Synthesis and in vitro assessment of the reactivation profile of clinically available oximes on the acetylcholinesterase model inhibited by A-230 nerve agent surrogate

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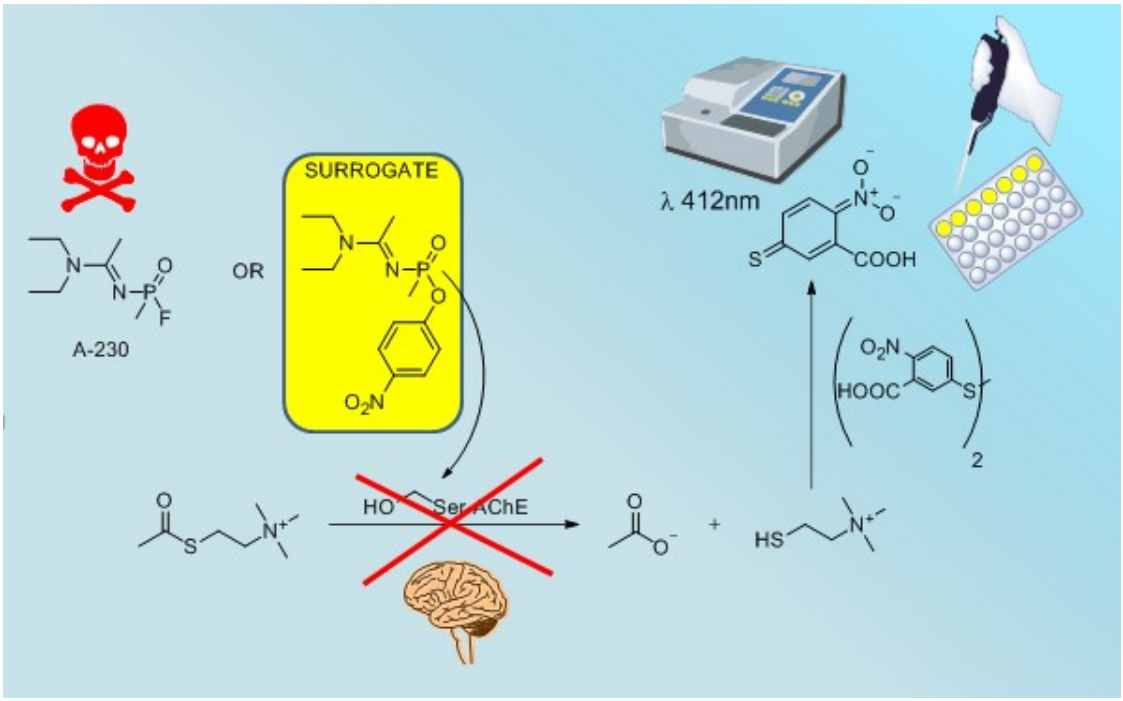
Graphical Abstract



Abstract

The risk of use of toxic chemicals for unlawful acts has been matter of concern for different governments and multilateral agencies. The Organisation for the Prohibition of Chemical Weapons (OPCW), which oversees the implementation of the Chemical Weapons Convention (CWC), considering recent events employing chemical warfare agents as means of assassination, has recently included in the CWC "Annex on Chemicals" some organophosphorus compounds that are regarded asactingin a similar fashion to the classical G- and V-series of nerve agents, inhibiting the pivotal enzyme acetylcholinesterase. Therefore, knowledge of the activity of the pyridinium oximes, the sole class of clinically available acetylcholinesterase reactivators so far, is plainly justified. In this paper, continuing our research efforts in the Medicinal Chemistry on this class of toxic chemicals, we synthesized an A-230 nerve agent surrogate and applied a modified Ellman's assay in order to evaluate its ability to inhibit our enzymatic model, acetylcholinesterase from *Electrophorus eel*, and if the clinically available antidotes are able to rescue the enzyme activity for the purpose of relating the findings to the previously disclosed *in silico* data for the authentic nerve agent and other studies with similar A-series surrogates. Our experimental data indicates that pralidoxime is the most efficient compound for reactivation of acetylcholinesterase inhibited by A-230 surrogate, which is the opposite to the *in silico* data previously disclosed.

Keywords: A-230, Nerve Agent Surrogates, Acetylcholinesterase, Antidotes, Chemical Weapons Convention.

Introduction

Chemical warfare agents are toxic substances used to harm or incapacitate humans and animals, existing since the dawn of society, being used in conflicts since ancient times (Nepovimova and Kuca 2018, 2019). In order to avoid the use of such abhorrent method of warfare, different international agreements have been signed. However, they could not deterthe use and the further development of such toxic substances (Bajgar et al. 2009; Eckert 1991; Goliszek 2004; Fry et al. 2006; Worek et al. 2005). Misuse of toxic chemicals in conflicts and terrorist acts led the international community to discuss a more comprehensive agreement oncontrol and prohibition of use of chemicals as method of warfare, among other issues. Actions taken culminated in the Chemical Weapons Convention (CWC), which entered into force in May 1997. CWC also gave rise to the Organization for the Prohibition of Chemical Weapons (OPCW), the international watchdog responsible for verifying CWC compliance and implementation, as well as assisting non-signatory countries in preparing to join to the CWC (OPCW 1997).

Among the chemical warfare agents, the class of nerve agents poses a risk due to the high toxicity of its compounds, with a proven use in the past years in different occasions, as method of warfare during the late stages of the Iran-Iraq War (1980-1988) and the ongoing Syrian Civil War (2011-), or in terrorist plots, for example, events that took place at the Tokio Subway in 1995, at the Kuala Lumpur International Airport in 2017, in the United Kingdom in 2018 and in Russia in 2020 (OPCW 2020). In these incidents, G-, V-and A-series nerve agents have been disclosed as toxic chemicals used. Examples of such compounds are depicted in the Figure 1: tabun (**1**, GA), sarin (**2**, GB), VX (**3**), A-230 (**4**) and A-242(**5**)(França et al. 2019;Nepovimova and Kuca 2018;Noga and Jurowski 2023). Besides, events that took place in the UK have prompted technical discussion on the inclusion of some organophosphorus and carbamates in the CWC "Annex on Chemicals" (OPCW 2024).

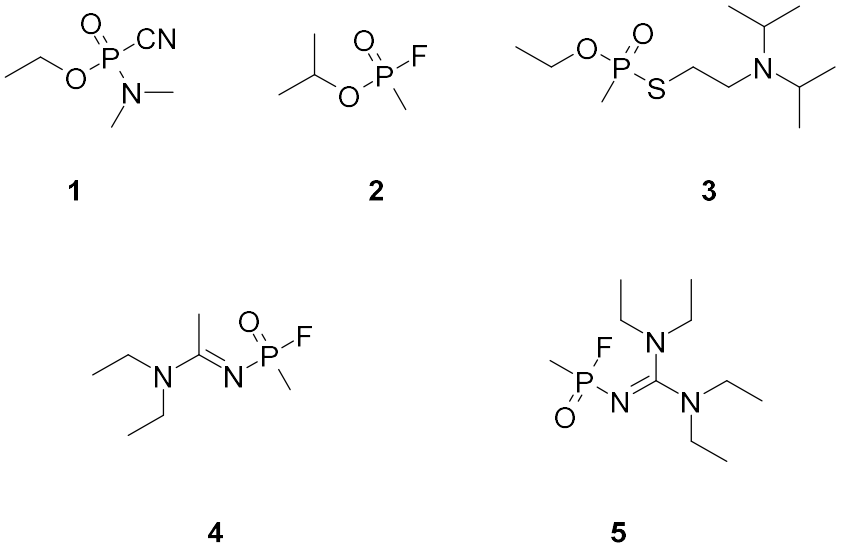


Fig. 1. Structure of some nerve agents.

Nerve agents are potent neurotoxins that target acetylcholinesterase (AChE), a pivotal enzyme for parasympathetic neurotransmission, present in the brain, neuromuscular junctions and erythrocytes. Current knowledge on their molecular mechanism of action reveals that they covalently bind to the hydroxyl group of the serine residue of the AChE catalytic triad (Ser_{203}-His_{447}-Glu_{334}) at hydrolytic site, leading to the accumulation of neurotransmitter (and enzyme substrate) acetylcholine (ACh) and triggering a cholinergic crisis by overstimulation of cholinergic innervations. Common symptoms of AChE inhibition are salivation, lacrimation, urinary incontinence, gastrointestinal disturbances, emesis (vomiting), and miosis (pupil constriction), accompanied by muscle spasms (Taylor et al. 1992; Holstege et al. 1997; Geißler 2002). This outcome can be fatal depending on the way and dose of exposure, as well delayed administration of medical countermeasures. Another important biological effect occurs when OP-AChE adduct undergoes to hydrolysis or dealkylation, resulting in the so-called aged form of the enzyme (Nepovimova and Kuca 2018). Once aging takes place, the enzyme is permanently inactivated and no current therapy can restore its activity (Kuča and Pohanka 2010; Sharma et al. 2015). Figure 2 illustrates a proposed inhibition by an A-agent (4), in accordance with the current understanding on organophosphorus nerve agents (Nepovimova and Kuca 2018; Cavalcante et al. 2020; Quinn 1987; Dvir et al. 2010; Santos et al. 2022).

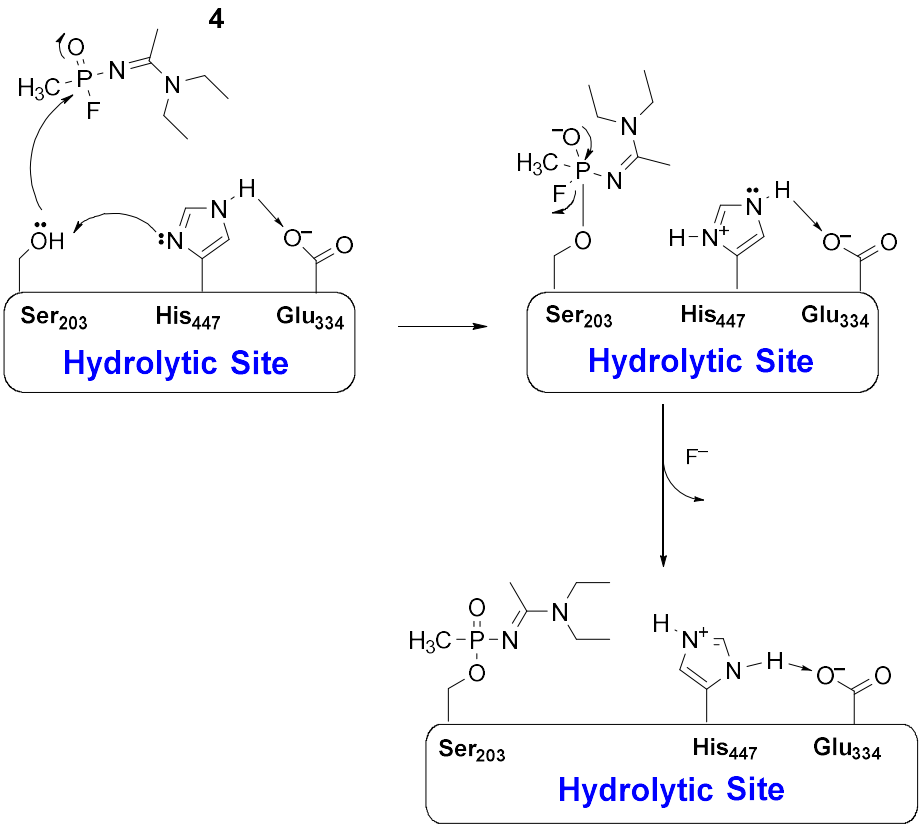


Fig. 2. Proposed mechanism of AChE inhibition by A-230 nerve agent.

IDQBRN/CTEx has been an OPCW Designated Laboratory for analysis of environmental samples since 2021. Our research group has recently been publishing some data on the inhibition of acetylcholinesterase caused by nerve agents' surrogates, which provide the same enzyme adduct but are safer to handle compared to authentic nerve agents. There have also been studies on reactivation promoted by clinically available oximes, currently approved medical countermeasures for nerve agents, and in-house synthesized reactivators candidates (França et al. 2023; Santos et al. 2022; Cavalcante et al. 2018a, 2019; Kitagawa et al. 2021, 2023). Considering the recent events employing A-series of nerve agents have taken place and the scarce information about their toxic profile, this work presents an one-pot, small scale synthesis and purification method of an A-230 surrogate (**6**) and the ability of named reactivators, pralidoxime (**7**), trimedoxime (**8**), obidoxime (**9**), asoxime (**10**) and methoxime (**11**), shown in Figure 3, to rescue AChE activity in our Ellman's assay conditions, in order to verify if the designed surrogate could be useful as a toxicological tool and clinically available pyridinium oximes might be regarded as valid countermeasures.

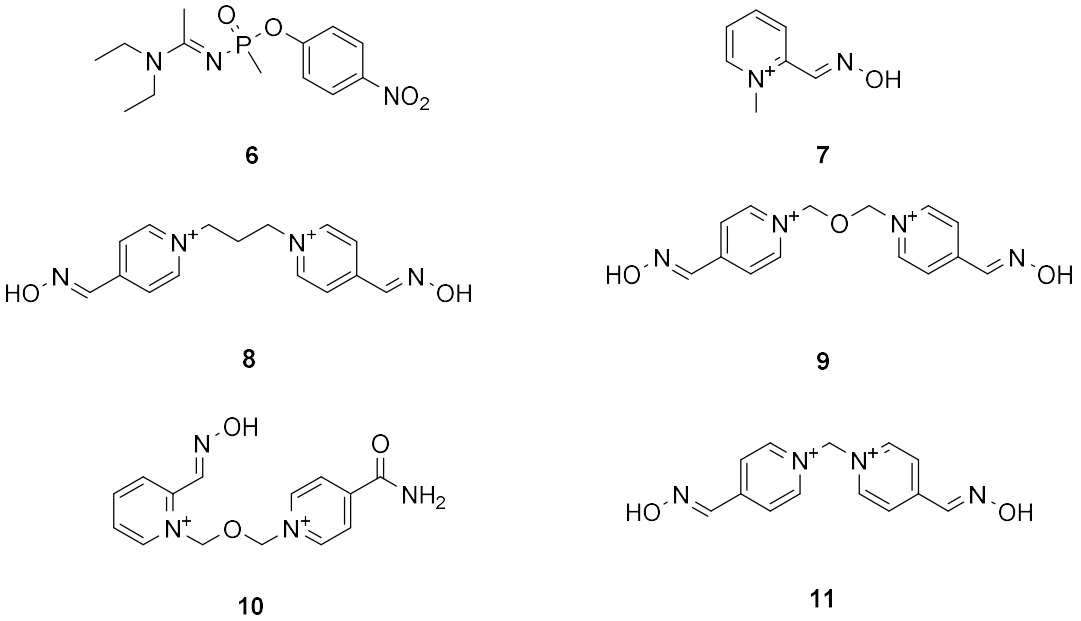
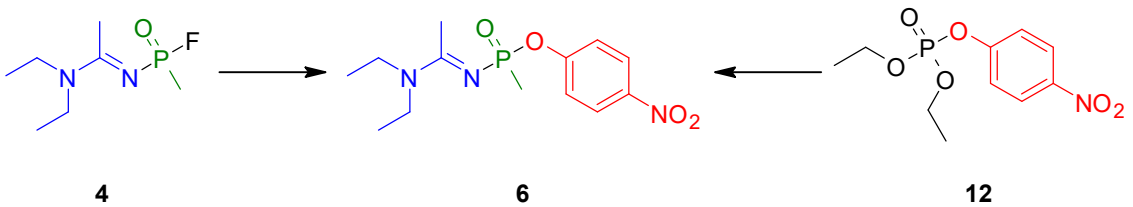


Fig. 3. A-230 surrogate (**6**) and oximes used in this work.

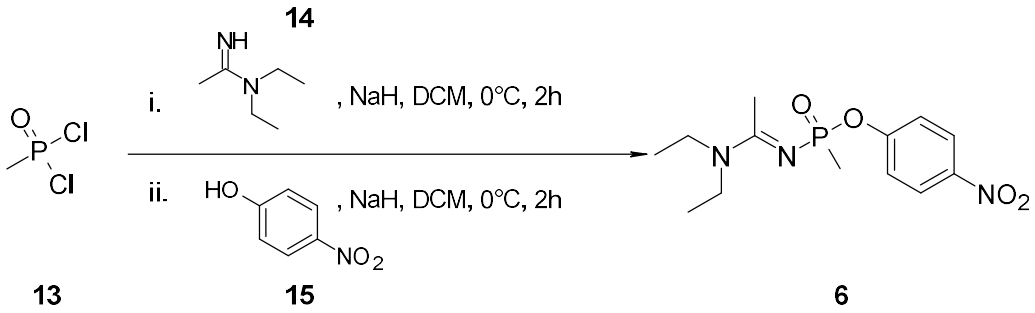
Results

Design, synthesis and purification method of the A-230 surrogate:4-nitrophenyl (E)-N-(1-(diethylamino)ethylidene)-P-methylphosphonamidate (ANMP,6)

The design of the A-230 surrogate (**6**) followed our previous work (França et al. 2023; Santos et al. 2022; Cavalcante et al. 2018a, 2019; Kitagawa et al. 2021, 2023). Basically, the surrogate can be understood as a hybrid of pesticide paraoxon (**12**) and actual nerve agent A-230 (**4**), where the 4-nitrophenolate moiety replaces fluoride as the leaving group, requirement for addition-elimination reaction with hydroxyl group of the Ser_{203} residue at the hydrolytic site (Scheme 1). In order to obtain better analytical data, for the first time we opted for a purification method using preparative thin layer chromatography, which proved successful. The one-pot synthesis route to ANMP (**6**) is given in the Scheme 2.

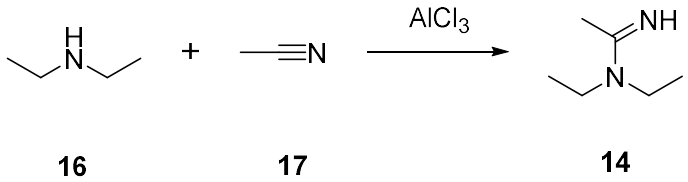


Scheme 1. Design approach to A-230 surrogate ANMP (**6**).



Scheme 2. Synthesis route to A-230 surrogate ANMP (**6**).

The starting material, methylphosphonic dichloride (**13**), firstly was coupled to *in situ* generated sodium salt of *N,N*-diethylacetimidamide (**14**). The reaction was monitored by LC-MS analysis and when dichloride was deemed as consumed, sodium 4-nitrophenolate (**15**) was added. After two hours, a sample from the reaction mixture was taken, filtered, diluted in acetonitrile and analyzed by LC-MS (ESI+) (See Figure 4 and Scheme 4), confirming the formation of target compound (**6**,*m/z* 314, $[M+H]^+$). As *N,N*-diethylacetimidamide (**14**) was not commercially available, it was also synthesized from diethylamine (**16**) and acetonitrile (**17**) using the procedure disclosed in the literature with slight modifications (Scheme 3, Jalani et al. 2013).



Scheme 3. Synthetic route to *N,N*-diethylacetimidamide (**14**).

Aiming to obtain improved NMR data, the crude reaction mixture was filtered through a 0.22µm syringe filter to remove solids, then the filtrate was evaporated. The residue obtained was carefully purified by preparative thin layer chromatography (silica gel, methanol:ethyl acetate 1:9 v/v), with ANMP (**6**) having a R_F of 0.53 and the major impurities being 4-nitrophenol (**15**, R_F 0.80) and *N,N*-diethylacetimidamide (**14**,R_F 0.00). Spots obtained were duly checked by LC-MS (ESI+). It is necessary to emphasize that all synthesis and purification procedures were carried out with appropriate safety measures by highly trained personnel.

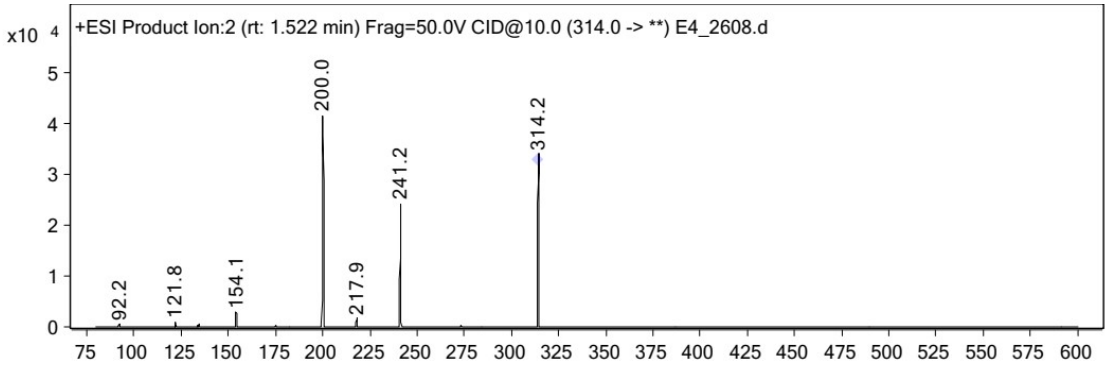
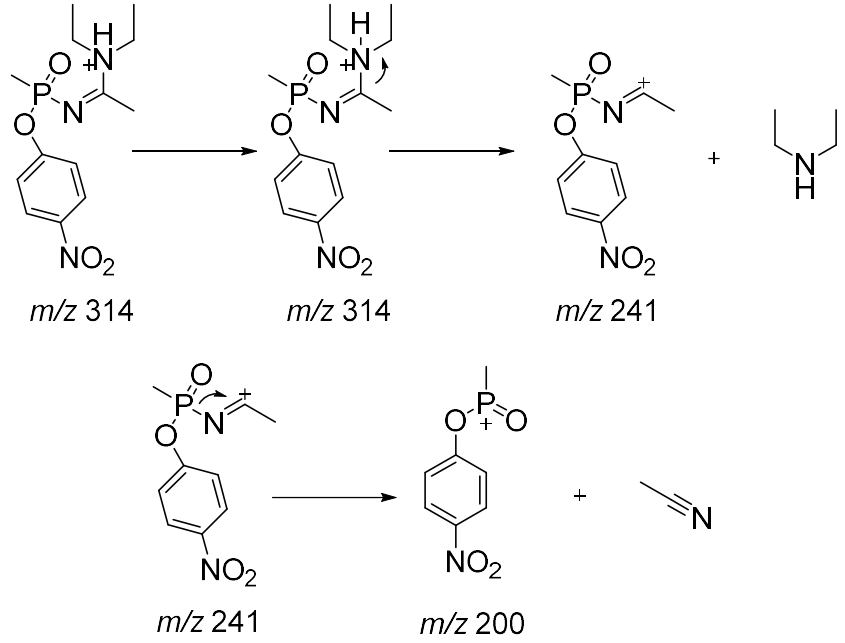


Fig. 4. LC-MS (ESI+) of A-230 surrogate ANMP (**6**).



Scheme 4. Proposed fragmentation pathways to A-230 surrogate ANMP (6) by ESI+.

Analysis of NMR data confirmed the structure of target compound ANMP (**6**). ^{1}H-NMR data showed the characteristic doublet at δ 1.66 ppm related to the $^{2}J_{H,P}$ coupling (methyl group attached to phosphorus atom, 16.56Hz), as well as the complex signals of methylene groups of the ethyl moiety at δ 3.36ppm and doublets of the 4-nitrophenyl moiety at δ 7.36 and 8.19ppm. Regarding the ^{31}P-NMR, an intense signal at δ 27.93ppm, allowing to estimate the purity higher than 95%, which was then considered for *in vitro* assays. NMR data are available in Figures S1-S4of the supplementary material.

Ellman's tests for ANMP *in vitro*activity (AChE inhibition, reactivation and aging estimation)

In order to verify whether purified ANMP (**6**) could be a convenient substitute for the development of antidotes for treatment of A-230 poisoning, a modified Ellman spectrophotometric assay was used to estimate the inhibition caused by ANMP and the reactivation using some clinical oximes (Cavalcante et al. 2018b) and a commercial acetylcholinesterase source (*Electrophorus eel*) was used as enzymatic model. First, four test solutions were prepared to estimate the IC_{50} (0.56µM) and define which one could yield the highest AChE inhibition at the lowest concentration after 10 min of incubation, being the results given in Table 1. Based on the results, the selected concentration for reactivation assays was 2µmol/L^{-1}, also confirming that this surrogate is a valid toxicological tool for this kind of assessment.

Table 1 *In vitro* inhibition results using the purified A-230 surrogate ANMP (**6**).

Concentration(µmol/L)	**3**	**0.90**	**0.30**	**0.09**
Inhibition (%)	98± 0	72± 0	25± 3	0± 4

Results expressed as the percentage of inhibition (mean ± standard deviation).

By using an approach similar to our previous papers', we evaluated the potential of the pyridinium oximes, some available as countermeasures for emergencies involving AChE inhibitors at a maximum concentration of 100 µmol/L (Tattersall 1993; Bajgar 2004; Bajgar et al. 2007). Considering the tested compounds, pralidoxime (**7**), trimedoxime (**8**), obidoxime (**9**), asoxime (**10**) and methoxime (**11**), pralidoxime did exhibit the best reactivation profile in the test conditions (Table 2). Therefore, pralidoxime was also chosen for aging estimation of the ANMP-AChE adduct.

Table 2 *In vitro* reactivation results of AChE by tested oximes.

Oxime	**1 µmol/L**	**10 µmol/L**	**100 µmol/L**
Pralidoxime (**7**)	2 ± 0	7 ± 0	27 ± 0
Trimedoxime (**8**)	0 ± 0	2 ± 0	16 ± 1
Obidoxime (**9**)	1 ± 1	1 ± 0	10 ± 3
Asoxime (**10**)	0 ± 0	0 ± 0	1 ± 0
Methoxime (**11**)	0 ± 0	0 ± 0	1 ± 0

Results expressed as the percentage of inhibition (mean ± standard deviation).

Regarding the aging assessment, performed after identifying pralidoxime (**7**) as the most active reactivator in our Ellman's assay conditions, AChE inhibition for 10 min and 24 h with surrogate led to similar reactivation rates, suggesting that A-230 surrogate ANMP (**6**) did not age AChE (Table 3).

Table 3 *In vitro* estimation of AChE aging using pralidoxime as reactivator at 100 µmol/L.

Inhibition Time	**10 min**	**24 h**
Reactivation Rate	27 ± 0	28 ± 1

Results expressed as the percentage of inhibition (mean ± standard deviation).

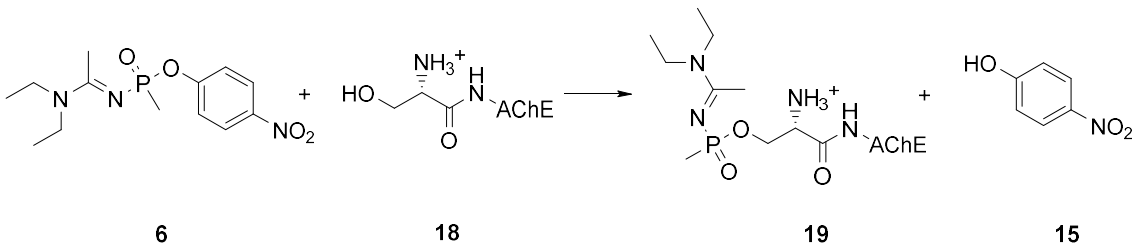
Discussion

The design and synthesis procedure used for preparation of A-230 surrogate ANMP (**6**) was based on our previous publication regarding similar study with A-242 surrogate (Santos et al. 2022), with the replacement of triethylamine with sodium hydride as base. That was justified by experimental results, since triethylamine, albeit a volatile compound, disturbed the LC-MS analyses. Sodium hydride not only cancelled that effect but also had two more positive features: the slight excess afforded the reactive anionic forms of *N,N*-diethylacetimidamide (**14**) and 4-nitrophenol (**15**) employed in the one-pot reaction, and the mitigation of the hurdles that humidity could cause, due to the hydrolysis side reactions that could lead to the consumption of methylphosphonic dichloride (**13**). Such procedure has been applied in the synthesis of reference chemicals for an internal compound library directed to CWC verification purposes and OPCW Official Proficiency Tests, as IDQBRN/CTEx is an OPCW Designated Laboratory for analysis of environmental samples. This information underscores the full preparedness of personnel and infrastructure involved in this study. This experimental approach also reduced the number of operations to be performed, presenting benefits due to minimal risk of exposure to the toxic target compound. Figure 5 portrays the apparatus used for synthesis of surrogates.

Fig. 5. Apparatus for the synthesis of A-230 surrogate ANMP (**6**).

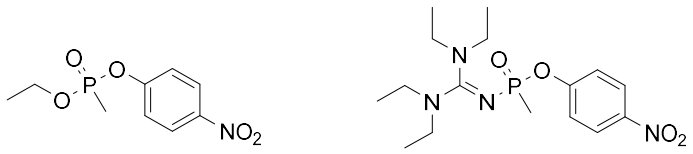
After running the second step for 2h, LC-UV-MS analysis showed a peak (λ = 205nm, ESI+) at *m/z* 314, related to the protonated form of ANMP's molecular ion. Visually, the development of a yellow to orange color means that the reaction was carried out as expected, with no bisphosphoramido substitution side-product found. The reaction was then filtered through a 0.22µm syringe filter and evaporated for the purification step. Efforts to obtain the purest possible compound were aimed at acquiring improved analytical data, intended for submission to convenient databases, and its use in enzymatic assays. Since the synthesis scale was small to reduce risks, preparative thin layer chromatography was employed for purification. The eluent was 10% methanol/ ethyl acetate mixture and the stationary phase was silica gel. 10mg of the A-230 surrogate ANMP (**6**) was obtained with a high purity, in accordance with the ^{31}P-NMR analysis.

As previously stated in published research (Nepovimova and Kuca 2018; Cavalcante et al. 2020; Quinn 1987; Dvir et al. 2010; Santos et al. 2022) and based on the common sense of reaction pathways of nerve agents with their biological target (AChE), it is expected that the hydroxyl group of Ser_{203} reacts with ANMP (**6**), affording an adduct (**19**) identical to that for authentic phosphonamidofluoridate agent A-230 (**4**), as represented in the Scheme 5 (Vieira et al. 2023).



Scheme 5. Proposed reaction of ANMP (**6**) with hydroxyl group of AChESer_{203}(**19**).

In comparison to experimental data in literature, ANMP (**6**) is 2.2-fold less toxic than VX surrogate NEMP (**20**, IC_{50} 0.25 μM) and presents similar toxicity to A-242 surrogate NTMGMP (**21**, IC_{50} 0.44 μM), both shown in Figure 6 (Kitagawa et al. 2023, Santos et al. 2022). This corroborates the theoretical results in the literature (Santos et al. 2022), suggesting a weaker binding energy between the enzyme and phosphonoamidates, when compared to phosphonates. Moreover, theoretical electrophilic of phosphorus atom was calculated, demonstrating a greater reactivity of V- series than a-series OP (Vieira et al. 2021). It is also observed in experimental data in literature (Hrabinova et al. 2024) that VX has a greater inhibitory potential than A-234, confirming the toxicological correlation between the neurotoxic agents in Schedule 1 of the CWC and their surrogates, demonstrating the potency and usefulness of these kind of nerve agent surrogate scaffold for toxicological assessments.



20 **21**

Fig. 6. Structures of NEMP (**20**) and NTMGMP (**21**).

Results on reactivation revealed the best performance of pralidoxime (**7**), suggesting that, for this surrogate, the size of reactivator might be of relevance to access the phosphonylated hydroxyl group at AChE hydrolytic site. These results did not agree with previous *in silico* data disclosed (Malinak et al. 2018; Vieira et al. 2023). Both of their *in silico* studies indicatedthat pralidoxime (**7**) was not the most efficient oxime for rescuing AChE-inhibited by phosphoramidate-related nerve agents, as opposed to what our workfoundin the *in vitro* assay (see Table 2). Furthermore, experimental data in literature suggests trimedoxime (**8**) as reactivator of AChE inhibited by A-242 surrogate, demonstrating different toxicological proprieties among A-series compounds. All this shows that the research on novel AChE reactivators must be continued, as there is no “Universal Antidote” available to treat poisoning caused by different AChE inhibitors, although oximes still having their value for emergencies.

Another important conclusion is that A-230 surrogate ANMP (**6**) did not cause AChE aging, as reactivation promoted by pralidoxime (**7**) did not change when measured after 10 min or after 24 h of enzyme inhibition (see Table 3). This outcome

ratifies the need for moiety in OP structure that favor formation of carbocations to justify aging mechanism by dealkylation. Otherwise, aging mechanisms by hydrolysis may not occur due to steric hindrance and less electrophilic of phosphoramidate as ANMP. Lastly, this work also suggests that pralidoxime (**7**) might be the first choice in poisoning involving similar compounds.

Materials and Methods

General Information

All chemicals used in this work were purchased from commercial suppliers and used as received, unless otherwise stated. Acetylcholinesterase from *Electrophorus eel* (*Ee*AChE, C2888,Type V-S, 1000 U/mg protein), pralidoxime iodide, acetylthiocholine iodide (ATCI), DTNB (Ellman's reagent,5,5′-dithio-bis-(2-nitrobenzoic acid)), dimethyl sulfoxide (DMSO, dry, biological assay grade), diethylamine, *N,N*-dimethylformamide (DMF), aluminum chloride, sodium hydride (60% w/w in mineral oil), 4-nitrophenol,sodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate, anhydrous sodium sulfate, 4Å molecular sieves and calcium hydride were purchased from SigmaAldrich Brazil (São Paulo, Brazil). Dichloromethane was purchased from Anidrol (Diadema,São Paulo, Brazil), dried over calcium hydride under inert atmosphere, distilledand stored over 4Å molecular sieves. Methylphosphonic dichloride, trimedoxime dibromide, obidoxime dichloride, pralidoxime mesylate and methoxime dichloride were synthesized in-house, and analytical data were compatible with the literature. HPLC-grade acetonitrile for chromatographic analysis was purchased from MerckBrasil (São Paulo, Brazil). HPLC-grade formic acid was purchased from AppliChem (Darmstadt, Germany). Deuterated chloroform and DMSO-*d*6 (1%tetramethylsilane as internal standard) were purchased from Cambridge Isotopes Laboratories (Tewksbury, Massachusetts, USA). Purified water was obtained from a MilliporeMilli-Q system (18.2 MΩ.cm at 25 °C, Millipore Brazil,São Paulo, Brazil).

NMR data were acquired from Bruker NMR Avance III Plus 400 MHz UltraShield, using TopSpin 4.1.4 for data processing. The reference for ^{1}H and ^{13}C nuclei was tetramethylsilane (TMS). For ^{31}P, the reference was triphenyl phosphate (TPP), via external reference OPCW Validation Group Working Database (VGWD) was used for data.

Liquid chromatography coupled to ultraviolet detection and mass spectrometry (LC-UV-MS) and LC-MS/QqQ (liquid chromatography tandem mass spectrometry) data were obtained from an Agilent 1260 LC Infinity system equipped with a 6410B triple quadrupole (QqQ) mass spectrometer detector and Agilent 1260 DAD (Billerica, MA, USA). The analyses were performed using an ACE Generix C18 column, 50mm x 2.1mm x 1.8µm (ACE Advanced Chromatography Technologies Ltd., UK). The elution conditions used were as follows: gradient A (0.1% formic acid in water): B (0.1% formic acid in methanol), 0 min = 10:90, 3 min = 10:90, 7 min = 90:10, 10 min = 90:10, 10.10 min = 10:90, 12 min = 10:90. Total time = 12 min, column temperature 25°C. Flow rate 0.050 mL/min and injection volume 5µL of sample. The mass spectrometer conditions were: ionization gas temperature 350°C, ionization gas flow rate 10L/min, nebulizer 25psi, SCAN m/z range from 50 to 400, precursor ion mode, fragmentor 50V, capillary 4000V, and collision energy 10eV.

Agilent MassHunter Workstation software, versions B.08.02 build 8.2.8260.0 and B.08.00 build 8.0.8208.0 (Agilent Technologies, Santa Clara, California, USA) wereused for LC-MS/QqQ data acquisition and processing.

SpectraMaxPlus 384 microplate reader (Molecular Devices, San Jose,CA, USA) was used in all in vitro assays. Disposable 96-wellmicroplates were purchased from Kasvi Brasil (São José dos Pinhais, Paraná, Brazil). Gilson single channel pipettes were purchased from Gilson Inc. (Middleton, WI, USA) and Eppendorf 8-channel pipettes were acquired from Eppendorf Brasil (São Paulo, Brazil). Ellman's tests were performed in triplicate, in three different assays, by at least two different operators, measured at 25 ± 2°C (Cavalcante et al. 2018b). Microsoft Excel 2010 was used for all calculations.

12mL Q-Tube™ sets were purchased from Q-Labtech LLC (East Lyme, CT, USA). EasyMax 102 Basic reactor used in syntheses was purchased from Mettler-Toledo Brasil (São Paulo, Brazil). Solvents were removed by using a Yamato RE-301 Rotary Evaporator, model RE301 from Yamato USA (Santa Clara, CA, USA).

A-230 surrogate ANMP (**6**) was purified by preparative thin layer chromatography (TLC) using a KP-SIL 2.5x7.5 cm TLC plates coated with silica gel F_{254} were purchased from Biotage (São Paulo, Brazil).

All disposable materials and glassware in contact with organophosphorus compounds were decontaminated with aqueous solution containing 10% w/v NaOH and 10% w/v NaClO for 48 h at room temperature in a fume hood before correct destination.

Synthesis of Compounds

Important Information: The synthetic procedure for surrogates must be conducted under strict safety requirements due to the toxicity of the target compounds. All personnel involved must be well trained in the techniques,and operations must be carried out in an efficient fume hood. All glassware and disposable materials must be decontaminated before proper destination.

Synthesis of *N,N*-diethylacetamidine (Jalani et al. 2013)

In a slight adaptation from literature, into a 250-mL round-bottomed flask, 47 mL of acetonitrile (900 mmol) was added, following cooling to 10°C with the aid of an ice bath. Then, 60 g of aluminum chloride (450 mmol) was slowly added (30 min), maintaining the temperature below 30°C. Afterwards, 47.3 mL of diethylamine (450 mmol) was added dropwise, keeping the temperature below 35°C. The system was cooled again to 10°C. The processes of adding aluminum chloride and diethylamine were then repeated, using the same amounts. After the 2nd addition of reagents, the system was heated to 120°C for 30 minutes. Then, the system was heated for 25 minutes at 140°C. The temperature was maintained between 140-145°C for 1 h. After this time, the reaction was cooled to 70°C and then poured over ice, keeping the temperature below 15°C and the mixture stirred. Then, 400 mL of dichloromethane was added, followed by slow addition of 400 mL of 40% w/v aqueous solution of sodium hydroxide. The mixture was stirred for additional 15 minutes and poured into a separatory funnel. The organic layer was collected and the aqueous phase was again extracted with 400 mL of dichloromethane. The organic extracts were combined, dried over anhydrous sodium sulfate and filtrated. Finally, the solvent was removed by vacuum evaporation, yielding a dark brown deliquescent solid. The solid was dissolved in CH_2Cl_2 and taken to the rotary evaporator at room temperature for 1 hour. Then, portions of 1 mL of hexane were added to assist in the removal of volatiles, which was carried on for 1 hour to eliminate any traces of solvents.

Subsequently, the obtained solid was subjected to lyophilization process to remove any residual moisture, by cooling to 0°C under high vacuum and kept at

constant temperature for 24 h. The lyophilized *N,N*-diethylacetamidine was stored in a bottle with a nitrogen atmosphere and kept in a desiccator.

Preparation of A-230 Surrogate ANMP (6)

Into a 12-mL sealed tube (Q-Tube™) equipped with a magnetic stirrer, 50 mg of methylphosphonic dichloride (0.376 mmol) was weighed and 1 mL of dry CH_2Cl_2 was added (tube A). The tube was sealed with a septum and the atmosphere was purged 5 times with nitrogen, followed by it cooling to 0°C and stirring at 500 rpm in Mettler Toledo EasyMax102 Reactor. In a 12-mL screw-capped vial containing 1 mL of dry CH_2Cl_2, 43 mg of *N,N*-diethylacetamidine (0.376 mmol) and 0.376 mmol of the sodium hydride (60% w/w in mineral oil) were subsequently added cautiously (hydrogen gas evolved). After that, the vial was flushed with nitrogen 3 times and centrifugated. The supernatant was collected with a syringe and added into the tube A. Then, the system was kept stirring for additional 2 h at 0°C. After this period (followed by LC-MS analysis, consumption of methylphosphonic dichloride), a suspension prepared in another 12mL screw-capped vial containing 1 mL of dry CH_2Cl_2, 70.6 mg (0.508 mmol) of 4-nitrophenol and 20 mg (0.508 mmol) of sodium hydride were added into tube A, with the reaction kept at 0°C for additional 2 h.

After this time (total of 4 h, reaction progress was checked with LC-UV-MS), the mixture was warmed to room temperature, filtered through a 0.22µm syringe filter and evaporated until a residue is formed. The residue was applied onto a thin layer chromatography plate KP-SIL silica gel 2.5x7.5 cm and eluted with 10% methanol: ethyl acetate. The spots were checked by LC-UV-MS after careful removal from the TLC plate, solubilization in methanol and filtration. Fractions containing the title compound were treated accordingly and evaporated to yield a yellow to orange oil.

Ellman's tests for ANMP *in vitro* activity (AChE inhibition, reactivation and aging estimation)

For the assessment of inhibition profile of our surrogate and the ability of oximes to reactivate AChE, we performed our modified Ellman's assay, used with slight modifications from the original publication (Cavalcante et al 2018b) using disposable 96-well microplates, with a maximum volume of 200 µL and UV absorbance

read at 412 nm for each well. After addition of the enzyme substrate (ATCI), UV absorbances were was read for 1 h (intervals of 5 min), with measurements obtained in triplicate, performed by at least two different operators, with mean values used for final calculations. Equation 1 was used to calculate the enzyme inhibition (%I), as Equation 2 was used to calculate the enzyme reactivation (%R).

$$\%I = 100\ x\ \left(\frac{A_0 - A_I}{A_0}\right) (Eq.1)$$

$$\%R = 100\ x\ \left[\left(\frac{A_0 - A_R}{A_0 - A_I}\right)\right] (Eq.2)$$

Briefly, the negative control (maximum activity of the enzyme) was measured by pipetting 30 µL of phosphate buffer solution (PBS, pH 7.60±0.10), 70 µL of AChE solution (2.14 U/mL in each well, prepared in PBS) and 80 µL of DTNB 0.4 mg/mL (in PBS). Then, 20 µL of 1 mmol/L ATCI (in PBS) was added, followed by UV measurements (A_0).

The inhibition assay (positive control) was conducted using four solutions of purified A-230 surrogate ANMP (**6**) in absolute isopropanol (final concentration of the inhibitor in each well: 3, 0.9, 0.3 and 0.09 µmol/L) and determined the level of enzyme inhibition after 10 min of incubation (inhibition reaction). The concentrated that afforded 85–95% of inhibition was then selected for reactivation studies using pyridinium oximes. It was measured by pipetting 20 µL of PBS, 70 µL of AChE solution, 80 µL of DTNB solution and 10 µL of inhibitor solution (in the four concentrations mentioned above). After 10 min of inhibition, 20 µL of ATCI solution was added, followed by UV (A_I). For reactivation studies using the clinically available oximes (prepared in three different concentrations in PBS, final concentrations in each well: 100, 10 and 1 µmol/L, prepared from stock solutions in DMSO), 70 µL of *Ee*AChE solution, 80 µL of DTNB solution and 10 µL of inhibitor solution were pipetted, followed by 10 min of inhibition reaction. After this time, 20 µL of solutions of the standard antidotes were added, and 30 min were waited for the reactivation reaction. Lastly, 20 µL of ATCI solution were added, and the absorbance was read to calculate the enzyme reactivation (A_R).

For estimation of AChE aging caused by the A-230 surrogate ANMP (**6**), *Ee*AChE was reactivated following the reactivation procedure after enzyme inhibition for 10 min and 24 h using pralidoxime (**7**) at 100 µmol/L as a reactivator. The difference in reactivation rates was considered the aging estimation.

Abbreviations

ACh – Acetylcholine

AChE – Acetylcholinesterase

ANMP – 4-Nitrophenyl (E)-N-(1-(diethylamino)ethylidene)-P-methylphosphonamidate

ATCI – Acetylthiocholine Iodide

CWC – Chemical Weapons Convention

DAD – Diode Array Detector

DTNB – Ellman's reagent,5,5′-dithio-bis-(2-nitrobenzoic acid)

*Ee*AChE – Acetylcholinesterase from *Electrophorus eel*

ESI – Electrospray Ionization

GC-MS – Gas Chromatography – Mass Spectrometry

IC_{50} – Half-Maximal Inhibitory Concentration

LC-MS(QqQ) – Liquid Chromatography – Tandem Mass Spectrometry

NMR – Nuclear Magnetic Resonance

OPCW – Organisation for the Prohibition of Chemical Weapons

PBS – Phosphate Buffer Saline

UV – Ultraviolet

Disclaimer

Authors and Publisher should not be held responsible or accountable for accidents that may occur from incorrect handling of toxic organophosphorus compounds or misuse, in disagreement with the Chemical Weapons Convention. Readers must access the website of the Organisation for the Prohibition of Chemical Weapons (OPCW, www.opcw.org) to obtain further information about the legal framework for research in certain aspects of the organophosphorus chemistry.

Consent for Publication

All Authors agree with the published version of this manuscript.

Authorship Contribution

L.B.B.: conceptualization, synthesis of compounds, spectral data acquisition, writing - original draft, review and editing, data analysis. **C.V.N.B.**: spectral data acquisition, writing - review and editing. **P.A.G.B.**: synthesis of compounds, writing - review and editing. **K.K.**: writing - review and editing. **S.F.A.C.**: conceptualization, synthesis of compounds, data analysis, spectral data acquisition, original draft review and editing. **R.B.S.**: writing - review and editing. **A.L.S.L.**: writing - review and editing. **D.A.S.K.**: conceptualization, *in vitro* assays, data analysis, writing - review and editing.

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Conflict of Interest

The Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Material

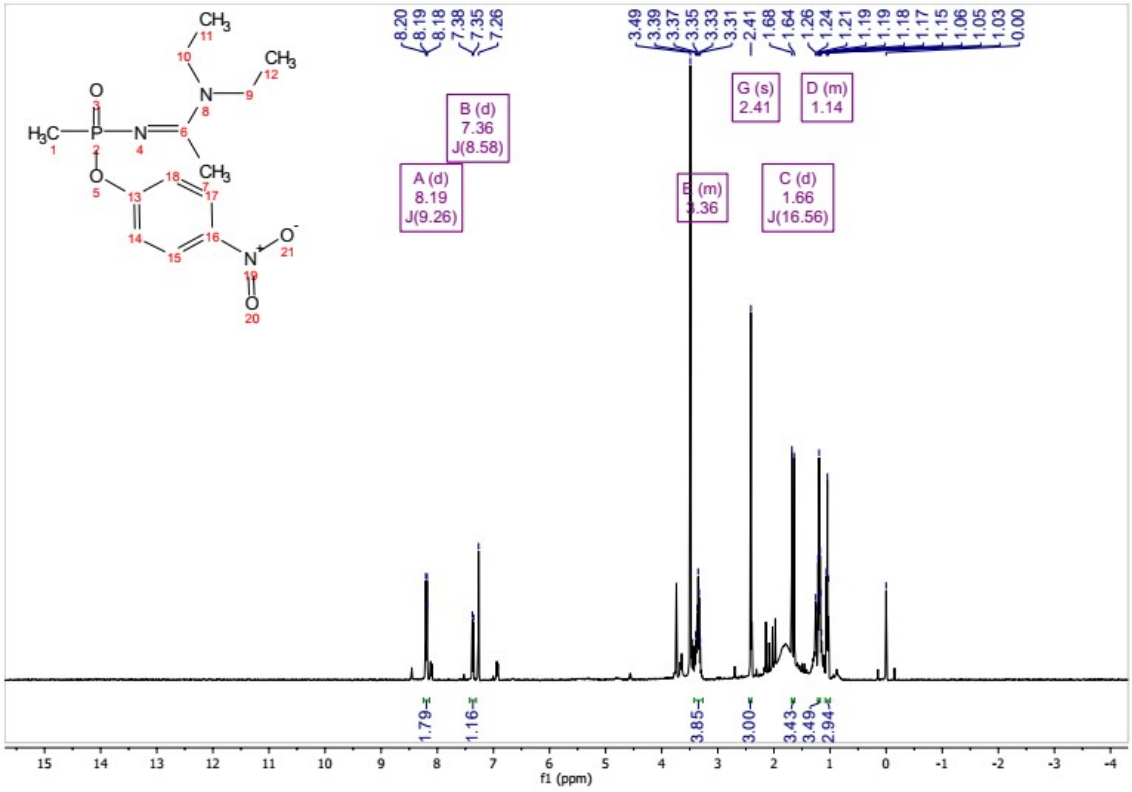

Fig. S1. 1H NMR spectrum of A-230 surrogate ANMP in $CDCl_3$

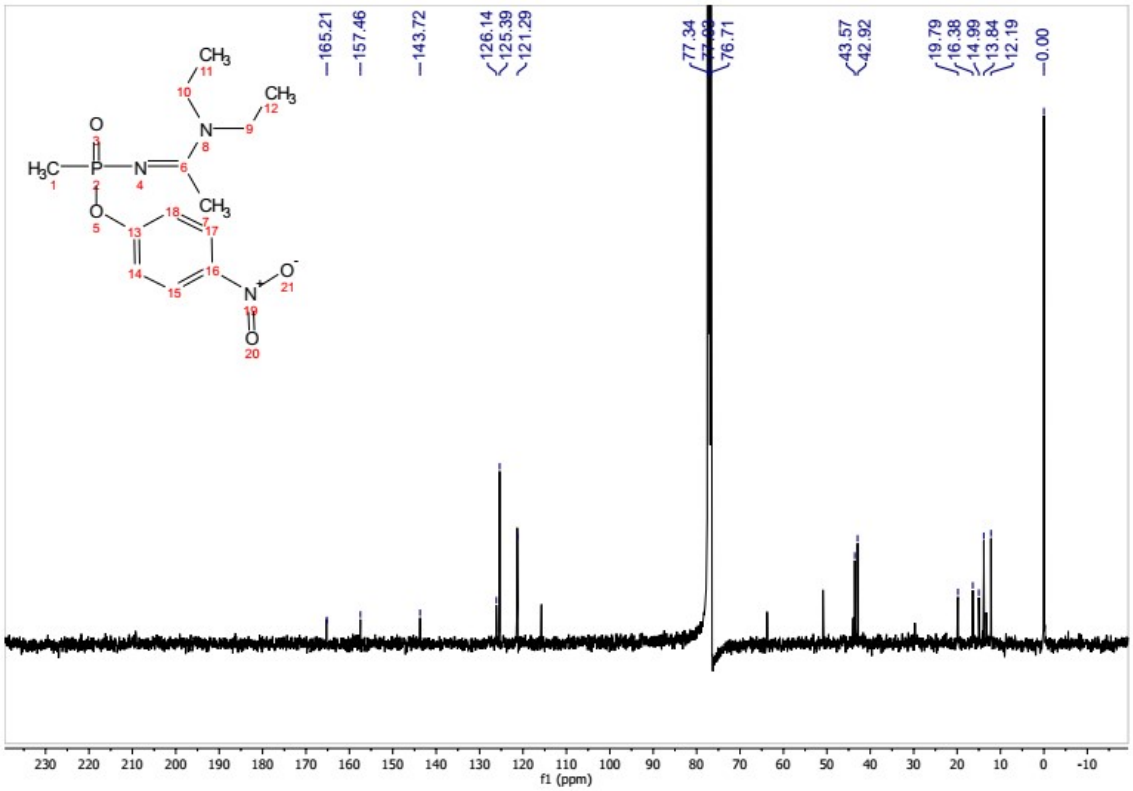

Fig. S2. 13C NMR spectrum of A-230 surrogate ANMP in $CDCl_3$

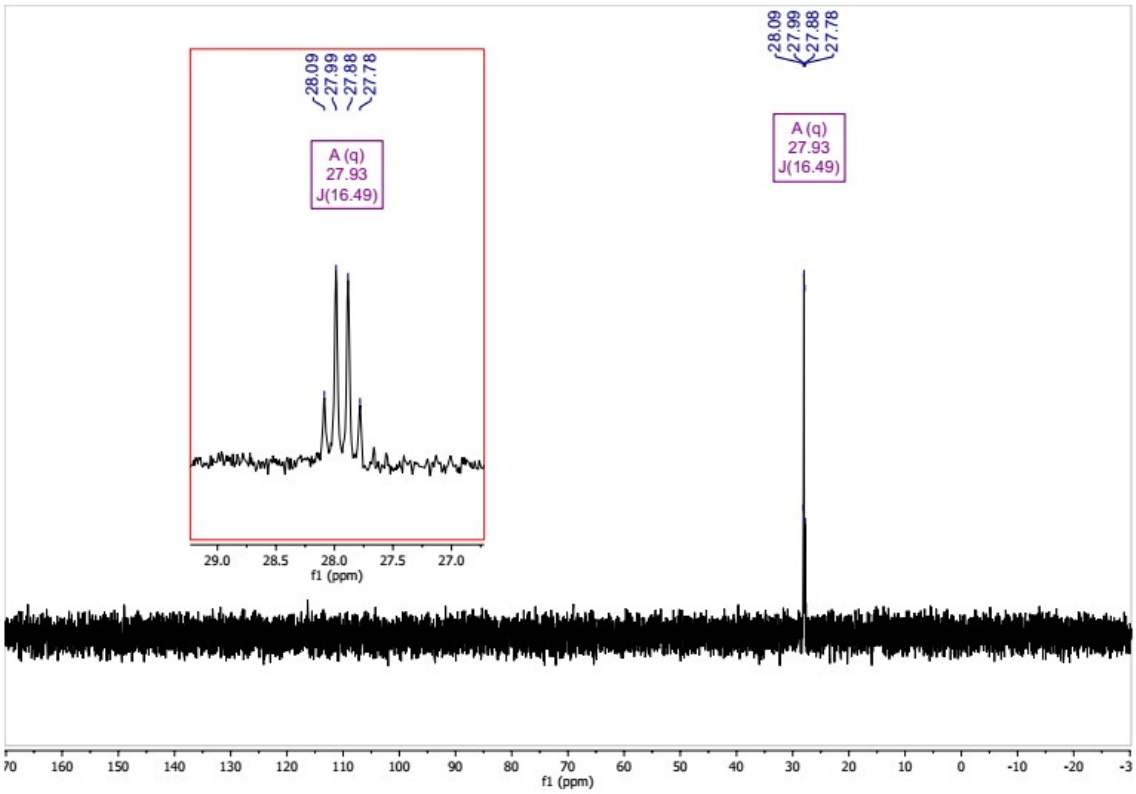

Fig. S3. 31P NMR spectrum of A-230 surrogate ANMP in $CDCl_3$

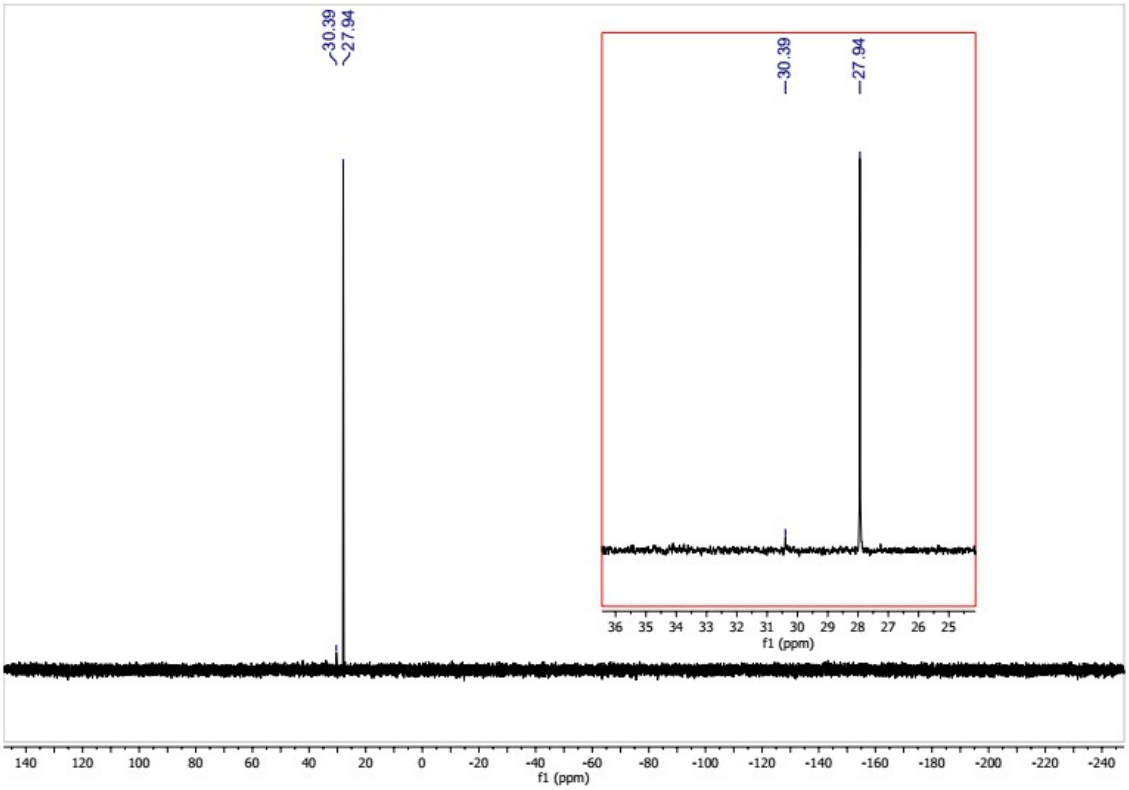

Fig. S4. 31P NMR spectrum of A-230 surrogate ANMP in $CDCl_3$