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Phosphorylation of Tyrosine and Tyr-Thr-Lys Tripeptide with Cyclohexylmethyl- and (Deuteromethyl)phosphonochloridates

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Abstract—Methods for phosphorylation of tyrosine and Tyr-Thr-Lys tripeptide with cyclohexylmethyl(deuteromethyl)-phosphonochloridates have been developed. These products can be used as reference compounds in the analysis of blood plasma of patients presumably exposed to cyclohexylmethylphosphonofluoridate (cyclosarin) action. Conditions for the separation and purification of the synthesized intermediates by means of chromatography have been determined and optimized, allowing high-purity phosphorylated products at the final stage of the synthesis.

Keywords: biomarker, tripeptide, phosphorylation

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Cyclohexylmethylphosphonofluoridate (cyclosarin) is among the alkyl(alkyl)phosphonofluoridates included in Schedule 1 of the chemicals of Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on Their Destruction [1], development, production, stockpiling, and use of which are completely prohibited. The Work Instruction for the Reporting of the Results of the OPCW Biomedical Proficiency Tests by Organization for the Prohibition of Chemical Weapons (OPCW) [2] prescribes determination of biomarkers of high-priority alkyl(alkyl)-phosphonofluoridates in the biomedical specimens and elucidation of their exact structure. The samples of blood plasma of a human exposed to cyclosarin were provided to the involved national laboratories during the 4th official biomedical test by OPCW (2019). The experience of that and previous biomedical tests by OPCW unambiguously confirmed that reliable identification of the biomarkers of human exposure to alkyl(alkyl)phosphonofluoridates including cyclosarin demanded the availability of high-pure reference samples. The matter might include the products of cyclosarin in vivo interaction with tyrosine or the Tyr-Thr-Lys tripeptides [3, 4]. This study aimed to elaborate a synthetic method of preparation of high-pure reference samples of those compounds including the deuterated analogs.

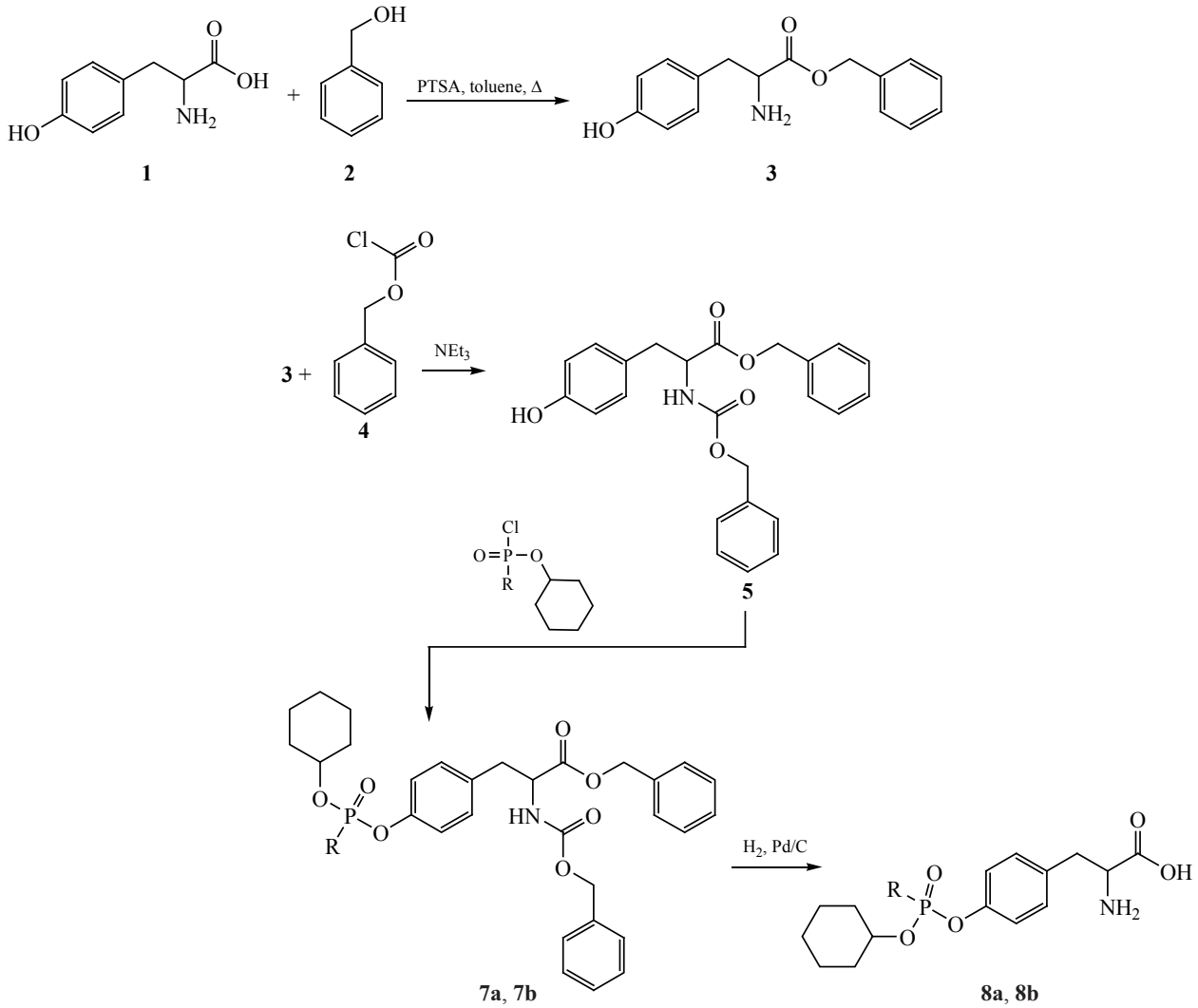
Phosphorylated derivatives of tyrosine was performed via Scheme 1 involving sequential protection of carboxylic and amine groups of tyrosine according to [5, 6], phosphorylation with cyclohexylmethylphosphonic or cyclohexyldeuteromethylphosphonic acid chlorides, and deprotection affording the target phosphorylate tyrosines **8a**, **8b**.

Protection of L-tyrosine **1** was performed in two stages. L-Tyrosine **1** was first treated with benzyl alcohol **2** in the presence of stoichiometric amount of *p*-toluenesulfonic acid to obtain benzyl ester of L-tyrosine as the *p*-toluenesulfonate **3**. Further interaction of salt **3** with carboxybenzyl chloride **4** in the presence of 2 eq. of triethylamine led to benzyl ester of *N*-carboxybenzyl-L-tyrosine **5** as white crystalline powder.

Cyclohexylmethyl(deuteromethyl)phosphonochroridates **6a**, **6b** used in the phosphorylation were prepared as described elsewhere [13].

In contrast to the earlier published procedure [4], phosphorylation of benzyl ester of *N*-carboxybenzyl-

Scheme 1.



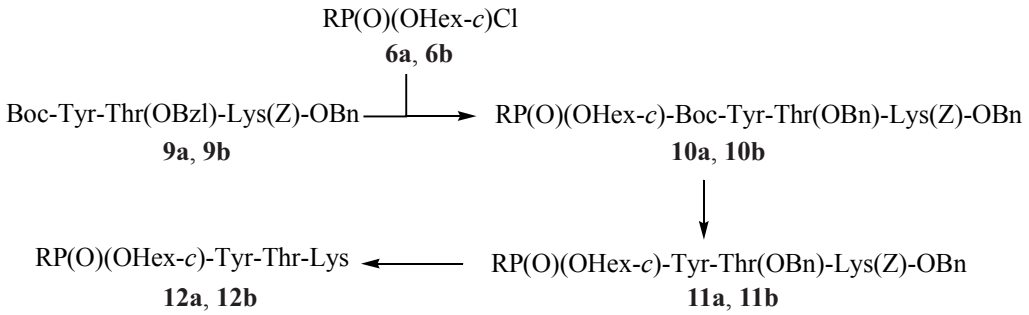
L-tyrosine **5** with cyclohexylmethyl(deuteromethyl)-phosphonochloridates was performed upon the treatment of compound 5 with sodium hydride in acetonitrile. According to the chromatography and NMR spectroscopy data, phosphorylated benzyl esters of *N*-carboxybenzyl-L-tyrosine **7a**, **7b** obtained as viscous liquids contained many admixtures. The esters were purified by means of column chromatography (SiO_2, eluent—methylene chloride–ethyl acetate, 1 : 1). The products with purity exceeding 90% were thus obtained. The final stage consisted in benzyl and carboxybenzyl deprotection of the phosphorylated L-tyrosine **7a**, **7b** via catalytic hydrogenation (10% Pd/C) with molecular hydrogen under atmospheric pressure. The target products **8a**, **8b** were obtained as white crystalline powders readily soluble in water and ethanol and poorly soluble in methylene chloride and acetonitrile.

The Tyr-Thr-Lys tripeptide phosphorylated with cyclohexylmethyl(deuteromethyl)phosphonochloridates was prepared via Scheme 2 including the interaction of cyclohexylmethylphosphonic or cyclohexyldeutero-methylphosphonic acid chlorides with free phenolic OH group of tyrosine unit in the protected Boc-Tyr-Thr(OBzl)-Lys(Z)-OBn tripeptide, followed by deprotection.

The starting protected tripeptide Boc-Tyr-Thr(OBn)-Lys(Z)-OBn **9** was obtained via conventional liquid-phase peptide synthesis [8–12] as described in Ref. [14].

The interaction of tripeptide **9** with cyclohexyl-methyl(deuteromethyl)phosphonochloridates was performed via the treatment of suspension of compound **9** in acetonitrile with sodium hydride at 5°C, followed by the addition of cyclohexylmethyl(deuteromethyl)-phosphonochloridate **6a**, **6b** and the mixture keeping

Scheme 2.



R = CH_3 (**a**), CD_3 (**b**).

at 20°C during 2 h. The obtained adducts **10a**, **10b** were purified via column chromatography on silica gel, followed by sequential Boc and benzyl groups deprotection to afford the target compounds **11a**, **11b** and **12a**, **12b**, as described elsewhere [14].

^{1}H and ^{31}P NMR spectroscopy as well as LS–MS study of the obtained compounds during storage revealed that they were stable during 3 months and were thus suitable as reference samples in the analysis of biomedical specimens.

EXPERIMENTAL

^{1}H and ^{31}P NMR spectra were recorded using a QONE AS 400 spectrometer at 298 K (399.85 and 161.86 MHz, respectively). The reactions course was monitored via thin-layer chromatography on Marchery-Naglel AlugramSILG/UV254 plates detected using an Ultramag-K254/365 instrument at 254 nm. High-resolution mass spectra were recorded using an Agilent Infinity 1260/Thermo Scientific Orbitrap Fusion Lumos HPLC–MS analytical station in the electrospray ionization mode.

The phosphorylated tripeptide was prepared from the protected amino acids (AKos, Germany).

Benzyl ester of L-tyrosine, *p*-toluenesulfonate (3). A mixture of 10 g (0.055 mol) of L-tyrosine **1**, 25 mL of benzyl alcohol **2**, 120 mL of toluene, and 12.6 g (0.066 mol) of *p*-toluenesulfonic acid monohydrate was refluxed with a Dean–Stark apparatus during 7 h until the removal of water was complete. The mixture was cooled down to ambient, the precipitate was filtered off, washed with diethyl ether, dried in air, and recrystallized from ethanol. Yield 20.7 g (85%), white crystals.

Benzyl ester of *N*-carboxybenzyl-L-tyrosine (5). 5 g (0.0495 mol) of triethylamine was added to a solution of 10.9 g (0.0246 mol) of *p*-toluenesulfonate **3** in 100 mL of methanol. The mixture was cooled to 0°C, and 4.2 g (0.0246 mol) of carboxybenzyl chloride **4** was added. The reaction mixture was stirred during 8 h at 0°C and then heated up to ambient. The mixture was poured into 100 mL of water and extracted with diethyl ether (3× 100 mL), the extract was washed with water and saturated solution of NaCl, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was recrystallized from a hexane–ethanol mixture (2 : 1). Yield 7.93 g (80%), white crystals, mp 102–104°C.

General procedure for synthesis of compounds 7a, 7b. 120 mg (0.005 mol) of NaH washed with hexane to remove mineral oil was added to a 0.0042 M solution of compound **5** in 40 mL of anhydrous acetonitrile at cooling to 5°C and stirring. The obtained mixture was stirred during 30 min at room temperature, then 0.005 mol of cyclohexylmethyl(deuteromethyl)-phosphonic acid chloride **6a**, **6b** was added, and the mixture was stirred during 1 h monitoring the reaction course by TLC. The mixture was evaporated under vacuum to half of the volume, poured into 40 mL of water, and extracted with chloroform (3×30 mL). The extract was washed with 20 mL of water, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was subject to chromatography on a 150×30 mm column (SiO_2, GeduranSi 60 40–63 μm, Merck) with methylene chloride–ethyl acetate (1 : 1) as eluent. Products **7a**, **7b** with 96% purity were isolated.

***O*-(*O*-Cyclohexyl)methylphosphonate of *N*-carboxybenzyl-L-tyrosine benzyl ester (7a).** Yield 1.59 g (67%). ^{31}P NMR spectrum (DMSO-d_6): δ_P 26.87 ppm. Mass spectrum, *m/z*: 566. 6023 $[M + H]^+$.

***O*-(*O*-Cyclohexyl)deuteromethylphosphonate of *N*-carboxybenzyl-L-tyrosine benzyl ester (7b).**

Yield 1.51 g (63%). ^{31}P NMR spectrum (DMSO-d_6): δ_P 27.02 ppm. Mass spectrum, *m/z*: 569. 6197 $[M+H]^+$.

General procedure for synthesis of compounds 8a, 8b. 1.59 g of compound **7a** (or 1.51 g of compound **7b**), 50 mL of ethanol, and 100 mg of 10% palladium on active coal were charged into an apparatus for normal-pressure hydrogenation. The apparatus was filled with hydrogen and shaken during 8 h, bubbling hydrogen slowly. The catalyst was filtered off, and the solvent was removed under vacuum. The residue was triturated with 5 mL of acetonitrile, filtered, and washed with 10 mL of diethyl ether on a filter. *O*-[*O*-cyclohexylmethyl(deuteromethyl)-phosphonyl]-L-tyrosine **8a**, **8b** with purify of 94% were obtained.

***O*-(*O*-Cyclohexylmethylphosphonyl)-L-tyrosine (8a)**. Yield 0.625 g (65%), white crystals. ^{31}P NMR spectrum (D_2O): δ_P 31.76 ppm. Mass spectrum, *m/z*: 342.3477 $[M+H]^+$.

***O*-(*O*-Cyclohexyldeuteromethylphosphonyl)-L-tyrosine (8b)**. Yield 0.641 g (70%), white crystals. ^{31}P NMR spectrum (D_2O): δ_P 31.92 ppm. Mass spectrum, *m/z*: 345.3651 $[M+H]^+$.

The protected tripeptide Boc-Tyr-Thr(OBn)-Lys(Z)-OBn **9** was prepared as described by us earlier [14]. The procedure for its phosphorylation with cyclohexylmethyl(deuteromethyl)phosphonochloridate and deprotection are given below.

General procedure for synthesis of compounds 10a, 10b. 0.017 g (0.0007 mol) of NaH washed with hexane to remove mineral oil was added to a suspension of 0.412 g (0.0005 mol) of protected tripeptide **9** in 40 mL of anhydrous acetonitrile at stirring and cooling to 5°C. The mixture was stirred during 30 min at room temperature, 0.0006 mol of compound **6a**, **6b** in 10 mL of anhydrous acetonitrile was added, and the mixture was stirred during 2 h monitoring the reaction course by TLC. The mixture was evaporated under vacuum to half of the volume, poured into 40 mL of water, and extracted with chloroform (3×30 mL). The extract was washed with 20 mL of water, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was subject to chromatography on a 150×30 mm column (SiO_2, GeduranSi 60 40–63 μm, Merck) with methylene chloride–ethyl acetate (3 : 1) as eluent. Products **10a**, **10b** with 86% purity were isolated (^{31}P NMR data).

Benzyl-N^2-(*O*-benzyl-*N*-{2-[(*tert*-butoxycarbonyl)-amino]-3-(4-{[(cyclohexyloxy)(methyl)phosphoryl]-oxy}phenylpropanoyl)threonyl})-N^6-[(benzyloxy)-carbonyl]lysinate (10a). Yield 0.417 g (84.6%), semicrystalline mass. ^{31}P NMR spectrum (DMSO-d_6): δ_P 26.75 ppm. Mass spectrum, *m/z*: 986.1168 $[M+H]^+$.

Benzyl-N^2-(*O*-benzyl-*N*-{2-[(*tert*-butoxycarbonyl)-amino]-3-(4-{[(cyclohexyloxy)(deuteromethyl)-phosphoryl]oxy}phenylpropanoyl)threonyl})-N^6-[(benzyloxy)carbonyl]lysinate (10b). Yield 0.424 g (85.8%), semicrystalline mass. ^{31}P NMR spectrum (DMSO-d_6): δ_P 26.92 ppm. Mass spectrum, *m/z*: 989.1342 $[M+H]^+$.

General procedure for synthesis of compounds 11a, 11b. 10 mL of a 15% solution of anhydrous HCl in dioxane was added to 0.424 g of the phosphorylated tripeptide **10a**, **10b**. The mixture was stirred at room temperature during 1 h, and the solvent was evaporated under vacuum. 40 mL od ethyl acetate was added to the residue, and the solvent was washed with 5% solution of sodium hydrocarbonate (2×10 mL) and with 10 mL of saturated solution od sodium chloride in water. After drying over sodium sulfate and distilling off the solvent, the residue (amorphous white mass) was subject to hydrogenolysis without purification for the benzyl groups deprotection.

Benzyl-N^2-{*N*-[2-amino-3-(4-{[(cyclohexyloxy)-(methyl)phosphoryl]oxy}phenyl)propanoyl]-*O*-benzylthreonyl}-N^6-[(benzyloxy)carbonyl]lysinate (11a). ^{31}P NMR spectrum (DMSO-d_6): δ_P 26.65 ppm. Mass spectrum, *m/z*: 886.0009 $[M+H]^+$.

Benzyl-N^2-{*N*-[2-amino-3-(4-{[(cyclohexyloxy)-(deuteromethyl)phosphoryl]oxy}phenyl)propanoyl]-*O*-benzylthreonyl}-N^6-[(benzyloxy)carbonyl]lysinate (11b). ^{31}P NMR spectrum (DMSO-d_6): δ_P 26.83 ppm. Mass spectrum, *m/z*: 889.1838 $[M+H]^+$.

General procedure for synthesis of compounds 12a, 12b. The Bn-protected phosphorylated tripeptide **11a, 11b**, 50 mL of ethanol, and 50 mg of freshly prepared palladium hydroxide were charged into an apparatus for normal-pressure hydrogenation. The apparatus was filled with hydrogen and shaken during 36 h, bubbling hydrogen slowly. After that, 50 mg of freshly prepared palladium hydroxide was added and the hydrogenation was performed further during 36 h. The reaction course was monitored by TLC each 6 h. The catalyst was filtered off, and the solvent was removed under vacuum. The residue was triturated with 5 mL of acetonitrile, filtered, and washed with 10 mL of diethyl ether on a filter. The *O*-phosphorylated tripeptides **12a**, **12b** were obtained as

white fine-crystalline mass with purity of 70% (HPLC–MS data). Further purification (to 95% purity) was performed via preparative HPLC.

[2-Amino-3-(4-{[(cyclohexyloxy)(methyl)phosphoryl]oxy}phenyl)propanoyl]threonyllysine (12a) Yield 0.157 g (65%), white crystals. ^{31}P NMR spectrum (D_2O), δ_P, ppm: 31.60, 31.66. Mass spectrum: *m/z*: 571.6239 $[M + H]^+$.

[2-Amino-3-(4-{[(cyclohexyloxy)(deuteromethyl)-phosphoryl]oxy}phenyl)propanoyl]threonyllysine (12b). Yield 0.173 g (70%), white crystals. ^{31}P NMR spectrum (D_2O), δ_P, ppm: 31.75, 31.81. Mass spectrum, *m/z*: 574.6413 $[M + H]^+$.

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CONFLICT OF INTERESTS

No conflict of interest was declared by the authors.

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