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Synthesis of the Tripeptides Tyr–Thr–Lys Phosphorylated with Isopropyl Methyl- and (Deuteromethyl)phosphonochloridates as Reference Standards for the Analysis of Biomedical Samples

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Abstract—A procedure for the phosphorylation of the tripeptide Tyr−Thr−Lys with isopropyl methyl- or (deuteromethyl)phosphonochloridate is developed. The phosphorylated tripeptides are intended for use as reference standards in the analysis of blood samples of people suspected to have been exposed to acetylcholinesterase inhibitors. Conditions of chromatographic separation and purification of the synthesized compounds are determined and optimized, which ensures the preparation of high-purity phosphorylated tripeptides.

Keywords: biomarker, tripeptide, phosphorylation; NMR spectroscopy

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The acetylcholinesterase inhibitors alkyl and methylphosphonofluoridates are listed in Schedule 1 of the Chemical Weapons Convention (hereinafter, Convention) [1]. Their development, production, accumulation and use are subject to strict control.

Over the past years, the Organization for the Prohibition of Chemical Weapons in its activities puts special emphasis on the analysis of biomedical samples in its activities in the monitoring of compliance with the Convention. The source of these samples are people and/or animals suspected to have been exposed to such chemicals.

There are some publications, where phosphorylation products of the tyrosine–threonine–lysine tripeptide have been mentioned as the possible markers of exposure of people and animals to alkyl methylphosphonofluoridates [2, 3]. However, scarce information on these compounds has so far been available. As the first step toward isolation of phosphorylated tripeptides from blood plasma and study of their properties we considered it expedient to develop a method of synthesis of these compounds, including isotopically labeled derivatives, as reference standards for HPLC–MS/MS analysis of plasma samples.

In the present work we have developed a method of synthesis of methyl- and deuterophosphonoyl derivatives of the tripeptide Tyr–Thr–Lys, as reference standards intended for use in the analysis of plasma samples from patients suspected to have been exposed to isopropyl methylphosphonofluoridate (sarin).

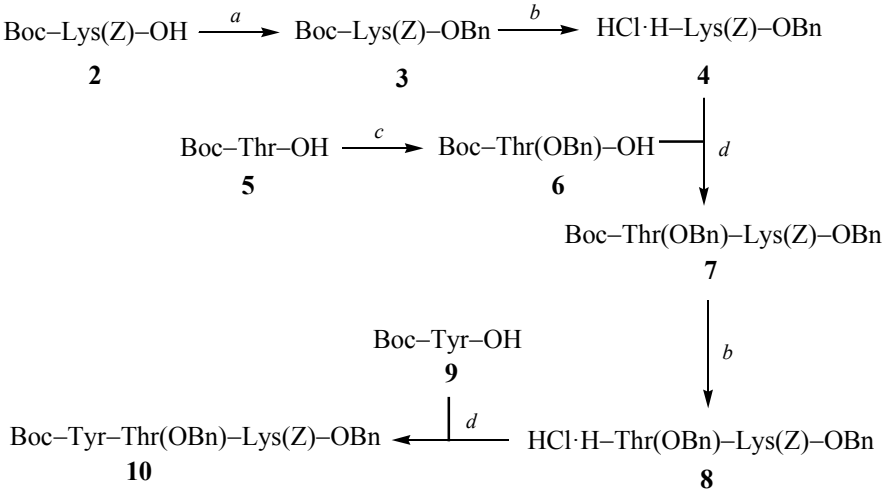
The synthesis of the target phosphorylated (phosphonoyl) tripeptide derivatives involved the reaction of isopropyl methyl- and deuteromethyl-phosphonochloridates with the free phenolic hydroxyl of tyrosine incorporated in the protected tyrosine–threonine–lysine Boc–Tyr–Thr(OBn)–Lys(Z)OBn and the subsequent deprotection.

Protected tripeptide **10** required for phosphorylation was synthesized by classical liquid-phase peptide synthesis [4–9], following Scheme 1, and purified by preparative HPLC. Isopropyl methyl- and deuteromethyl-phosphonochloridates were synthesized by the procedures described in [10, 11], starting from triisopropyl phosphite (Scheme 2).

A suspension of tripeptide **10** in acetonitrile was pretreated with NaH at 5°C, after which phosphonoyl chloride **15a** or **15b** was added, and the mixture was left to stand for 2 h at 20°C. Phosphorylation products **16a** and **16b** were purified by column chromatography on silica gel.

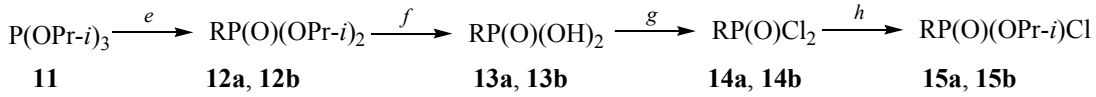
The protective groups were removed consecutively. First, by treatment with 15% HC1 in dioxane we removed

Scheme 1.



a: BnCl, K_2CO_3, DMF; *b*: 15% HCl in dioxane; *c*: BnCl, NaH, DMF; *d*: HOBt (NMM is *N*-methylmorpholine and HOBt is *N*-hydroxybenzotriazole).

Scheme 2.

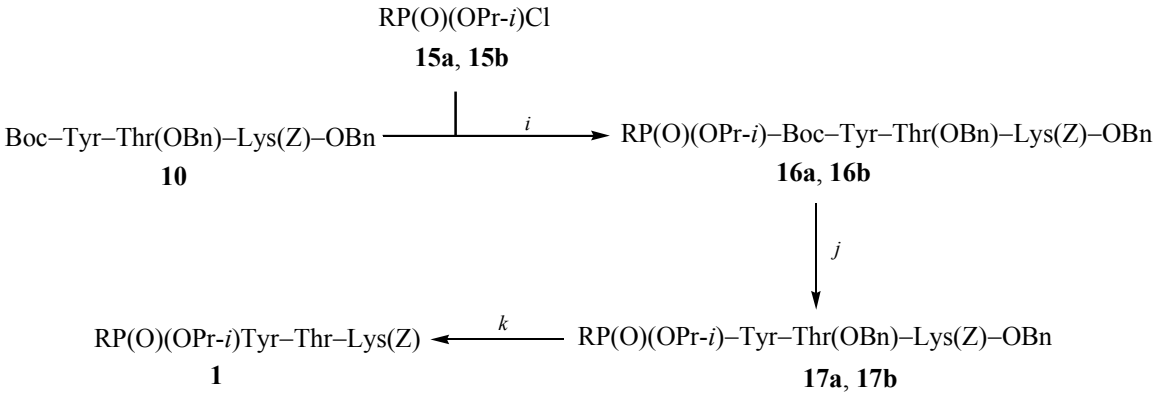


R = CH_3 (**a**), CD_3 (**b**); *e*: CH_3I or CD_3I, *t*, °C, 2 h; *f*: H_2O, H^+; *g*: $SOCl_2$; *h*: *i*-PrOH, NEt_3.

the Boc protective group from the tyrosine amino group to obtain compounds **17a** and **17b** as yellow viscous oils. According to NMR and HPLC-MS, the products did not require further purification and subjected to catalytic hydrogenation to remove the benzyl protective groups. Hydrogenolysis in the presence of a Pd/C catalyst failed to remove benzyl protection. Benzyl groups could be removed one after another by hydrogenolysis in the presence of a freshly prepared PdOH in ethanol at an atmospheric pressure of hydrogen. The reaction progress was monitored by HPLC-MS every 6 h. Traces of the target products were detected in 12 h. In 36 h, an additional portion of the catalyst was added to complete reaction. Benzyl groups were completely removed in 72 h (Scheme 3).

After filtration and removal of the solvent, the purity of phosphorylated compounds **1a** and **1b** was about

Scheme 3.



R = CH_3 (**a**), CD_3 (**b**); *i*: NaH, ACN, 5–20°C; *j*: 15% HCl in dioxane; *k*: H_2, $Pd(OH)_2$.

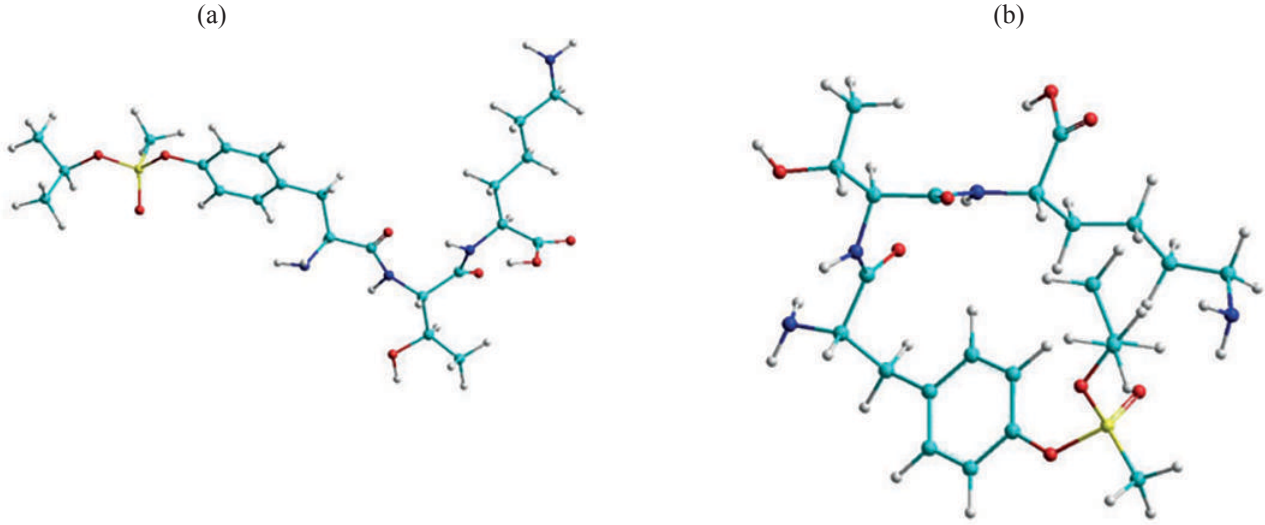


Fig. 1. Predicted conformations of the phosphorylated Tyr–Thr–Lys tripeptide: (a) linear conformer and (b) folded conformer.

~70%. The products were further purified by preparative HPLC–MS and isolated with a purity of more than 95% as white crystals.

The ^{31}P NMR spectra of phosphorylated tripeptides **1a** and **1b** displayed pseudodublets [J = 7 (**1a**) and 10 Hz (**1b**)] rather than singlets like in the spectra of phosphorylated tripeptides **16a**, **16b**, **17a**, and **17b**. Taking into account that the measurement conditions excluded any P–H coupling and the signals in the pseudoulets had different intensities, we suggested that compounds **1a** and **1b** were mixtures of conformers. Calculations using Hyperchem-7 software method of molecular mechanics MM3 and semiempirical molecular orbital program PM3 predicted that the linear conformer (Fig. 1a) is energetically less favored that folded (Fig. 1b).

It was shown by ^{1}H and ^{31}P NMR spectroscopy and HPLC–MS that compounds **1a** and **1b** were stable for 3 months, and, therefore, can be used as reference standards in biomedical sample analysis.

EXPERIMENTAL

The ^{1}H and ^{31}P NMR spectra were registered on a QOne AS 400 spectrometer at 399.85 and 161.86 MHz, respectively, and at 298 K. The proton chemical shifts were referred to residual proton signals of the deuterated solvent, and the ^{31}P chemical shifts, to external H_3PO_4. Thin-layer chromatography was performed on Marchery–Naglel AlugramSILG/UV254 plates using an Ul'tramag-K254/365 UV lamp (λ = 254 nm) for spot visualization.

The high-resolution mass spectra were obtained on an Agilent Infinity 1260/Thermo Scientific Orbitrap Fusion Lumos HPLC–MS system operated in the ESI mode.

Protected amino acids were obtained from AKos (Germany) and triisopropyl phosphite, methyl iodide, and deuteromethyl iodide were obtained from Sigma–Aldrich.

Boc–Lys(Z)OBn (3). Freshly distilled benzyl chloride, 1.4 g (11 mmol) and 1.65 g (1.2 mmol) of K_2CO_3 were added to a solution of 3.8 g (10 mmol) of Boc-Lys(Z)–OH **2** in 40 mL of DMF. The reaction mixture was stirred for 12 h at room temperature, after which it was diluted with 70 mL of water, and the reaction product was extracted with ethyl acetate (5×30 mL), washed with 5% $NaHCO_3$ (2×20 mL), 20 mL of water, and 20 mL of saturated NaCl and dried over Na_2SO_4. The solvent was removed in a vacuum. Yield 4.7 g (85%), light-colored thick oil.

HCl·H-Lys(Z)OBn (4). To 4.7 g of compound **3**, a 15% solution of HCl in dioxane, 25 mL, was added, and the mixture was stirred for 2 h at room temperature. The solvent was removed in a vacuum, and the residue was triturated with 20 mL of ether and dried in air to constant weight. Yield 3.72 g (100%), white crystals.

Boc-Thr(OBn)OH (6). To a solution of 2.4 g (20 mmol) of Boc-Thr–OH **5** in 100 mL of DMF, 1.7 g (44 mmol) of 65% NaH was added at 0°C. When hydrogen no longer evolved, 2.8 g (22 mmol) of freshly distilled benzyl chloride was added. The reaction mixture was stirred for 5 h at 25–30°C, and the solvent was removed in a vacuum. The residue was dissolved in 100 mL of water,

the solution was acidified with 3 N HCl to pH 3.5, and the product was extracted with ethyl acetate (5×30 mL). The organic layer was washed with 20 mL of water, 20 mL of saturated NaCl, and dried over Na_2SO_4. The solvent was removed in a vacuum, and the residue was triturated with hexane. Yield 3.02 g (49%), white crystals.

Boc–Thr(OBn)–Lys(Z)OBn (7). A solution of 2.06 g (10 mmol) of dicyclohexylcarbodiimide in 10 mL of DMF was added dropwise to a cold (0°C) solution of 3.72 g (9.2 mmol) of compound **4**, 2.84 g (9.2 mmol) of compound **6**, 1.53 g (10 mmol) of *N*-hydroxybenzothriazole, and 1.0 g (10 mmol) of *N*-methylmorpholine in 50 mL of DMF. The reaction mixture was stirred for 1 h at 0°C and then for 12 h at room temperature. A 5% solution of citric acid, 100 mL , was added, and the reaction product was extracted with ethyl acetate (5×30 mL), washed with 5% $NaHCO_3$ (2×20 mL), 20 mL of water, and 10 mL of saturated NaCl and dried over Na_2SO_4. The solvent was removed in a vacuum, and the residue was triturated with 20 mL of hexane. Yield 5.86 g (96.2%), light-colored crystals.

HCl·H–Thr(OBn)–Lys(Z)OBn (8). To 5.86 g (8.86 mmol) of compound **7**, a 15% solution of HCl in dioxane, 25 mL, was added. The mixture was stirred for 2 h at room temperature, the solvent was removed in a vacuum, and the residue was triturated with 20 mL of ether and dried in air to constant weight. Yield 4.9 g (99%), white crystals.

Boc–Tyr–Thr(OBn)–Lys(Z)OBn (10). A solution of 2.06 g (10 mmol) of dicyclohexylcarbodiimide in 10 mL of DM was added dropwise to a solution of 4.9 g (8.8 mmol) of compound **8**, 2.75 g (9.8 mmol) of Boc–Tyr–OH **9**, 1.53 g (10 mmol) of *N*-hydroxybenzothriazole, and 1.1 g (11 mmol) of *N*-methylmorpholine in 80 mL of DMF. The reaction mixture was stirred for 1 h at 0°C and then for 12 h at room temperature. A 5% solution of citric acid, 100 mL, was added, and the reaction product was extracted with ethyl acetate (5×30 mL), washed with 20 mL of water, 20 mL of 5% $NaHCO_3$, and 10 mL of saturated NaCl and dried over Na_2SO_4. The solvent was removed in a vacuum, and the residue was triturated with 20 mL of hexane. Yield 4.1 g (49.8%). ^{1}H NMR spectrum (DMSO-d_6), δ, ppm: 1.09 d (3H, $\underline{CH_3}$CH, J = 6.1 Hz), 1.34 s [9H, $(\underline{CH_3})_3$C], 1.20–1.39 m (4H, CH_2CH_2), 2.85 t, 2.91 d (2H, $\underline{CH_2}$), 2.92 m (2H, CH_2N), 4.15 m, 4.29 m, 4.32 m, 4.41 m (4H, CH), 4.44 s, 4.99 s, 5.12 s (6H, $\underline{CH_2}$Ar), 6.63 d (2H, $\underline{Ar}$OH, J = 8.3 Hz), 7.03 d (2H, $\underline{Ar}CH_2$, J = 8.3 Hz), 7.04 d (1H, NH, J = 6.4 Hz), 7.23–7.33 m (15H, Ar), 7.79 d (1H, NH, J = 8.8 Hz), 8.23 d (1H, NH, J = 7,1 Hz), 9.15 s (1H, OH). HRMS, *m/z*: 825.9661 [M + H].

RP(O)(OPr-*i*)–Boc–Tyr–Thr(OBn)–Lys(Z)OBn (16a, 16b). Sodium hydride, 0.17 g (0.0007 mol), prewashed with hexane to remove mineral oil, was added with stirring to a coled (5°C) suspension of 0.412 g (0.0005 mol) of compound **10** in 40 mL of anhydrous acetonitrile. The mixture was stirred for 30 min at room temperature, after which 0.0006 mol phosphonoyl chloride **15a** or **15b** in 10 mL of anhydrous acetonitrile was added, and stirring was continued for an additional 2 h. The reaction progress was monitored by TLC. The reaction mixture was reduced by half in a vacuum and poured into 40 mL of water. The reaction product was extracted with chloroform (3×30 mL), and the extract was washed with 20 mL of water, dried over $MgSO_4$, and concentrated in a vacuum to leave a colorless viscous liquid. The reaction products were isolated by chromatography on a column (150×30 mm) of silica gel (GeduranSi 60, 40–63 μm, Merck), eluent ethyl acetate–methylene chloride (3 : 1), elution rate 1 mL/min. The purity of the products after column chromatography was 85–87 % (by ^{31}P NMR).

CH_3P(O)(OPr-*i*)–Boc–Tyr–Thr(OBn)–Lys(Z)–OBn (16a). Yield 0.372 g (76 %), semicrystalline material. ^{31}P NMR spectrum ($CDCl_3$): δ_P 26.70 ppm. HRMS, *m/z*: 946.0530 [M + H].

CD_3P(O)(OPr-*i*)–Boc–Tyr–Thr(OBn)–Lys(Z)–OBn (16b). Yield 0.399 g (81%), pale yellow semicrystalline material. ^{31}P NMR spectrum ($CDCl_3$): δ_P 26.79 ppm. HRMS, *m/z*: 949.0714 [M + H].

RP(O)(OPr-*i*)–Tyr–Thr(OBn)–Lys(Z)–OBn (17a, 17b). A 15% solution of anhydrous HCl in dioxane, 10 mL, was added to phosphorylated peptide **16a** or **16b**. The mixture was stirred for 1 h at room temperature, and the solvent was removed in a vacuum. The residue was dissolved in 40 mL of ethyl acetate, the solution was washed with 5% $NaHCO_3$ (2×20 mL) and 10 mL of saturated NaCl, and dried over Na_2SO_4. The solvent was removed to leave an amorphous white material, which purification was subjected to hydrogenolysis to remove benzyl protective groups.

CH_3P(O)(OPr-*i*)-Tyr-Thr(OBn)-Lys(Z)OBn (17a). ^{31}P NMR spectrum ($CDCl_3$): δ_P 26.76 ppm. HRMS, *m/z*: 845.9371 [M + H].

CD_3P(O)(OPr-*i*)–Tyr–Thr(OBn)–Lys(Z)OBn (17b). ^{31}P NMR spectrum: δ_P 26.92 ppm. HRMS, *m/z*: 848.9556 [M + H].

RP(O)(OPr-*i*)–Tyr–Thr–Lys (1a, 1b). Protected phosphorylated tripeptide **17a** or **17b**, 50 mL of ethanol, and 50 mg of freshly prepared palladium hydroxide were placed in a hydrogenation apparatus at atmospheric pressure. The device was filled with hydrogen and shaken for 36 h, passing a slow stream of hydrogen, then 50 mg of freshly prepared palladium hydroxide was added and hydrogenation was continued for 36 h. The reaction was monitored every 6 h using HPLC–MS. The catalyst was filtered off, the solvent was removed in a vacuum, and the residue was triturated with 5 mL of acetonitrile, filtered off, and on the filter with 10 mL of diethyl ether to obtain phosphorylated tripeptides as white fine crystals, purity ca. 70% (by HPLC–MS). Further purification was performed by preparative HPLC.

CH_3P(O)(OPr-*i*)–Tyr–Thr–Lys (1a). Yield 0.135 g (65%). ^{31}P NMR spectrum (D_2O), ppm: δ_P 31.54, 31.59. White crystals. HRMS, *m/z*: 531.2581 [*M* + H].

CD_3P(O)(OPr-*i*)-Tyr-Thr-Lys (1b). Yield 0.142 g (63%). ^{31}P NMR spectrum (D_2O), ppm: δ_P 31.72, 31.78. White crystals. HRMS, *m/z*: 534.2755 [*M* + H].

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

REFERENCES

1. *Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction*. https://www.opcw.org/chemical-weapons-convention.
2. Black, R.M. and Read, R.W., *Arch. Toxicol.*, 2013, vol. 87, no. 3, p. 421. https://doi.org/ 10.1007/s00204-012-1005-1
3. Williams,N.H.,Harrison,J.M.,Read,R.W.,andBlack,R.M., *Arch. Toxicol.*, 2007, vol. 81, no. 9, p. 627. https://doi.org/10.1007/s00204-007-0191-8
4. Sato, A., Yoshida, M., and Hara, S., *Chem. Commun*., 2008, no. 46, p. 6242. https://doi.org/10.1039/B814804J
5. Gershkovich, A.A. and Kibirev, V.K., *Sintez peptidov. Reagenty i metody* (Synthesis of Peptides. Reagents and Methods), Kiev: Naukova Dumka, 1987.
6. Balaev, A.N., Osipov, V.N., Okhmanovich, K.A., and Fedorov, V.E., *Pharm. Chem. J*., 2014, vol. 48, no. 3, p. 217. https://doi.org/10.1007/s11094-014-1080-0
7. Balaev, A.N., Osipov, V.N., and Fedorov, V.E., *Pharm. Chem. J*., 2014, vol. 48, p. 340. https://doi.org/10.1007/s11094-014-1106-7
8. Balaev, A.N., Osipov, V.N., Okhmanovich, K.A., Ruchko, Ye.A., Kolotaev, A.V., and Khachatryan, D.S., *Russ. Chem. Bull*., 2016, vol. 65, no. 11, p. 2766. https://doi.org/10.1007/s11172-016-1651-1
9. Balaev, A.N., Osipov, V.N., Okhmanovich, K.A., Ruchko, Ye.A., Baryshnikova, M.A., and Khachatryan, D.S., *Russ. Chem. Bull*., 2016, vol. 65, no. 12, p. 2948. https://doi.org/10.1007/s11172-016-1683-6
10. Bennet, A.J., Kovach, I.M, and Bibbs, J.A., *J. Am. Chem. Soc*., 1989, vol. 111, p. 6424 https://doi.org/10.1021/ja00198a067
11. Briseno-Roa, L., Hill, J., Notman, S., Sellers, D., Smith, A.P., Timperley, C.M., Wetherell, J., Williams, N.H., Williams, G.R., Fersht, A.R., and Griffiths, A.D., *J. Med. Chem*., 2006, vol. 49, p. 246. https://doi.org/10.1021/jm050518j