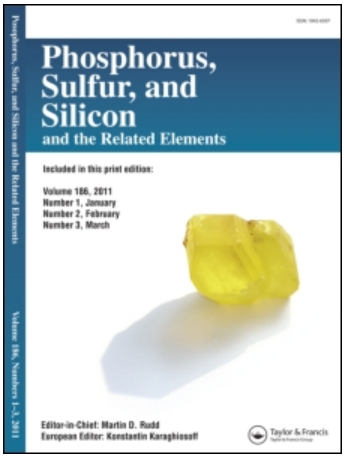

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CHEMICAL AND BIOCHEMICAL STUDIES ON THE CONVERSION OF ALKYLATING AGENTS TO PHOSPHOROTHIOLATES AND THEIR SUBSEQUENT SEQUESTRATION BY CHOLINESTERASES

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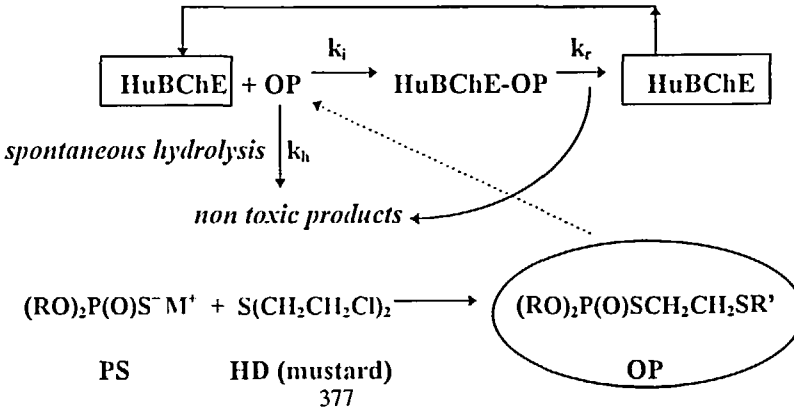
Abstract In endeavor to develop a universal, mild decontamination solution of vesicants and nerve agents, we have examined the possibility of transforming bis(β-chloroethyl)sulfide (sulfur mustard; HD) to an organophosphorus (OP) compound that will have a significant preference for inhibition of human butyrylcholinesterase (HuBChE) compared with acetylcholinesterase (AChE).

Key Words Human butyrylcholinesterase, sulfur mustard, nerve agents, inhibition, reactivation, hydrolysis.

INTRODUCTION

A new strategy for the development of a universal bio-detoxification solution of alkylating agents (e.g., mustards) and anti-AChE poisons (e.g., nerve agents), is based on a mixture of, a) human butyrylcholinesterase (HuBChE), b) suitable reactivator of the phosphoylated enzyme , and c) phosphorothiolate salt that rapidly transforms mustards to non toxic anti-HuBChE compounds. Reactions **a** and **b** depict the chemical and biochemical principles that underlie this approach. The presence of HuBChE together with suitable reactivator permits the reuse of the reactivated enzyme to catalytically hydrolyze both mustards and nerve agents.



EXPERIMENTAL PROCEDURES

The dicyclohexylammonium salt of $(n\text{-PrO})_2P(O)SH$ (I) was prepared according to a general procedure described by Pelchowicz and Leader [1] and recrystalized from ethyl acetate (mp 158°C). δ ^{31}P 52.3 ppm ($CDCl_3$; quintet, $J_{P\text{-}O\text{-}CH} = 7.6$ Hz).

Isolation of products of reaction of I with mustard in aqueous solution.
Bis(β-chloroethyl)sulfide (HD; 20 mg, 126 μmol) was delivered dropwise from a micropipetor to a stirred solution of I (100 mg, 260 μmol) in 40 ml water at ambient temperature. The suspended droplets gradually dissolved with the concomitant appearance of a light precipitate. The mixture was allowed to stand overnight with continuous stirring at room temperature. The precipitate was filtered off and the major components in solution extracted into ethyl acetate and chromatographed on silica gel column using chloroform as the eluent.

NMR spectra were determined with a GN 300WB NMR instrument (General Electric) at 300 (^1H) and 121.65 (^{31}P) MHz. ^1H and ^{31}P NMR chemical shifts were assigned to TMS and trimethyl phosphate, respectively. MS was performed on a VG 70SEQ hybrid mass spectrometer, using the 'in beam' probe introduction technique.

Inhibition and reactivation of cholinesterases. The bimolecular rate constants of the inhibition of HuBChE and fetal bovine serum acetylcholinesterase (FBS-AChE) was determined as described before [2]. Inhibitor's stock solutions (0.4 mM) were made in CH_3CN, and the actual concentration was determined by measuring the amount of thiol released in 0.1 N NaOH [3]. Enzyme solution was made in 50 mM phosphate buffer pH 8.0. Reactivation of OP-ChE conjugates was initiated by dilution of the inhibited enzyme into 0.1 M of 2-(hydroximinomethyl)-1-methylpyridinium methanesulfonate (P2S), or mono-isonitrosoacetone (MINA) in phosphate buffer, pH7.30-7.95, at 25°C.

RESULTS AND DISCUSSION

Elucidation of the structure of (n-PrO)$_2$P(O)S-mustard adducts.
The reaction of I with mustard gave two major products (II and III) in high yield. Purification to homogeneity was achieved by column chromatography. The front peak was identified as the bis-phosphoryl mustard II followed by the mono-phosphoryl adduct III.

$$(CH_3CH_2CH_2O)_2P(O)S\underset{a}{CH_2}\underset{b}{CH_2}S\underset{b}{CH_2}\underset{a}{CH_2}SP(O)(OCH_2CH_2CH_3)_2$$

II

$$(CH_3CH_2CH_2O)_2P(O)S\underset{a'}{CH_2}\underset{b'}{CH_2}S\underset{c}{CH_2}\underset{d}{CH_2}OH$$

III

FIGURE 1. Structures of bis- (II) and mono-(III) phosphorylated mustard.

Thus, ^{31}P NMR chemical shifts of **II** (δ, 24.16 ppm) and of **III** (δ, 24.44 ppm), is consistent with alkylkation of the P-SH moiety of the starting material **I** (δ, 52.3 ppm).

The analysis of the ^{1}H NMR spectra revealed essentially similar chemical shifts and line multiplicity for both compounds with one major difference: the multiplicity of one set of the two methylene lines assigned to protons *a* and *b* in the symmetric molecule **II**, was reduced in **III**, as expected, to a first order triplets (Fig. 2, hydrogens *c* and *d*).

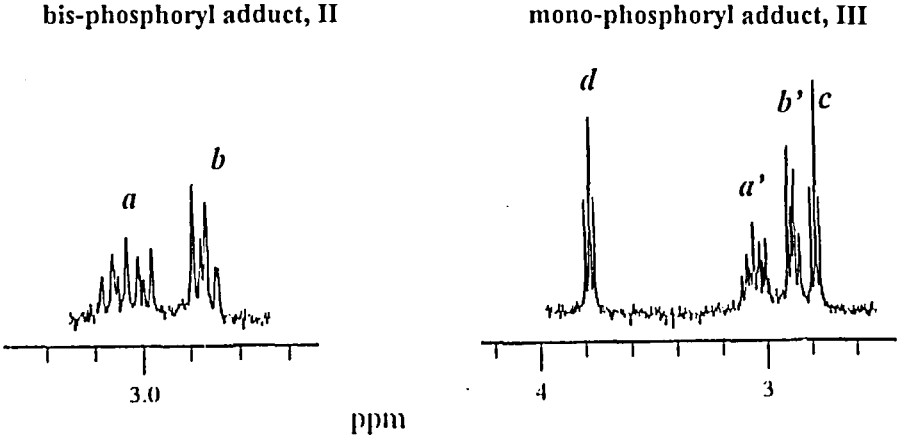


FIGURE 2. Excerpt from ^{1}H NMR spectra of **II** and **III**. Lines assignment is in accordance with Fig. 1.

MS (CI) showed strong quasi-molecular ions $[M+H]^+$ of m/z of 483 and 303 for **II**, and **III**, respectively. When either **II** or **III** were transferred to 2% NaOD, the ^{31}P NMR signal shifted upfieled (δ, -2.24 ppm), suggesting that the phosphorothiolates were hydrolyzed to the mono-acid $(CH_3CH_2CH_2O)_2P(O)OH$. All these observations are consistent with the proposed structures of **II** and **III**.

Inhibition and reactivation of HuBChE and FBS-AChE.

The bimolecular rate constants of the inhibition of HuBChE and FBS-AChE by **II** and **III** are summarized in Table I.

TABLE I

Bimolecular rate constants of the inhibition of ChEs by **II** and **III** (k_i, $x10^6$ $M^{-1}min^{-1}$)

Phosphorylated mustard	HuBChE	FBS-AChE	k_{HuBChE}/k_{AChE}
II	69	3.1	22
III	3.8	0.11	35

The potency of the bis-phosphoryl adduct **II** in inhibition of HuBChE and AChE is 18- and 28-fold higher, respectively, than that of **III**, due to structural differences in the S-alkyl side chain. It appears that the second phosphoryl moiety enhances the inhibition relative to **III** by a decrease of approximately 1.9 kcal/mol in energy barrier. This is

attributed to more productive interactions of II compared with III, with amino acids at the entrance to the catalytic gorge. Either inhibitor phosphorylated HuBChE faster than AChE. This selectivity is likely to arise from differences in the amino acid residues that are lining the active site gorge of HuBChE and AChE [4, 5]. The release of constraint in the active site of HuBChE due to replacement of aromatic with aliphatic side chains, suggest that bulky alkyl groups of OP inhibitors are accommodated by HuBChE more comfortably than by AChE.

Reactivation of HuBChE inhibited by II and III, provides evidence that the same OP-HuBChE conjugate was obtained irrespective of the S-alkyl moiety. More than 93% of the phosphorylated enzyme by either inhibitor, were reactivated by 0.1 M P2S at $t_{1/2}$ 5.6 to 5.8 min.

To demonstrate the feasibility of hydrolysis of OPs catalyzed by use of HuBChE-reactivator mixture, molar excess of III over HuBChE was added to enzyme solution containing 0.1 M of either P2S or MINA (Fig. 3A). Despite the large stoichiometric excess of III over the enzyme, the inhibition of HuBChE at steady state did not exceed 70%. Since the hydrolysis of III was < 1%/h, the recovery of enzyme activity suggests that reaction *a* is essentially correct. The time course of enzyme activity shown in Fig. 3A indicates that the overall detoxification rate of a given organophosphoyl-bound moiety, depends on the nature of the reactivator. From the data shown in Fig. 3B it is also evident that the efficacy of the proposed mixture can be enhanced by selecting suitable PS salt.

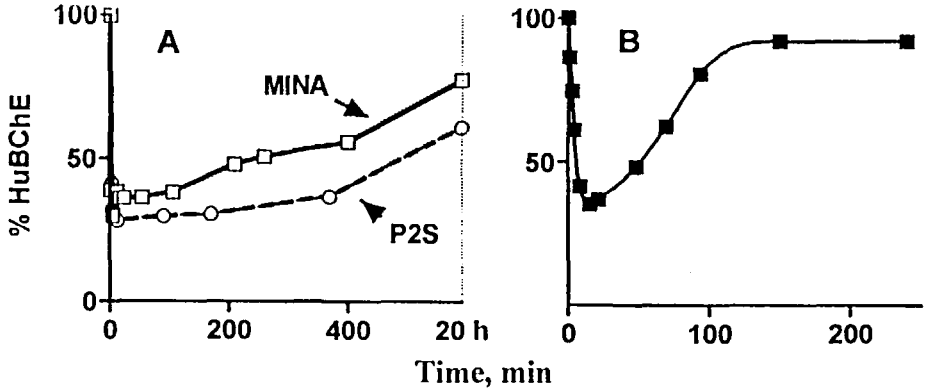


FIGURE 3. Time course of enzyme activity of HuBChE-reactivator solutions following the addition of stoichiometric excess of OPs. Panel A, III to HuBChE ratio = 15 (pH 7.95, 25°C). Both reactivators were at 0.1 M; Panel B, reactivator: 0.1 M P2S; $CH_3P(O)(OC_2H_5)SCH_2CH_2N(isoPr)_2$ to HuBChE ratio = 8 (pH 7.3, 25°C).

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