Supplementary Material (ESI) for Chemical Communications

SUPPORTING INFORMATION

Toward the Discovery of Potent Inhibitors of Botulinum Neurotoxin A: Development of a Robust LC-MS Based Assay Operational from Low to Subnanomolar Enzyme Concentrations

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S2-S3: Experimental procedures.
S3-S4: ^{1}H and ^{13}C NMR of compound (**1**).

Inhibitor Synthesis

1-adamantane-N-hydroxyacetamide (**1**, ANH) was synthesized as described previously[8] Briefly, O-Chlorotrityl hydroxyl amine resin (1 eq) pre-swollen in DMF: CH_2Cl_2 (1:1), adamantane acetic acid (5 eq.), DIC (5 eq.) and Cl-HOBt (5 eq.) were combined and the reaction shaken overnight at rt. Next, the resin was washed with CH_2Cl_2:DMF. Product was released by treatment with 1:20 TFA:CH_2Cl_2 sol. followed by shaking for 1 h. The solution phase was then collected and concentrated. The crude product mixture was purified by flash chromatography on silica gel using 1:1 EtOAc/CH_2Cl_2 as an eluant to afford the desired hydroxamate (1) in 20% yield. ^{1}H NMR (600 MHz, MeOD) δ (ppm) 1.66 (9H, m), 1.74 (3H, m), 1.83 (2H,s), 1.96 (3H, s). ^{13}C NMR (125 MHz, MeOD) δ (ppm) 29.7 (3 x CH), 33.3 (Cquaternary), 37.4 (3 x CH2), 43.2 (3 x CH2), 47.9 (CH2). ESI-TOF calc. $C_{12}H_{19}NO_2$ [M+H+]: 210.1488, found: 210.1490.

Peptide Synthesis

SNAP-25 (141-206) was synthesized and purified as previously described.[11] H_2N-Arg-[Ala(1-^{13}C)]-Thr-Lys-Met-[Leu(1-^{13}C)]-[Gly(1-^{13}C]-Ser-[Gly(1-^{13}C)]-COOH was prepared by stepwise solid-phase peptide synthesis using standard protocols for Fmoc chemistry.[19] The ^{13}C-labeled amino acids were purchased from Cambridge Isotope Laboratories. The product was purified via preparative RP-HPLC using a Vydac 218TP101522 column at a flow rate of 10 ml/min, with detection at 214 nm during a linear gradient of 0-40% B over 40 min. Solvent A is 0.1% TFA in water and B is 0.09% TFA in acetonitrile.

Assay for BoNT/A LC activity with SNAP-25 (141-206)

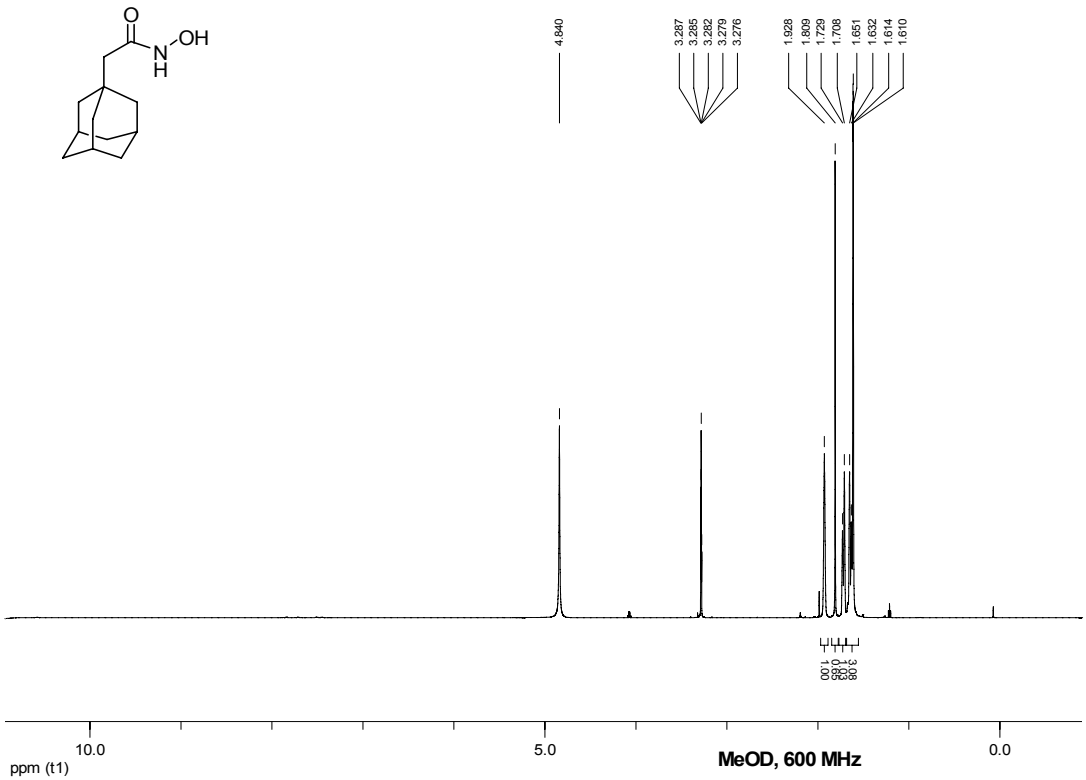
BoNT/A LC at 2 nM was assayed at 22.5 °C, pH 7.4, in 40 mM HEPES in 150 µl volumes with a total DMSO concentration of 2%. At timed intervals, ranging from 10 min to 60 min, 25 µl aliquots were withdrawn and quenched by the addition of 3 µl of 15% aqueous TFA, SIL standard was added to a 1 µM final concentration. Sample analysis was by use of an Agilent 1100 LC MS system. A 20 µl sample was injected onto a Zorbax 300SB-C8 column (4.6x50 mm, 5 µm, Agilent Technologies) subjected to a gradient (A to B where A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile) of 2.5% B from 0 to 2.50 min, 2.5% B to 97.5% B from 2.50 to 10 min, and 97.5% B from 10 to 13 min at a constant flow rate of 0.5 ml/min. A column-solvent equilibration time of 4 min was conducted prior to the next sample analysis. Mass spectral acquisition included a solvent front delay of 2.50 min. Operational parameters were: positive ion single ion monitoring of m/z 460.9 and 462.9 corresponding to the M+2 peak of the reaction product and SIL respectively, nitrogen as a nebulizing and drying gas (20 psi, 3 l/min), HV capillary voltage at 4000 V and the drying gas temperature to 300 °C. Run analysis and quantitation was by use of Chemstation software (Agilent). Enzyme velocities were determined from a linear fit of product formation versus incubation time. The kinetic constants, K_M and k_{cat}, were determined by a nonlinear least squares fit of the Michaelis-Menten equation to enzyme velocities versus substrate concentrations. The inhibition constant (K_i) for compound (**1**) was obtained

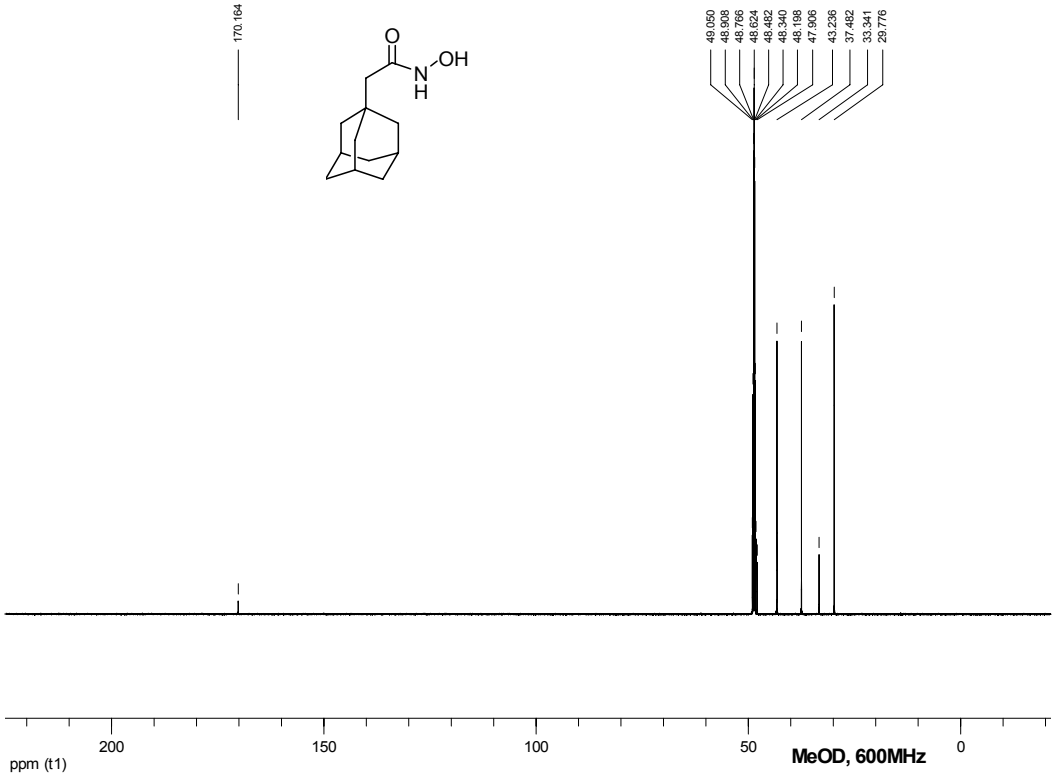
from a nonlinear least squares global fit of the competitive inhibition equation to the initial rates of product formation for a matrix of substrate and inhibitor concentrations bracketing K_M(apparent) and K_i(apparent).

Assay for BoNT/A activity with SNAPtide™

The assay was performed as described previously,[20] Tween 20 at 0.01% wt/vol was used as detergent and the BoNT/A LC concentration was 100 nM.

Corrections for the inner filter effect were performed as described in Liu et al.[13]





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2 Eubanks, L.M.; Hixon, M.S.; Jin, W.; Hong, S.; Clancy, C.M.; Tepp, W.H.; Baldwin, M.R.; Malizio, C.J.; Goodnough, M.C.; Barbieri, J.T.; Johnson, E.A.; Boger, D.L.; Dickerson, T.J.; Janda, K.D. *Proc. Natl. Acad. Sci. U.S.A.* 2007, **104**, 2602.