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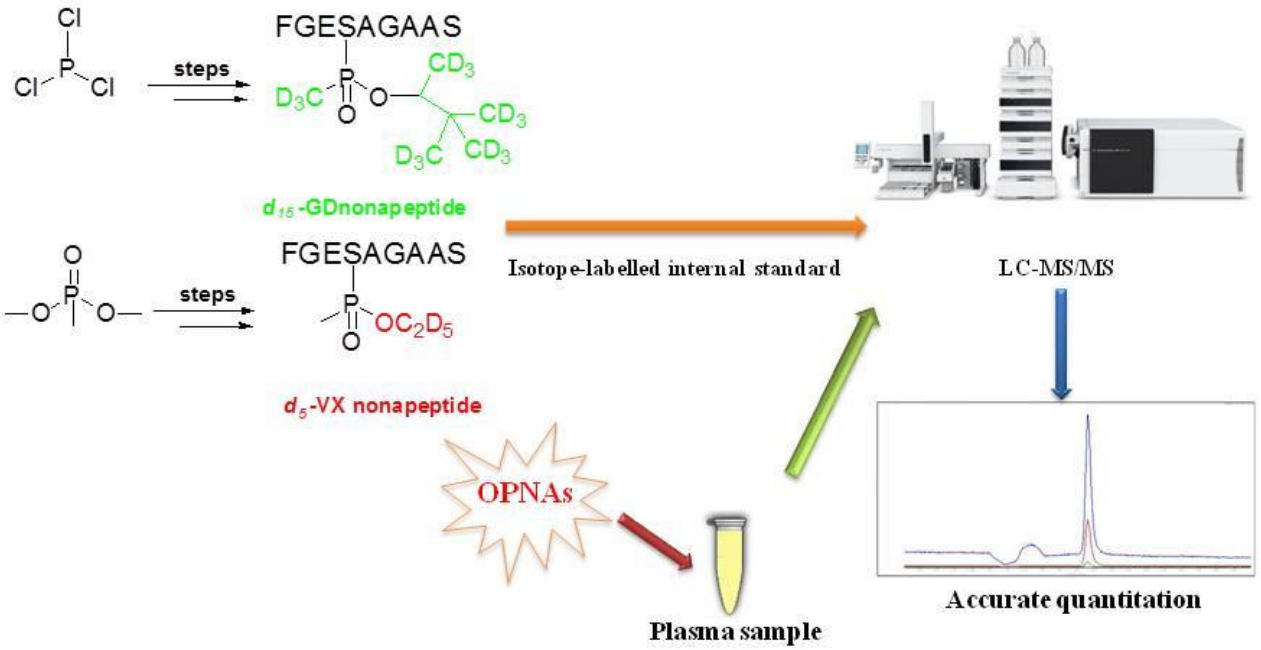
The deuterated nonapeptides synthesized could be used as the internal standard for LC-MS/MS isotope-dilution detection for OPNAs exposure.



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ABSTRACT

An efficient synthesis of d_5-VX adducted nonapeptide and d_{15}-GD adducted nonapeptide *via* solid-phase approach has been developed. The deuterated peptides could be used as the isotope-labeled internal standard for LC-MS/MS detecting the BuChE-OPNA biomarkers. This method also offers an access to the synthesis and detection of other phosphorylated nonapeptides.

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1. Introduction

Organophosphates (OP compounds) with anti-cholinesterase activity have been widely used as pesticides and incidentally as chemical warfare agents. Among the OP compounds, organophosphorus nerve agents (OPNAs) are a series of alkylphosphonic esters compound with signature phosphorus-carbon bond which could inhibit the enzymatic activity of acetylcholin esterase (AChE)[1] upon even low levels of exposure. As is known, The AChE is a neurotransmitter responsible for the conduction of nerve impulses at cholinergic synapses, which plays an important role in the hydrolysis of acetylcholine in vivo. Acute toxic effects of OPNAs exposure are due to AChE inhibition caused by phosphylation of a serine residue in the active site of the enzyme which will result in uncontrolled increase of acetylcholine that overstimulates cholinergic receptors. This will cause muscle twitching, miosis, convulsions, respiratory arrest, seizures, and even death.[2] Because of the high toxicity and proliferation in terrorist attacks and warfare, OPNAs is still one of the most potential threats to human life and modern societies, e.g. sarin or its similar compounds attack in Tokyo, Matsumoto and Syrian.[3] Therefore, new rapid, sensitive, and reliable methods are urgently needed for retrospective detection of exposure to and handling of these agents.

Basically, two approaches have been explored to detect exposure to OPNAs involving mass spectrometry technology. The conventional method developed from 1990s is based on measurement of the OPNAs hydrolysis products in vivo, e.g., O-alkyl methylphosphonicacids. Methods for analysis of these compounds are based on GC-MS or on LC-MS[4]. However, the serious shortcoming of these method is that the hydrolysis products can't be detained in vivo for a relatively long life-time. Actually, it is estimated that ~90% of the compounds will be excreted in urinary within 2-3 days which increase the difficulty in the analysis of the trace exposure to OPNAs[5]. In contrast, the techniques used nowadays relying on enzymatic digestion of the adducts combined by cholinesterase and OPNAs followed by LC-MS/MS analysis provide better limits of detection, greater specificity, and afford positive identification of the OPNAs. As a kind of the cholinesterase and stoichiometric bioscavenger of OPNAs, the butyrylcholine esterase (BuChE) which is readily available in serum could form a relatively stable covalent adduct combined with OPNAs has a lifetime for at least 16 days[6] and mainly exists in blood. Thus, the advantage of the BuChE aforementioned make it an ideal biomarker for monitoring OPNAs exposure.

With the US military codes GB, GD and VX respectively, there are three predominate compounds among the OPNAs presented as follows: O-isopropyl methylphosphonofluridate, O-pinacolyl methylphosphonofluridate and O-ethyl, S-2-diisopropyl-amino ethyl methylphosphonothiolate. The structure of these three compounds are shown in Figure 1. As the biomarkers formed by OPNAs and BuChE can be converted to the phosphorylated nonapeptide[7] compounds with similar structures through the pepsin digestion (Figure 1), only the obvious structural difference are shown between the peptides originated from VX and GD in alkoxy moieties.

In recent years, the developed method for the verification of the OPNAs exposure was based on high performance liquid chromatography isotope dilution tandem mass spectrometry quantitative detection of the phosphorylated nonapeptide. Although the analysis of the nonapeptides biomarkers in blood samples plays an important role in forensic investigations of the alleged use of nerve agents currently, to the best of our knowledge, there is no literature reporting the synthesis method of the isotope-labeled internal standard nonapeptides. Herein, we envisioned to synthesize brand new d_5-VX and d_{15}-GD nonapeptides with unique alkoxy moieties (Figure 1) which could be used as the isotope-labeled internal standard of BuChE-OPNAs biomarkers via LC-MS/MS detecting.

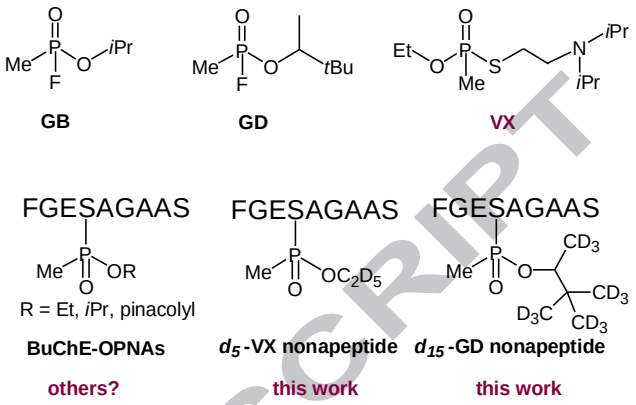


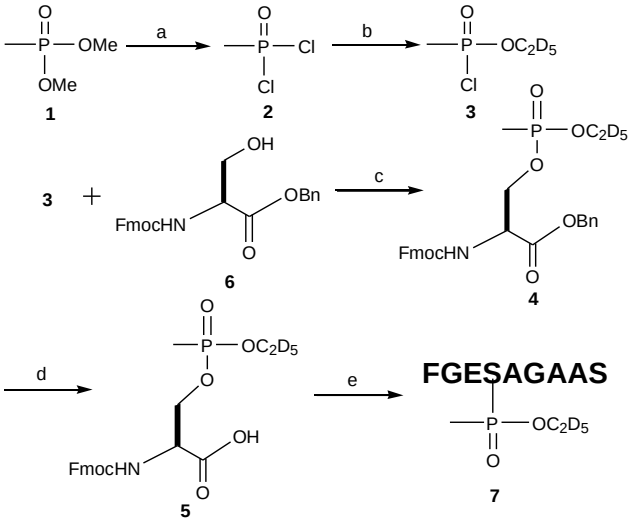
Figure 1 Structures of prominent organophosphorus nerve agents, their nonapeptides derived from the pepsin digestion of the BChE-OPNA adducts, the isotope-labeled internal standard of d_5-VX nonapeptide and d_{15}-GD nonapetide.

2. Synthesis procedures for the d_5-VX nonapeptide and d_{15}-GD nonapetide

The synthesis procedures of the d_5-VX adducted nonapeptide and d_{15}-GD adducted nonapeptide are presented in Figure 2 and 3, respectively. As the starting material to synthesize the d_5-VX adducted nonapeptide, dimethyl methylphosphonate was treated with 2.5 equivalent sulfoxide chloride at 140 ºC for 6 hours, affording methylphosphonic dichloride **2**[8] in the 90% yield. Subsequent monoesterfication with d_6-Ethanol and triethylamine lead to d_5-Ethyl methylphosphonic monocloridate **3** in 36% yield[9]. The chemical **3** must be purified by carefully distillation under vacuum to prevent the undesired polymerization. d_5-Fmoc-serine(O-ethyl methylphosphonate) benzyl ester **4**[10] was obtained in the 60% yield by reacting with the protected serine **6** in the presence of DMAP and triethylamine. Four equivalent of the intermediate **3** was used during the esterfication because of the low reactivity. Finally, target molecule **5,** which is the important precursor before Solid-Phase synthesis, was formed by the Pd/C hydrogenation of **4**[10] with 80% yield.

While the synthetic procedures of the d_{15}-GD adducted nonapeptide started from phosphorus trichloride. Phosphorus trichloride was treated with three equivalent d_4-methanol in -15 °C to afford d_9-trimethyl phosphite in 40% yield. d_9-Dimethyl methylphosphonate was obtained in 95% yield via the subsequent Arbuzov rearrangement reaction triggered by adding 1% d_3-iodomethane[11]. d_9-Dimethyl methylphosphonate was treated with exess equivalent sulfoxide chloride at 140 ºC for 6 hours, affording d_3-methylphosphonic dichloride **11** in 90% yield[8]. Intermediate **13** was obtained in the 50% yield via bi-molecular reduction of d_6-acetone in amalgam formed by mercuric chloride and magnesium turnings[12]. Subsequent Pinacol rearrangement reaction with 3 mol/L sulfuric acid lead to d_{12}-3,3-dimethyl-2-butanone **14** in 60% yield[13]. The chemical **15** was formed in 80% yield by potassium borohydride reduction. Subsequent monoesterfication with intermediate and triethylamine lead to d_{15}-Pinacolyl methylphosphonic monocloridate **16** in 60% yield[14]. d_{15}-Fmoc-serine(O-pinacolyl methylphosphonate) benzyl ester **17**[10] was obtained in the 60% yield by reacting with the protected serine **6** in the presence of DMAP and triethylamine. Also four equivalent of the intermediate **16** was used during the

esterfication because of the low reactivity. Finally, target molecule **18,** which is the important precursor before Solid-Phase synthesis, was formed by the Pd/C hydrogenation of **17**[10] with 80% yield .

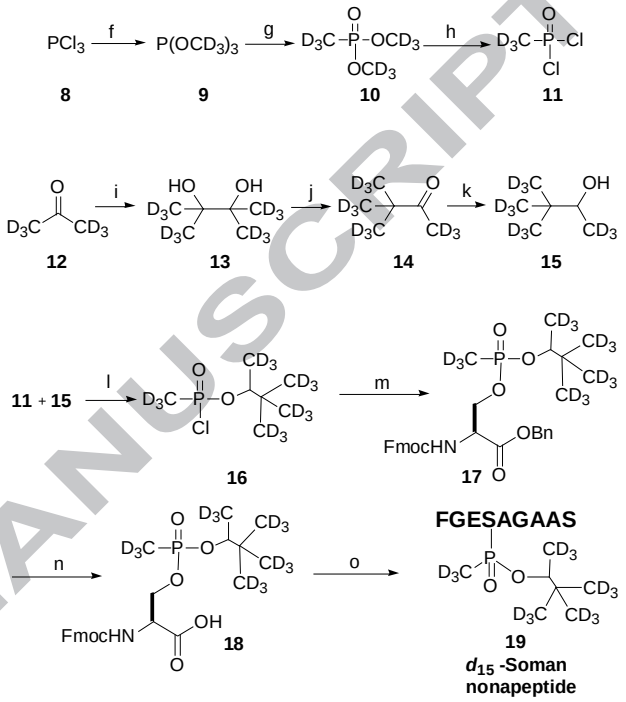


(a) $SOCl_2$ (2.5 equiv.), 140 °C, 90% yield; (b) C_2D_5OD (1 eqiuv.), Et_3N, bezene, 36% yield; (c) reagent **6** (0.25 equiv.), Et_3N, DMAP, bezene, 0 °C, 75% yield; (d) H_2, 10% Pd/C, THF/MeOH, 80% yield; (e) manual Solid-phase peptide synthesis procedure

Figure 2 Synthesis route of d5-Vx nonapeptide

Solid-phase peptide synthesis was done manually from C-terminal to N-terminal to enhance the coupling efficiency. The d_5-VX adducted nonapeptide was synthesized using a universal method for peptides preparation on a Fmoc-Ser (tBu)-Wang resin and HBTU/DIPEA activation. Compared with the method reported before[10], it takes less time for the resin elution to obtain the target peptide and the method could be used in practical synthesis. Protected amino acids used in the peptide synthesis included Fmoc-glutamic acid with the tert-butyl ester on the sidechain carboxylic acid and Fmoc protection of the other residues without side-chain protection. Piperidine/DMF (25%) was used to de-protect the Fmoc group. The rest of the amino acids residues were induced onto the resin in sequence by HBTU/DIPEA activation. Kaiser test was used to ensure the connection with right amino acid residue upon every step. After this point, cleavage from the resin was achieved by treating the peptide resin with the solution containing 68.5% TFA, 10% 1,2-ethanedithiol, 10% thioanisole, 5% phenol, 3.5% double-distilled water and 1% triisopropylsilane for 3 h at room temperature. The crude product of the phosphonylated peptide which could be precipitated by anhydrous ether, was further purified by reversed-phase HPLC on a C18 column (0.05% TFA–water–2% acetonitrile). Peptide purity was >90% and was verified by HPLC-DAD, NMR and high resolution mass spectrometry analysis.

However, the d_{15}-GD adducted nonapeptide was failed to be obtained via the Solid-phase peptide synthesis procedure for the d_5-VX adducted nonapeptide. Maybe the Pinacolyl moiety is susceptible to elimination or readily to leave in 68.5% TFA and this phenomenon would be in accord with the study that the GD-BuChE adduct is quite unstable in vivo and easy to lose the pinacolyl moiety which is called "aging" process[6a]. Alternatively, we employed the reaction condition containing 2-chlorotrityl resin and 1% TFA instead[10].Protected amino acids used in the peptide synthesis included Fmoc-glutamic acid with the isopropyl phenyl ester on the side chain carboxylic acid and Fmoc protection of other residues without side-chain protection. Cleavage from the resin was achieved by treating the peptide resin with 1% TFA–CH_2Cl_2 containing 2% triisopropylsilane overnight at room temperature. The crude product of the phosphonylated peptide which could be precipitated by anhydrous ether, was further purified by reversed-phase HPLC on a C18 column (0.05% TFA–water–2% acetonitrile). Peptide purity was >90% and was verified by analysis of HPLC-DAD, NMR and high resolution mass spectrometry.



(f) CD_3OD (1 equiv.), -15 °C, 40% yield; (g) CD_3I(1% eqiuv.) , 50 °C, 95% yield; (h) SOCl2 (2.5 equiv.), 140 °C, 90% yield; (i) magnesium amalgam, 50~60 °C, 50% yield; (j) 3 mol/L sulfuric acid, 107 °C , 60% yield; (k) potassium borohydride (1 equiv.), 80 °C, 80% yield; (l) reagent **15** (1 equiv.), Et_3N, bezene, 60% yield; (m) reagent **6** (0.25 equiv.), Et_3N, DMAP, bezene, 0 °C, 75% yield; (n) H_2, 10% Pd/C, THF/MeOH, 80% yield; (o) manual Solid-phase peptide synthesis procedure

Figure 3 Synthesis route of d_{15}-GD nonapeptide

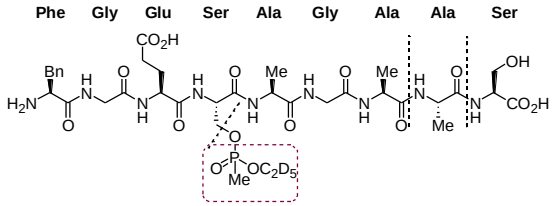


Figure 4 Structures of the d_5-VX adducted nonapeptide and fragmentation sites denoted with dashed lines.

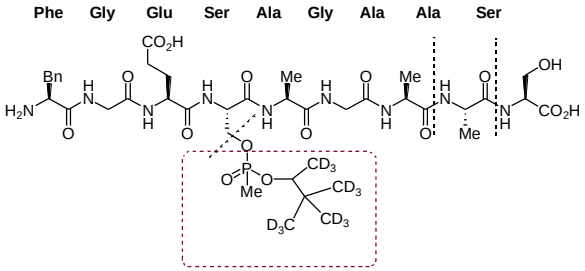


Figure 5 Structures of the d_{15}-GD adducted nonapeptide and fragmentation sites are denoted with dashed lines.

3. The mass spectrometry fragment pathway of the d_5-VX nonapeptide and d_{15}-GD nonapetide

The fragmentation of the d_5-VX and d_{15}-GD adducted nonapeptides (Figure 4 and Figure 5) was observed by Precursor-to-Product Ion Transitions Monitored using Multiple Reaction Monitoring (MRM). Interestingly, The mass spectrometry fragment pathways of the d_5-VX and d_{15}-GD nonapetide were identified with the isotopically enriched nonapeptides biomarker in the similar structure[7c]. The protonated, peptide-adduct fragments show losses of the adducted nerve agent, the C-terminal serine and the C-terminal alanine. It showed the same fragmentation pattern as the d_5-VX-BuChE and d_{15}-GD-BuChE nonapeptide[7c]. As is showed in the Figure 6 and 7, the precursor ion for the chemical 7 (m/z 907.3968) exhibited product ions derived from a loss of 129 Da that corresponded to the loss of the d_5-VX moiety (m/z 778.3364); further losses of the terminal serine (m/z 673.2932), and the alanine (m/z 602.2554) residue were also exhibited. While the precursor ion for the chemical 19 (m/z 973.5225) exhibited product ions derived from a loss of 195 Da that corresponds to the loss of the d_{15}-GD moiety (m/z 778.3371); further losses of the terminal serine (m/z 673.2937), and the alanine (m/z 602.2562) residue were also exhibited. Additionally, we find the dehydration impurities for d_{15}-GD adducted nonapeptides. As is metioned in reference[10], the compound generated via the elimination of OP-serine and addition of piperidine process was found (calcd for $C_{38}H_{58}N_{10}O_{13}$: 863.4258).

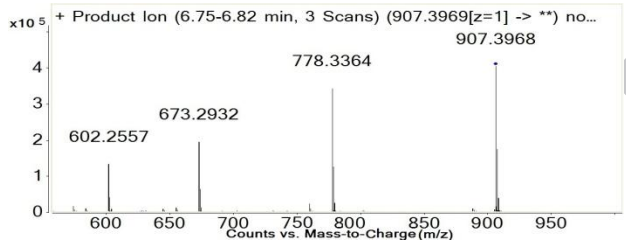


Figure 6 Structures of the d_5-VX adducted nonapeptide and fragmentation sites denoted with dashed lines.

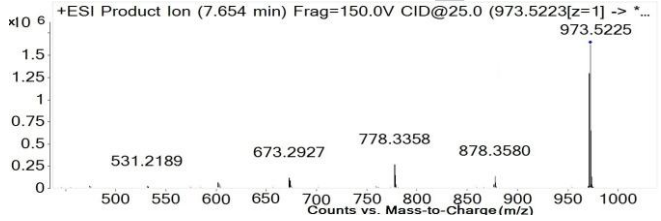


Figure 7 Structures of the d_{15}-GD adducted nonapeptide and fragmentation sites denoted with dashed lines.

4. Co

In summary, the synthesis approaches of d_5-VX and d_{15}-GD adducted nonapeptide via solid-phase has been developed. The MS/MS fracture manners of the d_5-VX and d_{15}-GD nonapeptides are as same as the VX-BuChE nonapeptide[7c]. So the brand new compound d_5-VX and d_{15}-GD peptide could be used as the substitute of the isotope-labeled internal standard reported before for LC-MS/MS to monitor the OPNAs exposure. Additionally, the synthesis material d_6-Ethanol and d_{12}-3,3-Dimethyl-2-butanol used in the procedure has the property of the easier availability than the deuterated amino acid used in the former literature[5]. The superiority of the nonapeptides described in the paper is that the convenience in the synthesis procedure. Although the procedures involve in many steps, the synthesis for the previously reported internal standards may also follow it actually except for the d_{15}-pinacolyl methylphosphonic monochloridate synthesis. Additionally to our knowledge, there is no literature reporting the synthesis method to these compouds, and we used the deuterated alkyl phosphonic momochloridate to substitute for the isotope labeling peptides to synthesize the target nonapeptides. They can be used as brand new internal standards for the retrospective analysis after the OPNAs exposure. This method may also find applications in the synthesis and detection of other phosphorylated nonapeptides.

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15. The target compound d_5-VX adducted nonapeptide was synthesized via the Solid-phase peptide synthesis manually from C-terminal to N-terminal to enhance the coupling efficiency. The peptides were synthesized using Fmoc chemistry on a Fmoc-Ser (tBu)-Wang resin and HBTU/DIPEA activation. Piperidine/DMF (25%) was used to de-protect the Fmoc group. The rest of the amino acid residues were induced onto the resin in sequence by HBTU/DIPEA activation. Kaiser test was used to ensure the connection with right amino acid residue upon every step. After

this point, cleavage from the resin was achieved by treating the peptide resin with the solution containing 68.5% TFA, 10% 1,2-ethanedithiol, 10% thioanisole, 5% phenol, 3.5% double-distilled water and 1% triisopropylsilane for 3h at room temperature. The crude product of the phosphorylated peptide which could be precipitated by anhydrous ether, was further purified by reversed-phase HPLC on a C18 column (0.05% TFA–water–2% acetonitrile). Peptide purity was>90% by analysis of HPLC-DAD. It was also verified by NMR and high resolution mass spectrometry. ESI+-HRMS data showed the molecular weight 907.3968 as the formular $C_{36}H_{51}D_5PN_9O_{16}$ and the ^{1}H NMR (599.7 MHz, D_2O) data was showed as follows: δ =1.28 (t, J = 7.4 Hz, 9H), δ = 1.47 (d, $^{2}J_{H\text{-}C\text{-}P}$ = 17.6 Hz, 3H), δ = 1.80-2.11 (m, 6H), δ = 2.37 (t, J = 7.1 Hz, 2H), δ = 3.12 (m, 3H), δ = 3.75-3.88 (m, 6H), δ = 4.20-4.35 (m, 9H), δ = 4.55 (s, 1H), δ = 7.18 (d, J = 7.2 Hz, 2H), δ = 7.26-7.31 (m, 3H). While $^{13}C\{^{1}H\}$ NMR (150.8 MHz, D_2O) data was showed as follows: δ = 177.1, 175.0, 174.7, 174.6, 173.5, 173.3, 170.9, 170.7, 169.8, 169.6, 133.6, 129.3, 129.1, 128.0, 64.2, 64.1, 61.2, 61.1, 55.1, 54.4, 53.45, 53.40, 53.35, 53.1, 49.9, 49.5, 49.4, 42.2, 36.7, 30.0, 26.1, 16.7, 16,4, 9.4, 8.5. $^{31}P\{^{1}H\}$ NMR (242.8 MHz, D_2O) data: δ = 35.9.

16. The target compound d_{15}-GD adducted nonapeptide was synthesized using Fmoc chemistry on a 2-chlorotrityl resin and HBTU/DIPEA activation. Protected amino acids used in the peptide synthesis included Fmoc-glutamic acid with the isopropyl phenyl ester on the side chain carboxylic acid and Fmoc protection of other residues without side-chain protection. Cleavage from the resin was achieved by treating the peptide resin with 1% TFA–CH2Cl2 containing 2% triisopropylsilane overnight at room temperature. The crude product of the phosphonylated peptide which could be precipitated by anhydrous ether, was further purified by reversed-phase HPLC on a C18 column (0.05% TFA–water–2% acetonitrile). Peptide purity was >90% and was verified by analysis of HPLC-DAD, NMR and high resolution mass spectrometry. ^{1}H NMR (599.7 MHz, D_2O): δ =1.28 (m, 9H), δ =1.95 (m, 5H), δ = 2.36 (t, J = 7.1 Hz, 2H), δ =3.13 (m, 3H), δ = 3.75-3.87 (m, 7H), δ = 4.19-4.31 (m, 8H), δ = 7.18 (d, J = 7.1 Hz, 2H), δ = 7.29 (m, 3H), $^{31}P\{^{1}H\}$ (242.8 MHz, D_2O): δ = 34.52, 34.60, 34.95, 35.01. ESI+-HRMS: m/z $(M+1)^{+}$ calcd for $C_{40}H_{49}D_{15}PN_9O16$: 973.5223 ; found: 973.5225.

Highlights in the paper

1. The two target compounds reported in the paper are brand new.
4. We present a new practical synthesis method for the d_5-VX nonapeptides.
3. The mass spectrometry fragment pathways are identical as the biomarkers.
4. They are new deuterated internal standards in the analysis of OPNAs exposure.