Canavanine versus arginine: Prospects for cell-penetrating peptides

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General experimental

Materials. Commercial compounds were from Sigma–Aldrich (St. Louis, MO), Chem Impex (Wood Dale, IL), or Iris Biotech GmbH (Marktredwitz, Germany) and were used without further purification. Bis(2-ethylhexyl) hydrogen phosphate was converted to its sodium salt by adding NaOH (1 equiv) to an aqueous solution of the phosphate followed by lyophilization. Ac-Arg-NH_2·HOAc (**1·HOAc**) was converted to Ac-Arg-NH_2·HCl (**1·HCl**) by cation ion-exchange chromatography using the procedure that produced Ac-Cav-NH_2·HCl (**2·HCl**), *vide infra*.

Conditions. All procedures were performed in air at ambient temperature (~22 °C) and pressure (1.0 atm) unless specified otherwise.

Solvent removal. The phrase "concentrated under reduced pressure" refers to the removal of solvents and other volatile materials using a rotary evaporator while maintaining a water-bath temperature at 40 °C. Residual solvent was removed from samples at high vacuum (<0.1 Torr), which refers to the vacuum achieved by a mechanical belt-drive oil pump, or through lyophilization (freeze-drying) using a Labconco FreeZone freeze dryer.

Chromatography. Chemical reactions were monitored by thin-layer chromatography (TLC) using EMD 250 µm silica gel 60-F_{254} plates and visualization with UV-illumination or $KMnO_4$-staining, or by LC–MS with an ESI Agilent 6125B mass spectrometer. Flash chromatography was performed with a Biotage Isolera automated purification system using prepacked and re-packed SNAP KP silica gel columns.

Instrumentation. ^{1}H-NMR and ^{13}C-NMR spectra for compound characterization were obtained with Bruker spectrometers, and HRMS data were obtained with an Agilent 6545 Q-ToF mass spectrometer at the Department of Chemistry Instrumentation Facility at the Massachusetts Institute of Technology.

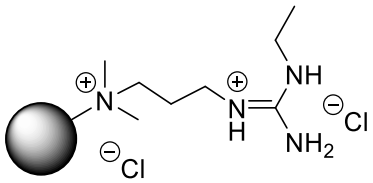
Octanol–water partitioning experiments

Ac-Cav-NH_2·HCl (5 mg, 0.02 mmol) was placed in four separate vials and dissolved in D_2O (1.0 mL) to make 0.02 M solutions. The pH of the solutions was adjusted to 7.4 (pD 7.0) or 3.5 (pD 3.1) by the addition of highly concentrated NaOD and DCl solutions in 1-µL increments. A 200-µL aliquot was taken from each vial for NMR analysis. Subsequently, sodium bis(2-ethylhexyl) phosphate (13.8 mg, 0.04 mmol, 2.5 equiv of Ac-Cav-NH_2·HCl) was added to two vials, and then 800 µL of octanol was added to each vial. The vials were shaken vigorously for 5 min, and then subjected to centrifugation for 10–20 min. Once the layers had separated, a 200-µL aliquot was removed carefully from the aqueous layers with a pipette and added to a new tube. A solution of 0.2 M pyridine in D_2O (20 µL) was added to each tube, the tubes were shaken, and the solutions were transferred to 3-mm NMR tubes for analysis. This procedure was repeated with sodium dodecylsulfate and sodium dodecanoate, as well as with Ac-Arg-NH_2·HCl and each anionic lipid.

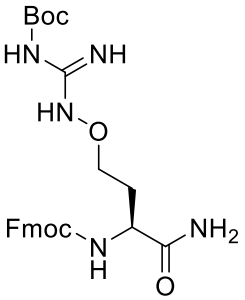
^{1}H-NMR spectra were collected of the aliquots. Spectral integrations were referenced to the signal of the *para*-hydrogen of pyridine, and the integration of the signal from the H^α proton of the

amino acid residue was measured. The relative integrations pre- and post-wash were compared to each other. For both amino acid residues, the spectra following the octanol washes showed a higher integration. This increase was likely the result of an increase in the residual water signal from octanol protons exchanging with D_2O and influencing the integration of the H^{α} proton by altering the local baseline (Figure S1). Because the amount of additional water in the post-octanol and octanol + lipid washes is likely to be the same, we evaluated the extent of partitioning by determining the difference between the octanol and octanol + lipid washes and averaging the values from two replicates.

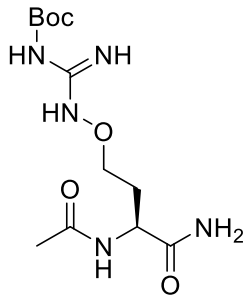
Chemical synthesis



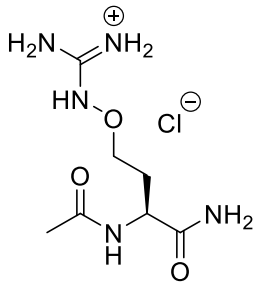
Dimethylaminopropyl-ethylguanidine resin. Polymer-bound 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), 200–400 mesh, ~1.4 mmol/g loading (1.0 g) was suspended in a solution of 7 N NH_4Cl in MeOH (6 mL). A solution of 4 M HCl in dioxane (375 µL, 1.5 mmol) was added, and the resulting suspension was stirred. As a solution-phase surrogate, EDC (267.4 mg, 1.4 mmol) was dissolved concurrently in 7 N NH_4Cl in MeOH (6 mL). A solution of 4 M HCl in dioxane (375 µL, 1.5 mmol) was added, and the resulting solution was stirred. The progress of the latter reaction was monitored by LC–MS. When the signal for the guanidine product had appeared and the signal for the starting material had disappeared, the resin beads were filtered and dried (and the solution-phase reaction mixture was discarded). The resin was packed into a pipette column and rinsed with water (3×), 1 M HCl (3×), and water again (3×).



Fmoc-Cav(Boc)-NH_2 (4). Ammonium bicarbonate (277.66 mg, 3.51 mmol) and Boc_2O (152.81 mg, 0.7 mmol) were dissolved in pyridine (2.5 mL, 0.20 mol). Fmoc-Cav(Boc)-OH (**3**) (250 mg, 0.5 mmol) was added, and the mixture was stirred for 6 h. The reaction mixture was left to dry under a stream of air overnight. The product was purified by column chromatography on silica gel with a gradient of 1–5% v/v MeOH in DCM to produce a white fluffy solid (209 mg, 84% yield). **^{1}H NMR** (600 MHz, MeOD, δ): 7.81 (d, J = 7.5 Hz, 2H), 7.68 (t, J = 6.4 Hz, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.33 (t, J = 7.3 Hz, 2H), 4.45–4.35 (m, 2H), 4.30 (dd, J = 9.4, 4.8 Hz, 1H), 4.24 (t, J = 6.6 Hz, 1H), 3.89 (m, 2H), 2.18 (m, 1H), 1.91 (m, 1H), 1.48 (s, 9H). **^{13}C NMR** (600 MHz, MeOD, δ): 176.16, 157.09, 153.81, 143.91, 143.84, 141.20, 127.38, 126.79, 124.82, 119.51, 80.90, 68.81, 66.57, 52.26, 47.04, 31.12, 27.05. **HRMS** *m/z* calcd for $C_{25}H_{31}N_5O_6$ $[M + H]^+$, 498.2353; found 498.2345.



Ac-Cav(Boc)-NH$_2$ (5). Fmoc-Cav(Boc)-NH$_2$ (**4**) (280 mg, 0.56 mmol) was dissolved in 2.0 M dimethylamine in THF (3 mL), and the resulting solution was stirred for 2 h. The mixture was concentrated under reduced pressure before being suspended in water and vacuum-filtered to remove Fmoc byproducts. The filtrate was lyophilized to yield a white solid. The mixture was then dissolved in MeOH (5.5 mL), and Amberlyst-A21 tertiary amine resin (1 g) and acetic anhydride (529 µL, 5.6 mmol) were added. The mixture was stirred for 3 h, filtered, concentrated under reduced pressure, and purified by column chromatography on silica gel in 20% v/v MeOH in DCM to produce a white foam (144 mg, 81% yield). **^{1}H NMR** (600 MHz, MeOD, δ): 4.51 (dd, J = 8.9, 5.1 Hz, 1H), 3.93 (m, 2H), 2.19 (ddt, J = 10.7, 7.8, 5.2 Hz, 1H), 2.01 (s, 3H), 1.93 (ddt, J = 14.4, 9.0, 5.4 Hz, 1H), 1.50 (s, 9H). **^{13}C NMR** (600 MHz, MeOD, δ): 176.81, 173.47, 154.27, 153.96, 83.63, 71.72, 51.70, 32.10, 28.32, 22.58. **HRMS** *m/z* cald for $C_{12}H_{23}N_5O_5$ [M + H]$^+$, 318.1777; found, 318.1771.



Ac-Cav-NH$_2$·HCl (2·HCl): Ac-Cav(Boc)-NH$_2$ (**5**) (144 mg, 0.45 mmol) was dissolved in a solution of TFA (1.0 mL) and MeOH (50 µL), and the resulting solution was stirred for 1 h. The mixture was dried under a stream of air and then under reduced pressure. The residue was dissolved in water, and the resulting solution was lyophilized to remove excess TFA. The residue was redissolved in water, and the resulting solution was flushed through a pipette column of dimethylaminopropyl-ethylguanidine resin that had been charged with HCl and lyophilized to produce a clear solid (97 mg, >95% yield). **^{1}H NMR** (600 MHz, DMSO-d_6, δ): 11.16 (s, 1H), 8.20 (d, J = 8.1 Hz, 1H), 7.70 (s, 4H), 7.55 (s, 1H), 7.13 (s, 1H), 4.29 (td, J = 8.7, 5.0 Hz, 1H), 3.80 (t, J = 6.5 Hz, 3H), 2.03 (dtd, J = 14.1, 7.0, 4.9 Hz, 1H), 1.80 (ddt, J = 14.7, 9.3, 5.9 Hz, 1H). **^{13}C NMR** (600 MHz, DMSO-d_6, δ): 174.19, 170.51, 158.79, 73.62, 49.86, 30.56, 22.94. **HRMS** *m/z* calcd for $C_7H_{15}N_5O_3$ [M + H]$^+$, 218.1253; found, 218.1245.

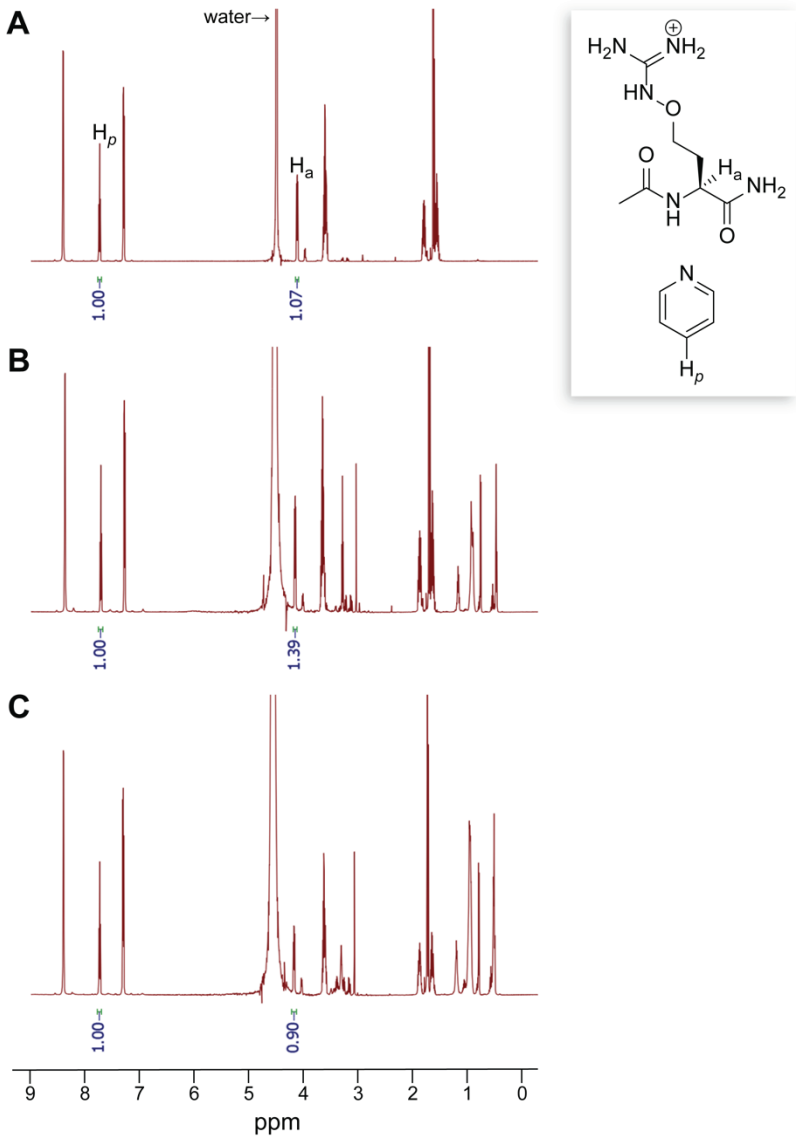


Figure S1. Representative ^{1}H NMR spectra of the aqueous layer from the octanol–water partitioning of Ac-Cav-NH_2·HCl (**2·HCl**) into octanol in the absence or presence of sodium bis(2-ethylhexyl) phosphate (lipid **7**), showing 65% (= 0.90/1.39) remaining in the aqueous layer post-octanol + lipid wash. A. Pre-wash. B. Post-wash with octanol. C. Post-wash with octanol containing lipid **7**.

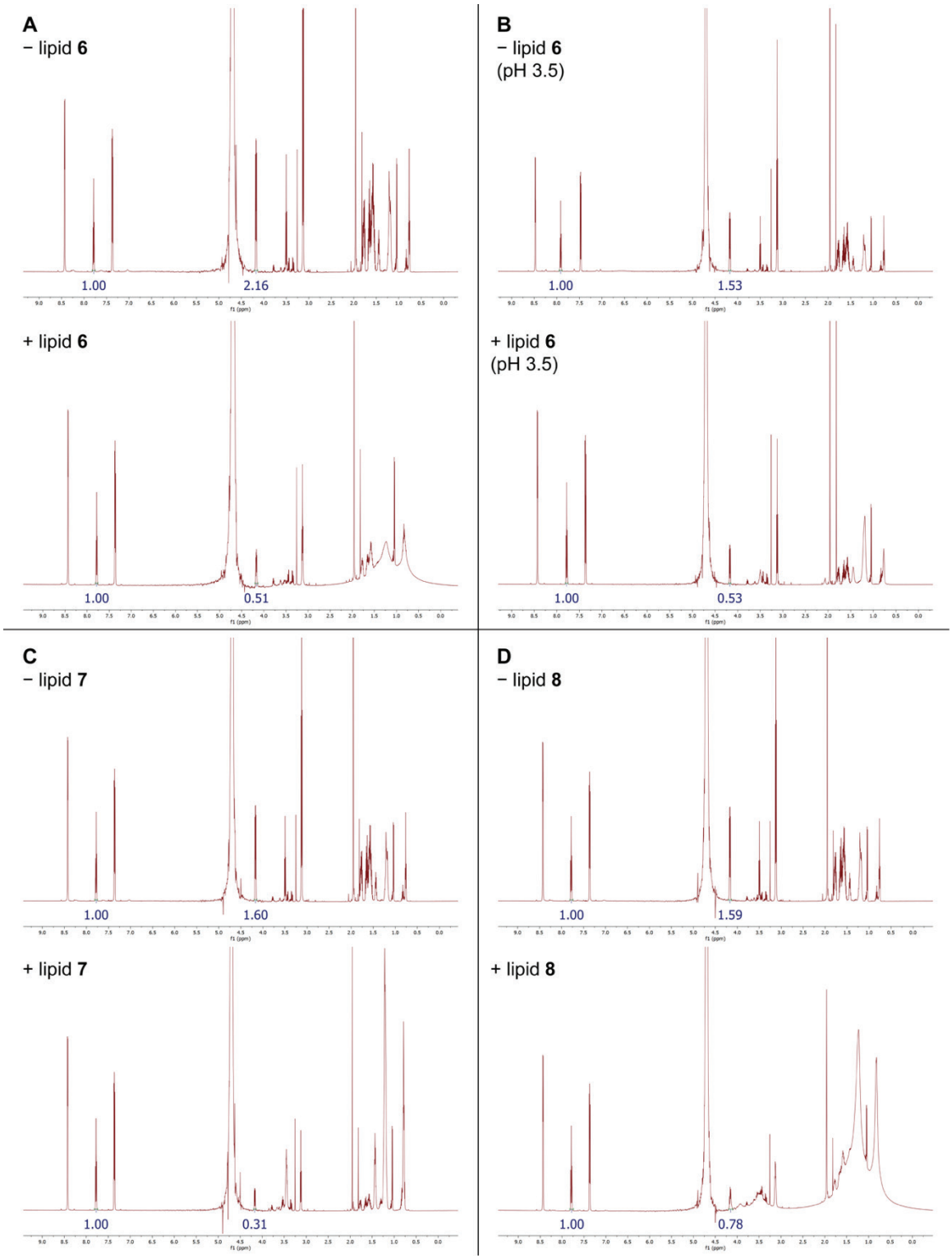


Figure S2. ^{1}H NMR spectra from the partitioning of Ac-Arg-NH_2·HCl (**1·HCl**) into octanol alone (top) and octanol with a lipid (bottom). A. **1·HCl** and lipid **6** (carboxylate). B. **1·HCl** and lipid **6** at pH 3.5. C. **1·HCl** and lipid **7** (phosphate). D. **1·HCl** and lipid **8** (sulfate). Experiments were performed in duplicate; one data set is shown.

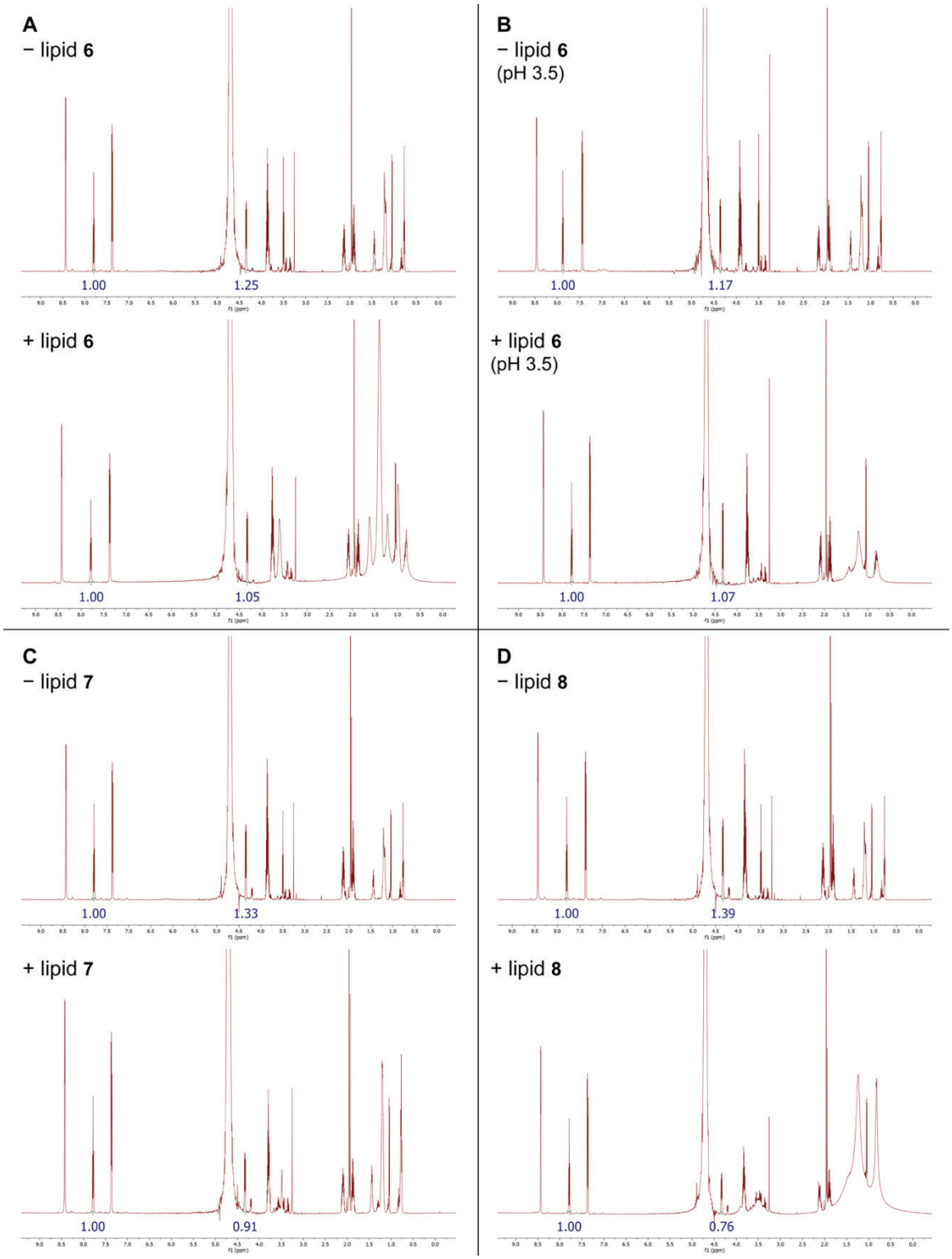


Figure S3. ^{1}H NMR spectra from the partitioning of Ac-Cav-NH_2·HCl (**2·HCl**) into octanol alone (top) and octanol with a lipid (bottom). A. **2·HCl** and lipid **6** (carboxylate). B. **2·HCl** and lipid **6** at pH 3.5. C. **2·HCl** and lipid **7** (phosphate). D. **2·HCl** and lipid **8** (sulfate). Experiments were performed in duplicate; one data set is shown.

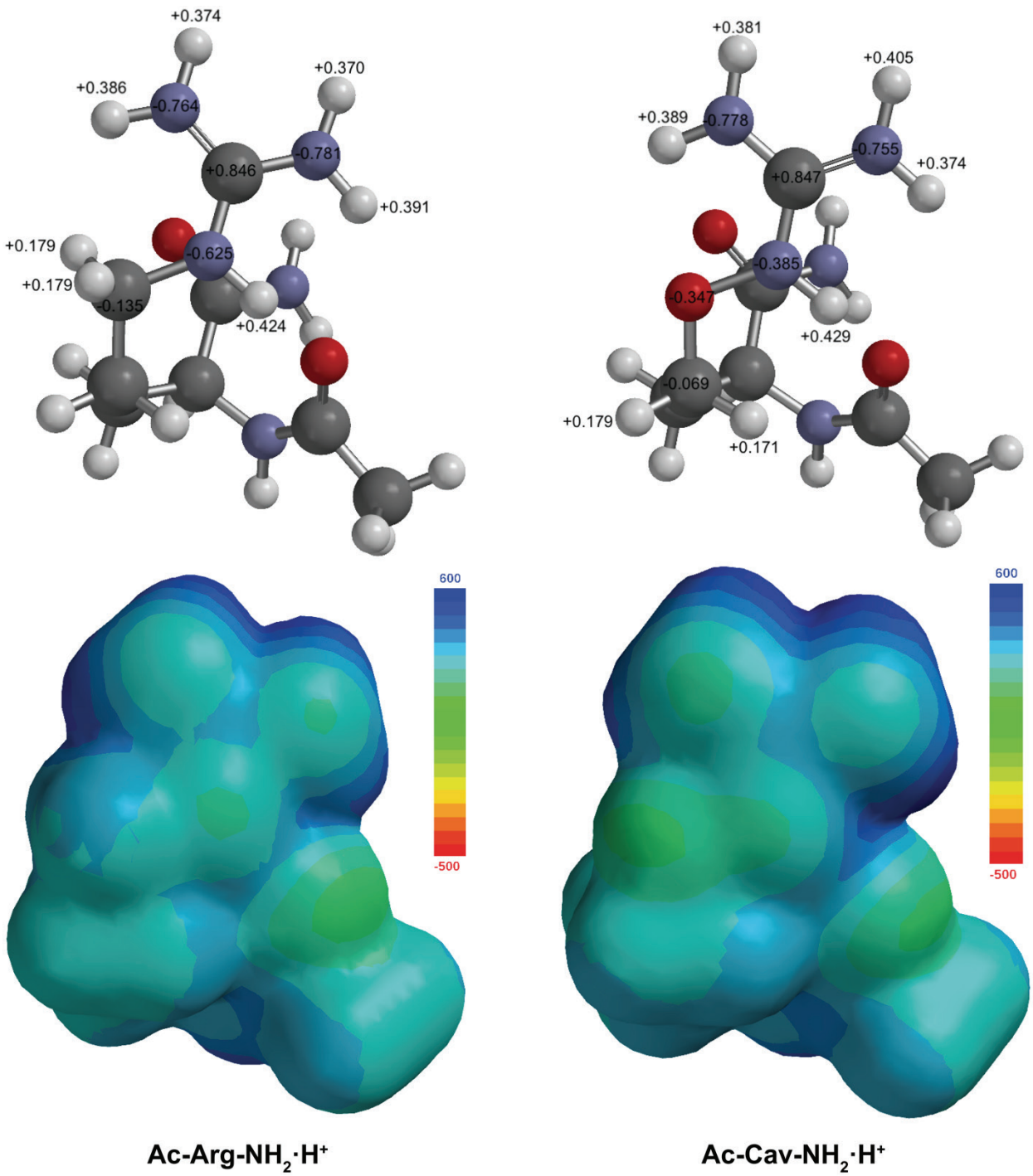


Figure S4. Calculation with Spartan '18 (Wavefunction, Irvine, CA) showing the electron density on atoms in Ac-Arg-$NH_2 \cdot H^+$ (left) and Ac-Cav-$NH_2 \cdot H^+$ (right).