# NEW FRET SUBSTRATE FOR BOTULINUM NEUROTOXIN TYPE A

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## ABSTRACT

**Purpose of Study:** A new FRET peptide substrate for botulinum toxin type A (BoNT/A), marketed as SNAPtide® flP6 (DABCYL/5-IAF) Prod #523, U.S. Patent pending #61/252,675, was evaluated and compared to SNAPtide® (FITC/DABCYL) Prod #521.

**Methods Used:** For kinetic studies, initial rates of cleavage were measured at room temperature (RT) for both SNAPtides® after addition of 5 nM BoNT/A light chain (LC), Prod #610A, in Assay Buffer: 50 mM HEPES, pH 7.4 containing 0.05% TWEEN 20. The cleavage product was analyzed using HPLC with fluorescence detection. A standard curve was generated to convert the peak areas to nanomolar of cleaved product. The kinetic data was obtained from plots of initial rates expressed as nM/min versus concentration of FRET substrate. Results were calculated from nonlinear regression analysis using the Michaelis-Menton equation and Kaleidagraph software.

**Summary of Results:** The kinetic data indicate that at RT the new SNAPtide® flP6, Prod #523 has a considerably lower $K_m$ value, 3.8 µM for #523 versus >200 µM for #521, and that SNAPtide® flP6, Prod #523 is cleaved significantly more efficiently than SNAPtide®, Prod #521. The rate of cleavage for SNAPtide® flP6, Prod #523, was 13.5 RFU/sec versus 4.8 RFU/sec for SNAPtide®, Prod #521 using 8µM substrate, with 10 nM BoNT/A LC at 37°C. Both substrates show a linear fluorescence response up to approximately 8 µM substrate. SNAPtide®, Prod #521 is more sensitive using BoNT/A LC for cleavage. At 37°C, 12.5 ng/ml can be detected after 2.5 hrs and 1.56 ng/ml can be detected after overnight digest with SNAPtide® flP6, Prod #523. With SNAPtide®, Prod #521, 0.6 ng/ml is detected at 2.5 hrs and 0.26 ng/ml after overnight digestion.

**Conclusions:** The data indicate that SNAPtide® flP6, Prod #523 is an ideal substrate for kinetic studies and for rapid inhibitor screening.

## INTRODUCTION

The botulinum neurotoxins are among the most potent toxins in nature. They are synthesized as single 150 kDa polypeptide chains which are subsequently cleaved to produce a 100 kDa heavy chain and a 50 kDa light chain linked by a disulfide bond. Four of the seven immunologically distinct serotypes, A, B, E, and F, cause botulism in humans. The 50 kDa light chain of each serotype is a zinc endoprotease that cleaves a single target protein which is essential for synaptic vesicle membrane fusion. This inhibits neurotransmitter release which leads to muscular paralysis.

These secreted toxins are regarded as major biological warfare threats. Due to their extreme potency and lethality, detection of these toxins requires a highly sensitive and reliable assay. Measurement of proteolytic activity provides a potentially sensitive and direct means for detecting these potent toxins. Substantial signal amplification can be expected as a result of catalytic turnover of the substrate.

Peptide substrates for botulinum toxin type A (BoNT/A), SNAPtide®, have been designed based on the native synaptosomal substrate SNAP-25. These fluorescence resonance energy transfer (FRET) peptides contain a fluorescent group at one end and a suitable chromogenic acceptor group at the other. Fluorescence is quenched initially by intramolecular energy transfer between the donor/acceptor pair. Upon cleavage, the fluorescence is recovered.

The cleavage of two SNAPtide® substrate peptides by BoNT/A light chain (LC), Prod #610A and BoNT/A holotoxin, Prod #130A, were evaluated and compared. These substrates are marketed as SNAPtide®(FITC/DABCYL), Prod # 521, and SNAPtide® flP6 (DABCYL/5-IAF), Prod #523, U.S. Patent pending #61/252,675.

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## MATERIALS and METHODS

SNAPtide® substrates (Products #521 and #523), Unquenched Calibration Peptide for SNAPtide® 521 (Prod #528), BoNT/A LC (Product #610A), and BoNT/A holotoxin (Prod #130A) are products of List Biological Laboratories, Inc. Trimethylamine oxide (TMAO) was purchased from Acros Organics.

***Fluorimetric Assay:*** Continuous assays were performed on a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) using Greiner FLUO-TRAC black flat-bottomed plates (E&K Scientific, Campbell, CA). Stock solutions of the FRET substrates were made at 2.5 mM in dimethyl sulfoxide (DMSO). Final dilutions were made in the appropriate buffer. Plates were equilibrated at 25°C or 37°C for 15 min prior to initiation of the reaction. For all experiments the time-dependent increase in fluorescence intensity was monitored at 25°C or 37°C. The excitation wavelength was set to 490 nm and emission to 523 nm with a cutoff at 495 nm. Details of each reaction are given below or in the figure legend.

***Buffer Optimization:*** FRET assays were performed to test the activity of BoNT/A LC with SNAPtide® flP6, Prod #523 as a function of pH, Tween-20, TMAO concentration and temperature, and compared to SNAPtide®, Prod #521. Cleavage of both substrates by BoNT/A holotoxin as a function of zinc concentration was also examined. Initial velocities of cleavage in RFU/sec were evaluated and compared for each assay in order to determine the optimum buffer conditions for the reaction (**Figure 1 and Table 1, 2**).

***Linearity:*** The fluorescence intensity as a function of SNAPtide® flP6, Prod #523 and SNAPtide® Prod #521 concentration using 10 nM BoNT/A LC was examined. The titration experiment was performed in the buffer optimized for cleavage by BoNT/A LC: 50 mM HEPES, pH 7.4, 0.05% Tween-20. Both SNAPtides® were prepared at 30, 24, 20, 15, 12, 10, 7.5, 6, 5, 3, 2 5,and 1.25 µM concentrations. Following equilibration, the cleavage reaction was initiated with addition of BoNT/A LC. Initial velocities of cleavage were plotted against SNAPtide® concentration **(Figure 2)**.

***Sensitivity and LOD:*** BoNT/A LC titration experiment was performed in 50 mM HEPES, pH 7.4, 0.05% Tween-20, using 8 µM SNAPtide®. Eleven serial dilutions of BoNT/A LC concentrations was prepared starting at 2 nM. Following equilibration, the cleavage reaction was initiated with addition of 8 µM SNAPtide®. Relative fluorescence units (RFU) obtained after an overnight digest are plotted against BoNT/A LC concentration **(Figure 3)**.

The cleavage of SNAPtide® flP6 Prod #523 (8µM) by a series of BoNT/A LC concentrations was monitored by HPLC using a monolithic Swift RP-All, 4.6 x 50 mm reverse phase column. Solvent A was 0.1% TFA and solvent B was 100% acetonitrile containing 0.1% TFA. The elution was accomplished using a linear gradient of 14-70% B in 3 min with a flow rate of 1.5 ml/min. The pH of the column effluent was made basic prior to detection, using the Hitachi fluorescence detector with excitation set to 488 nm and emission at 520 nm, to increase the sensitivity. The injection volume was 20 µl. The results are shown in **Figure 4**.

The limit of detection (LOD) is the minimum concentration of BoNT/A LC that can be measured with 99% confidence that the concentration present is greater than zero under the conditions using this fluorogenic assay. Previous data indicated that SNAPtide®, Prod #521 allowed more sensitive detection of the BoNT/A light chain so this substrate was used in the LOD experiments. A calibration curve was generated by measuring the initial rate of proteolysis as a function of enzyme concentration. Eight replicates of a concentration estimated to be slightly greater than the expected detection limit were performed. Each analysis was calculated as pg/ml using the calibration curve. The detection limit was calculated as 2.998 (Student's t-Distribution with 99% confidence and 7 degrees of freedom) times the standard deviation of the 8 replicates. The LOD assays were performed with BoNT/A LC in the presence and absence of TMAO using SNAPtide®, Prod #521 at 8 µM. The reaction was monitored at 37°C every 30 minutes for 5 hours and then left overnight at room temperature. The results are given in **Table 3**.

## METHODS (continue)

***Kinetic parameter evaluations:*** Dilutions of SNAPtide® (Product #521 and #523) were prepared at the appropriate concentrations in 50 mM HEPES, pH 7.4 containing 0.05 % Tween-20 (ASSAY BUFFER) from a 250 µM substrate in the same buffer. The BoNT/A LC was reconstituted in the ASSAY BUFFER as a 0.25 µM solution; final concentration was 5 nM.

HPLC was performed using a SWIFT, monolithic RP-all reverse phase column, 4.6 x 50 mm (Dionex, currently Thermo Scientific) attached to a Varian HPLC system (Agilent, Santa Clara, Ca.). Solvent A was 0.1%TFA and solvent B was 100% acetonitrile containing 0.1% TFA. A linear gradient from 15 to 70% B in 3 minutes with a flow of 1.5 ml/min was used. Total duration per run was 8.5 minutes. The column effluent was monitored at 510 nm, the absorbance maximum for FITC. The injection volume was 20 µl. The intensity for the cleaved fragment was converted to nanomoles of cleaved product obtained from a standard curve generated from totally cleaved substrate at concentrations from 0.1 to 2.0 nM. The kinetic data was analyzed using the Michaelis-Menton equation from KaleidaGraph software.

***Inner Filter Effect Correction:*** Dilutions of SNAPtide®, Prod #521, were prepared in ASSAY BUFFER to achieve concentrations ranging from 1.25 to 30 µM. Fluorescence end point readings of Prod #521 were recorded at each concentration. In order to determine the inner filter effect at each substrate concentration another set of end point fluorescence (RFU) readings were recorded after addition of 0.2 µM Unquenched Calibration Peptide for SNAPtide® 521, Prod #528. Fluorescence intensity obtained for Prod #521 was then subtracted from the fluorescence intensity obtained for Prod #521 containing 0.2 µM Prod #528 in order to obtain the contribution to the fluorescence due to Prod #528. The decrease in fluorescence of Prod #528 in the presence of Prod #521 reflects the inner filter effect**.** A correction factor is obtained for each SNAPtide®, Prod #521 concentration:

$$\text{correction factor} = \frac{\text{RFU (Prod \#528) at each [Prod \#521]}}{\text{RFU (Prod \#528)}}$$

Initial reaction rates were obtained for each substrate concentration after addition of 5 nM BoNT/A LC. The rates were corrected and the kinetic data was analyzed using the Michaelis-Menton equation from KaleidaGraph software.

## RESULTS

### A. Buffer Optimization

**BoNT/A Light Chain**: The optimum buffer was determined as 50 mM HEPES, pH 7.4, containing 0.05% TWEEN 20 (ASSAY BUFFER). Rates measured at 37°C were slightly higher than those obtained at 25°C.

As shown in **Figure 1 and Table 1**, TMAO affects the two SNAPtides® differently. The rate of cleavage of Prod #523 is decreased in the presence of TMAO and at 3 M TMAO the rate is decreased to the level seen for Prod #521 in the absence of osmolyte. The reverse is seen when TMAO is added to Prod #521. An initial rate of cleavage similar to that observed for Prod #523 without TMAO, is achieved with Prod #521 in the presence of 2 M TMAO. Since the base fluorescence in RFU's changes in the presence of TMAO, increasing (~1.4x) for Prod # 521, and decreasing (~4.5 x) for Prod #523 (data not shown), the effect of the osmolyte may be due to a conformational change, beneficial for Prod #521 and detrimental for Prod #523.

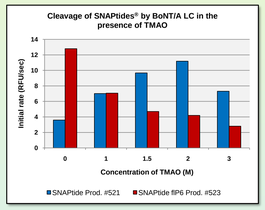


**Figure 1**: The effect of the osmolyte, TMAO, on the initial rate of cleavage, as expressed in relative fluorescence units (RFU) per sec, for 5 µM SNAPtide®, Prod #521 or #523 by 10 nM BoNT/A light chain at 37°C in ASSAY BUFFER, is plotted as a function of TMAO concentration.

**Table 1:** Rate of cleavage of 8 µM SNAPtide® by 10 nM BoNT/A LC at 37°C

| | RFU/sec | |
|---|---|---|
| **SNAPtide® Prod #** | **+ 2 M TMAO** | **No TMAO** |
| **521** | 13.42 | 4.76 |
| **523** | 4.62 | 13.53 |

**BoNT/A Holotoxin:** Digestion of both SNAPtides® was conducted at several pH values (7.4, 8.0), concentrations of substrate ( 2.5, 5, 8 µM) and zinc concentrations (0.3, 0.6, 0.75, and 1.2 mM $ZnCl_2$ and $ZnSO_4$) to determine the optimum buffer. The rate of cleavage for both SNAPtides® was optimum in 20 mM HEPES, pH 7.4, 0.3 mM $ZnCl_2$, 1.25 mM DTT, and 0.1% TWEEN 20. The BoNT/A holotoxin was activated by incubation at 37°C for 30 min in 20 mM HEPES, pH 8.0, 0.3 mM $ZnCl_2$, 5 mM DTT, and 0.1% TWEEN 20. To maximize the solubility of both substrates, the first dilution in the reaction buffer is at 50 µM, followed by a 1:10 dilution to obtain 5 µM. The cleavage of 5 µM SNAPtides® by 5 nM BoNT/A holotoxin in optimum buffer at 37°C for 5 hrs is shown in **Table 2.** The rate of cleavage of SNAPtide® flP6, Prod #523, by the holotoxin is faster than for Prod #521. Also, the RFU value before cleavage is significantly lower for Prod #523, indicating that this FRET substrate is quenched more efficiently than Prod #521.

**Table 2:** Cleavage of 5 µM SNAPtide® by 5 nM BoNT/A at 37°C.

| SNAPtide®, Prod # | Initial rate (RFU/sec) | Δ RFU* | $RFU_{final}/RFU_{initial}$ |
|---|---|---|---|
| **521** | 0.942 | 10959 | 6 |
| **523** | 1.56 | 12524 | 29 |

*$RFU_{\text{after 5 hrs. digestion}} - RFU_{initial}$.

### B. Linearity

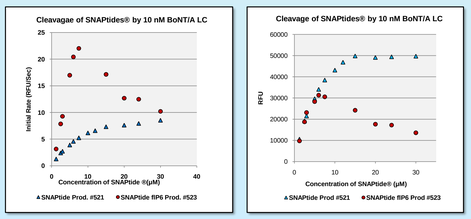


**Figure 2:** Plot of the initial rate (RFU/sec), left, or the RFU, right, as a function of SNAPtide® concentration (µM) for both Prod #523 and #521. The substrates were digested for 4.3 hrs in ASSAY BUFFER at 37°C with 10 nM BoNT/A LC.

The fluorescence response for both SNAPtides® is linear to 8 µM. The initial rate of cleavage is significantly higher for Prod #523 as shown in the plot on the left. At the same time the RFU values at concentrations above 8 µM continue to increase for Prod #521 while the values decrease significantly for Prod #523. For both peptides this effect is most likely due to an inner filter effect.

### C. Sensitivity and LOD

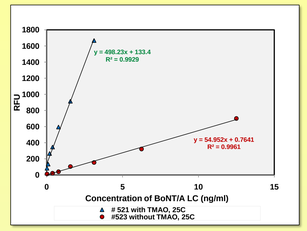


**Figure 3:** A plot of RFU versus BoNT/A LC concentration demonstrates that the cleavage reaction for both SNAPtides® is linearly proportional to the light chain concentration in this range. The LOD for Prod #521 was determined and is given in **Table 3.**

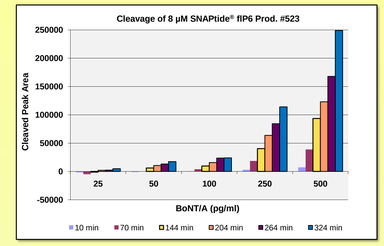


**Figure 4:** The cleavage of SNAPtide® flP6 Prod. #523 (8µM) by a series of BoNT/A LC concentrations monitored by HPLC using an Hitachi fluorescence detector as described in Materials and Methods. As little as 25 pg/ml Light Chain can be detected quantitatively in less than 3.5 hrs. using SNAPtide® flP6 Prod. #523 with this HPLC method.

**Table 3. LOD for SNAPtide®, Prod #521**

| | + TMAO | | No TMAO | |
|---|---|---|---|---|
| **Digestion time** | **4.5 hr** | **24.5 hr** | **4.5 hr** | **24 hr** |
| **LOD (pg/ml)** | 165 | 161 | 345 | 264 |

As indicated in Table 3, the LOD for SNAPtide® Prod #521 is improved in the presence of 2.3 M TMAO.

### D. Kinetic Measurements

Initial rates of cleavage were measured at room temperature (RT) for SNAPtide®, Prod #521 and #523 after addition of 5 nM BoNT/A LC in Assay Buffer. The cleavage product was analyzed using HPLC and a standard curve generated to convert the peak areas to nanomolar cleaved product.

For Prod #521, initial rates were also measured in Assay Buffer containing 2.3 M of the osmolyte, trimethylamine oxide (TMAO), at both room temperature and 37°C using a fluorescence plate reader. An appropriate peptide, the cleaved FITC-containing N-terminal, Prod #528, was used to correct for the inner filter effect. The rate is expressed as RFU/sec for these data sets.

The kinetic data was obtained from plots of initial rates expressed as nM/min for the HPLC data or RFU/sec for the platereader data. Results were calculated from nonlinear regression analysis using the Michaelis-Menton equation and Kaleidagraph software (see **Figure 5**). Kinetic parameters are reported in **Table 4**.

The data indicate that at RT, SNAPtide® flP6, Prod #523 has a lower $K_m$ value and is cleaved significantly more efficiently than Prod #521. Interestingly, the $K_m$ values estimated for Prod #521 upon addition of 2.3 M TMAO to the assay buffer at both 37°C and RT are comparable to that observed for Prod #523 in assay buffer alone at RT.

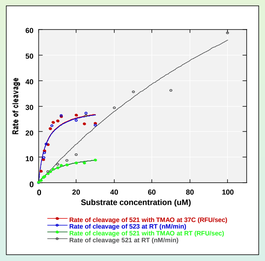


**Figure 5:** SNAPtide® (Prod. #521 and #523) initial hydrolysis rates at different substrate concentrations. Rates for Prod #521, reported as RFU/sec, were obtained in the presence of 2.3 M TMAO using the fluorescence microplate reader and are corrected for the inner filter effect as described in Materials and Methods. For Prod #523, the curves were generated from HPLC peak intensities and a standard curve was used to convert the peak areas to nmoles of cleaved product. Estimated $K_m$ values are given in **Table 4**.

**Table 4: Kinetic Parameters**

| | Dissociation constant, $K_m$ (µM) | |
|---|---|---|
| **SNAPtide® Prod** | **RT** | **37°C** |
| **#523** | 3.80 ± 1.11 | NM |
| **#521** | >200 | NM |
| **#521** (Assay Buffer + 2.3 M TMAO) | 3.96 ± 2.4 | 3.82 ± 1.09 |

## CONCLUSIONS

- SNAPtide® flP6, Prod #523, is cleaved ~ 4 times more efficiently than SNAPtide® , Prod #521, by BoNT/A LC.
- Addition of 2.3 M TMAO increases the initial rate of cleavage of SNAPtide®, Prod #521, by BoNT/A LC to the level observed for SNAPtide® flP6, Prod #523 without this osmolyte.
- SNAPtide® flP6, Prod #523 is cleaved more efficiently than Prod #521 by BoNT/A holotoxin. After 5 hours digestion, a ~ 30 fold increase in RFU values is observed with Prod #523 while only a 6-fold increase is detected with Prod #521.
- Both SNAPtides® exhibit a linear fluorescence response up to 8 µM. It is suggested that 5 µM be used in digestion reactions with both BoNT/A holotoxin and light chain.
- SNAPtide®, Prod #521, exhibits an LOD of ~160 pg/ml BoNT/A LC after 4.5 hours of digestion. Using HPLC with fluorescence detection, 25 pg/ml of BoNT/A LC is detected using SNAPtide® flP6, Prod #523.
- The dissociation constant, $K_m$ (µM), for SNAPtide® flP6, Prod #523 is 3.8 ± 1.11 versus >200 for SNAPtide® , Prod #521. SNAPtide® flP6, Prod #523 is ideally suited for kinetic studies.