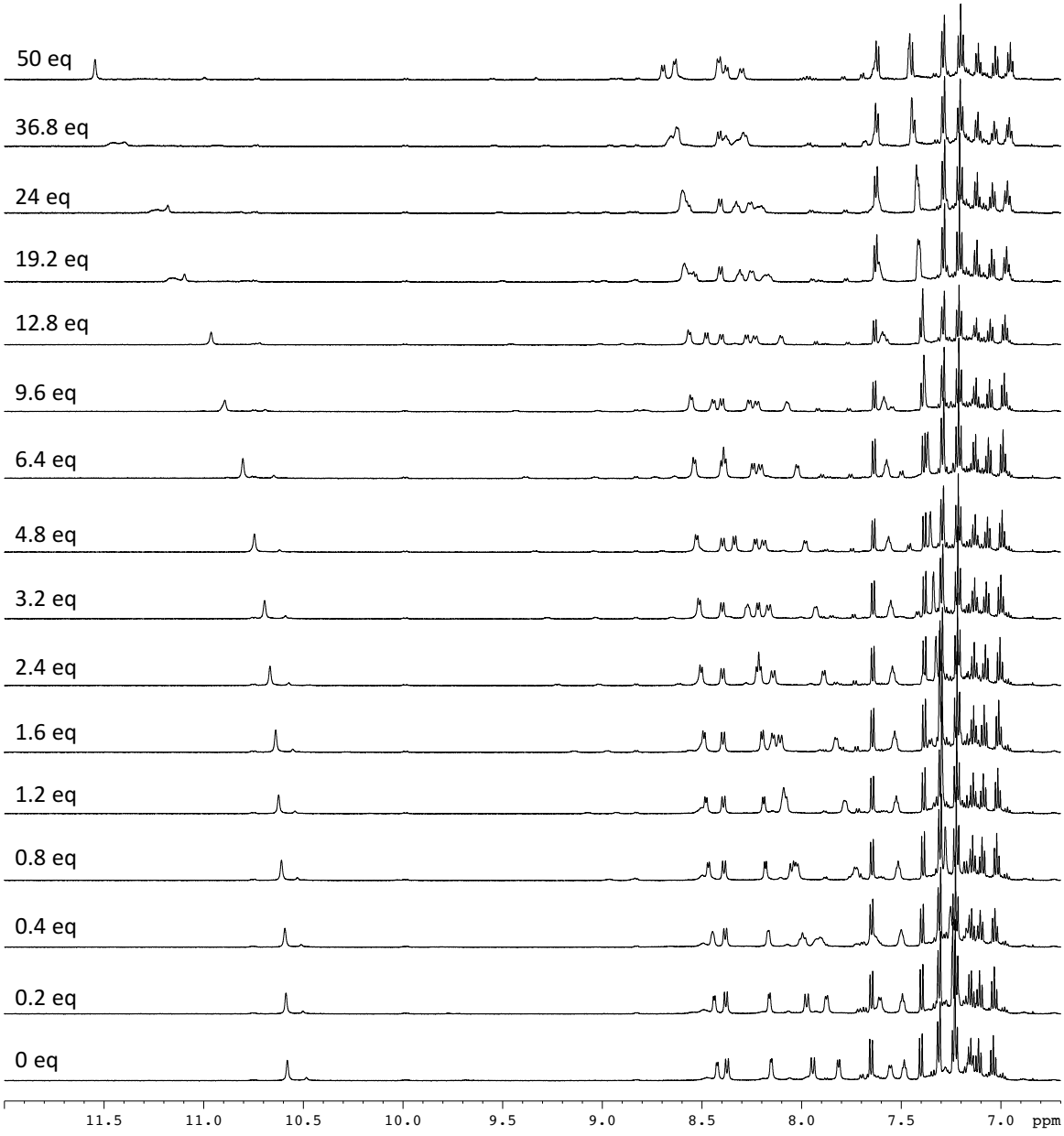
$$\Delta\delta = \frac{\delta_{\Delta HG} K_1[G]_0[H] + 2\delta_{\Delta HG_2} K_1 K_2 [G]_0[H]^2}{[H]_0(1 + K_1[H] + K_1 K_2 [H]^2)} \qquad \text{Eq. (S3)}$$

2.2. ^{1}H NMR titration stackplots of compound 1

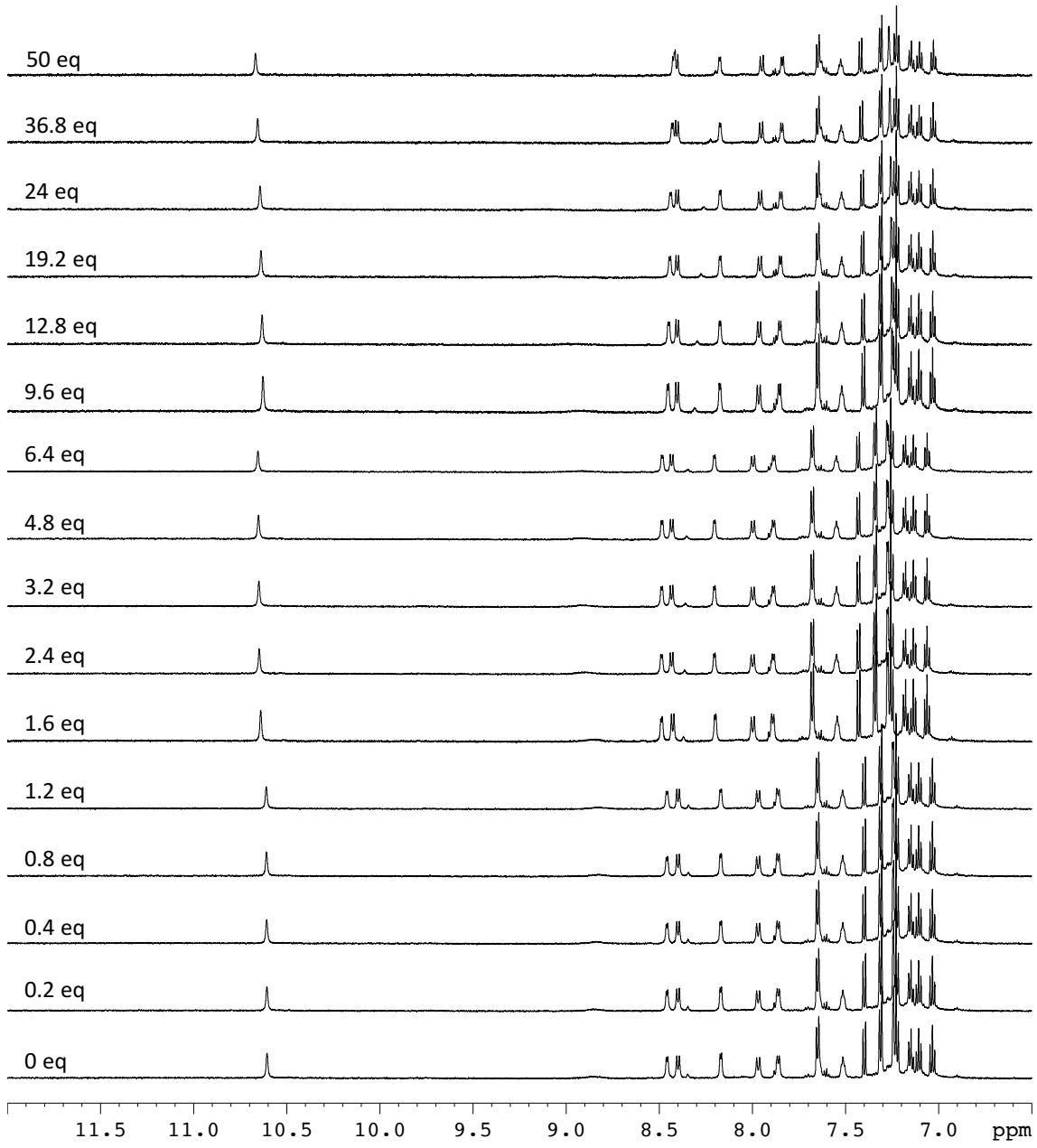
TBABr titration



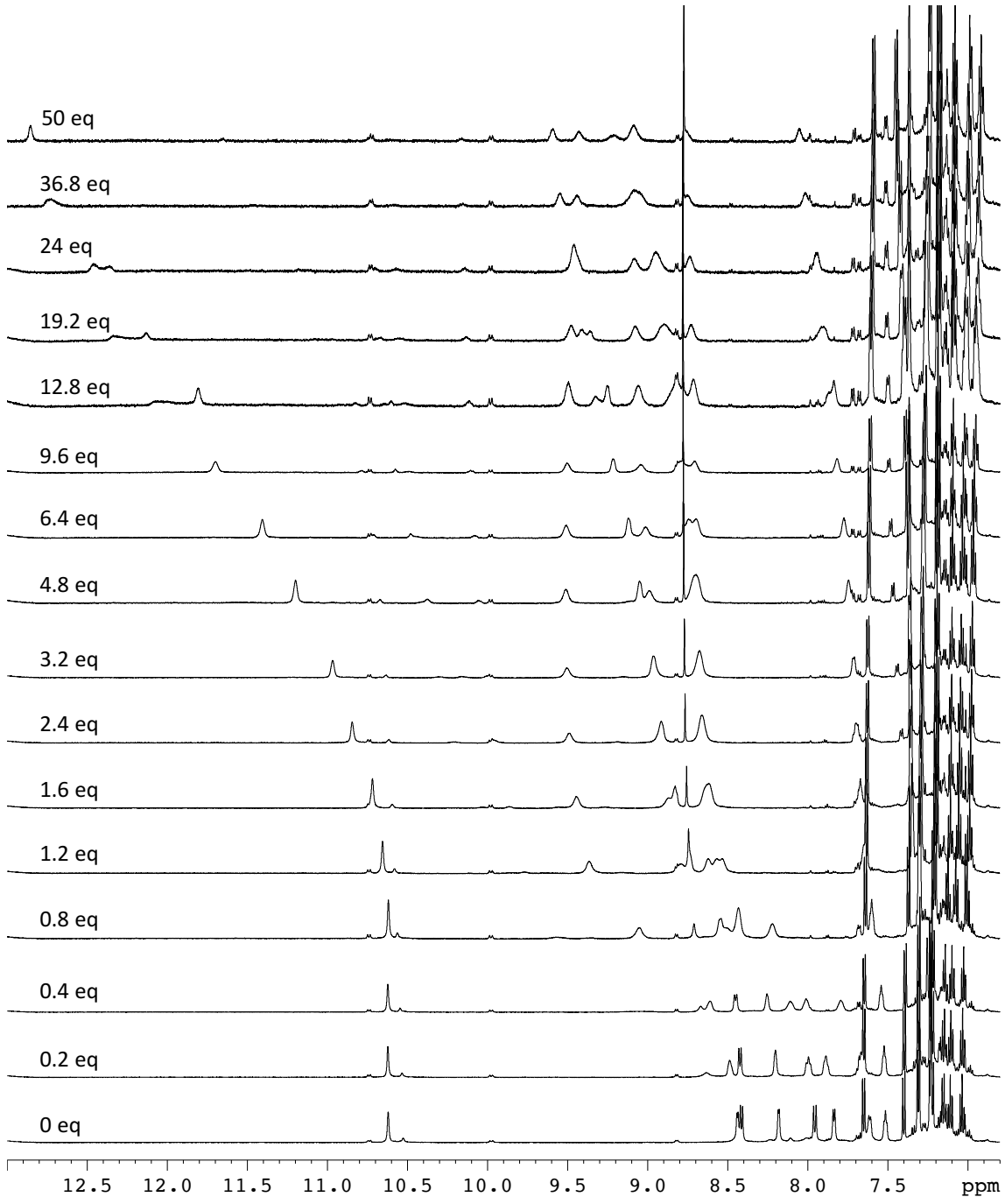
TBACl titration



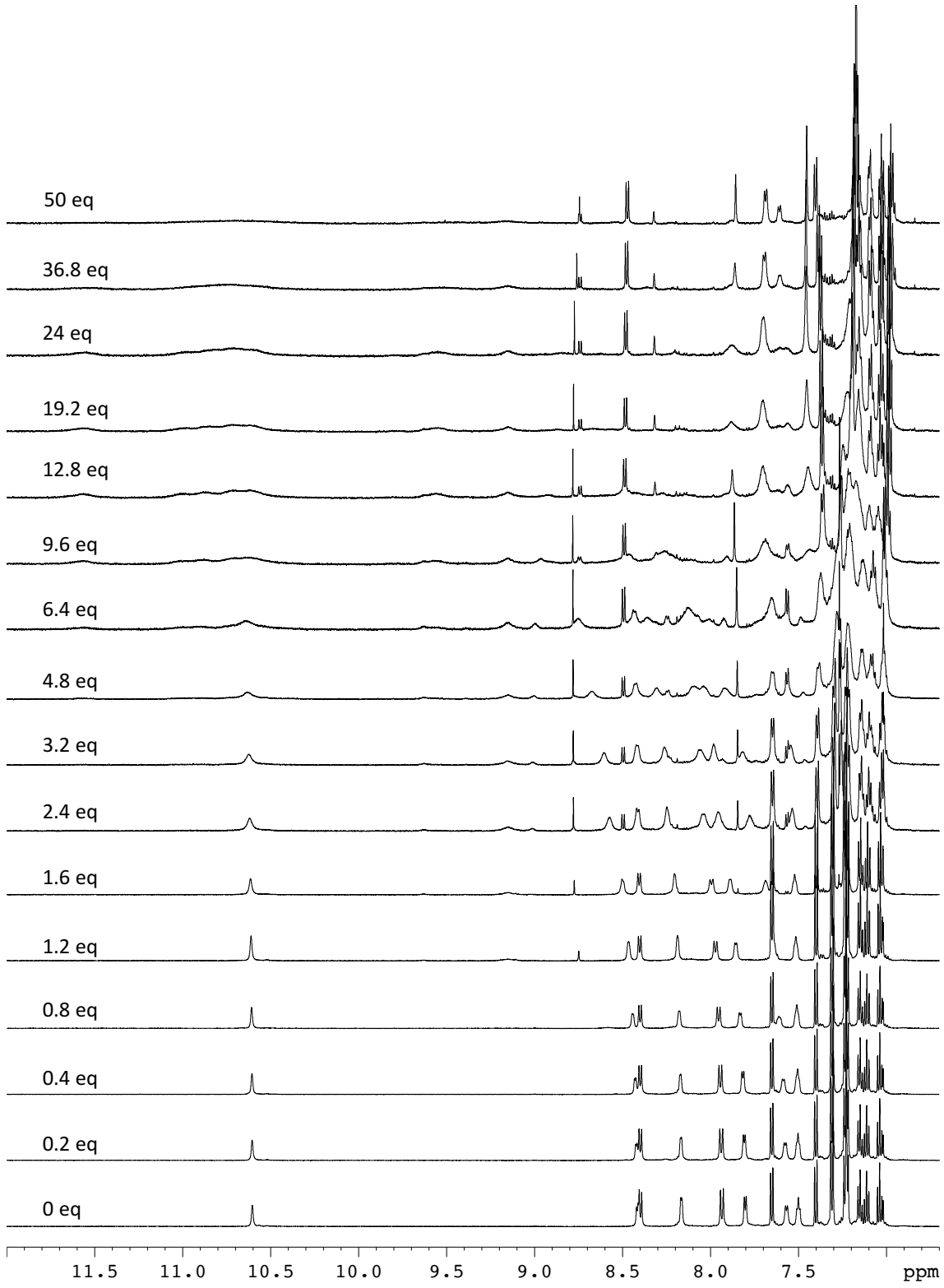
TBAI titration



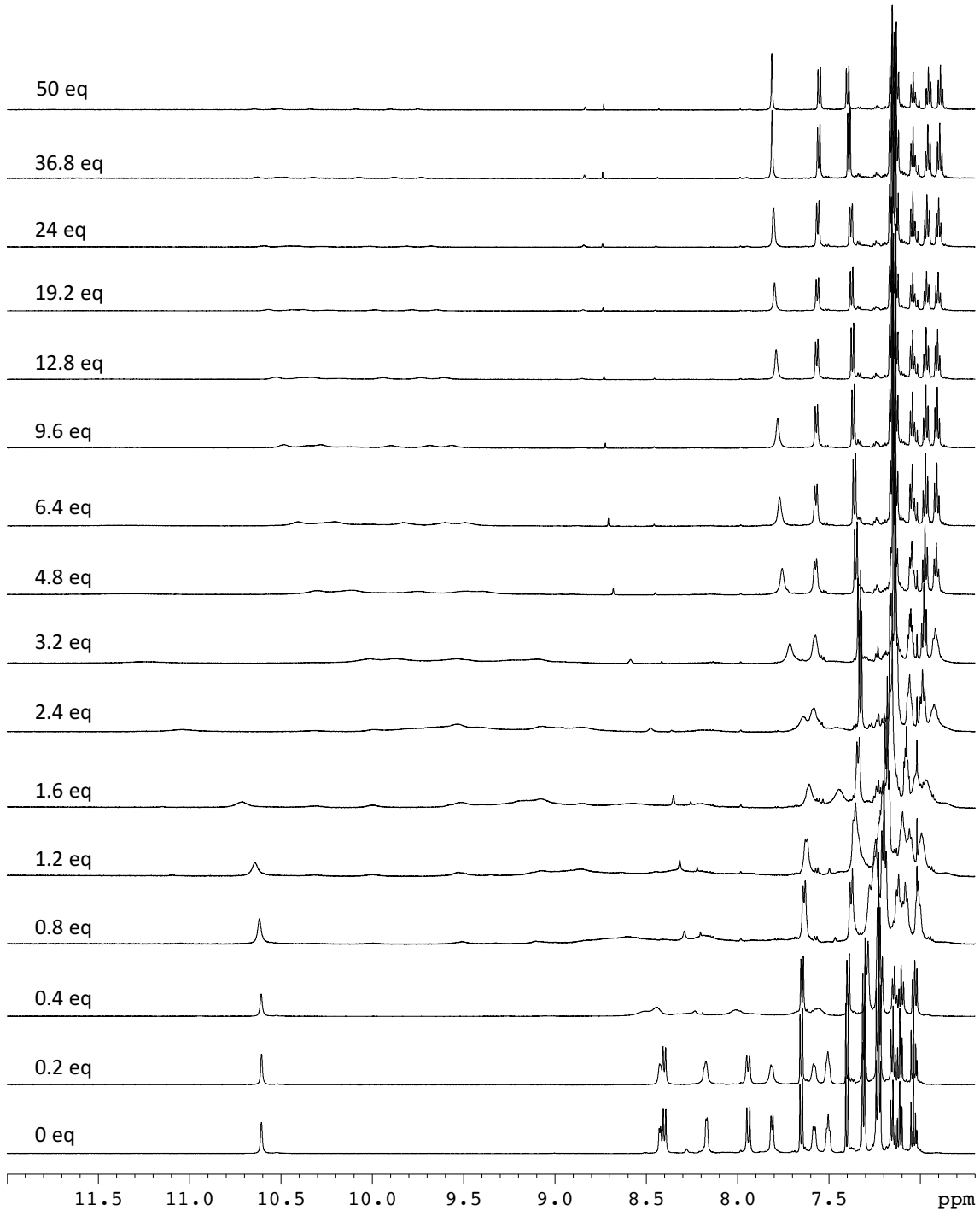
TBAOAc titration



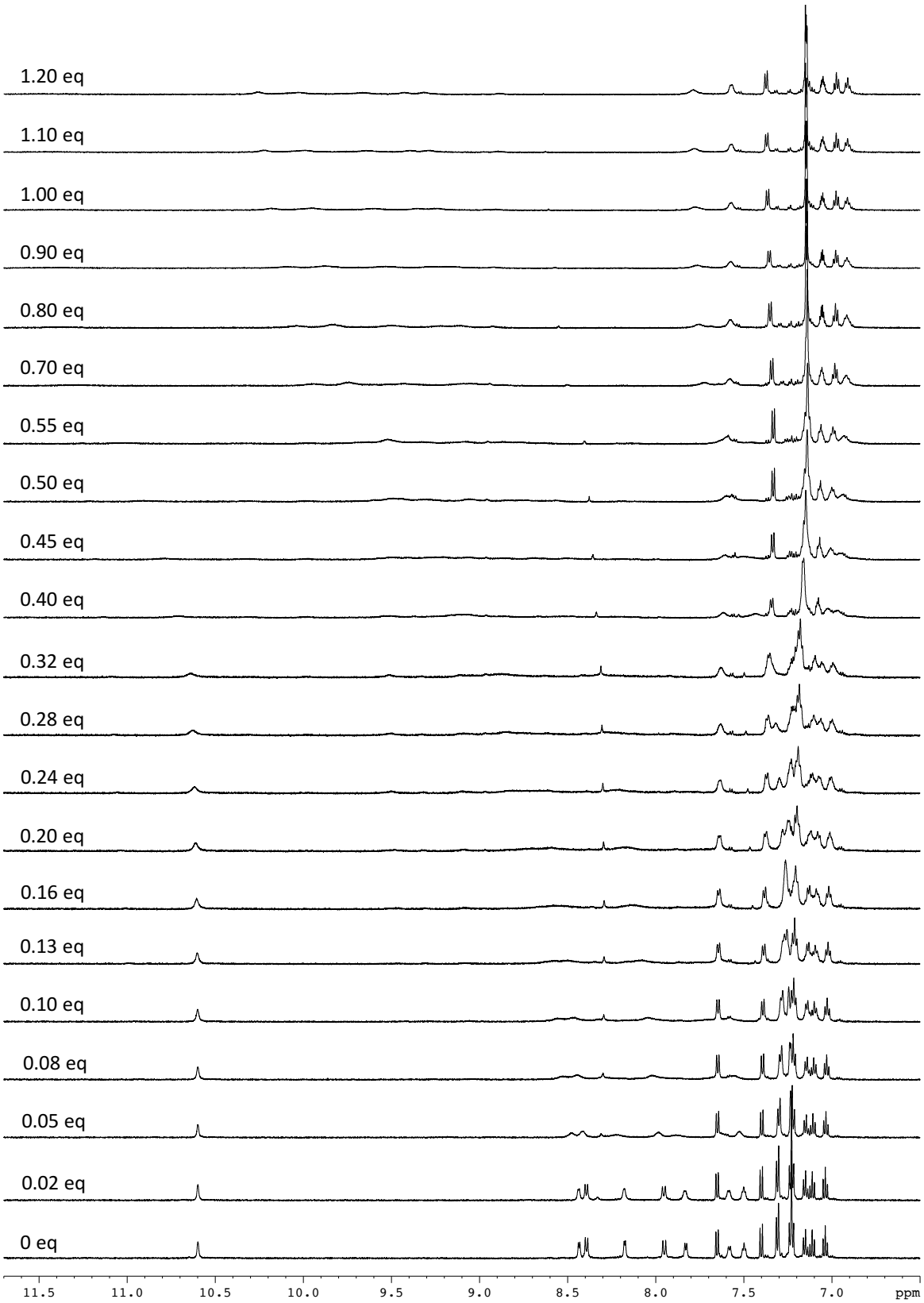
Tetraetylammonium hydrogencarbonate titration



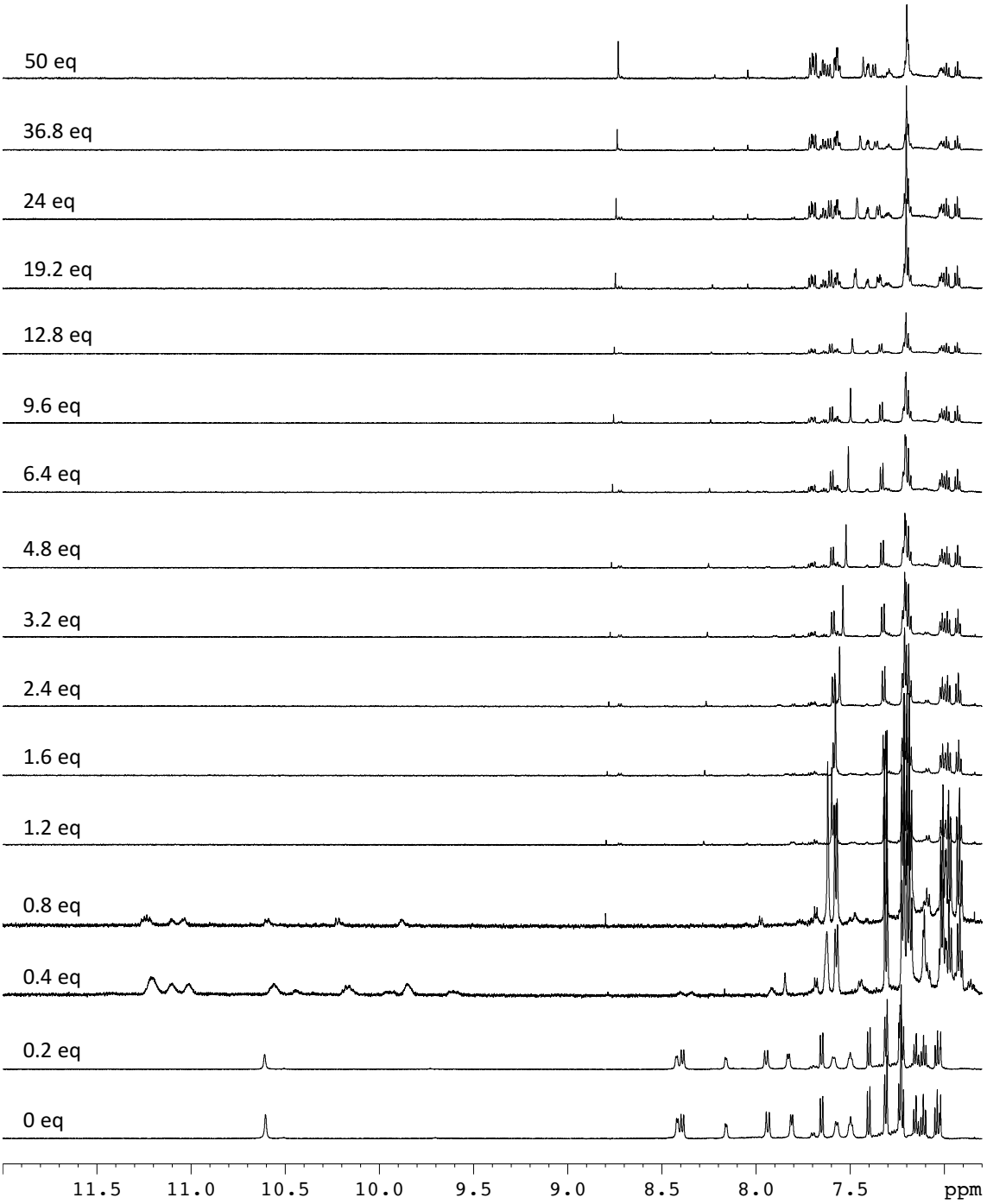
TBAH$_2$PO$_4$ titration



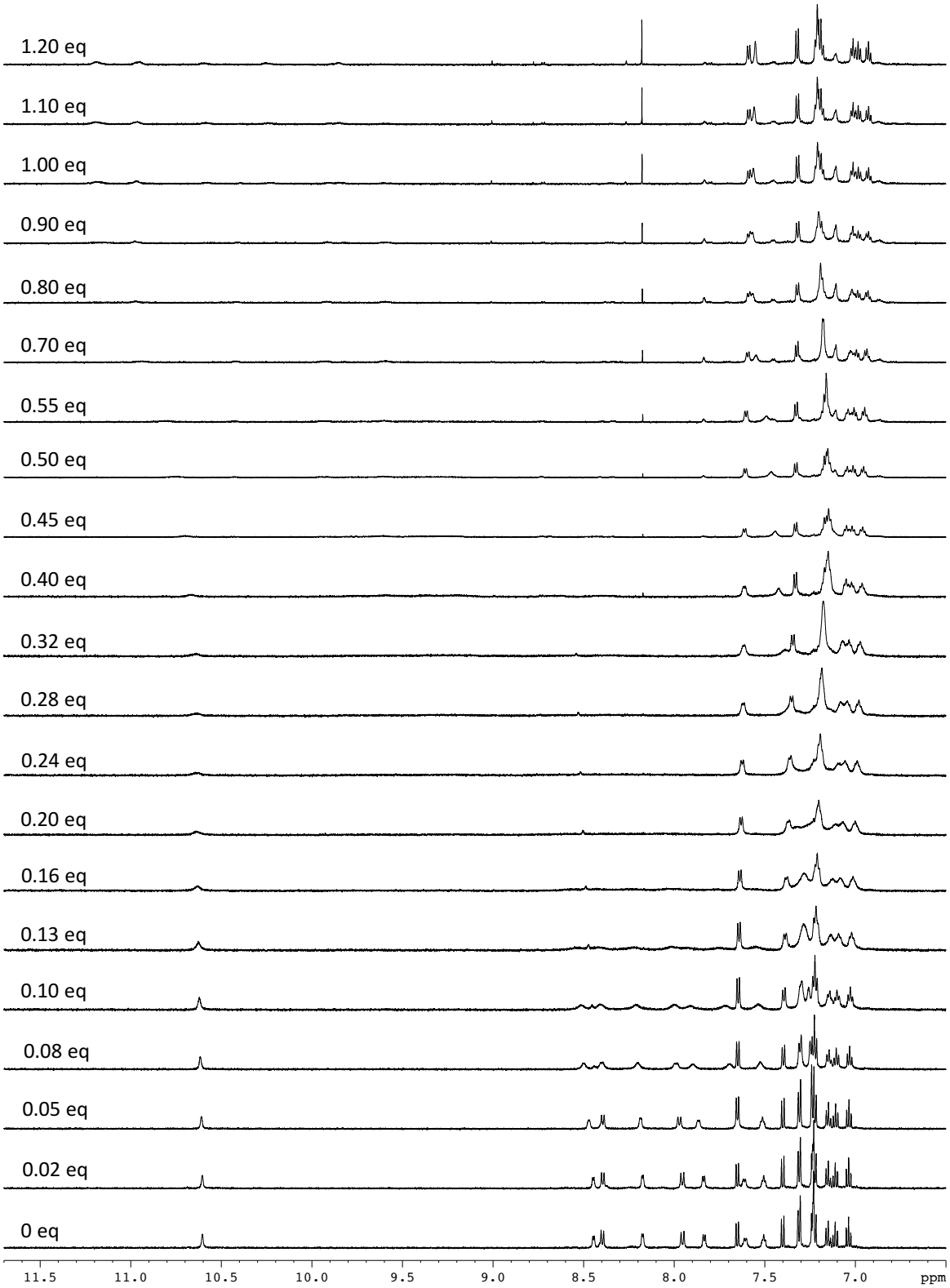
TBAH$_2$PO$_4$ titration (0–1.2 eq.)



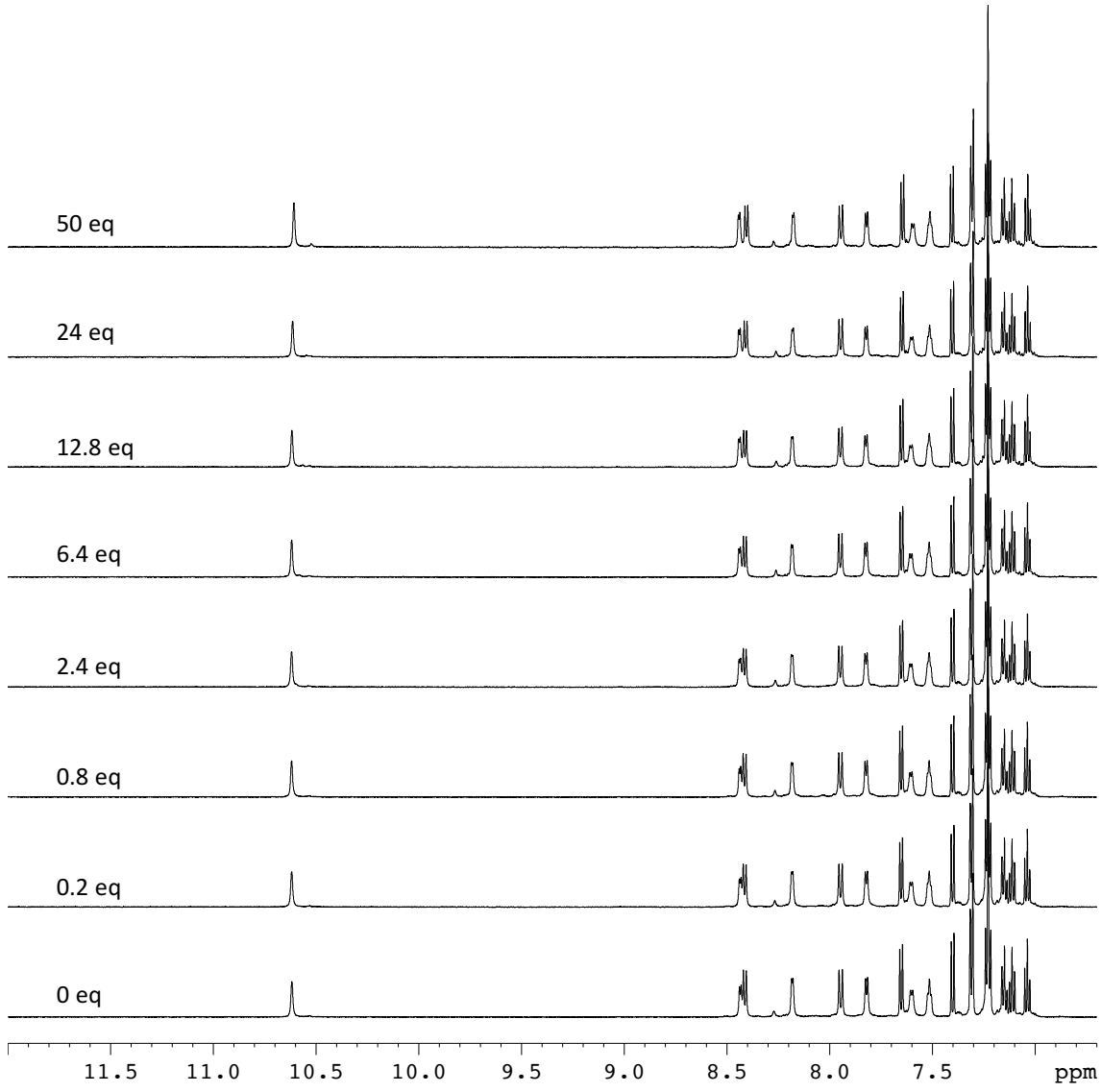
(TBA)$_3$HP$_2$O$_7$ titration



(TBA)$_3$HP$_2$O$_7$ titration (0–1.2 eq.)



TBAPF$_6$ titration



3. Binding data for compounds 2–5 with Cl^-

Compound	Binding model	K_1 (M^{-1})	K_2 (M^{-1})	cov_{fit} (10^{-3})	α[a]
1	1:1	164	–	96.2	–
	1:2	1058	17	1.81	0.064
2	1:1	14	–	14.9	–
	1:2	157	2.85	1.91	0.73
3	1:1	83	–	9.31	–
	1:2	277	10.1	0.44	0.15
4	1:1	104	–	133	–
	1:2	489	2.76	0.56	0.023
5	1:1	49	–	13.2	–
	1:2	247	6.08	0.12	0.098

4. Links to online repositories of binding data

The raw input data, the calculated fit, statistical information and associated information can be accessed via the www.supramolecular.org database through the unique links below (copy/paste into a web browser):

Compound **1**–Br^- binding:
http://app.supramolecular.org/bindfit/view/0ccab474-d45a-434d-8436-9e6c27508cc7

Compound **1**–Cl^- binding:
http://app.supramolecular.org/bindfit/view/0f862d5b-395f-4601-ac60-9e5f24fa5fd2

Compound **1**–HCO_3^- binding:
http://app.supramolecular.org/bindfit/view/6ffcdef5-930c-4631-b10a-357112dfbfd6

Compound **1**–AcO^- binding:
http://app.supramolecular.org/bindfit/view/880ad86b-8e5a-46ed-a555-bcbbe7e781ec

Compound **2**–Cl^- binding:
http://app.supramolecular.org/bindfit/view/fc709398-8a27-48b6-a610-f02ee3856356

Compound **3**–Cl^- binding:
http://app.supramolecular.org/bindfit/view/2557e19f-349f-463c-b867-49d8b28164ac

Compound **4**–Cl^- binding:
http://app.supramolecular.org/bindfit/view/e5578177-89df-4622-a739-12e6737a5a4e

Compound **5**–Cl^- binding:
http://app.supramolecular.org/bindfit/view/241d4228-d7d9-49a1-a67d-cf15f0e01171

5. Transmembrane Chloride Transport Study

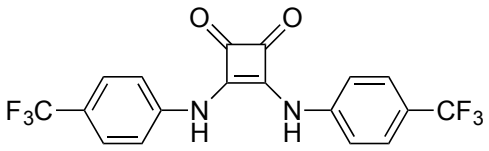
Base-pulse HPTS assays[1] were conducted using POPC LUVs (mean diameter 200 nm) loaded with the ratiometric pH fluorescence probe HPTS (1 mM). The HPTS-loaded POPC LUVs were prepared from a chloroform solution of POPC evaporated in a round-bottom flask and the lipid film formed was dried under vacuum for at least 12 h. The lipid film was hydrated by vortexing with the intervesicular solution containing HPTS (1 mM) and *N*-methyl-D-glucamine (NMDG) chloride (100 mM), buffered with HEPES (10 mM) at pH 7.0. The lipid suspension was subjected to nine freeze/thaw cycles and then extruded 25 times through a 200 nm polycarbonate membrane. The unencapsulated HPTS was removed by size exclusion chromatography on a Sephadex G-25 column using a HPTS free external solution (NMDG chloride (100 mM), buffered with HEPES (10 mM) at pH 7.0) as the eluent.

For each measurement, a concentrated vesicle stock suspension (lipid concentration ~10 mM) was diluted using the external solution to obtain a 2.5 mL suspension containing 0.1 mM of lipid. At the beginning of each measurement, 5 μL of a DMSO solution of the assisting protonophore (gramicidin D (50 μM), final concentration of 0.1 μM) was added to the vesicle suspension and followed by a 5 μL DMSO solution of unguisin A (500 μM, final concentration of 1 μM). To this mixture, a base pulse (25 μL of 0.5 M NaOH, final base of concentration 5 mM) was added to generate a transmembrane pH gradient, and the fluorescence ratio of HPTS (λ_{ex} = 460 nm, λ_{em} = 510 nm divided by λ_{ex} = 403 nm, λ_{em} = 510 nm) was recorded on a Varian Cary Eclipse fluorescence spectrophotometer. At 200 s, a detergent (25 μL of Triton X-100 (11 w%) in 7:1 (v/v) H_2O:DMSO) was added to lyse the vesicles, hence equilibrate the pH gradient to normalise the assay. Data are collected and reported from the average of three repeats. The fractional fluorescence intensity (I_f) was calculated using the following equation:

$$I_f = \frac{R_t - R_0}{R_d - R_0}$$

Where R_t is fluorescence ratio at a given time, R_0 is the is the fluorescence ratio at 0 s and R_d is the fluorescence ratio after the addition of detergent and full equilibration of the pH gradient has occurred.

The assay is repeated with DMSO as a control and a previously reported transport squaramide[2] (10 μM, final concentration of 0.02 μM) for comparison.



squaramide

References

1 (a) Wu, X.; Judd, Luke W.; Howe, Ethan N. W.; Withecombe, Anne M.; Soto-Cerrato, V.; Li, H.; Busschaert, N.; Valkenier, H.; Pérez-Tomás, R.; Sheppard, David N.; Jiang, Y.-B.; Davis, Anthony P.; Gale, Philip A. *Chem* **2016**, *1*, 127-146. (b) Matile, S.; Sakai, N. In *Analytical Methods in Supramolecular Chemistry*; 2 ed.; Schalley, C. A., Ed.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2007; pp 711-742.

2 (a) Busschaert, N.; Kirby, I. L.; Young, S.; Coles, S. J.; Horton, P. N.; Light, M. E.; Gale, P. A. *Angew. Chem. Int. Ed.* **2012**, *51*, 4426-4430; (b) N. Busschaert, S.-H. Park, K.-H. Baek, Y.P. Choi, J. Park, E.N.W. Howe, J.R. Hiscock, L.E. Karagiannidis, I. Marques, V. Félix, W. Namkung, J.L. Sessler, P.A. Gale and I. Shin, Nature Chem., DOI: 10.1038/nchem.2706