Ethyl Octylphosphonofluoridate and Analogs: Optimized Inhibitors of Neuropathy Target Esterase

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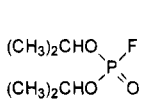
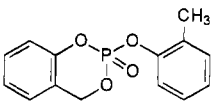
The relation between organophosphorus-induced delayed neuropathy (OPIDN) and brain neuropathy target esterase (NTE) inhibition is further examined in hens by structure–activity studies leading to the most potent in vitro NTE inhibitors known, which are then examined for their neuropathic effects in vivo in hens. The principal compounds studied are alkyl alkylphosphonofluoridates and dialkyl phosphorofluoridates. Potencies that exceed those of any previous inhibitors under the standard in vitro NTE assay condition are achieved with alkyl octylphosphonofluoridates (ethyl, isopropyl, 2-chloroethyl, 2-bromoethyl, 2-iodoethyl, and 3-iodopropyl), 2-iodoethyl hexylphosphonofluoridate, and dialkyl phosphorofluoridates [ethyl, nonyl; di(2-iodoethyl); di(3-iodopropyl); dipentyl]. The concentration for 50% NTE inhibition (I_{50}) of these compounds is 0.04–0.14 nM. Thirty-eight less active analogs including aryl phosphonates and aryl phosphates give I_{50}s of 0.27–4730 nM. For highest potency the summation of length of the alkyl and alkoxy groups on phosphorus should be 12–16 atoms (carbons, oxygens, and phosphorus) (a terminal iodo substituent in this relationship is equivalent to a propyl group). In general, the phosphonofluoridates and phosphorofluoridates are more active than analogs with leaving groups other than fluorine, *i.e.*, phenoxy, 4-nitrophenoxy, 4-cyanophenoxy, 3,4-dichlorophenoxy, and 4*H*-1,3,2-benzodioxaphosphorin. Considering the exceptional potencies of ethyl and 2-iodoethyl octylphosphonofluoridates (I_{50}s of 0.04 and 0.09 nM, respectively), it is not surprising that at ip doses of 10–30 mg/kg they inhibit brain NTE by 82–97% 48 h after treatment. However, unexpectedly, only the ethyl but not the 2-iodoethyl compound induces OPIDN, possibly associated with the greater ease of aging for NTE inhibited with the ethyl than the 2-iodoethyl compound (as observed in vitro both spontaneously and on induction by potassium fluoride). The high potency of ethyl octylphosphonofluoridate and several analogs as NTE inhibitors suggests that they are useful probes in determining the toxicological features of this secondary lesion for organophosphorus poisoning.

Introduction

Knowledge of the mechanisms by which organophosphorus (OP)[1] compounds induce a type of delayed neuropathy (OPIDN), involving inhibition of neuropathy target esterase (NTE), is based primarily on studies with two types of toxicants differing considerably in structure. The first type includes alkyl alkylphosphonofluoridates and dialkyl phosphorofluoridates which are nerve gases or model compounds thereof, as exemplified by diisopropyl phosphorofluoridate (DFP) (*1–3*). The second class consists of 2-substituted-4*H*-1,3,2-benzodioxaphosphorin 2-oxides (2-substituted-BDPOs) such as the 2-(2-methylphenoxy) analog, which is the metabolic activation product of tri-*o*-cresyl phosphate (TOCP) (*4–6*). DFP is only moderately active as an in vitro inhibitor of NTE, with a concentration for 50% inhibition (I_{50}) of 566–700 nM (*7, 8*), whereas the TOCP metabolite is considerably more active (I_{50} 29–60 nM) (*5, 9*). Both compounds induce OPIDN signs in hens at ip or im doses of 0.25–5 mg/kg (*8, 10*).



DFP



TOCP metabolite

Several studies indicate the importance of the chain length of alkyl and alkoxy groups on phosphorus in conferring activity for OPIDN. In the homologous series of dialkyl phosphorofluoridates, the optimal potency is achieved with propyl and butyl, which are active at 0.25–0.5 mg/kg (*8*). The enhancing effects of extended dialkyl chain lengths are also evident in the dialkyl 2,2-dichlorovinyl and 4-nitrophenyl phosphates and asymmetric alkyl 4-nitrophenyl alkylphosphonates, although none of the compounds examined show an I_{50} of <1 nM (*8, 11*). The potency of dialkyl phosphates for OPIDN and NTE inhibition is influenced not only by the chain length but also by the presence of 2-chloroethyl substituents (*8, 12*). However, the effects of haloalkyl moieties have not been systematically examined.

Induction of OPIDN requires a high level of NTE inhibition in vivo, in most cases coupled with permanent block of the enzyme active site; *i.e.*, aging of phosphorylated NTE, usually involving dealkylation, appears to be a critical step whereas reactivation of the inhibited

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[1] Abbreviations: BDPO, 4*H*-1,3,2-benzodioxaphosphorin 2-oxide; CI, chemical ionization; DFP, diisopropyl phosphorofluoridate; EI, electron impact; I_{50}, concentration for 50% inhibition; NTE, neuropathy target esterase; OP, organophosphorus; OPIDN, organophosphorus-induced delayed neuropathy; TOCP, tri-*o*-cresyl phosphate.

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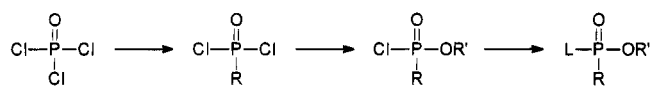

Figure 1. Synthetic pathway for phosphorofluoridates and aryl phosphates. Phosphonofluoridates and aryl phosphonates were similarly prepared from $RP(O)Cl_2$. L = F, aryloxy; R = alkyl, phenyl, alkoxy; R′= alkyl, aryl.

enzyme by dephosphorylation is associated with the absence of OPIDN signs (*13*). It is therefore important to evaluate new toxicants for both inhibitory potency (I_{50}) and propensity to undergo reactivation or aging of the inhibited enzyme.

The present study, designed to better understand OPIDN, involves three steps: optimize the NTE inhibitors by synthesis of higher alkyl homologs and haloalkyl analogs of phospho- and phosphorofluoridates to possibly achieve enhanced potency in vitro; compare the effectiveness of the optimized ethyl and 2-iodoethyl octylphosphonofluoridates as inhibitors of NTE in vivo and delayed neurotoxicants; evaluate the specificity of these OPs as NTE inhibitors in terms of reactivation or aging of the phosphorylated enzyme.

Materials and Methods

Caution: *The OP compounds described herein are known or suspected to be acute and/or delayed neurotoxicants. Their preparation and use should be under careful containment conditions. They can be destroyed by treatment with aqueous NaOH.*

Spectroscopy. NMR spectra were recorded for $CDCl_3$ solutions with a Bruker AM-300 spectrometer. Chemical shifts (δ) are reported for ^1H at 300 MHz relative to internal tetramethylsilane and for ^{31}P at 121.5 MHz relative to external trimethyl phosphate. Mass spectra were acquired by GC/MS with the Hewlett-Packard 5971A or 5985B instrument in the electron impact (EI) mode (70 eV, 200 °C) or the chemical ionization (CI) mode (200 eV, methane, 0.8 Torr, 130 °C).

Syntheses (Figure 1). The synthesis routes were selected to minimize side reactions that might yield inhibitory impurities, but this possibility cannot be ruled out in all cases.

For synthesis of the title compound ethyl octylphosphonofluoridate (**12**), a solution of octylphosphonic dichloride (231 mg, 1 mmol) in methylene chloride (10 mL) was cooled to −60 °C with a dry ice−acetone bath, and then ethanol (46 mg, 1 mmol) and triethylamine (101 mg, 1 mmol) in methylene chloride (3 mL) were added slowly. The mixture was stirred for 30 min at room temperature followed by addition of 20% KF aqueous solution (10 mL) containing benzyltriethylammonium chloride (~10 mg) and further reaction for 1 h. The methylene chloride phase was separated and dried (Na_2SO_4), and after filtration and evaporation, the residual oil was purified on a single TLC plate (silica gel, 20 × 20 × 0.1 cm) to give **12** (R_f = 0.55 band faintly evident on solvent evaporation). Yield: 160 mg, 0.71 mmol, 71%.

Other phosphonofluoridates (**1**−**11** and **13**−**21**) were synthesized similarly using the required alkyl- or phenylphosphonic dichloride and alcohol (or phenol). In the same manner, phosphorofluoridates **22** and **25**−**32** were prepared by mixing phosphorus oxychloride with 2 equiv of the corresponding alcohol and triethylamine, whereas **23** and **24** were synthesized from ethyl phosphorodichloridate and equivalent alcohol and triethylamine. Alkylphosphonic dichlorides that were not commercially available were synthesized in two ways: by reaction of triethyl phosphite with appropriate 1-haloalkanes to form diethyl alkylphosphonates which were hydrolyzed to phosphonic acids and chlorinated with phosphorus pentachloride (*14*); by reaction of alkyl chlorides with phosphorus trichloride and aluminium chloride and then with H_2O, as described before (*5*).

Aryl phosphonates **33**−**40** and aryl phosphates **41**−**45** were synthesized by analogous methods to those described above for the phospho- and phosphorofluoridates, respectively, except in the last step, instead of aqueous KF, equivalent amounts of the corresponding phenol and triethylamine were used as methylene chloride solutions.

BDPO analogs **46**−**49** were prepared by reaction of 2-hydroxybenzyl alcohol with equivalent phosphorus oxychloride in methylene chloride in the presence of 2 equiv of triethylamine to give 2-chloro-BDPO, whose chloro substituent was then replaced by an alkoxy group on reaction with equivalent amounts of the corresponding alcohol and triethylamine (*5*). Phenoxy-, phenyl-, and octyl-BDPOs used in this study were from previous preparations (*5*).

The yields were 55−74% for phosphonofluoridates, 41−58% for phosphorofluoridates, 47−63% for aryl phosphonates, and 39−54% for aryl phosphates. The structure of each OP compound used for bioassay was confirmed by ^1H and ^{31}P NMR and GC/MS analyses, and the purity was estimated to be >97% on the basis of ^1H and ^{31}P NMR integration.

Buffers. 50 mM Tris−0.2 mM EDTA was adjusted to pH 8.0 with 12 M HCl, and then a portion was mixed with 50 mM citric acid−0.2 mM EDTA to obtain pH 5.2 or 6.0; these buffers are subsequently identified only by pH.

Assay of NTE Inhibition in Vitro. Frozen hen brains (Pel-Freez Biologicals, Rogers, AR, stored at −80 °C) were thawed, and a homogenate was prepared at 10% (w/v) in pH 8 buffer as the NTE source. NTE was considered to be that portion of the phenyl valerate-hydrolyzing activity which was insensitive to paraoxon but sensitive to mipafox. The OP inhibitory potency as $I_{50} \pm$ SD (based on 2−4 replicates) was determined by the standard NTE assay procedure (*15*) which involves the following: incubation of the homogenate and an OP test compound (added in acetone at 2% final concentration) for 20 min at pH 8 and 37 °C in the presence of paraoxon (40 μM) (sample A) or of paraoxon (40 μM) plus mipafox (50 μM) (sample B); an additional 15 min incubation with the substrate phenyl valerate; determination of the liberated phenol by coupling with 4-aminoantipyrine and absorbance measurement at 510 nm; calculating NTE activity as the difference between samples A and B; comparing controls with preparations containing the test OP to determine the percent inhibition.

The OP compounds examined are irreversible inhibitors acting by phosphorylating the active site of NTE, and as such their I_{50} values are dependent on incubation time (*7*), which is held constant in the present study at the standard protocol time of 20 min (*15*).

Assay of NTE Inhibition in Vivo and OPIDN. Adult hens (1.4−1.9 kg) were administered individual test compounds at 3, 10, 20, and 30 mg/kg by the ip route with corn oil (1 mL/kg) as the carrier vehicle. Control birds received corn oil only. Each hen was housed in a separate cage with food and water available *ad libitum*. For determining NTE inhibition in vivo, control and OP-treated birds were decapitated 2 days after treatment and the whole brains were homogenized and assayed immediately as above. NTE activity in vivo for OP treatments is given as percentage compared to that for the corresponding control birds based on 2−3 replicate assays. The signs of OPIDN were rated in another group of hens at 15 days after treatment.

Spontaneous Reactivation. Hen brain homogenate was preincubated for 0.5 or 24 h with an OP test compound at 3 times its I_{50} value before performing the standard NTE assay as above. Compound **15** was also tested at higher concentrations. NTE activity was related to the corresponding control at each time period using three replicate assays. By assuming the inhibition was complete at 0.5 h, the increase of NTE activity by 24 h was considered to be spontaneous reactivation. For compounds **12** and **15**, the assay frequency was increased and the maximal preincubation time was extended to 4 days to determine the initial rate and ultimate degree of reactivation of NTE activity.

KF-Promoted Reactivation. The previously-established procedure (*2*) was used with some modifications. Hen brain homogenate was incubated at pH 8 with 200 μM benzenesulfonyl fluoride (in place of paraoxon) for 20 min and then with pH 6.0 buffer only (P) or this buffer plus mipafox (M) (200 μM) for 5 min; excess inhibitor was removed by centrifugation (30000*g*, 20 min) and adding back pH 5.2 buffer. Pairs of P and M samples were incubated consecutively under the following conditions: 10 min with either acetone only or an OP in acetone

Table 1. Phosphonofluoridates, Phosphorofluoridates, Aryl Phosphonates, and Aryl Phosphates Studied and Their NMR and MS Data and Potency as Neuropathy Target Esterase Inhibitors in Vitro

no.	LP(O)(R)OR′ L	R	R′	NMR, ppm ^{31}P	^{1}H(POCH_2)	CI-MS, m/z base ion	MH$^+$ (int)	NTE $I_{50} \pm$ SD, nM
			Phosphonofluoridates[a]					
1	F	$ClCH_2$	nC_9H_{19}	9.8, 18.7	4.31	133	259 (5)	2570 ± 35
2	F	C_2H_5	C_2H_5	26.1, 34.9	4.27	141	141 (100)	82 ± 1
3	F	C_2H_5	ClC_2H_4	26.7, 35.5	4.41	175	175 (100)	18 ± 1
4	F	C_2H_5	BrC_2H_4	26.5, 35.4	4.46	219	219 (100)	10 ± 2
5	F	C_2H_5	IC_2H_4	26.2, 35.0	4.40	155	267 (1)	5.0 ± 0.1
6	F	C_2H_5	nC_5H_{11}	25.9, 34.7	4.19	113	183 (21)	4.6 ± 0.1
7	F	C_2H_5	nC_7H_{15}	26.0, 34.8	4.18	113	211 (10)	1.3 ± 0.1
8	F	C_2H_5	nC_9H_{19}	26.0, 34.8	4.18	113	239 (12)	0.31 ± 0.01
9	F	nC_4H_9	IC_2H_4	25.4, 34.2	4.42	155	295 (25)	0.28 ± 0.02
10	F	nC_4H_9	C_6H_5	20.8, 29.8		217	217 (100)	0.27 ± 0.01
11	F	nC_6H_{13}	IC_2H_4	25.4, 34.3	4.41	155	323 (18)	0.13 ± 0.01
12	F	nC_8H_{17}	C_2H_5	25.2, 34.0	4.26	225	225 (100)	0.04 ± 0.01
13	F	nC_8H_{17}	ClC_2H_4	25.8, 34.7	4.40	259	259 (100)	0.07 ± 0.01
14	F	nC_8H_{17}	BrC_2H_4	25.7, 34.6	4.46	303	303 (100)	0.11 ± 0.02
15	F	nC_8H_{17}	IC_2H_4	25.3, 34.2	4.39	155	351 (78)	0.09 ± 0.01
16	F	nC_8H_{17}	iC_3H_7	24.1, 32.9	4.90(POCH)	197	239 (12)	0.05 ± 0
17	F	nC_8H_{17}	$I(CH_2)_3$	25.5, 34.4	4.22	197	365 (76)	0.12 ± 0.01
18	F	nC_8H_{17}	$TsOC_2H_4$	25.9, 34.4	4.36	91[b]	394 (4)[b]	0.92 ± 0.10
19	F	C_6H_5	IC_2H_4	10.3, 19.0	4.47	155	315 (18)	17 ± 2
20	F	C_6H_5	$(CH_3)_3CCH_2$	10.2, 18.8	3.92	161	231 (2)	4.5 ± 0.1
21	F	$C_6H_5CH_2$	IC_2H_4	18.3, 27.3	4.30	155	329 (23)	0.29 ± 0.03
			Phosphorofluoridates[a]					
22	F	C_2H_5O	C_2H_5	−15.4, −7.3	4.27	157	157 (100)	206 ± 14
23	F	C_2H_5O	IC_2H_4	−16.4, −8.3	4.20, 4.36	155	283 (42)	0.99 ± 0.01
24	F	C_2H_5O	nC_9H_{19}	−15.3, −7.2	4.18, 4.28	129	255 (8)	0.07 ± 0.01
25	F	ClC_2H_4O	ClC_2H_4	−16.5, −8.4	4.30	225	225 (100)	1.6 ± 0.1
26	F	BrC_2H_4O	BrC_2H_4	−16.8, −8.6	4.35	107	315 (42)	0.66 ± 0.02
27	F	IC_2H_4O	IC_2H_4	−17.3, −9.1	4.39	155	409 (1)	0.14 ± 0.02
28	F	nC_3H_7O	nC_3H_7	−15.2, −7.1	4.15	101	185 (31)	3.7 ± 0.1
29	F	$I(CH_2)_3O$	$I(CH_2)_3$	−15.8, −7.7	4.27	169	473 (5)	0.09 ± 0.01
30	F	$nC_5H_{11}O$	nC_5H_{11}	−15.1, −7.1	4.17	101	241 (28)	0.12 ± 0.01
31	F	$nC_7H_{15}O$	nC_7H_{15}	−15.1, −7.1	4.17	57	297 (8)	0.96 ± 0.05
32	F	$nC_9H_{19}O$	nC_9H_{19}	−15.1, −7.1	4.17	127	353 (7)	13 ± 1
			Aryl Phosphonates					
33	$4CNC_6H_4O$	nC_4H_9	IC_2H_4	28.4	4.35	394	422 (15)	168 ± 26
34	$3,4Cl_2C_6H_3O$	nC_4H_9	nC_4H_9	28.1	4.10	283	339 (77)	136 ± 4
35	$4NO_2C_6H_4O$	nC_4H_9	CH_3	29.6	3.83(POCH_3)	274	274 (100)	4020 ± 726
36	$4NO_2C_6H_4O$	nC_4H_9	C_2H_5	28.2	4.17	57	288 (9)	4100 ± 690
37	C_6H_5O	nC_8H_{17}	C_2H_5	27.3	4.17	94[b]	298 (10)[b]	480 ± 110
38	C_6H_5O	nC_8H_{17}	C_6H_5	23.8		94[b]	346 (26)[b]	320 ± 20
39	$4NO_2C_6H_4O$	nC_8H_{17}	C_2H_5	28.3	4.20	217[b]	343 (4)[b]	2.3 ± 0.8
40	$4NO_2C_6H_4O$	nC_8H_{17}	$4NO_2C_6H_4$	25.6		139[b]	436 (8)[b]	5.6 ± 0.8
			Aryl Phosphates					
41	$4NO_2C_6H_4O$	BrC_2H_4O	BrC_2H_4	−10.5	4.51	109[b]	433 (0.5)[b]	645 ± 36
42	$4NO_2C_6H_4O$	IC_2H_4O	IC_2H_4	−10.9	4.40	155[b]	527 (0)[b]	88 ± 25
43	$4NO_2C_6H_4O$	$nC_5H_{11}O$	nC_5H_{11}	−9.2	4.17	220[b]	359 (0.7)[b]	82 ± 2
44	$4NO_2C_6H_4O$	$nC_7H_{15}O$	nC_7H_{15}	−9.2	4.16	220[b]	415 (0.2)[b]	958 ± 25
45	$4NO_2C_6H_4O$	$nC_9H_{19}O$	nC_9H_{19}	−9.2	4.17	220[b]	471 (0.1)[b]	4730 ± 12
46	OC_6H_4-2-CH_2O		C_2H_5	−11.8	4.26, 5.35	122[b]	214 (32)[b]	1590 ± 99
47	OC_6H_4-2-CH_2O		ClC_2H_4	−12.1	4.40, 5.38	249	249 (100)	37 ± 1
48	OC_6H_4-2-CH_2O		BrC_2H_4	−12.2	4.45, 5.40	293	293 (100)	21 ± 1
49	OC_6H_4-2-CH_2O		IC_2H_4	−12.5	4.37, 5.38	186[b]	340 (0.5)[b]	10 ± 1

[a] The two numbers for the ^{31}P NMR chemical shift represent a P−F doublet (coupling constant ~1000 Hz). [b] Data given as base ion and M$^+$ from EI-MS.

for inhibition of NTE; 20 min with a 25-fold excess volume of buffer (pH 5.2) for aging of the inhibited NTE; 20 min with a 2.5-fold excess volume of either pH 5.2 buffer only or the same buffer that contained KF (200 mM final concentration) for reactivation of the inhibited but not aged NTE. After centrifugation (30000g, 1 h), the pellets were resuspended in buffer (pH 8.0), further preincubated if specifically stated, and finally assayed with phenyl valerate for 15 min to determine NTE activities, which were calculated as percentages compared to the corresponding controls based on 2−4 replicates. The increased NTE activities for samples treated with KF in comparison with their paired KF-free samples were considered to be due to reactivation by KF. That portion of the initially-inhibited NTE that was not reactivatable by KF was referred to as aged NTE.

Housefly Toxicity Assays. Adult female houseflies (*Musca domestica* L., SCR strain, ~20 mg/each) were treated with the test OP compounds as solutions in measured drops of acetone (0.22 μL) applied to the ventrum of the abdomen. Piperonyl butoxide (PB) pretreatment was performed similarly at 5 μg/fly and 2 h prior to the OP compounds. LD_{50} values were determined after 24 h at 25 °C.

Results

NMR and MS Characterization (Table 1). Phosphonofluoridates **1**−**21** and phosphorofluoridates **22**−**32** show characteristic ^{31}P doublets with chemical shifts in the range of 10−35 ppm and −7 to −17 ppm, respectively, and large P-F coupling constants (~1000 Hz). The ^{31}P

Table 2. Effects of Alkyl Chain Length and Halogen Substituents on Potency of Alkyl and 2-Haloethyl Phosphonates and Phosphates as NTE Inhibitors

	NTE I_{50}, nM						
	R′ = alkyl				R′ = 2-haloethyl		
structure	C_2H_5	nC_5H_{11}	nC_7H_{15}	nC_9H_{19}	ClC_2H_4	BrC_2H_4	IC_2H_4
One OR′ Substituent							
$FP(O)(C_2H_5)OR'$	82	4.6	1.3	0.31	18	10	5.0
OC_6H_4-2-$CH_2OP(O)OR'$	1591	11[a]	1.7[a]	0.89[a]	37	21	10
Two OR′ Substituents							
$FP(O)(OR')_2$	206	0.12	0.96	13	1.6	0.66	0.14
$4NO_2C_6H_4OP(O)(OR')_2$	>100 000[b]	82	958	4730	10 000[b]	645	88

[a] Data from ref 5. [b] Data from ref 8.

signals of aryl phosphonates **33**–**40** and aryl phosphates **41**–**49** appear as singlets in regions analogous to those of the corresponding phosphonofluoridate and phosphorofluoridate series, respectively. Protons in the $POCH_2$ moiety of these molecules resonate at 3.9–4.5 ppm, except for those in the ring of BDPO analogs which appear at 5.4 ppm. More generally, the ^1H NMR spectra of all of the compounds agree well with their structures in terms of chemical shifts and signal area integration.

The MS data tabulated reflect the variety of structural features of the OPs examined, with intensities for MH^+ or M^+ ranging from 0% to 100% and with m/z of the base ion appearing as 20–100% of that for the parent molecule. Small MH^+ or M^+ peaks are often observed for chemicals having one or two 2-iodoethyl or 4-nitrophenyl substituents or when determinations are made in the EI mode, probably due in each case to low compound volatility, thereby requiring higher temperatures for vaporization leading to decomposition.

Potency of Phosphonofluoridates 1–21 and Phosphorofluoridates 22–32 as NTE Inhibitors in Vitro (Table 1). In the phosphonofluoridate series, the alkyl ethylphosphonofluoridates (**2**–**8**) increase in potency (*i.e.*, have lower I_{50}s) with increasing alkyl chain size and length from 82 nM for the ethyl analog to 0.31 nM for the nonyl compound whereas the (chloromethyl)phosphonofluoridate (**1**) is of very low activity (2570 nM) even with the nonyl substituent. Butyl- and hexylphosphonofluoridates **9** and **11**, respectively, are 18- and 38-fold more potent than the ethylphosphonofluoridate analog (**5**), and another butylphosphonofluoridate (**10**) is also quite active. Seven compounds in the octylphosphonofluoridate series are characterized by very high potency for the ethyl and haloalkyl compounds (0.04–0.12 nM) (**12**–**17**) or moderately active for the tosyl derivative (**18**). The most active analog is ethyl octylphosphonofluoridate (**12**) with an I_{50} of 0.04 nM. Phenylphosphonofluoridates **19** and **20** are much less active than benzylphosphonofluoridate **21**.

Relative to the phosphorofluoridates, the ethyl series (**22**–**24**) increases in potency with chain length. The di-(2-haloalkyl) compounds are more potent with increasing halogen size (**25**–**27**) and chain length (**29**). The effect of chain length is clearly illustrated in the dialkyl series, with a marked potency increase for propyl (**28**) to pentyl (**30**), then a decrease for heptyl (**31**) and nonyl (**32**).

Potency of Aryl Phosphonates 33–40 and Aryl Phosphates 41–49 as NTE Inhibitors in Vitro (Table 1). Butylphosphonates **33**–**36** and octylphosphonates **37** and **38** are of low activity, possibly due to short chain length (**35**, **36**) or poor leaving group (**33**, **34**, **37**, **38**). Nitrophenyl compounds **39** and **40** give improved I_{50}s of 2–6 nM compared with their phenyl analogs **37** and **38**. Nitrophenyl phosphates **41**–**45** are of low activity (I_{50}s 82–4730 nM) as is ethoxy-BDPO (**46**), but the 2-haloethoxy-BDPOs (**47**–**49**) are more effective (I_{50}s 10–37 nM).

Table 3. Potency of Ethyl and 2-Iodoethyl Octylphosphonofluoridates as NTE Inhibitors in Vivo and Delayed Neurotoxicants

compound (no.)	dose, mg/kg	NTE inhibition in vivo, %[a]	OPIDN rating[b]
$FP(O)(nC_8H_{17})OC_2H_5$ (**12**)	3	47, 56	−, −
	10	82, 86, 87	−, ++
	20	89, 92	++, ++,[c] ++[c]
$FP(O)(nC_8H_{17})OC_2H_4I$ (**15**)	30	88, 91, 97	−, −, −, −
phenoxy-BDPO	3	79, 82, 50,[d] 81[d]	++, ±,[d] +[d]

[a] Individual hens 2 days after ip administration. [b] Individual hens 15 days after ip administration: −, normal; ±, occasional stumbling and wing-drooping; +, staggering gait, landing awkwardly when dropped from 60-cm height, resting often; ++, stand on limbs, move by shuffling on hocks. [c] Data for 30 mg/kg in the same experiment. [d] Data from ref 5.

Effects of Alkyl Chain Length and Halogen Substituents on Potency of Alkyl and 2-Haloethyl Phosphonates and Phosphates as NTE Inhibitors (Table 2). The optimal alkyl (R′) chain length among the series examined depends on the other substituents of the molecule, *i.e.*, nonyl in the BDPOs and ethylphosphonofluoridates with one OR′ substituent and pentyl in the dialkyl phosphorofluoridates and nitrophenyl phosphates with two OR′ substituents. The potency of the 2-haloethyl compounds always increases in the order Cl < Br < I, and interestingly, in every case the iodoethyl and pentyl analogs are of almost identical potency, *i.e.*, the iodo substituent is equivalent to a propyl moiety in conferring activity.

Potency of Ethyl and 2-Iodoethyl Octylphosphonofluoridates as NTE Inhibitors in Vivo and Delayed Neurotoxicants (Table 3). Ethyl and 2-iodoethyl octylphosphonofluoridates were compared for NTE inhibition in vivo and OPIDN in hens. As a positive control, phenoxy-BDPO inhibited hen brain NTE activity by 50–82% and induced OPIDN at 3 mg/kg. At the same dose, ethyl octylphosphonofluoridate (**12**) was less active and inhibited NTE by 47–56% and did not produce OPIDN. However, **12** is clearly neuropathic at 10 and 20 mg/kg, which inhibited NTE by 82–87% and 89–92%, respectively. The iodoethyl analog (**15**) at 30 mg/kg, although inhibiting NTE by 88–97%, surprisingly did not yield OPIDN signs.

Spontaneous Reactivation of NTE Inhibited by Alkyl and Haloalkyl Phosphono- and Phosphorofluoridates. The structural features of the fluorophosphorus compounds greatly influence the ease of spontaneous reactivation during the period of 0.5–24 h following NTE inhibition (Table 4). 2-Iodoethyl phosphonofluoridates **11**, **15**, **19**, and **21** generally result in more extensive spontaneous reactivation than octylphosphonofluoridates without an iodoethyl substituent (**12**–**14**,

Table 4. Spontaneous and KF-Promoted Reactivation of NTE Inhibited by Alkyl and Haloalkyl Phosphono- and Phosphorofluoridates

no.	FP(O)(R)OR′ R	R′	reactivation, % ± SD spontaneous[a]	KF promoted[b]
		2-Iodoethyl Phosphonofluoridates		
15	nC_8H_{17}	IC_2H_4	41 ± 3[c]	47 ± 9
11	nC_6H_{13}	IC_2H_4	27 ± 5	36 ± 6
19	C_6H_5	IC_2H_4	27 ± 8	
21	$C_6H_5CH_2$	IC_2H_4	13 ± 6	
		Octylphosphonofluoridates		
14	nC_8H_{17}	BrC_2H_4	9 ± 1	
13	nC_8H_{17}	ClC_2H_4	6 ± 3	27 ± 1
18	nC_8H_{17}	$TsOC_2H_4$	5 ± 3	
12	nC_8H_{17}	C_2H_5	4 ± 2	−9 ± 2
		Other Iodoalkyl Phosphono- and Phosphorofluoridates		
17	nC_8H_{17}	$I(CH_2)_3$	2 ± 4	
29	$I(CH_2)_3O$	$I(CH_2)_3$	−3 ± 5	
27	IC_2H_4O	IC_2H_4	−12 ± 2	−8 ± 3

[a] Spontaneous reactivation refers to percent of control NTE activity at 24 h minus that at 0.5 h. OP concentrations are 3 times the I_{50} in standard NTE assay, which gave 33−63% of control NTE activity at 0.5 h under conditions of spontaneous reactivation assay. [b] KF-promoted reactivation refers to percent of control NTE activity with KF minus that without KF. OP concentrations are 1000 times the I_{50} in standard NTE assay, which gave 2−11% of control NTE activity without KF under conditions of KF-promoted reactivation assay. No KF-induced reactivation occurs with octyl-BDPO, phenyl-BDPO, and FP(O)(nC_4H_9)OC_2H_5 (**10**). [c] Reactivation ranged from 37% to 41% at 3, 6, and 12 times the I_{50}.

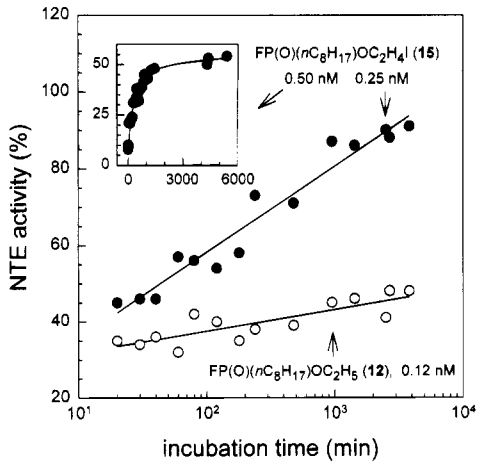

Figure 2. Spontaneous reactivation of NTE inhibited by ethyl octylphosphonofluoridate (**12**, 0.12 nM) and its 2-iodoethyl analog (**15**, 0.25 and 0.50 nM). The log scale is used without mechanistic implications to illustrate rate differences.

18). The di(2-iodoethyl) and 3-iodopropyl compounds (**17, 27, 29**) result in little or no spontaneous reactivation.

Iodoethyl compound **15** at three different concentrations results in similar levels of spontaneous reactivation (37−41%), indicating that some aging also occurs (Table4). The rate and ultimate extent of reactivation were therefore examined in more detail with ethyl and 2-iodoethyl octylphosphonofluoridates. Spontaneous reactivation is about 4-fold greater for the 2-iodoethyl (**15**) compared to the ethyl analog (**12**) (Figure 2). On extending the incubation time, it is apparent that a change in rate takes place after about 50% NTE reactivation at 20 h with little further change in activity after this time, attributable to the competing aging reaction (Figure 2).

KF-Promoted Reactivation of NTE Inhibited by Alkyl and Haloalkyl Phosphono- and Phosphorofluoridates under Various Incubation Conditions. KF-promoted reactivation of NTE, almost completely inhibited by phosphonofluoridates, is most extensive with

Table 5. KF-Promoted Reactivation of NTE Inhibited by Ethyl and 2-Iodoethyl Octylphosphonofluoridates under Various Incubation Conditions

preincubation, min[a]	NTE activity, % 5 min aging −KF	+KF	react.	20 min aging −KF	+KF	react.
	Ethyl Octylphosphonofluoridate (**12**)[b]					
0	8	33	25 ± 5[c]	7	−2	−9 ± 2
60	9	29	20 ± 6	9	9	0 ± 0
180	10	28	18 ± 3	10	11	1 ± 4
	2-Iodoethyl Octylphosphonofluoridate (**15**)[b]					
0	9	97	88 ± 7	6	53	47 ± 9
60	17	79	62 ± 4	11	50	39 ± 6
180	29	79	50 ± 5	23	50	27 ± 2

[a] Time after aging period prior to addition of phenyl valerate for NTE assay. [b] OP concentrations are 1000 times the I_{50} in the standard assay. [c] Mean ± SD.

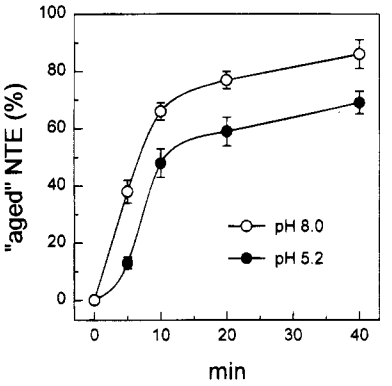

Figure 3. Aging of NTE after various times at pH 5.2 and 8.0 following inhibition by 2-iodoethyl octylphosphonofluoridate (**15**).

iodoethyl analog **15** and least with ethyl analog **12** (Table 4). These two compounds were then evaluated for aging of the inhibited NTE assayed as KF-promoted reactivation with various preincubation times (Table 5). The preincubation time does not affect the reactivation with the ethyl phosphonate but has a significant effect with the 2-iodoethyl phosphonate. In the latter case, the longer the preincubation time the less the reactivation, *i.e.*, the greater the aging; a smaller portion can be reactivated with longer aging time (20 vs 5 min). The rate and extent of aging of NTE inhibited with 2-iodoethyl octylphosphonofluoridate, based on KF-promoted reactivatability, are dependent on the pH, with a significantly greater rate at pH 8.0 than 5.2 (Figure 3).

Insecticidal Activity. When applied topically to houseflies, ethyl octylphosphonofluoridate is moderately toxic (LD_{50} 5 μg/g with PB and 15 μg/g without PB), and the potency of the analogs decreases in the order of ethyl (**12**) > chloroethyl (**13**) > bromoethyl (**14**) ≫ iodoethyl (**15**) and tosyloxyethyl (**18**).

Discussion

One goal of this study was to prepare improved probes for NTE by further optimizing the alkyl alkylphosphonofluoridates and dialkyl phosphorofluoridates. This was clearly achieved since 19 of the 49 compounds examined have I_{50} values of <1 nM, which greatly extends the list of inhibitors of such high potency (*5, 6*). More importantly, 11 of these fluoridates have I_{50} values below 0.18 nM [the previous record achieved with nonyl-BDPO (*5*)], and the most potent has an I_{50} of 0.04 nM. This potency may be approaching the theoretical maximum for equimolar reaction of the OP with the enzyme since

the calculated concentration of NTE (155 kDa) under the assay conditions (3.26 mg of brain/mL) falls in the range of 0.009–0.025 nM using data from one study (0.44–1.2 μg of NTE/g brain) (*16*) and 0.034 nM based on results from another experiment (1.6 μg of NTE/g brain) (*17*).

The optimal chain length of the phosphorylating moiety is 12–16 atoms for the summation of carbons, oxygens, and phosphorus in each series; compounds with haloalkyl substituents are a special case considered later. Phosphonofluoridates are generally more potent than phosphorofluoridates, and although less active, the aryl phosphonates are superior to the aryl phosphates. These results are consistent with earlier findings in the BDPO series (*5, 6*). In comparing the phosphorofluoridates with the same or different alkyl substituents, the position of phosphorus in the chain is not a critical factor, suggesting the importance of the overall volume or hydrophobicity of the molecule.

Haloethyl substituents influence the potency in different ways depending on the overall molecular size. With the optimized ethyl octylphosphonofluoridate, addition of a halogen to the ethyl group always reduces potency. In contrast, when the overall length of the alkyl and alkoxy groups on phosphorus is relatively small, the introduction of a halogen increases the potency in the order ethyl < chloroethyl < bromoethyl < iodoethyl. In comparing the 2-haloethyl and alkyl phosphonates and phosphates examined, the corresponding iodoethyl and pentyl derivatives give almost the same I_{50} values, indicating the substituent equivalency of iodo and propyl groups, probably due to their similar steric effects and hydrophobicity (*18*). Analogous relationships may also exist in these series for terminal chloro vs methyl and bromo vs ethyl substituents.

Iodoethyl and ethyl octylphosphonofluoridates are quite similar to each other in potency as NTE inhibitors in vitro and in vivo, yet unexpectedly, the iodoethyl analog gives no neurotoxic signs at a dose 3-fold higher than the neurotoxic dose of the ethyl compound. This apparent anomaly may be related to greater reactivation of NTE inhibited by the iodoethyl than by the ethyl compound based on in vitro comparisons. Enhanced reactivation and decreased aging with the iodoethyl relative to the ethyl compound are probably attributable to steric hindrance conferred by the iodo substituent. The iodoethyl group is thereby protected from cleavage on attack by a nucleophile in an S_N2 mechanism to give the dealkylated and aged form of NTE, consistent with an earlier suggestion for soman-inhibited NTE (*19*).

Aging of inhibited NTE is considered to be an important factor for OPIDN. Aging, measured as resistance to KF reactivation, appears to occur rapidly with BDPO analogs, alkyl phosphonofluoridates, and haloalkyl phosphorofluoridates but not with 2-iodoethyl phosphonofluoridates. Incomplete reactivation with racemic compounds in the latter series may be due to differential aging of NTE inhibited by the two enantiomers, as noted earlier with ethyl 4-nitrophenyl phenylphosphonate (*20*). Findings in this study including the pH effect are generally consistent with previous investigations on different OPs using KF as the reactivating agent (*2, 19*).

Ethyl octylphosphonofluoridate and its high potency analogs are new and useful probes for investigations on NTE inhibition, reactivation, aging, and, most importantly, its physiological functions.

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