Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 1523–1526

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis of Fluorescent Probes Directed to the Active Site Gorge of Acetylcholinesterase

Jennifer R. Saltmarsh,[a] Aileen E. Boyd,[b] Oscar P. Rodriguez,[a] Zoran Radić,[b] Palmer Taylor[b] and Charles M. Thompson[a,c,*]

[a]*Department of Chemistry, The University of Montana, Missoula, MT 59812, USA*
[b]*Department of Pharmacology 0636, University of California, San Diego, La Jolla, CA 92093-0636, USA*
[c]*Department of Pharmaceutical Sciences, The University of Montana, Missoula, MT 59812, USA*

Received 6 March 2000; accepted 25 April 2000

Abstract—Six organophosphorus compounds linked to fluorophore groups were prepared in an effort to selectively modify the active site of acetylcholinesterase and deliver probes to the gorge region. Two compounds that vary by the length of a methylene (CH_2) group, pyrene-$SO_2NH(CH_2)_nNHC(O)CH_2CH_2P(O)(OEt)(F)$ (where $n=2$ or 3) were found to be potent, irreversible inhibitors of recombinant mouse AChE ($K_i \cong 10^5$ M^{-1} min^{-1}). Size exclusion chromatography afforded a fluorescently-labeled cholinesterase conjugate.

Introduction

The crystal structure of acetylcholinesterase (AChE),[1] the enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh),[2] has provided many insights into key structural elements and motifs central to its catalytic mechanism and mode of ACh processing. One of the more interesting structural features of AChE revealed from the crystal structure and predicted in part by fluorescence experiments[3] is the presence of a long, narrow hydrophobic gorge approximately 20 Å deep leading to the active site. Key to understanding the remarkably high turnover rate and specific activity of AChE is the manner in which substrates and inhibitors traverse this passageway to reach the catalytic center. Selective placement of a reporter group into the gorge region would help elucidate the properties and dynamics of this unique portal. The aim of this work was to design, synthesize, and evaluate the anticholinesterase activity of pyrene- or dansyl-containing organophosphates[3] that covalently attach to the active site serine and place fluorescent reporter groups within the gorge region. As such, the resultant fluorescence data would permit examination of solvent exposure and segmental motion of the reporter group.

Design

The primary objective was to develop and evaluate AChE inhibitor molecules (Fig. 1) capable of anchoring a fluorophore at predictable depths within the protein. To seat the fluorescent probe, an organophosphate (OP) group was selected to covalently modify the active site serine hydroxyl of AChE[4] thereby placing the probe in the gorge region but removed from the immediate vicinity of the catalytic serine. Spacer groups were used to position the reactive phosphorus atom 16–19 Å from the further edge of the fluorophore moiety. Other considerations used in the design of the target molecules (Fig. 1) include the need for: (a) small phosphoester alkyl groups (R = Me, Et) and leaving groups to reduce steric interactions at the active site, (b) spacer variations in length and chemical composition, (c) a non-hydrolyzable linkage tethering the phosphorus group to the fluorophore to obviate post-inhibitory reactions such as aging or

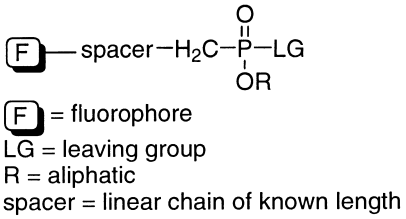
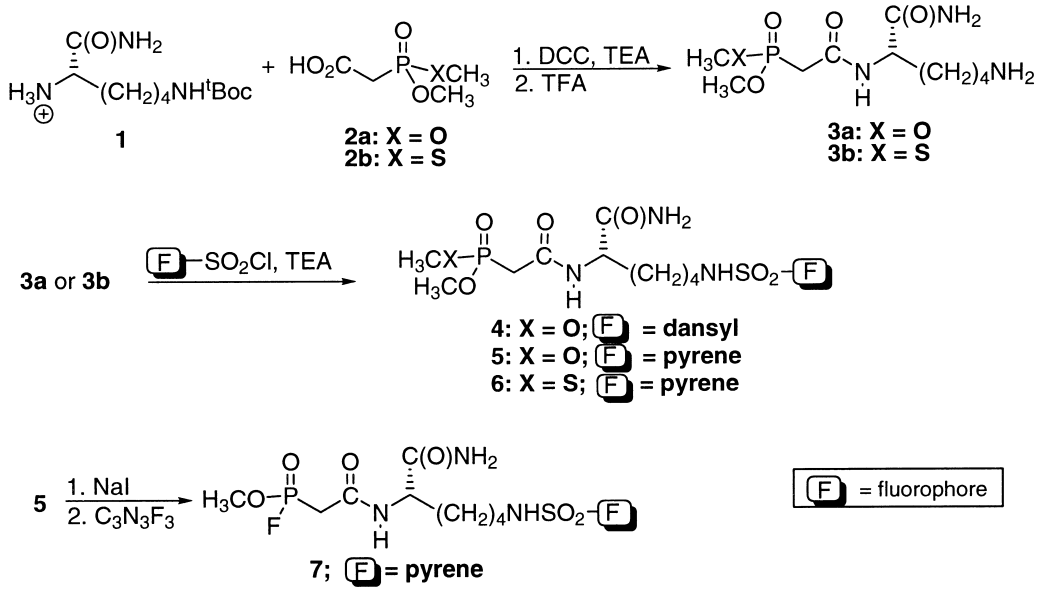


Figure 1. Proposed design for OP-based inhibitors tethered to a reporter group.

*Corresponding author. Tel.: +1-406-243-4643; fax: +1-406-243-4227; e-mail: cmthomp@selway.umt.edu

0960-894X/00/$ - see front matter
PII: S0960-894X(00)00275-4



Scheme 1. Synthesis of OP-based probes (**4**, **5**, **6** and **7**) using lysineamide as the linker.

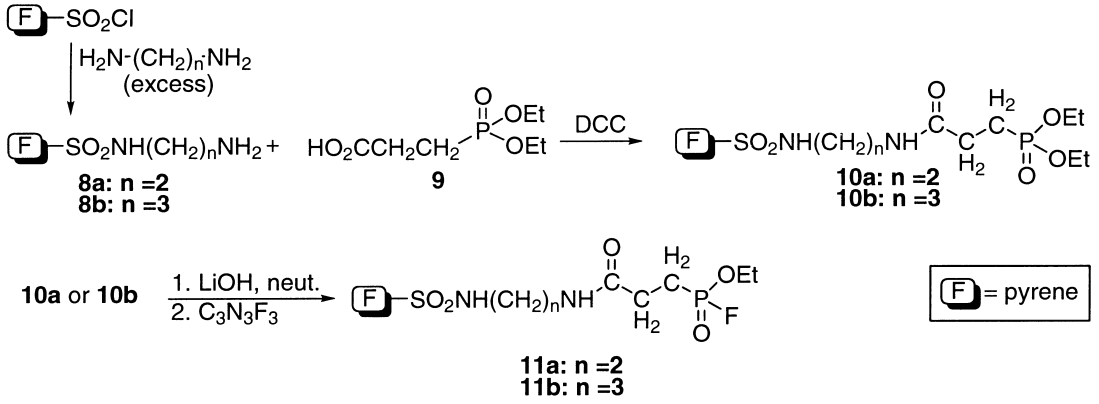
dealkylation and (d) fluorophores which show emission properties characteristic of their immediate chemical environment and segmental motion. Additional bonds between the fluorophore, spacer and OP moieties were planned as sulfonamide/amide to ensure stability and chemical similarity to protein structure.

Synthesis[5]

The first synthesis utilized commercially available *N*(ε)-ᵗBoc-L-lysineamide **1** as the spacer group since attachment of OP and fluorophore to the amine groups would afford probe-containing molecules of suitable length and chemical composition. The syntheses of compounds **4**–**7** are outlined in Scheme 1.

Dimethyl phosphonoacetic acid **2a**[6] was coupled to the amino group of *N*(ε)-ᵗBoc-L-lysineamide hydrochloride using dicyclohexylcarbodiimide (DCC) and triethylamine (TEA). Removal of the ᵗBoc group with trifluoroacetic acid (TFA) and coupling to dansyl chloride or pyrene-sulfonyl chloride in the presence of TEA afforded probe molecules **4** and **5**, respectively. In an identical synthetic sequence, *O*,*S*-dimethyl phosphonoacetic acid **2b**[7] was used to prepare probe **6** in which the SMe moiety was expected to function as the leaving group upon reaction with AChE.[8] Compound **5** was dealkylated at the P-OCH_3 with sodium iodide (NaI, acetone, reflux) and then converted to the phosphonofluoridate **7**[10] with cyanuric fluoride ($C_3N_3F_3$)[9] in DMSO. The conversion of **5** to **7** using cyanuric fluoride was near quantitative and was easily monitored by ^{31}P NMR, namely, the 5 ppm downfield shift (relative to the phosphorus monoacid) and formation of the characteristic P-F coupling constant ($J_{P-F} = 1055$ Hz).

A second synthetic approach was undertaken in an effort to provide additional options in the target molecule length and concurrently circumvent problems in the phosphorylation of AChE by probes **4**–**7** (vide infra). Scheme 2 shows the synthetic route to probes **11a** and **11b** using simple diamine spacer groups linked to pyrene and 3-(diethylphosphono)propanoic acid **9** as the reactive OP precursor.



Scheme 2. Synthesis of OP-based pyrene probes (**11a** and **11b**) using diamines as the linkers.

Pyrenesulfonyl chloride was reacted with excess ethylene diamine or 1,3-diaminopropane to afford pyrenesulfonamido intermediates **8a** and **8b**, respectively. These intermediates were coupled to 3-(diethylphosphono)propanoic acid **9** (prepared from acrylic acid and triethylphosphite)[11] using DCC to afford the 2-carbon spacer product **10a** and three-carbon spacer product, **10b**. The phosphorus ester groups were changed to ethyl in this series to reduce the possibility of post-inhibitory hydrolysis reactions. Hydrolysis of the phosphorus diethyl ester to the monoacid was accomplished with LiOH/THF-H_2O. Use of other base combinations led to either no reaction or hydrolysis of both ester groups. Dealkylation using sodium iodide failed to provide the monoacid. Following neutralization with HCl, the two-carbon spacer and three-carbon spacer monoacids were converted to the phosphonofluoridates **11a** (δ 37.1; $J_{P\text{-}F} = 1057$ Hz) and **11b** (δ 37.3; $J_{P\text{-}F} = 1057$ Hz) using cyanuric fluoride in DMSO. All phosphonofluoridates were generated in DMSO and used directly in inhibition studies without further purification.

Inhibition of AChE by Probes 4–7, 11a and 11b

To demonstrate that the target compounds **4**–**7**, **11a** and **11b** placed the fluorophore portion of the inhibitor into the active site gorge it was necessary to first show that they were covalent inhibitors of AChE.[12] Compounds **5**, **6** and **7** bear identical spacers and pyrene fluorophores but vary in the phosphorus leaving group. As a result, differences were expected in the K_i (inhibition rate constant) despite the fact that identical inhibitor-AChE conjugates would result. While modest anticholinesterase inhibition was achieved against recombinant mouse acetylcholinesterase (mAChE)[13] by compounds **5**–**7** in the 5–40 μM range, they were virtually ineffectual against electric eel acetylcholinesterase (EEAChE). Inhibition of mAChE by compounds **6** and **7** led to apparent reactivation (spontaneous scission of the serine-phosphate bond)[14] that may be due to noncovalent interactions between these inhibitors and mAChE or alternatively, spontaneous hydrolysis. The dansyl analogue **4** was also a weak inhibitor indicating that the fluorophore size was not a major influence in the poor inhibition profiles of **5**–**7**.

Branching at the spacer group (lysine chiral center) of compounds **4**–**7**, which could diminish entry and access to the active site, was seen as one potential problem to overcome. Additionally, the phosphonoacetate group contains an enolizeable methylene that could both reduce the phosphoryl reactivity and increase reactivation. Two variations were made in the construction of **11a** and **11b** to address these problems. Aliphatic diamine spacer groups replaced lysine to eliminate branching and 3-phosphonopropanoic acid was used in place of phosphonoacetic acid to reduce enolization. Compounds **11a** and **11b** ($K_i = 1.0 \times 10^5$ $M^{-1}min^{-1}$) were found to be excellent irreversible inhibitors of wild-type (WT) mAChE. These values compare well with related phosphonate inhibitors.[15] Molecular modeling[16] of the chain-extended forms of these two inhibitor probes indicates that the interatomic distances from phosphorus atom to fluorophore edge are 17.8–18.1 and 19.3–19.4 Å, respectively. Since the diameter of the pyrene group is approximately 7.0 Å, inhibitors **11a** and **11b** center the fluorophore group at average distances of 14.4 and 15.9 Å from the active site and 4 to 5.5 Å internal to the outer rim of the gorge. Despite excellent inhibitory activity against WT mAChE, compounds **11a** and **11b** were poor inhibitors of EEAChE. We are currently exploring this species-dependent difference.

To demonstrate that these new inhibitors became covalently conjugated to the enzyme and resulted in an extrinsically fluorescent enzyme, as designed, an excess of **11b** was reacted with recombinant mAChE until >95% inhibition was achieved. Free inhibitor was separated from phosphonylated enzyme by means of size exclusion chromatography (Sephadex G-25), and the fractions containing protein were pooled and analyzed spectroscopically. Fractions containing protein showed an absorbance maxima at 352 nm in a ratio to the protein aromatic side-chain absorption suggesting near stoichiometric conjugation. Furthermore, fluorescence emission spectra upon excitation at 352 nm yielded the characteristic emission profile of pyrene compounds.

Conclusion

Novel inhibitor structures that selectively position a fluorophore group into the gorge region of AChE have been synthesized. Studies are currently underway using inhibitors **11a** and **11b** to probe the environment and dynamics of the gorge region of AChE using fluorescence techniques.

Acknowledgements

C.M.T. thanks the University of Montana Sponsored Research Programs Award, the National Science Foundation (MCB-9808372 and CHE-9807469) and National Institutes of Health (NS38248) for financial support. The NMR used in this study was purchased with funding from the NSF (CHE9302468). P.T. thanks the United States Public Health Service (GM 18360) and the Department of Army Medical Defense (17-1-8014) for financial support. A.E.B. was supported by predoctoral fellowships from the Lucille P. Markey Charitable Trust and ASERT.

References and Notes

1. (a) Sussman, J. L.; Harel, M.; Frolow, F.; Oefner, C.; Goldman, A.; Toker, L.; Silman, I. *Science* **1991**, *253*, 872. (b) Bourne, Y.; Taylor, P.; Marchot, P. *Cell* **1995**, *83*, 503.
2. For reviews of acetylcholinesterase see: (a) Quinn, D. *Chem. Rev.* **1987**, *87*, 955. (b) Taylor, P. In *The Pharmacological Basis of Therapeutics, 9th Edition*; McGraw Hill: New York, 1996; pp 161–176. (c) Rosenberry, T. L. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1975**, *43*, 103.
3. For examples of OP inhibitors containing fluorophore groups see: (a) Steinberg, N.; van der Drift, A. C. M.; Grun-

wald, J.; Segall, Y.; Shirin, E.; Haas, E.; Ashani, Y.; Silman, I. *Biochemistry* **1989**, *28*, 1248. (b) Amatai, G.; Ashani, Y.; Gafni, A.; Silman, I. *Biochemistry* **1982**, *21*, 2060. (c) Berman, H. A.; Taylor, P. *Biochemistry* **1978**, *17*, 1704. (d) Epstein, D. J.; Berman, H. A.; Taylor, P. *Biochemistry* **1979**, *18*, 4749.

4. For reviews of acetylcholinesterase inactivation by organophosphates see: (a) Jarv, J. *Bioorg. Chem.* **1984**, *12*, 259. (b) Ecobichon, D. J. In *Pesticides and Neurological Diseases*; CRC: Boca Raton, 1982: pp 151–203. (c) Eto, M. *Organophosphorus Pesticides; Organic and Biological Chemistry;* CRC: Florida, 1974. (d) Fukuto, T. R. *Bull. Wld. Health Org.* **1971**, *44*, 31.

5. All new compounds were fully characterized by spectral and elemental or mass spectral analyses.

6. Dimethyl phosphonoacetic acid was prepared by acid hydrolysis (CF_3CO_2H, rt, 4–6 h) of *tert*-butyl dimethyl phosphonoacetate (Aldrich).

7. *O*,*S*-Dimethyl phosphonoacetic acid was prepared from *tert*-butyl dimethyl phosphonoacetate using the following sequence in 40% overall yield: (1) thionation with Lawesson's reagent (toluene, reflux, 24 h), (2) $EtOC(S)S^-K^+$/MeOH reflux then CH_3I, (3) CF_3CO_2H, rt, 4–6 h. For representative experimentals see: Thompson, C. M.; Frick, J. A.; Natke, B. E.; Hansen, L. K. *Chem. Res. Toxicol.* **1989**, *2*, 386.

8. For examples of thiolates as leaving groups see: (a) Thompson, C. M.; Fukuto, T. R. *J. Agric. Food Chem.* **1982**, *30*, 282. (b) Thompson, C. M. In *Organophosphorus Insecticides: Chemistry, Fate and Effects*; Academic: San Diego, 1992, pp 19–46. (c) Thompson, C. M.; Berkman, C. E.; Ryu, S.; Jackson, J. A.; Quinn, D. A.; Larsen, A. In *Rev. Pestic. Toxicol.*; Toxicology Communications: Raleigh, 1993; Vol. 2, pp 131–146.

9. (a) Olah, G. A.; Nojima, M.; Kerkes, I. *Synthesis* **1973**, 487. (b) Kokotos, G.; Noula, C. *J. Org. Chem.* **1996**, *61*, 6994. (c) Carpino, L. A.; Sadat-Aalaee, D.; Chao, H. G.; DeSelms, R. H. *J. Am. Chem. Soc.* **1990**, *112*, 9651.

10. NOTE: Phosphonofluoridates are likely to be neurotoxic and should only be handled by qualified personnel in a well-ventilated hood. The anticholinesterase activity of the organophosphates described in this study can be destroyed by stirring the compounds with 1 M NaOH. For a general review of phosphonofluoridate nerve gas agents see: Benshop, H. P.; De Jong, L. P. A. *Acc. Chem. Res.* **1988**, *21*, 368.

11. Dixon, H. B. F.; Sparkes, M. J. *Biochem. J.* **1974**, *141*, 715.

12. Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherston, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88.

13. Bourne, Y.; Taylor, P.; Bougis, P. E.; Marchot, P. *J. Biol. Chem.* **1999**, *274*, 2963.

14. Ecobichon, D. J. In *Pesticides and Neurological Diseases*; CRC: Boca Raton, 1982; pp 151–203.

15. Hosea, N.; Berman, H. A.; Taylor, P. *Biochemistry* **1995**, *34*, 11528.

16. Spartan Pro, Wavefunction Inc., Irvine, CA. Modeling of active site probes was conducted with S_P-enantiomers which directs the smaller substituent to the acyl pocket and the phosphoryl oxygen to the oxyanion hole.