Botulinum Neurotoxin A Protease: Discovery of Natural Product Exosite Inhibitors

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Supporting Information

General

Natural products: D-chicoric acid (**I1**), caftaric acid (**I3**) and chlorogenic acid (**I4**) were purchased from ChromaDex. Synthetic L-Chicoric acid (**I1'**) was purchased from Sigma Aldrich. Hydroxamates (**I2** and **I5**) were synthesized and described previously.[1]

Assay for BoNT/A Light Chain Activity with SNAP-25 (141-206)[2]

BoNT/A LC at 75 pM was assayed at 20°C, pH 7.4, in 40 mM HEPES in 200 µl volumes with a total DMSO concentration of 2%. At timed intervals, ranging from 20 min to 40 min, 25 µl aliquots were withdrawn and quenched by the addition of 3 µl of 15% aqueous TFA, ^{13}C – labeled standard was added to a 1 µM final concentration. Sample analysis was done 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 2.5 min, 2.5 % B to 97.5 % B from 2.5 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 2.5 min. Operational parameters were: positive single ion monitoring of m/z 460.9 and 462.9 corresponding to the M+2 peak of the reaction product and labeled internal standard respectively, nitrogen as a nebulizing and drying gas (20 psi, 3 l/min), HV capillary voltage at 4 kV 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 inhibition constants, K_U and K_C, were determined by a non-linear least squares global fit of the equation 1 to the initial rates of product formation for a matrix of substrate and inhibitor concentrations bracketing K_M(apparent) and K_I(apparent).

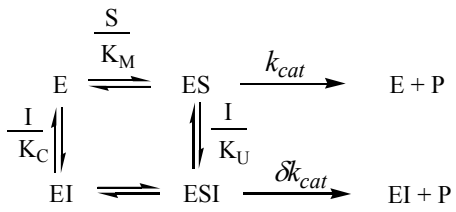
Table S1. Values of fractional catalytic activity (δ) at saturating [I] and inhibition constants for chicoric acid, caftaric acid and chlorogenic acid.

Entry	Compound*	δ	K_U / µM	K_C / µM
1	**I1**	0.42 ± 0.04	1.6 ± 0.3	0.7 ± 0.1
2	**I1'**	0.48 ± 0.05	2.3 ± 0.5	0.8 ± 0.1
3	**I3**	0.20 ± 0.06	20 ± 7	26 ± 7
4	**I4**	0.30 ± 0.04	39 ± 9	100 ± 40

*) **I1** = D-Chicoric acid, **I1'** = L-Chicoric acid, **I3** = Caftaric acid, **I4** = Chlorogenic acid

Derivation of the rate equation for chicoric acid (I1) inhibition

Scheme S1. Chicoric Acid Mechanism of Inhibition



Where I = chicoric acid, $K_C = K_I$(competitive), $K_U = K_I$(uncompetitive), δ = the fractional catalytic activity at saturating [I] while S, K_M, K_I, k_{cat} & V_{MAX} have their usual definitions.

$E_{Total} = E + EI + ES + ESI$ kinetically relevant species

$$E_{Total} = \left(1 + \frac{I}{K_C} + \frac{S}{K_M} + \frac{S}{K_M}\frac{I}{K_U}\right)E$$ expressed in terms of steady state parameters[3]

$$\frac{E}{E_{Total}} = \frac{1}{1 + \frac{I}{K_C} + \frac{S}{K_M}\left(1 + \frac{I}{K_U}\right)}$$ fractionation of E with respect to [I] and [S]

$$v = k_{cat}(ES + \delta\, ESI)$$ the rate equation

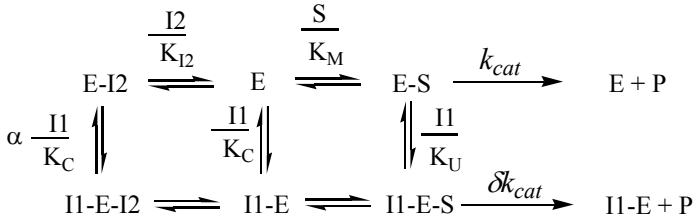
$$v = k_{cat}\frac{S}{K_M}\left(1 + \delta\frac{I}{K_U}\right)E$$ expressed in terms of E

$$v = \frac{k_{cat}\frac{S}{K_M}\left(1 + \delta\frac{I}{K_U}\right)}{1 + \frac{I}{K_C} + \frac{S}{K_M}\left(1 + \frac{I}{K_U}\right)}E_{Total}$$ expressing E in terms of E_{Total}

$$v = \frac{V_{MAX}S\left(1 + \delta\frac{I}{K_U}\right)}{K_M\left(1 + \frac{I}{K_C}\right) + S\left(1 + \frac{I}{K_U}\right)}$$ (Eq.1) expressed in Michaelis - Menten form

Derivation of the rate equation for the chicoric acid (I1) - active site inhibitor (I2) combination

Scheme S2. Active site inhibitor (**I2**) in combination with chicoric acid (**I1**) depicted as two synergistic inhibitors with chicoric acid displaying partial inhibition.



Where I1 = chicoric acid (**I1**), I2 = active site inhibitor (**I2**), K_C = K_I(competitive) of chicoric acid, K_U = K_I(uncompetitive) of chicoric acid, K_{I2} = K_I of **I2**, $K'_X = K_X * S/(S+K_M)$ i.e.; K_I(apparent) at a given [S], α is a synergistic parameter reflecting the difference of affinity for chicoric acid binding in the presence and absence of **I2**, δ = the fractional catalytic activity at saturating [**I1**], Vo = the velocity in the absence of inhibitors, while S, K_M, K_I, k_{cat} & V_{MAX} have their usual definitions.

$$E_{Total} = E + EI2 + EI1I2 + EI1 + ES + ESI1 \quad \text{kinetically relevant species}$$

$$E_{Total} = \left(1 + \frac{I2}{K_{I2}} + \frac{I1}{K_C} + \alpha\frac{I2}{K_{I2}}\frac{I1}{K_C} + \frac{S}{K_M} + \frac{S}{K_M}\frac{I1}{K_U}\right)E \quad \text{expressed in terms of steady state parameters}^3$$

$$\frac{E}{E_{Total}} = \frac{1}{1 + \frac{I2}{K_{I2}} + \frac{I1}{K_C} + \alpha\frac{I2}{K_{I2}}\frac{I1}{K_C} + \frac{S}{K_M}\left(1 + \frac{I1}{K_U}\right)} \quad \text{fractionation of E with respect to [I2], [I1] and [S]}$$

$$v = k_{cat}\left(ES + \delta\, ESI1\right) \quad \text{the rate equation}$$

$$v = k_{cat}\frac{S}{K_M}\left(1 + \delta\frac{I1}{K_U}\right)E \quad \text{expressed in terms of E}$$

$$v = \frac{k_{cat}\frac{S}{K_M}\left(1 + \delta\frac{I1}{K_U}\right)}{1 + \frac{I2}{K_{I2}} + \frac{I1}{K_C} + \alpha\frac{I2}{K_{I2}}\frac{I1}{K_C} + \frac{S}{K_M}\left(1 + \frac{I1}{K_U}\right)}E_{Total} \quad \text{expressing E in terms of } E_{Total}$$

$$v = \frac{V_{MAX}S\left(1 + \delta\frac{I1}{K_U}\right)}{K_M\left(1 + \frac{I2}{K_{I2}} + \frac{I1}{K_C} + \alpha\frac{I2}{K_{I2}}\frac{I1}{K_C}\right) + \frac{S}{K_M}\left(1 + \frac{I1}{K_U}\right)} \quad \text{expressed in Michaelis - Menten form}$$

$$\frac{v}{Vo} = \frac{\dfrac{V_{MAX}\mathrm{S}\left(1+\delta\dfrac{\mathrm{I1}}{\mathrm{K_U}}\right)}{\mathrm{K_M}\left(1+\dfrac{\mathrm{I2}}{\mathrm{K_{I2}}}+\dfrac{\mathrm{I1}}{\mathrm{K_C}}+\alpha\dfrac{\mathrm{I2}}{\mathrm{K_{I2}}}\dfrac{\mathrm{I1}}{\mathrm{K_C}}\right)+\dfrac{\mathrm{S}}{\mathrm{K_M}}\left(1+\dfrac{\mathrm{I1}}{\mathrm{K_U}}\right)}}{\dfrac{V_{MAX}\mathrm{S}}{\mathrm{K_M}+\mathrm{S}}}$$ relative rate in presence and absence of inhibitors

$$\frac{v}{Vo} = \frac{\left(\mathrm{K_M}+\mathrm{S}\right)\left(1+\delta\dfrac{\mathrm{I1}}{\mathrm{K_U}}\right)}{\mathrm{K_M}\left(1+\dfrac{\mathrm{I2}}{\mathrm{K_{I2}}}+\dfrac{\mathrm{I1}}{\mathrm{K_C}}+\alpha\dfrac{\mathrm{I2}}{\mathrm{K_{I2}}}\dfrac{\mathrm{I1}}{\mathrm{K_C}}\right)+\dfrac{\mathrm{S}}{\mathrm{K_M}}\left(1+\dfrac{\mathrm{I1}}{\mathrm{K_U}}\right)}$$ simplifing $\dfrac{\dfrac{\mathrm{a}}{\mathrm{b}}}{\dfrac{\mathrm{c}}{\mathrm{d}}} = \dfrac{\mathrm{ad}}{\mathrm{bc}}$

$$\frac{v}{Vo} = \frac{1+\delta\dfrac{\mathrm{I1}}{\mathrm{K_U}}}{1+\dfrac{\mathrm{I2}}{\mathrm{K'_{I2}}}+\dfrac{\mathrm{I1}}{\mathrm{K'_C}}+\alpha\dfrac{\mathrm{I2}}{\mathrm{K'_{I2}}}\dfrac{\mathrm{I1}}{\mathrm{K'_C}}+\dfrac{\mathrm{S}}{\mathrm{K_M}+\mathrm{S}}\left(\dfrac{\mathrm{I1}}{\mathrm{K_U}}\right)}$$ (Eq. 3) dividing through by $\mathrm{S}+\mathrm{K_M}$

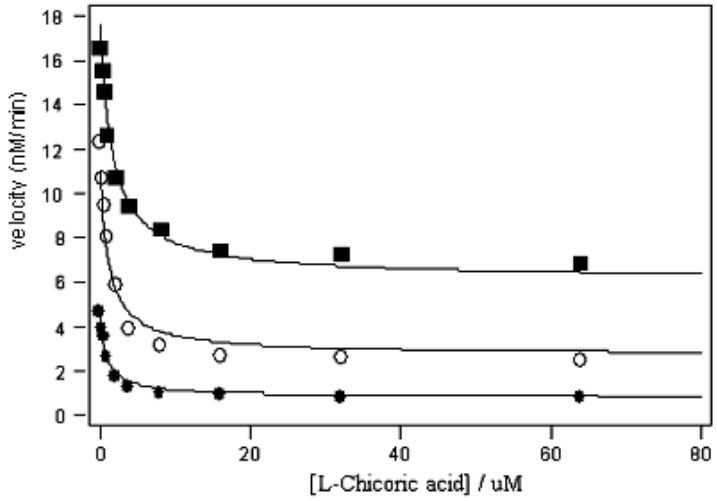


Figure S1. BoNT/A LC catalysis at varied concentrations of substrate and L-chicoric acid as inhibitor.

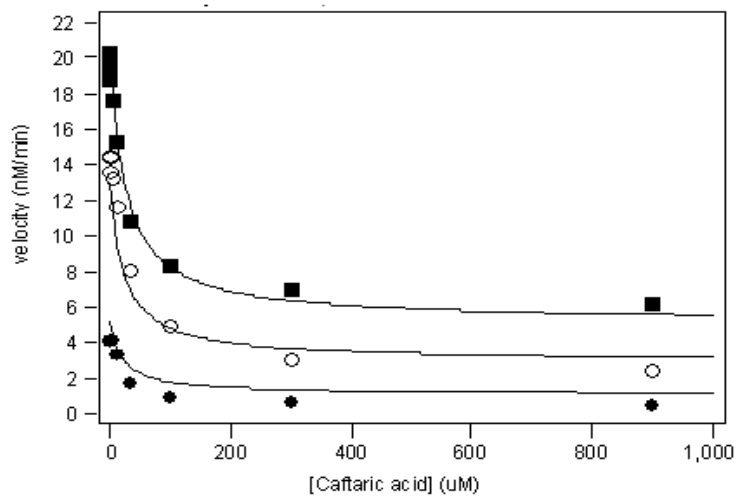


Figure S2. BoNT/A LC catalysis at varied concentrations of substrate and caftaric acid as inhibitor.

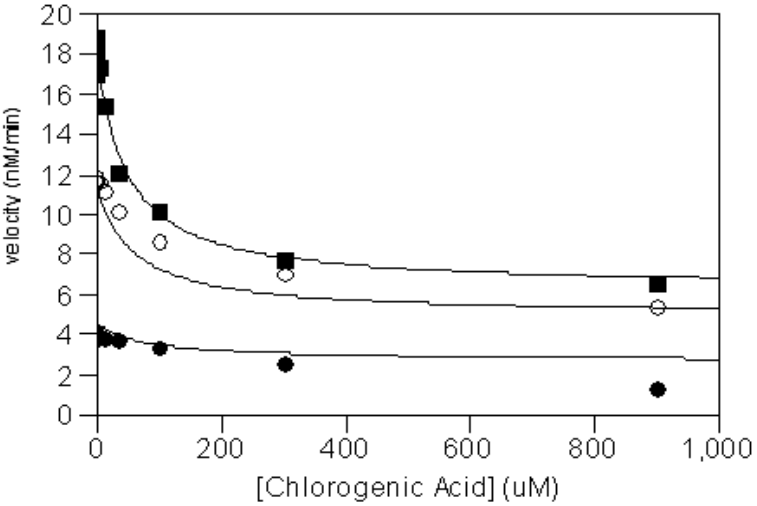


Figure S3. BoNT/A LC catalysis at varied concentrations of substrate and chlorogenic acid as inhibitor.

1) Boldt, G. E.; Kennedy, J. P.; Janda, K. D. *Organic Letters* **2006**, 8, 1729-1732.
2) Čapková, K.; Hixon, M.S.; McAllister, L.A.; Janda, K.D. *Chem. Commun,* **2008**, 14, 3525-3527.
3) Cha, S. *J Biol Chem* **1968**, 243, 820-825