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(54) Title: PENTAPEPTIDES CONTAINING 3-[2-(2-QUINOLINYL)BENZOXAZOL-5-YL]ALANINE, A PHARMACEUTICAL COMPOSITION AND THEIR APPLICATION

(57) Abstract: The present invention relates to novel compounds, a pharmaceutical composition and the application of the novel compounds for the manufacture of a therapeutic agent for the treatment of diseases caused by bacterial and/or fungal activity.



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5 **Pentapeptides containing 3-[2-(2-quinolinyl)benzoxazol-5-yl]alanine,
a pharmaceutical composition and their application**

10 The present invention relates to novel pentapeptides, a pharmaceutical composition and their application for the manufacture of a therapeutic agent intended for the treatment of diseases caused by bacterial and/or fungal activity.

15 The continued search for novel effective medicines is inextricably linked with civilisation development, which at the same time is one of the causes for the spread of diseases, particularly infectious diseases. For the past few decades an increasing number of antibiotics have been used to combat these diseases. Nowadays, however, using natural antibiotics (e.g. penicillins, tetracyclines) would often be a losing battle,
20 as the microorganisms that were once susceptible to them have developed mechanisms of resistance. Also, the widespread use of antibiotics until the mid-20th century exerted a selective pressure that gave an advantage to resistant strains leading to their expansion over time. This expansion has made treatment of diseases with antibiotics increasingly difficult, particularly in the cases of the so-called nosocomial
25 infections. There is therefore a continued need for novel antimicrobial substances. In addition to the synthesis of completely novel compounds, extensive research efforts are being made to search for novel peptide antibiotics. One of the frequently used modifications in this type of peptides to increase their biological activity involves incorporation of an unnatural α -amino acid resulting in antibiotic molecule being
30 composed of the proteinogenic and the non-proteinogenic portions. Benzoxazole and its derivatives are a good example of such non-proteinaceous fragment (Fig.), mainly because of the numerous literature reports on the wide range of biological activities displayed by compounds containing the benzoxazole moiety.

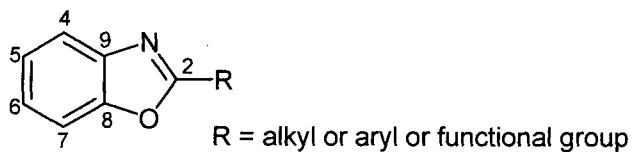


Fig. The structure of benzoxazole showing the numbering of ring atoms

The benzoxazole ring is found in the naturally occurring biologically active compounds, such as: calcimycin (an antibiotic produced by *Streptomyces chartreusensis*) and cesomycin (an antibiotic of the calcimycin class that has been isolated from *Frankia*, a genus within Actinomycetales); UK-1, UK-2 and UK-3 (respectively, a cytotoxic and two antifungal metabolites from *Streptomyces* sp. 517-02); and antituberculous and/or antifungal and bacteriocidal alkaloids isolated from the coral species *Pseudopterogorgia elisabethae* or from the sea sponges of the *Dysidea* genus.

The published literature contains multiple examples of reports on various applications of synthetic derivatives of benzoxazole in medicine, with the overall conclusion being that their efficacy is generally determined by the substituents present in the molecule of a given compound. A large group of benzoxazole derivatives show antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus subtilis*) and/or Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*), and/or antifungal activity (*Candida albicans*, *Candida krusei*, *Candida glabrata*, *Candida-6*, *Candida-51*, *Aspergillus niger*, *Aspergillus flavus*), which in some cases is higher than the activity of the control medicines. These compounds are therefore characterised by a relatively wide spectrum of activity that largely depends on the type of substituent in the benzoxazole ring and its location, with substitution at positions 2 and 5 being of particular significance (Fig.). In the case of *Pseudomonas aeruginosa*, a Gram-negative bacterium that is very often resistant to conventional antibiotics, 6-methyl-2-(2-chlorophenyl)benzoxazole (MIC = 25 µg/ml), 5-phenylacetamide-2-phenylbenzoxazole (MIC = 25 µg/ml), 2-chlorobenzamide-2-phenylbenzoxazole (MIC = 25 µg/ml) and 5-propyloxybenzamide-2-phenylbenzoxazole (MIC = 25 µg/ml) show higher activity than

5 the control medicines: tetracycline (MIC = 50 µg/ml) and streptomycin (MIC = 100 µg/ml).

Similarly, 1-[2-(benzoxazol-2-yl)ethoxy]-2,6-bis(p-chlorophenyl)-3-methylpiperidin-4-one (MIC = 6.25 µg/ml) shows higher activity against *Streptococcus faecalis* than do penicillin (MIC = 25 µg/ml) and streptomycin (MIC = 12.5 µg/ml).

10 A benzoxazole derivative that shows higher activity against the pathogenic fungi *Candida-51*, *Aspergillus niger* and *Aspergillus flavus* than does amphotericin B (MIC = 25 µg/ml for *Candida-51* and MIC = 50 µg/ml for *A. niger* and *A. flavus*) is 1-[2-(benzoxazol-2-yl)ethoxy]-2,6-bis(p-methoxyphenyl)-3-methylpiperidin-4-one (MIC = 12.5 µg/ml for *Candida-51* and MIC = 25 µg/ml for *A. niger* and *A. flavus*).

15 Furthermore, incorporation of benzoxazole derivatives in the structure of the glycopeptide antibiotic mannopeptimycin-β has been shown to increase the activity of most of the resulting compounds versus the activity of the parent compound. They have been showed to be active against multiple strains of *Staphylococcus aureus* (including methicillin-resistant strains), strains of *Enterococcus* (including vancomycin-resistant strains), and *Streptococcus* (including penicillin-resistant strains).

Tuberculosis is one of the most prevalent infectious diseases. It is caused by *Mycobacterium tuberculosis*. Many people are carriers of the latent form of the bacterium, which is a dangerous source of remission after completion of treatment. There is therefore an unmet need for novel classes of antituberculous drugs that

25 would be capable of providing complete cure without the need to undergo many months of antibiotic treatment. Benzoxazole derivatives are good candidates for such drugs, given the fact that the 2-substituted lipophilic 5,7-di-tert-butylbenzoxazole derivatives have been shown to be active against *M. tuberculosis* and several non-tuberculous opportunistic mycobacterial strains (*Mycobacterium kansasii*,

30 *Mycobacterium avium* 80/72 and 152/74), against which the first antituberculous drug isoniazid was inactive. In the case of antituberculous drugs, compound lipophilicity plays a very important role, given the very low permeability of the cell membrane and the very thick and compacted cell wall in these bacteria. In the case of the above benzoxazole derivatives, the presence of tert-butyl groups in the molecule increases its

35 lipophilicity, although it has been observed that their activity is more affected by their chemical structure than by their lipophilicity. 5,7-di-tert-butyl-2-(pyridin-4-

5 yl)benzoxazole and 5,7-di-tert-butyl-2-styrylbenzoxazole have been shown to display the highest activity against the test strains. It has also been observed that compounds that are active against non-tuberculous strains are also toxic to the human taste cell line HTC-8. Activity against *M. tuberculosis* CNCTC My 331/88, *Mycobacterium kansasii* CNCTC My 235/80 i 6509/96 and *Mycobacterium avium* CNCTC My 330/88 have also
10 been demonstrated for 2-benzylsulfanylbenzoxazole derivatives, with the activity increasing when the phenyl ring contains substituents, particularly two nitryl groups or one thioamide group. It has also been demonstrated that compounds with the substituent at position 4 of the phenyl ring display higher activity than their analogues with the substituent at position 3.

15 Despite the relatively large number of literature reports on the antimicrobial activity of benzoxazole derivatives, detailed information on the mechanism of their action is lacking. It has, however, been determined that the first phase of benzoxazole and 2-methylbenzoxazole metabolism in rabbits most likely involves degradation of the oxazole ring (the C-O bond) as a result of mild hydrolysis with subsequent
20 formation of o-formamidophenol and o-acetamidophenol, respectively.

The present invention comprises five novel pentapeptides, each containing the
25 non-proteinogenic amino acid 3-[2-(2-quinoliny)benzoxazol-5-yl]alanine in their sequence (Fig. 1). These compounds show antimicrobial activity at concentrations ranging from several to less than 100 micrograms per millilitre (Table 2). One of them, H-Box(2Q)-His-Ile-His-Met-NH₂, shows selective activity against a single yeast species, *Pichia pastoris*. The other four pentapeptides display antimicrobial activity against
30 Gram-positive bacteria (*Bacillus subtilis* 168), including pathogenic strains (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 25923), and against yeast (*Pichia pastoris*). Although the activity of the pentapeptides is comparable to the activity of the currently used commercial antibiotics, the different chemical structure of the pentapeptides
35 (particularly the presence of the non-proteinogenic fragment based on the benzoxazolylalanine skeleton) renders the currently isolated multidrug resistant bacterial strains susceptible to these compounds. These compounds have been shown

5 to display considerable activity against 6 multi-drug resistant strains of *S. aureus* – clinical isolates from patients (Fig. 5). It has also been shown that the multidrug resistant bacteria under investigation do not acquire resistance to the invented pentapeptides for about 150 generations with the exception of one pentapeptide, namely H-Box(2Q)-Leu-Arg-Trp-Phe-NH₂, and only against one strain (where a
10 reduction in activity of about 50% was observed). These results suggest that these compounds could potentially be used in continuous treatment without risking a rapid emergence of resistance to the antibiotic among the targeted bacteria. Furthermore, the pentapeptides (H-Box(2Q)-His-Leu-His-Ile-NH₂, H-Box(2Q)-His-Leu-His-Phe-NH₂, H-Box(2Q)-Leu-Arg-Trp-Phe-NH₂) do not show any significant cytotoxicity in the
15 healthy human embryonic kidney cell line Hek293 or the neoplastic mouse fibroblast cell line A9 within the concentration ranges at which they display antimicrobial activity, which suggests that it will be possible to safely use these compounds in living organisms.

20

The non-proteinogenic fragment used in the pentapeptides (the benzoxazole ring) is relatively common in various types of biologically active compounds and also in this case it is biologically active, as previously demonstrated in microbiological assays using 3-[2-(2-quinolinyl)benzoxazol-5-yl]alanine methyl ester. Combining a
25 benzoxazole derivative with a peptide chain increases the biological activity of the former, which consists in inhibiting the growth of both prokaryotic and eukaryotic microorganisms. The fact that the presence of an appropriate peptide sequence is responsible for increasing the activity of the compound has been demonstrated during investigation of dipeptides obtained by modification of the carboxyl or the amine
30 group of benzoxazolyalanine with specific proteinogenic amino acids (except cysteine). This has also been corroborated by further research, as a result of which libraries of pentapeptides (6859 compounds each) were obtained by methods of combinatorial chemistry. As a result of deconvolution of the libraries of pentapeptides the most active peptide sequences were identified, with the pentapeptides described
35 above being some of them. Effective concentrations ranging from several to less than

- 5 100 micrograms per millilitre allow us to suspect that it is possible to use these compounds as antibiotics.

The present invention relates to novel pentapeptides whose general formula is as follows:

H-Box(2Q) A₁A₂A₃A₄NH₂

where the individual substituents are:

H-Box(2Q) is 3-[2-(2-quinoliny)]benzoxazol-5-yl]alanine

- A₁A₂A₃A₄ is His-Leu-His-Ile (1)
15 A₁A₂A₃A₄ is His-Leu-His-Phe (2)
A₁A₂A₃A₄ is Leu-Arg-Trp-Phe (3)
A₁A₂A₃A₄ is His-Ile-His-Ala (4)
A₁A₂A₃A₄ is His-Ile-His-Met (5)

- 20 A pharmaceutical composition which contains a novel pentapeptide described above and at least one pharmaceutically acceptable carrier and/or diluent.

The composition has antibacterial and/or antifungal application.

- Application of the novel pentapeptides defined above for the manufacture of a therapeutic agent intended for the treatment of diseases caused by bacterial and/or
25 fungal activity.

Application of the novel pentapeptides defined above for the manufacture of a therapeutic agent which advantageously displays antibacterial activity against multidrug resistant strains, including *Staphylococcus aureus*.

- Application of the novel pentapeptides defined above for the manufacture of a
30 therapeutic agent advantageously used in continuous treatment.

The novel compounds to which the present invention relates have the following advantages:

- 35 • Relatively high antimicrobial activity (low MIC values — overlapping with the MIC values of the currently used antibiotics)

- 5 • Activity against pathogenic strains of Gram-positive bacteria (particularly *S. aureus*), including multidrug resistant strains — potential application of the compounds to combat the so-called nosocomial infections
- Slow emergence of resistance in pathogenic strains of *S. aureus* to the compounds — potential application in continuous treatment
- 10 • No cytotoxicity of the compounds, within concentration ranges consistent with MIC, in a healthy cell line
- Relatively simple method of obtaining the compounds

15

Description of the figures:

Fig. 1 depicts the structures of the pentapeptides containing 3-[2-(2-quinolinyl)benzoxazol-5-yl]alanine.

20 **Fig. 2** depicts a diagram of the synthetic pathway of N-(tert-butoxycarbonyl)-3-[2-(2-quinolinyl)benzoxazol-5-yl]alanine.

Fig. 3 depicts a simplified diagram of the synthetic pathway of the pentapeptides.

Fig. 4 depicts cell viability after incubation with specific compounds (Z2 = H-Box(2Q)-Leu-Arg-Trp-Phe-NH₂, Z10 = H-Box(2Q)-His-Leu-His-Ile-NH₂, Z11 = H-Box(2Q)-His-Leu-His-Phe-NH₂, 0 – D = consecutive dilutions of a given compound).

25 **Fig. 5** depicts examples of Petri dishes after incubation of multidrug resistant strains of *Staphylococcus aureus* (435 (A), 128/0 (B), 1322 (C), 1863 (D)) with solutions of the pentapeptides in 96% ethanol (Z2 = H-Box(2Q)-Leu-Arg-Trp-Phe-NH₂; Z10 = H-Box(2Q)-His-Leu-His-Ile-NH₂; Z11 = H-Box(2Q)-His-Leu-His-Phe-NH₂; CM = chloramphenicol (commercial antibiotic); EtOH = 96% ethanol (solvent)). Each dot represents the place where 1 µl of the solution of a specific compound of a specific concentration was applied (in decreasing order from right to left in individual rows— Z2 [6 mg/ml; 0.6 mg/ml; 0.06 mg/ml; 0.006 mg/ml], Z10 [8 mg/ml; 0.8 mg/ml; 0.08 mg/ml; 0.008 mg/ml], and for Z11 [5 mg/ml; 0.5 mg/ml; 0.05 mg/ml; 0.005 mg/ml]).

35 **Fig. 6** depicts Petri dishes after incubation of the multidrug resistant strain of *Staphylococcus aureus* 435 with solutions of the pentapeptides in DMSO (Z2 = H-Box(2Q)-Leu-Arg-Trp-Phe-NH₂; Z10 = H-Box(2Q)-His-Leu-His-Ile-NH₂; Z11 = H-Box(2Q)-

- 5 *His-Leu-His-Phe-NH₂*; 435(k) – control strain; 435 Z2, Z10 and Z11 – strains cultured in the presence of sublethal quantities of the pentapeptide Z2, Z10 and Z11, respectively). Each dot represents the place where 1 µl of the solution of a specific compound of a specific concentration was applied (in decreasing order from right to left in individual rows).
- 10 **Fig. 7** depicts Petri dishes after incubation of the multidrug resistant strain of *Staphylococcus aureus* 128 with solutions of the pentapeptides in DMSO (Z2 = H-Box(2Q)-Leu-Arg-Trp-Phe-NH₂; Z10 = H-Box(2Q)-His-Leu-His-Ile-NH₂; Z11 = H-Box(2Q)-His-Leu-His-Phe-NH₂; (k) – control strain; 128 Z10 and 632 Z10 – strains cultured in the presence of sublethal quantities of the pentapeptide Z10). Each dot represents the
- 15 place where 1 µl of the solution of a specific compound of a specific concentration was applied (in decreasing order from right to left in individual rows).

- 20 The present invention is illustrated by the following example of execution, which is not a limitation of the invention in any way

Example

- 25 a) Synthesis of a non-proteinogenic amino acid

The first step of the synthesis involved the obtaining of a non-proteinogenic amino acid (*N*-(*tert*-butoxycarbonyl)-3-[2-(2-quinolinyl)benzoxazol-5-yl]alanine) (Fig. 2) according to the procedure described in the literature which is based on the oxidative cyclisation of a Schiff base with lead tetraacetate in dimethylsulfoxide [Guzow *et al.*, *Tetrahedron* 58 (2002) 2201]. *N*-(*tert*-butoxycarbonyl)-3-nitrotyrosine (1 eq.) is used as the substrate and is dissolved in methanol and placed on a magnetic stirrer. A suspension of palladium on activated charcoal (10%, but 5% is also acceptable) in methanol is then added. While the solution continues to be stirred, hydrogen gas is

30 passed through the solution for about 3 hours. The reaction is carried out at room temperature and its progress is monitored by thin-layer chromatography (TLC; Merck Kieselgel 60 F₂₅₄ silica gel plates; visualisation of the spots using a UV lamp at 254 nm

5 and 366 nm) using methylene chloride–methanol–acetic acid (100:10:1, v/v/v) ($R_f = 0.91$ [substrate], $R_f = 0.36$ [product]) as the eluent system. After completion of the reaction the catalyst is filtered out using a hard fluted filter, and the filtrate is evaporated using a rotary evaporator, yielding *N*-(tert-butoxycarbonyl)-3-aminotyrosine in the form of a brown oil which is dissolved in anhydrous ethanol and
10 placed on a magnetic stirrer. While the solution is being stirred, a solution of quinoline-2-carboxaldehyde (1 eq.) in anhydrous ethanol is added, as a result of which, several minutes later, a fine yellow precipitate of Schiff base precipitates. The reaction is carried out for about 24 hours at room temperature and its progress is monitored by TLC (eluent: ethyl acetate, $R_f = 0.80$ [aldehyde], $R_f = 0.08$ [Schiff base]). The solvent is
15 then evaporated using a rotary evaporator, yielding a yellow-brown solid which is dissolved in DMSO. While the Schiff base solution is being stirred by a magnetic stirrer, lead tetraacetate (1.5 eq.) is added to the solution, as a result of which the solution changes its colour from yellow to dark red. The reaction is carried out at room temperature for about 1 hour and its progress is monitored by TLC (eluent: ethyl
20 acetate, $R_f = 0.17$ [product]). The reaction mixture is then transferred to the separator, ethyl acetate and saturated aqueous sodium chloride solution are added, and the layers are separated. The organic layer is washed three times with saturated aqueous sodium chloride solution and then it is dried with anhydrous magnesium sulfate. The drying agent is then filtered out using a fluted filter of intermediate hardness, and the
25 filtrate is evaporated using a rotary evaporator, yielding a dark brown oil, which is purified by column chromatography, using silica gel (Kieselgel 60, 0.040-0.063 mm, Merck) as the stationary phase and ethyl acetate–acetic acid (100:1, v/v) as the mobile phase. The resulting product is crystallised from ethyl acetate–petroleum ether, as a result of which, at a yield of 30%, *N*-(tert-butoxycarbonyl)-3-[2-(2-quinolinyl)benzoxazol-5-yl]alanine (Boc-Box(2Q)-OH) is obtained in the form of an off-
30 white solid body. The purity of the compound is verified by TLC ($R_f = 0.51$ – using ethyl acetate–acetic acid [100:1, v/v]) and reversed-phase high-performance liquid chromatography (RP-HPLC), using the analytical column Phenomenex® Jupiter (4.6 x 250 mm, C18, 5 μ m) and a diode array detector (Shimadzu, with the detection
35 wavelengths set to 223 nm, 254 nm and 320 nm) with the mobile phase flow rate of 1 ml/min. The mobile phase is a linear gradient from 0 to 100% B over 60 minutes (A =

- 5 0.1% aqueous trifluoroacetic acid solution, B = 80% acetonitrile in phase A, $t_R = 42.5$ min). The compound is identified based on a mass spectrum (Bruker Biflex III (MALDI TOF) spectrometer, $m/z = 434.1 [M+H]^+$, $M=433$ g/mol).

b) Synthesis of the pentapeptides

10 The pentapeptides are produced through solid-phase peptide synthesis using the Fmoc/tBu protection scheme (Fig. 3). An appropriate weighed-out amount of a polymer (an amide resin, e.g. TentaGel S RAM [Rapp Polymere, Germany]) preloaded with the first Fmoc-protected amino acid (Fmoc-Ala, Fmoc-Ile, Fmoc-Met or Fmoc-Phe)

15 is transferred into a reaction vessel (a syringe equipped with a glass frit, a vessel for manual peptide synthesis or a column if an automated peptide synthesiser is used). An unloaded amide resin may also be used for the synthesis, in which case it needs to be loaded with Fmoc-Ala, Fmoc-Ile, Fmoc-Met or Fmoc-Phe, respectively, using an appropriate procedure and the resulting loading density must be determined. The

20 resin is then prepared by shaking for 15 minutes in methylene chloride (DCM), as a result of which it swells. The solvent is then removed from the resin by filtration. The next step involves Fmoc deprotection of the amino acid at position X_1 , followed by acylation of the amino acid with the next residue, which is carried out according to the following procedure:

25 • **Fmoc deprotection of position X_1**

1. Resin pre-wash:

methylene chloride (DCM)–N,N-dimethylformamide (DMF) (1:1, v/v) 1 x 2 min
N,N-dimethylformamide (DMF) 2 x 2 min

2. Fmoc deprotection:

20% piperidine in DMF 1 x 3 min
1 x 8 min

3. Resin wash:

DMF 3 x 2 min
DMF–isopropanol (iPrOH) (1:1, v/v) 1 x 2 min
isopropanol (iPrOH) 3 x 2 min
DCM–iPrOH (1:1, v/v) 1 x 2 min
DCM 3 x 2 min

5 **4. Chloranil test for the presence of free amino groups:**

	resin beads	several
	anhydrous acetaldehyde	50 μ l
	saturated solution of p-chloranil in toluene	100 μ l
10	reaction time	approx. 10 min

Result: green beads (positive)	-	free amino groups present
colourless beads (negative)	-	free amino groups absent

15 If the test is positive, the next step follows (acylation), and if the test is negative, the procedure is repeated.

• **Acylation of position X₁**

20 **1. Pre-wash:**

DCM-DMF (1:1, v/v) 1 x 2 min

25 **2. Acylation: the active ester method**

Fmoc-amino acid-HOBt-TBTU-DIPEA at a molar ratio of 1:1:1:2 is dissolved in DCM-DMF (1:1, v/v), added to the resin and shaken for about 1.5 hours at room temperature (HOBt – N-hydroxybenzotriazole; TBTU – O-(1H-benzotriazol-1a-yl)-N,N,N',N'- tetramethyluronium tetrafluoroborate; DIPEA – N,N'-diisopropylethylamine).

A 3-fold excess of Fmoc-amino acid (Fmoc-His(Trt)-OH or Fmoc-Trp(Boc)-OH, respectively) relative to the resin loading density is used for the reaction.

35 **3. Resin wash:**

	DMF	3 x 2 min
	DMF-iPrOH (1:1, v/v)	1 x 2 min
40	iPrOH	3 x 2 min
	DCM-iPrOH (1:1, v/v)	1 x 2 min
	DCM	3 x 2 min

45 **4. Chloranil test for the presence of free amino groups:**

Positive — the procedure is repeated using a 2-fold excess of the Fmoc-amino acid and a different coupling method (e.g. the symmetrical anhydride method: Fmoc-amino acid-HOBt-N,N'-diisopropylcarbodiimide (DIPCI) 1:1:1 or the active ester method: Fmoc-

5 amino acid-HOBt-HBTU (O-(1H-benzotriazol-1a-yl)-N,N,N',N'-tetramethyluronium hexafluoroborate)-DIPEA 1:1:1:2).

Negative — proceed to the next step

10 5. Final resin wash

DCM-methanol (MeOH) (1:1, v/v) 1 x 2 min

methanol (MeOH) 3 x 2 min

15 6. Drying of the resin in a vacuum desiccator over P₂O₅ and KOH.

The next amino acid residues are added at positions X₃ (Fmoc-Ile-OH, Fmoc-Leu-OH or Fmoc-Arg(Pbf)-OH), X₄ (Fmoc-His(Trt)-OH or Fmoc-Leu-OH) and X₅ (Boc-Box(2Q)-OH) using the same method as for the acylation at X₁, except that when a
20 benzoxazolylalanine derivative is being added, a lower amount is used (i.e. equimolar amount relative to the resin loading density).

After all the amino acid residues have been added the final cleavage of the pentapeptide from the resin is performed. This is achieved by shaking the peptidyl-resin in trifluoroacetic acid-phenol-water-triisopropylsilane (88:5:5:2, v/v/v/v) for
25 about 1.5 hours. The polymer is then filtered off by washing it twice with a small amount of trifluoroacetic acid. The resulting filtrate is concentrated on a rotary evaporator and the residue is treated with cold diethyl ether to precipitate the peptide. The precipitated peptides are centrifuged or filtered off using a Schott funnel, dissolved in water (or in water with the addition of a small amount of acetic acid) and
30 freeze-dried.

The resulting compounds are purified using reversed-phase high-performance liquid chromatography (semi-preparative column from Supelco (Supelcosil™ SPLC-ABZ, 10x250 mm, C-18, 5 μm), UV detection (λ=330 nm), flow rate = 4 ml/min), and identified on the basis of mass spectra (Table 1).

35

- 5 Table 1. Conditions for the purification of the pentapeptides, their retention times, reaction efficacy, the obtained m/z values and the calculated molar masses of the compounds.

Pentapeptide sequence	Purification			Identification	
	Mobile phase	t _R [min]*	efficacy [%]	[M] ⁺ [m/z]	M [g/mol]
H-Box(2Q)-His-Leu-His-Ile-NH ₂	isocratic 30% B	29.3	43.2	833.3	833.0
H-Box(2Q)-His-Leu-His-Phe-NH ₂	isocratic 30% B	30.3	39.1	867.3	867.0
H-Box(2Q)-Leu-Arg-Trp-Phe-NH ₂	isocratic 35 % B	34.0	34.5	935.8	935.1
H-Box(2Q)-His-Ile-His-Ala-NH ₂	isocratic 20 % B	25.7	29.5	791.4	790.9
H-Box(2Q)-His-Ile-His-Met-NH ₂	isocratic 25 % B	27.8	29.2	851.4	851.0

* A linear gradient from 0 to 100 % B over 60 minutes (A = 0.1% aqueous trifluoroacetic acid solution, B = 80% acetonitrile in phase A)

c) Determination of MIC values of the pentapeptides

The minimum inhibitory concentration (MIC; Table 2) values are determined in a liquid culture assays in 96-well sterile plates, according to the double dilution method. In each series of the wells, increasing concentrations of the test compound are placed (50 µl of the solution of the compound in sterile water per well), to which 50 µl of bacterial culture is added (*Bacillus subtilis* 168 or *Enterococcus faecalis* ATCC 29212 or *Staphylococcus aureus* ATCC 29213 or *Staphylococcus aureus* ATCC 25923) or yeast culture (*Pichia pastoris*) at an optical density of about 0,01 and $\lambda = 570$ nm (approximately 10^5 cells per well). The testing is performed in sterile Mueller-Hinton Broth, which in the case of the yeast additionally contains 4% glucose. The resulting samples are incubated overnight at 25°C (yeast) or 37°C (bacteria), and the optical density of the culture is determined at $\lambda = 570$ nm. Two batches of the same concentrations (2 runs) are prepared for each compound, with the wells filled with the culture medium containing specific concentrations of the test compounds being the negative control and the wells filled with the culture medium inoculated with the bacterial or yeast culture being the positive control. The lowest concentration that

- 5 inhibits the growth of bacteria or yeast is the minimum inhibitory concentration (MIC) (Table 2).

Table 2. Minimal inhibitory concentration (MIC) values of the pentapeptides for selected strains of Gram-positive bacteria and for the yeast species *Pichia pastoris*.

Compound	MIC [$\mu\text{g/ml}$]				
	<i>B. subtilis</i> 168	<i>E. faecalis</i> ATCC 29212	<i>S. aureus</i> ATCC 29213	<i>S. aureus</i> ATCC 25923	<i>P. pastoris</i>
H-Box(2Q)-His-Leu-His-Ile-NH ₂	7.5	41.5	26.8	41.5	6.6
H-Box(2Q)-His-Leu-His-Phe-NH ₂	9.5	52.5	na	na	8.4
H-Box(2Q)-Leu-Arg-Trp-Phe-NH ₂	15.8	na	60	na	14
H-Box(2Q)-His-Ile-His-Ala-NH ₂	108	135	na	na	84
H-Box(2Q)-His-Ile-His-Met-NH ₂	na	-	-	-	22

na – no activity

d) Cytotoxicity testing

Cytotoxicity of the compounds is assessed with *in vitro* methods on healthy cell lines (human embryonic kidney cell line Hek293) and on neoplastic cell lines (mouse fibroblast cell line A9). The cells are cultured as monolayers in DMEM at a high glucose concentration with the addition of 1% solution of antibiotics (penicillin + streptomycin), 1% glutamine, NaHCO₃ (3.7 g/l) and 10% fetal bovine serum (FBS) in an incubator at 37°C in an atmosphere of 5% CO₂. The culture medium is replaced every two days. Confluent colonies are passaged and a cell suspension of a density of 5×10⁴ cells/ml is prepared for testing. The suspension is then plated into 96-well ELISA plates and incubated for 24 hours. Serial dilutions of the test compounds in DMSO are then prepared to improve their solubility and added to the cell-containing plates (the addition of DMSO did not affect cell viability, as demonstrated experimentally). The cells with the appropriate doses of the test substances are incubated for about 44 hours. The cell viability and proliferation is quantified using the WST-1 assay, which is a sensitive and accurate colorimetric method based on the ability of mitochondrial dehydrogenases to convert a yellow substrate, namely WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-phenyl sodium disulfonate) into orange-coloured formazan, whose quantity in the aqueous solution is directly proportionate to

5 the viable cell count. For this purpose 10 μ l of WST-1, diluted four times in a phosphate buffer (PBS), are added to each well in the plate and incubated in an incubator at 37°C in an atmosphere of 5% CO₂. Four hours later absorbance at a wavelength of 450 nm is read. Cell viability is expressed as a percentage viability of exposed cells relative to controls. It has been demonstrated that for concentrations
10 consistent with MIC the cell viability decrease relative to controls does not exceed 20% (Fig. 4).

e) Testing antibacterial activity against multidrug resistant strains

15 The activity of the compounds against 6 multidrug resistant strains of *S. aureus*, which originated from the collection of the Molecular Diagnostics Laboratory, Intercollegiate Faculty of Biotechnology of the University of Gdansk and the Medical University of Gdansk, and were clinical isolates from patients, was tested using the method of antimicrobial susceptibility testing on a solid medium. 1 μ l or 2 μ l of the solution of the
20 test compound in DMSO or in 96% ethanol are transferred onto a plate with Mueller Hinton broth solidified with agar onto which an appropriate bacterial strain was spread plated, and the plates are incubated at 37°C for 24 or 48 hours. The solvent alone, namely 96% ethanol or DMSO, respectively, is transferred as a control solution. A 5 mg/ml solution of chloramphenicol in 96% ethanol is used as positive control.
25 Sensitivity of the strain is determined from the size of the growth inhibition zone (the so-called halo) around the site of application of the test substance. All the strains that were investigated proved sensitive to each of the 3 investigated peptides (Fig. 5).

f) Testing of the resistance emergence rate

30

The rates at which the multidrug resistant bacteria (128/O, 435, 632, 659, 1863) acquire resistance to the test compounds are determined by growing the relevant bacterial strains in Mueller-Hinton Broth in the presence of sublethal quantities of these compounds (H-Box(2Q)-His-Leu-His-Ile-NH₂ – 10 μ g/ml, H-Box(2Q)-His-Leu-His-Phe-NH₂ and H-Box(2Q)-Leu-Arg-Trp-Phe-NH₂ – 30 μ g/ml). The bacteria are grown for
35 about 150 generations and the measurement of activity of each of the compounds against these strains and against the control strains, using the method of antimicrobial

5 susceptibility testing, is repeated. 1 µl of individual concentrations of the solutions of the test compounds in DMSO are transferred onto plates with Mueller Hinton broth solidified with agar onto which an appropriate bacterial strain treated with the compound or the control strain was spread plated. The plates are incubated overnight at 37°C, and the size of the growth inhibition zone at the compound application site is
10 determined. No significant decrease in activity, compared to the activity against the control strains, is observed for any of the compounds. There is one exception, namely strain 435, in the case of which it has been observed that the activity of the compound H-Box(2Q)-Leu-Arg-Trp-Phe-NH₂ decreases by about 50% compared to the control strain (Figs. 6 and 7).

15

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5

Patent claims

1. Novel pentapeptides whose general formula is as follows:

H-Box(2Q) A₁A₂A₃A₄NH₂

10

where the individual substituents are:

H-Box(2Q) is 3-[2-(2-quinolinyl)benzoxazol-5-yl]alanine

A₁A₂A₃A₄ is His-Leu-His-Ile (1)

15

A₁A₂A₃A₄ is His-Leu-His-Phe (2)

A₁A₂A₃A₄ is Leu-Arg-Trp-Phe (3)

A₁A₂A₃A₄ is His-Ile-His-Ala (4)

A₁A₂A₃A₄ is His-Ile-His-Met (5)

20

2. A pharmaceutical composition characterised in that it contains a novel pentapeptide defined in claim 1 and at least one pharmaceutically acceptable carrier and/or diluent.

3. The composition according to claim 1 characterised in that it has antibacterial and/or antifungal application.

25

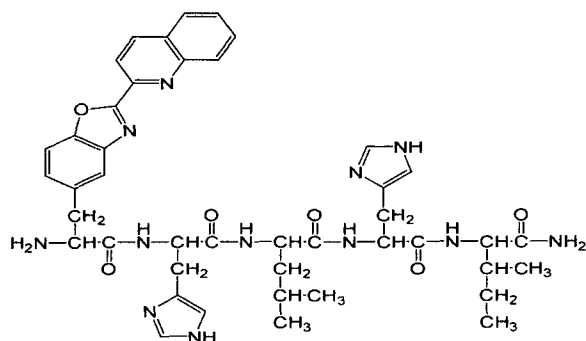
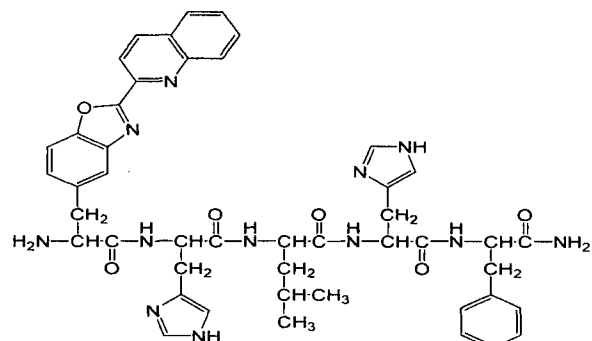
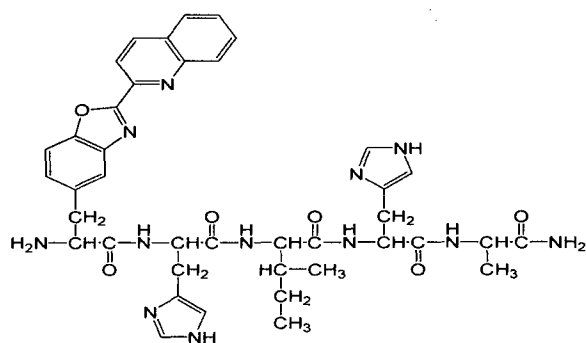
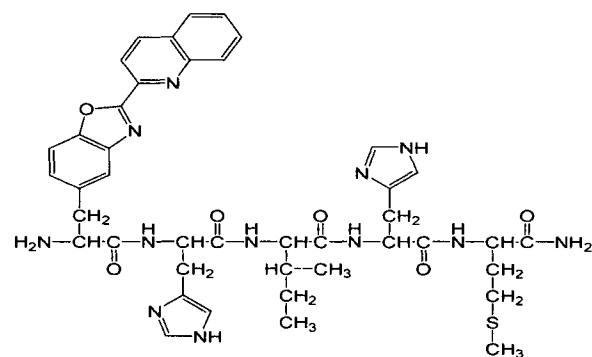
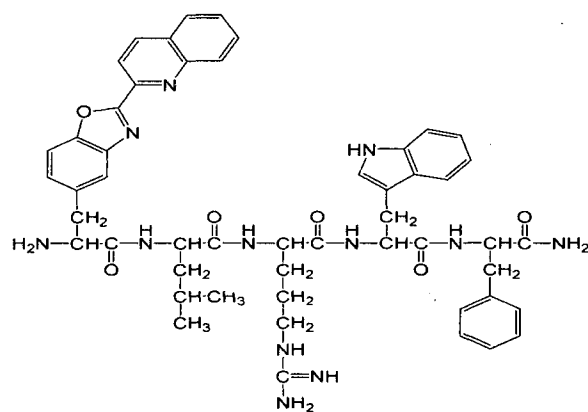
4. Application of the novel pentapeptides defined in claim 1 for the manufacture of a therapeutic agent intended for the treatment of diseases caused by bacterial and/or fungal activity.

5. The application according to claim 4 characterised in that the manufactured therapeutic agent advantageously displays antibacterial activity against multidrug resistant strains, including *Staphylococcus aureus*.

30

6. The application according to claims 4 and 5 characterised in that the manufactured therapeutic agent is advantageously used in continuous treatment.

35

**H-Box(2Q)-His-Leu-His-Ile-NH₂****H-Box(2Q)-His-Leu-His-Phe-NH₂****H-Box(2Q)-His-Ile-His-Ala-NH₂****H-Box(2Q)-His-Ile-His-Met-NH₂****H-Box(2Q)-Leu-Arg-Trp-Phe-NH₂**

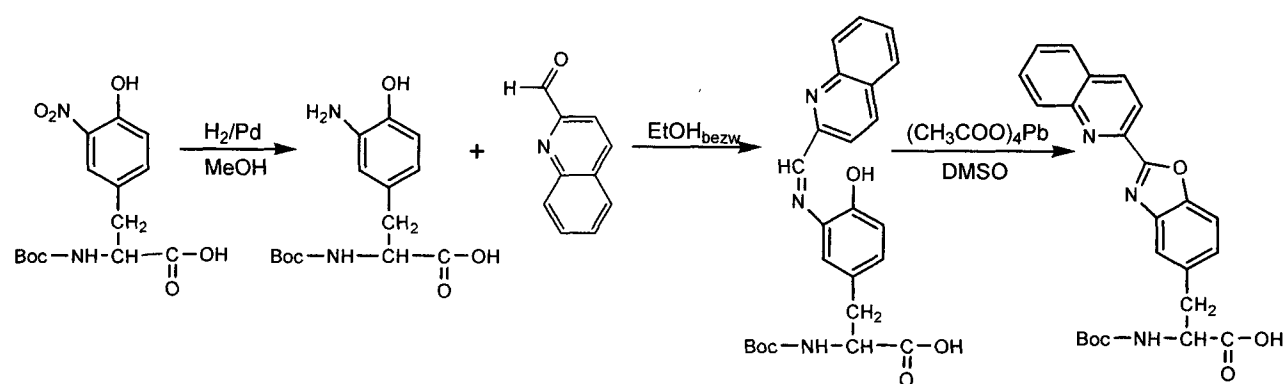
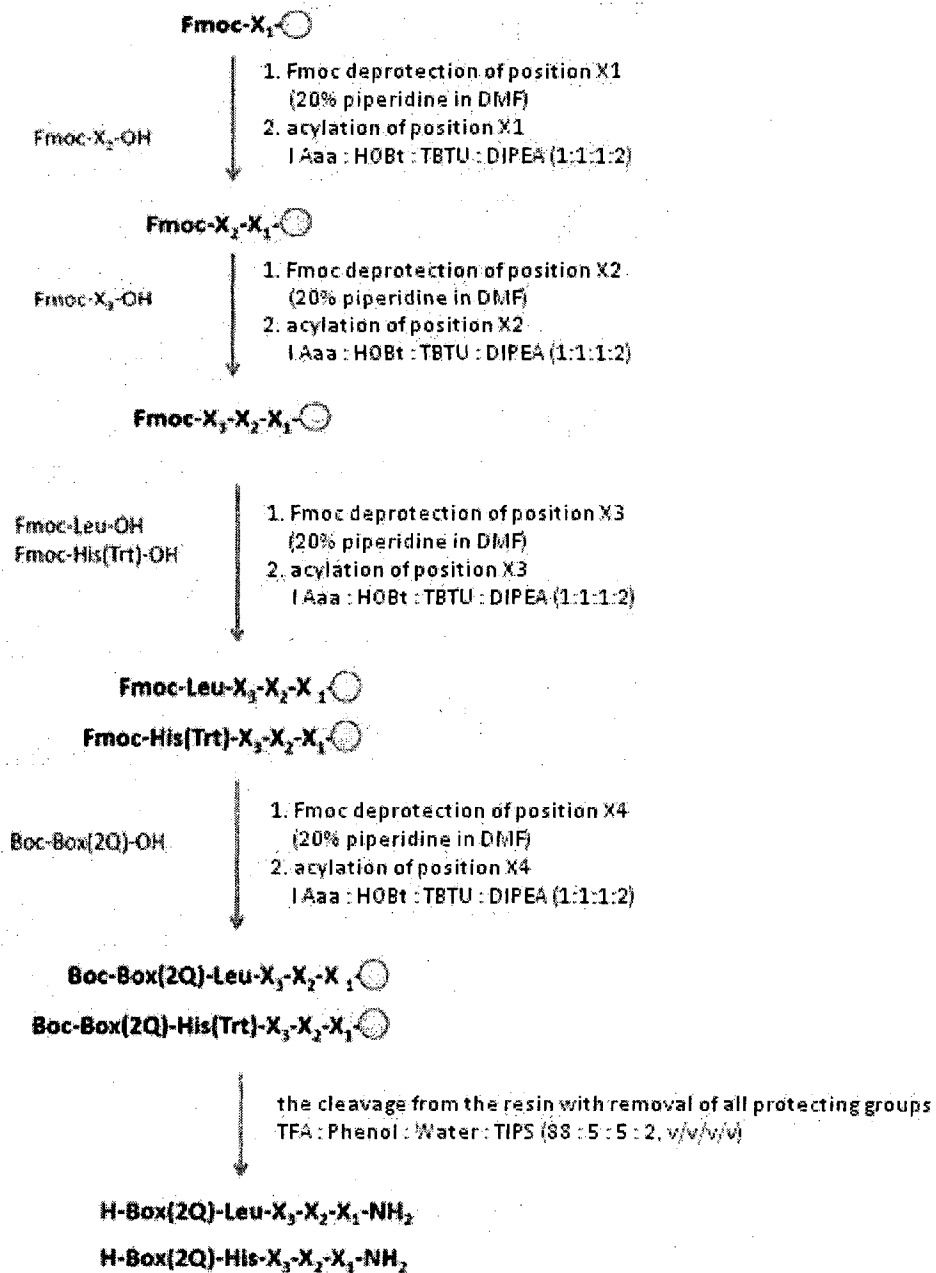
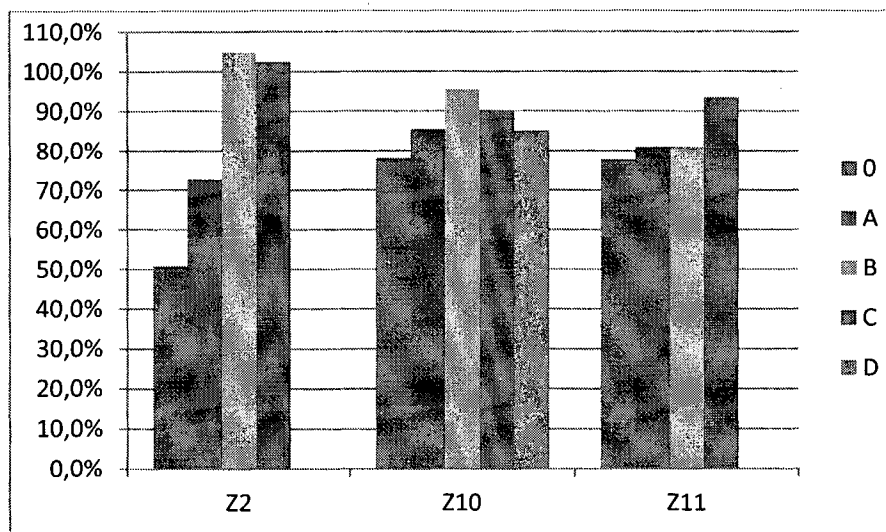
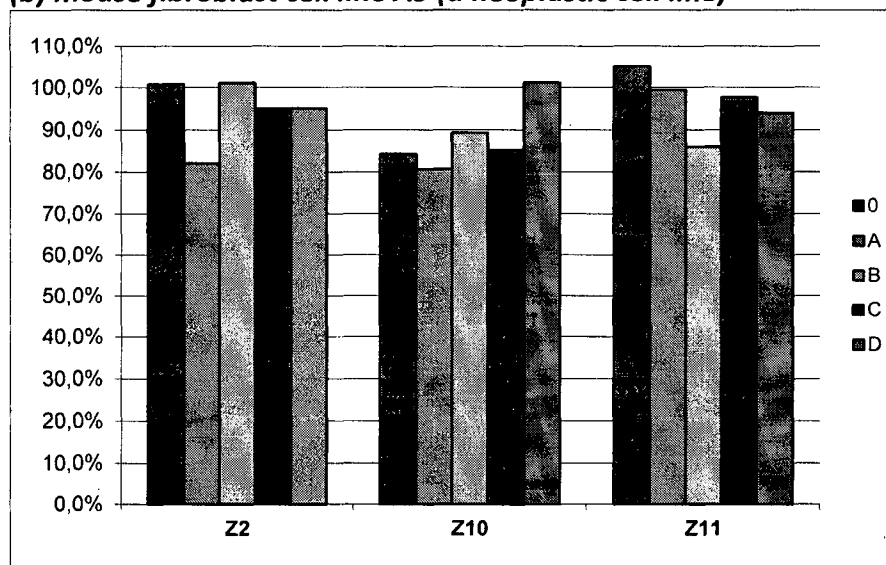


Fig. 2



X₃ = Arg, Ile or His; X₂ = His or Trp; X₁ = Ala, Ile, Met or Phe

Fig. 3

(a) human embryonic kidney cell line Hek293 (a healthy cell line)**(b) mouse fibroblast cell line A9 (a neoplastic cell line)****Fig. 4**

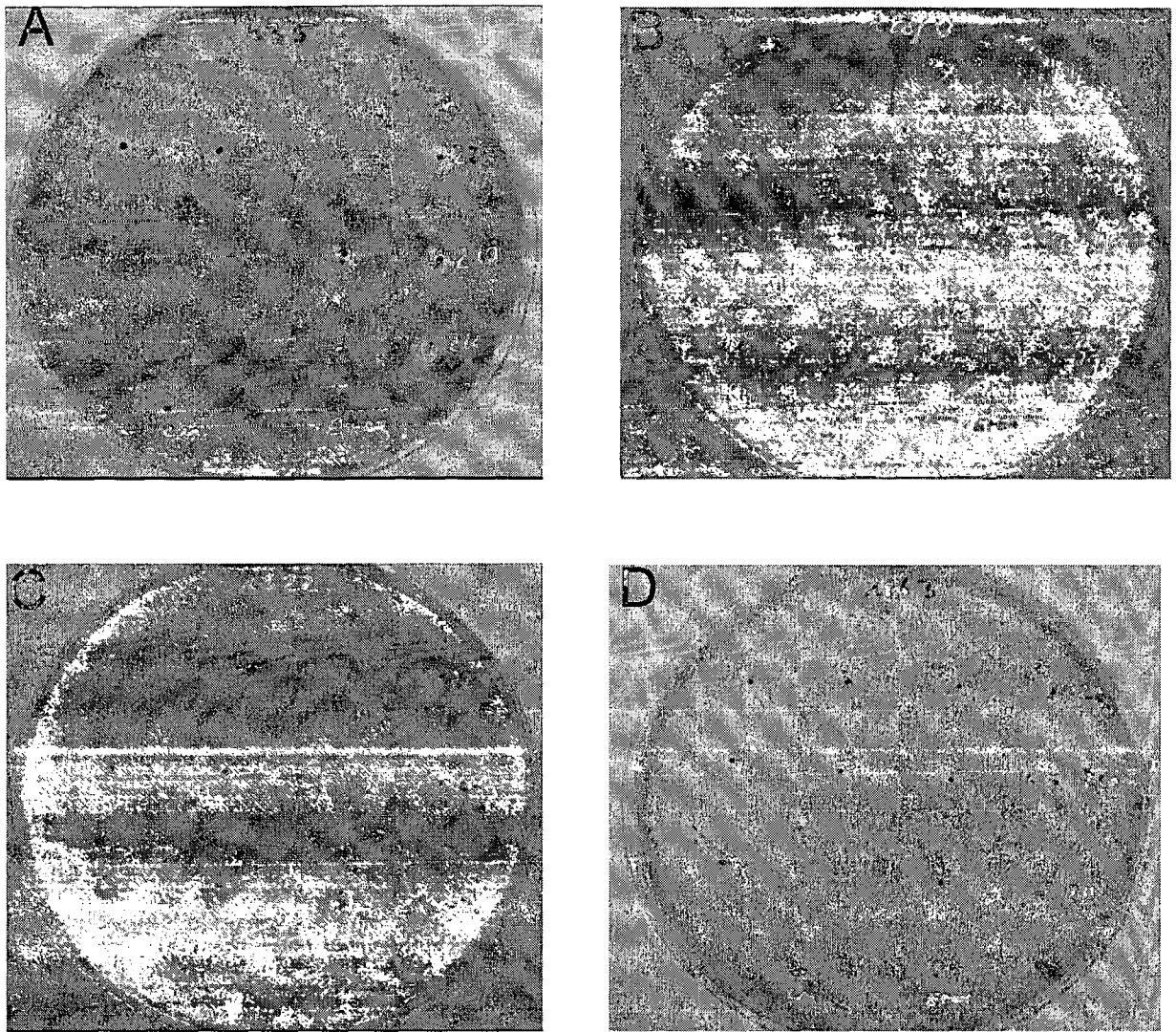


Fig. 5

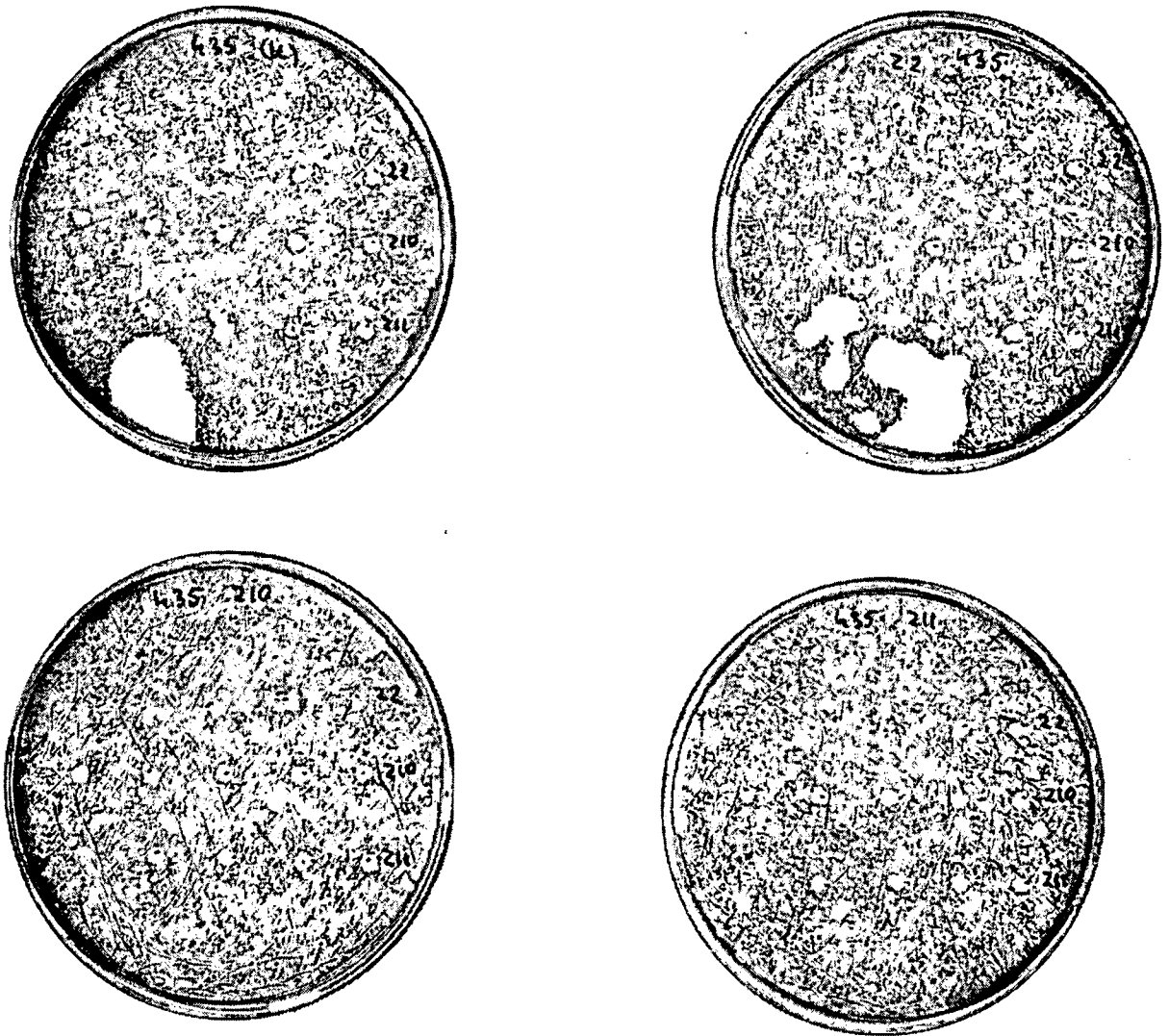


Fig. 6

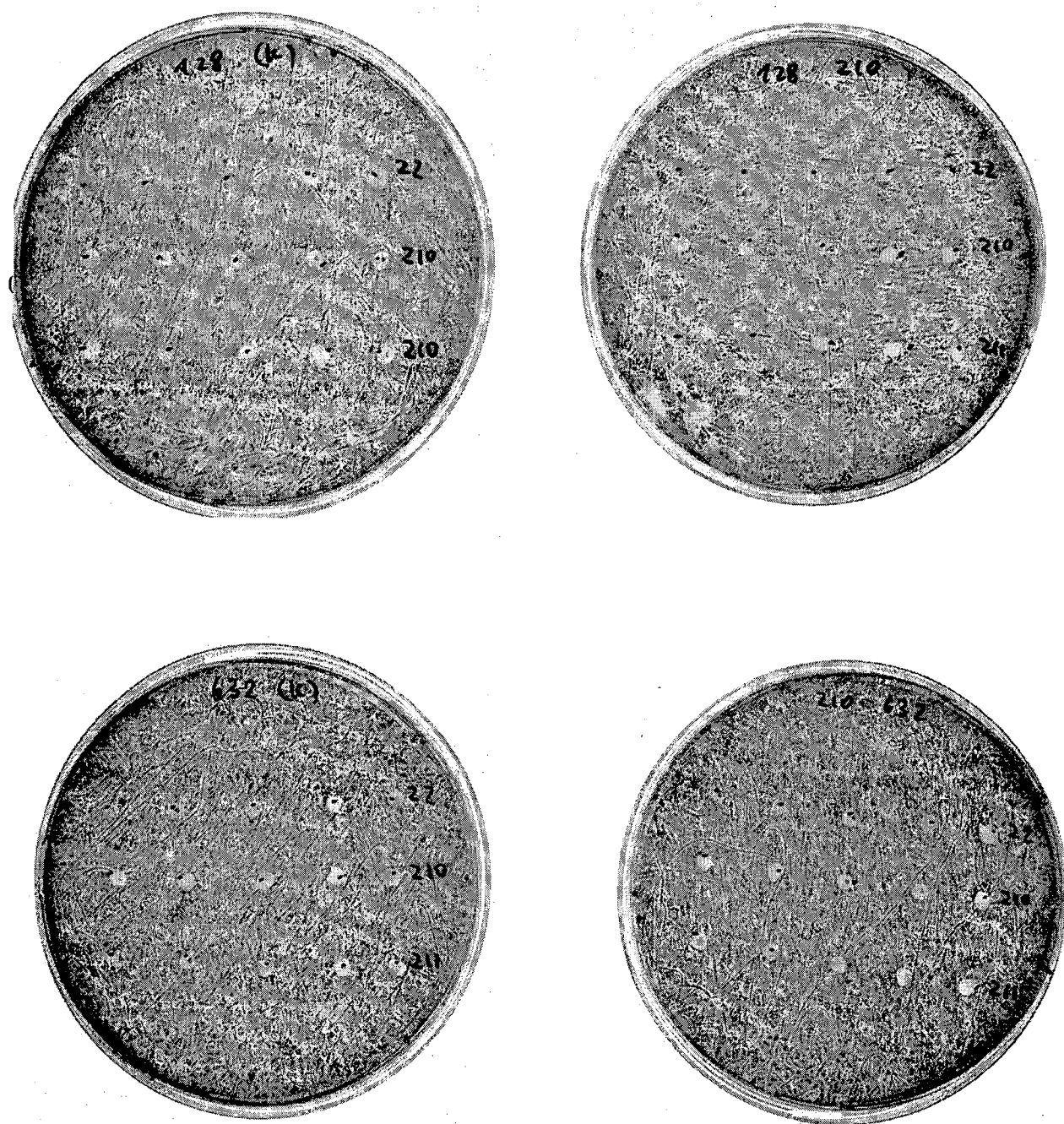


Fig. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2015/000011A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K7/06
ADD.

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

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)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GUZOW K; BEJNAROWIICZ J; SIERADZAN A; OBUCHOWSKI M; WICZK W: "P20/III: Modification of the position 5 of 3-(2-benzoxazol-5-yl)alanine derivatives and its influence on their antimicrobial activity", 20TH POLISH PEPTIDE SYMPOSIUM; WTADYSTAWOWO, POLAND; SEPTEMBER 6-10 2009,, 6 October 2009 (2009-10-06), page 82, XP002741969, the whole document ----- -/--	1-6



Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

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Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No

PCT/PL2015/000011

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BEJNAROWICZ J B; GUZOW K; OBUCHOWSKI M; WICZK W: "Antimicrobial activity of dipeptides with 3-[2-(8-quinoliny)benzoxazol-5-yl]alanine ", JOURNAL OF PEPTIDE SCIENCE; 31ST EUROPEAN PEPTIDE SYMPOSIUM (EPS), vol. 16, no. Suppl. 1, 5 September 2010 (2010-09-05), pages 146-146, XP002741970, the whole document	1-6
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A	----- US 2013/338341 A1 (SEKARAN SHAMALA DEVI [MY] ET AL) 19 December 2013 (2013-12-19) Abstract; peptide SEQ. ID. No.:5	1-6
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/PL2015/000011

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2013338341 A1	19-12-2013	MY 151307 A US 2013338341 A1	05-05-2014 19-12-2013
