

Novel diagnostic marker for prostate cancer

The present invention relates to chemical compounds for use in the diagnosis of prostate cancer. In particular, the present invention relates to chromogenic peptides suitable for the
5 detection of proteolytic enzymes within a sample.

Background of the invention

Prostate cancer is the second leading cause of male cancer deaths in the western population, following lung cancer. Although the disease is typically diagnosed in men over the
10 age of 65, its impact is still significant in that the average life span of a man who dies from prostate cancer is reduced by 9-10 years. If discovered, early prostate cancer can now be cured with surgery in approximately 90% of cases. However, the disease is slowly fatal once the tumor spreads outside the area of the gland and forms distant metastases. Early detection and accurate staging are therefore of great importance for the accurate choice of therapy. There is
15 an unmet need for reliable, fast and uncomplicated diagnostic methods for the detection of prostate cancer. Earlier diagnosis would enhance the chances for surgical treatment, which could significantly extend the patient's survival.

The process of initiation, growth and dissemination of cancer cells involves many factors including a number of proteolytic enzymes. This group of proteins is able to hydrolyze
20 proteins and peptides into smaller fragments. Proteolytic enzymes mediate degradation of the extracellular matrix, thus allowing cancer cells to colonize new tissues, and enable the formation of new blood vessels (angiogenesis), which promotes efficient delivery of nutrients to the tumour. In addition, proteolytic enzymes are present as a result of the death of healthy cells due to the tumour growth process. All these processes form a profile of proteolytic
25 enzymes that is characteristic for a tumour.

Chromogenic peptide molecules that break down under the influence of proteolytic enzymes, causing a change or increase in the colour of the solution being tested, have been described previously. This chromogenic effect is a consequence of the release of a chromophore (e.g. 4-nitroanilide or 5-amino-2-nitrobenzoic acid).

30 This type of peptide derivative is known from the following publications:

1. Erlanger BF, Kokowsky N, Cohen W. The preparation and properties of two new chromogenic substrates of trypsin. Arch Biochem Biophys. 1961 Nov;95:271-8.

2. Hojo K, Maeda M, Iguchi S, Smith T, Okamoto H, Kawasaki K. Amino acids and peptides. XXXV. Facile preparation of p-nitroanilide analogs by the solid-phase method. Chem Pharm Bull (Tokyo). 2000 Nov;48(11):1740-4.

Preferred compounds according to the invention were developed in such a way that a
5 signal, e.g. an increase in colour in the range of 380-440 nm, can be detected, if the compound is contacted with a urine sample of a person with prostate cancer. This effect does not occur if the compound is contacted with a urine sample of a healthy person or a patient diagnosed with another type of cancer.

The present inventors have discovered that such a signal, e.g. from a released
10 chromogenic compound or a fluorescent signal after separation of a fluorescent donor/acceptor pair can be used to detect the presence of proteolytic enzymes in the urine of a subject having prostate cancer. Enzymatic hydrolysis of the compounds leads to the generation of a detectable difference, e.g. the release of free chromophore molecules (ANB-NH₂ – amide of 5-amino-2-nitrobenzoic acid or pNA – para-nitroaniline, respectively), which show absorbance at 320-480
15 nm.

The compounds of the present invention provide *inter alia*: (i) a fast and non-invasive diagnostic method for the detection of prostate cancer, (ii) a method that is suitable for the early detection of prostate cancer, (iii) a method that can be successfully used for screening for prostate cancer, (iv) a full diagnostic process at an early stage of cancer development to
20 introduce more effective treatment.

Summary of the invention

In a first aspect, the present invention relates to a compound characterized by formula
1:

25 X1-Trp-Glu-Gly-Asn-X2 (formula 1),

wherein cleavage of the compound into a fragment 1 comprising X1 and a fragment 2 comprising X2 generates a detectable signal. The compound comprises the tetrapeptide Trp-Glu-Gly-Asn according to SEQ ID NO: 1.

In a second aspect, the present invention relates to an *in vitro* method for detecting
30 protease activity in a subject's body fluid, comprising contacting the body fluid with the compound according to the first aspect of the invention and detecting a signal, wherein the body fluid may comprise a hydrolytic enzyme, in particular a protease, derived from prostate cancer cells.

In a third aspect, the present invention relates to a kit comprising the compound according to the first aspect of the invention and a measurement buffer.

In a fourth aspect, the present invention relates to use of the compound according to the first aspect of the invention, the method according to the second aspect of the invention or the kit according to the third aspect of the invention for the detection of prostate cancer, or for
5 monitoring a subject that is suspected of having prostate cancer, has an increased risk of developing prostate cancer, or has had prostate cancer.

In a fifth aspect, the present invention relates to the use of the compound according to the first aspect of the invention in a method of treating prostate cancer, the method comprising
10 the steps of (a) carrying out the method according to the second aspect of the invention, and (b) treating prostate cancer in a subject for which protease activity, in particular increased protease activity has been detected in step (a).

Detailed description of the invention

15 Before the present invention is described in detail below, it is to be understood that this invention is not limited to the particular methodology, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the appended claims. Unless defined otherwise, all
20 technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art. Preferably, the terms used herein are defined as described in "A multilingual glossary of biotechnological terms: (IUPAC Recommendations)", Leuenberger, H.G.W, Nagel, B. and Kölbl, H. eds. (1995), Helvetica Chimica Acta, CH-4010 Basel, Switzerland).

25 Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.), whether supra or infra, are hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

30 To practice the present invention, unless otherwise indicated, conventional methods of chemistry, biochemistry, and recombinant DNA techniques are employed which are explained in the literature in the field (cf., e.g., *Molecular Cloning: A Laboratory Manual*, 2nd Edition, J. Sambrook *et al.* eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989).

Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. As used in this
5 specification and the appended claims, the singular forms “a”, “an”, and “the” include plural referents, unless the content clearly dictates otherwise.

In the following, the elements of the present invention will be described. These elements are listed with specific embodiments, however, it should be understood that they may be combined in any manner and in any number to create additional embodiments. The variously
10 described examples and preferred embodiments should not be construed to limit the present invention to only the explicitly described embodiments. This description should be understood to support and encompass embodiments, which combine the explicitly described embodiments with any number of the disclosed and/or preferred elements. Furthermore, any permutations and combinations of all described elements in this application should be considered disclosed
15 by the description of the present application unless the context indicates otherwise.

The terms and abbreviations used in the patent description and claims are to be understood as follows:

In the context of the present invention, the expression “chromophore” is used to refer to a compound with “chromogenic properties”. The expression “chromogenic properties” refers
20 to the ability of a compound to form a coloured product.

In the context of the present invention, the expression “fluorophore” is used to refer to a compound with “fluorogenic properties”. The expression “fluorogenic properties” refers to the ability of a compound to form a fluorescence emitting product.

In the context of the present invention, NMP refers to *N*-methylpyrrolidone.

25 In the context of the present invention, DMF refers to dimethylformamide.

In the context of the present invention, DCM refers to methylene chloride.

In the context of the present invention, pNA refers to 4-nitroaniline, which may also be referred to as para-nitroaniline.

In the context of the present invention, ABZ refers to 2-aminobenzoic acid.

30 In the context of the present invention, ANB-NH₂ refers to amide of 5-amino-2-nitrobenzoic acid.

In the context of the present invention, AFC refers to 7-amido-4-trifluoromethylcoumarin.

In the context of the present invention, Boc refers to tert-butyloxycarbonyl group.

In the context of the present invention, Fmoc refers to 9-fluorenylmethoxycarbonyl group.

In the context of the present invention, TFA refers to trifluoroacetic acid.

In the context of the present invention, the term “prostate cancer” is used in the broadest
5 sense and refers to all cancers that start in the prostate. It includes the subtypes adenocarcinoma, sarcoma, small cell carcinoma, neuroendocrine tumor (other than small cell carcinoma), and transitional cell carcinomas. It also includes the following stages (as defined by the corresponding TNM classification(s) in brackets): stage I (T1, N0, M0, Gleason score 6 or less, PSA less than 10; or T2a, N0, M0, Gleason score 6 or less, PSA less than 10), stage IIA (T1,
10 N0, M0, Gleason score of 7, PSA less than 20; T1, N0, M0, Gleason score of 6 or less, PSA at least 10 but less than 20; or T2a or T2b, N0, M0, Gleason score of 7 or less, PSA less than 20), stage IIB (T2c, N0, M0, any Gleason score, any PSA; T1 or T2, N0, M0, any Gleason score, PSA of 20 or more; or T1 or T2, N0, M0, Gleason score of 8 or higher, any PSA), stage III (T3, N0, M0, any Gleason score, any PSA) and stage IV (T4, N0, M0, any Gleason score, any PSA;
15 any T, N1, M0, any Gleason score, any PSA; or any T, any N, M1, any Gleason score, any PSA).

Peptides according to the present invention preferably chromogenic or fluorogenic peptides can be obtained by carrying out peptide synthesis on a solid support as known in the art. The solid support can be in the form of a resin having a Fmoc group, which is removed
20 during the course of the reaction. The resin used to carry out this process should be properly prepared. The preparation of this resin comprises increasing its volume by repeated washing with hydrophobic solvents.

The Fmoc protecting group must be removed from the resin by washing with a 20% solvent solution.

25 Known processes of obtaining chromogenic peptides involve the attachment of individual components under appropriate time and stoichiometric conditions. This attachment process consists of subsequent stages, in which individual elements (amino acid derivatives) are attached, residues are washed off, and protecting groups are removed and washed again. This cycle is repeated for each amino acid residue. The resulting peptide is separated from the
30 resin by reaction under acidic conditions. Subsequently, the solution is separated from the resin by filtration, and then the peptide is precipitated from the obtained solution with a non-polar solvent. The peptide sediment is then centrifuged.

The known method of obtaining chromogenic peptides is based on the process carried out on a solid support and partly in a buffer. An amide resin is used as a solid support, and a mixture of hydrophobic solvents is used as a solution.

The compounds according to the invention were prepared using the method described
5 in Hojo *et al.*, Chem Pharm Bull (Tokyo), 2000. A detailed description of the synthesis can be found below in the examples section.

In a first aspect, the invention provides a compound characterized by formula 1: X1-Trp-Glu-Gly-Asn -X2 (formula 1), wherein cleavage of the compound into a fragment 1 comprising X1 and a fragment 2 comprising X2 generates a detectable signal.

10 In preferred embodiments, the sequence Trp-Glu-Gly-Asn is accessible for a hydrolytic enzyme, in particular a hydrolytic enzyme cleaving the compound into X1-Trp-Glu-Gly-Asn-OH (fragment 1) and NH₂-X2 (fragment 2).

The hydrolytic enzyme may be a protease or a combination of several proteases.

The detectable signal generated upon cleavage of the compound may be selected from
15 various suitable signals known to the skilled person. In preferred embodiments, the detectable signal is an optical signal.

In preferred embodiments, X1 comprises or consists of a component C1 and X2 comprises or consists of a component C2, and the detectable signal is generated upon spatial separation of C1 and C2, i.e. by hydrolytic cleaving of the peptide Trp-Glu-Gly-Asn.

20 In addition to C1, X1 may e.g. comprise one or more amino acids on either side of C1. In addition to C2, X2 may e.g. comprise one or more amino acids on either side of C2. Thus, before cleavage, C1 and C2 may be separated by an amino acid sequence of 4 to 20 amino acids or 5 to 10 amino acids comprising or consisting of Trp-Glu-Gly-Asn. In preferred embodiments, C1 and C2 are separated by no more than 10 amino acids.

25 In preferred embodiments, the detectable signal is a change of absorption or fluorescence. Said change may be an increase or a decrease. In preferred embodiments, the detectable signal is an increase in absorbance intensity at 300-500 nm, in particular 380-430 nm.

In preferred embodiments, one of C1 and C2, is a chromophore having an absorption
30 maximum 1 (AM1) at a wavelength 1, and the compound has an absorption maximum 2 (AM2) at a wavelength 2 that is different from wavelength 1. Thus, if absorption is measured at wavelength 2 before and after cleavage of the compound, an increase in absorption will be detected.

It is particularly preferred that upon cleavage, the chromophore is released in free form. Upon cleavage, fragment 1 consists of X1-Trp-Glu-Gly-Asn, while fragment 2 consists only of NH₂-X2. Thus, in preferred embodiments, the chromophore is C2 rather than C1. In instances where C2 is a chromophore, it is preferred that X2 consists of C2 or consists essentially of C2. 5 X1 on the other hand may comprise additional amino acids on either side of C1, so that before cleavage, C1 and C2 may be separated by an amino acid sequence longer than Trp-Glu-Gly-Asn.

In preferred embodiments, the chromophore is selected from para-nitroaniline (pNA), amide of 5-amino-2-nitrobenzoic acid (ANB-NH₂), 7-amido-4-trifluoromethylcoumarin (AFC) 10 and 3-nitro L-tyrosine (Tyr3-NO₂). In preferred embodiments, the chromophore is para-nitroaniline (pNA). In preferred embodiments, the chromophore is an amide of 5-amino-2-nitrobenzoic acid (ANB-NH₂).

In preferred embodiments, C1 and C2 are a pair of a fluorescence donor and a fluorescence acceptor. In instances where C1 and C2 are a fluorescence donor and acceptor pair 15 and the compound is used in a method that measures a change of fluorescence upon cleavage of the compound, it is preferred that C1 and C2 are separated by no more than 10 amino acids in order to guarantee efficient quenching of the fluorescence donor by the fluorescence acceptor. The skilled person is aware that the crucial parameter is the distance between fluorescence donor and fluorescence acceptor. Thus, in instances where the amino acid 20 sequence separating C1 and C2 is folded into a condensed or twisted secondary structure, resulting in a proximity of C1 and C2 that is closer than in the case of a linear linker, an even longer spacer between C1 and C2 may be allowed.

In preferred embodiments, the pair of C1 and C2 is selected from the group consisting of 2-aminobenzoic acid (ABZ)/pNA, ABZ/ANB-NH₂, ABZ/DNP, ABZ/EDDNP, 25 EDANS/DABCYL, TAM/DANSYL, ABZ/Tyr(3-NO₂), in particular the pair of C1 and C2 is selected from ABZ/pNA and ABZ/ANB-NH₂. It is preferable that C1 and C2 are characterized by a molecular weight of less than 500 g/mol, particularly less than 400 g/mol, more particularly less than 300 g/mol, even more particularly between 100 and 200 g/mol.

The pair of C1 and C2 may also be a protein fluorescence donor and acceptor pair 30 selected from the group consisting of BFP/GFP, BFP/CFP, BFP/YFP, BFP/DsRed, CFP/GFP, CFP/YFP, CFP/mVenus, CeFP/ YFP, CeFP/mVenus, CeFP/mCitrine, CFP/dsRed, CFP/mCherry, mTurquoise/mVenus, GFP/YFP, GFP/DsRed, GFP/RFP, Clover/mRuby, Cy3/C5, Alexa 488/Alexa 555, and FITC/TRITC. The skilled person is aware how to select

suitable protein fluorescence donor and acceptor pairs based on their emission and absorbance spectra.

In instances where C1 and C2 are a protein fluorescence donor and acceptor pair, it has to be guaranteed that the relatively large size of the proteins does not impede the cleavage of the compound, and that the sequence Trp-Glu-Gly-Asn is accessible for cleavage by a hydrolytic enzyme. This can be attained, e.g. by increasing the lengths of the amino acid sequence comprising the peptide according to SEQ ID NO:1.

In preferred embodiments, the compound is characterized by formula 2:

ABZ- Trp-Glu-Gly-Asn -ANB-NH₂ (formula 2).

In preferred embodiments, the compound is characterized by formula 3:

ABZ- Trp-Glu-Gly-Asn-pNA (formula 3).

Upon cleavage of the compounds characterized by formula 2 or 3, free chromophore molecules are released (ANB-NH₂ or pNA, respectively). Thus, an increase of absorbance intensity can be detected at 380-430 nm. In addition, cleavage results in spatial separation of a fluorescence donor (ABZ) from a fluorescence acceptor (ANB-NH₂ or pNA, respectively). Thus, fluorescence emitted from ABZ is no longer quenched and an increase of fluorescence intensity can be detected at 420 nm.

In a second aspect, the invention provides an *in vitro* method for detecting protease activity in a subject's body fluid comprising contacting the body fluid with the compound according to the first aspect of the invention and detecting a signal, wherein the body fluid may comprise a hydrolytic enzyme, in particular a protease, derived from prostate cancer cells.

In preferred embodiments, the presence of protease activity in the body fluid indicates the presence of prostate cancer and the absence of protease activity in the body fluid indicates the absence of prostate cancer.

In preferred embodiments, the invention provides a method for the diagnosis of prostate cancer.

In preferred embodiments, the body fluid is selected from blood or urine. In preferred embodiments, the body fluid is urine. Surprisingly, the inventors found that the compound according to the first aspect of the invention is capable of detecting hydrolytic enzyme activity in a urine sample. The hydrolytic enzyme activity is significantly increased in subjects diagnosed with prostate cancer compared to healthy subjects (Fig. 1, 2).

In preferred embodiments, the subject has an increased risk of developing prostate cancer, is suspected of having prostate cancer, has had prostate cancer, or has prostate cancer.

In preferred embodiments, the compound is provided at a concentration of 0.1-10 mg/ml, particularly 0.25-7.5 mg/ml, more particularly 0.5-5 mg/ml, more particularly 0.75-2 mg/ml, even more particularly about 1 mg/ml, in a measurement buffer having neutral or alkaline pH, preferably a pH between 6.8 and 8.5, more preferably physiological pH, and the
5 body fluid sample is added to the compound at a ratio of 1:2 to 1:10, particularly 1:3 to 1:8, more particularly 1:4 to 1:6, even more particularly about 1:5.

In the context of the present specification, the expression "neutral pH" refers to a pH of approximately 7.0. In the context of the present specification, the expression "physiological pH" refers to a pH of approximately 7.4.

10 In preferred embodiments, detecting the signal comprises measuring absorbance or fluorescence.

In preferred embodiments, detecting the signal comprises measuring absorbance intensity at 300-500 nm, in particular 380-430 nm, preferably for 40-60 min at 25-40°C, in particular at 36-38°C.

15 In preferred embodiments, an increase in absorbance indicates the presence of hydrolytic enzyme activity.

In a third aspect, the invention provides a kit comprising a compound according to the first aspect of the invention and a measurement buffer.

In a fourth aspect, the invention provides the use of the compound according to the first
20 aspect, the method according to the second aspect or the kit according to the third aspect for the detection of prostate cancer, or for monitoring a subject that has an increased risk of developing prostate cancer, is suspected of having prostate cancer or has had prostate cancer.

Depending on what the method of the second aspect is to be used for, the term "subject" may have different limitations. For example, if the method is to be used for detecting prostate cancer or screening subjects for prostate cancer, the subject is not known to have prostate
25 cancer, i.e. it may or may not have prostate cancer. In this example, the subject preferably has an increased risk of developing or is suspected to have prostate cancer, or has had prostate cancer (i.e. has been cured of detectable prostate cancer). "Increased risk" means that one or more risk factors for cancer generally or for the prostate cancer can be attributed to the subject,
30 preferably as defined by the American Cancer Society for cancer generally or for prostate cancer. Examples of risk factors for prostate cancer are: age of 50 or older (in particular 65 or older), male gender, ethnicity (in particular African American and Caribbean men of African ancestry), family history of prostate cancer (in particular in first degree relatives), having an inherited syndrome or predisposition (such as a mutation of the BRCA1 or BRCA2 gene, or

Lynch syndrome), diet (in particular rich in red meat or high-fat dairy products), obesity, tobacco consumption (in particular smoking), occupation (in particular fire fighters), inflammation of the prostate, sexually transmitted infections (in particular gonorrhoea or chlamydia), and vasectomy.

- 5 The prostate cancer may be of any subtype and stage as defined above, i.e. the presence or absence of any subtype and/or stage can be detected.

 In a preferred embodiment, the presence of a significant amount of protease activity, or of an amount larger than in a control, indicates the presence of prostate cancer, and the absence of a significant amount of protease activity, or of an amount equal to or smaller than in a control,
10 indicates the absence of prostate cancer.

 In a particular embodiment, the method of the second aspect further comprises confirming the detection of prostate cancer by using one or more further means for detecting prostate cancer. The further means may be a cancer marker (or "biomarker") or a conventional (non-marker) detection means. The cancer marker can for example be a DNA methylation
15 marker, a mutation marker (e.g. SNP), an antigen marker, a protein marker, a miRNA marker, a cancer specific metabolite, or an expression marker. The conventional means can for example be a biopsy (e.g. visual biopsy examination with or without staining methods for example for protein or expression markers), an imaging technique or a physical, e.g. tactile examination. In preferred embodiments, the further means for prostate cancer detection is selected from the
20 group consisting of PSA test, DRE test, PCA3 test, ultrasound (in particular transrectal ultrasound), biopsy, MRI, MRI fusion biopsy, and CT scan.

 The term "is indicative for" or "indicates" as used herein refers to an act of identifying or specifying the thing to be indicated. As will be understood by persons skilled in the art, such assessment normally may not be correct for 100% of the subjects, although it preferably is
25 correct. The term, however, requires that a correct indication can be made for a statistically significant part of the subjects. Whether a part is statistically significant can be determined easily by the person skilled in the art using several well-known statistical evaluation tools, for example, determination of confidence intervals, determination of p values, Student's t-test, Mann-Whitney test, etc. Details are provided in Dowdy and Wearden, Statistics for Research,
30 John Wiley & Sons, New York 1983. The preferred confidence intervals are at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%. The p values are preferably 0.05, 0.01, or 0.005.

 The phrase "method for detecting the presence or absence" as used herein with regard to prostate cancer refers to a determination whether the subject has the cancer or not. As will

be understood by persons skilled in the art, such assessment normally may not be correct for 100% of the subjects, although it preferably is correct. The term, however, requires that a correct indication can be made for a statistically significant part of the subjects. For a description of statistic significance and suitable confidence intervals and p values, see above.

5 The term "diagnosis" as used herein refers to a determination whether a subject does or does not have cancer. A diagnosis by analysis of protease activity as described herein may be supplemented with a further means as described herein to confirm the cancer detected with the analysis of protease activity. As will be understood by persons skilled in the art, the diagnosis normally may not be correct for 100% of the subjects, although it preferably is correct. The
10 term, however, requires that a correct diagnosis can be made for a statistically significant part of the subjects. For a description of statistic significance and suitable confidence intervals and p values, see above.

 The phrase "screening a population of subjects" as used herein with regard to the cancer of the specification refers to the use of the method of the first aspect with samples of a
15 population of subjects. Preferably, the subjects have an increased risk for, are suspected of having, or have had the cancer. In particular, one or more of the risk factors recited herein can be attributed to the subjects of the population. In a specific embodiment, the same one or more risk factors can be attributed to all subjects of the population. For example, the population may consist of subjects characterized by heavy alcohol use and/or tobacco consumption. It is to be
20 understood that the term "screening" refers to a diagnosis as described above for subjects of the population, and is preferably confirmed using a further means as described herein. As will be understood by persons skilled in the art, the screening result normally may not be correct for 100% of the subjects, although it preferably is correct. The term, however, requires that a correct screening result can be achieved for a statistically significant part of the subjects. For a
25 description of statistic significance and suitable confidence intervals and p values, see above.

 The term "monitoring" as used herein refers to the accompaniment of a diagnosed cancer during a treatment procedure or during a certain period of time, typically during at least 1 week, 2 weeks, 3 weeks, 4 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 1
year, 2 years, 3 years, 5 years, 10 years, or any other period of time. The term "accompaniment"
30 means that states of and, in particular, changes of these states of a cancer may be detected based on the amount of protease activity, particular based on changes in the amount in any type of periodical time segment, determined e.g., daily or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 times per month (no more than one determination per day) over the course of the treatment, which may be up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15 or 24 months. Amounts or changes in

the amounts can also be determined at treatment specific events, e.g. before and/or after every treatment cycle or drug/therapy administration. A cycle is the time between one round of treatment until the start of the next round. Cancer treatment is usually not a single treatment, but a course of treatments. A course usually takes between 3 to 6 months, but can be more or less than that. During a course of treatment, there are usually between 4 to 8 cycles of treatment. Usually a cycle of treatment includes a treatment break to allow the body to recover. As will be understood by persons skilled in the art, the result of the monitoring normally may not be correct for 100% of the subjects, although it preferably is correct. The term, however, requires that a correct result of the monitoring can be achieved for a statistically significant part of the subjects.

For a description of statistic significance and suitable confidence intervals and p values, see above.

In a fifth aspect, the invention provides the use of the compound according to the first aspect of the invention in a method of treating prostate cancer, the method comprising the steps of carrying out a method according to the second aspect of the invention, and treating prostate cancer in a subject for which protease activity, in particular increased protease activity, has been detected.

In another aspect, the invention provides a method of treating prostate cancer, comprising carrying out a method according to the second aspect of the invention, and treating prostate cancer in a subject for which protease activity, in particular increased protease activity, has been detected.

The term "treatment" or "treating" with respect to cancer as used herein refers to a therapeutic treatment, wherein the goal is to reduce progression of cancer. Beneficial or desired clinical results include, but are not limited to, release of symptoms, reduction of the length of the disease, stabilized pathological state (specifically not deteriorated), slowing down of the disease's progression, improving the pathological state and/or remission (both partial and total), preferably detectable. A successful treatment does not necessarily mean cure, but it can also mean a prolonged survival, compared to the expected survival if the treatment is not applied. In a preferred embodiment, the treatment is a first line treatment, i.e. the cancer was not treated previously. Cancer treatment involves a treatment regimen.

The term "treatment regimen" as used herein refers to how the subject is treated in view of the disease and available procedures and medication. Non-limiting examples of cancer treatment regimens are chemotherapy, surgery and/or irradiation or combinations thereof. The early detection of cancer the present invention enables allows in particular for a surgical treatment, especially for a curative resection. In particular, the term "treatment regimen" refers

to administering one or more anti-cancer agents or therapies as defined below. The term "anti-cancer agent or therapy" as used herein refers to chemical, physical or biological agents or therapies, or surgery, including combinations thereof, with antiproliferative, antioncogenic and/or carcinostatic properties.

5 A chemical anti-cancer agent or therapy may be selected from the group consisting of alkylating agents, antimetabolites, plant alkaloids and terpenoids and topoisomerase inhibitors. Preferably, the alkylating agents are platinum-based compounds. In one embodiment, the platinum-based compounds are selected from the group consisting of cisplatin, oxaliplatin, eptaplatin, lobaplatin, nedaplatin, carboplatin, iproplatin, tetraplatin, lobaplatin, DCP, PLD-
10 147, JMI 18, JM216, JM335, and satraplatin.

A physical anti-cancer agent or therapy may be selected from the group consisting of radiation therapy (e.g. curative radiotherapy, adjuvant radiotherapy, palliative radiotherapy, teloradiotherapy, brachytherapy or metabolic radiotherapy), phototherapy (using, e.g. hematoporphyrin or photofrin II), and hyperthermia.

15 Surgery may be a curative resection, palliative surgery, preventive surgery or cytoreductive surgery. Typically, it involves an excision, e.g. intracapsular excision, marginal, extensive excision or radical excision as described in Baron and Valin (Rec. Med. Vet, Special Canc. 1990; 11(166):999-1007).

A biological anti-cancer agent or therapy may be selected from the group consisting of
20 antibodies (e.g. antibodies stimulating an immune response destroying cancer cells such as rituximab or alemtuzumab, antibodies stimulating an immune response by binding to receptors of immune cells an inhibiting signals that prevent the immune cell to attack "own" cells, such as ipilimumab, antibodies interfering with the action of proteins necessary for tumor growth such as bevacizumab, cetuximab or panitumumab, or antibodies conjugated to a drug,
25 preferably a cell-killing substance like a toxin, chemotherapeutic or radioactive molecule, such as Y-ibritumomab tiuxetan, I-tositumomab or ado-trastuzumab emtansine), cytokines (e.g. interferons or interleukins such as INF-alpha and IL-2), vaccines (e.g. vaccines comprising cancer-associated antigens, such as sipuleucel-T), oncolytic viruses (e.g. naturally oncolytic viruses such as reovirus, Newcastle disease virus or mumps virus, or viruses genetically
30 engineered viruses such as measles virus, adenovirus, vaccinia virus or herpes virus preferentially targeting cells carrying cancer-associated antigens), gene therapy agents (e.g. DNA or RNA replacing an altered tumor suppressor, blocking the expression of an oncogene, improving a subject's immune system, making cancer cells more sensitive to chemotherapy, radiotherapy or other treatments, inducing cellular suicide or conferring an anti-angiogenic

effect) and adoptive T cells (e.g. subject-harvested tumor-invading T-cells selected for antitumor activity, or subject-harvested T-cells genetically modified to recognize a cancer-associated antigen).

In one embodiment, the one or more anti-cancer drugs is/are selected from the group

5 consisting of Abiraterone Acetate, ABVD, ABVE, ABVE-PC, AC, AC-T, ADE, Ado-Trastuzumab Emtansine, Afatinib Dimaleate, Aldesleukin, Alemtuzumab, Aminolevulinic Acid, Anastrozole, Aprepitant, Arsenic Trioxide, Asparaginase Erwinia chrysanthemi, Axitinib, Azacitidine, BEACOPP, Belinostat, Bendamustine Hydrochloride, BEP, Bevacizumab, Bexarotene, Bicalutamide, Bleomycin, Bortezomib, Bosutinib, Brentuximab

10 Vedotin, Busulfan, Cabazitaxel, Cabozantinib-S-Malate, CAFCapecitabine, CAPOX, Carboplatin, CARBOPLATIN-TAXOL, Carfilzomib, Carmustine, Carmustine Implant, Ceritinib, Cetuximab, Chlorambucil, CHLORAMBUCIL-PREDNISONE, CHOP, Cisplatin, Clofarabine, CMF, COPP, COPP-ABV, Crizotinib, CVP, Cyclophosphamide, Cytarabine, Cytarabine, Liposomal, Dabrafenib, Dacarbazine, Dactinomycin, Dasatinib, Daunorubicin

15 Hydrochloride, Decitabine, Degarelix, Denileukin Diftitox, Denosumab, Dexrazoxane Hydrochloride, Docetaxel, Doxorubicin Hydrochloride, Doxorubicin Hydrochloride Liposome, Eltrombopag Olamine, Enzalutamide, Epirubicin Hydrochloride, EPOCH, Eribulin Mesylate, Erlotinib Hydrochloride, Etoposide Phosphate, Everolimus, Exemestane, FEC, Filgrastim, Fludarabine Phosphate, Fluorouracil, FU-LV, Fulvestrant, Gefitinib, Gemcitabine

20 Hydrochloride, GEMCITABINE-CISPLATIN, GEMCITABINE-OXALIPLATIN, Gemtuzumab Ozogamicin, Glucarpidase, Goserelin Acetate, HPV Bivalent Vaccine, Recombinant HPV Quadrivalent Vaccine, Hyper-CVAD, Ibritumomab Tiuxetan, Ibrutinib, ICE, Idelalisib, Ifosfamide, Imatinib, Mesylate, Imiquimod, Iodine 131 Tositumomab and Tositumomab, Ipilimumab, Irinotecan Hydrochloride, Ixabepilone, Lapatinib Ditosylate,

25 Lenalidomide, Letrozole, Leucovorin Calcium, Leuprolide Acetate, Liposomal Cytarabine, Lomustine, Mechlorethamine Hydrochloride, Megestrol Acetate, Mercaptopurine, Mesna, Methotrexate, Mitomycin C, Mitoxantrone Hydrochloride, MOPP, Nelarabine, Nilotinib, Obinutuzumab, Ofatumumab, Omacetaxine Mepesuccinate, OEPA, OFF, OPPA, Oxaliplatin, Paclitaxel, Paclitaxel Albumin-stabilized Nanoparticle Formulation, PAD, Palifermin,

30 Palonosetron Hydrochloride, Pamidronate Disodium, Panitumumab, Pazopanib Hydrochloride, Pegaspargase, Peginterferon Alfa-2b, Pembrolizumab, Pemetrexed Disodium, Pertuzumab, Plerixafor, Pomalidomide, Ponatinib Hydrochloride, Pralatrexate, Prednisone, Procarbazine Hydrochloride, Radium 223 Dichloride, Raloxifene Hydrochloride, Ramucirumab, Rasburicase, R-CHOP, R-CVP, Recombinant HPV Bivalent Vaccine, Recombinant HPV

Quadrivalent Vaccine, Recombinant Interferon Alfa-2b, Regorafenib, Rituximab, Romidepsin, Romiplostim, Ruxolitinib Phosphate, Siltuximab, Sipuleucel-T, Sorafenib Tosylate, STANFORD V, Sunitinib Malate, TAC, Talc, Tamoxifen Citrate, Temozolomide, Temsirolimus, Thalidomide, Topotecan Hydrochloride, Toremifene, Tositumomab and I 131 Iodine Tositumomab, TPF, Trametinib, Trastuzumab, Vandetanib, VAMP, VeIP, Vemurafenib, Vinblastine Sulfate, Vincristine Sulfate, Vincristine Sulfate Liposome, Vinorelbine Tartrate, Vismodegib, Vorinostat, XELOX, Ziv-Aflibercept, and Zoledronic Acid or salts thereof.

In another aspect, the invention provides a method of producing the compound according to the first aspect of the invention.

In preferred embodiments, the compound characterized by the formula ABZ1-Trp2-Glu3-Gly4-Asn5-ANB-NH26, where ABZ is 2-aminobenzoic acid, and ANB-NH2 is an amide of 5-amino-2-nitrobenzoic acid, is produced according to a process performed on a resin carrier, preferably having the Fmoc group. Before initiating the process, the carrier is prepared: its volume is increased by repeated washing with hydrophobic solvents, preferably dimethylformamide, methylene chloride or N-methylpyrrolidone, and removing the Fmoc protecting group, preferably by washing with a 10-30% solution of piperidine in solvents such as dimethylformamide, methylene chloride or N-methylpyrrolidone. Next, the process is carried out in subsequent stages:

- a) Deposition of 5-amino-2-nitrobenzoic acid ANB on the resin is preceded by washing the carrier with a 3-6% solution of N-methylmorpholine (NMM) in DMF, followed by DMF. Next, a solution of ANB in DMF is prepared, to which TBTU, DMAP and finally diisopropylethylamine (DIPEA) are added in the following excess in relation to the polymer deposition: ANB/TBTU/DMAP/DIPEA, 3:3:2:6. The obtained mixture is added to the resin and mixed until homogeneous, then the resin is filtered off under reduced pressure and washed with solvents such as DMF, DCM and isopropanol. Next, the binding of ANB to the resin is continued by using hexafluorophosphate-O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium (HATU), followed by hexafluorophosphate-O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium (HBTU) in excess, and after finishing, the carrier is washed successively with DMF, DCM and isopropanol, and gently dried,
- b) Attachment of the amino acid residue to ANB is carried out using the reaction with the amino acid derivative of Fmoc-Asn(Trt)-OH, at least a five-fold molar excess of the amino acid derivative in relation to the resin is dissolved in anhydrous pyridine and

5 contacted with the resin with deposited ANB. Next, the whole is cooled to a temperature not lower than 20°C and POCl₃ is added in a ratio of 1:1 to the amount of amino acid derivative used, and mixed. The mixing process is carried out at room temperature and then at an elevated temperature; after the reaction is completed, the resin is filtered off under reduced pressure, washed with DMF and MeOH and gently dried; the obtained intermediate compound is subjected to the process of acylation.

10 c) Acylation of the resulting intermediate compound is carried out using an amino acid derivative, preferably Fmoc-Gly-OH, and then Fmoc-Glu(OtBu)-OH followed by Fmoc-Trp(Trt)-OH and finally by Boz-Abz-OH. Acylation is carried out in stages from residue 6 to 1, using diisopropylcarbodiimide as a coupling agent, which is used in excess. At the end of each step, the resin is washed with DMF and (preferably) subjected to the chloroanil test, in which the attachment of the amino acid derivative is monitored.

15 d) Removing the Fmoc protecting group is carried out by washing with a 10-30% piperidine solution in DMF, and subsequent washing with each of the solvents: DMF, isopropanol and methylene chloride.

20 e) Separation of the peptide from the resin is carried out using the mixture: TFA, phenol, water, and TIPS, while maintaining a ratio of 88:5:5:2 v/v/v/v, respectively; the mixture is stirred for at least one hour, preferably for three hours, and the resulting precipitate is filtered off under reduced pressure, washed with diethyl ether, and the resulting peptide is centrifuged.

f) Preparation of the finished product is carried out by dissolving the peptide in water by means of ultrasound, and lyophilisation.

25 In preferred embodiments, the compound characterized by the ABZ1-Trp2-Glu3-Gly4-Asn5-pNA6, where ABZ is 2-aminobenzoic acid, and pNA is paranitroanilide, is produced according to a process performed on a resin carrier, preferably having the Fmoc group. Before initiating the process, the carrier is prepared: its volume is increased by repeated washing with hydrophobic solvents, preferably dimethylformamide, methylene chloride or N-methylpyrrolidone, and removing the Fmoc protecting group, preferably by washing with a 10-30% solution of piperidine in solvents such as dimethylformamide, methylene chloride or N-methylpyrrolidone; then the process is carried out in subsequent stages:

30 a) Attachment of the third amino acid residue (Fmoc-Gly) to the resin is carried out using the appropriate amino acid derivative at a 9-fold molar excess in relation to the resin

deposition, which is dissolved in anhydrous methylene chloride. The mixture is then stirred for at least two hours at room temperature; after completing the reaction, the resin is filtered off under reduced pressure and washed with DMF and MeOH, and then dried.

- 5 b) In the subsequent steps, attachment of the amino acid residue, hereinafter referred to as acylation, is performed, and the derivative of Fmoc-Glu(OtBu)-OH is used, followed by Fmoc-Trp(Trt)-OH and Boc-ABZ-OH; each step is preceded by washing the resin with DMF, for preferably 5 minutes; in subsequent attachments, a coupling agent is used, preferably diisopropylcarbodiimide, which is used in excess. This procedure is repeated twice, and after each step the resin is washed with DMF, and preferably subjected to the chloroanil test, in which the attachment of the amino acid derivative is monitored.
- 10
- c) Removal of the Fmoc protecting group is carried out by washing with a 10-30% piperidine solution in DMF, and subsequent washing with each of the solvents: DMF, isopropanol and methylene chloride.
- 15
- d) Repeating steps b) to c) until the compound (ABZ1-Trp(Trt)2-Glu(OtBu)3-Gly4-OH) is obtained; synthesis is carried out from residue 6 to 3, and then the resulting compound is detached from the carrier using the mixture: TFA: phenol: water: TIPS in the proportions 88:5:5:2, v/v/v/v, respectively, while stirring. After at least two hours, the contents of the flask are filtered off under reduced pressure, and the precipitate is washed with diethyl ether. The precipitate is then centrifuged for preferably 20 minutes, dissolved in water by means of ultrasound, and then lyophilised.
- 20
- e) Fmoc-Asn(Trt)-pNA synthesis is carried out in stages; in the first stage, 2 mmol of Fmoc-Asn(Trt) is dissolved in anhydrous tetrahydrofuran (THF) in the presence of 2 mmol of N-methylmorpholine (NMM), and the carboxyl group of the amino acid derivative is activated with 2 mmol of isobutyl chloride. After 10 minutes of activation, 3 mmol of p-nitroaniline are added, and the reaction is carried out for (preferably) 2 hours at a temperature (preferably) of -15°C, followed by a day at room temperature. When the reaction is completed, the solvent is evaporated and the dry residue is dissolved in ethyl acetate; then the resulting solution is washed successively with saturated aqueous NaCl solution, 10% citric acid, 5% sodium bicarbonate, and dried over anhydrous sodium sulphate; ethyl acetate is distilled under reduced pressure, and the dry residue is dried.
- 25
- 30

- 5 f) Combining the protected peptide ABZ1-Trp(Trt)2-Glu(OtBu)3-Gly4-OH with paranitroanilide Asn(Trt) is based on the following process: the protected peptide ABZ1-Trp(Trt)2-Glu(OtBu)3-Gly4-OH is dissolved in a small amount of DCM, and then activated with TFFH (tetramethylfluoroformamide) for preferably 30 minutes at a preferable temperature of 0°C, after which a catalytic amount of DMAP and Fmoc-Asn(Trt)5-pNA6 is added. The reaction is preferably carried out for 24 hours at room temperature, after which the solvent is evaporated, and the resulting solution is poured with the mixture removing the side protection: TFA: phenol: water: TIPS (88:5:5:2, v/v/v/v), and mixed for preferably 3 hours.
- 10 g) The finished product is prepared by dissolving the peptide in water using ultrasound, and then subjecting to lyophilisation.

Brief description of the figures

- 15 Fig. 1 shows the chromatographic analysis (A) along with mass spectrometry spectra (C) of substrate ABZ- Trp-Glu-Gly-Asn-ANB-NH₂ and the same for ABZ- Trp-Glu-Gly-Asn-pNA (B) and (D).

- 20 Fig. 2 shows RP HPLC analysis of a randomly selected system containing urine from a person diagnosed with prostate cancer. Both compounds (1) (ABZ-Trp-Glu-Gly-Asn-ANB-NH₂, fig 2A) and (2) (ABZ-Trp-Glu-Gly-Asn-pNA, fig 2B) break down into the ABZ-Trp-Glu-Gly-Asn-OH peptide fragment and chromophore (ANB-NH₂ or pNA, respectively). A: ABZ- Trp-Glu-Gly-Asn-ANB-NH₂ substrate in 200 mM Tris-HCl buffer, pH 8.0. B: Hydrolysis of ABZ- Trp-Glu-Gly-Asn -pNA substrate in urine of patients diagnosed with prostate cancer.

- 25 Fig. 3 shows the rate of hydrolysis of substrate (1) ABZ- Trp-Glu-Gly-Asn-ANB-NH₂ in urine samples of patients diagnosed with cancer (samples 1-25) and in urine from healthy individuals (26-35). Arabic numbers indicate the number of the selected urine sample. Figure 1 shows that all samples 1-25 disintegrated, but for samples 11,12,23,24,25, the degradation of the ABZ-Thr-Thr-Ala-Arg-ANB-NH₂ substrate occurred more efficiently than for materials 18 or 22. This result may be due to the difference in activity and amount of enzymes responsible for proteolysis. In addition, Figure 3 shows that incubation of a solution of the compound with formula 2 with urine samples from
- 30

healthy individuals (without a diagnosed cancer) does not lead to an increase in absorbance, and thus no hydrolysis of the test compound occurs. The result indicates the absence of proteolytic enzymes characteristic of prostate cancer.

Fig. 4 shows the rate of substrate hydrolysis (2) ABZ-Trp-Glu-Gly-Asn-pNA in urine samples of patients diagnosed with cancer (samples 1-25) and in urine from healthy individuals (26-35). Arabic numbers indicate the number of the selected urine sample. A similar result as described above for Fig. 3 is obtained.

Fig. 5 shows the incubation of substrate (1) ABZ- Trp-Glu-Gly-Asn-ANB-NH₂ with urine originated from various kind of malignancies(pancrease, liver, bowel, lung, bile duct, stomach and prostate). The absorbance increase is observed only for urine of patient diagnosed with prostate cancer.

Examples

The invention is illustrated by the following, non-limiting examples of implementation.

Example 1: Synthesis of compound ABZ¹-Trp²-Glu³-Gly⁴-Asn⁵-ANB-NH₂⁶

1. Preparation of the chromogenic peptide

a) The first stage of the synthesis was to obtain a chromogenic peptide by solid phase synthesis – on a solid carrier, using Fmoc/tBu chemistry, i.e. using the protection.

A compound with the sequence ABZ¹- Trp²-Glu³-Gly⁴-Asn⁵-ANB-NH₂⁶, where ABZ is 2-aminobenzoic acid, ANB-NH₂ is amide of 5-amino-2-benzoic acid, and ANB is 5-amino-2-benzoic acid, obtained in the process of chemical synthesis in the solid phase using the following amino acid derivatives.

Boc-ABZ, Fmoc-Trp(Trt), Fmoc-Glu(OtBu), Fmoc Gly Fmoc-Asn(Trt), ANB

The synthesis of the compound, i.e. a diagnostic marker for the detection of prostate cancer, whose diagnosis is associated with the hydrolysis of this compound under the influence of proteolytic enzymes, was carried out on a solid carrier enabling the conversion of 5-amino-2-benzoic acid into ANB-NH₂ amide:

– e.g. amide resin, for instance TentaGel S RAM from RAPP Polymere (Germany), e.g. with a deposit of 0.23 mmol/g.

It is possible to use other commercially available amide resins, including Rink Amide (Germany).

The compound was synthesized manually using a laboratory shaker. For most of the steps, a 25 mL sintered syringe for solid phase synthesis was used as a reactor.

- 5 All the obtained final compounds contained ABZ 2-aminobenzoic acid in position 1 of their sequence (i.e. at the N-terminus), and ANB 5-amino-2-nitrobenzoic acid molecule in position 6 (at the C-terminus). ABZ acts as a fluorescence donor, while ANB (5-amino-2-benzoic acid) acts as a fluorescence quencher and chromophore. The peptides contained at least (and preferably) one reactive site in their sequence, located between the amino acid residues Arg-
10 ANB-NH₂: at the 5 position of the compound. The synthesis, involving the attachment of amino acid derivatives, is carried out from the residue 6 to 1, i.e. from the C- to N-terminus.

b) Deposition of ANB on TentaGel S RAM resin:

- Peptide synthesis was performed on TentaGel S RAM resin (Rapp Polymere) with a deposition of 0.23 mmol/g. In the first stage, the resin was prepared, including its loosening by the wash
15 cycle. Subsequently, the Fmoc amino group protection was removed from the carrier with a 20% solution of piperidine in NMP, and a solvent wash cycle was carried out. To confirm the presence of free amino groups, a chloranil test was performed.

Solvent wash cycle:

DMF 1 x 10 minutes

- 20 IsOH 1 x 10 minutes

DCM 1 x 10 minutes

Removal of Fmoc protection:

DMF 1 x 5 minutes

20% piperidine in NMP 1 x 3 minutes

- 25 20% piperidine in NMP 1 x 8 minutes

Solvent wash cycle:

DMF 3 x 2 minutes

IsOH 3 x 2 minutes

DCM 3 x 2 minutes

c) Chloranil test:

The chloranil test consisted of transferring (using a spatula) several grains of resin from the reactor – a syringe, into a glass ampoule, to which 100 μ L saturated solution of p-chloranil in
5 toluene and 50 μ L fresh acetaldehyde were added. After 10 minutes, the control of grains colour was carried out.

At this stage, after performing the test, a green grain colour was obtained, which indicated the presence of free amino groups. After confirming the removal of 9-fluorenylmethoxycarbonyl protection from the resin, it was possible to proceed to the next stage, the attachment of the
10 ANB derivative (5-amino-2-nitrobenzoic acid)

d) Deposition of 5-amino-2-nitrobenzoic acid on a solid carrier

The first step in the synthesis of the peptide library – a mixture of peptides, was ANB deposition on 1 g of resin. Before attaching the chromophore, the resin used for the reaction was washed with the following solvents: DMF, DCM and again DMF, after which the Fmoc protection was
15 removed from the carrier functional group. One cycle of removing Fmoc protection included the following steps:

Removal of Fmoc protection:

20% piperidine in NMP 1 x 3 minutes

20% piperidine in NMP 1 x 8 minutes

20 e) Washing

DMF 3 x 2 minutes

IsOH 3 x 2 minutes

DCM 3 x 2 minutes

f) Chloranil test for the presence of free amino groups.

25 The resin with a free amino group was washed with a 5% solution of *N*-methylmorpholine (NMM) in DMF, followed by DMF. The procedure of removing Fmoc protection and the wash cycle were performed in a Merrifield vessel. In a separate flask, ANB was dissolved in DMF, and TBTU, DMAP, and finally diisopropylethylamine (DIPEA) were subsequently added in the following excess in relation to polymer deposition:

ANB/TBTU/DMA/DIPEA, 3:3:2:6. The mixture thus prepared was added to the resin and stirred for 3 hours. The resin was filtered off under reduced pressure, washed with DMF, DCM and isopropanol, and the entire acylation procedure was repeated twice. Hexafluorophosphate-*O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium (HATU), and then
5 hexafluorophosphate-*O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium (HBTU) were used to carry out subsequent reactions of ANB attachment to the resin. In the last step, the resin was washed successively with DMF, DCM and isopropanol, and air dried.

g) Attachment of the C-terminal amino acid residue to ANB

The corresponding amino acid derivative (9-fold molar excess relative to resin deposition) was
10 dissolved in pyridine and transferred to the flask containing the resin with ANB deposited. The whole was cooled to -15°C (ice bath: 1 part by weight NH₄Cl, 1 part by weight NaNO₃, 1 part by weight ice). After reaching the desired temperature, POCl₃ was added (in a 1:1 ratio to the amount of amino acid derivative used) and the whole was stirred on a magnetic stirrer: 20 minutes at -15°C, 30 minutes at room temperature, and 6 hours at 40°C (oil bath). After
15 completing the reaction, the resin was filtered off under reduced pressure, washed with DMF and MeOH, and left to dry.

In the next step, the residue (alanine) was attached in the P2 position.

Every attachment of amino acid residues was preceded by washing the resin with DMF for 5 minutes. Diisopropylcarbodiimide was used as a coupling agent in subsequent attachments. The
20 procedure was repeated twice.

After each acylation, a resin wash cycle started, followed by the chloranil test to monitor the attachment of the amino acid derivative to the free amino groups of the resin.

Solvent wash cycle:

DMF 3 x 2 minutes

25 IsOH 3 x 2 minutes

DCM 3 x 2 minutes

Chloranil test:

As a result of the tests, after the first two couplings, the colour of the grains was first green and then grey, so it was necessary to carry out another acylation, as a result of which the grains of

the resin tested with the chloranil test were colourless. This indicated the attachment of ANB to TentaGel S RAM resin, which enabled moving to the next stage of peptide synthesis.

h) Attachment of further protected amino acid residues:

- 5 The resin together with the attached ANB residue in the reactor was washed with DMF, followed by deprotection of the Fmoc from the amino group to attach the protected amino acid alanine derivative.

Removal of Fmoc protection:

DMF 1 x 5 minutes

20% piperidine in NMP 1 x 3 minutes

- 10 20% piperidine in NMP 1 x 8 minutes

Solvent wash cycle:

DMF 3 x 2 minutes

IsOH 3 x 2 minutes

DCM 3 x 2 minutes

- 15 Chloranil test:

The chloranil test produced a positive result, as evidenced by the green colour of the resin grains. This enabled moving to the next stage – attachment of the Fmoc-Thr (tBu)-OH amino acid residue.

Attachment of the amino acid derivative:

- 20 The process of coupling was preceded by washing the resin in DMF. The composition of the coupling mixture remained unchanged when attaching the protected glutamic acid residue.

At the end of each acylation, a solvent wash cycle was performed according to the given procedure, which was followed by a chloranil test for the presence of free amino groups in the solution.

- 25 Solvent wash cycle:

DMF 3 x 2 minutes

IsOH 3 x 2 minutes

DCM 3 x 2 minutes

Chloranil test:

The resin grains during the test carried out after the second acylation were colourless, which enabled moving to the next stage of the synthesis, i.e. the introduction of another protected amino acid derivative – threonine and 2-aminobenzoic acid molecule. The coupling processes followed the procedure discussed earlier.

Tests carried out after attaching the above-mentioned residues showed positive results: the resin grains were colourless.

10 2. Removal of the peptide from the carrier

After synthesis, the amide of ABZ-Thr-Thr-Ala-Arg-ANB-NH₂ peptide was removed from the carrier, along with the simultaneous removal of the side protection with the mixture: TFA: phenol: water: TIPS (88:5:5:2, v/v/v/v) in a round-bottomed flask on a magnetic stirrer.

After 3 hours, the contents of the flask were filtered off under reduced pressure on a sintered (Schott) funnel and washed with diethyl ether. The resulting sediment was centrifuged on a SIGMA 2K30 centrifuge (Laboratory Centrifuges) for 20 minutes. The precipitate obtained after centrifugation is dissolved in water by means of ultrasound and lyophilised.

Identity/characteristics of a new compound – HPLC analysis, MS

HPLC conditions: RP Bio Wide Pore Supelco C8 250 mm 4 mm column, A phase system 0.1% TFA in water, B: 80% acetonitrile in A), flow rate 1 ml/min, UV detection at 226 nm.

Obtaining the compound was confirmed.

Example 2: Preparation of the compound with the formula: ABZ1-Thr2-Thr3-Ala4-Arg5-pNA6

The process is carried out in a similar manner to that described in Example 1, except that the corresponding amino acid derivatives and additional substituents are used, and the process is carried out partly in solution and partly on a solid carrier.

Preparation of p-nitroanilide Ala

- a) The first stage of the synthesis was to obtain a protected peptide by solid phase synthesis using Fmoc/tBu chemistry.

ABZ¹-Thr(tBu)²-Thr(tBu)³-Ala⁴-OH compound, where ABZ is 2-aminobenzoic acid, was obtained by solid phase chemical synthesis using the following amino acid derivatives:

- 5 Boc-ABZ, Fmoc-Thr(tBu), Fmoc-Ala.

The compound was synthesized on a solid carrier:

- 2-chloro-chlorotriyl resin, e.g. from Iris BIOTECH GMBH (Germany), with deposition of 1.6 mmol Cl/g groups.

- 10 The compound was synthesized manually using a laboratory shaker. Throughout all stages, a 25 ml sintered syringe for solid phase synthesis was used as the reactor.

- Peptide synthesis was carried out on a carrier: 2-chloro-chlorotriyl resin, e.g. from Iris BIOTECH GMBH (Germany), with deposition of 1.6 mmol Cl/g groups. In the first stage, the resin was loosened in a wash cycle. Subsequently, the Fmoc amino group protection was removed from the carrier with a 20% solution of piperidine in NMP. Then a solvent wash cycle was carried out. To confirm the presence of free amino groups, a chloranil test was performed.

Solvent wash cycle:

DMF 1 x 10 minutes

IsOH 1 x 10 minutes

DCM 1 x 10 minutes

- 20 Removal of Fmoc protection:

DMF 1 x 5 minutes

20% piperidine in NMP 1 x 3 minutes

20% piperidine in NMP 1 x 8 minutes

Solvent wash cycle:

- 25 DMF 3 x 2 minutes

IsOH 3 x 2 minutes

DCM 3 x 2 minutes

b) Chloranil test:

The chloranil test consisted of transferring (with a spatula) several grains of resin from the reactor – a syringe, into a glass ampoule, and adding 100 μ L saturated solution of p-chloranil in toluene and 50 μ L fresh acetaldehyde. After 10 minutes, the control of grains colour was carried out.

At this stage, after the test, a green colour of grains was obtained, which indicated the presence of free amino groups. After confirming the removal of the 9-fluorenylmethoxycarbonyl protection from the resin, the attachment of Fmoc-Ala derivative was initiated.

c) Embedding Fmoc-Ala on a solid carrier

The first step in the synthesis of the peptide library was the deposition of Fmoc-Ala on 1 g of resin. Before attachment of the amino acid derivative, the resin used for the reaction was washed with the following solvents: DMF (dimethylformamide), DCM (methylene chloride) and again with DMF, after which the Fmoc-protection was removed from the carrier functional group. One cycle of Fmoc-protection removal included the following steps:

Removal of Fmoc protection:

20% piperidine in NMP 1 x 3 minutes

20% piperidine in NMP 1 x 8 minutes

Washing

DMF 3 x 2 minutes

IsOH 3 x 2 minutes

DCM 3 x 2 minutes

Chloranil test for the presence of free amino groups.

The resin with a free amino group was washed with DMF. In a separate flask, Fmoc-Ala was dissolved in DMF. Next, TBTU, DMAP, and finally diisopropylethylamine (DIPEA) were added in excess to the polymer deposition: Fmoc-Pro/TBTU/DMAP/DIPEA, 3:3:2:6. The obtained mixture was added to the resin and stirred for 3 hours. The resin was filtered off under reduced pressure, washed with DMF, DCM and isopropanol, and the entire acylation procedure was repeated twice. Hexafluorophosphate-*O*-(7-azabenzotriazol-1-yl) -*N,N,N',N'*-tetramethyluronium (HATU), followed by hexafluorophosphate-*O*-(benzotriazol-1-yl)-

N,N,N',N'-tetramethyluronium (HBTU) were used to conduct subsequent reactions of Fmoc-Pro attachment to the resin. In the last step, the resin was washed successively with DMF, DCM and isopropanol, and air dried.

c. Attachment of further protected amino acid residues:

- 5 The resin together with the attached Fmoc-Ala residue in the reactor was washed with DMF, followed by deprotection of Fmoc from the amino group to attach the protected threonine derivative.

Removal of Fmoc protection:

DMF 1 x 5 minutes

- 10 20% piperidine in NMP 1 x 3 minutes

20% piperidine in NMP 1 x 8 minutes

Solvent wash cycle:

DMF 3 x 2 minutes

IsOH 3 x 2 minutes

- 15 DCM 3 x 2 minutes

Chloranil test:

The chloranil test produced a positive result, as evidenced by the green colour of the resin grains. This enabled moving to the next stage – attachment of the Fmoc-Thr (tBu)-OH amino acid residue.

- 20 Attachment of the amino acid derivative:

The process of coupling was preceded by washing the resin in DMF. The composition of the coupling mixture remained unchanged when attaching the protected glutamic acid residue.

At the end of each acylation, a solvent wash cycle was performed according to the given procedure, which was followed by a chloranil test for the presence of free amino groups in solution.

25

Solvent wash cycle:

DMF 3 x 2 minutes

IsOH 3 x 2 minutes

DCM 3 x 2 minutes

Chloranil test:

5 The resin grains during the test carried out after the second acylation were colourless, which enabled moving to the next stage of the synthesis, i.e. the introduction of another protected amino acid derivative – threonine and 2-aminobenzoic acid molecule. The coupling processes followed the procedure discussed earlier.

Tests carried out after attaching the above-mentioned residues showed positive results: the resin grains were colourless.

10 d) Removal of peptide from the carrier while maintaining side group protection

After completing the synthesis, the protected ABZ-Thr(tBu)-Th (tBu)-Ala-OH peptide was removed from the carrier, retaining the side protection with the mixture: acetic acid: TFE (trifluoroethanol): DCM (2:2:6, v/v/v) in a round-bottomed flask on a magnetic stirrer.

15 After 2 hours, the contents of the flask were filtered off under reduced pressure on sintered (Schott) funnels, washing with the astringent mixture. The solution was washed with hexane (1:10 v/v), evaporated under reduced pressure and then lyophilised.

e) Chemical synthesis of paranitroanilide Arg derivatives

20 The mixed anhydrides method was used to synthesize Fmoc-Arg(Pbf)-pNA. In the first step, 2 mmol of Fmoc-Arg (Pbf) were dissolved in anhydrous tetrahydrofuran (THF) in the presence of 2 mmol of N-methylmorpholine (NMM). The carboxyl group of the amino acid derivative was activated with 2 mmol of isobutyl chloride. After 10 minutes of activation, 3 mmol of p-nitroaniline were added. Reactions were carried out for 2 hours at -15°C, and then for 24 hours at room temperature. After completing the reaction, the solvent evaporated and the dry residue was dissolved in ethyl acetate. The resulting solution was washed successively with saturated
25 aqueous NaCl solution, 10% citric acid, and 5% sodium bicarbonate. The resulting solution was dried over anhydrous sodium sulphate, ethyl acetate was distilled off under reduced pressure, and the dry residue was dried in a vacuum desiccator over P₂O₅ and KOH.

f) Coupling of the protected peptide with paranitroanilide Arg (Pbf)

30 The protected ABZ¹-Thr(tBu)²-Thr (tBu)³-Ala⁴-OH peptide was dissolved in a small amount of DCM, and subsequently activated with TFFH (tetramethylfluoroformamide) for 30 minutes

at 0°C. Then a catalytic amount of DMAP and Arg (Pbf)-pNA was added. The reaction was carried out for 24 hours at room temperature, after which the solvent evaporated. The resulting solution was poured with the mixture removing the side protection: TFA: phenol: water: TIPS (88:5:5:2, v/v/v/v) and mixed in a round bottom flask on a magnetic stirrer for 3 hours.

- 5 After this time, cold diethyl ether was added to the flask, and the resulting precipitate was centrifuged in a high speed centrifuge at 5,000 rpm for 20 minutes. The precipitate obtained after centrifugation was dissolved in water by means of ultrasound and then lyophilised.

Identity/characteristics of a new compound – HPLC analysis, MS

HPLC conditions: RP Bio Wide Pore Supelco C8 250 mm 4 mm column, A phase system 0.1%

- 10 TFA in water, B: 80% acetonitrile in A), flow rate 1 mL/min, UV detection at 226 nm. Obtaining of the compound was confirmed.

Assay condition

- The study on the application of new compounds was performed on a group of 25 patients diagnosed with prostate cancer. For this purpose, the compound with formula: ABZ-Trp-Glu-Gly-Asn-ANB-NH₂ or ABZ-Trp-Glu-Gly-Asn-pNA was dissolved in dimethyl sulfoxide (at a concentration of 0.5 mg/mL); 50 µL of this solution was mixed with 120 µL buffer (200 mM Tris-HCl, pH 8.0) and 80 µL of urine of a person with prostate cancer. The measurement was made in a 96-well plate designed for measuring absorbance, and each sample was analysed in triplicate at 37°C. The duration of the measurement was 60 minutes. During the measurement, 15 the wavelength characteristic of the released chromophore (ANB-NH₂ or pNA) was monitored at 405 nm (range 380-430 nm). 20

- As a result of the measurement, the colour of the solution increased over time in all urine samples from patients diagnosed with prostate cancer. The observed absorbance increase over time was different for each of the tested samples. A different effect was obtained for 15 samples of healthy people, as none of the 15 urine samples tested had an increase in absorbance in the diagnostic range. 25

- The analysis confirmed the use of the compounds, according to the examples, in the diagnosis of prostate cancer. The mechanism of action of the new compound is based on its enzymatic hydrolysis in such a place, which leads to the release of free chromophore molecules, 30 respectively, ANB-NH₂ – amide of 5-amino-2-nitrobenzoic acid