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Research Paper

New Safe Method for Preparation of Sarin-Exposed Human Erythrocytes Acetylcholinesterase Using Non-Toxic and Stable Sarin Analogue Isopropyl *p*-Nitrophenyl Methylphosphonate and its Application to Evaluation of Nerve Agent Antidotes

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Introduction. A non-toxic and stable sarin analogue, isopropyl *p*-nitrophenyl methylphosphonate (INMP), was synthesized for safe preparation of sarin-exposed acetylcholinesterase (AChE).
Results and Discussion. This agent was stable for years, able to be handled in an ordinary laboratory without special care, and its 50% inhibitory concentration (IC_{50}) on 0.04 U/ml human erythrocytes AChE was 15 nM. This reagent was thought to be especially useful since it enables experiments that require sarin-inhibited AChE, such as the development of antidotes for sarin, in a usual laboratory. To demonstrate the usefulness of this method, 40 known and novel pyridinealdoxime methiodide (PAM)-type oxime antidotes were synthesized, and their reactivation activities to INMP-exposed AChE and structure–activities correlation were studied.
Conclusion. Among the antidotes tested in this experiment except for 2-PAM, the compound found to have the highest reactivation activity, was the novel hydrophobic 2-PAM-type compound, 2-[(hydroxyimino)methyl]-1-[4-(*tert*-butyl)benzyl] pyridinium bromide.

KEY WORDS: acetylcholinesterase; antidote; isopropyl *p*-nitrophenyl methylphosphonate (INMP); PAM; sarin.

INTRODUCTION

In Japan, in 1994 and 1995 ordinary citizens suffered from two horrifying terrorist attacks with an extremely toxic nerve agent, sarin (isopropyl methylphosphonofluoridate, **1a** in Fig. 1).

In these attacks, 19 people were killed and over 5,000 injured (1–3), and we previously reported a new method for detection of the hydrolysis products of sarin from the blood and brain of victims (4–6). In Japan, synthesis and use of sarin are strictly controlled by the Chemical Warfare Convention, even for basic studies. This strongly prevents general researchers from studying the pathophysiology of sarin poisoning and developing antidotes for nerve agents. We have already reported a preparation method for sarin-exposed human erythrocytes by using bis(isopropyl methylphosphonate; BIMP) instead of extremely dangerous sarin itself (4). Unfortunately, BIMP is still toxic, and readily hydrolyzed during storage; thus, it must be freshly prepared for experimental use. In this paper, we report a new, safe and convenient method for preparation of sarin-exposed human erythrocytes acetylcholinesterase (AChE) using a stable and non-toxic sarin analogue, isopropyl *p*-nitrophenyl methylphosphonate (INMP, **1b** in Fig. 1).

According to our previous work, at the 1995 sarin attack in the Tokyo subway, there were several cases of victims dying by sarin intoxication after the antidote 2-pyridinealdoxime methiodide (2-PAM, **2a** in Fig. 1) was administered (5). Their plasma AChE activities had been recovered by 2-PAM administration, but brain AChE activities could not be recovered (5), even though 2-PAM is reported to penetrate the blood–brain barrier (BBB) somewhat (7). On the other

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ABBREVIATIONS: AChE, acetylcholinesterase; BBB, blood–brain barrier; BIMP, bis(isopropyl methylphosphonate); CH_2Cl_2, dichloromethane; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMAP, dimethylaminopyridine; IC_{50}, 50% inhibitory concentration; IMPA, methylphosphonic acid monoisopropyl ester; INMP, isopropyl *p*-nitrophenyl methylphosphonate; LC–ESI-MS, liquid chromatography–electrospray mass spectrometry; NMR, nuclear magnetic resonance; PAM, pyridinealdoxime methiodide; pK_a, dissociation constant; RT, room temperature; THF, tetrahydrofuran; TLC, thin-layer chromatography.

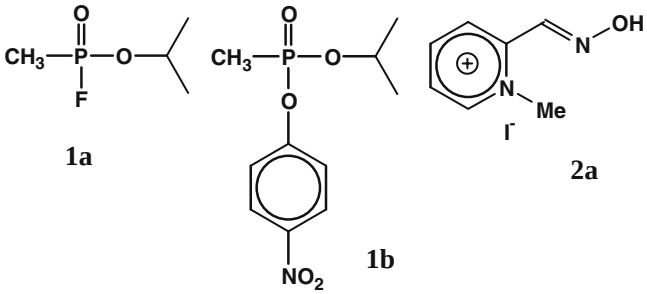


Fig. 1. The chemical structures of sarin (**1a**), INMP (**1b**) and 2-PAM (**2a**).

hand, it is known that some 4-PAM homologues with long aliphatic hydrocarbon chains and high lipophilicities can permeate the BBB and reactivate sarin-inhibited brain AChE (8). Therefore, a basic study of BBB-permeable PAM analogues seems necessary to protect ordinary citizens from another such terrorist attack. In this paper, 23 known and novel homologues of 2-, 3- and 4-PAM were synthesized, and their reactivation activities to sarin-exposed human erythrocytes AChE prepared by using INMP were examined. On the other hand, some *N*-benzyl PAM analogues with lipophilic structures are also known to have high reactivation activities (9). Thus, 17 known and novel alkylated benzyl and homobenzyl analogues of PAM were also synthesized and examined as reactivators for sarin-inhibited AChE. On the basis of all the results of the experiments described above, the structure–activity correlation of PAM analogues was also discussed.

MATERIALS AND METHODS

Chemicals and Reagents

Human erythrocyte AChE and 2-PAM were purchased from Sigma (St. Louis, MO, USA). All other reagents were of the highest grade available commercially.

Instruments

Measurements of 600 MHz ^{1}H and 151 MHz ^{13}C nuclear magnetic resonance (NMR) were performed with JEOL ECP600 (JEOL Ltd., Tokyo, Japan). Analyses by low- and high-resolution liquid chromatography–electrospray mass spectrometry (LC–ESI-MS) were performed with JEOL LCmate (JEOL Ltd.).

Preparation of INMP

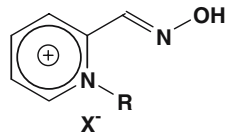
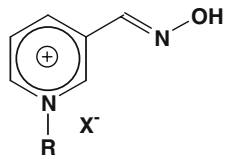
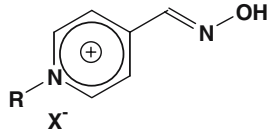
Methylphosphonic acid monoisopropyl ester (IMPA) was prepared as we previously reported (10). To a stirred solution of 309 mg of dicyclohexylcarbodiimide (DCC) in 2 ml of dry dichloromethane (CH_2Cl_2), a solution of 200 mg (1.45 mmol) of IMPA in 2 ml of CH_2Cl_2 was added at room temperature (RT). To this was added a solution of 17.7 mg (0.145 mmol) of dimethylaminopyridine (DMAP) in 1 ml of CH_2Cl_2 dropwise at RT. A large amount of white precipitate formed. To this was added a solution of 222 mg (1.59 mmol) of *p*-nitrophenol in 10 ml of CH_2Cl_2 dropwise to give a yellowish emulsion. To dissolve 222 mg of *p*-nitrophenol, more than 7–8 ml of CH_2Cl_2 was required. The reaction was monitored by thin-layer chromatography (TLC) using Merck Kieselgel F_{254} silica-gel plate (0.25 mm thickness) as the stationary phase, *n*-hexane-ethyl acetate (2:1) as the mobile phase, and ultraviolet absorption at 254 nm as the detecting method. The R_f values of INMP and *p*-nitrophenol were 0.4 and 0.8, respectively. The mixture was further stirred for 48 h at RT until the spot of *p*-nitrophenol on TLC had mostly disappeared. The mixture was concentrated by evaporation in vacuo and re-dissolved in 2 ml of CH_2Cl_2. Most of the dicyclohexylurea (DCU) that formed in the reaction deposited as a thick oil and was easily removed by filtration with filter paper, but still remained in the filtrate. The CH_2Cl_2 filtrate was left for 12 h, and then further deposited DCU was filtered again. The final filtrate was evaporated in vacuo and carefully chromatographed on a silica-gel column (*n*-hexane-ethyl acetate) to give 339 mg of INMP (90.3% yield) as a pale yellow oil. Six hundred megahertz ^{1}H NMR: δ 1.28 (d, 3 H, $J = 6.0$ Hz, isopropyl CH_3), 1.37 (d, 3 H, $J = 6.0$ Hz, isopropyl CH_3), 1.68 (d, 3 H, $^{2}J_{P-C-H} = 17.6$ Hz, P-CH_3), (dsept, 1 H, $J = 6.0$ Hz, isopropyl CH), 7.37–7.41 (m, 2H, 2,6-CH of 4-nitrophenoxy group), 8.22–8.26 (m, 2H, 3,5-CH of 4-nitrophenoxy group). One hundred fifty-one megahertz ^{13}C NMR: δ 12.0 (isopropyl CH_3), 13.0 (isopropyl CH_3), 24.0 (d, $^{1}J_{P-C} = 21.9$ Hz, P-CH_3), 72.3 (isopropyl CH), 121.1 (2,6-C of 4-nitrophenoxy group), 125.7 (3,5-C of 4-nitrophenoxy group), 144.6 (4-C of 4-nitrophenoxy group), 155.7 (1-C of 4-nitrophenoxy group).

Neat INMP was stable for years at −20°C in a refrigerator. For the AChE inhibition experiment, a freshly prepared stock solution of INMP of various concentrations in methanol stored at −20°C was diluted with 0.1 M phosphate buffer (pH 7.0) and used as the inhibitor reagent.

Preparation of 2-PAM Analogue

PAM analogues listed in Table I except 2-PAM (**2a**) itself were synthesized as follows: To a stirred solution of 1.00 g (8.19 mmol) of 2-, 3- or 4-pyridinealdoxime in 5 ml of tetrahydrofuran (THF), two molar equivalents (16.4 mmol) of corresponding alkyl bromides or four molar equivalents (32.8 mmol) of chlorides or iodides were added dropwise at RT. The reaction was monitored by TLC using Merck Kieselgel F_{254} silica-gel plate (0.25 mm thickness) as the stationary phase, ethyl acetate as the mobile phase, and ultraviolet absorption at 254 nm as the detecting method. The R_f values of 2-, 3- and 4-pyridinealdoxime were 0.5–0.6. The mixtures were refluxed until the spot of pyridinealdoximes on TLC disappeared. If the reaction was too slow, it was stopped at 12 h. Typical reaction times were 8–12 h for alkyl halides and 15 min for benzyl bromides. Formed 2-PAM analogues that had side chains longer than *n*-butyl group deposited as thick oils during the reaction. In these cases, the supernatants were removed after cooling to RT, 5 ml of fresh THF was added and the mixtures were refluxed with vigorous stirring for 1 h. This process was repeated three times to remove excess reagents completely. After supernatants were finally removed, the remaining thick oils were allowed to cool to RT and left until they crystallized. After crystallization, they were recrystallized from THF-ethanol or *n*-hexane-THF. Other reaction products precipitated as powders. They were collected by filtration and recrystallized

Table I. Synthesized PAM Analogues

Compound Type		No.	R	X	Yield (%)
 2-PAM analogues	Original 2-PAM	**2a**	methyl (2-PAM)	I	-
	Linear side chain type	**2b**	ethyl	I	18
		2c	*n*-butyl	Br	8
		2d	*n*-hexyl	Br	6
		2e	*n*-octyl	Br	5
		2f	*n*-decyl	Br	1
		2g	*n*-octadecyl	Br	1
	Branched side chain type	**2h**	isoamyl	Br	6
		2i	2-ethylhexyl	Br	no reaction
	Benzyl type	**2j**	benzyl (Benzyl-P2A)	Br	12
		2k	*p*-methylbenzyl	Br	1
		2l	*p-tert*-butylbenzyl	Br	17
	Homobenzyl type	**2m**	2-phenylethyl	Br	5
		2n	3-phenylpropyl	Br	1
		2o	4-phenylbutyl	Cl	no reaction
 3-PAM analogues	Linear side chain type	**3b**	ethyl	I	98
		3c	*n*-butyl	Br	83
		3d	*n*-hexyl	Br	93
		3e	*n*-octyl	Br	100
		3f	*n*-decyl	Br	61
		3g	*n*-octadecyl	Br	24
	Branched side chain type	**3h**	isoamyl	Br	46
		3i	2-ethylhexyl	Br	9
	Benzyl type	**3j**	benzyl	Br	83
		3k	*p*-methylbenzyl	Br	99
		3l	*p-tert*-butylbenzyl	Br	47
	Homobenzyl type	**3m**	2-phenylethyl	Br	88
		3n	3-phenylpropyl	Br	27
		3o	4-phenylbutyl	Cl	5
 4-PAM analogues	Linear side chain type	**4b**	ethyl	I	96
		4c	*n*-butyl	Br	99
		4d	*n*-hexyl	Br	91
		4e	*n*-octyl (OPAB, SPK-3)	Br	76
		4f	*n*-decyl	Br	89
		4g	*n*-octadecyl	Br	16
	Branched side chain type	**4h**	isoamyl	Br	38
		4i	2-ethylhexyl	Br	7
	Benzyl type	**4j**	benzyl	Br	75
		4k	*p*-methylbenzyl	Br	82
		4l	*p-tert*-butylbenzyl	Br	81
	Homobenzyl type	**4m**	2-phenylethyl	Br	77
		4n	3-phenylpropyl	Br	57
		4o	4-phenylbutyl	Cl	2

from THF-ethanol or ethanol. All the 2-PAM analogues synthesized were characterized by 600 MHz ^{1}H and 151 MHz ^{13}C NMR, and low- and high-resolution LC–ESI-MS. The yields are also shown in Table I.

In Table I, the low yields of 2-PAM analogues were due to their very low reaction speed, which might be due to the steric hindrance of the 2-hydroxyiminomethyl group of 2-pyridinealdoxime. The yields of **3g** and **4g** were also low; this could be caused by their high solubilities in THF. The low yields of **3i** and **4i** seemed to be due to steric hindrance of 2-ethylhexyl bromide. Synthesized PAM analogues were used for reactivation experiments as an aqueous solution. Some water-insoluble analogues were initially dissolved in ethanol and diluted with water. Unfortunately, **4g** was insoluble in water–ethanol (1:1) and could not be used for this experiment.

Inhibitory Effect of INMP on AChE

Twenty-five microliters of 0.2, 2, 20, 200, 2,000 and 20,000 nM INMP (for inhibition) or 0.1 M phosphate buffer (pH 7.0, for positive control) were added to 25 μl of 0.02 U human erythrocytes AChE in 0.1 M phosphate buffer (pH 7.0). This mixture was incubated at 25°C for 30 min. Then, 50 μl of distilled water was added, and the remaining AChE activities were measured by the method of Ellman *et al.* (11). AChE activities relative to the positive control at the various INMP concentrations are shown in Fig. 2. From Fig. 2, the

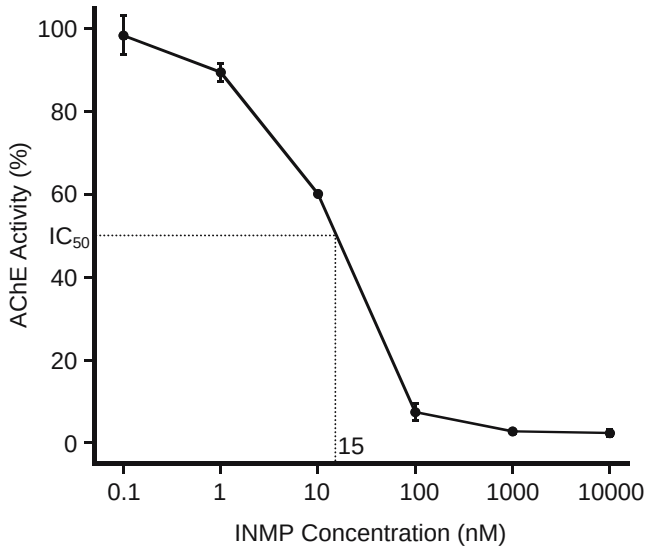


Fig. 2. Inhibitory effect of INMP to human erythrocyte AChE.

50% inhibitory concentration (IC_{50}) of INMP to 0.04 U/ml human erythrocytes AChE was determined to be 15 nM.

Reactivation of Inhibited AChE with 2-PAM

Twenty-five microliters of 30 nM INMP was added to 25 µl of 0.02 U human erythrocyte AChE and incubated at 25°C for 30 min to form 50%-inhibited AChE solution. This was used as a negative control in the subsequent experiments. To this was added 50 µl of aqueous solution of 2-PAM at various concentrations, and the mixture was incubated at 25°C for 15 min; then the recovered AChE activities were measured by the method of Ellman *et al.* (11). When the final concentration of 2-PAM solution was 150 µM, 50%-inhibited AChE was reactivated to 75% activity in relation to the positive control. Thus, the final concentrations of other PAM analogues were also fixed to 150 µM for comparison with AChE activities in response to 2-PAM.

Reactivation of Inhibited AChE with PAM Analogues

Twenty-five microliters of 30 nM INMP was added to 25 µl of 0.02 U human erythrocyte AChE and incubated at 25°C for 30 min. To this was added 50 µl of 300 µM solutions of PAM analogues, and the mixture was incubated at 25°C for 15 min; then the recovered AChE activities were measured by the method of Ellman *et al.* (11). Three samples were assayed for each data point for calculation of standard deviations.

RESULTS AND DISCUSSION

AChE reactivation activities of the synthesized 39 PAM analogues are shown in Table II. The activities are shown as the relative activities relative to 2-PAM (2-PAM: 100%). To evaluate the branched side chain analogues, alkylbenzyl analogues and benzyl homologues, the relative activities of those compounds to the common linear side chain analogues that have the same or similar carbon numbers in the side chain are also shown in Table II.

In Table II, 29 of 39 PAM analogues showed AChE reactivation activities and the activities varied from 3 to 92% of the activity of 2-PAM. Concerning to branched side chain analogues, alkylbenzyl analogues and benzyl homologues, 13 of 22 analogues showed AChE reactivation activities, seven of them showed the similar activities (95 to 117%) and three of them showed higher activities (164 to 278%) to the linear side chain analogues that have the same or similar carbon numbers in the side chain. To discuss about the result and the structure–activity correlation in detail, the result was separated by compound types and studied as follows.

Reactivation Activities of PAM Analogues with Linear Side Chain to INMP-Inhibited AChE

AChE reactivation activities of PAM analogues with linear aliphatic side chains (**2a**–**2g**, **3b**–**3g**, **4b**–**4f**) relative to 2-PAM in Table II are extracted and shown in Fig. 3. In Fig. 3, 2-PAM itself showed the best recovery activity, and the activities of 2-PAM analogues were reduced by elongation of the side chain. This could be explained as a result of the decrease of the dissociation constant (pK_a) with the increase of lipophilicity of analogues (12) or the increase in steric hindrance due to the side chain which may prevent the reaction of oxime group and inhibited AChE. All the 3-PAM analogues showed low recovery activities, consistent with a prior report (13). Recovery activities of all the 4-PAM analogues were more than moderate and increased by side chain elongation. This is also consistent with prior reports on *in vitro* or *in vivo* experiments (8,12–14). **4e**, as known as "SPK-3" (8) or "OPAB" (14), is a well-known antidote able to reactivate sarin-inhibited brain AChE (8) and also known as potent decontamination agent for organophosphorus compounds by its micellar property (13). It is reported that **4e** showed mostly equal reactivation activities to 2-PAM for blood AChE of sarin-inhibited mice (8); by contrast, it showed 46% relative activity to 2-PAM in Fig. 3. This probably reflects the difference between the *in vivo* and *in vitro* experiments, and the difference in experimental conditions of reactivation.

Reactivation Activities of PAM Analogues with Branched Side Chain to INMP-Inhibited AChE

Relative AChE reactivation activities of PAM analogues with branched aliphatic side chains (**2h**, **3h**–**3i**, **4h**–**4i**) in Table II are extracted and shown in Fig. 4. The results of PAM analogues with linear side chains are also shown in Fig. 4 for comparison (error bars omitted). A novel compound, 2-[(hydroxyimino)methyl]-1-isoamylpyridinium bromide (**2h**), showed intermediate recovery activity between **2c** and **2d**, and its behavior was just the same as that expected for an *n*-amyl analogue. 2-[(hydroxyimino)methyl]-1-[2-ethylhexyl] pyridinium bromide (**4i**), which is also novel compound, showed the same activity as **4e**. In these cases, the recovery activities to sarin-inhibited AChE seemed to depend on the number of carbons and lipophilicities of the side chains, not the length of the side chains. For unknown reasons, novel compounds **3h**, **3i** and **4h** did not show recovery activities. Considering the low yields of **2h**, **3h**–**3i**, **4h**–**4i** in Table I, PAM analogues with branched side chains were thought to have no advantages over linear side chain analogues.

Table II. AChE reactivation activities of synthesized PAM Analogues

Compound type		No.	R	X	Carbons in side chain	Relative activity to 2-PAM (%)	S.D. (%, $n = 3$)	Relative activity to linear side chain analogue (%)[a]
2-PAM analogues	Linear side chain type	**2b**	Ethyl	I	2	92	9.5	
		2c	*n*-butyl	Br	4	58	1.5	
		2d	*n*-hexyl	Br	6	39	2.2	
		2e	*n*-octyl	Br	8	34	14	
		2f	*n*-decyl	Br	10	23	0.8	
		2g	*n*-octadecyl	Br	18	–[b]	–	
	Branched side chain type	**2h**	Isoamyl	Br	5	48	8.0	99[c]
	Benzyl type	**2j**	Benzyl	Br	7	39	0.7	108[d]
		2k	*p*-methylbenzyl	Br	8	55	6.6	164
		2l	*p-tert*-butylbenzyl	Br	11	64	1.1	278[e]
	Homobenzyl type	**2m**	2-phenylethyl	Br	8	5.3	2.5	16
		2n	3-phenylpropyl	Br	9	–	–	–
3-PAM analogues	Linear side chain type	**3b**	Ethyl	I	2	11	0.5	
		3c	*n*-butyl	Br	4	8.4	1.8	
		3d	*n*-hexyl	Br	6	11	3.2	
		3e	*n*-octyl	Br	8	5.4	7.2	
		3f	*n*-decyl	Br	10	3.6	3.2	
		3g	*n*-octadecyl	Br	18	–	–	
	Branched side chain type	**3h**	Isoamyl	Br	5	–	–	–
		3i	2-ethylhexyl	Br	8	–	–	–
	Benzyl type	**3j**	Benzyl	Br	7	–	–	–
		3k	*p*-methylbenzyl	Br	8	9.0	3.9	167
		3l	*p-tert*-butylbenzyl	Br	11	15	3.7	4.2[e]
	Homobenzyl type	**3m**	2-phenylethyl	Br	8	–	–	–
		3n	3-phenylpropyl	Br	9	–	–	–
		3o	4-phenylbutyl	Cl	10	4.2	11	117
4-PAM analogues	Linear side chain type	**4b**	Ethyl	I	2	29	11	
		4c	*n*-butyl	Br	4	30	8.9	
		4d	*n*-hexyl	Br	6	43	6.3	
		4e	*n*-octyl	Br	8	46	0.4	
		4f	*n*-decyl	Br	10	59	1.2	
	Branched side chain type	**4h**	Isoamyl	Br	5	–	–	–
		4i	2-ethylhexyl	Br	8	47	5.6	101
	Benzyl type	**4j**	Benzyl	Br	7	–	–	–
		4k	*p*-methylbenzyl	Br	8	48	5.2	104
		4l	*p-tert*-butylbenzyl	Br	11	30	1.5	51[e]
	Homobenzyl type	**4m**	2-phenylethyl	Br	8	44	4.6	95
		4n	3-phenylpropyl	Br	9	–	–	–
		4o	4-phenylbutyl	Cl	10	3.1	2.0	5.3

[a] Compared with the activities of linear side chain analogues that have the same number of carbons in the side chains.
[b] '–' No AChE reactivation activities were observed.
[c] Compared with average of the activities of **2c** and **2d.**
[d] Compared with average of the activities of **2d** and **2e.**
[e] Compared with the activities of **2f**, **3f** and **4f**, respectively.

Reactivation Activities of *N*-alkylbenzyl PAM Analogues to INMP-Inhibited AChE

Relative AChE reactivation activities of *N*-alkylbenzyl PAM analogues (**2j**–**2l**, **3j**–**3l**, **4j**–**4l**) in Table II are extracted and shown in Fig. 5. The results of PAM analogues with linear side chains are also shown in Fig. 5 for comparison (error bars omitted). **2j**, 2-[(hydroxyimino)methyl]-1-benzylpyridinium bromide, known as "Benzyl-P2A," is a well-known antidote for *N*,*N*-dimethyl *O*-ethyl phosphoramidecyanidate

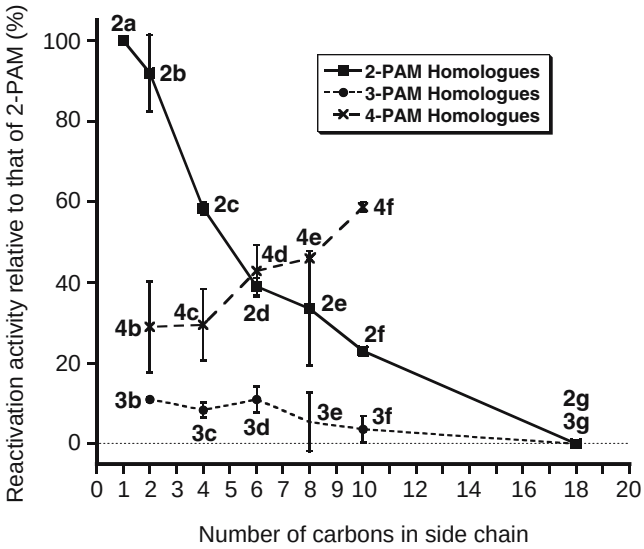


Fig. 3. Relative AChE reactivation activities of PAM analogues with linear alkyl side chains to 2-PAM.

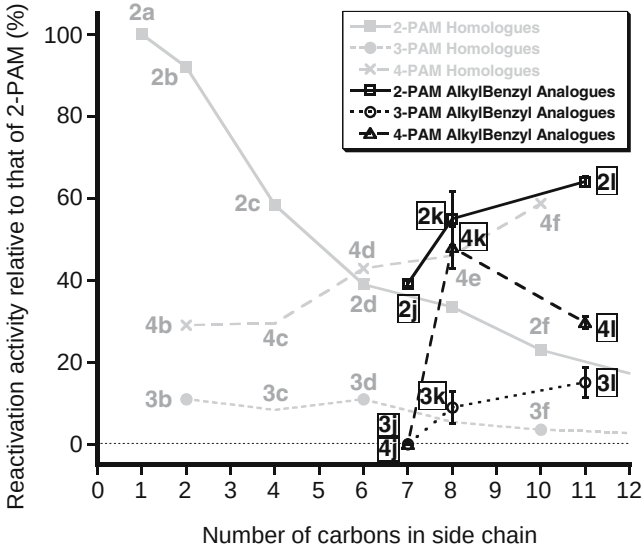


Fig. 5. Relative AChE reactivation activities of *N*-alkylbenzyl PAM analogues to 2-PAM.

(tabun) (9). In this experiment, **2j** showed moderate recovery activity in Fig. 5. **2k**, **2l** and **4k** showed higher reactivation activities than the known compounds **2j** ("Benzyl-P2A") and **4e** ("OPAB," "SPK-3"). **2k**, known as less potent activator for tabun-exposed bovine AChE than **2j** (9), unexpectedly showed a stronger recovery effect for INMP-inhibited AChE than **2j** in Fig. 5. A novel compound, 2-[(hydroxyimino)-methyl]-1-[4-(*tert*-butyl)benzyl] pyridinium bromide (**2l**), was found to have the highest reactivation activity among the antidotes tested in this experiment except for 2-PAM itself. As demonstrated in this case, the INMP method enables rapid assay of reactivation activities of a large number of newly synthesized compounds, and helps rapid screening and evaluation of antidotes. The difference between this result and that of the prior report (9) might be based on the difference between sarin and tabun, or *in vitro* and *in vivo* experiments. In Fig. 5, 3-PAM analogues **3j**–**3l** showed low reactivation activities, as in Figs. 3 and 4. **3j** and **4j** showed almost no reactivation activities, and this was also reported in the prior report. Generally, the results in Fig. 5 showed similarity to those of the prior reactivation experiment on tabun-inhibited bovine AChE (9).

Reactivation Activities of *N*-benzyl PAM Homologues to INMP-Inhibited AChE

Relative AChE reactivation activities of *N*-benzyl PAM homologues (**2m**–**2n**, **3m**–**3o**, **4m**–**4o**) in Table II are extracted and shown in Fig. 6. The results of PAM analogues with linear side chains are also shown in Fig. 5 for

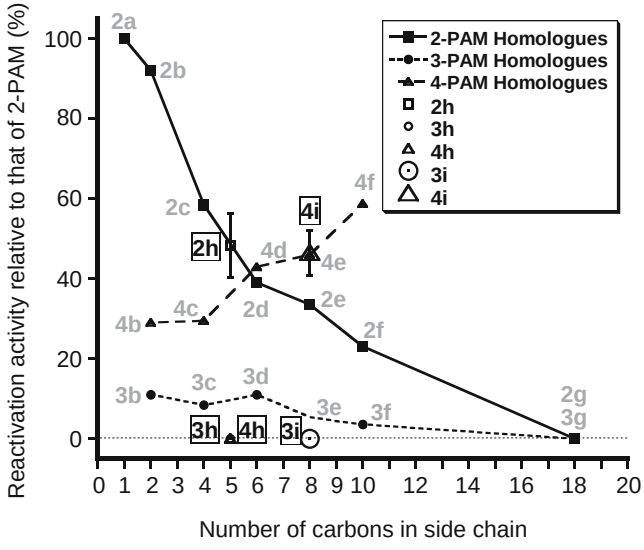


Fig. 4. Relative AChE reactivation activities of PAM analogues with branched alkyl side chains to 2-PAM.

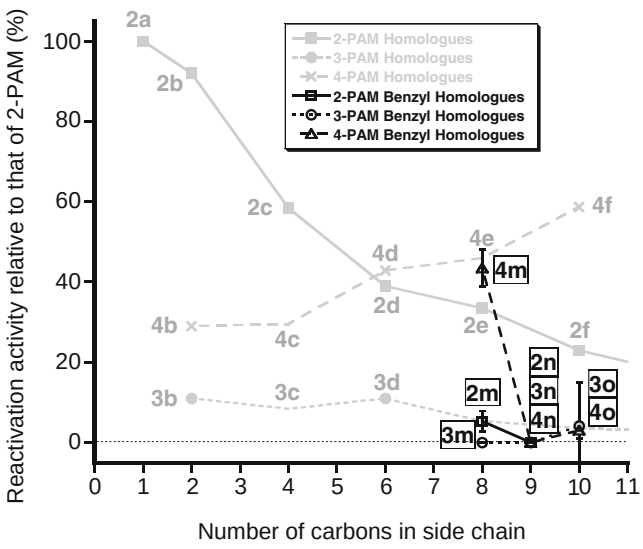


Fig. 6. Relative AChE reactivation activities of *N*-benzyl PAM homologues to 2-PAM.

comparison (error bars omitted). **2m** showed low reactivation activities, and this result is similar to that of the tabun-inhibited bovine AChE experiment (9). In this series, only the novel compound **4m** showed equal reactivation activity to the known antidote **4e** ("OPAB," "SPK-3"). In this case, similar to **2h** and **4i** in Fig. 4, the number of carbons in the side chain seemed to be more important for reactivation activity than chain length, and it seemed not to be so important whether the substituents were aliphatic or aromatic.

CONCLUSION

A non-toxic sarin analogue INMP was found to enable safe and easy preparation of sarin-exposed AChE. This agent could be easily handled, and its IC_{50} on 0.04 U/ml human erythrocytes AChE was 15 nM. To demonstrate the usefulness of this reagent, 40 hydrophobic PAM-type oxime antidotes were synthesized, and their reactivation activities to sarin-exposed AChE were examined in order to demonstrate their abilities for easy development of BBB-permeable antidotes for sarin poisoning in normal laboratories. In general, the results of the recovery experiment were similar to those of the experiments previously reported using real sarin or tabun. Structure–activity correlation studies showed some interesting properties of PAM analogues. 2-PAM showed the best recovery activity; the activities of its homologues were reduced by side chain elongation. In contrast, 4-PAM showed moderate reactivation activities, and the activities of its homologues increased by side chain elongation. All the 3-PAM analogues showed poor reactivation activities. Regarding the side chains of PAM analogues, linear hydrocarbons seemed to be more suitable than branched hydrocarbons in terms of both activity and availability. All the alkylbenzyl analogues of 2-PAM and some alkylbenzyl analogues of 4-PAM tested showed strong reactivation activities. In the *N*-benzyl PAM homologues tested, only the 4-phenethyl analogue showed strong activity. According to the collective results, the reactivation activities of the oximes tested seemed to depend on side chain carbon number and lipophilicity, not the chain length. Among the antidotes tested in this experiment except for 2-PAM, the novel hydrophobic 2-PAM-type compound 2-[(hydroxyimino)methyl]-1-[4-(*tert*-butyl)benzyl] pyridinium bromide was found to have the highest reactivation activity. As demonstrated in this experiment, the INMP method enabled rapid assay of reactivation activities of a large number of newly synthesized compounds, and helped rapid screening and evaluation of antidotes.

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