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Evaluation of Phosphoramidon and Three Synthetic Phosphonates for Inhibition of Botulinum Neurotoxin B Catalytic Activity†‡

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Three putative metalloprotease inhibitors were synthesized and tested for their ability to inhibit the catalytic activity of botulinum neurotoxin B light chain (BoNT/B LC). The compounds were designed to emulate the naturally occurring metalloprotease inhibitor phosphoramidon, which has been reported to be a weak antagonist of BoNT/B action. All three analogs contained the dipeptide Phe-Glu in place of Leu-Trp of phosphoramidon and possessed a phenyl, ethyl or methyl group in place of the rhamnose sugar of the parent compound. The inhibitors were evaluated in a cell-free assay based on the detection of a fluorescent product following cleavage of a 50-mer synaptobrevin peptide ([Pya^{88}] S 39–88) by BoNT/B LC. This peptide corresponds to the hydrophilic core of synaptobrevin-2 and contains a fluorescent analog L-pyrenylalanine (Pya) in place of Tyr^{88}. Cleavage of [Pya^{88}] S 39–88 by BoNT/B LC gives rise to fragments of 38 and 12 amino acid residues. Quantification of BoNT/B-mediated substrate cleavage was achieved by separating the 12-mer fragment (FETSAAKLKRK-Pya) that contains the C-terminal fluorophore and measuring fluorescence at 377 nm. The results indicate that the phenyl-substituted synthetic compound ICD 2821 was slightly more active than phosphoramidon, but analogs with methyl or ethyl substitutions were relatively inactive. These findings suggest that phosphonate monoesters may be useful for providing insights into the structural requirement of BoNT/B protease inhibitors.

INTRODUCTION

The botulinum neurotoxins (BoNTs) comprise a group of seven dichain protein toxins, A–G, secreted by the bacterium *Clostridium botulinum*.[1] Each serotype consists of a ca. 100-kDa heavy chain with two functional domains: a C-terminal domain for binding to ectoacceptors on cholinergic nerve endings and an N-terminal domain for the cytosolic translocation of the toxin. The heavy chain is associated with a ca. 50 kDa light chain (LC) that is responsible for inhibition of acetylcholine release.[2]

Exposure to BoNT leads to the potentially fatal condition of botulism. The symptoms of intoxication generally begin with a gradual onset of weakness in muscles innervated by the cranial nerves, manifested as diplopia, dysphagia and dysarthria. Symptoms progress to generalized muscle weakness and difficulty in breathing that in severe cases culminate in respiratory failure and death.[3] The pathophysiology of BoNT intoxication is thought to be a consequence of cleavage of one of three proteins that are required for the docking of synaptic vesicles with active zones in the nerve terminal: synaptobrevin, syntaxin and SNAP-25. Cleavage of these proteins results from the zinc metalloprotease activity of the BoNT LC.[4–6] Botulinum neurotoxin light chains possess a characteristic HEXXH sequence that is a universal feature of zinc metalloproteases. The LCs bind zinc in a 1:1 stoichiometric ratio, require zinc for proteolytic activity and can be inhibited by exposure to chelators such as *N*,*N*,*N*′*N*′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN).[5]

The present effort represents a rational approach towards the design of selective metalloprotease inhibitors for BoNT/B as a basis for subsequent development of effective therapeutic agents. The inhibitors were designed to emulate the structure of phosphoramidon, a naturally occurring metalloprotease inhibitor with demonstrated, albeit weak, anti-BoNT activity at the mouse neuromuscular junction.[7] Two essential features of phosphoramidon were incorporated in the design of the synthetic compounds: a phosphonate functional group for interacting with the active site zinc and a dipeptide core for active site recognition.[8] The dipeptide Phe-Glu was selected because point mutation studies suggested that Phe^{77} plays a key role in the susceptibility of human synaptobrevin to cleavage by BoNT/B

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LC.[9] The compounds (Fig. 1) were tested for inhibitory activity by monitoring cleavage of a fluorescent peptide that encompasses residues 39–88 of human synaptobrevin-2 ([Pya^{88}] S 39–88). Two of the analogs (ICD 2820-ethoxy and ICD 2822-methoxy) were found to be relatively inactive. The phenoxy analog (ICD 2821), however, was more potent than phosphoramidon. These results suggest that phosphonate monoesters may be useful for establishing structure–activity requirements of BoNT/B LC inhibitors.

EXPERIMENTAL

Synthesis of analogs

Preparation of phosphoramidon analogs is difficult because of the synthetic challenges associated with the phosphorus-linked glycopeptide. However, it was reported that replacement of the rhamnose moiety in phosphoramidon by small alkoxy groups yielded metalloprotease inhibitors with potencies similar to that of the parent compound.[8] Such rhamnose-free phosphoramidon analogs provided more feasible targets and were therefore selected for synthesis in the current study. The essential modification of the phosphoramidon molecule was the replacement of Leu-Trp by Phe-Glu so that the dipeptide sequence of the analogs would correspond to residues adjacent to the BoNT/B are cleavage site of synaptobrevin-2.[4] The synthetic scheme for the preparation of the methoxy and ethoxy target compounds (**9a** and **9b**) is analogous to the methodology described recently by Bertenshaw *et al.*[8] and is outlined in Fig. 2. Preparation of the phenoxy analog (**9c**) required a different protection–deprotection strategy and is illustrated in Fig. 3.

Peptides **3a** and **3b** were prepared by coupling commercially available *t*-Boc-L-phenylalanine (**1**) with L-glutamic acid, dimethyl ester (**2a**) or dibenzyl ester (**2b**), respectively, using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.[10] Coupling was followed by removal of the *t*-butoxycarbonyl protecting group with trifluoracetic acid (TFA), then addition of *p*-toluenesulfonic acid to give **4a** and **4b** as tosylate salts.[11] Chlorophosphates **7a** and **7b** were prepared by addition of phenol (**6a**) to ethyl dichlorophosphate (**5a**) and methyl dichlorophosphate (**5b**), respectively. Intermediates **8a** and **8b** were then prepared by phosphorylation of **4a** with chlorophosphates **7a** and **7b**, respectively. The protected phosphorylated dipeptides were hydrolyzed with 1.5 M aqueous LiOH to give **9a** and **9b** as hydrated trilithium salts containing excess LiCl (**9a**: Analysis calculated for $C_{16}H_{20}Li_3N_2O_8P \cdot 1.7\ LiCl \cdot 2.75\ H_2O$: C, 35.47; H, 4.74. Found: C, 35.49, 35.40; H, 4.70, 4.72; **9b**: Analysis calculated for $C_{15}H_{18}Li_3N_2O_8P \cdot 0.55\ CH_3CN \cdot 1.5\ LiCl \cdot 1.9\ H_2O$: C, 36.73; H, 4.49; N, 6.78. Found: C, 36.78, 36.68; H, 4.53, 4.54; N, 6.82, 6.78).

Chlorophosphate (**7c**) was prepared by addition of benzyl alcohol (**6b**) to phenyl dichlorophosphate (**5c**). Intermediate **8c** was then prepared by phosphorylation of **4b** with chlorophosphate (**7c**). This tribenzyl-protected phosphorylated dipeptide was debenzylated by transfer hydrogenation with 10% palladium on carbon, employing ammonium formate as the source of hydrogen,[12] to give **9c** as a hydrated diammonium salt (**9c**: Analysis calculated for $C_{20}H_{29}N_4O_8P \cdot 0.8\ H_2O$: C, 48.15; H, 6.18; N, 11.23. Found: C, 48.15, 48.05; H, 5.77, 5.82; N, 10.61, 10.58).

The identity of the compounds was confirmed by ^{1}H-NMR, ^{31}P-NMR [**9a**: ^{31}P-NMR (D_2O), δ 6.56 (s, 1P); **9b**: ^{31}P-NMR (D_2O), δ 7.74 (s, 1P); **9c**: ^{31}P-NMR (D_2O), δ 2.67 (s, 1P)], and (mass spectrum analysis [**9a**: electrospray MS, *m*/*z* 401 (M-H); **9b**: electrospray MS, *m*/*z* 387 (M-H); **9c**: electrospray MS, *m*/*z* 449 (M-H)].[13]

Evaluation of inhibitory activity

A recently described fluorescence assay was used for investigating the putative BoNT/B LC inhibitors.[14] The assay is based on measuring reductions in the rate of cleavage of a 50 amino acid peptide that spans residues 39–88 of human synaptobrevin-2 and contains a modified tyrosine fluorophore (pyrenylalanine, Pya) on residue 88 ([Pya^{88}] S 39–88). The approach used in the present study was similar to that reported previously.[14,15] Briefly, the reactions were carried out at 22° C in a 100-μl volume. Pure BoNT/B LC (60 nM) was preincubated with 50 μM $ZnSO_4$, 1 mM dithiothreitol and 20 mM HEPES (pH 7.1) for 30 min followed by a 30-min incubation with [Pya^{88}] S 39–88 (10 μM). The above LC concentration and incubation time are within the linear range of the assay.[14] The reaction was stopped by addition of 0.9 ml of 0.1% trifluoroacetic acid (TFA, v/v) in 72% methanol (v/v). The blank contained HEPES and [Pya^{88}] S 39–88 but lacked BoNT/B LC and $ZnSO_4$. Stock solutions of the phosphoramidon analogs were dissolved in 20 mM HEPES at pH 7.1 and added to the preincubation mixture.

During incubation, BoNT/B LC cleaves [Pya^{88}] S 39–88 between Gln^{76} and Phe^{77}, resulting in fragments of 38 and 12 residues, with the latter containing the Pya fluorophore. The 12-mer [Pya^{88}] S 77–88 fluorescent cleavage product was separated from the intact substrate and the 38-mer non-fluorescent fragment using C18 Sep-Pak Vac matrix columns (Waters Corp., Milford, MA). Elution of [Pya^{88}] S 77–88 was accomplished by use of 65% methanol/0.1% TFA solution. Soleihac *et al.*[14] reported that over 99% of the fluorescent 12-mer cleavage product is selectively eluted under similar experimental conditions. Fractional inhibition was calculated at λ_{ex} = 343 nm/λ_{em} = 377 nm using the formula 1–(I–B)/(C–B), where *I* and *C* represent fluorescence in the presence and absence of inhibitor, respectively, and *B* is the fluorescence of the blank solution.

Materials

Methanol (Optima™ spectrophotometric grade) was obtained from Fisher Scientific Co. (Pittsburgh, PA). Trifluoroacetic acid (peptide synthesis grade) was obtained from Millipore, Inc. (Beverly, MA). Pure BoNT/B LC was prepared by Drs Eric Johnson and Michael Goodnough (Food Research Institute, Madison, WI). Ultrapure $ZnSO_4$ and HEPES were acquired from Sigma-Aldrich Corp. (St. Louis, MO) and TPEN was

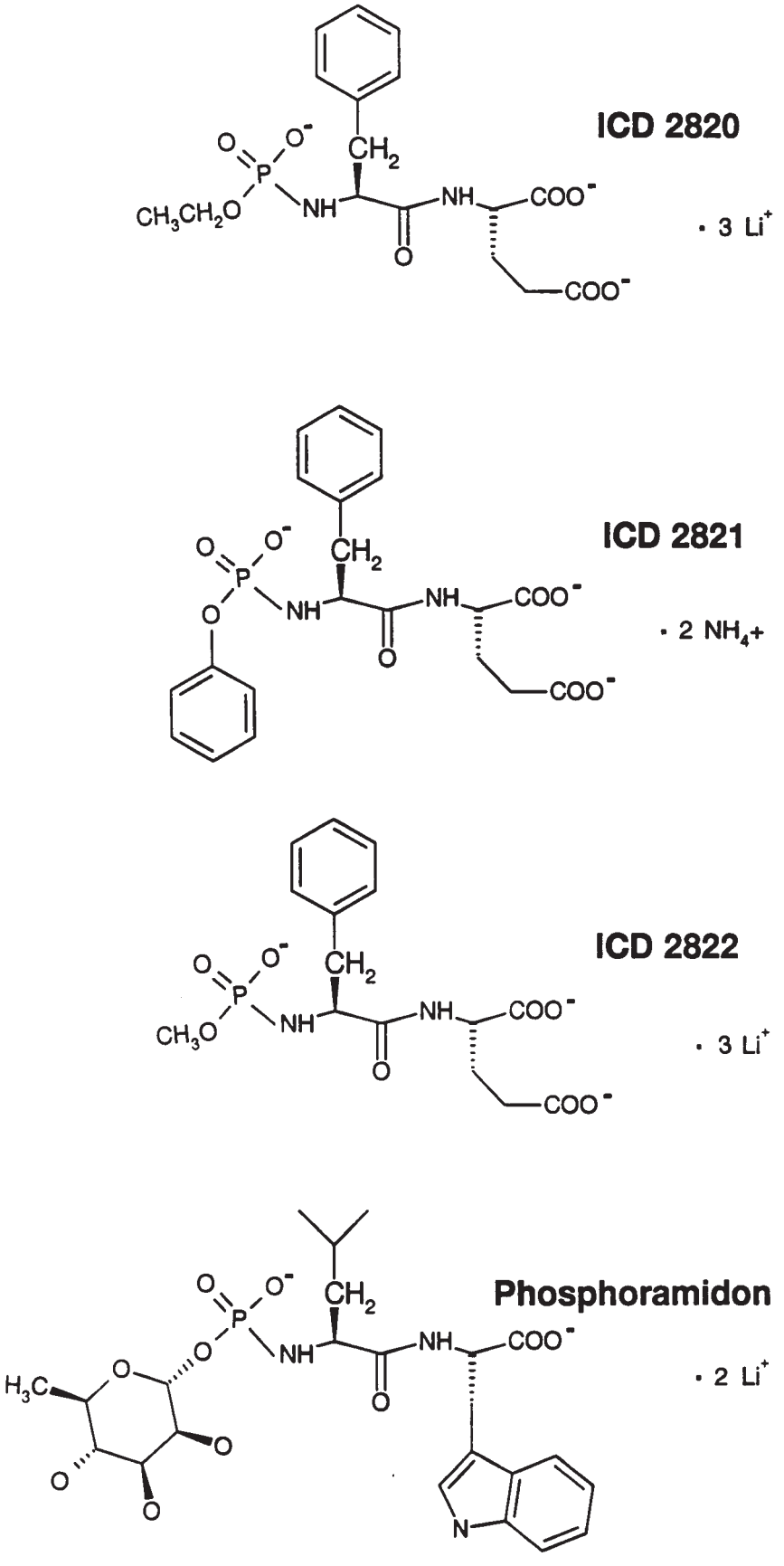

Figure 1. Structural formulae of ICD 2820, ICD 2821, ICD 2822 and phosphoramidon. The synthetic inhibitors are dipeptides containing Phe-Glu in which the N-terminal nitrogen of Phe is bound to a phosphorus atom and the substituents are attached to one of the phosphonate oxygens. The synthetic compounds are identical except for the nature of the substituent, which consists of an ethyl (ICD 2820), phenyl (ICD 2821) or methyl (ICD 2822) group. Phosphoramidon differs from the synthetic compounds in the composition of the dipeptide (Leu-Trp in place of Phe-Glu) and by the presence of a rhamnose sugar.

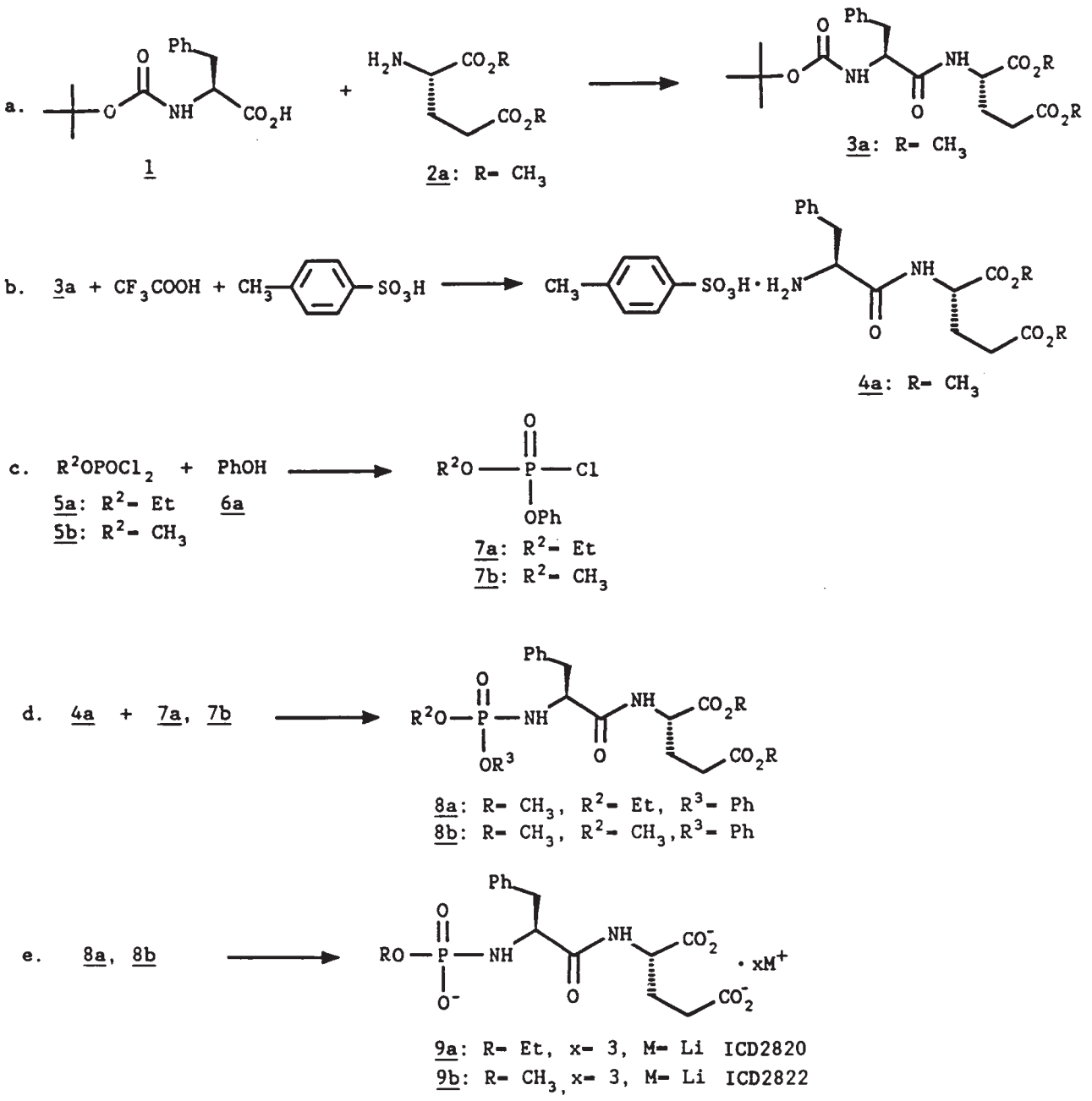

Figure 2. Scheme for synthesis of ICD 2820 (L-glutamic acid, *N*-(*N*-(ethoxyhydroxyphosphinyl)-L-phenylalanyl)-, trilithium salt) and ICD 2822 (L-glutamic acid, *N*-[*N*-(methoxyhydroxyphosphinyl)-L-phenylalanyl]-, trilithium salt).

purchased from Calbiochem (San Diego, CA). All other chemicals were reagent grade or higher.

RESULTS

Effect of $ZnSO_4$ on BoNT/B-mediated substrate cleavage

In previous *in vitro* assays, it has often been unclear whether exogenous zinc was required to achieve optimal catalytic rates for BoNT LC. To address this question, we examined the cleavage of [Pya^{88}] S 39–88 in the absence of added zinc and in the presence of 10 μM–5 mM $ZnSO_4$. The results are illustrated in Fig. 4. As is clear from the convergence of the fluorescence spectra, cleavage of [Pya^{88}] S 39–88 was not altered significantly by addition of zinc in the range 10 μM–1 mM. The observation that proteolytic activity does not require exogenous zinc is consistent with the high affinity of BoNT LC for the active site zinc.[5]

Increasing the zinc concentration to 5 mM, however, led to a marked inhibition of [Pya^{88}] S 39–88 cleavage. Inhibition by high zinc concentrations has been observed in previous studies but its mechanism remains unclear.[16,17] Although supplemental zinc was clearly not required for BoNT/B LC activity, 50 μM $ZnSO_4$ was routinely included in the incubation mixture to avoid complications from potential chelator actions of test agents or buffers.[18]

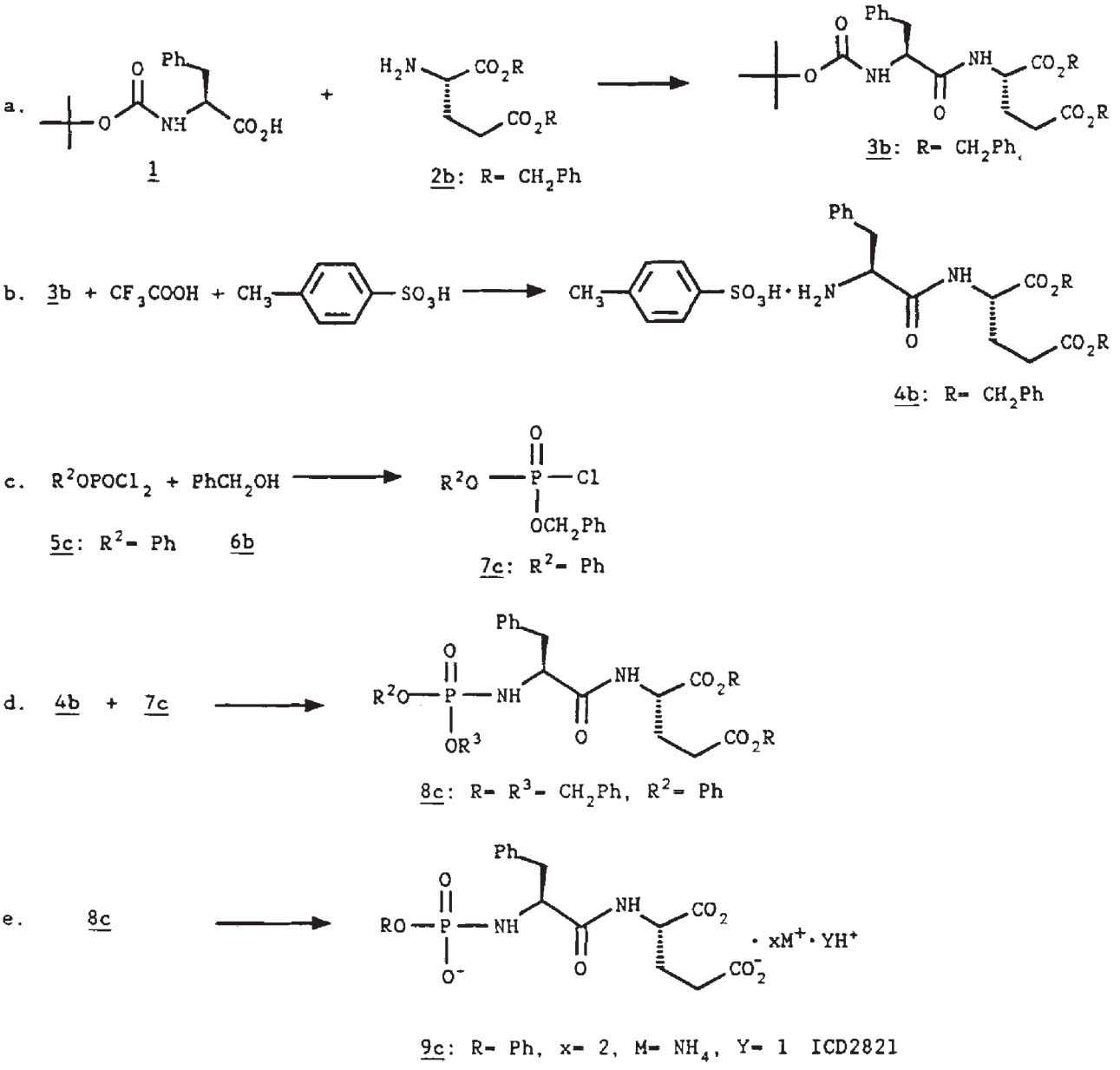

Figure 3. Scheme for synthesis of ICD 2821 (L-glutamic acid, *N*-[*N*-(phenoxyhydroxyphosphinyl)-L-phenylalanyl]-, diammonium salt).

Effect of TPEN on BoNT/B-mediated substrate cleavage

To examine the consequence of removing zinc that is tightly bound to the active site of BoNT/B LC, experiments similar to those described above were carried out in the presence of TPEN, a chelator with high affinity for transition metals such as zinc.[18] Addition of TPEN to the reaction mixture was expected to inhibit [Pya^{88}] S 39–88 proteolysis by chelating zinc that is bound to the BoNT/B LC active site. A marked inhibition of BoNT/B catalytic activity was indeed observed, as illustrated in Fig. 5. In the presence of TPEN, there was a concentration-dependent decrease in fluorescence intensity, indicating inhibition of BoNT/B-mediated proteolysis. The TPEN concentrations of 20, 30, 40 and 50 μM reduced the fluorescence intensity at 377 nm to 77, 68, 53 and 18% of control, respectively (Fig. 5). These results are consistent with the requirement of zinc for BoNT/B-mediated proteolysis[5] and serve to validate the assay for unknown inhibitors.

Effect of phosphoramidon and synthetic analogs on [Pya^{88}] S 39–88 cleavage

Phosphoramidon and three synthetic phosphonate monoester compounds were examined for their ability to inhibit cleavage of [Pya^{88}] S 39–88. Figure 6 shows concentration–response data for the effects of phosphoramidon and synthetic phosphonate monoester compounds on BoNT/B LC-mediated cleavage of [Pya^{88}] S 39–88. As with TPEN, the compounds were incubated with BoNT/B LC for 30 min prior to addition of substrate. Phosphoramidon was found to be a weak inhibitor of BoNT/B LC, requiring 4 mM to produce a detectable inhibition and 14.7 ± 1.2 mM (mean ± SEM) to achieve half-maximal inhibition of [Pya^{88}] S 39–88 catalysis. Synthetic compound ICD 2821 was more potent than phosphoramidon, with an IC_{50} of 8.1 ± 1.7 mM. This difference was significant by the Student's *t*-test ($P < 0.05$). The analogs with either an ethyl (ICD 2820) or a methyl (ICD 2822) substitution displayed little inhibitory activity up to the highest concentration tested (12 mM).

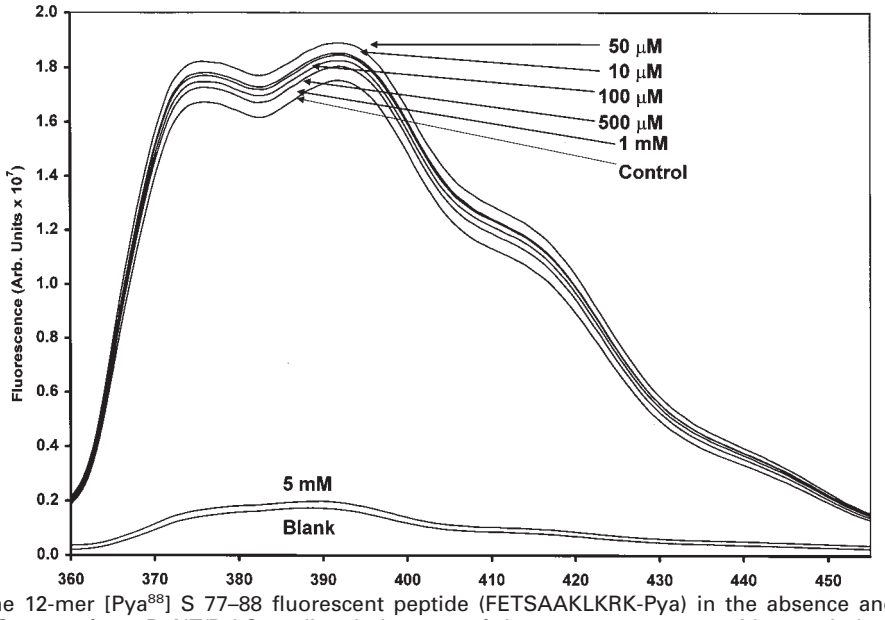

Figure 4. Spectra of the 12-mer [Pya[88]] S 77–88 fluorescent peptide (FETSAAKLKRK-Pya) in the absence and presence of $ZnSO_4$. Production of [Pya[88]] S 77–88 from BoNT/B LC-mediated cleavage of the 50-mer parent peptide was independent of exogenous zinc up to 1 mM but underwent marked inhibition after addition of 5 mM $ZnSO_4$.

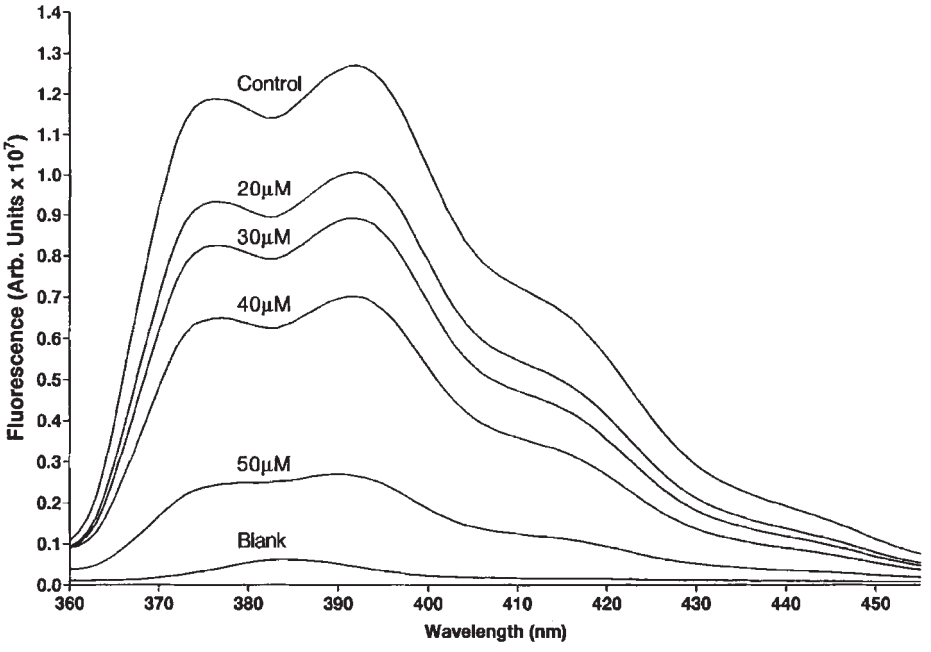

Figure 5. Fluorescence spectra of the 12-mer [Pya[88]] S 77–88 cleavage product in the absence and presence of the metal chelator TPEN. The progressive reduction in substrate cleavage with increasing TPEN concentration was attributed to removal of zinc bound to the active site of BoNT/B LC. Zinc sulfate was omitted from the incubation media in this experiment.

DISCUSSION

The results of the present investigation suggest that it is possible to inhibit the catalytic activity of BoNT/B LC by using small dipetide metalloprotease inhibitors. The synthetic compounds ICD 2820, ICD 2821 and ICD 2822 were modeled on the structure of phosphoramidon. This naturally occurring metalloprotease inhibitor was selected as the starting point for the current synthesis for two compelling reasons: phosphoramidon was demonstrated to delay the onset of paralysis following exposure of mouse diaphragm muscles to BoNT/B;[7] and the small size of phosphoramidon makes this compound an attractive candidate for drug development. When examined in the current assay, which measures inhibition of the metalloprotease activity of BoNT/B LC directly, phosphoramidon was found to be a relatively poor inhibitor, effective only in the millimolar range.

In designing inhibitors, it was assumed that the low potency of phosphoramidon may reflect its inability to fit optimally into the active site of BoNT/B LC, because its dipeptide composition (Leu-Trp) differs markedly from Phe-Glu, which is adjacent to the cleavage site of the substrate, synaptobrevin-2. A potential solution was to synthesize compounds of comparable

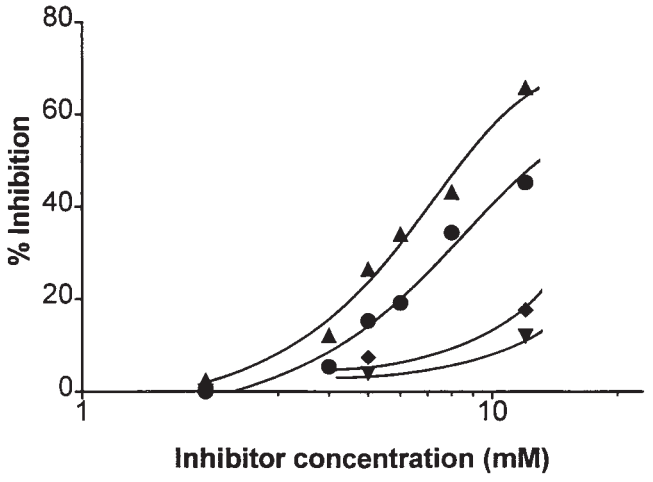

Figure 6. Concentration–response data for the inhibition of BoNT/B LC activity by phosphoramidon (●), ICD 2820 (▼), ICD 2821 (▲) and ICD 2822 (◆). The symbols represent means of four determinations. The curves for phosphoramidon and ICD 2821 were fit by non-linear regression with IC_{50} values of 14.7 ± 1.2 and 8.1 ± 1.7 mM, respectively. The fits to ICD 2820 and ICD 2822 data are arbitrary.

size with a similar phosphonate functional group to that found in phosphoramidon but with a dipeptide sequence optimized to interact with the active site of BoNT/B LC. Coupling of a rhamnose sugar was not attempted in the synthetic compounds because the reaction is highly unfavorable: instead, a methyl, ethyl or phenyl group was attached to this position based on reports that such substitutions led to little loss of activity in matrix metalloproteases.[8]

Of the three analogs tested in the present study, those with small alkyl substituents were found to be relatively inactive even at the highest concentration tested (12 mM), whereas the compound with a phenyl substituent was more potent than phosphoramidon (Fig. 6), suggesting that an aromatic or bulky group may be important for activity. These results, coupled with the high potency displayed by the isocoumarin analog ICD 1578 against BoNT/B LC,[15] suggest the existence of an aromatic binding pocket near the active site of BoNT/B LC. The relatively low potency of ICD 2821 in spite of its correct dipeptide sequence (Figure 1) suggests that the complementary sites for the dipeptide may not be accessible in the native toxin.[19] Future synthetic efforts exploiting the features of the first-generation lead compounds should give rise to more specific and more potent inhibitors of BoNT/B LC. The initial albeit moderate success of this approach suggests that a pharmacological treatment for BoNT intoxication is a realistic goal.

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