

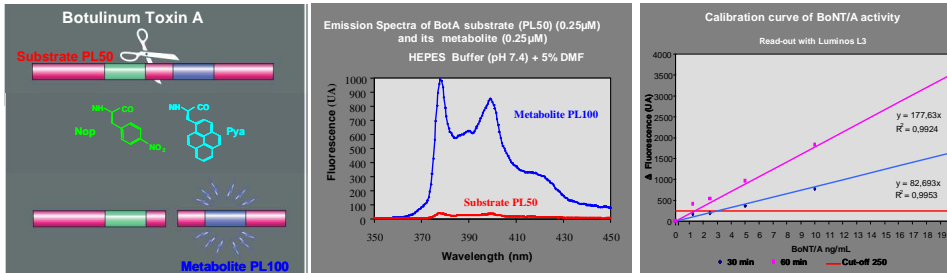
# Design of fluorogenic peptide substrates for Botulinum Toxin A

## Quantification and identification of novel cooperative sites for substrate binding

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Botulinum toxins (BoNTs) cause paralysis, BoNT type A being the most toxic substance known to man. BoNT/A is a disulfide-linked di-chain toxin. The heavy chain, HC carries the receptor binding and membrane translocation domain, while the light chain, LC, is a zinc metalloprotease inhibiting neurotransmitter release through the specific cleavage of SNAP-25 a component of the exocytosis complex. Based on this protease activity and using the Internal Collision-Induced Fluorescence Quenching technique, Pharmaleads has developed a high-affinity mimetic peptide substrate used in an extremely specific, rapid, sensitive and reliable quantification technique designated EzyBot<sup>®</sup>. This technique, useful for the detection of bioterrorist contamination as well as for the quantification of therapeutic toxins, is based on the introduction of a highly fluorescent pyrenylalanine (Pya) along with a nitro-phenylalanine (Nop) repressor residue on each side of the cleavage site. Based on the SNAP-25 sequence, peptides of various lengths encompassing the cleavage site flanked with the Pya and Nop residues were designed. More specifically, a minimal 17-mer and a longer 48-mer peptide reaching the previously identified  $\alpha$ -exosite<sup>2</sup> were designed with the latter showing a significantly higher affinity for BoNT/A. Enzymatic assay and peptide mapping reveal that this increased affinity is mainly due to the connecting peptide sequence located between the N terminal end of the 17-mer and the  $\alpha$ -exosite, thus bringing to light a new cooperative exosite for BoNT/A substrate binding, as previously shown for TeNT<sup>3</sup> and also suggested from the BoNT/A three-dimensional structure<sup>2</sup>.

### 1. THE ENZYMATIC ASSAY: OVERVIEW



The enzymatic assay is:

**Sensitive and Robust** – the high affinity of PL50 (higher than SNAP-25) together with the extremely intense fluorescence released by the Pyrenylalanine fluorescent moiety allows the detection of 2ng/mL of BoNT/A.

**Easy to perform** – one-step detection complete in hour.

Product distributed worldwide for field detection of Botulinum Toxins under the brand name EzyBot<sup>®</sup>

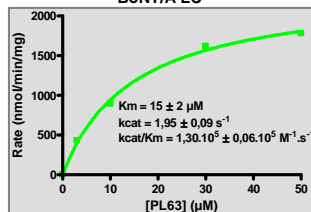


### 2. DEVELOPMENT OF THE PL50 SUBSTRATE

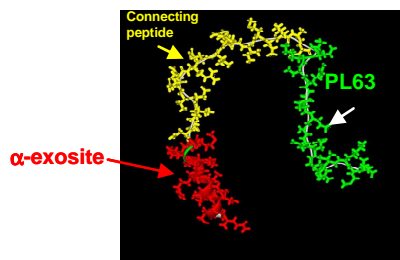
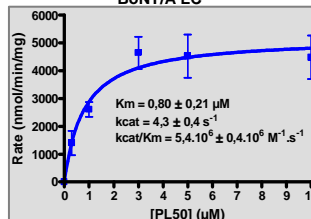
<sup>156</sup>AcIIGNLRHMLDMGNEIDTQNRQIDRIMEKADSNKTRIDEANNopRAPyaKNIELNH2<sup>203</sup> (PL50)

<sup>187</sup>AcSNKTRIDEANNopRAPyaKNIELNH2<sup>203</sup> (PL63)

Kinetic parameters of PL63 cleavage by BoNT/A LC



Kinetic parameters of PL50 cleavage by BoNT/A LC



Experimental procedure:

Kinetic parameters were studied using BoNT/A LC (List Biological) at either 25ng/mL for PL63 or 10 ng/mL for PL50, with increasing concentrations of either peptide substrate in reaction buffer (20 mM Hepes, pH 7.4, 200µM ZnSO<sub>4</sub>, 5mM DTT, 1 mg/mL BSA). Reactions with PL63 and PL50 were incubated at 37°C for either 60 or 10 minutes respectively. Fluorescence was read on a Berthold Tinsckle fluorimeter  $\lambda_{ex}$ =340 nm,  $\lambda_{em}$ =405, lamp energy=10000.

Botulinum toxin A exclusively cleaves SNAP25, a membrane protein involved in vesicle docking and neurotransmitter release. Moreover, it cleaves this 206 amino-acid protein at a single amide bond, Q<sup>197</sup>-R<sup>198</sup>.

Based on the sequence of SNAP 25, we have designed specific BoNT/A substrates. Starting from the « minimal » active site binding region surrounding the cleavage site<sup>2,4</sup>, we synthesized a 17 mer quenched peptide termed PL63. Study of the kinetic parameters of this substrate towards BoNT/A LC reveals that it binds the toxin with an affinity of about 15µM whereas its catalytic constant is of 1.95 10<sup>5</sup> M<sup>-1</sup>.s<sup>-1</sup>.

In order to see whether extending the substrate to the  $\alpha$ -exosite identified by Breidenbach and Brunger<sup>2</sup> could impact the kinetic parameters of the substrate, we synthesized the PL50 quenched peptide which covers amino acids 156 to 203 of the SNAP-25 peptide sequence.

The PL50 substrate bound the BoNT/A toxin with an affinity of 0,8 µM, i.e. with almost a 20 fold increase compared to PL63. Although the catalytic constant was not significantly modified, the resulting specificity constant of PL50 was increased by more than 40 fold.

These results show that extending the PL63 peptide substrate to the  $\alpha$ -exosite sequence does not modify the enzyme activity, but considerably increases substrate binding, as suggested by Breidenbach and Brunger<sup>2</sup>.

### 3. COOPERATIVE HYDROLYSIS OF PL50 THROUGH EXOSITE RECOGNITION

<sup>152</sup>QVSGIIGNLRHMLDMG<sup>167</sup>

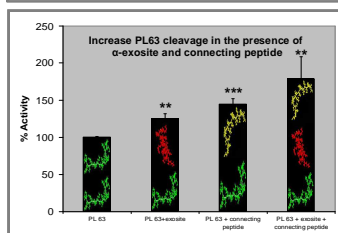
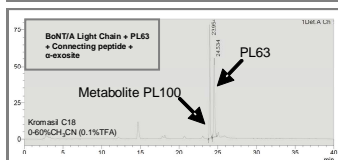
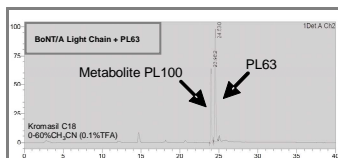
$\alpha$ -exosite

<sup>168</sup>NEIDTQNRQIDRIMEKAD<sup>186</sup>

Connecting peptide

<sup>187</sup>AcSNKTRIDEANNopRAPyaKNIELNH2<sup>203</sup>

PL 63



In order to directly assess the effect of adding the  $\alpha$ -exosite into the PL50 substrate, peptide mapping experiments were undertaken.

Experimental procedure

Peptides corresponding to the  $\alpha$ -exosite (SNAP-25 aa152-167, in red) as well as to the intermediate or connecting peptide (SNAP-25 aa 168-186, in yellow) were added alone or in combination to the « minimal » PL63 substrate. Cleavage was measured by HPLC.

The LC toxin (50 ng/mL) was pre-incubated with 200 µM of either connecting peptide or  $\alpha$ -exosite, or both, for 60 minutes at 37°C. PL63 substrate (10µM) was added and the reaction left to proceed at 37°C for 5 hours. The reactions were analyzed by HPLC (343 nm).

Study of the kinetic parameters of PL63 alone or with the  $\alpha$ -exosite and connecting peptides shows a slight increase of affinity of PL63 towards the BoNT/A LC in the latter conditions suggesting a function for both these peptides in substrate binding.

PL63	PL63 + $\alpha$ -exosite + connecting peptide
Km = 19 ± 4µM	Km = 11 ± 4µM
kcat = 0.74 ± 0.06 s <sup>-1</sup>	kcat = 0.44 ± 0.05 s <sup>-1</sup>
kcat/Km = 3.9.10 <sup>4</sup> ± 0.3.10 <sup>4</sup> M <sup>-1</sup> .s <sup>-1</sup>	kcat/Km = 4.0.10 <sup>4</sup> ± 0.4.10 <sup>4</sup> M <sup>-1</sup> .s <sup>-1</sup>

Conclusion

Internal fluorescence quenching was used to design PL50, providing the most simple and sensitive assay (EzyBot<sup>®</sup>) reported to date for the detection and quantification of BoNT/A. Cooperative hydrolysis of clostridial neurotoxin has previously been shown for TeNT<sup>3</sup>, and also proposed to occur for BoNT/A. In this study, we have explored the existence of such a mechanism in BoNT/A by using a « minimal » substrate in addition to two peptides corresponding to the  $\alpha$ -exosite and to the connecting peptide. HPLC analysis demonstrated a slight improvement of the hydrolysis although less important than for TeNT<sup>3</sup>. This phenomenon probably plays a role in the exquisite selectivity of clostridial neurotoxins towards their substrate.

<sup>1</sup>Fournié-Zaluski MC et al. (2005) PCT WO 2005121354  
<sup>2</sup>Breidenbach & Brunger (2004) Nature, 432:925

<sup>3</sup>Cornille et al. (1997) JBC, 272:3459  
<sup>4</sup>Chen & Barbieri (2006) JBC, 281:10906

