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Synthesis and Evaluation of Compound 35 as a Potential Botulinum Neurotoxin Inhibitor

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14. ABSTRACT

A pseudotripeptide inhibitor designated as compound 35 was synthesized and tested for its ability to inhibit the catalytic activity of botulinum neurotoxin serotype A (BoNT/A) or serotype B (BoNT/B). Catalytic activity was assessed using a fluorescent microplate assay in which an immobilized substrate for BoNT/A (SNAP-25 peptide) or BoNT/B (synaptobrevin peptide) was released into the medium following addition of the appropriate BoNT light chain (LC). Compound 35 was able to inhibit both LCs with an IC_{50} of 1 μM for BoNT/A and 7 μM for BoNT/B. Compound 35 is the first inhibitor that has been found to be effective against two serotypes of BoNT; these two serotypes are responsible for most human intoxications.

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ABSTRACT

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1. INTRODUCTION

The botulinum neurotoxins (BoNTs) are a group of seven antigenically distinct protein toxins (A-G) produced by the anaerobic bacterium *Clostridium botulinum* (Simpson, 1989). These neurotoxins inhibit the release of acetylcholine (ACh) from motor nerve endings, leading to muscle paralysis and death (Middlebrook 1995; Adler *et al.*, 2001). Each of the seven serotypes of BoNT consists of a light chain (LC) of ~50 kDa and a heavy chain (HC) of ~100 kDa linked by a single disulfide bond and non-covalent interactions (Singh and DasGupta, 1989). The LC has Zn^{2+} metalloprotease activity and cleaves one of three proteins associated with transmitter release: synaptobrevin, SNAP-25 or syntaxin (Binz *et al.*, 1994; Tonello *et al.*, 1996; Schoch *et al*., 2001). The HC is responsible for the binding of the toxin to the nerve terminal and for internalization of the LC into the cytosol (Simpson, 1989; Dolly *et al.*, 1990; Montecucco and Schiavo, 1993).

Exposure to BoNT leads to generalized muscle weakness, paralysis and potentially lethal respiratory collapse (Simpson, 1989). At the present time, treatment of BoNT intoxication consists of infusion of serotype-specific equine antitoxin and critical care (Roblot *et al.*, 1994). There are a number of limitations to the use of equine-derived antitoxin including shortages of supply, a high incidence of hypersensitive reactions and a narrow window of opportunity that is generally confined to the onset of symptoms (Shapiro *et al.*, 1998; Adler *et al.*, 2001). In addition to antitoxin, an investigational vaccine is used for prophylaxis of high risk populations, but adequate protection requires a series of three vaccinations over several months followed by a booster at the end of one year (Middlebrook, 1995).

Although an urgent need for pharmacological treatments has been recognized for over a decade (Simpson, 1989; Adler *et al*., 1995), there are still no effective drug therapies for botulinum intoxication. In principle, multiple sites in the pathway of BoNT action could be exploited for developing pharmacological treatments. These include antagonism of the binding of BoNT to the nerve terminal, inhibition of internalization of the toxin and blockade of the zinc metalloprotease activity of the LC (Simpson, 1989; Adler *et al.*, 2001; Simpson, 2004). Of these, blockade of the catalytic activity appears to be the most promising since it can potentially be effective after intoxication even in symptomatic individuals. The current study describes our efforts to develop an active site inhibitor for BoNT/A and BoNT/B. Our lead compound was based on a pseudotripeptide scaffold (compound 35, Anne *et al*., 2003, Blommaert *et al*., 2004). Compound 35 was found to be effective in inhibiting the catalytic activities of BoNT/A and BoNT/B LCs in cell-free enzymatic assays. The interactions between compound 35 and the toxin active site were explored by molecular docking studies, and these have provided insight into the structural features for synthesis of more effective compounds.

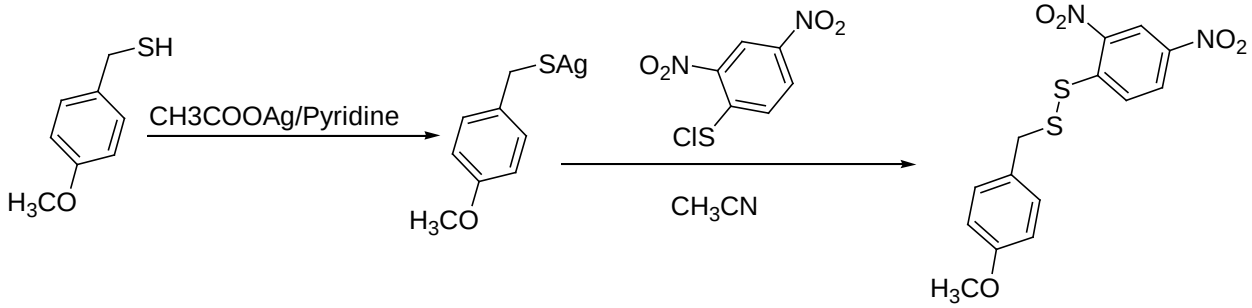
2. METHODS

2.1 Synthesis of Compound 35

2.1.1. General

For all anhydrous reactions, glassware was dried in the oven overnight at 120 ºC prior to its use. All anhydrous reactions were run under nitrogen atmosphere. Melting points (uncorrected) were determined in open tubes using a capillary melting point apparatus. Thin layer chromatography (TLC) was conducted using silica gel plates with UV and/or iodine visualization and the same solvent system which was used for column chromatography unless otherwise stated. Column chromatographic separations were effected on Fisher silica gel (40-63 µm) with indicated solvents. Flash chromatography was conducted using the Isco CombiFlash Companion system with Isco Redisep normal phase columns.

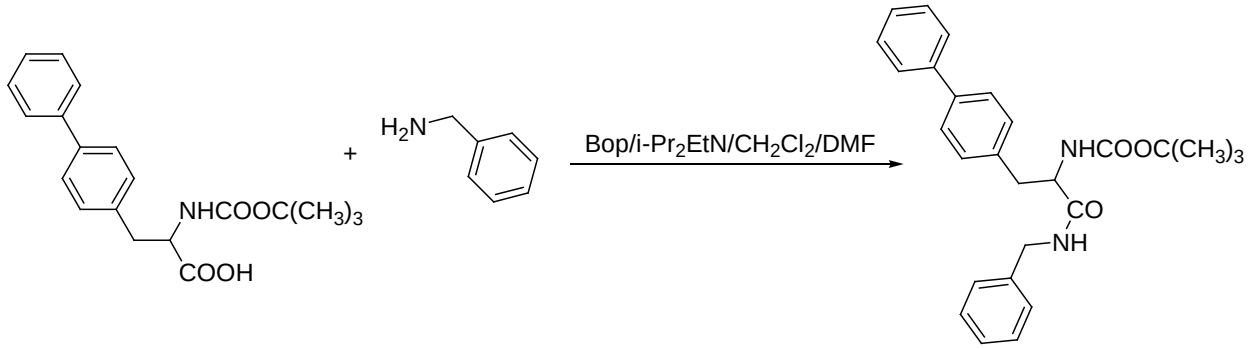
2.1.2. 4-Methoxybenzyl 2,4-dinitrophenyl disulfide (KP1)



A solution of 5.00 g (0.0324 mol) of 4-methoxy-α-toluenethiol in 15 ml of pyridine was added to a solution of 5.41 g (0.0324 mol) of silver acetate in 120 ml of pyridine with stirring. The reaction mixture was stirred for 2 h at room temperature (RT). The mixture was filtered and the precipitate was washed with hot water (5 x 50 ml). The washed silver thiolate was dried and used without further purification for obtaining 4-methoxybenzyl 2,4-dinitrophenyl disulfide.

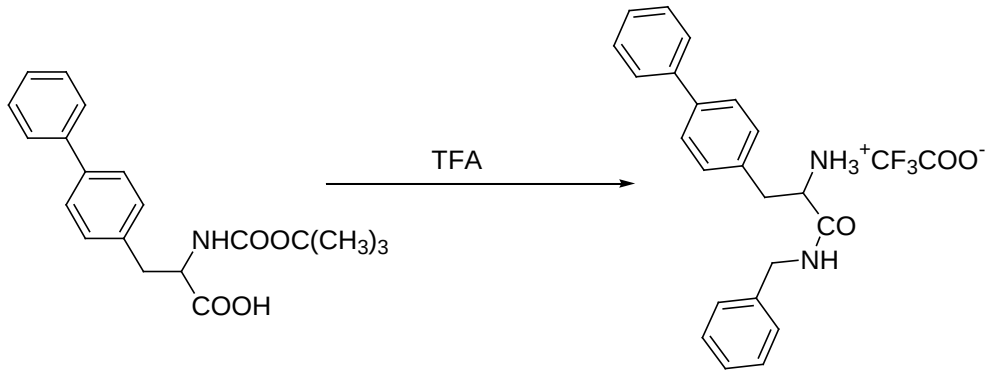
Eight grams (0.0306 mol) of dried silver thiolate was added to a solution of 7.18 g (0.0306 mol) 2,4-dinitrobenzenesulfenyl chloride in 300 ml of acetonitrile. The addition was conducted in an ice bath. The reaction mixture was stirred for 3 h at the same temperature. The mixture was filtered, the precipitate was washed with hot CH_3OH (4 X 70 ml) and combined filtrates were evaporated by rotary evaporator. The residue was purified by column chromatography (eluent: CH_3OH) to yield 7.01 g (0.0199 mol, 61.4%) of 4-methoxybenzyl 2,4-dinitrophenyl disulfide as yellow crystals.

2.1.3. 2-(tert-Butoxycarbonylamino)-2-(4-Biphenylyl)-N-(phenylmethyl)-propanamide (KP3)



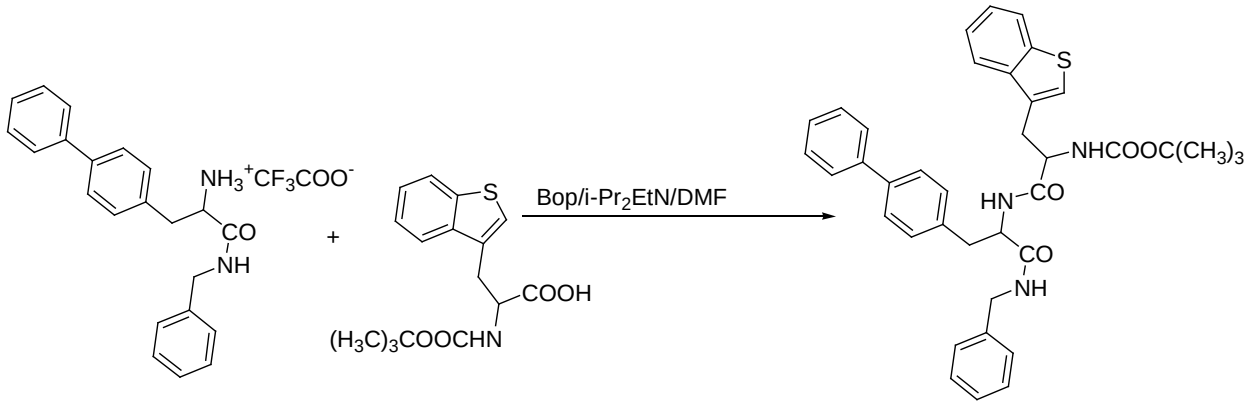
To a solution of 0.500 g (0.00146 mol) 3-(4-biphenylyl)-*N*-(*tert*-butoxycarbonyl)-L-alanine in 5 ml of CH_2Cl_2 and 15 ml of DMF was added successively 0.188 g (0.00175 mol) of benzylamine, 0.774 g (0.00175 mol) of benztriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and 0.660 g (0.00511 mol) of diisopropylethylamine. The reaction was stirred for 12 h at RT. Then 30 mL of CH_2Cl_2 was added to the reaction mixture, and the organic layer was washed with water (3 X 40 ml). The organic layer was dried over $MgSO_4$ and filtered. The solvent from filtrate was evaporated by rotary evaporator, and the residue was purified by flash chromatography (eluent: AcOEt/CH_2Cl_2 [1.5; v:v]) to yield 0.423 g (0.000982 mol, 67.3%) of desired compound as white crystals.

2.1.4. 2-Amino-2-(4-Biphenylyl)-N-phenylmethyl)-propanamide (KP5)



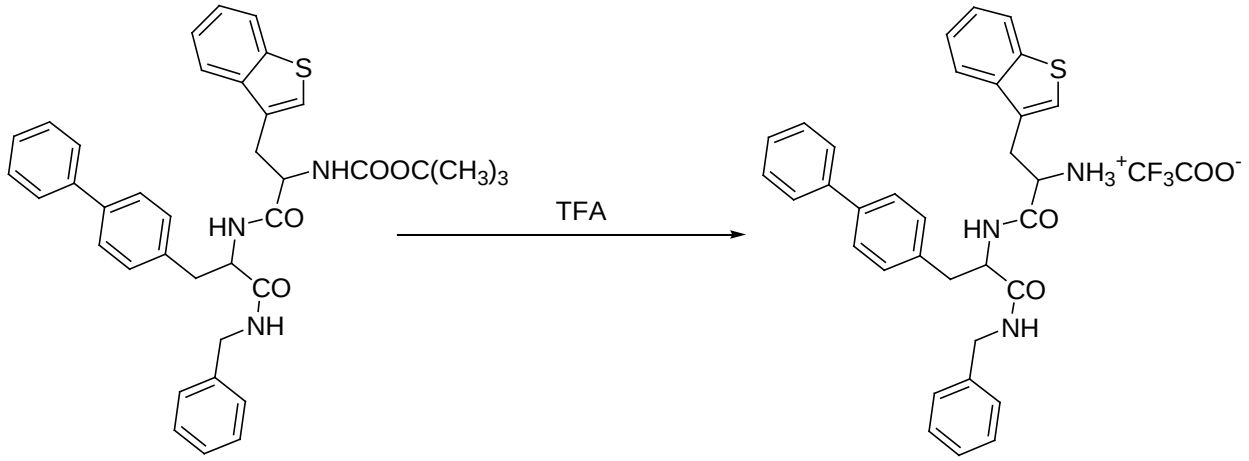
A solution of 0.400 g (0.000929 mol) of carbamic acid [(1S)-1-([1,1'-biphenyl]-4ylmethyl)-2-oxo-2-[(phenylmethyl)amino]ethyl]-,1,1-dimethylethyl ester in 5 ml of CF_3COOH was stirred for 2 h in an ice bath. Then stirring was continued at RT for 1 h. Trifluoroacetic acid was evaporated by rotary evaporator, and the residue was triturated with a mixture of 15 ml of hexane and 15 ml of ether. The mixture was filtered, and the precipitate was dried under vacuum to yield 0.410 g (0.000922 mol, 99.2%) of the product as white crystals.

2.1.5. (2S)-N-[(1S)-2-Biphenyl-4-yl-1-benzylcarbamoyl-ethyl]-2-tert-butoxycarbonylamino-3-benzo[b]thiophen-3-yl-propanamide (KP7)



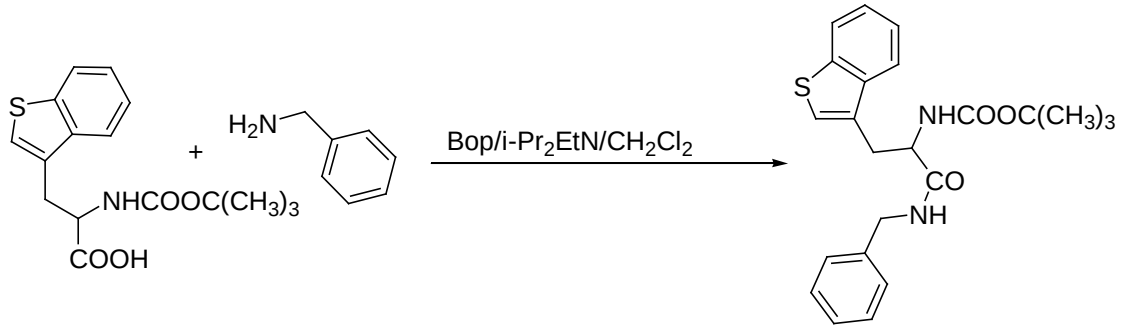
To a solution of 0.400 g (0.000900 mol) of 2-amino-2-(4-biphenylyl)-*N*-(phenylmethyl)-propanamide in 15 ml of DMF was added successively 0.390 g (0.00121 mol) of BOC-3-(3-benzothienyl)-L-alanine, 0.536 g (0.00121 mol) of BOP, and 0.550 g (0.00426 mol) of diisopropylethylamine. The reaction was stirred for 12 h at RT. Then 50 ml of CH_2Cl_2 was added to the reaction mixture, and the organic layer was washed with water (3 X 50 ml). The organic layer was dried over MgSO4 and filtered. The solvent from filtrate was evaporated by rotary evaporator, and the residue was purified by flash chromatography (eluent: AcOEt/CH_2Cl_2 [1:5, v:v]) to yield 0.476 g (0.000751 mol, 83.4%) of desired compound as white crystals.

2.1.6. (2S)-N-[(1S)-2-Biphenyl-4-yl-1-benzylcarbamoyl-ethyl]-2-amino-3-benzo[b]thiophen-3-yl-propanamide (KP9)



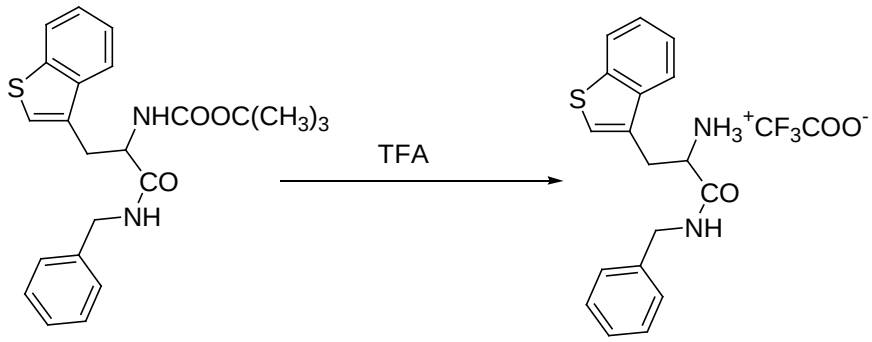
A solution of 0.450 g (0.000710 mol) of (2S)-*N*-[(1S)-2-biphenyl-4-yl-1-benzylcarbamolyl-ethyl]-2-*tert*-butoxycarbonylamino-3-benzo[b]thiophen-3-yl-propanamide in 5 ml of CF_3COOH was stirred for 2 h in an ice bath. Stirring was continued at RT for 1 h. Trifluoroacetic acid was evaporated by rotary evaporator, and the residue was triturated with a mixture of 10 ml of hexane and 10 ml of ether. The mixture was filtered, and the precipitate was dried under vacuum to yield 0.423 g (0.000653 mol, 92%) of the product as white crystals.

2.1.7. 2-(tert-Butoxycarbonylamino)-2-(3-benzothienyl)-N-(phenylmethyl)-propanamide (KP11)



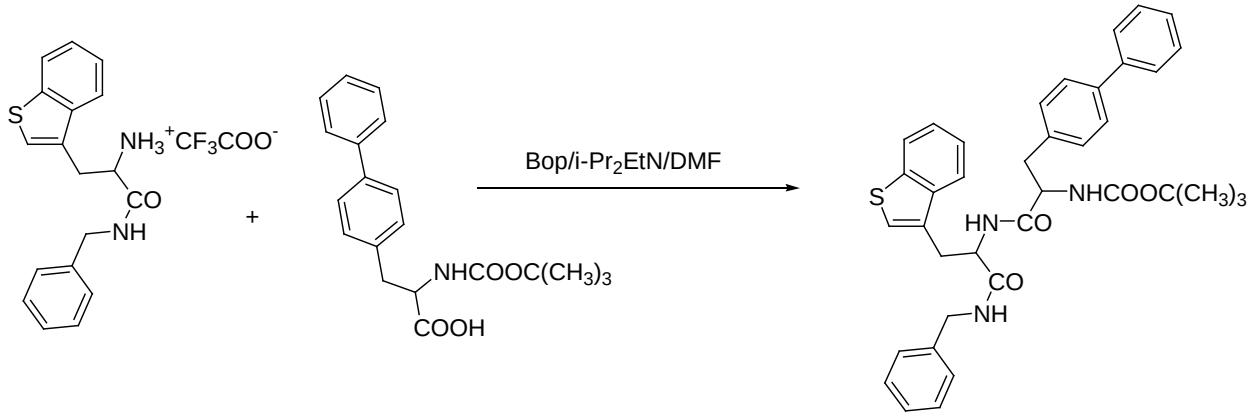
To a solution of 1.00 g (0.00311 mol) of BOC-3-(3-benzothienyl)-L-alanine in 10 ml of DMF was added successively 0.400 g (0.00373 mol) of benzylamine, 1.65 g (0.00373 mol) of BOP, and 1.41 g (0.0109 mol) of diisopropylethylamine. The reaction was stirred for 12 h at RT. Fifty ml of CH_2Cl_2 was added to the reaction mixture, and the organic layer was washed with water (3 x 40 ml). The organic layer was dried over $MgSO_4$ and filtered. The solvent from filtrate was evaporated by rotary evaporator, and the residue was purified by flash chromatography (eluent: AcOEt/CH_3Cl_2 [1:5; v:v]) to yield 1.145 g (0.00279 mol, 89%) of the compound as white crystals.

2.1.8. 2-(Amino)-2-(3-benzothienyl)-N-(phenylmethyl)-propanamide (KP13)



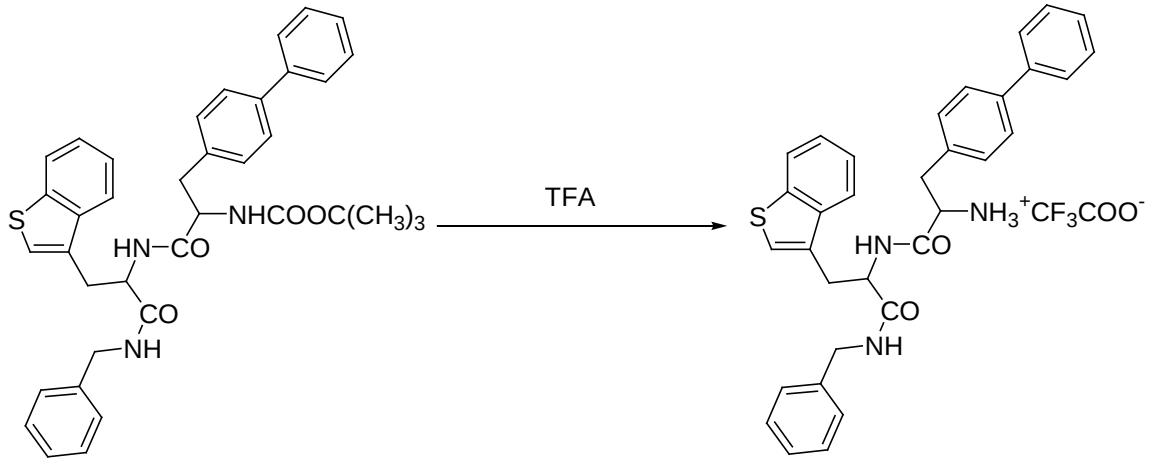
A solution of 1.12 g (0.00273 mol) of 2-(*tert*-butoxycarbonylamino)-2-(3-benzothienyl)-*N*-(phenylmethyl)-propanamide in 5 ml of CF_3COOH was stirred for 2 h in an ice bath. Stirring was continued at RT for 1 h. Trifluoroacetic acid was evaporated by rotary evaporator, and the residue was triturated with a mixture of 10 ml of hexane and 10 mL of ether. The mixture was filtered, and the precipitate was dried under vacuum to yield 0.951 g (0.00224 mol, 82.1%) of the product as white crystals.

2.1.9. (2S)-N-[(1S)-2-Benzo[b]thiophen-3-yl-1-benzylcarbamoyl-ethyl]-2-tert-butoxycarbonylamino-3-biphenyl-4-yl-propanamide (KP15)



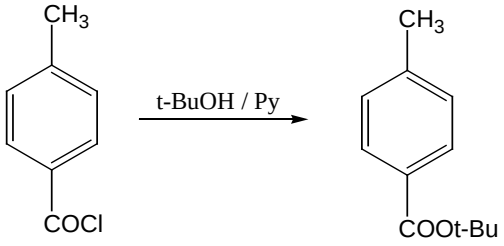
To a solution of 0.900 g (0.00212 mol) of 2-(amino)-2-(3-benzothienyl)-*N*-(phenylmethyl)-propanamide in 15 ml of DMF was added successively 0.765 g (0.00224 mol) of 3-(4-biphenylyl)-*N*-(*tert*-butoxycarbonyl)-L-alanine, 0.991 g (0.00224 mol) of BOP, and 0.844 g (0.00653 mol) of diisopropylethylamine. The reaction was stirred for 12 h at RT. Then 80 ml of CH_2Cl_2 was added to the reaction mixture, and the organic layer was washed with water (3 x 70 ml). The organic layer was dried over $MgSO_4$ and filtered. The solvent from filtrate was evaporated by rotary evaporator, and the residue was purified by flash chromatography (eluent: AcOEt/CH_2Cl_2 [1:5; v:v]) to yield 1.01 g (0.00159 mol, 75%) of the compound as white crystals.

2.1.10. (2S)-N-[(1S)-2-Benzo[b]thiophen-3-yl-1-benxylcarbamoyl-ethyl]-2-amino-3-biphenyl-4-yl-propanamide (KP17)



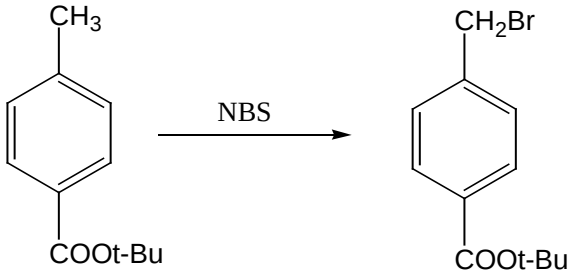
A solution of 0.900 g (0.00142 mol) of (2S)-*N*-[(1S)-2-benzo[b]thiophen-3-yl-1-benzylcarbamoyl-ethyl]-2-*tert*-butoxycarbonylamino-3-biphenyl-4-yl-propanamide in 5 ml of CF_3COOH was stirred for 1 h in an ice bath. Stirring was continued at RT for 1 h. Trifluoroacetic acid was evaporated by rotary evaporator, and the residue was triturated with a mixture of 10 ml of hexane and 10 ml of ether. The mixture was filtered, and the precipitate was dried under vacuum to yield 0.790 g (0.00122 mol, 85.9%) of the product as white crystals.

2.1.11. tert-Butyltoluate (VF-1)



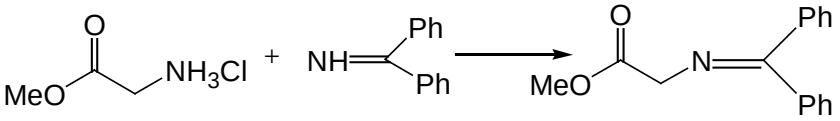
Under an inert atmosphere, toluic acid chloride 66.7 g (0.433 mol) was added to a solution of 0.264 g (0.00216 mol) of 4-dimethylaminopyridine in *tert*-BuOH (42 ml) and pyridine (42 ml). After 24 hours, water (100 ml) and ethyl acetate (170 ml) were added. The organic layer was separated and washed with 0.5 N HCl (2 x 50 ml) and 5% $NaHCO_3$ (2 x 50 ml) and then dried over Na_2SO_4. The drying agent was removed and the solvent was evaporated by rotary evaporator. The residue was distillated under vacuum. A bp of 70-72 ºC (0.75 torr) and yield of 76.0 g (0.0396 mol, 91.4%) was obtained.

2.1.12. tert-Butyl α-Bromotoluate (VF-3)



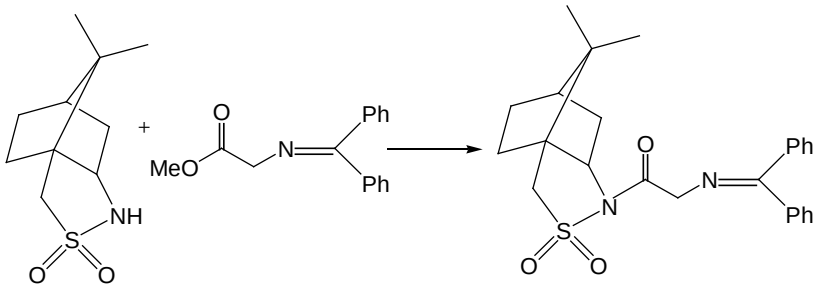
A mixture of 19.2 g of *tert*-butyltoluate ester (0.1 mol), *N*-bromosuccinimide (17.8 g, 0.1 mol) and dibenyoyl peroxide (0.2 g) in CCl_4 (100 ml) was refluxed with stirring for 4 h. The reaction mixture was filtered and the precipitate was washed with CCl_4 (2 x 50 ml). The combined organic layers were evaporated by rotary evaporator. The residue was crystallized from hexane/AcOEt (9:1; v:v) to yield 19.5 g (0.072 mol, 72%) of *tert*-butyl α-bromotoluate with a mp of 50-52 ºC

2.1.13. Methyl N-(diphenylmethylene)glicinate (VF-5)



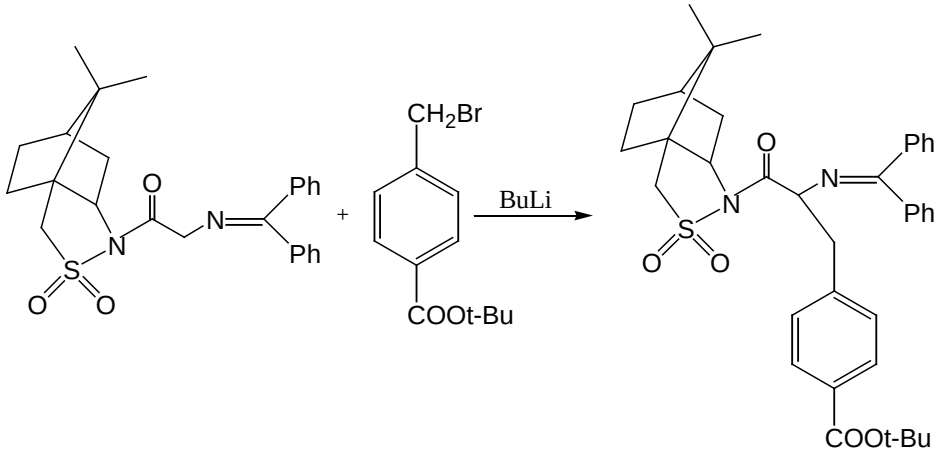
A mixture of 15.0 g of benzopenone imine (0.0828 mol) and 10.4 g of glycinemethyl ether hydrochloride (0.0828 mol) in methylene chloride (300 ml) was stirred at RT for 12 h. The reaction mixture was filtered and the filtrate was evaporated by rotary evaporator. Diethyl ether (300 ml) was added to the residue, and the ether layer was washed with water (2 X 50 ml), dried over $MgSO_4$, and filtered. The filtrate was evaporated by rotary evaporator, and the crystalline material was washed with hexane (3 X 20 ml) to yield 15.9 g (0.0629 mol, 76.0%) of methyl *N*-(diphenylmethylene)glicinate with a mp of 42.5-43.0 ºC.

2.1.14. N-(Diphenylmethylene)glicinesultam (VF-27)



Eighty-seven ml of 1M triethylaluminium (0.087 mol) in hexane was added, dropwise and with stirring, to a solution of 15.0 g (0.0697 mol) (-)-2,10-camphorsultam in toluene (175 ml). Addition was conducted at RT and required 30 min. The mixture was stirred additionally for 15 min. A solution of 25 g (0.0987 mol) methyl *N*-(diphenylmethylene)-glycinate (VF-5) in toluene (125 ml) was added dropwise for 30 min, and the mixture was heated at 60 ºC for 64 h. The reaction mixture was allowed to cool to RT. The solution was poured into a mixture of ice (300 g) and CH_2Cl_2 (300 ml). The resulting mixture was filtered through silica gel. Silica gel was washed with CH_2Cl_2 (2 x 100 ml). The combined organic layer was dried over $MgSO_4$, filtered and concentrated by rotary evaporator to yield 29.1 g (0.0667 mol) of *N*-(Diphenylmethylene)glicinesultam as a yellow oil (yield 95.7%).

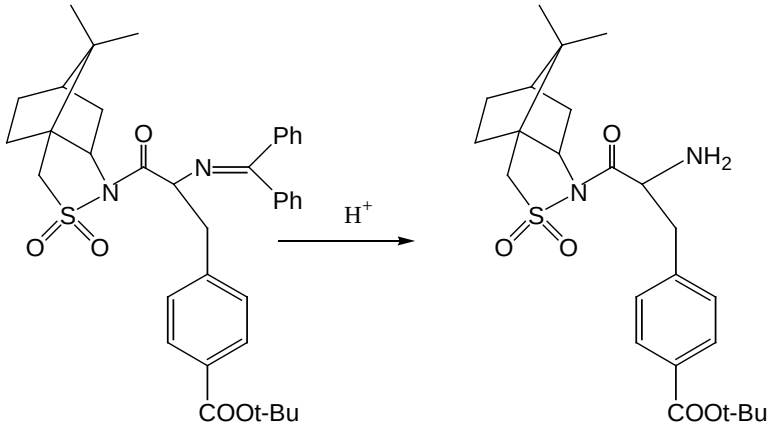
2.1.15. 4-[2-(Benzhydrylidenenamino)-3-(10,10-dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0^{1,5}]dec-4-yl)-3-oxopropyl]benzoic acid tert-butyl ester (VF-29).



A solution of 2.5 M n-BuLi in hexane (31.5 ml, 0.0790 mol) was added, dropwise and with stirring, to a solution of 29.10 g (0.0667 mol) of *N*-(diphenylmethylene)glicinesultam in 350 ml anhydrous THF. The addition was conducted at -78 ºC for 30 min (acetone/dry ice bath). The mixture was stirred for 60 min at -78 ºC. A solution of 26.25 g (0.0970 mol) of *tert*-butyl α-bromotoluate in 52.5 ml of HMPA and 175 ml of THF was added for 45 min. The mixture was allowed to warm to 0 ºC under stirring for 4 h, and 150 ml of 10% NH_4Cl was added to the mixture. The water layer was washed with ether (2 X 150 ml). The organic layers were combined, dried over Na_2SO_4 and filtered. The solvent was evaporated by rotary evaporator, and the residue was purified by column chromatography (eluent: 1 EtOAc/Cyclohexane [5:95; v:v]; 2

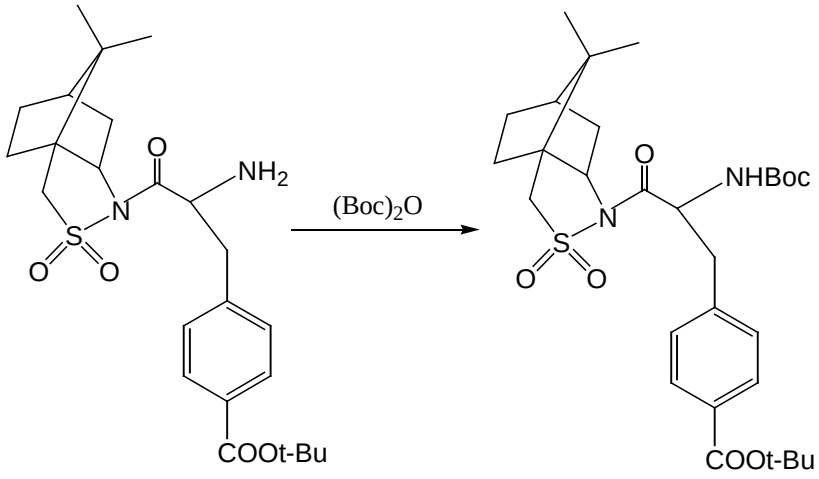
EtOAc/Cyclohexane [1:1; v:v], R_f 0.56) to yield 39.03 g of 4-[2-(benzhydrylideneamino)-3-(10,10-dimethyl-3,3-dioxo-3λ6-thia-4-aza-tricyclo[5.2.1.$0^{1.5}$]dec-4-yl)-3-oxopropyl]-benzoic acid *tert*-butyl ester (0.0623 mol, 93.4%) as a yellow oil.

2.1.16. 4-[(2-Amino)-3-(10,10-dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.$0^{1.5}$]dec-4-yl)-3-oxopropyl]benzoic acid tert-butyl ester (VF-31)



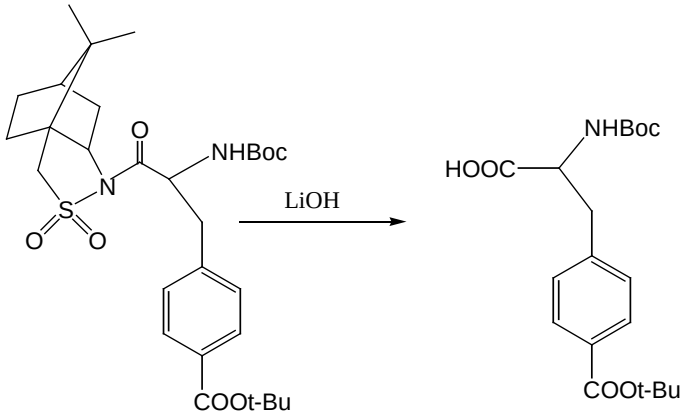
An aqueous solution of 10% citric acid (500 ml) was added to a solution of 39.03 g of 4-[2-benzhydrylidenenamino)-3-(10,10-dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo-[5.2.1.$0^{1.5}$]dec-4-yl)-3-oxopropyl]benzoic acid *tert*-butyl ester (0.0623 mol) in 500 ml THF. The mixture was stirred at RT for 8.5 h. THF was evaporated by rotary evaporator, the residue-aqueous layer was washed with ether (2 X 150 ml) and the pH was adjusted to 8.0 by adding a saturated solution of $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2 (3 X 100 ml). The organic layer was dried over Na_2SO_4 and concentrated to yield 22.18 g (0.0520 mol, 83.5%) of 4-[(2-amino)-3-(10,10-dimethyle-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.$0^{1.5}$]dec-4-yl)-3-oxopropyl]benzoic acid *tert*-butyl ester as a yellow oil.

2.1.17. 4-[(2-tert-Butoxycarbonylamino)-3-(10,10-dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.$0^{1.5}$]dec-4-yl)-3-oxopropyl]benzoic acid tert-butyl ester (VF-33)



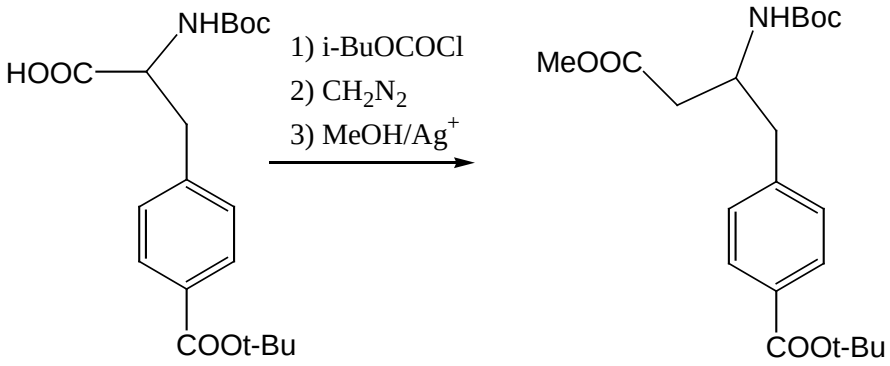
A solution of 12.20 g (0.0554 mol) of di-*tert*-butyldicarbonate in 60 ml of CH_2Cl_2 was added, dropwise and with stirring, to a solution of 22.18 g (0.0520 mol) of 4-[2-amino)-3-(10,10-dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0$^{1.5}$]dec-4-yl)-3-oxopropyl]benzoic acid *tert*-butyl ester and 13.0 ml of triethylamine in 220 ml of CH_2Cl_2. The addition was conducted for 30 min at -15 ºC (acetone/dry ice). The reaction mixture was stirred for 12 h at RT. The solvent was removed by rotary evaporator to yield 27.10 g of 4-[(2-*tert*-butoxycarbonylamino)-3-(10,10-dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0$^{1.5}$]dec-4-yl)-3-oxopropyl]benzoic acid *tert*-butyl ester (0.0482 mol, 92.6%) as a yellow oil.

2.1.18. 4-(2-tert-butoxycarbonylamino-2-carboxyethyl)benzoic acid tert-butyl ester (VF-35)



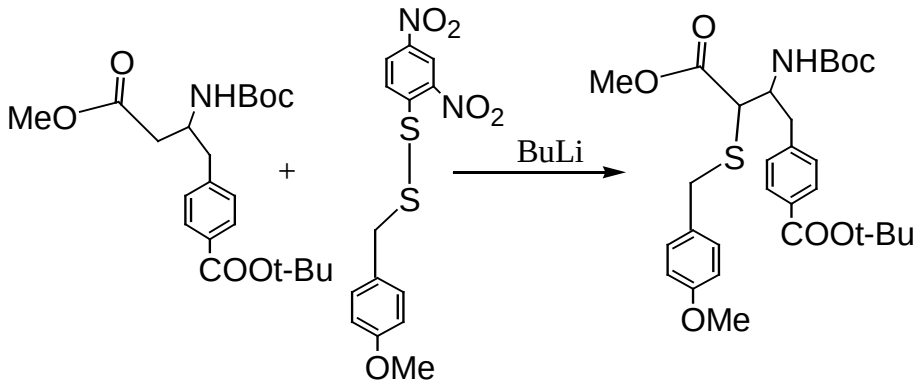
A mixture of 27.10 g 4-[(2-*tert*-butoxycarbonylamino)-3-(10,10-dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0$^{1.5}$]dec-4-yl)-3-oxopropyl]benzoic acid *tert*-butyl ester of (0.04815 mol), 4.63 g (0.19425 mol) of LiOH, 21.36 g (0.24589 mol) of LiBr and 6.33 g (0.00136 mol) of $(n\text{-}Bu)_4NBr$ in 500 ml acetonitrile was stirred 20 h at RT. The solvent was removed by rotary evaporator. Added to the residue at 0 ºC were 120 ml 10% H_2SO_4 and 400 ml ether. The organic layer was separated and washed with a saturated NaCl solution (3 X 70 ml). The organic layer was dried over $MgSO_4$ and filtered. The filtrate was evaporated by rotary evaporator, and the residue was purified by chromatography (eluent: $CHCl_3$, R_f 0.12). Evaporations of the solvent under vacuum yielded 11.84 g (0.03240 mol, 67.3%) of 4-(2-*tert*-butoxycarbonylamino-2-carboxyethyl)benzoic acid *tert*-butyl ester as a yellow oil.

2.1.19. 4-(2-tert-Butoxycarbonylamino-3-(methoxycarbonylpropyl)benzoic acid tert-butyl ester (VF-17)



Isobutyl chloroformate 0.336 g (0.00246 mol) was added to a solution of 0.900 g of 4-(2-*tert*-butoxycarbonylamino-2-carbozyethyl)benzoic acid *tert*-butyl ester (0.00246 mol) and 0.249 g (0.00247 mol) *N*-methylmorpholine in 7 ml of THF at -20 ºC. The mixture was stirred for 15 min and filtered. A solution of diazomethane (0.02000 mol) in 50 ml of ether was added to the filtrate in an ice bath. The reaction mixture was stirred for 2 h at RT. The solvent was evaporated under vacuum, and 40 ml of methanol, 0.040 g (0.00017 mol) of silver benzoate and 1.4 ml of triethylamine were added to the residue. The reaction mixture was stirred for 12 h and then filtered through silica gel. The solvent was evaporated under vacuum, and the residue was dissolved in THF and dried over Na_2SO_4. Evaporation of THF by rotary evaporator yielded 0.900 g of 4-(2-*tert*-butoxycarbonylamino-3-(methoxycarbonylpropyl)benzoic acid *tert*-butyl ester (0.00228 mol, 92.7%) as a yellowish oil.

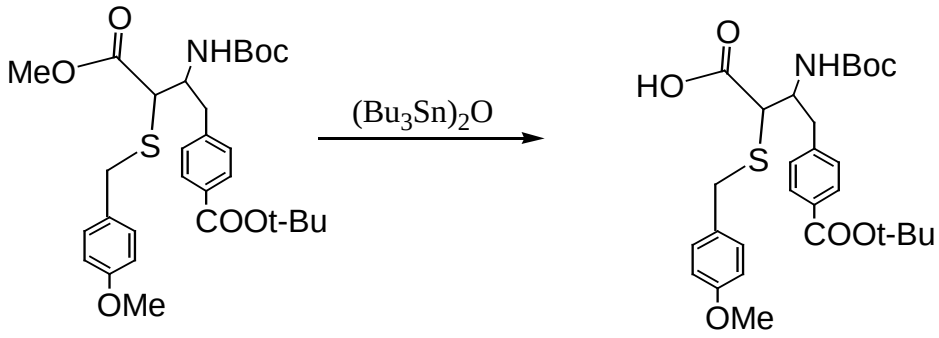
2.1.20. 4-[2-tert-Butoxycarbonylamino-3-[[(4-methoxyphenyl)methyl]thio]-3-methoxycarbonylpropyl] benzoic acid tert-butyl ester (VF-39)



A solution of 2.5 M n-BuLi in hexane (6.65 ml, 0.01664 mol) was added, dropwise and with stirring, to a solution of 2.675 g (0.01656 mol) of hexamethyl-disilazane in 40 ml THF. The addition was conducted for 15 min in an ice bath. The reaction mixture was stirred additionally for 15 min and then cooled to -70 ºC (acetone/dry ice). A solution of 4-(2-*tert*-butoxycarbonylamino-3-(methoxy-carbonylpropyl)benzoic acid *tert*-butyl ester (2.30 g, 0.00584 mmol) in 20 mL THF was added dropwise. The reaction mixture was stirred for 90 min at -70 ºC. The 4-methoxybenzyl 2,4-dinitrophenyl disulfide (2.90 g, 0.00821 mol) and 2 ml of HMPA were added. The mixture was stirred for 75 min at -70

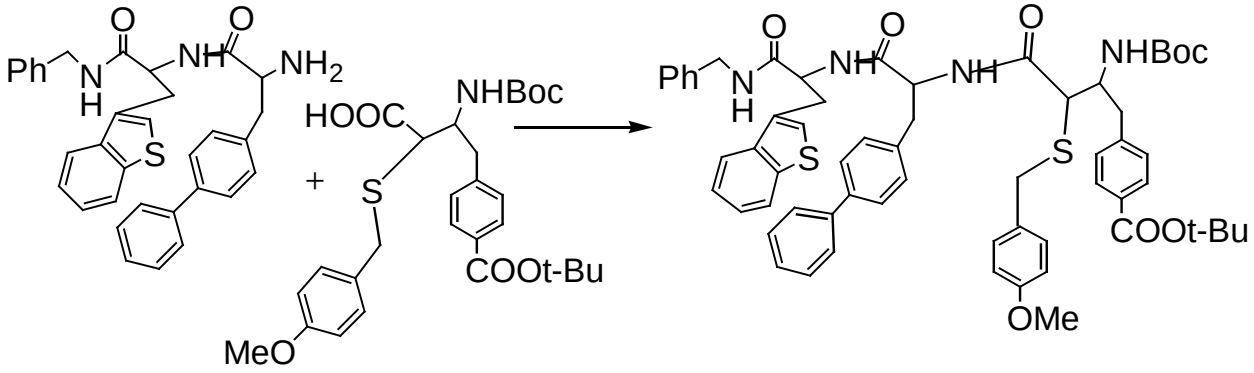
ºC. The reaction was quenched with 10% NH_4Cl (30 ml) and 75 ml EtOAc was added. The organic layer was filtered through silica gel and dried over Na_2SO_4. The filtrate was evaporated under vacuum, and the residue was purified by column chromatography (eluent: ethyl acetate/methylene chloride/hexane [1.5:1.5:7; v:v]). The solvent was evaporated under vacuum, and the yellow oil was crystallized from hexane to yield 1.198 g (0.00220 mol, 37.6%) of 4-[2-*tert*-butoxy-carbonylamino-3-[[4-methoxyphenyl)methyl]thio]-3-methoxycarbonylpropyl] benzoic acid *tert*-butyl ester as white crystals with a mp of 80.0-81.5 ºC.

2.1.21. 4-[2-tert-Butoxycarbonylamino-3-carboxy-3-[[(4-methoxyphenyl)methyl]thio]propyl] benzoic acid tert-butyl ester (VF-41)



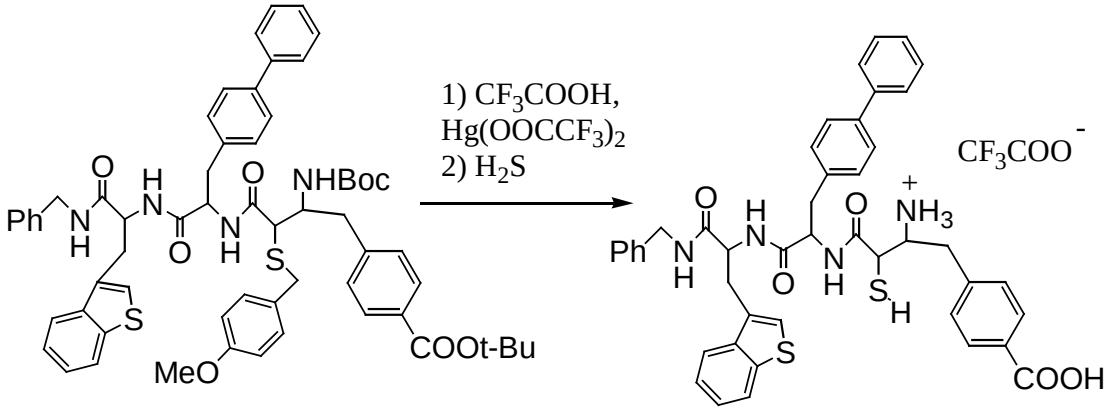
A solution of 1.00 g (0.00181 mol) of 4-[2-*tert*-butoxycarbonylamino-3-[[4-methoxyphenyl)methyl]thio]-3-methoxycarbonylpropyl] benzoic acid *tert*-butyl ester and 6.40 g (0.01071 mol) of bis(tributyl-tin)oxide in 60 ml of toluene was stirred for 72 h at 95 ºC. Toluene was evaporated by rotary evaporator and 30 ml 1M HCl was added. The mixture was stirred for 1.5 h at RT and 70 ml CH_2Cl_2 was added. The water layer was extracted by CH_2Cl_2 (3 X 20 ml). Combined organic layers were dried over $MgSO_4$. The mixture was filtered, the filtrate was evaporated by rotary evaporator and the residue was purified by column chromatography (eluent: hexane/AcOEt/AcOH [8:2:0.5, v:v]). The fraction with R_f = 0.33 was collected, and the solvent was evaporated to yield 0.704 g (0.00132 mol, 73.2%) of 4-[2-*tert*-butoxycarbonylamino-3-carboxy-3-[(4-methoxy-phenyl)methyl]thiopropyl] benzoic acid *tert*-butyl ester as a yellow oil.

2.1.22. 4-[(2S)-2-tert-Butoxycarbonylamino-3-[1-(2S)-2-benzo[b]thiophen-3-yl-1-benzylcarbamoylethylcarbamoyl]-(2S)-2-biphenyl-4-yl-ethylcarbamoyl]-3(S)-[(4-methoxyphenyl)methyl]thiopropyl] benzoic acid tert-butyl ester (VF-45)



To a solution of (2S)-N-[(1S)-2-benzo[b]thiophen-3-yl-1-benzylcarbamoyl-ethyl]-2-amino-3-biphenyl-4-yl-propanamide trifluoroacetic acid salt (1.359 g, 0.00209 mol) in 45 ml of DMF was added 1.000 g (0.00185 mol) of 4-[2-*tert*-butoxycarbonylamino-3-carboxy-3-[(4-methoxyphenyl)methyl]thiopropyl] benzoic acid *tert*-butyl ester, 1.131 g (0.00256 mol) of BOP, and 1.060 g (0.00818 mol) of diisopropylethylamine. The reaction was stirred for 16 h at RT, and DMF was evaporated by rotary evaporator. The residue was washed with 0.5% $KHSO_4$ (2 X 10 ml) and centrifuged, and then by water (2 X 10 ml), EtOH (2 x 10 ml), and 10 ml $CHCl_3$. The precipitate was suspended in ethanol (30 ml) for 12 h, then filtered and dried under vacuum for 10 h (0.03 torr, 80 ºC) to yield 1.420 g (0.00136 mol, 73.5 %) of 4-[(2S)-2-*tert*-butoxycarbonylamino-3-[1-(2S)-2-benzo[b]thoiphen-3-yl-1-benzylcarbamoylethylcarbamoyl]-(2S)-2-biphenyl-4-yl-ethylcarbamoyl]3(S)-[(4-methoxyphenyl)methyl]thiopropyl]benzoic acid *tert*-butyl ester as a white solid.

2.1.23. 4-[(2S)-2-amino-3-[1-(2S)-2-benzo[b]thiophen-3-yl-1-benzylcarbamoylethylcarbamoyl]-(2S)-2-biphenyl-4-yl-ethylcarbamoyl]-3(S)-propylthiol] benzoic acid trifluoroacetic acid salt (VF-47)



A solution of 0.510 g (0.00049 mol) of 4-[(2S)-2-*tert*-butoxycarbonylamino-3-[1-(2S)-2-benzo[b]thophen-3-yl-1-benzylcarbamoylethylcarbamoyl]-(2S)-2-biphenyl-4-yl-ethylcarbamoyl]-3(S)-[(4-methoxyphenyl)methyl]thiopropyl] benzoic acid *tert*-butyl ester, 0.7 ml (0.00642 mol) of anisole and CF_3COOH (8 ml) was stirred under N_2 for 1.5 h at RT. After the mixture was cooled to 0 ºC (ice bath), 0.230 g (0.00054 mol) $Hg(CF_3COO)_2$ was added and stirred for 70 min at RT. Water (1 ml) was added and H_2S was bubbled through the solution for 30 min. The resulting mercuric sulfide was removed by centrifugation, and the solution was filtered through Celite 503. Solvent was evaporated by rotary evaporator, and the residue dried 24 h under vacuum (0.05 torr). The residue was purified by chromatography (eluent: 1 EtOAc; 2 EtOAc/TFA [98:2; v:v]). Middle fractions were combined, and the solvent was evaporated by rotary evaporator to yield 0.220 g of the white solid, which was dissolved in EtOH (6 ml), and 18 ml of water was added. Precipitate was removed by centrifugation and dried 24 h (0.05 torr, 80 ºC) to yield 0.090 g (0.00010 mol, 20.4%) of 4-[(2S)-2-amino-3-[1-(2S)-2-benzo[b]thiophen-3-yl-1-benzylcarbamoylethylcarbamoyl]-(2S)-2-biphenyl-4-yl-ethylcarbamoyl]-3(S)-propylthiol] benzoic acid (Compound 35) triflouroacetic acid salt as white solid.

2.2. Mass Spectra

All mass spectra were obtained by the W.M. Keck FT-ICR-MS laboratory at the Mayo Proteomics Research Center (Mayo Clinic and Foundation, Rochester, MN). Compound 35 was analyzed by electrospray ionization mass spectrometry (ESI-MS) in positive mode for mass determination.

2.3. BoNT Fluorescent Microplate Assay

BoNT/A and BoNT/B LC activities were determined using a fluorescent microplate assay (Schmidt *et al*., 2001). BoNT/A and BoNT/B LCs were pre-incubated with various concentrations of compound 35 for 30 min at either RT or 37 °C. Assay buffer was added to the microplate, and an initial fluorescence was measured with a SpectraMax GeminiXS multiwell fluorometer (Molecular Devices, Sunnyvale, CA). All assay conditions included 40 mM HEPES pH 7.3 and 50 μM $ZnSO_4$. Enzyme concentrations in the assay were 60 nM BoNT/A recombinant LC (rLC), 80 nM BoNT/A purified LC (pLC), 180 nM BoNT/B rLC or 100 nM BoNT/B pLC. BoNT LC was incubated in the microplate for 1 h at 37 °C in the dark without agitation, and fluorescence was measured in the same microplate. At the end of the incubation, 40 μg of trypsin was added to each well, and the plate was incubated at 37 °C for 30 min in the dark to determine the total fluorescence released. Initial fluorescence was subtracted from the BoNT rLC-induced fluorescence, and this was normalized to total fluorescence. Four replicates were averaged and standard deviation was determined. GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA) was used to fit the data with the sigmoidal dose-response equation.

2.4. Molecular Modeling

Crystal structures 1XTG and 1S0F of botulinum toxin light chains were obtained from the protein data bank (www.rcsb.org). Automated docking was performed with Autodock 3.0.5 and Autodock Tools (Scripps Research Institute, La Jolla, CA). Homology modeling, molecular modeling and rendering were performed with insight/discover/homology software (Accelrys Inc., San Diego, CA). Computed logD was calculated with Marvin software (Chemaxon, Budapest, Hungary).

2.5. Materials

BoNT/A and BoNT/B rLCs were a gift from Dr. Leonard A. Smith (USAMRIID) and were purified as previously described (Ahmed and Smith, 2000). BoNT/A and BoNT/B pLCs were purified from holotoxin and purchased from Metabiologics (Madison, WI).

3. RESULTS

3.1 Synthesis and Verification of Compound 35

Compound 35 was first described by Anne *et al.* (2003a) as a potent inhibitor of BoNT/B. It was synthesized by our group to further investigate its potential use as a therapeutic countermeasure against BoNT intoxication. Ninety mg of compound 35 as the triflouroacetic acid (TFA) salt was produced from the synthesis described in section 2.1. The molecular weight of compound 35 was verified by ESI-MS (Fig. 1). Under non-

reducing conditions, two molecular species are observed at mass-to-charge ratios (m/z) of 1539.51 and 770.25 (Fig. 1A). The molecular species at m/z 770.25 is in agreement with the molecular weight of compound 35 without the TFA ion. A dimer of compound 35 is consistent with the species observed at m/z 1539.51. Alternatively, the molecular mass of the compound synthesized may be 1539.51, and the species observed at m/z 770.25 represents doubly charged molecules. To address this possibility, the compound was reduced with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) prior to ESI-MS analysis. Only one species was observed at m/z 771.26 (Fig. 1B), suggesting that the species observed at m/z 1539.51 was a compound 35 dimer. ^{1}H NMR and ^{13}C NMR analysis supports the presence of a compound 35 dimer and verified the correct structure for compound 35 (data not shown).

3.2 Effect of Compound 35 on BoNT/A activity

Compound 35 was originally developed as an inhibitor of BoNT/B and was reported to be a weaker inhibitor of BoNT/A (Anne *et al*., 2003a). To test the efficacy of the compound 35 synthesized by our group, we determined its ability to inhibit BoNT/A and BoNT/B catalytic activity using a fluorescent microplate assay. BoNT/A rLC was incubated at RT for 30 min with varying concentrations of compound 35 prior to addition to micro wells containing fluorescent SNAP-25 peptide. Compound 35 inhibition of BoNT/A rLC was complete at concentrations of ≥ 5 μM (Fig. 2). The IC50 for inhibition of BoNT/A rLC was 1.1 μM, which is similar to the previously reported IC50 of 2 μM (Anne *et al*., 2003a) for inhibition of type A rLC. Inhibition of BoNT/A rLC by compound 35 was independently verified with a fluorescent assay developed by Veritas (Rockville, MD) that uses full-length SNAP-25 as substrate. This assay yielded an IC_{50} of 412 nM for inhibition of BoNT/A rLC by compound 35 (data not shown).

3.3 Effect of Compound 35 on BoNT/B activity

Inhibition of BoNT/B LC was determined using a similar microplate assay with a fluorescent synaptobreven peptide as substrate. BoNT/B rLC was incubated with varying concentrations of compound 35 at RT and 37 °C for 30 min prior to assay for activity. The IC50 was ~7 μM and independent of temperature (data not shown). The ability of compound 35 to inhibit BoNT/B pLC was also examined in the presence and absence of DTT to determine effect of the dimer form of compound 35 on inhibition. Based on NMR data, approximately 15% of the compound exists as a dimer due to spontaneous formation of disulfide linkages. In the absence of DTT, compound 35 inhibited BoNT/B pLC with an IC50 of 5.4 μM (Fig. 3A). The reduced compound 35 displayed a slightly higher IC50 of 7 μM (Fig. 3B), suggesting that dimerization of compound 35 may increase its potency as an inhibitor.

3.4 Effect of Compound 35 on Molecular Modeling

The binding mode of compound 35 was determined by comparison of various derivatives and their inhibition constants as reported by Anne *et al.* (2003a, 2003b). Crystal structure 1XTG (BoNT/A) was prepared for docking by removing crystallographic waters and the cocrystallized SNAP-25 substrate. It was submitted with Autodock tools to Autodock 3.0.5 on a Silicon Graphics computer at the National Cancer Institute's Advanced Biomedical Computing Center in Frederick, Maryland. The Lamarckian search algorithm was used with default parameters. Twenty potential

dockings were found. Based on reports that the bis-disulfide dimer of compound 35 (compound 4) as well as other dimers of related compounds was equally effective or more effective than the monomers (Anne *et al*., 2003a), the bound conformations of compound 35 for which the free sulfhydryl group interacted with the active site zinc of BoNT were rejected. Since the free amine group in compound 17, a compound 35 analog, was not required for binding (Anne *et al*., 2003b), binding interactions involving this free amine were also rejected. The resulting docking revealed one suitable candidate for compound 35 interaction with BoNT/A LC (Fig. 4).

Similar attempts to dock compound 35 to BoNT/B LC were unsuccessful, most likely due to the absence of the hydrophobic biphenyl binding pocket (present in BoNT/A) in all BoNT/B crystal structures. However, a brief survey of the other serotypes shows that this region is highly flexible and crystallizes in a number of positions, even within the same serotype. As a result, a homology model of BoNT/B LC was constructed from a structure of the BoNT/B LC (1S0F) where residues 243-265 were forced into a homologous conformation occupied by residues 236-259 of the BoNT/A crystal structure 1XTG (Fig. 5). Two residues of the modified 1S0F (K432 and I433) were manually rotated to accommodate the new structure. Y372 was also manually rotated to accommodate compound 35 initial docking. R67 was slightly rotated to remove close contacts. In this model, BoNT/B LC adopts a similar conformation to the compound 35-docked BoNT/A LC. However, steric hindrance from Y54 and a less hydrophobic biphenyl binding pocket are apparent in the active site of BoNT/B LC. Both BoNT/A and BoNT/B compound 35 dockings display the binding of the active site zinc by the carboxyl of compound 35's toluyl group. Compound 35 cyclizes along its major axis, and the toluyl aromatic ring contacts the benzyl aromatic ring on the other end. While the sulfhydryl does not participate in the binding, it does create steric hindrance for the rotation of the toluyl group, thus stiffening the molecule. The major binding contributions from compound 35 are the shape of its cyclized main axis which fills the active site and the binding of the biphenyl group in a relatively hydrophobic pocket. No hydrogen bonds were apparent from either of these dockings. Based on the docking results, compound 35 is predicted to be a better inhibitor of BoNT/A than BoNT/B, supporting the experimental results.

Figure 1. ESI-MS analysis of compound 35. Compound 35 was analyzed by ESI-MS in the absence (A) or presence (B) of the reducing agent TCEP. Mass-to-charge ratios are shown on the x-axis and percent intensity is shown on the y-axis.

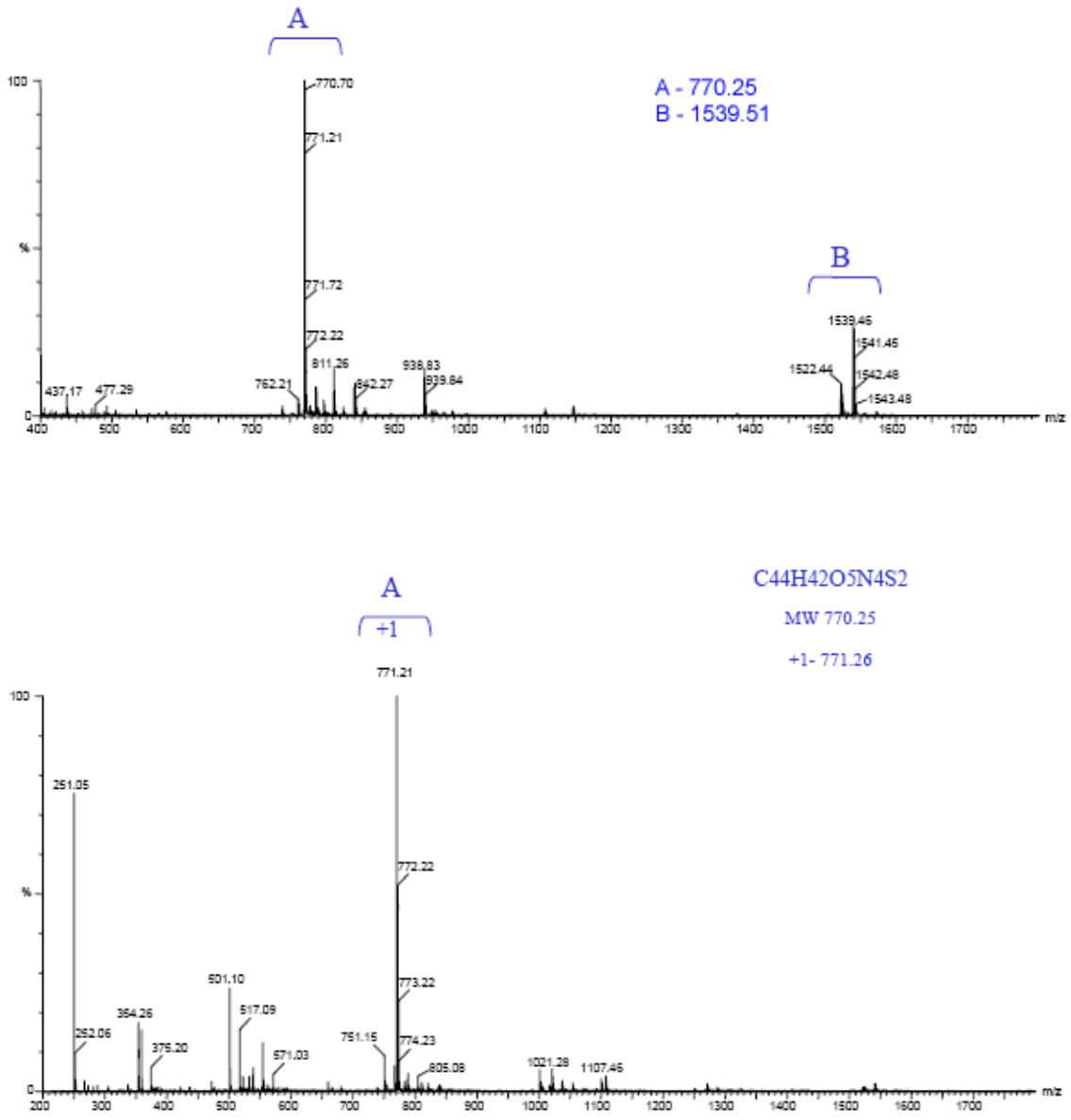


Figure 2. Compound 35 inhibition of BoNT/A rLC. BoNT/A rLC was incubated with varying concentrations of compound 35 at RT for 30 min prior to enzymatic analysis. Error bars represent the standard deviation for four replicates. GraphPad Prism version 3.02 was used to fit the data with the sigmoidal dose-response equation (IC50 = 1.1 μM, R^2 = 0.9948).

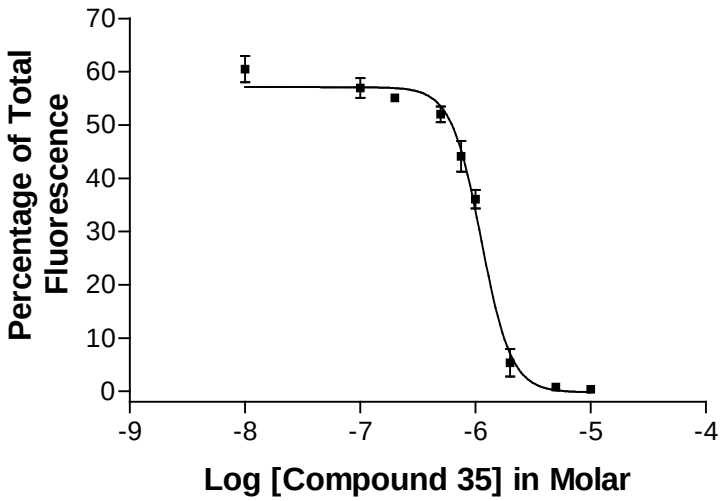
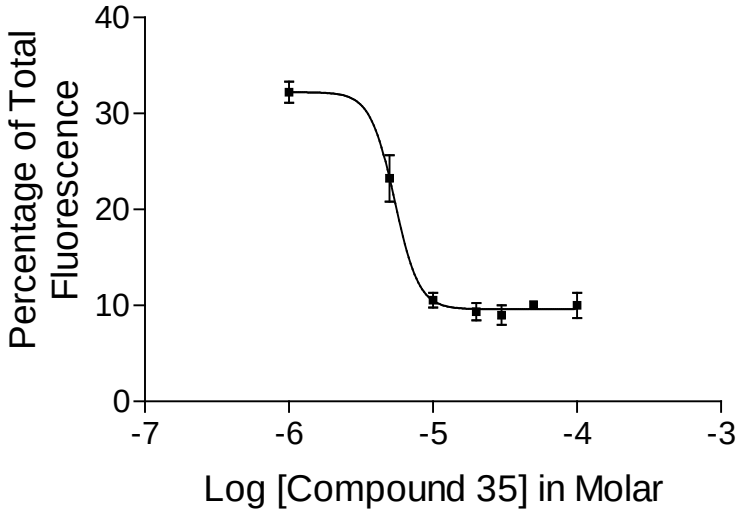


Figure 3: Compound 35 inhibition of BoNT/B pLC in the absence and presence of DTT. BoNT/B pLC was incubated with varying concentrations of compound 35 in the absence (A) or presence (B) of 100 μM DTT at 37 °C for 30 min prior to the microplate assay. Error bars represent the standard deviation of four replicates. GraphPad Prism version 3.02 was used to fit the data with the sigmoidal dose-response equation. The IC50 values of compound 35 were 5.4 μM (R^2 = 0.9983) and 7 μM (R^2 = 0.9983) in the absence and presence of DTT respectively.



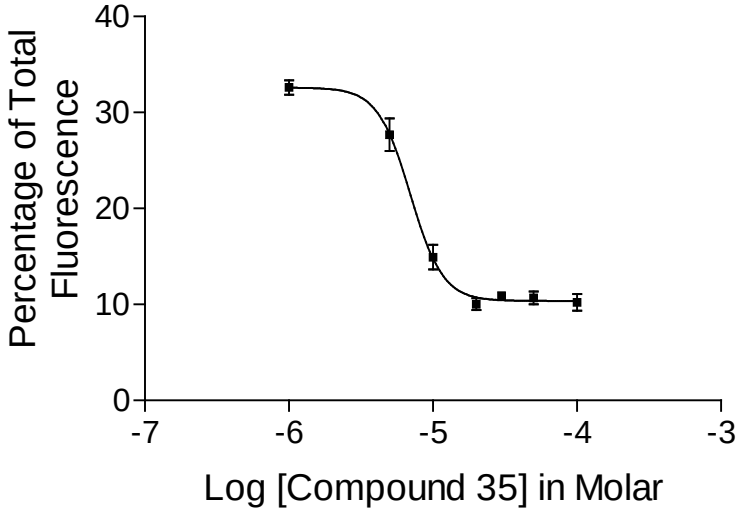


Figure 4. Depiction of the active site of BoNT/A LC (from structure 1XTG) with compound 35 docked using autodock. Residues displayed are within 2.5 Åof compound 35. The active site zinc atom is rendered CPK and colored magenta. Compound 35 is rendered ball and stick, while all atoms are colored by atom type. Note the near cyclization of the toluyl and benzyl groups of compound 35 and the stacked aromatic ring interaction of the biphenyl group with Y249 of the LC.

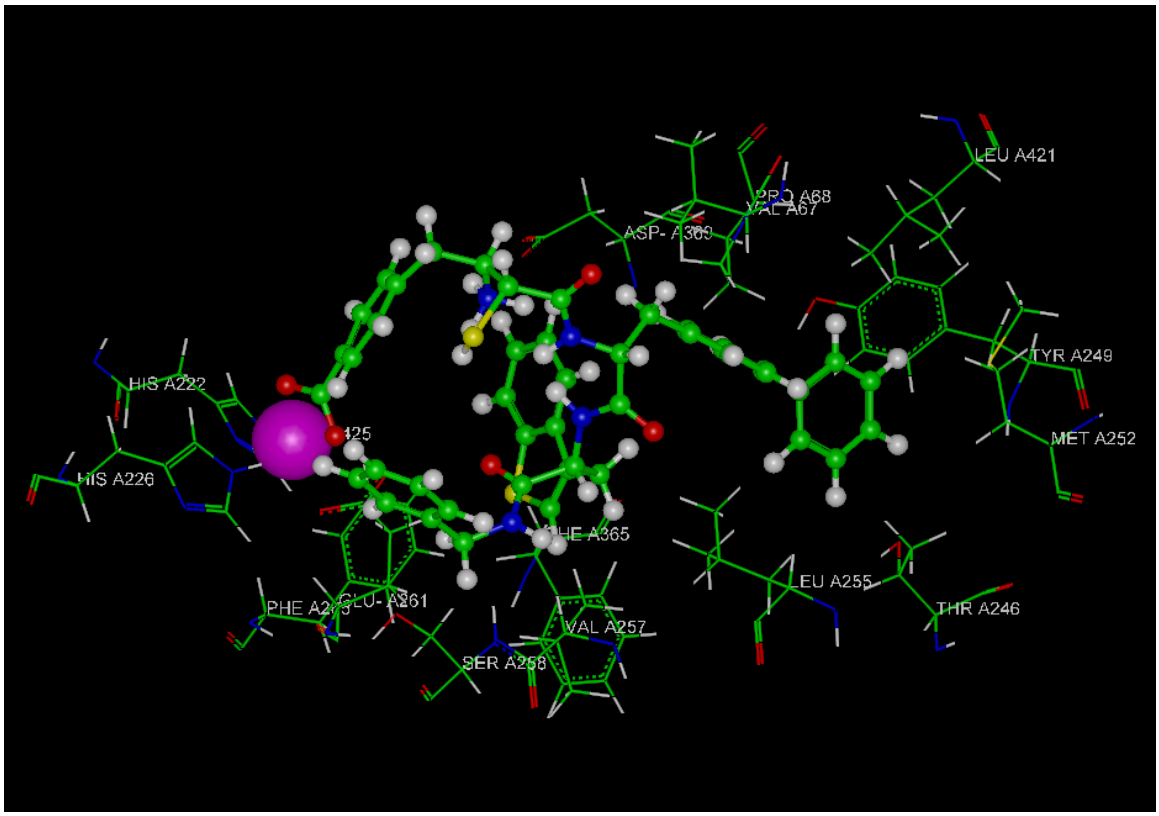
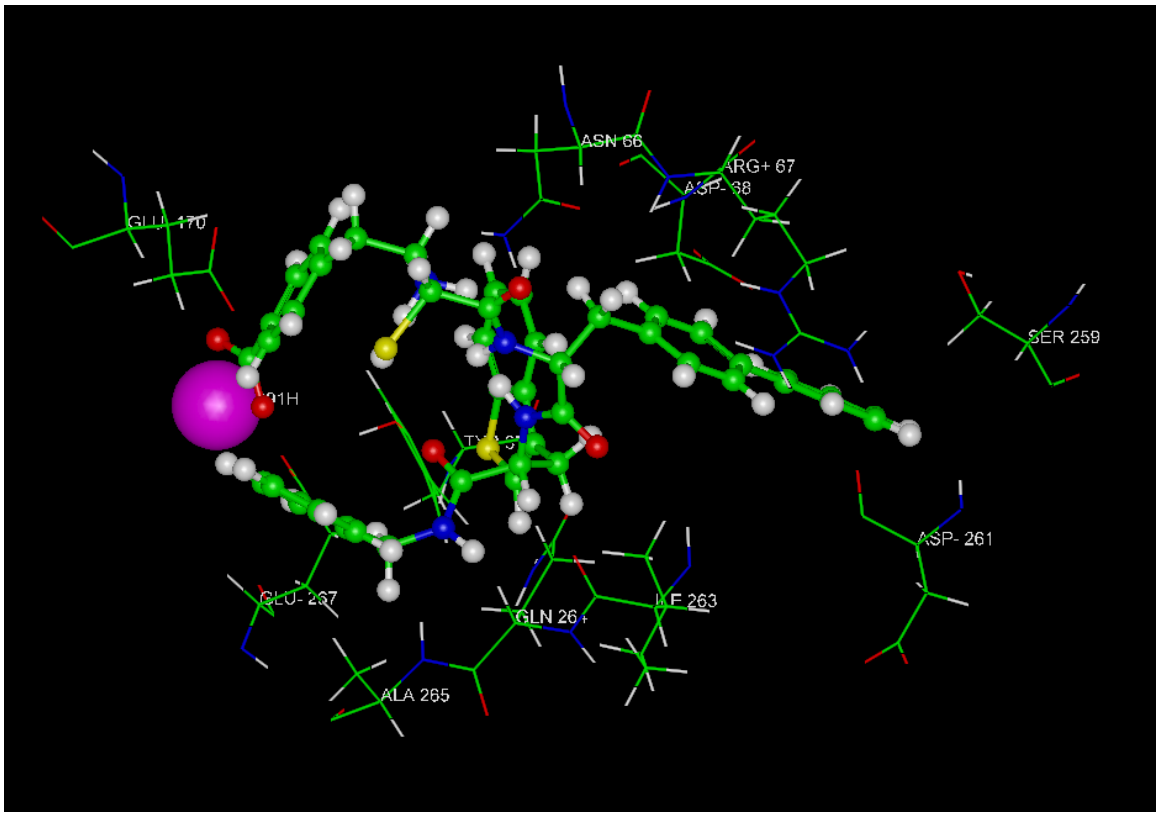


Figure 5. Depiction of the active site of BoNT/B LC (from structure 1S0F) which is modeled by homology to structure 1XTG as described in Results section 3.4. Coloring and rendering are identical to Figure 4 above. Note, the biphenyl group does not have close binding partners as in the docking into 1XTG. This may be a result of the homology modeling. However, R67 intrudes into the biphenyl binding pocket, most likely weakening the binding energy.



4. DISCUSSION

A promising candidate, designated as compound 35, was synthesized according to methods published in Roques' laboratory (Anne *et al.*, 2003a, b; Blommaert *et al.*, 2004) and evaluated for its ability to inhibit the metalloprotease activities of BoNT/A and BoNT/B LCs. De novo synthesis was attempted, since large quantities of the compound would be needed for subsequent evaluation for safety and efficacy in cell culture systems (Sheridan *et al.*, 1999) and in animal models (Adler *et al.*, 2001). Initial tests of compound 35 as reported here were encouraging, since it was effective against the two serotypes that produce most human outbreaks of botulism (Shapiro *et al.*, 1998). Efficacy against multiple serotypes is important since it will greatly facilitate drug development (Adler *et al*., 2001).

Our results with inhibition of BoNT/A LC by compound 35 were similar to those reported by Anne *et al*. (2003a). However, for inhibition of BoNT/B LC, there is a large numerical discrepancy between the present results and those published by Anne *et al.* (2003a, b). The latter authors reported that compound 35 inhibits BoNT/B LC in the range of 10-20 nM, whereas in the current study, the IC50 was determined to be 5.4-7 μM, depending on conditions (Fig. 3). The reasons for this discrepancy are not clear but are currently being investigated. The most direct way to resolve this difference is a mutual exchange and testing of compound 35 samples in each laboratory.

In spite of this unresolved issue, compound 35 may still prove to be an effective inhibitor of BoNT/B, since drug development is complex and potency is only one of several criteria. Thus an effective BoNT inhibitor must be able to penetrate the nerve terminal membrane, be free of acute and chronic toxicity and persist for as long as the toxin remains active in the nerve terminal (Adler *et al*., 2001; Keller and Neale, 2001; Simpson, 2004). Thus far, we have confirmed efficacy for compound 35 in a cell-free system. Due to the charges on compound 35 from carboxyl and amino groups, compound 35 is unlikely to be membrane permeable. Membrane permeability can be enhanced, however, by removing the amine group, which Anne *et al*. (2003b) found not to be required for activity. Although the other charged group (carboxyl) is essential for activity and cannot be removed, it can be reversibly shielded. The most reasonable approach is to generate an acetyoxymethylester derivative, which would mask the negative charge, allow membrane permeation, and be removed by esterases in the cytoplasm. This strategy has been successfully used for allowing internalization of negatively charged calcium indicators (Ayman *et al.*, 2001). Analogs of compound 35 with increased membrane permeability are currently under synthesis and will be tested in primary spinal cord cells (Sheridan *et al*., 1999) and in isolated mouse phrenic nerve hemidiaphragm preparations (Adler *et al*., 1995) for efficacy and toxicity. If these compounds are found to be effective and non-toxic, analogs of compound 35 may form the basis for a future pharmacological treatment for botulinum intoxication.

5. FUTURE DIRECTIONS

One important inference that can be drawn from the table of compound 35 analogs is that monomers that are dimerized across their free sulfhydryls are better inhibitors of BoNT/B. Since the free sulfhydryl makes a major contribution to these poorly water soluble compounds, the dimers are expected to have even less aqueous solubility. Based on the modeling studies, the sulfhydryl group is most likely free and can be easily derivatized. Derivatizing compound 35 with Oregon Green 488 (Molecular Probes, CA) would improve its properties without compromising its ability to inhibit botulinum toxins. This derivative would improve the calculated logD to ~2 at neutral pH. This would make the compound more water soluble and more amenable to cocrystallization. In addition, the presence of the Oregon Green 488 fluorescent tag, in conjunction with deletion of the amine group and shielding of the carboxyl group, would allow for optical monitoring of the inhibitor in BoNT-intoxicated cells.

6. REFERENCES

Adler M, Scovil J, Parker G, Lebeda F, Piotrowski J, Deshpande SS. (1995) Antagonism of botulinum toxin-induced muscle weakness by 3,4-diaminopyridine in rat phrenic nerve-hemidiaphragm preparations. Toxicon 33, 527-537.

Adler M, Oyler GA, Keller JE, Lebeda FJ (2001) Pharmacological countermeasures for botulinum intoxication. In: Somani S, Romano J, eds. Chemical Warfare Agents: Toxicity at Low Levels. Boca Raton: CRC Press, 373-391.

Ahmed SA and Smith, LA (2000) Light chain of botulinum A neurotoxin expressed as an inclusion body from a synthetic gene is catalytically and functionally active. J Protein Chem 19, 475–487.

Anne C, Blommaert A, Turcaud S, Martin AS, Meudal H, Roques BP (2003a) Thio-derived disulfides as potent inhibitors of botulinum neurotoxin type B: implications for zinc interaction. Bioorg Med Chem 11, 4655-4660.

Anne C, Turcaud S, Quancard J, Teffo F, Meudal H, Fournie-Zaluski MC, Roques BP (2003b) Development of potent inhibitors of botulinum neurotoxin type B. J Med Chem 46, 4648-4656.

Ayman S, Gibson A, McFadzean I, Reynolds M and Wallace P. (2001). Inhibition of capacitative calcium entry is not obligatory for relaxation of the mouse anococcygeus by the NO/cyclic GMP signalling pathway. Brit J Pharmacol 132, 807-814.

Binz, T, Blasi J, Yamasaki S, Baumeister A, Link E, Sudhof TC, Jahn R, Neimann, H (1994) Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. J Biol Chem 269, 1617-1620.

Blommaert A, Turcaud S, Anne C and Roques BP (2004) Small tripeptide surrogates with low nanomolar affinity as potent inhibitors of the botulinum neurotoxin B metallo-proteolytic activity. Bioorg Med Chem 12, 3055-3062.

Dolly JO, Ashton AC, McInnes C, Wadsworth JDF, Poulain B, Tauc L, Shone CC, Melling J (1990) Clues to the multi-phasic inhibitory action of botulinum neurotoxins on release of transmitters. J Physiol (Paris) 84, 237-246.

Keller JE, Neale EA (2001) The role of the synaptic protein SNAP-25 in the potency of botulinum neurotoxin type A. J. Biol Chem 276, 13476-13482.

Middlebrook JL (1995) Protection strategies against botulinum toxin. Adv Exp Med Biol 383, 93-98.

Montecucco C and Schiavo G (1993) Tetanus and botulism neurotoxins: a new group of zinc proteases. Trends Biochem Sci 18, 324-327.

Roblot P, Roblot F, Fauchere JL, Devilleger A, Marechaud R, Breux JP, Grollier G, Becq-Giraudon B (1994) Retrospective study of 108 cases of botulism in Poitiers, France. J Med Microbiol 40, 379-384.

Schmidt JJ, Stafford RG, Millard CB (2001) High-throughput assays for botulinum neurotoxin proteolytic activity: serotypes A, B, D, and F. Anal Biochem 296, 130-137.

Schoch S, Deak F, Konigstorfer A, Mozahayeva M, Sara Y, Sudhof TC and Kavalali ET. (2001) SNARE function analyzed in synaptobrevin/VAMP knockout mice. Science 294, 1015-1016.

Shapiro RL, Hatheway C and Swerdlow DL (1998) Botulism in the United States: a clinical and epidemiological review. Ann Intern Med 129, 221-228.
Sheridan RE, Deshpande SS, Smith T (1999) Comparison of *in vivo* and *in vitro* mouse assays for botulinum toxin type A. J Appl Toxicol 19, S29-S33.
Simpson LL (1989) Peripheral actions of the botulinum toxins. In: Simpson LL, ed. Botulinum Neurotoxin and Tetanus Toxin. New York: Academic Press, 153-178.
Simpson LL (2004) Identification of the major steps in botulinum toxin action. Annu Rev Pharmacol Toxicol 44, 167-193.
Singh BR and DasGupta BR (1989) Changes in the molecular topography of the light and heavy chains of type A botulinum neurotoxin following their separation. Biophys Chem 34, 259-267.
Tonello F, Morante S, Rossetto O, Schiavo G and Montecucco C (1996) Tetanus and botulism neurotoxins: a novel group of zinc-endopeptidases. Adv Exp Ned Biol 389, 251-260.