EXPERIMENTAL WORKS

1,3-OXAZOL-4-YLPHOSPHONIUM SALTS
AS NEW NON-PEPTIDE INHIBITORS OF FURIN

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A series of novel triphenylphosphonium derivatives of 1,3-oxazole containing at C2 and C5-positions electron withdrawing or electron-donating groups were synthesized and characterized by ^{1}H, ^{31}P NMR and IR spectroscopy, element analysis and chromato-mass spectrometry. These compounds were found to be a new class of non-peptide inhibitors of furin. Depending on the chemical structure, they inactivated enzyme at micromolar level by mechanism of competitive, non-competitive or mixed inhibition. Evaluation of the synthesized derivatives as furin inhibitors showed that among the triphenylphosphonium salts studied by us, oxazole 12 containing 2,4-dichlorophenyl- in the C2-position and MeS-group at C5 is the most active (K_i = 1.57 μM) competitive inhibitor of furin. Our results provided evidence that chemical modification of 1,3-oxazole-4-yl-triphenylphosphonium salts may be useful for developing new more potent and selective inhibitors of furin.

K e y w o r d s: furin, inhibitors, 1,3-oxazole derivatives, triphenylphosphonium salts, mechanism of inhibition.

The enzyme furin (EC 3.4.21.75) is a multi-domain, calcium-dependent serine proprotein convertase subtilisin/kexin type, which converts inactive precursors of proteins into mature biologically active products: hormones, growth factors, receptors, enzymes, adhesive molecules, ion channels, blood coagulation factors, etc. [1-3]. Furin is involved not only in normal physiological processes, but also in the development of a number of pathological conditions: infectious diseases, neoplastic formations, cardiovascular diseases, obesity, diabetes, neurological and cognitive dysfunctions, as well as impaired reproduction and bone mineralization [4-6]. Therefore, the enzyme is considered as a promising target for the synthesis of its inhibitors and the creation of modern drugs based on them [3-6]. The search for potential inhibitors of furin among low-molecular weight compounds of a non-peptide nature is an important area of biochemical research, since such compounds exhibit increased metabolic and proteolytic stability [7, 8]. In addition, they are easily accessible by synthetic methods, and by structural modifications of these molecules it is easy to carry out a structure-activity relationship analysis. In recent years, different classes of compounds have been discovered that are capable of blocking the activity of proprotein convertases and, in particular, of furin [7-10].

Earlier, we have studied the inhibitory effect of some derivatives of thiazole and pyrazole and found that, under the experimental conditions, they inhibited the enzyme by 15-90% [11]. Continuing the search for new non-peptide inhibitors of furin, we paid attention to 1,3-oxazole derivatives. Zhang et

al. note in a recent review [12], that oxazole compounds are readily able to bind with a variety of enzymes and receptors in biological systems via diverse non-covalent interactions, and thus display versatile biological activities [12]. These compounds are of particular interest, firstly, due to the presence of the oxazole moiety in various natural compounds [13], and, secondly, due to the fact that they are used as precursors in various chemical transformations [14, 15]. Thus, the diverse oxazole-containing heterocycles are highly useful derivatives from synthetic and medicinal as well as pharmaceutical aspects. They were found to possess antibacterial, antifungal, anticancer, anti-inflammatory properties [14-16]. The heterocycle was shown to play a vital role in synthesis and manufacture of varies drugs as brain-derived neurotrophic factor inducers [17], anticancer agents [18-21], antidepressants and anticonvulsants [22], compounds with antimicrobial, antiviral and antifungal activities [23-28], as well as antidiabetic [29] activity.

To create new bioregulators, chemists use usually diverse chemical modifications of the initial structures by various pharmacophoric groups. One of these groups is the lipophilic triphenyl-phosphonium (TPP) ion [30-32]. Indeed, the derivatives, bearing quaternary phosphonium salts were reported to show high antimicrobial activities [33-35] so that these cations were considered as a new generation of effective antiseptics. As pharmacological agents, TPP salts found to have also antiglycemic properties [36] and antiproliferative [36], antioxidant [37], antiviral activities [34]. It is important that the phosphonium cation itself does not exhibit cytotoxicity [38].

Since, furin recognizes in its natural substrates a portion of the polypeptide chain enriched in basic amino acid residues: -Arg-Xn-(Lys/Arg)-Arg-↓, where $n = 0, 2, 4, 6$, and X is any amino acid except Cys [4], then positively charged substituents are introduced into the structure of furin inhibitors: a guanidine group or its mimetics, for example, amidine, amidinohydrazone, aminomethylbenzamidine and other groups [7-10].

The aim of the work is design, synthesis, and evaluation of some derivatives of 1,3-oxazol-4-yltriphenylphosphonium salts as new non-peptide furin inhibitors.

Materials and Methods

Reagents and preparations. The fluorogenic substrate Boc-Arg-Val-Arg-Arg-AMC was purchased from Bachem (Switzerland), and truncated recombinant human furin (2000 units/ml) was obtained from New England BioLabs (USA). Commercial furin solution was diluted 80-fold with HEPES-buffer (pH 7.3; 100 mM HEPES, 1 mM $CaCl_2$, 0.5% Triton X-100 and 1 mM β-mercaptoethanol) and used then in the enzymatic reaction. One unit of furin activity was defined as quantity of the enzyme, that under standard conditions, cleaved off 1 picamol of 7-amino-4-methylcoumarin (AMC) in 1 min.

EDTA, β-mercaptoethanol, HEPES, Triton X-100 were purchased from Sigma Aldrich.

General method for the synthesis of triphenylphosphonium derivatives of 1,3-oxazole. [2-R-4-(Triphenylphosphonium)-1,3-oxazol-5-yl] sulfanides **1-5** were synthesized according to the known method [39] from the starting N-(1,2,2,2-tetrachloroethyl)amides **A**. These compounds were converted into phosphonium chlorides **B** by reaction with triphenylphosphine running in benzene by heating to 70-80 °C. Interaction of phosphonium salts **B** with an excess of sodium hydrosulfide gave [2-R-4-(triphenylphosphonium)-1,3-oxazol-5-yl] sulfanides **1-5** (Scheme 1). Sequential interaction the compound **3** with 4-chlorodiazonium chloride and sodium perchlorate formed phosphonium salt **6**, which has been converted to the corresponding sulfone **7**. Alkylation of compounds **4**, **5** with alkyl iodides leads to phosphonium salts **8**, **12**. Treatment of the compound **4** with phenacyl chloride and the subsequent with sodium perchlorate gives the phosphonium salt **9**. The sequential processing of the compound **4** with chlorine and sodium perchlorate derives 5-chlorosubstituted oxazole **11**.

2,2-Dichloro-1-(4-aroylamino)ethenyltriphenylphosphonium chlorides **B** were used to synthesize 5-N-substituted oxazoles **10**, **13** (Scheme 2). The reactions were performed out at 20-25 °C by mixing reagents **B** and hydrazine hydrate, or dimethylamine in methanol. The resulting (1,3-oxazol-4-yl)triphenylphosphonium chlorides were converted into the corresponding perchlorates **10**, **13**.

Chemistry: synthetic procedures and spectroscopic data of compounds. ^{1}H, ^{31}P NMR spectra were obtained on a Bruker AVANCE DRX-400 spectrometer (TMS as internal reference or 85% phosphoric acid as external reference) in DMSO-d_6. IR spectra were recorded on a Vertex 70 spectrometer in KBr pellets. GC-MS spectra were recorded on an LC-MS system - HPLC Agilent 1100 Series equipped with a dinode array detector Agilent LC\MSD SL. Parameters of GC-MS analy-
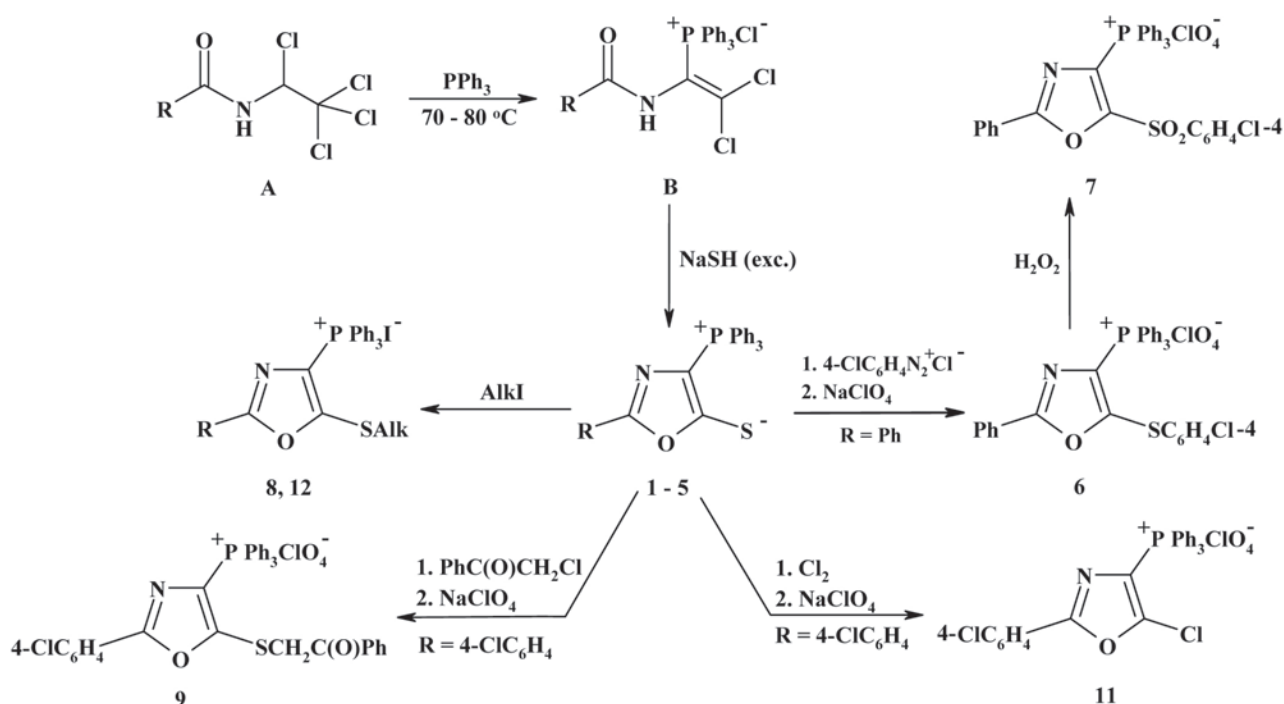
*Scheme 1. Synthesis of [2-R-4-(triphenylphosphonium)-1,3-oxazol-5-yl]-sulfanides (**1-5**) and (1,3-oxazol-4-yl)-triphenylphosphonium salts (**6-9**, **11**, **12**)*
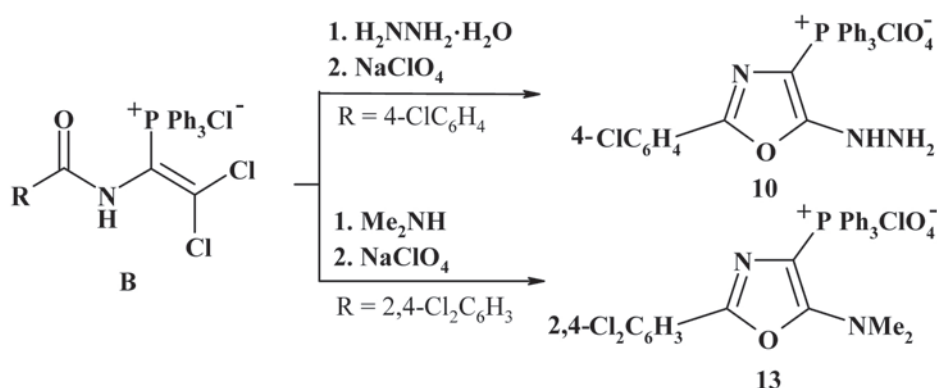
*Scheme 2. Synthesis of (1,3-oxazol-4-yl)-triphenylphosphonium salts (**10**, **13**)*

sis: Zorbax SB - C18 column (1.8 μm, 4.6×15 mm, PN 821975-932), solvent water – acetonitrile mixture (95 : 5), 0.1% of aqueous trifluoroacetic acid; eluent flow 3 ml/min; injection volume 1 μl; UV detecting at 215, 254, 265 nm; chemical ionization at atmospheric pressure (APCI), scan range m/z 80 - 1000. Elemental analysis was carried out in the Analytical Laboratory of the V.P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry of the National Academy of Sciences of Ukraine by manual methods. The carbon and hydrogen contents were determined using the Pregl gravimetric method, while nitrogen was determined using the Duma's gasometrical micromethod. Sulfur was determined by the Scheininger titrimetric method, chlorine content was determined by the mercurometric method, phosphorus content was determined by the colorimetric method [40]. Uncorrected melting points were determined on a Fisher–Johns apparatus. Reactions and purity of the products were monitored by thin-layer chromatography on Silufol UV-254 plates using 9:1 (v/v) chloroform–methanol as eluent. All reagents and solvents were purchased from Aldrich and used as received.

[2-R-4-(Triphenylphosphonium)-1,3-oxazol-5-yl]sulfanides (1-5) were synthesized following a procedure described in the literature [39].

[5-(4-Chlorophenylthio)-2-phenyl-1,3-oxazol-4-yl]triphenylphosphonium perchlorate (6) was synthesized as previously described [41].

[5-(4-Chlorophenylsulfonyl)-2-phenyl-1,3-oxazol-4-yl]triphenylphosphonium perchlorate (7) was synthesized following a procedure described in the literature [42].

[2-(4-Chlorophenyl)-5-ethylthio-1,3-oxazol-4-yl]triphenylphosphonium iodide (8) was synthesized following a procedure described in the literature [43].

Colorless powder, m.p. 206-208 °C (EtOH). IR (KBr, ν_{max}, cm^{-1}): 1107, 1402, 1434, 1474, 1602, 3048. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 1.22 (t, 3H, J_{HH} = 7.5 Hz, CH$_3$), 3.13 (q, 2H, J_{HH} = 7.5 Hz, CH$_2$), 7.68 (d, 2H, J_{HH} = 8.0 Hz, ArH), 7.92-7.78 (m, 12H, ArH), 8.00-7.94 (m, 3H, ArH), 8.06 (d, 2H, J_{HH} = 8.0 Hz, ArH). ^{31}P NMR (202 MHz, DMSO-d$_6$): δ 10.55. ESI-MS, m/z: 500 [M-I]$^+$. Found, %: C, 55.47%; H, 3.85%; Cl, 5.65%; N, 2.23%; P, 4.93%; S, 5.11%. Calc. for C$_{29}$H$_{24}$ClINOPS: C, 55.40%; H, 3.85%; Cl, 5.7%; N, 2.20%; P, 4.80%; S, 5.20%.

[2-(4-Chlorophenyl)-5-(2-oxo-2-phenylethylsulphanyl)-1,3-oxazol-4-yl]triphenylphosphonium perchlorate (9) was synthesized following a procedure described in the literature [43].

[2-(4-Chlorophenyl)-5-hydrazino-1,3-oxazol-4-yl]triphenylphosphonium perchlorate (10) was synthesized following a procedure described in the literature [44].

[2-(4-Chlorophenyl)-5-chloro-1,3-oxazol-4-yl]triphenylphosphonium perchlorate (11) was synthesized as previously described [45].

[2-(2,4-Dichlorophenyl)-5-methylthio-1,3-oxazol-4-yl]triphenylphosphonium iodide (12) was synthesized following a procedure described in the literature [43].

Colorless powder, m.p. 166-168 °C (EtOH). IR (KBr, ν_{max}, cm^{-1}): 691, 1107, 1432, 1467, 1589, 3043. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 2.64 (s, 3H, CH$_3$), 7.68 (dd, 1H, J_{HH} = 1.5 Hz, J_{HH} = 6.5 Hz, ArH), 8.02-7.78 (m, 16H, ArH), 8.17 (d, 1H, J_{HH} = 8.0 Hz, ArH). ^{31}P NMR (202 MHz, DMSO-d$_6$): δ 10.00. ESI-MS, m/z: 520 [M-I]$^+$. Found, %: C, 51.87%; H, 3.26%; Cl, 10.94%; N, 2.16%; P, 4.78%; S, 4.95%. Calc. for C$_{28}$H$_{21}$Cl$_2$INOPS: C, 51.80%; H, 3.25%; Cl, 10.80%; N, 2.20%; P, 4.75%; S, 4.85%.

[2-(2,4-Dichlorophenyl)-5-dimethylamino-1,3-oxazol-4-yl]triphenylphosphonium perchlorate (13). To a solution of 1-(2,4-dichlorobenzoylamino)-2,2-dichloro-ethenyltriphenyl-phosphonium chloride (0.01 mol) [39] in 40 ml of methanol, a solution of dimethylamine (0.035 mol) in 50 ml methanol was added. The mixture was kept at 20-25 °C for 24 h, and then 10 ml of a saturated aqueous solution of sodium perchlorate was added. The formed precipitate was filtered off and recrystallized from ethanol.

Colorless powder, m.p. 226-228 °C. IR (KBr, ν_{max}, cm^{-1}): 1086, 1102, 1422, 1434, 1476, 1624. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 2.68 (s, 6H, 2CH$_3$), 7.59 (dd, 1H, J_{HH} = 2.0 Hz, J_{HH} = 6.5 Hz, ArH), 7.82-7.74 (m, 7H, ArH), 7.96-7.83 (m, 10H, ArH). ^{31}P NMR (202 MHz, DMSO-d$_6$): δ 14.56. ESI-MS, m/z: 517 [M-ClO$_4$]$^+$. Found, %: C, 67.19%; H, 4.67%; Cl, 13.68%; N, 5.40%; P, 5.97%. Calc. for C$_{29}$H$_{24}$Cl$_2$N$_2$OP: C, 67.10%; H, 4.70%; Cl, 13.52%; N, 5.40%; P, 5.90%.

Biochemistry: Determination of furin activity. 10 μl of the diluted furin solution containing 0.25 unit of enzymatic activity were incubated for 1 h at 37 °C with Boc-Arg-Val-Arg-Arg-AMC (final concentration 75-250 μM) in a buffer pH 7.3 with a total sample volume 150 μl. The reaction was stopped by adding 2 ml EDTA (initial concentration 5 mM) and relative fluorescence was detected with PTI Quanta Master 40 spectrofluorometer (Canada) (excitation, 380 nm; emission, 460 nm; both band widths 2 nm). The readings were recorded over 60 sec.

Michaels-Menten constant values were determined using Lineweaver-Burk plots from three independent experiments.

Determination of inhibitory effects of the studied compounds. To prepare a stock solution (concentration 7.5 mM), a sample of the corresponding phosphonium salt was dissolved in DMSO. The stock solution was then diluted to the concentration required to inhibit furin. The enzyme solution (0.25 unit of activity), a buffer pH 7.3 and the studied inhibitor solution were incubated at room temperature for 30 min. Then a solution of the fluorogenic substrate was added to reach the final concentration of 100 μM and enzymatic reaction was run for 1 h at 37 °C. The total volume of the mixture was 150 μl.

The reaction was terminated by adding 2 ml EDTA solution and the quantity of the released AMC was measured as described above. Enzyme activity in the absence of the studied compounds was

assumed to be 100%. Inhibition constants K_i were determined from the Lineweaver-Burk plots.

Data analysis and plotting were carried out using Origin Professional 8.0 software (OriginLab). At least two measurements were used for each point. The experimental error did not exceed 10% of the measured value.

Results and Discussion

The structure of the synthesized triphenylphosphonium derivatives of 1,3-oxazole corresponds to the results of elemental analysis and the data of NMR and IR spectroscopy.

The inhibitory activity of phosphonium salts (Table 1) varies in the experimental conditions from 15% (compound **1**) to 95% (compound **12**) and depends on the chemical nature of the substituents at the C2- and C5-positions of the oxazole cycle. For example, replacement of the hydrogen atom in C2-Me of oxazole **1** with a hydrophobic phenyl group (compound **2**) causes an increase in the inhibitory effect almost fourfold (compound **2** inactivates furin by 55%). The introduction of Ph directly into the C2-position (compound **3**) causes an even greater increase in inhibitory activity (84% of enzyme inactivation). The antifurin activity of betaine **4**, which contains at C2-position a hydrophobic electron-withdrawing 4-chlorophenyl group, increases to ~ 91%. But the presence of a second chlorine atom in this benzene ring (compound **5**) does not increase the degree of furin inactivation (87% inhibition). Alkylation of the sulfur atom (compound **3**) by 4-chlorophenyl group causes a drop in the activity of the compound **6**, which inactivates the enzyme by only 34%. Oxidation of the thiol group of oxazole **6** to a sulfo-group (compound **7**), which is able to give additional hydrogen bonds with furin, increases the inhibitory effect (inactivation by ~57%), which, however, does not reach the level of the phosphonium salt **3**. The alkylation of the sulfur atom of the betaine **4** by an electron-donating Et-group leads to a slight decrease in the inhibitory effect: compound **8** inhibits furin by 85%. An even greater decrease of antifurin activity is observed for the compound **9**. Despite the fact that C5-substituent of oxazole **9** contains an oxygen atom, which is capable of forming an additional hydrogen bond and Ph can realize additional hydrophobic contacts with furin, the enzyme is inactivated only by 73%. Replacement of the EtS-group presenting at the C5-position (com-

pound **8**) with hydrazine moiety (compound **10**) does not affect practically the activity of furin, while the introduction of a chlorine atom in this position (compound **11**) reduces the inhibitory effect (inactivation by ~57%). The presence of 2,4-dichlorophenyl moiety in C2 and MeS-group in the C5-position of oxazole (compound **12**) causes the highest degree of furin inactivation (95%.) However, the replacement of the MeS-group at the C5-position of the phosphonium salt **12** by Me_2N- leads to a decrease in antifurin activity (inactivation by ~71%), which is apparently due to spatial difficulties.

For a number of active compounds, the values of inhibition constants (K_i) were calculated from the graphs of Lineweaver-Burk and they are presented in Table 2. These data indicate that the most active inhibitors (K_i = 1.57-1.64 μM) are phosphonium salts containing electron-withdrawing substituents in the C2-position of oxazole (4-chlorophenyl- or 2,4-dichlorophenyl-), and at C5-position have donor groups (CH_3S- or H_2N-NH-). It is seen that the most active compound **12** is a competitive inhibitor of furin. Since the positively charged triphenylphosphonium group is very bulky, it is unlikely that it can fit in the pocket of the furin active center. It can be speculated that a flat uncharged 2,4-dichlorophenyl group accommodates into this pocket and that is what causes competitive inactivation of the enzyme.

Betaine **4** interacts with furin by the mechanism of non-competitive inhibition. Triphenylphosphonium salts containing at C2 p-chlorophenyl-group, and in the C5 position having an electron donating group (EtS- or H_2N-NH-) inactivate furin by the mechanism of mixed inhibition. (Table 2).

Fig. 1 shows examples of the corresponding graphs of Lineweaver-Burk plots, which allow determining the mechanism of furin inhibition.

Thus, as a result of the work, novel triphenylphosphonium derivatives of 1,3-oxazole were synthesized. They were found to be a new class of nonpeptide inhibitors of furin which retard the enzyme at the micromolar level. Depending on the chemical structure, they inactivate furin by the mechanism of competitive, non-competitive or mixed inhibition.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

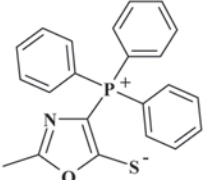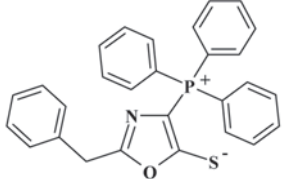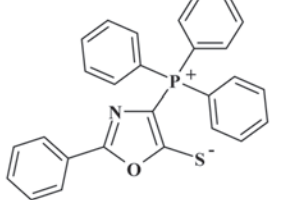*T a b l e   1. Screening of triphenylphosphonium derivatives of 1,3-oxazole as furin inhibitors*
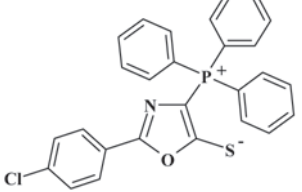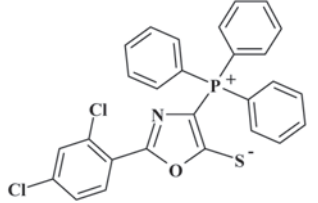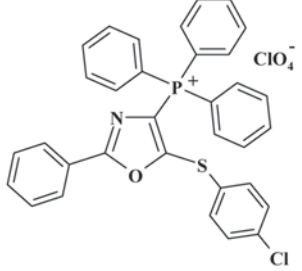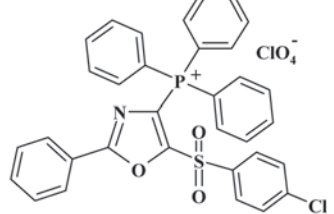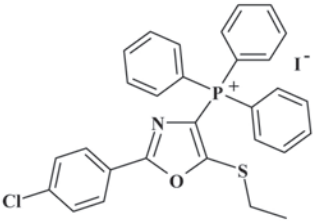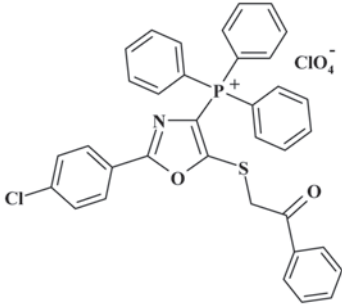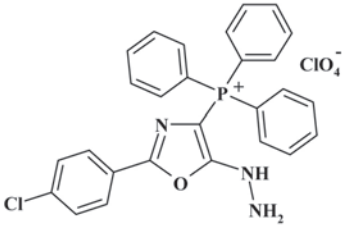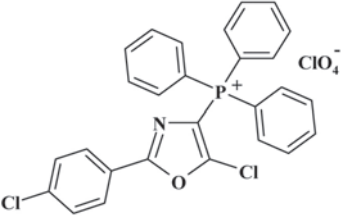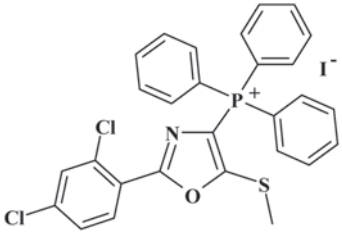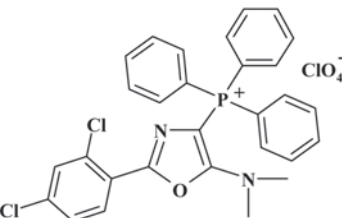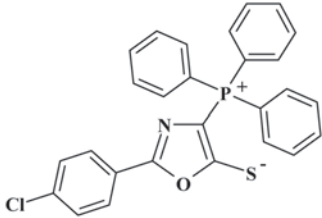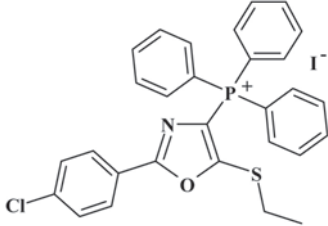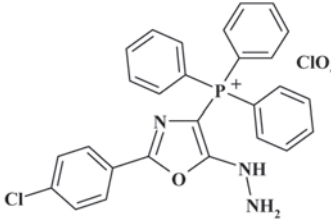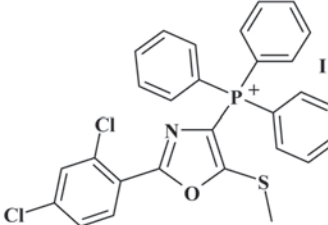
Compound number	Structure of compound	Inhibition of furin, %
1		15.2
2		55.1
3		84.3
4		90.7
5		87.0
6		34.2
7		56.8

T a b l e 1. Continue

8		85.2
9		73.1
10		86.2
11		56.6
12		95.1
13		70.5

*Screening conditions: The concentration of the fluorogenic substrate was 100 μM and the inhibitor under study was 500 μM; the total volume of the incubation medium was 150 μl, the sample contained 0.25 units of furin activity; the reaction was carried out for 1 hour at pH = 7.3 and 37 °C

T a b l e 2. Structure and properties of triphenylphosphonium derivatives of 1,3-oxazole as furin inhibitors

Number	Structure of compound	K_i, μM	Mechanism of inhibition
4		2.25	Non-competitive
8		3.04	Mixed inhibition
10		1.64	Mixed inhibition
12		1.57	Competitive

1,3-ОКСАЗОЛ-4-ІЛФОСФОНІЄВІ СОЛІ ЯК НОВИЙ КЛАС НЕПЕПТИДНИХ ІНГІБІТОРІВ ФУРИНУ

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Синтезовано низку нових похідних 1,3-оксазол-4-ілфосфонієвих солей, які містять у С2- та С5-положеннях гетероциклу електронодонорні або електроноакцепторні групи. Сполуки охарактеризовано ¹Н, ³¹Р ЯМР- та ІЧ-спектроскопією, даними елементного аналізу та хромато-масспектрометрії. Знайдено, що ці азоли є новим класом непептидних інгібіторів фурину. Залежно від хімічної будови вони інактивують ензим за механізмом конкурентного, неконкурентного або змішаного інгібування. Оцінка синтезованих похідних як інгібіторів фурину показала, що серед трифенілфосфонієвих солей оксазол **12**, який містить у С2-положенні 2,4-дихлорфеніл-, а у С5 – MeS-групу, є найактивнішим (K_i = 1,57 мкМ) конкурентним інгібітором ензиму. Результати свідчать про те, що хімічна модифікація фосфонієвих похідних
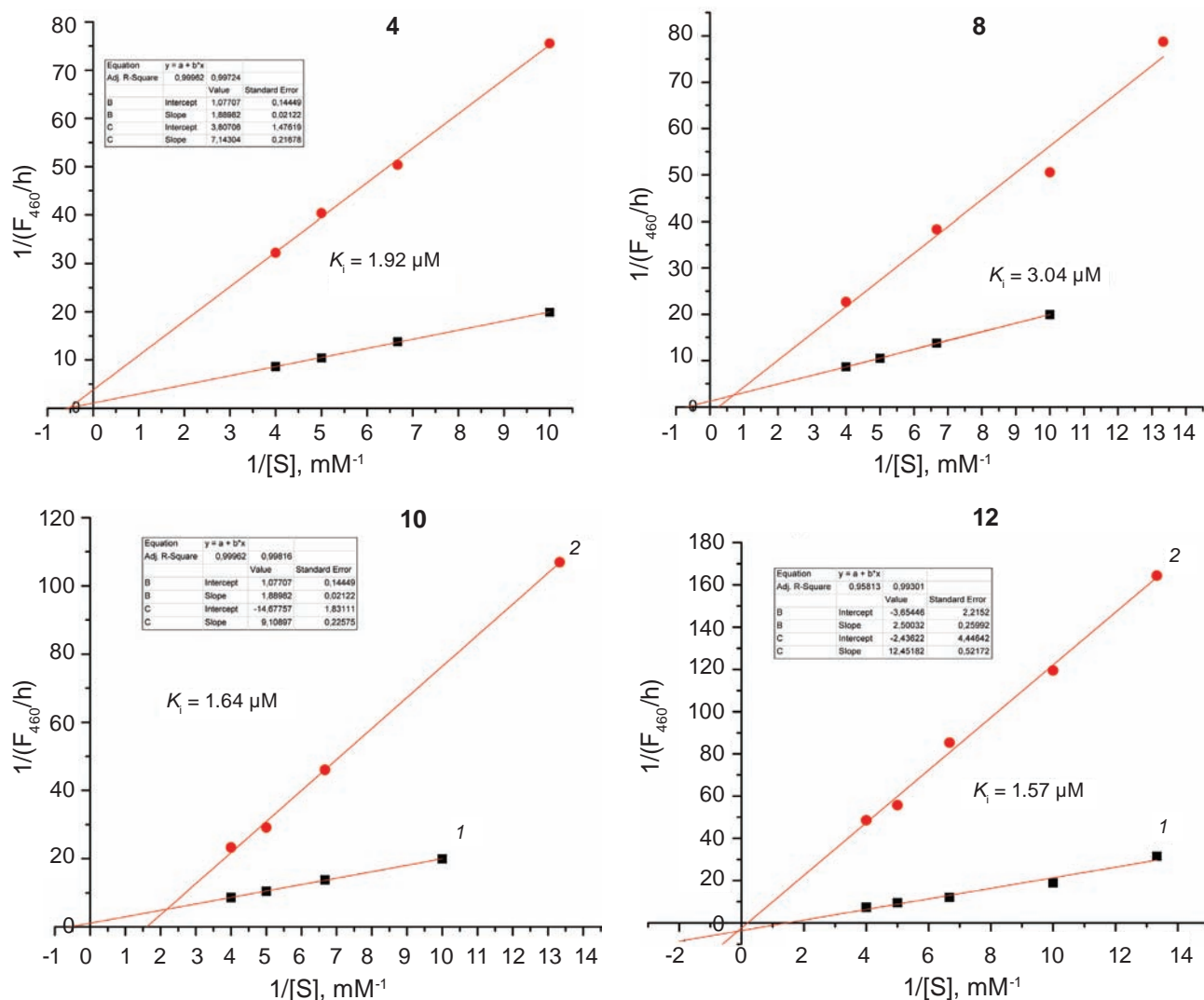
Fig. 1. Competitive, non-competitive and mixed inhibition of furin by triphenylphosphonium derivatives of 1,3-oxazole

оксазолу може бути корисною для створення по-тужніших та селективних інгібіторів фурину.

К л ю ч о в і   с л о в а: фурин, інгібітори, похідні 1,3-оксазолу, трифенілфосфонієві солі, механізм інгібування.

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