(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 July 2014 (24.07.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/112902 A1

(51) International Patent Classification:
C07K 7/06 (2006.01) *A61K 8/02* (2006.01)
A61K 38/08 (2006.01) *A61K 8/30* (2006.01)
A61K 8/44 (2006.01) *A61Q 19/08* (2006.01)

(21) International Application Number:
PCT/RU2014/000032

(22) International Filing Date:
20 January 2014 (20.01.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2013102410 21 January 2013 (21.01.2013) RU

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2014/112902 A1

(54) **Title**: PEPTIDE INHIBITORS OF NICOTINIC ACETYLCHOLINE RECEPTOR

(57) **Abstract**: The present invention refers to biochemistry, namely to new peptide compounds having the ability to selectively block the muscle-type nicotinic acetylcholine receptor. The claimed compounds have common formula (I): X1-X2-X3-Pro-X4-X5-X6, where X1 is chosen within H, Ac-, Palm-; X2 is chosen within Trp, Tyr, His, Phe, Pro; X3 is chosen within Trp, Tyr, His, Phe, Lys, Om, Dbu, Dpr, Arg; X5 is chosen within Trp, Tyr, His, Phe, Pro; X6 is chosen within -OH, $-NH_2$, $-OCH_3$, $-OC_2H_5$, $-NH-C_6H_5$. The invention can be applied in cosmetics for smoothing mimic and age-related wrinkles.

PEPTIDE INHIBITORS OF NICOTINIC ACETYLCHOLINE RECEPTOR.

Technical Field.

The present invention relates to biochemistry namely to new peptide compounds able to selectively block the muscle-type nicotinic acetylcholine receptor. More particularly the invention relates to applying those compounds for smoothing mimic and age-related wrinkles.

Background Art.

In the last decades the steady increased expectation of life in developed countries has been observed. In 2000 in the USA there were 13% of people older than 65 years, in 2030 the number is being expected to increase up to 20%. The demographical change demands accelerating efforts to design an effective medical treatment for senior people. This demand refers in full measure to the cosmetic dermatology which provides with roborant, anti-aging and sun-protecting agents as well as anti-wrinkle creams. Cosmetic and pharmaceutical companies often use peptides as active ingredients in such creams. Peptides and the cosmetics on their basement have different activity: stimulation of fibroblast activity, inhibiting of the enzymes involved in collagen degradation, angiogenesis stimulation, immunomodulating, regulating melanin production, neuromuscular transmission blockage. One of the most important drawbacks for topical application of the peptides in creams is their decreased ability to penetrate into the skin. In fact, the ability to penetrate into the skin depends on various factors such as physical and chemical conditions of the compound (dissociation constant [pKa], molecular size, stability, solubility and lipophilicity coefficient); penetrating time; sustainability, thickness and skin composition, skin metabolism; area, square and duration of the application (Ranade, V.V. Drug delivery systems. 6. Transdermal drug delivery. J. Clin. Pharmacol. 1991, 31, 401–418). A peptide is considered to be a suitable tool for topical application if it corresponds to the parameters listed below, however, it must be noted that all the parameters are empirical and not universal (Guy, R.H. Current status and future prospects of transdermal drug delivery. Pharm. Res. 1996, 13, 1765–1769):

1. Molecular mass less than 500 Da
2. Lipophilic coefficient value (logarithm of the distribution coefficient for the octanol/water system) from 1 to 3.
3. Melting temperature under 200 C
4. Good solubility in water (1mg/ml)
5. No or few polar centers.

Peptides used in cosmetology can be divided into four major groups: signal peptides, enzyme inhibitors, carrier peptides and blockers of neuromuscular transmission (Gorouhi F, Maibach HI. Role of topical peptides in preventing or treating aged skin. Int J Cosmet Sci. 2009 Oct;31(5):327-45). Despite the similar visible physiological effect after using the peptides from the four groups, the mechanism of their action differs significantly. The peptides from the group of blockers of neuromuscular transmission are therewith considered as a safe alternative for botulotoxin injections used for treatment of age-related and mimic wrinkles. Highly specific botox has many drawbacks such as high toxicity and therefore, the necessity of the exact dose amount calculation, essential dependence of product quality on manufacturing conditions, injected usage only.

Muscle contraction is a physiological process under which muscles undergo tension – shorten or lengthen providing mechanical work. This process provides animals and humans with the ability to spontaneous and nonspontaneous movements and directly connected with digestive, respiratory, defensive, secretory and other physiological processes. Unstriated muscle is responsible for nonspontaneous movement, for example ventricle or intestinal peristalsis, changes in tonus of blood vessels and urinary bladder. Striated muscle provides spontaneous movement – spatial motion, face mimics, breath, swallowing etc. Heart work is provided by the heart muscular contraction.

Muscles are formed by multinuclear muscular fibers each of them separately is not only cellular but also physiological unit owning to the presence of such a specific "contracting" element as myofibrils. Those filaments are gathered into the clusters of the first order; several first-order clusters combine and form clusters of the second order etc. leading to the muscle formation. As muscle contraction is initiated by an impulse coming from the central nervous system, there are areas on the muscle which are innervated with synaptic terminals of neuronal axons. The junction between the neuron and muscle fiber is called neuromuscular synapse (NMS).

The mechanism of muscle contraction can be divided into several main stages. The first stage is sending a stimulus from neurons in a form of action potential. Action potential spreads along the nerve fibril to its terminals on the muscle fibers which leads to acetylcholine (Ach) neurotransmitter release from the presynaptic part of NMS into the synaptic gap. This neurotransmitter affects only a part of the muscle fiber membrane (postsynaptic part of NMS) opening various acetylcholine gated channels – acetylcholine receptors (AChRs). In response to the opening of the channels, there is an increase in sodium concentration inside the muscle fiber

that leads to generation of action potential on cell membrane which is conducted longwise the muscle fiber membrane. Action potential depolarizes muscle membrane leading to calcium ions release from sarcoplasmic reticulum. Calcium ions directly initiate the process of muscle contraction. By means of calcium pump, calcium is actively pumped back into the sarcoplasmic reticulum leading to muscle relaxation.

The mechanism of acetylcholine release is well established. When neuronal impulse in a form of action potential reaches the termini of the motor neurons it opens calcium channels located on the presynaptic membrane of NMS. Local increase in intracellular calcium ion concentration is followed by calcium interaction with the proteins which provide fusion of synaptic vesicles containing neurotransmitter Ach with the plasma membrane. The process has also been studied in details and goes with the help of complex SNARE formed by the effective four-helix junction of three proteins – vesicle surface protein synaptobrevin, syntaxin and neuronal surface membrane protein SNAP-25. The complex formation leads to rapid fusion of the vesicle and plasma membranes and induces exocytosis of Ach into the synaptic gap. Ach molecules defuse throughout the gap with width 50-100 nm and reach postsynaptic membrane which is very sensitive to the transmitter due to the presence of highly specific receptors – AchRs. Their binding with Ach initiates opening of the channel; it causes dramatic influx of sodium inside the cell and a weaker flow of potassium out of the cell with the further depolarization of muscular membrane, calcium release and muscle contraction as described above.

The mechanism of neuronal impulse transmission through the postsynaptic part of NMS resulting from the binding of meurotransmitter Ach to the AChR and was also well studied. There are two major groups of AChRs – nicotinic (nAChRs) and muscarinic (mAChRs) which differ in their ability to bind with agonists. In particular, nAChRs were named for their ability to bind a natural plant alkaloid nicotine, and mAChRs – for the ability to bind muscarine alkaloid from poisonous mushrooms. NAChRs are ionotropic receptors which are permeable for specific ions after binding with a ligand. MAChR refers to a group of metabotropic receptors; it is a one-chain protein containing 7 transmembrane fragments associated with G-pritein. In this case, signal transmission after ligand binding follows many metabolic pathways.

At the molecular level nAChRs are oligomeric proteins consisting of 5 subunits. In membranes the 5 subunits are known to be pseudo symmetrically organized around the central axis where there is an ionic channel with diameter of 2.5 nm. These data were obtained for nAChR from the electrical organ of the electric ray *Torpedo*; this receptor has subunit composition $(\alpha 1)_2$-$\beta 1$-γ-δ

[Unwin N. Refined structure of the nicotinic acetylcholine receptor at 4 Å resolution. J Mol Biol 2005; 346: 967-89]. Up to present, 10 various subtypes of α-subunits (α1–α10) and 4 β –subunits (β1–β4) are discovered. All the subtypes of α- and β-subunits (except α1- and β1-) are revealed in neuronal type nAChRs expressed mainly in neurons of the central and/or peripheral nervous system in many cases even on the presynaptic membrane [Dani JA, Bertrand D. Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. Annu Rev Pharmacol Toxicol 2007; 47: 699-729]. Postsynaptic nAChRs of both animal and human neuromuscular synapses have composition $(\alpha1)_2$-β1-γ-δ (identical to the one for the receptor from electrical organ) but at the stage of the initial (embryotic) development of an organism. In the mature form γ-subunit is substituted with ε- [Yomoto N., Wakatsuki S., Sehara-Fujisawa A. 2005. The acetylcholine receptor gamma-to-epsilon switch occurs in individual endplates. Biochem. Biophys. Res. Commun. 331:1522-1527]. In the mature muscle, only nAChR with the structure $(\alpha1)_2$-β1-ε-δ is responsible for binding the neurotransmitter which leads to muscle contraction. Taking into account the highly homologous structure of all the nAChR sunbunits, their spatial pentameric channel composition in the membrane is considered to be similar too. Nowadays the nAChRs region for binding with the classical agonists and competitive antagonists is also discovered: two ligand binding regions are located in the area of the contact of major N-terminal extracellular domains of two α1- and neighboring γ(ε)- and δ-subunits of the receptor approximately in the middle part towards the membrane surface.

To provide the neuron and the muscle fiber with the fast information transmission, high concentration of AChR in essential areas of postsynaptic membrane of neuromuscular junction is needed. Therefore, aggregation of AChRs is crucial for normal functioning of neuromuscular junction [Hoch W. 1999. Formation of the neuromuscular junction. Agrin and its unusual receptors. Eur. J. Biochem. 265:1-10]. There are several proteins involved in the process of aggregation, mainly agrin, rapsin and kinase MuSK (Muscle-Specific Kinase). Developed earlier and being created nowadays new agents blocking neuromuscular transmission are aimed at disruption of normal functioning of either presynaptic membrane of NMS or postsynaptic part of a synapse.

Cosmetic industry has undertaken several various efforts to develop new compounds for the topic application during the treatment of mimic wrinkles to avoid side effects observed after botulinum toxin injections (Lupo MP, Cole AL. Cosmeceutical peptides. Dermatol Ther. 2007, Sep-Oct;20(5):343-9). Up to present, several peptide compounds are known to block neuromuscular transmission (Table 1)

Table 1. Amino acid sequence of the peptides blocking neuromuscular transmission, and their biological activities.

Name	Amino acid sequence	Activity
Argireline	Ac-Glu-Glu-Met-Gln-Arg-Arg-NH2	Inhibits formation of the complex SNARE and neurotransmitter release
Leuphasyl	H-Tyr-D-Ala-Gly-Phe-Leu-OH	Mimics the action of enkefalin – decreases neuronal excitation
Vialox	H-Gly-Pro-Arg-Pro-Ala-NH2	Competitive antagonist of nAChR
Syn-Ake	H-β-Ala-Pro-Dab-NHBzl	Competitive antagonist of nAChR
Inyline	not published	Competitive antagonist of MuSK

Peptide argireline is the top one within "cosmetic analogues of botox" (Blanes-Mira C., et al. A synthetic hexapeptide (Argireline) with anti-wrinkle activity. Int J Cosmet Sci. 2002; 24(5): 303–310). Appearing at the beginning of 2000, it rapidly gained the popularity – today it is used in many cosmetic drugs aimed at smoothing mimic wrinkles. Six amino acids of argireline repeat the protein fragment SNAP 25 necessary for synaptic axonal vesicle binding with the presynaptic membrane. In an axon, argireline competes with protein SNAP25 and inserts into the temporary complex SNARE instead of it – this complex is formed from several membrane proteins directly before synapse binds with the membrane, and it is necessary for successful exocytosis. A deficient complex cannot provide a good junction of vesicle with membrane, as a result transmitter release does not occur and muscle does not receive a signal to contract and stay relaxed.

Argireline analogues obtained from protein SNAP 25 and aimed at inhibiting neuromuscular transmission at synaptic level by competing with SNAP 25 for complex SNARE formation, are described in patents EP 1180524 и WO9734620.

Topic 30-day use of cream containing 10% of argireline studied on volunteers leads to 30% decrease in the depth of mimic wrinkles in comparison with 10% exposure of Placebo.

Pentapetide Leuphasyl developed by Lipotech company (Spain) mimics enkephalin action decreasing neuronal excitation by inhibiting Ca^{2+}-influx throughout the membrane and

decreasing Ca^{2+}-dependant transmitter release. Use of Leuphasyl in a combination with argireline enhances argireline effect by 1.5-fold.

Inyline is an acetyl hexapeptide developed by Lipotech company (Spain) (WO2011/009626) was created basing on the predicted structure of the binding area of agrin to MuSK (Muscle-Specific Kinase) obtained by molecular modeling. Inyline works as a competitive antagonist of MuSK at binding with agrin, which is essential for muscle contraction. Postsynaptic mechanism of decreasing muscle contraction helps to avoid appearance of mimic wrinkles. Studies on volunteers have shown that 28-day administration of 5% Inyline solution leads to reduction in the depth of the wrinkles in the area of "crows feet" on 14.9%.

Vialox (Pentapharm company, Switzerland) is a pentapeptide fragment of the neurotoxin waglerin-1 from the venom of the Temple Viper. Vialox has a curare-like activity and it is a competitive antagonist of the nAChR. When the peptide binds the receptor sodium ion channel keeps closed which disrupts neuronal impulse transmission and does not allow muscles to contract. Patent EP1809652 describes peptide antagonists of the acetylcholine receptor which work postsynaptically following the vaglerine-1-like mechanism by blocking neuronal transmission and preventing wrinkle appearance. Vialox can be used together with other cosmetic peptides (WO 2006/069608).

28-day application of the cream containing 5% Vialox reduces wrinkle size up to 49% and skin roughness up to 47%.

One more active cosmetic peptide Syn-ake (Pentapharm, Switzerland) is a reversible antagonist of the muscle-type nicotinic acetylcholine receptor (mnAChR). This tripeptide acts in a manner similar to waglerin-1, which prevents acetylcholine binding with the receptor and the channel activation. Therefore, the muscles stay relaxed (WO 2006/047900). The studies performed on volunteers showed that the 28-day application of the cream containing 4% Syn-Ake decreases depth of wrinkles on foreheads up to 52%.

Thus, the competitive antagonists of the mnAChR Vialox and Syn-Ake are the most effective peptides with anti-wrinkle activity.

However, none of the compounds developed by cosmetic or pharmaceutical companies can inhibit muscle contraction with the effectiveness similar to botulinum toxin. Therefore, the necessity for creating new compounds capable to inhibit muscular contraction and reaching better results in decreasing and emolliating wrinkles especially mimic ones, still exists.

Disclosure of Invention.

In this description the abbreviations used for the amino acids follow the recommendations of IUPAC-IUB (Eur.J.Biochem., 1984, 138:9-37; J.Biol.Chem., 1989, 264:633-673). Thus, for example, Lys represents NH_2-CH(CH_2-CH_2-CH_2-NH_2)-COOH, Lys- represents NH_2-CH(CH_2-CH_2-CH_2-NH_2)-CO-, -Lys represents -NH-CH(CH_2-CH_2-CH_2-NH_2)-COOH, -Lys- represents -NH-CH(CH_2-CH_2-CH_2-NH_2)-CO-. Therefore, the hyphen, which represents the peptide bound, eliminates the OH in the carboxyl group of the amino acid when situated to the right of the symbol, and eliminates the H of the alpha-amino-group of the amino acid when situated to the left of the symbol.

The abbreviation Ac- represents acetyl group (CH_3-CO-) and Palm- represents palmitoyl group (CH_3-$(CH_2)_{14}$-CO-).

The abbreviation Orn represents amino acid ornitine, Dbu-2,4-diaminobutyric acid, Dpr-2,3-diaminopropanoic acid, -NH-C_6H_5- benzilamide.

The term "cosmetically acceptable salts" of peptides in the context of the present invention means salts recognized for their use in human beings and include salts formed after addition of either inorganic compounds for example, litium, sodium, calcium, potassium, magnesium, copper, zinc etc., or organic compounds, for example, ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, arginine, lysine, or acid addition salts, for example, acetate, citrate, lactate, malonate, maleate, fumarate, benzoate, aspartate, glutamate, oleate, trifluoroacetate, oxalate, gluconate, or inorganic acids, for example, chloride, sulfate, borate, carbonate. Cosmetically acceptable salts of the peptides of the invention can be obtained by the standard methods (Berge S.M., Bighley L.D. and Monkhouse D.C. 1977, "Pharmaceutical Salts", J.Pharm.Sci. 66:1-19).

The present invention solves the problem of increasing the number of neuromuscular transmission peptide blockers applicable for use in cosmetology and dermatology.

Syn-Ake and Vialox are the analogues of the claimed peptide acting in a similar mechanism. Both peptides have been developed by the Swiss company "Pentapharm" on the basis of 21 amino acid component waglerin-1 obtained from the venom of the Temple Viper. Waglerin-1 is known to block muscular nAChR, hence it has an antagonistic activity (Weinstein S.A. et al. Characterization and amino acid sequences of two lethal peptides isolated from venom of Wagler's pit viper, *Trimeresurus wagleri*. Toxicon. 1991; 29(2):227-36). Short peptides on its basement do not manifest toxicity and at high concentrations keep blocking the same receptor. Recently (Utkin Y.N. et al. Azemiopsin from *Azemiops feae* Viper Venom, a Novel Polypeptide Ligand of Nicotinic Acetylcholine Receptor. J.Biol.Chem. 2012, Aug 3;287(32):27079-86)

polypeptide azemiopsin containing 21 amino acids (DNWWPKPPHQGPRPPRPRPKP) has been discovered in the venom of the Fea's Viper. It is also able to block muscular nAChRs. While carrying out the search of an active center of azemiopsin (fragment of the amino acid sequence mainly contributing into the ability to bind the nAChR) using electrophysiological methods (Fig.1), the range of overlapping pentapetide fragments of the initial toxin was tested (Fig.2). The ability to inhibit acetylcholine induced currents through the muscle-type AChR expressed in oocytes from the clawed frog *Xenopus laevis* was investigated. Preliminary tests of these fragments were carried out at concentration of 150 μg/ml. The results obtained are shown in Fig.2. Pentapeptide SEQ ID NO:1:WWPKP appeared to be the most active. Meanwhile, this peptide does not exhibit toxicity at the doses up to 30 mg/kg at intraperitoneal injection and at the doses up to 160 mg/kg at per oral application. The sequence of pentapetide fragment SEQ ID NO:1:WWPKP (hereafter Az3) was changed as described bellow: 1. Amid group is inserted at C-terminus (SEQ ID NO:41: H-Trp-Trp-Pro-Lys-Pro-NH_2, hereafter Az3-NH2); 2. Tryptophane residue was substituted with tyrosine residue: [Tyr1]Az3 (SEQ ID NO:3), [Tyr2]Az3 (SEQ ID NO:2) и [Tyr1,Tyr2]Az3 (SEQ ID NO:4); second amino acid residue was substituted with hydrophobic valine residue ([Val-2]Az3); 4. Lysine-4 was substituted for diaminobutyric acid SEQ ID NO:5: H-Trp-Trp-Pro-Dbu-Pro-OH ([Dbu4]Az3); 5. The peptide sequence was reduced to four and three amino acids:[des-Trp1]Az3, [des-Pro5]Az3, [des-Trp1,des-Pro5]Az3.

Retrosequences of peptides Az3 and [Tyr2]Az3: PKPWW and PKPYW respectively have been synthesized and tested. However their inhibiting activity towards the muscle receptor was reduced. The diagram of retropeptides testing and comparison with the activity of initial Az3 and [Tyr2]Az3 is shown on Fig.5. The peptides were tested in concentration of 1mM.

Additional experiments were carried out to compare the effectiveness of the blockage of currents through the receptor channel by fragments of azemiopsin and commercial samples of Syn-Ake and Vialox. The compounds were tested at concentration of 1 mM. The results are shown on Fig.3.

After performing the tests, it was established more appropriate to use an amid form of the pentapeptide fragment of azemiopsin with substitution of the second amino acid for tyrosine SEQ ID NO:42: H-Trp-Tyr-Pro-Lys-Pro-NH_2 (herein after [Tyr2]Az3-NH2).

For more detailed comparison of [Tyr2]Az3-NH2 with the most widespread commercial product Syn-Ake, the curves describing dependence of inhibiting activity on concentration were obtained as and shown in Fig.3. Peptide [Tyr2]Az3-NH2 blocks 50% of current (value IC50) at the

concentration of 23μM, whereas the same concentration for Syn-Ake is equal to 180 μM, therefore, the new peptide is eight times more functionally active than the commercial analogue. It is demonstrated by a histogram on Fig.5.

Compounds of the invention.

The task to obtain new compounds blocking mnAChR is achieved by means of the peptides with the structures described by the following formula (I): X1-X2-X3-Pro-X4-X5-X6, where

X1 is selected from H, Ac-, Palm-

X2 is selected from Trp, Tyr, His, Phe, Pro,

X3 is selected from Trp, Tyr, His, Phe, Lys, Orn, Dbu, Dpr, Arg

X4 is selected from Trp, Tyr, His, Phe, Lys, Orn, Dbu, Dpr, Arg

X5 is selected from Trp, Tyr, His, Phe, Pro

X6 is selected from –OH, $-NH_2$, $-OCH_3$, $-OC_2H_5$, $-NH-C_6H_5$

Preferably compounds of formula (I) are selected from the group containing:

SEQ ID NO:1: H-Trp-Trp-Pro-Lys-Pro-OH

SEQ ID NO:2: H-Trp-Tyr-Pro-Lys-Pro-OH

SEQ ID NO:3: H-Tyr-Trp-Pro-Lys-Pro-OH

SEQ ID NO:4: H-Tyr-Tyr-Pro-Lys-Pro-OH

SEQ ID NO:5: H-Trp-Trp-Pro-Dbu-Pro-OH

SEQ ID NO:6: H-Trp-Tyr-Pro-Dbu-Pro-OH

SEQ ID NO:7: H-Tyr-Trp-Pro-Dbu-Pro-OH

SEQ ID NO:8: H-Tyr-Tyr-Pro-Dbu-Pro-OH

SEQ ID NO:9: Ac-Trp-Trp-Pro-Lys-Pro-OH

SEQ ID NO:10: Ac-Trp-Tyr-Pro-Lys-Pro-OH

SEQ ID NO:11: Ac-Tyr-Trp-Pro-Lys-Pro-OH

SEQ ID NO:12: Ac-Tyr-Tyr-Pro-Lys-Pro-OH

SEQ ID NO:13: Ac-Trp-Trp-Pro-Dbu-Pro-OH

SEQ ID NO:14: Ac-Trp-Tyr-Pro-Dbu-Pro-OH

SEQ ID NO:15: Ac-Tyr-Trp-Pro-Dbu-Pro-OH

SEQ ID NO:16: Ac-Tyr-Tyr-Pro-Dbu-Pro-OH

SEQ ID NO:17: Ac-Trp-Trp-Pro-Lys-Pro-NH_2

SEQ ID NO:18: Ac-Trp-Tyr-Pro-Lys-Pro-NH_2

SEQ ID NO:19: Ac-Tyr-Trp-Pro-Lys-Pro-NH_2

SEQ ID NO:20: Ac-Tyr-Tyr-Pro-Lys-Pro-NH_2

SEQ ID NO:21: Ac -Trp-Trp-Pro-Dbu-Pro-NH_2

SEQ ID NO:22: Ac-Trp-Tyr-Pro-Dbu-Pro-NH_2

SEQ ID NO:23: Ac-Tyr-Trp-Pro-Dbu-Pro-NH_2

SEQ ID NO:24: Ac-Tyr-Tyr-Pro-Dbu-Pro-NH_2

SEQ ID NO:25: H-Trp-Trp-Pro-Lys-Pro-OCH_3

SEQ ID NO:26: H-Trp-Tyr-Pro-Lys-Pro-OCH_3

SEQ ID NO:27: H-Tyr-Trp-Pro-Lys-Pro-OCH_3

SEQ ID NO:28: H-Tyr-Tyr-Pro-Lys-Pro-OCH_3

SEQ ID NO:29: H-Trp-Trp-Pro-Dbu-Pro-OCH_3

SEQ ID NO:30: H-Trp-Tyr-Pro-Dbu-Pro-OCH_3

SEQ ID NO:31: H-Tyr-Trp-Pro-Dbu-Pro-OCH_3

SEQ ID NO:32: H-Tyr-Tyr-Pro-Dbu-Pro-OCH_3

SEQ ID NO:33: Ac-Trp-Trp-Pro-Lys-Pro-OCH_3

SEQ ID NO:34: Ac-Trp-Tyr-Pro-Lys-Pro-OCH_3

SEQ ID NO:35: Ac-Tyr-Trp-Pro-Lys-Pro-OCH_3

SEQ ID NO:36: Ac-Tyr-Tyr-Pro-Lys-Pro-OCH_3

SEQ ID NO:37: Ac-Trp-Trp-Pro-Dbu-Pro-OCH_3

SEQ ID NO:38: Ac-Trp-Tyr-Pro-Dbu-Pro-OCH_3

SEQ ID NO:39: Ac-Tyr-Trp-Pro-Dbu-Pro-OCH_3

SEQ ID NO:40: Ac-Tyr-Tyr-Pro-Dbu-Pro-OCH_3

SEQ ID NO:41: H-Trp-Trp-Pro-Lys-Pro-NH_2

SEQ ID NO:42: H-Trp-Tyr-Pro-Lys-Pro-NH_2

SEQ ID NO:43: H-Tyr-Trp-Pro-Lys-Pro-NH_2

SEQ ID NO:44: H-Tyr-Tyr-Pro-Lys-Pro-NH_2

SEQ ID NO:45: H-Trp-Trp-Pro-Dbu-Pro-NH_2

SEQ ID NO:46: H-Trp-Tyr-Pro-Dbu-Pro-NH_2

SEQ ID NO:47: H-Tyr-Trp-Pro-Dbu-Pro-NH_2

SEQ ID NO:48: H-Tyr-Tyr-Pro-Dbu-Pro-NH_2

The peptides from the invention can consist of stereoisomers or mixures of stereoisomers of the amino acids, i.e. amino acids can have L- and/or D-configuration or exist as a racemic mixture independently in each position.

Technical result of the target invention is reached by means of such characteristics of the new peptide as: selective blockage of mnACChR, lack of toxic effects and lack of allergic reactions.

As the size of the claimed peptides is small and they contain 5 amino acid residues they can be obtained by means of chemical synthesis. The claimed peptides are new compounds and do not have similar homologues.

The peptides can be used in cosmetology in a cosmetic composition in the form of cream, lotion or gel for face skin application for decreasing the depth of mimic and age-related wrinkles. Weight concentration of the peptide is within 0.01-5%. Except the compound with general formula (I) such cosmetic composition can contain at least one additional component chosen from: amino acids, proteins, protein hydrolysates, growth factors, enzymes, enzyme inhibitors, peptides, polysaccharides, pyrimidines, purines, nucleotides, nucleosides, carboxylic acids, fat acids, lipids, sphingolipids, flavonoids, phenols, polyphenols, terpenes, alkaloids, benzofurans, polyalcohols, antimicrobial component, antimicrobial peptides, vitamins, provitamins, retinoids, carotenoids, chelating agents, antioxidants, agents improving skin permeability.

Brief Description of Drawings:

Fig.1. Example of registration of the current through the muscle-type nAChR heterologously expressed in oocytes from clawed frog *Xenopus laevis,* in response to 20 μM acetylcholine application. Each application of acetylcholine in the presence of or without a ligand is marked with black square above. The control current, to the left end, is compared with the current obtained in the presence of 50 μM azemiopsin. The arrow shows the time of the beginning of incubation. Time interval between acetylcholine applications is 5 minutes. Ccomplete blockage of the muscle receptor activity by azemiopsin is demonstrated.

Fig.2. Comparison of the effectiveness of the current inhibition through the muscle-type nAChR channel by overlapping pentameric fragments of azemiopsin and commercially available compounds Syn-Ake and Vialox. 100% refers to complete blockage of the current, 0% refers to the unblocked current. All the compounds are taken at concentration of 150 μg/ml.

Fig.3. Comparison of the effectiveness of the current inhibition through the muscle-type nAChR channel by various derivatives of azemiopsin and commercially available compounds Syn-Ake and Vialox. The tested compounds are taken at concentration of 1mM. 100% refers to complete blockage of the current, 0% refers to the unblocked current in response to application of 20 μM acetylcholine.

Fig.4. Comparison of the curves describing dependence of inhibiting activity on concentration for SEQ ID NO:41 ([2Tyr]Az3NH2) and commercial drug Syn-ake. Absciss axis represents

decimal logarithm of ligand concentrations, ordinate represents current through receptor channel (% of control) in response to application of 20 μM acetylcholine.

Fig.5. Comparison of concentrations for half inhibition of the current through the muscle-type nAChR channel. Ordinates represents concentration of tested peptides ([Tyr2]Az3-NH2 и Synake).

Fig.6. Comparison of effectiveness of inhibition of the current through the muscle-type nAChR channel by peptides Az3-NH2, [2Tyr]Az3-NH2 and their retro-sequences taken at concentration of 1mM. 100% refers to complete blockage of the current, 0% refers to the control current

Modes for Carrying Out the Invention.

Example 1. Chemical synthesis of peptide SEQ ID NO:1: H-Trp-Trp-Pro-Lys-Pro-OH.

Coupling of the first amino acid to the polymer (P). Preparation of Fmoc-Pro-P (Fmoc=9-fluorenylmethyloxycarbonyl).

200 mg 2-chlorotritylchloride containing 1.0 mmole hydroxyl groups per gram is washed with dry methylene chloride. 374 mg (1.1 mmoles) Fmoc-Pro-OH and 374 μl (2,2 mmole) diisopropylethylamine are dissolved in 5 ml methylene chloride, the solution is stirred 5 min and added to the polymer. The reaction is carried out for one hour at room temperature and stirring. After the end of the reaction, the polymer is filtered and washed with methylene chloride, ethanol and dimethylformamide three times.

Description of one synthetic cycle for elongation of peptide chain.

Preparation of Fmoc-Lys(Boc)-Pro-P :

a) the peptidyl-polymer obtained as described above is treated with 20% solution of piperidine in dimethylformamide for 20 min. Then the polymer is washed for 2 min with 5 ml dimethylformamide two times, for 5 min with 5 ml dioxane-water mixture (2:1) and five times for 2 min with 5 ml dimethylformamide again.

b) 505 mg (1.1 mmole) Fmoc-Lys(Boc)-OH, 150 mg 1-hydroxybenzotriazole (1.1 mmoles) and 170 μl (1.1 mmoles) N,N'-diisopropylcarbodiimide are dissolved in 5 mldimethylformamide, the solution is stirred for 10 min at 0° C and added to the polymer. The reaction is carried out for 4 hours with regular stirring. At the end of the

reaction process the polymer is filtered off, washed with dimethylformamide and treated with 5 ml mixture Ac_2O-pyridine-dimethylformamide (20:20:60) for one hour, then the polymer is washed with dimethylformamide, isopropanole and again dimethylformamide.

The polypeptide chain synthesis is carried out manually in glass flowing reactor (2x20 cm) using the protocol which follows below for each synthetic cycle (taking 8-10 ml of solvent for 400 mg of starting polymer), when carrying out the reaction of condensation (operation 6). The volume of the reaction mixture is 5-7 ml:

1. DMFA (dimethylformamide) (5 × 2 min);
2. 20% piperidine in ДМФА (20 min);
3. DMFA (3 × 2 min);
4. dioxane-water, 2:1, (2 × 5 min);
5. DMFA (5 × 2 min);
6. Condensation reaction: 5 molar equivalents of activated Fmoc-amino acid (4 h);
7. DMFA (3 × 2 min);
8. acetylation: Ac_2O–pyridine– dimethylformamide, 20:20:60, (1 h);
9. DMFA (3 × 2 min);
10. isopropanole (3 × 2 min);

To activate Fmoc-amino acid derivatives by DIPCDI/HOBT (N,N'-diisopropylcarbodiimide /1- hydroxybenzotriazole) mixture, 170 µl DIPCDI (1,1 mmoles, 5 equivalents) in 4 ml DMFA are added to the solution of 1.1 mmoles (5 equivalents) of Fmoc-protected aminoacid and 150 mg (1,1 mmoles, 5 equivalents) HOBt in 4 ml DMFA, the solution is stirred for 10 min.

The completeness of condensation reaction after operation 6 of synthetic protocol is controlled by ninhydrin test; in case of N-terminal proline, isatin test is used.

The following amino acid derivatives were used for synthesis: Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Trp(Boc)-OH.

Cleavage of peptide from polymer.

For the reaction of peptide cleavage from polymer and simultaneous removal of side chain blocking groups, 800 mg peptidyl-polymer was used. 15 ml mixture of TFA (trifluoroacetic acid) with water (97.5:2.5 V/V) is added to the peptidyl-polymer and

suspension is stirred for 2 hours, then the solution of the peptide obtained is filtered of the polymer, the polymer is washed with 5 ml TFA, and extra TFA is removed from combined solution by evaporation under reduced pressure. The peptide is precipitated by addition of 100 ml of ethyl ester, filtered and washed with the ester (5 × 20 ml). Precipitate is dissolved in 5 ml 10% acetic acid for 20 min, filtered and the residue was washed with 5 ml 10% acetic acid. The peptide solution obtained is freeze-dried and desalted by gel-filtration on the Sephadex G-10 column (2,5 × 60 sm) equilibrated with 0.1 M acetic acid. The peptide is purified by means of reversed-phase HPLC using a gradient of acetonitrile in water (from 10% to 35% in 75 min) in the presence of 0.1% acetic acid at the flow rate 3 ml/min, eluate absorbance is detected at 226 nm. The fractions corresponding to the main absorbance peak are collected and freeze-dried. The molecular mass of the peptide determined by mass-spectrometry is 713.8 Da, calculated molecular mass – 712.8 Da.

Example 2. Estimation of acute toxicity for peptide H-Trp-Trp-Pro-Lys-Pro-OH.

Estimation of acute toxicity (LD_{50}) after intraperitoneal administration of peptide H-Trp-Trp-Pro-Lys-Pro-OH was carried out on 40 male Balb mice weighting 20-23 g, separated into five equal groups. Mice were intraperitoneally injected with the peptide at doses of 1 mg/kg, 3 mg/kg, 10 mg/kg and 30 mg/kg in 0.2 ml sterile 0.9% NaCl solution. Control animals were injected with same volume of 0.9% NaCl . The results of the experiments did not reveal any toxic effects of the peptide for any of the tested groups.

Investigation of acute toxicity (LD_{50} per os) after administration of the peptide directly into a stomach by a special catheter was conducted on 40 male Balb mice weighting 20-23 g. Animals were randomly divided into 5 groups. The drug was applied at doses 2.5 mg/kg, 10 mg/kg, 40 mg/kg, 160 mg/kg in 0.2 ml sterile solution of 0.9%. Control animals were injected with the same volume of 0.9% NaCl. After the administration of the studied peptide at concentrations given above into the stomach of the mice, no toxic effect was observed.

Example 3. Preparation of the cream containing peptide H-Trp-Trp-Pro-Lys-Pro-OH with the range of concentrations between 0.01 and 5%. Cream composition: H-Trp-Trp-Pro-Lys-Pro-OH -0.01%, 1% or 5%, glycerin-20%, stearic acid-2%, butylparaben -1%, glyceryl stearate - 1%, stearyl alcohol -2%, decyl oleate -3%, water up to 100%. All the ingridients except water and peptide H-Trp-Trp-Pro-Lys-Pro-OH, are mixed and heated up to 70°C till homogeneous clear mass is obtained. Then the mixture is cooled up to 40°C, and water solution of peptide H-Trp-Trp-Pro-Lys-Pro-OH is added to the mixture at constant stirring. The cream is cooled down slowly to the room temperature.

Example 4. Investigation of skin-irritating action of the preparation. The skin-irritating action was tested in two ways. In an acute single treatment, and subacute repeated daily treatment within 2 weeks, experiments were conducted on five healthy volunteers (3 women (aged 24, 29 and 51) and 2 men (aged 44 and 46). The cream samples containing 0.01%, 1% or 5% tested peptide were administered daily within two weeks on the area of a forehead and a forearm. Skin-irritating activity was observed neither under single exposure nor under sequential administration of the cosmetics.

Example 5. Electrophysiological studies were carried out on muscle-type nicotinic acetylcholine receptors (nAChRs) heterolitically expressed in oocytes from clawed frog *Xenopus laevis*. Adult clawed frog (no less than 8 cm size) was placed into the solution of *p*-aminobenzoic acid for 15-20 minutes till total immobilization. For taking the necessary amount of oocytes, incision of skin and abdominal muscle layer of the frog was made laterally to «bikini line» and a portion of ovary was exteriorized. Exteriorized portion of ovary was settled into ND96 buffer without potassium ions and was minced. If necessary, oocytes were additionally treated with collagenase solution. The post-surgical cuts on the animal were sewed using suture material from polyglycolide. Each clawed frog was operated no more than once in 6 weeks.

Expression of the receptor was performed using the following procedure: solution of the mixure of the plasmids containing inserts with processed genes of α1, β1, δ and ε-subunits of the muscle-type nAChR under CMV-promotor control were injected into nucleus of freshly prepared oocytes using Nanotech («Drummond scientific», USA) device, after which oocytes were incubated at 18° C for 48 hours.

The nAChR is a ligand-gated ionic channel. After binding with the agonist (acetylcholine or nicotine), the receptor sustains conformational changes as a result of which positively charged ions go through the open ion channel. As soon as electrodes are attached to cell membrane expressing the receptor the possibility to record ionic current through the opening receptor channels appears.

Oocytes were placed into the tank with electrodes connected with the amplifier Turbo-TEC 03X (NPI Electronics, USA); the tank had preliminary been filled up with buffer ND96. The experiments were carried out using two-electrode fixation potential configuration. Amplifier electrodes made from borosilicate glass and filled with 3 M KCl, were injected into an oocyte. Membrane potential was fixed at -70 mV. Oocytes expressing muscle-type nAChR were treated with 20 μM acetylcholine several times for 5 min until the current amplitude reached the

stationary level in response to acetylcholine application. Then, oocyte were incubated for 5-minute in the solution of the peptide tested. After five-minute incubation with the peptide, an oocyte was treated with 20 μM acetylcholine solution in the presence of the peptide. The experiments were repeated using different concentrations of the peptides tested on several oocytes from different preparations. The amplitude of the current in response to application of acetylcholine in the presence of the peptide tested was compared with the amplitude of the current in response to the control application (in the absence of the peptide) carried out before each experiment. Dependence of current amplitude (represented as percentage of control) on decimal logarithm of ligand concentration was built to reveal characteristics of the peptide tested. Estimation of the inhibition curves and values of EC50 was performed using the program Origin 7.5 and the «dose response» model.

CLAIMS:

1. Compound of general formula (I): X1-X2-X3-Pro-X4-X5-X6, wherein

   X1 is selected from the group formed by H, Ac-, Palm-

   X2 is selected from the group formed by Trp, Tyr, His, Phe, Pro,

   X3 is selected from the group formed by Trp, Tyr, His, Phe, Lys, Orn, Dab, Dap, Arg

   X4 is selected from the group formed by Trp, Tyr, His, Phe, Lys, Orn, Dab, Dap, Arg

   X5 is selected from the group formed by Trp, Tyr, His, Phe, Pro

   X6 is selected from the group formed by –OH, $-NH_2$, $-OCH_3$, $-OC_2H_5$, $-NH-C_6H_5$
2. Compound according to claim 1, wherein X1 is Ac-
3. Compound according to claim 1, wherein X6 is $-NH_2$
4. Compound according to claim 1, wherein X2 and X3 are both -Trp- or -Tyr-, X4 is -Lys- or -Dab-, X5 is -Pro-
5. Compound according to claim 1, wherein it consist of stereoisomers or mixtures of stereoisomers of the amino acids, i.e. amino acids can have L- and/or D-configuration or exist as a racemic mixture independently in each position.
6. Compound according to claim 1, wherein it is represented as cosmetically acceptable salts.
7. Compound according to claim 1, wherein it has amino acid sequence SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48
8. Use of a compound according to any claims 1-7 as the main active component for neuromuscular transmission blockage.
9. Use of a compound according to any claims 1-7 for preparation of cosmetic composition for topical use acceptable for smoothing mimic and age-related wrinkles.

10. Cosmetic composition comprising a compound of general formula (I) within the weight concentrations from 0.01 to 5%.

11. Cosmetic composition according to claim 10, wherein it comprises at least one compound of general formula (I) and at least one additional component selected from the amino acids, proteins, protein hydrolysates, growth factors, enzymes, enzyme inhibitors, peptides, oligosaccharides, polysaccharides, pyrimidines, purines, nucleotides, nucleosides, carboxylic acids, fat acids, alcohols, lipids, sphingolipids, flavonoids, terpenes, alkaloids, benzofurans, polyalcohols, antimicrobial component, antimicrobial peptides, vitamins, provitamins, retinoids, carotenoids, chelating agents, antioxidants, agents improving skin permeability.

12. Cosmetic composition according to claim 10, wherein it comprises a dermatologically acceptable carrier.

13. Cosmetic composition according to claim 10, wherein it is presented in a form of solution, dispersion, emulsion or liposoms.

14. Cosmetic composition according to claim 10, wherein it is presented in a form of lotion, milk, balm, cream, gel, gel forming polymers, sprays, and face masks.

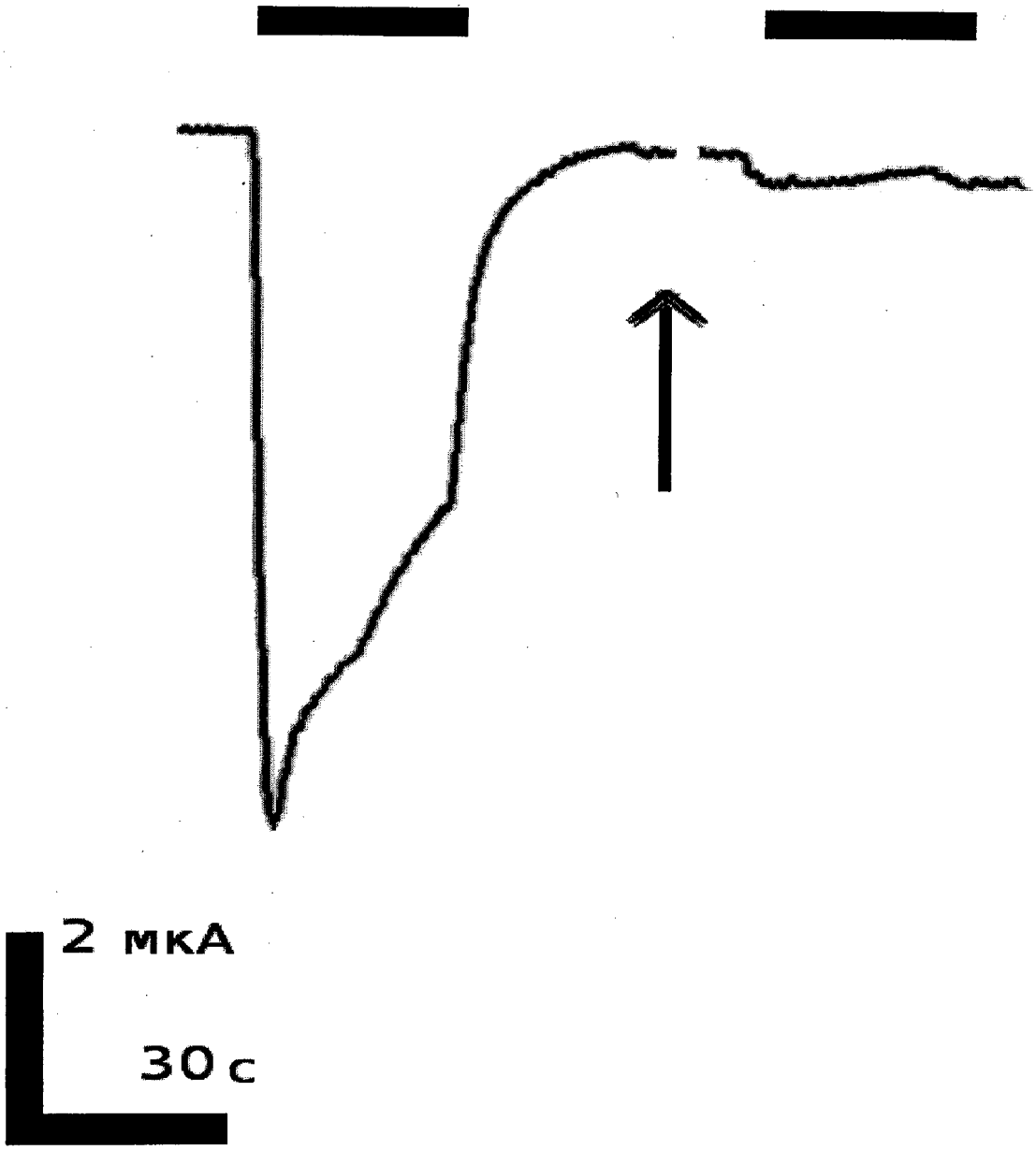


Fig.1

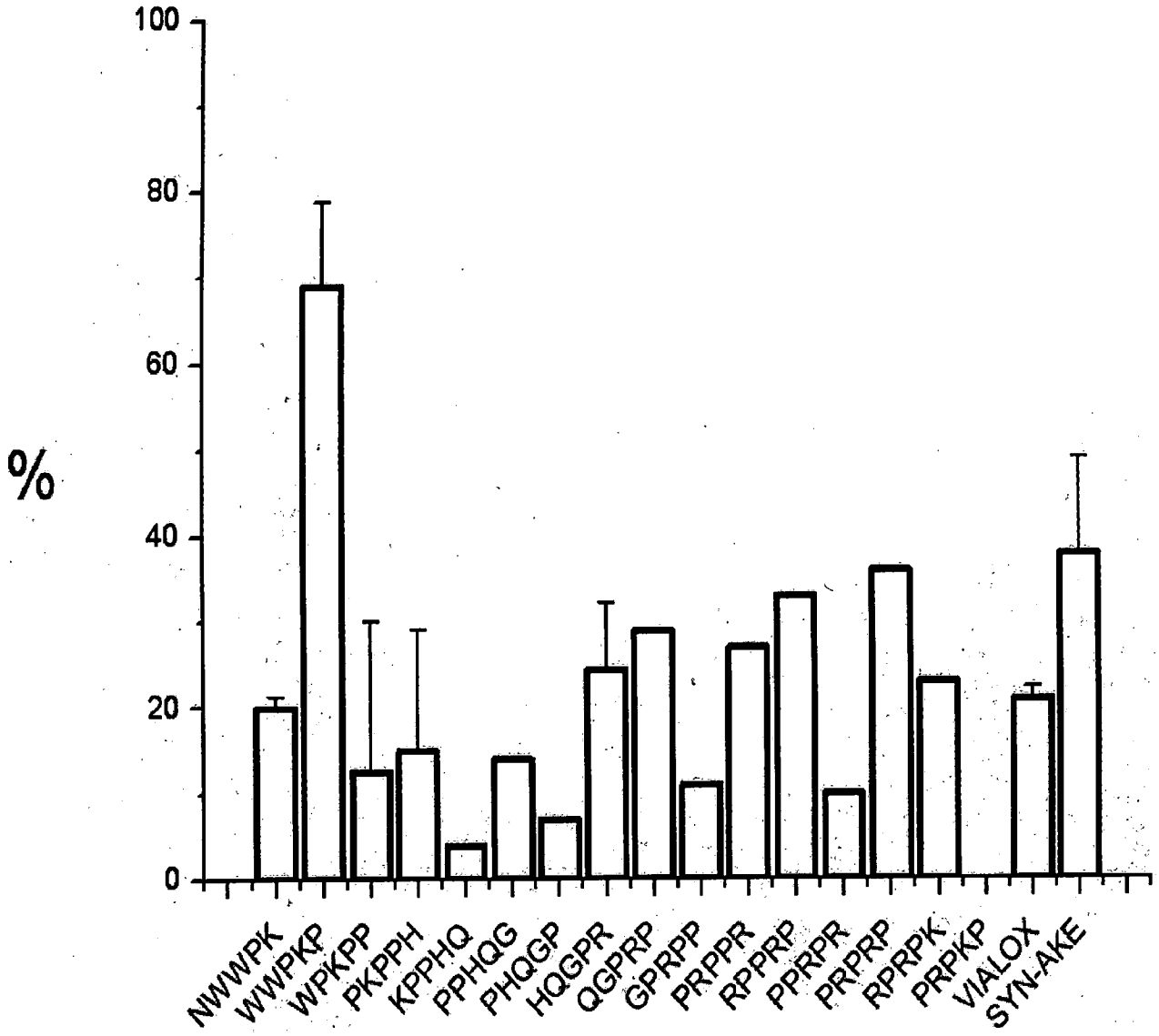
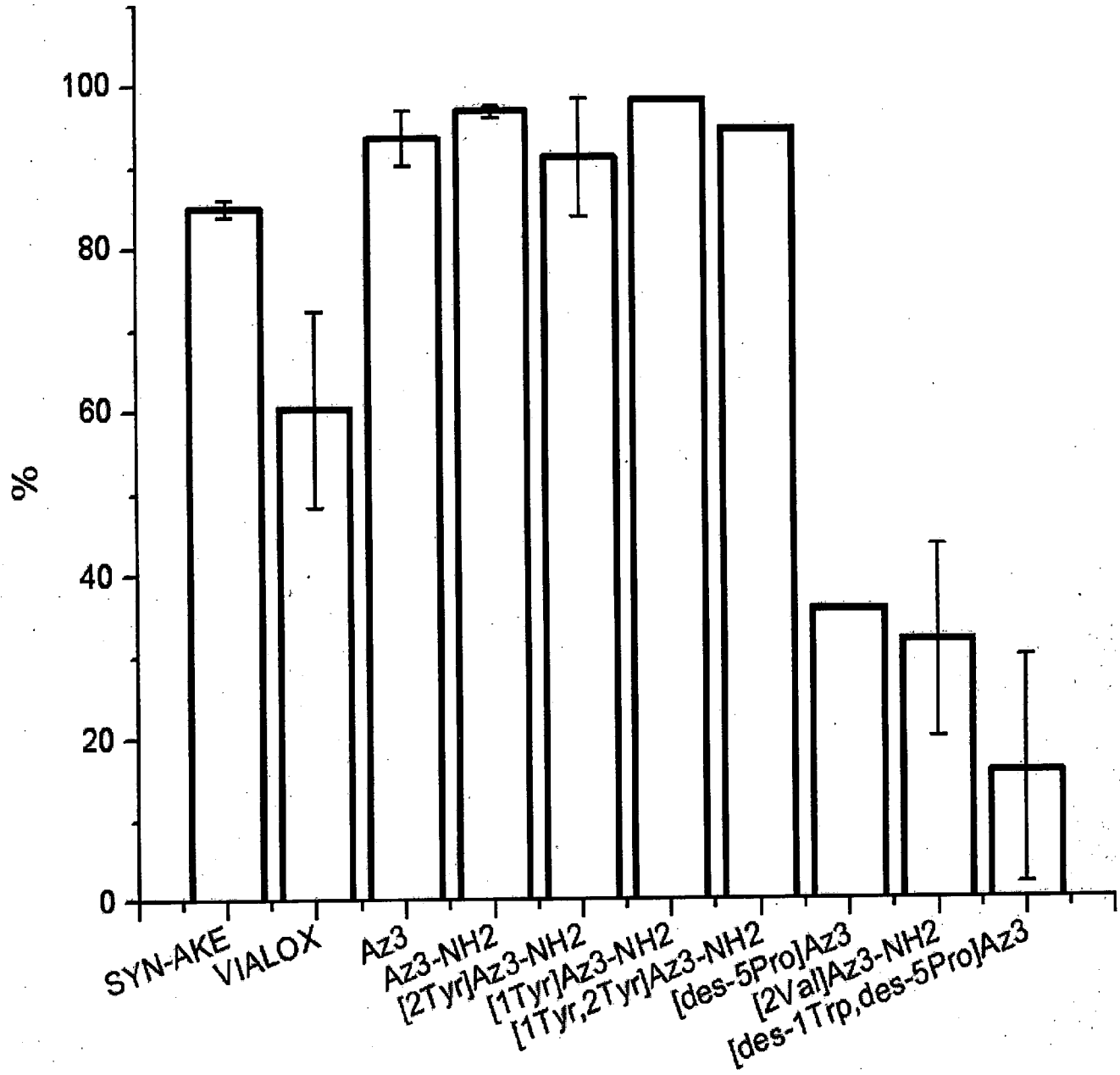


Fig.2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40
20
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%
SYN-AKE
VIALOX
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Az3-NH2
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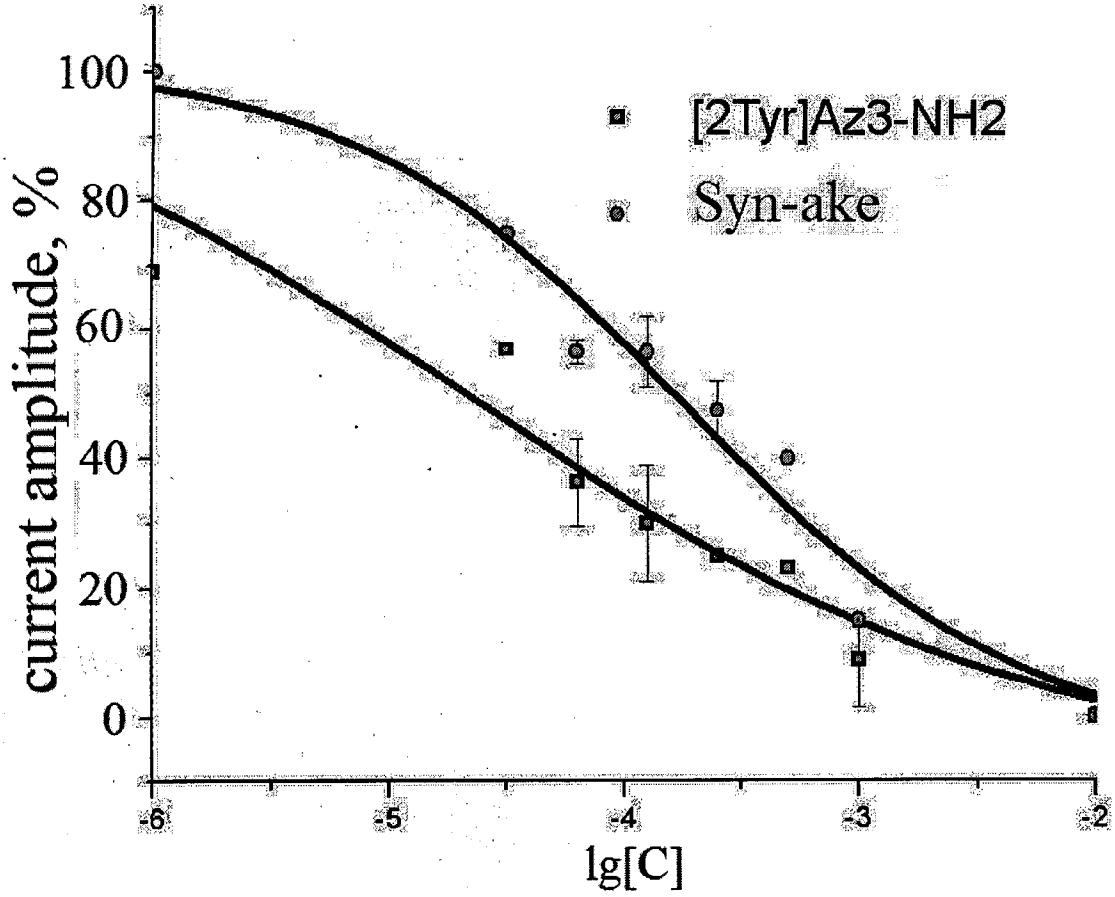
Fig.3


100
80
60
40
20
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current amplitude, %
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Syn-ake
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-5
-4
-3
-2
lg[C]

Fig.4

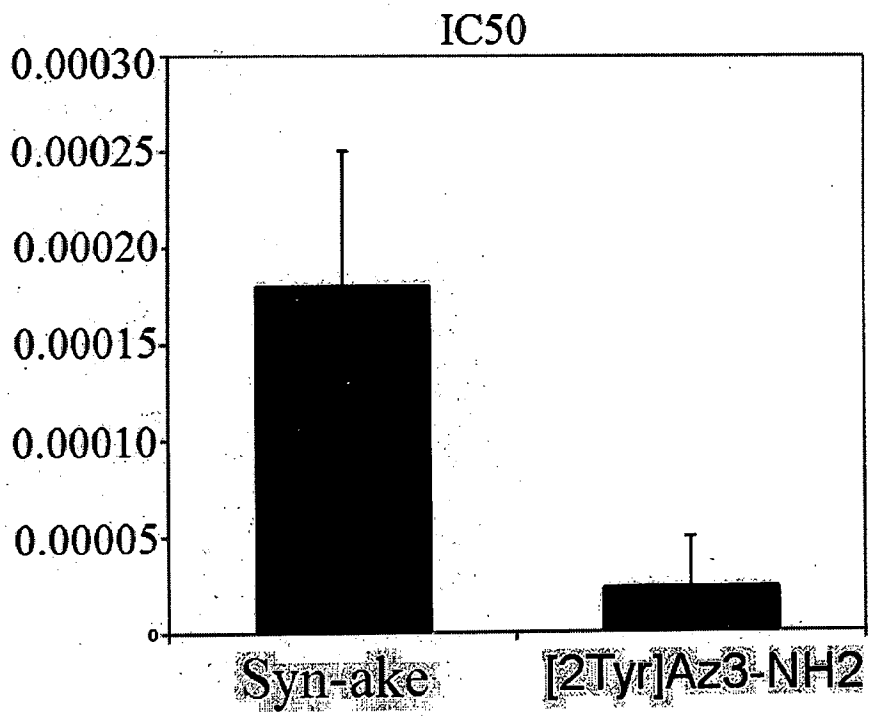

Fig.5

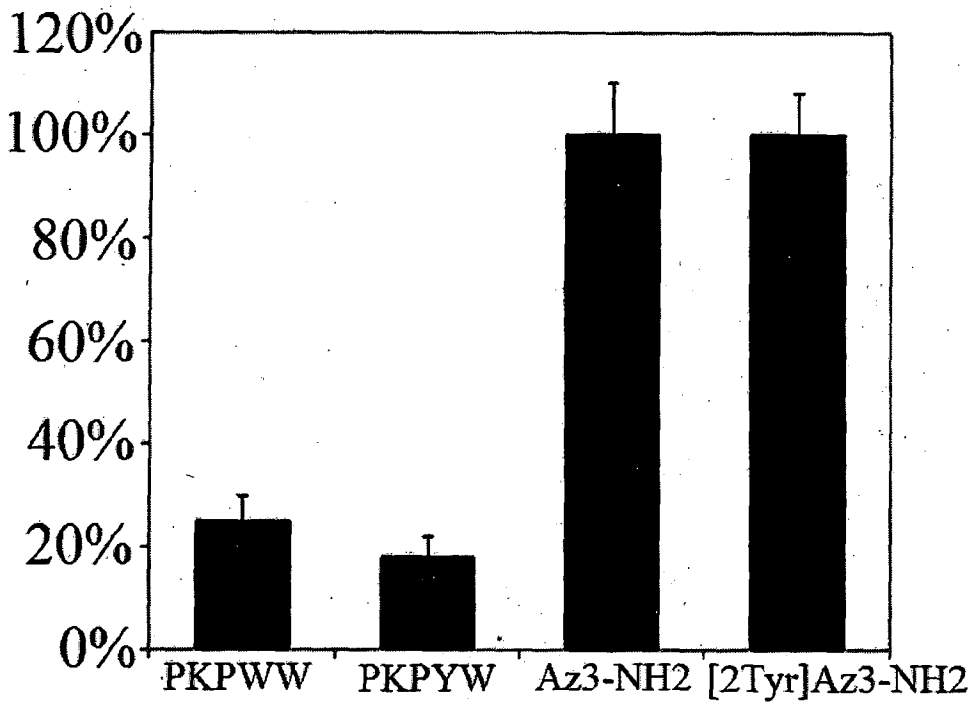

Fig.6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/RU 2014/000032

A. CLASSIFICATION OF SUBJECT MATTER

C07K 7/06 *(2006.01)*
A61K 38/08 *(2006.01)*
A61K 8/44 *(2006.01)*
A61K 8/02 *(2006.01)*
A61K 8/30 *(2006.01)*
A61Q 19/08 *(2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 7/06, A61K 38/08, 8/44 , 8/02, 8/30, A61Q 19/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PAJ, Esp@cent, PAJ, USPTO DB, WIPO, EAPATIS, PatSearch (RUPTO internal)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2009/0111731 A1 (PENTAPHARM AG) 30.04.2009 abstract, paragraph [0007], claims	1-14
A	Utkin Y.N. et al. Azemiopsin from Azemiops feae Viper Venom, a Novel Polypeptide Ligand of Nicotinic Acetylcholine Receptor, J. Biol. Chem., 2012, Vol.287,no.32, pp.27079-27086, abstract	1-14

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 May 2014 (21.05.2014)	10 June 2014 (10.06.2014)
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Authorized officer E.Tereshkina Telephone No. 495 531 65 15

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