Supplementary Material (ESI) for Chemical Communications

SUPPORTING INFORMATION

A Cross-over Inhibitor of the Botulinum Neurotoxin Light Chain B: A Natural Product Implicating an Exosite Mechanism of Action

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Chemicals and materials

All Fmoc protected amino acids and Fmoc-lys(Mtt)-OH were purchased from Novabiochemicals. Tentagel R NH_2 resin (90 µm) was purchased from Peptide International. Fmoc-Rink Linker was purchased from CS Bio (Code: RS-F002). Acetic anhydride, 7-methoxycoumarin-4-acetic acid, 1-fluoro-2,4-dinitrobenzene, tetrakis(triphenylphospine)palladium, piperidine (pip), trifluoroacetic acid (TFA), triisopropylsilane (TIS), diisopropylethylamine (DIEA), and Tween 20 were purchased from Aldrich. Fmoc-diaminopropionic acid (alloc)-OH (Dpa) was purchased from Senn Chemicals. 1,3-Diisopropylcarbodiimide (DIC) was purchased from Advanced Chemtech. 6-Chloro-1-hydroxybenzotriazole (6Cl-HOBt) was purchased from Ochem Incorporation. N,N-dimethylforamide, acetonitrile, dichloromethane, HEPES enzyme grade buffer and Greiner black flat bottom 96 well plates were purchased from Fisher. L-Chicoric acid was purchased from SIGMA (# C7243).

Synthesis of peptide substrates

The peptides were synthesized on a CS Bio peptide synthesizer (model CS136). In brief Tentagel R NH_2 resin (1 mmole) was swelled in CH_2Cl_2 followed by coupling with Fmoc-Rink Linker. After deprotection of the Fmoc, peptide substrates were constructed on the resin with activated mixtures of Fmoc protected amino acids (3 eq) and 6Cl-HOBt(3 eq.) and DIC (3 eq) in a 50 % mixture of DMSO and DMF. To the amino acid sequence was added Fmoc-lys(Mtt)-OH and Fmoc-Dpa(Alloc)-OH to facilitate coupling of the FRET pair. Coupling times where 1.5 hr, followed by multiple washes with DMF and CH_2Cl_2. The Fmoc protecting group was removed from amino acids attached to the resin by treatment with 20% pip/DMF for 10 min, with an additional treatment of 20% pip/DMF for 15 min, followed by washes with DMF and CH_2Cl_2. Growth of the peptide was monitored by test cleavages and subsequent LC/MS. After construction of the peptides the N-terminal Fmoc group was removed and the free amine was treated with acetic anhydride and collidine in CH_2Cl_2.

The FRET pair was added to the full length peptides via orthogonal protection strategies. The Alloc group was removed from diaminopropionic acid by treatment with $Pd(ph)_4$ (0) (0.1 eq) and phenylsilane (10 eq) in CH_2Cl_2. The resin was washed with DMF (2 x 50 ml) and treated with a 5% (w/w) sodium diethyldithiocarbamate solution in DMF for 30 min. This was followed by additional washes with DMF (5 x 50 ml) and CH_2Cl_2 (3 x 50 ml). To the deprotected resin was added a solution of 1-fluoro-2,4-dinitrobenzene (5 eq) and DIEA (5 eq) in DMF. The mixture was shaken in the dark for 24 hr. The resin was washed with DMF (3 x 50) and CH_2Cl_2 (2 x 50 ml).

The Mtt group was removed from lysine by treatment of the resin with 2 % TFA and 5 % TIS in CH_2Cl_2 for 10 min. This was repeated 4 times to completely remove the Mtt group and the resin was washed with DMF (5 x 50 ml) and CH_2Cl_2 (3 x 50 ml). After successful removal of Mtt the resin was treated with 5 % DIEA / DMF for 30 min and washed with DMF (5 x 50 ml) and CH_2Cl_2 (3 x 50 ml). To the resin was added a pre-activated solution of MCA (3 eq), 6Cl-HOBt (3 eq) and DIC (3eq) in 50 % DMSO/DMF and the resin was allowed to shake for 4 hr. The resin was washed with DMF (5 x 50 ml) and CH_2Cl_2 (3 x 50 ml).

The fully synthesized peptides were dried and cleaved using reagent K for 3.5 hr. The solution was filter and the resin was washed with additional TFA. The filtrate was added to a large excess of cold diethyl ether and the precipitate was collected by centrifugation of the mixture. Cold ether was again added to the precipitate and the mixture was centrifuged and the ether layer decanted. The crude peptide was dissolved in a solution of 5 % CH_3CN/water and 5 % AcOH. The solution was freeze dried to give an orange solid. The crude peptide was purified with a Shimadzu preparative HPLC using a 250 mm x 22 mm GRACE/Yydac column (218TP101522) running a linear gradient of 20 % CH_3CN/ H_2O to 70 % CH_3CN/ H_2O with 0.1 % TFA over 50 min.

High-resolution mass spectra (HRMS) were measured on a Bruker Fourier Transform Ion Cyclotron (FTICR) mass spectrometer (7.0 Tesra) by electron spray ionization (ESI) method.

VAMP44 yield 23 mg (2%) $C_{244}H_{381}N_{71}O_{78}$ M. W. 5557.06
HRMS (ESI-FTICR) calcd.: M+7 794.4083; Found: 794.4054 (-3.65 ppm)

VAMP48 yield 28 mg (2%) $C_{264}H_{417}N_{79}O_{83}$ M. W. 6025.62
HRMS (ESI-FTICR) calcd.: M+7 861.3056; Found: 861.3054 (-0.23 ppm)

VAMP52 yield 26 mg (2%) $C_{284}H_{451}N_{83}O_{89}S$ M. W. 6484.19
HRMS (ESI-FTICR) calcd.: M+8 811.0459; Found: 811.0441 (-2.22 ppm)

VAMP58 yield 31 mg (2%) $C_{312}H_{496}N_{90}O_{100}S$ M. W. 7139.88
HRMS (ESI-FTICR) calcd.: M+8 892.9606; Found: 892.9600 (-0.67 ppm)

Expression of recombinant LC/B (1-431)

A codon-optimized, ten-residue C-terminal truncated version of the *C. botulinum* serotype B light chain (LC/B (1-431)) was synthesized (GenScript, Piscataway, NJ) with N-terminal NdeI and C-terminal BamHI restriction sites, and cloned into the pET-15b vector (Novagen), which imparts an N-terminal 6xHis tag and thrombin cleavage site to the LC/B (1-431) and ampicillin resistance when transformed into BL21(DE3) *E. coli* (Invitrogen).

Purification of recombinant LC/B (1-431)

A starter culture of 250mL was grown in LB media at 37^{o}C overnight containing 100 μg/mL ampicillin. The culture was split evenly between 4 x 1L cultures of LB containing 100 μg/mL ampicillin and grown at 37^{o}C until OD_{600} = 0.6-1.0. IPTG was added to a final concentration of 1mM and flasks were transferred to 28^{o}C and allowed to incubate overnight. All bacterial incubations were conducted with shaking at 225 rpm. Cells were pelleted (~4 g of wet cell pellet /L culture) by centrifugation at 4^{o}C for 15 min at 6,000 rpm. The pellet was resuspended in 10 mL lysis buffer (25 mM Tris-Cl pH 8.0, 300 mM NaCl, 10 mM imidazole) per gram of wet cell pellet. Cells were lysed using a microfluidizer (Microfluidics, Newton, MA) at 18 kPa pressure. Lysate was centrifuged

at 4°C for 30 min at 38,000 rpm. The supernatant was filtered using a 0.22 μm syringe filter unit prior to applying to 2 x 5mL Ni-NTA columns (HisTrap HP columns, GE Life Sciences) for purification on the Akta FPLC system (GE Life Sciences). Protein was eluted via a step-wise imidazole gradient from 10 mM to 500 mM in lysis buffer with the major elution peak occurring at approximately 80 mM. Fractions were analyzed by SDS-PAGE, pooled and subsequently dialyzed overnight at 4°C in 2 L of elution buffer (20 mM Tris-HCl pH 8, 100 mM NaCl). Ammonium sulfate was added to the pooled fractions to a final concentration of 1 M. The resulting solution was filtered using a syringe filter unit (as above) and applied to a 5 mL HiTrap Butyl HP column (GE Lifesciences) pre-equilibrated with 1 M ammonium sulfate in elution buffer. Fractions were collected by applying a linear gradient from 1 M to 0 M ammonium sulfate in elution buffer with the major peaks eluting between 0.5 M and 0 M ammonium sulfate. Fractions were analyzed by SDS/PAGE, pooled, and subsequently dialyzed overnight at 4° C in 2 L of buffer containing 100 mM Bis-Tris, pH 6.5 and 200 mM NaCl. Finally, a buffer exchange equivalent to at least 1:200 was performed on the pooled LC/B431 fractions to remove additional ammonium sulfate from solution prior to concentrating (Amicon concentrator) to 60 mg/ml before adding an equal volume of anhydrous glycerol and storing at -20°C. The average final yield is 20mg/8g cell paste and >95% pure as judged by SDS-PAGE analysis.

Evaluation of chicoric acid with LC/B in a FRET-based assay

FRET-based enzyme assays were performed as previously described with minor modifications.[1] Briefly, assays contained 40 mM HEPES buffer pH 7.4, 15 μM substrate, and varying concentrations of chicoric acid in a final volume of 100 μl with a total DMSO concentration of 4 %. Reaction mixtures were preincubated for 5 min at 37 °C and initiated by the addition of 2.5 nM enzyme. Reactions were monitored over the course of 20 min at 37 °C and the initial velocities were determined from the linear region of each assay.

Assays for the detergent studies were conducted in the presence or absence of 0.05 % Tween 20.[2-3] Briefly, assays contained 40 mM HEPES buffer pH 7.4, 0.05 % Tween 20, 2.5 nM enzyme, and varying concentrations of chicoric acid in a final volume of 100 μl with a total DMSO concentration of 4 %. Reaction mixtures were preincubate for 5 min at 37 °C and initiated by the addition of 15 μM substrate. Reactions were monitored over the course of 20 min at 37 °C and the initial velocities were determined from the linear region of each assay.

Evaluation of chicoric acid with LC/B in a HPLC assay

HPLC enzyme assays were performed as previously described.[1] Briefly, assays contained 40 mM HEPES buffer pH 7.4, 15 μM substrate, and varying concentrations of chicoric acid in a final volume of 100 μl with a total DMSO concentration of 4 %. Reaction mixtures were preincubated for 5 min at 37 °C and initiated by the addition of 2.5 nM enzyme. Reactions were incubated for 20 min at 37 °C, 40 μl aliquots of the reaction were then quenched with 10 μl of glacial acetic acid, and the initial rates were determined by HPLC as previously described.[1]

Inhibition constants, IC_{50}'s were determined by a non-linear least squares global fit of the partial inhibition equation 1 to the initial rates of product formation at a constant concentration of substrate and varying concentrations of inhibitor. The α-constant describes the fractional velocity at saturating inhibitor.

$$v = V_o \frac{1 + \alpha \frac{[I]}{IC_{50}}}{1 + \frac{[I]}{IC_{50}}} \qquad \text{(Eq. 1)}$$

References

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2. B. K. Shoichet, *J Med Chem*, 2006, **49**, 7274-7277.
3. B. K. Shoichet, *Drug Discov Today*, 2006, **11**, 607-615.

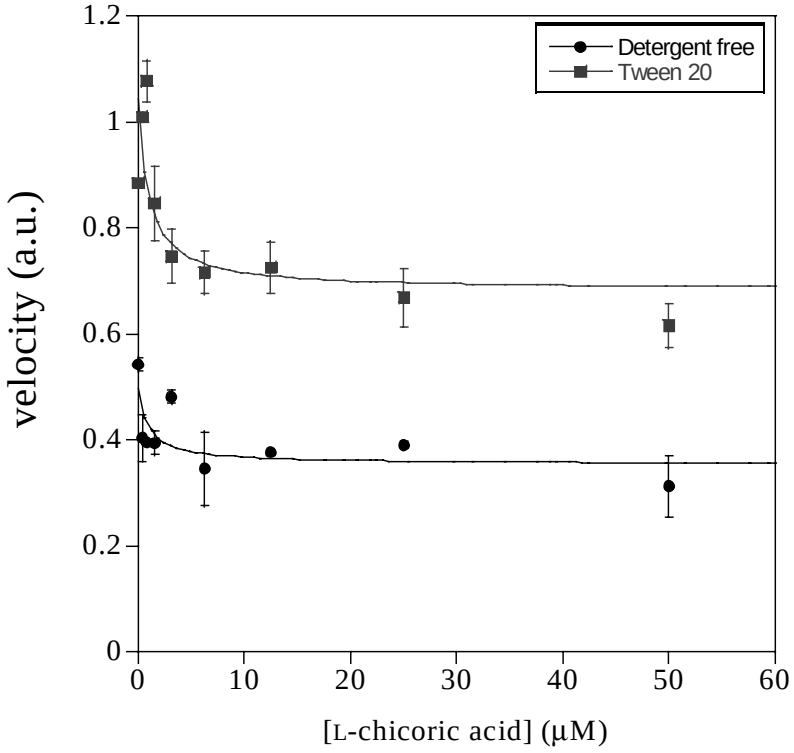

Figure S1. Inhibition studies with L-chicoric acid and LC/B in the presence and absence of detergent (Tween 20). Data was fit to the partial inhibition equation Eq. 1.