Use of thiopeptolides for determining the activity of ADAM-TS proteases

The present invention relates to the use of thiopeptolides of the formula R-$(Xaa)_n$-Pro-X-Gly-S-Y-Z-Gly-$(Xaa)_m$-R_1 (I) as substrate for determining the activity of ADAM-TS proteases and to a method for finding an ADAM-TS protease modulator, in particular an inhibitor.

An intact articular cartilage matrix is the decisive prerequisite for the functioning of all joints of the animal and human body. Damage to the articular cartilage leads to arthritic diseases such as osteoarthrosis (osteoarthritis) and rheumatism, which are characterized by dysfunction and finally immobility of the affected animal or human.

Among further diseases characterized by impaired matrix degradation must also be included the diverse forms of cancers, especially the metastasis of tumors.

It has been known for some time that matrix metalloproteases (MMPs) are involved in the degradation of the aggrecan and collagen in cartilage. These include for example the group of matrixins, which comprises all known MMPs from MMP-1, 2 etc, up to MMP-16. A further group, namely the proteases of the ADAM-TS- family (Nagase, H. et al. (2003), Arthritis Research Therapy, 5, 94 – 103), likewise plays a crucial role in the degradation of tissue matrix, resulting in damage to the cartilage matrix. The activity of these proteases, especially ADAM-TS 1, ADAM-TS 4 and ADAM-TS 5, which are also referred to as 'aggrecanases', is the cause of diseases characterized by impaired matrix degradation, such as osteoarthrosis, rheumatism and cancer. ADAM-TS is able to cleave in particular proteoglycan, but also other matrix constituents such as hyaluronan or collagen. Further effects of ADAM-TS 1, 4, 5 and 11, but also ADAM-TS 13, are crucial in inflammatory processes, angiogenesis, cell migration and blood clotting, or blood coagulation (Apte, S. S. (2004), The International Journal of Biochemistry & Cell Biology, 36, 981-985.

It is therefore an important task of pharmaceutical research on the one hand to be able to detect the enzymatic activity of the proteases involved in the diseases in the tissue

at risk or already diseased, e.g. cartilage tissue, or blood. However, on the other hand, it is also particularly important to develop pharmaceuticals able to inhibit single, a plurality of, or all relevant proteases.

The enzymatic (proteolytic) activity of the proteases involved can be measured in vitro by incubating the relevant protease with the appropriate high molecular weight matrix components, e.g. proteoglycan or collagen, and measuring the formation of the degradation products.

Various methods which regularly make elaborate procedures necessary, such as antibody recognition of specific cleavage sites, and mass spectrometric investigations, are available to the skilled worker for isolating and quantifying the heterogeneous degradation products, that is to say for example collagen fragments and protein fragments.

It has previously been described for example that a recombinant substrate which comprises important structural elements of the interglobular domain of natural aggrecan can be used to determine the activity of ADAM-TS 4. The recombinant aggrecan of molecular weight 72 kDa is expressed in COS cells. Determination of the aggrecanase activity requires, besides the use of the high molecular weight aggrecan molecule, further elaborate steps such as structural elements which the signal sequence of CD5, the FLAG epitope for the M1 monoclonal antibody determination, the hinge region of human IgG1, cDNA for the recombinant substrate mentioned, including vectors thereof, as described in detail in EP 0785 274 and also in Hörber, Chr. et al. (2000) Matrix Biology, 19, 533-543.

The use of shorter fragments of the aggrecan molecule has also been disclosed. WO 00/05256 reported that these peptide fragments may consist of 20 to 40 amino acid building blocks. However, it is particularly disadvantageous that peptides comprising fewer than 20 amino acids cannot be converted with aggrecanase. It has been possible to confirm this (see experimental section).

To determine the proteases of the matrixin family, according to the prior art low molecular weight molecules which can be obtained easily by synthesis are cleaved as substrates of the proteases to determine the enzymatic (proteolytic) activity, there being, because of the particular nature of these substrates, release by cleavage of, for example, an optical signal, ordinarily in the visible or ultraviolet wavelength range, which can be quantified.

A well-known example is (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-3-(2',4'-dinitro-phenyl)-L-2,3-diaminopropionyl-Ala-Arg-NH_2 (Bachem, Heidelberg, Germany) which was described by Knight, C. G. et al. (1992) FEBS, 296, 263-266 and which is cleaved by particular matrix metalloproteinases, and thus releases a measurable fluoroimetric signal which can be used to calculate the enzymic activity. It has thus been possible to confirm that it is possible to convert (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-3-(2',4'-dinitrophenyl)-L-2,3-diamino-propionyl-Ala-Arg-NH_2 with the proteases MMP-3 and MMP-8 (see experimental section). It was also possible to convert many further substrates of this type by MMP-3 and MMP-8.

It has also been disclosed that certain substrates from the thiopeptolide class of substances are converted by collagenase and membrane-associated matrix metalloproteinases, cf. EP 0149593 and US 2002/0142362. However, aggrecanase cleavage of a peptide truncated to 16 amino acids N-terminally up to the aggrecanase cleavage site no longer occurs.

It was also possible to confirm in some experiments that shorter-chain peptides, even if they comprise the sequence Glu-Ala, could not be cleaved by ADAM-TS. By contrast, it has been disclosed that the members of other protease families, e.g. the matrixins or the cathepsins, are able to cleave oligopeptides.

The use of oligopeptides in such experiments is particularly desired because they are easily obtainable by chemical synthesis. A further advantage of oligopeptides is that individual peptide building blocks can be chemically modified, e.g. can also be linked to

those chromophoric groups which permit cleavage of the peptide to be followed directly or indirectly by spectrometry, e.g. colorimetry.

In a corresponding manner it is also possible for the effect of enzyme inhibitors or activators easily to be determined by comparing the proteolytic activity of the relevant protease after addition of the inhibitor with the activity measured before addition of the inhibitor.

One example thereof are the thiopeptolides R-Pro-X-Gly-S-Y-Z-Gly-R1 (EP 0149593), which are cleaved by vertebrate collagenase, and acetyl-prolyl-leucyl-glycyl-[2-mercapto4-methyl-pentanoyl]-leucyl-glycyl-ethyl ester (US 2002/0142362), which is cleaved by certain matrixins, thus forming a free SH group which can be quantified by known methods, e.g. reaction with DTNB.

It has now been found according to the invention that the thiopeptolides R-$(Xaa)_n$-Pro-X-Gly-S-Y-Z-Gly-$(Xaa)_m$-R_1 of the formula (I) are cleaved by ADAM-TS proteases, in particular by ADAM-TS1, ADAM-TS4, ADAM-TS5, ADAM-TS11 and/or ADAM-TS13, especially by ADAM-TS1, ADAM-TS4 or ADAM-TS5, and can thus easily be detected via the free SH group. This is all the more surprising since the prior art reports that peptides comprising fewer than 16 amino acid units cannot be cleaved by ADAM-TS.

One aspect of the present invention therefore relates to the use of a thiopeptolide of the formula

R-$(Xaa)_n$-Pro-X-Gly-S-Y-Z-Gly-$(Xaa)_m$-R_1 (I),

where R is H or an N-protective group, preferably a carboxyl group, in particular of C_1-C_5-alkyls, especially of C_1-C_3-alkyls, particularly preferably an acetyl group,

Xaa = any naturally occurring amino acid,

n, m = identically or differently an integer from 0-2415, preferably from 0-35, in particular from 0-14, especially from 0-11 and very particularly preferably equal to 0,

X = Leu, Ile, Phe, Val, Gln, Ala,

Z = Leu, Ile, Phe, Val, Gln, Ala,

R_1 = terminal amide, carboxyl or ester group, preferably of C_1-C_5-alkyls, especially of C_1-C_3-alkyls, in particular an ethyl ester,

S-Y = R_2-CH-COO-,

where R_2 is the side chain of a naturally occurring amino acid, in particular

$-CH_2CH(CH_3)_2$,

$-CH(CH_3)C_2H_5$,

$-CH_2C_6H_5$,

$-CH(CH_3)_2$, or

$-CH_3$,

or a salt thereof, as a substrate for an ADAM-TS protease.

Suitable N-protective groups are generally all conventional amino acid protective groups such as, for example, Fmoc (9-fluoroenylmethyloxycarbonyl), Mtt (4-methyltrityl), Pmc (2,2,5,7,8-pentamethylchroman-6-sulfonyl), tBu (t-butyl), Boc (t-butyloxycarbonyl), Tos (tosyl), Mbzl (4-methylbenzyl), Bom (benzyloxymethyl), 2-chloro-Z (2-chlorobenzyloxycarbonyl) or For (formyl), as can be obtained for example from Bachem Distribution Services GmbH, Weil am Rhein.

A particularly preferred thiopeptolide of the formula (I) is one in which

X = Leu or Ala,

Z = Leu, Ala or Phe, and

R_2 = $-CH_2CH(CH_3)_2$.

In another preferred embodiment, $(Xaa)_n$ and/or $(Xaa)_m$ corresponds to the amino acid sequence shown in SEQ ID NO: 2, which represents the amino acid sequence for human aggrecan.

Further preferred thiopeptolides have the following structure:

Ac-Pro-Leu-Gly-S-Y-Leu-Gly-OC_2H_5,
in which R_2 = $CH_2CH(CH_3)_2$, and Ac is generally an acetyl group,

or

Ac-Pro-Leu-Gly-S-Y-Phe-Gly-OC_2H_5,
in which R_2 = $CH_2CH(CH_3)_2$,

or

Ac-Pro-Ala-Gly-S-Y-Phe-Gly-OC_2H_5,
in which R_2 = $CH_2CH(CH_3)_2$,

or

Ac-Pro-Ala-Gly-S-Y-Ala-Gly-OC_2H_5,
in which R_2 = $CH_2CH(CH_3)_2$.

All known ADAM-TS proteases are suitable according to the present invention, such as the ADAM-TS protease 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 , 17, 18, 19 and/or 20. The ADAM-TS proteases 1, 4, 5, 11 and/or 13 are preferred, especially the ADAM-TS protease 1, ADAM-TS protease 4 or ADAM-TS protease 5.

The use according to the invention is particularly suitable for determining the activity of an ADAM-TS protease, for purifying an ADAM-TS protease, for functional cloning of a

nucleotide sequence coding for an ADAM-TS protease, for finding an ADAM-TS protease modulator, in particular an ADAM-TS protease inhibitor, or for observing the onset or progress of a disease associated with damaged tissue matrix, in particular osteoarthritis, rheumatism, cancer, inflammations, angiogenesis, cell migration, blood clotting and/or blood coagulation, especially osteoarthritis, rheumatism or cancer. In all these methods, e.g. during the enzymatic purification of an ADAM-TS protease or after cloning of a gene coding for an ADAM-TS protease into a generally known expression vector, ultimately the activity of the ADAM-TS protease is measured with the aid of the described thiopeptolides.

The present invention therefore also relates to a method for determining the activity of an ADAM-TS protease, where the method comprises the following steps:

(a) incubating an ADAM-TS protease with a thiopeptolide substrate according to formula I or as described above in detail, and

(b) carrying out an activity measurement or determination of the ADAM-TS protease.

Suitable and preferred ADAM-TS proteases are the ADAM-TS proteases which have been described in detail above. The activity measurement or determination is preferably carried out by spectrophotometry.

In the determination by spectrophotometry there is usually employment of a detection reagent, in this case for thiol groups. Those which have proved advantageous in this connection are 4,4'-dithiodipyridine or, in particular, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), which is also known as Ellmann's reagent. However, it is also possible to employ any other suitable thiol-reactive reagents such as an iodoacetamide, e.g. 5-iodoacetamidofluorescein (5-IAF), a maleimide, e.g. fluorescein-5-maleimide, or other thiol-reactive reagents such as N,N'-didansyl-L-cystine or 5-(bromomethyl)fluorescein. Reagents of these types can be obtained for example from Invitrogen GmbH, Karlsruhe.

It is possible with the aid of said thiopeptolides to find in a suitable assay system particularly simply ADAM-TS protease modulators. Modulators mean according to the

present invention in particular ADAM-TS protease activators and especially ADAM-TS protease inhibitors.

A further aspect of the present invention therefore relates to a method for finding an ADAM-TS protease modulator, in particular an ADAM-TS protease inhibitor, in which the method comprises the following steps:

(a) incubating an ADAM-TS protease with a thiopeptolide substrate according to formula I or as described in detail above in the presence of a test compound and

(b) measuring or determining the influence of the test compound on the activity of the ADAM-TS protease.

Suitable and preferred ADAM-TS proteases are the ADAM-TS proteases which have been described in detail above. The activity measurement or determination is preferably carried out by spectrophotometry as already described in detail above. The compounds described above are in turn suitable as detection reagent for thiol groups, especially 4,4'-dithiodipyridine or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).

The test compound may be any conceivable chemical, biochemically, naturally occurring or synthetic, high or low molecular weight compound. It is particularly advantageous for the test compounds to be made available in the form of a chemical compound library from which the desired compound, e.g. an inhibitor of the tested ADAM-TS protease, can then be found with the aid of the method of the invention.

In a further preferred embodiment, the method of the invention is carried out on an array which particularly facilitates the finding and isolation of the desired compound. The use of a robot for carrying out the method of the invention likewise leads to a further facilitation and to an increase in the throughput and is therefore particularly advantageous. It is also possible with the aid of microfluidic technology, where appropriate combined with miniaturized plate recesses ("wells"), to miniaturize and further automate the assay system, which is in turn particularly advantageous. The method is generally employed in a high-throughput screening for an ADAM-TS protease modulator, in particular an ADAM-TS protease inhibitor.

Another aspect of the present invention, which is based on the method of the invention, relates to the manufacture of a medicament, which comprises the following steps:

(a) carrying out the abovementioned method for finding an ADAM-TS modulator, in particular an inhibitor,

(b) isolating a test substance found to be suitable in step (a), and

(c) formulating the test substance isolated in step (b) with one or more pharmaceutically acceptable carriers or adjuvants.

The pharmaceutically active compounds, preferably inhibitors, found with the aid of the method of the invention are particularly suitable for the treatment of a disease which is associated with damaged tissue matrix, in particular osteoarthritis, rheumatism, cancer, inflammations, angiogenesis, cell migration, blood clotting and/or blood coagulation, in particular osteoarthritis, rheumatism or cancer.

All known agents which are normally employed for pharmaceutical formulation are suitable as pharmaceutically acceptable carrier or adjuvant.

Examples are a sodium chloride solution, in particular an isotonic saline solution (0.9% strength sodium chloride solution), demineralized water, stabilizers such as protease inhibitors or nuclease inhibitors, preferably aprotinin, ε-aminocaproic acid or pepstatin A, or masking agents such as EDTA, gel formulations such as white petrolatum, low-viscosity paraffin and/or yellow wax, depending on the mode of administration.

Further suitable additives are for example detergents such as triton X-100 or sodium deoxycholate, but also polyols such as polyethylene glycol or glycerol, sugars such as sucrose or glucose, zwitterionic compounds such as amino acids, e.g. glycine or, in particular, taurine or betaine and/or a protein such as bovine serum albumin or human serum albumin. Detergents, polyols and/or zwitterionic compounds are particularly preferred.

The physiological buffer solution preferably has a pH of approximately 6.0-8.0, in particular a pH of approximately 6.8-7.8, especially a pH of approximately 7.4 and/or an osmolarity of approximately 200-400 milliosmole/liter, preferably approximately 290-310 milliosmole/liter. The pH of the medicament is generally adjusted with the aid of suitable organic or inorganic buffers, e.g. preferably with the aid of phosphate buffer, tris-buffer (tris(hydroxymethyl)aminomethane), HEPES buffer ([4-(2-hydroxy-ethyl)piperazino]ethanesulphonic acid) or MOPS buffer (3-morpholino-1-propanesulphonic acid). Choice of the appropriate buffer generally depends on the desired buffer molarity. Phosphate buffer is suitable for example for solutions for injection and infusion.

A further aspect of the present invention is a kit which is based on the thiopeptolide substrate of the invention according to formula I or as described above in detail and an ADAM-TS protease as described above and comprises where appropriate one or more buffers, e.g. TNCB buffer (see example). The buffer serves as stabilizing or reaction medium for carrying out the method of the invention. The kit preferably comprises in addition a detection reagent for thiol groups, e.g. at least one of the detection reagents described above, in particular 4,4'-dithiodipyridine and/or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). A further component of the kit may be instructions for use for carrying out the method of the invention, in particular the activity assay.

The following statements and examples are intended to explain the invention in detail without restricting it thereto:

Sequence listing

SEQ ID NO: 1 corresponds to the thiopeptolide according to formula (I)

SEQ ID NO: 2 represents the amino acid sequence of the aggrecan core protein precursor (cartilage-specific proteoglycan core protein, CSPCP or chondroitin sulfates proteoglycan core protein 1).

Examples

EXAMPLE 1 (COMPARATIVE EXAMPLE)

Assay conditions

The catalytic domains of the human recombinant MMP-3 and MMP-8 protein, MMP-3cd and MMP-8cd, can be purchased from, for example, Biomol International L. P. Pennsylvania, USA; catalog number SE-109 and SE-255 respectively. The truncated forms of ADAM-TS1 and ADAM-TS4 protease can likewise be purchased from, for example, Invitek, Gesellschaft für Biotechnik & Biodesign mbH, Berlin, Germany, catalog number 30400402 and 30400102, respectively.

Preparation of the buffers and solutions

TNCB buffer:

100mM tris(hydroxymethyl)aminomethane, adjusted to pH 7.5 with HCl

100mM NaCl

10mM $CaCl_2 \cdot 2H_2O$

0.015% Brij 35

Substrate solution:

10mM substrates (see Table) in DMSO. Immediately before use, 2 μL of the substrate stock solution were diluted with 130 μL of H_2O.

Enzyme solution:

MMP-3cd (2.3 μg/mL), MMP-8cd (0.6 μg/mL), ADAMTS-1 (2.3 μg/mL) and ADAMTS-4 (3.3 μg/mL) were diluted with TNCB buffer.

Assay procedure

10 μL of enzyme solution were mixed with 10 μL of H_2O, and the reaction was started by adding 10 μL of substrate solution.

Fluorometric analysis

The fluorescence was measured in a TECAN Spectrafluor Plus fluorescence apparatus; Exitation/Emission (see Table). This entailed measuring the fluorescence for 5 minutes on each occasion.

Results of comparative example 1

	+ : Increase in the optical signal was observed over 5 minutes					
Fluorimetric substance		$\lambda_{ex}/\lambda_{em}$	ADAMTS-4	ADAMTS-1	MMP-3cd	MMP-8cd
Dnp-P-L-G-L-W-A-R-NH2	Bachem: M-1855	280/355	-	-	+	-
Mca-G-K-P-I-L-F-F-R-L-K-(Dnp)-R-NH2	Sigma: M-0938	330/390	-	-	+	-
Mca-P-L-A-Q-A-V-Dap(Dnp)-R-S-S-S-R-NH2	Bachem: M-2255/ R&D: ES003	330/390	-	-	+	-
Mca-P-L-G-L-Dap(Dnp)-A-R-NH2	Bachem: M-1895	330/390	-	-	+	+
Mca-P-ß-cyclohexyl-A-G-Nva-H-A-Dpa-NH2	Calbiochem: 444235	330/390	-	-	+	+
Mca-R-P-K-P-V-E-Nval-W-R-K-(Dnp)-NH2	Bachem: M-2110/ R&D: ES002	330/390	-	-	+	-
Mca-R-P-L-A-L-W-R-Dap(Dnp)-NH2	Bachem: M-2390	330/390	-	-	+	+
Mca-P-L-A-	Bachem: M-	330/390	-	-	+	+

	+ : Increase in the optical signal was observed over 5 minutes					
C(Mob)-W-A-R-Dap(Dnp)-NH2	2510					
Mca-R-P-K-P-Y-A-Nva-Met-K(Dnp)-NH2	Bachem: M-2105	330/390	-	-	+	+
Mca-P-L-A-Nva-Dap(Dnp)-A-R-NH2	Bachem: M-2520	330/390	-	-	+	+
NBD-eAhx-R-P-K-P-L-A-Nva-W-K(DMACA)-NH2	Bachem: M-2300	350/465	-	-	+	+
Dnp-P-ß-cyclohexyl-A-G-C(Me)-H-A-K-(N-Me-Abz)-NH2	Bachem: M-2055	365/450	-	-	+	+

As expected, and in accordance with the state of the art, the MMP substrates were not converted by ADAMTS.

EXAMPLE 2

Preparation of the buffers and solutions

TNCB buffer (see Example 1)

Enzyme solution:
ADAMTS (Invitek Gesellschaft für Biotechnik & Biodesign mbH, Berlin, Germany)
5 µg of ADAMTS-1 were diluted with 2200 µL, and 5 µg of ADAMTS-4 were diluted with 600 µl of TNCB buffer.

Substrate solution
1) DTNB solution (5,5'-dithiobis(2-nitrobenzoic acid):
A 40 mM stock solution in DMSO was prepared:
Then 27.5 µL of DTNB stock solution were diluted with 522 µL of water.

2) Thiopeptilide solution: (Bachem Distribution Services GmbH, Weil am Rhein, Germany):
A 100 mM stock solution in DMSO was prepared. For use, 55 µL of thiopeptilide stock solution were diluted with 500 µL of TNCB buffer.
Immediately before use, 550 µl of DTNB were mixed with 550 µl of thiopeptilide.

Assay procedure
The measurements were carried out in 96-multiwell plates (half area plates, flat bottom, clear, polystyrene, No.3695) (Corning Costar, Acton, USA).

10 µL of enzyme solution and 10 µL of H_2O were mixed, and the reaction was started by adding 10 µL of substrate solution.

Colorimetric analysis

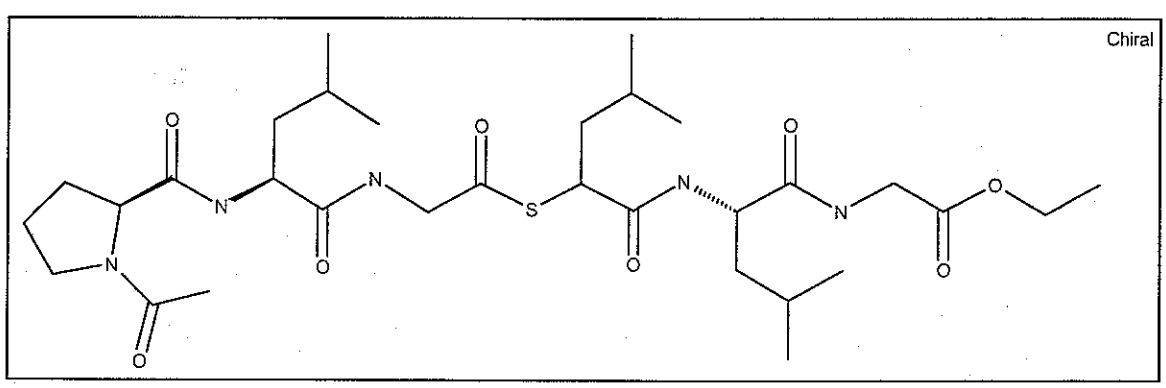
Microtiter plate photometer: Molecular Devices Sunnyvale, USA. SpectraMax 190.
The absorption was observed at a wavelength of 415 nm for 5 min.

Results for Example 2

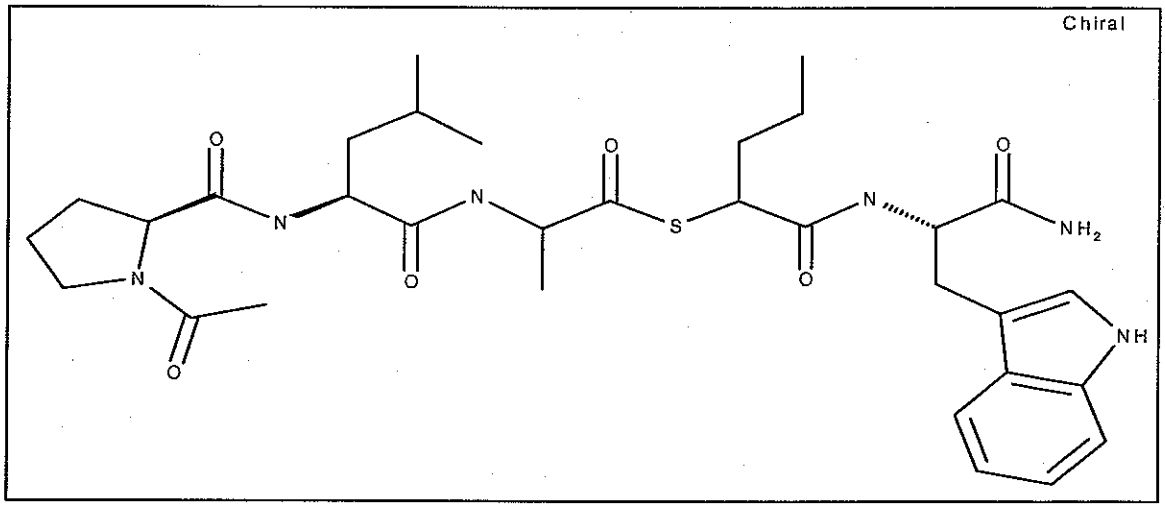
Colorimetric substrates in Ellman's reaction		λ (nm)	ADAMTS-4	ADAMTS-1	MMP-3cd	MMP-8cd
Ac-P-L-G-[(S)-2-mercapto-4-methylpentanoyl]-L-G-OEt	Bachem: H-7145	415	+	+	+	+
Ac-P-L-A-[(S)-2-mercapto-pentanoyl]-W-NH2	Bachem: H-1326	415	-	-	+	+

Compound 1 Bachem: H-7145	Compound 2 (Comparative example) Bachem: H-1326
(Synonyms)	(Synonyms)
Ac-P-L-G-^{S}L-L-G-OEt	Ac-P-L-A-^{S}Nva-W-NH2
Ac-P-L-G-[(S)-2-mercapto-4-methylpentanoyl]-L-G-OEt	Ac-P-L-A-[(S)-2-mercaptopentanoyl]-W-NH2
Ac-P-L-G-Sch[CH2CH(CH3)2]-CO-L-G-OC2H5	Ac-P-L-A-[2-mercaptopentanoyl]-W-NH2
Ac-P-L-G-[2-mercapto-4-methyl-pentanoyl]-L-G-OC2H5	

Structural formula of compound 1



Structural formula of compound 2 (comparative example)



Compound 2 was not converted by ADAMTS. However, surprisingly, compound 1 was converted by ADAMTS.

SEQ ID NO: 1

Xaa-Pro-Xaa-Gly-Xaa-Xaa-Gly-Xaa

SEQ ID NO: 2

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   1 MTTLLWVFVT LRVITAAVTV ETSDHDNSLS VSIPQPSPLR VLLGTSLTIP CYFIDPMHPV
  61 TTAPSTAPLA PRIKWSRVSK EKEVVLLVAT EGRVRVNSAY QDKVSLPNYP AIPSDATLEV
 121 QSLRSNDSGV YRCEVMHGIE DSEATLEVVV KGIVFHYRAI STRYTLDFDR AQRACLQNSA
 181 IIATPEQLQA AYEDGFHQCD AGWLADQTVR YPIHTPREGC YGDKDEFPGV RTYGIRDTNE
 241 TYDVYCFAEE MEGEVFYATS PEKFTFQEAA NECRRLGARL ATTGHVYLAW QAGMDMCSAG
 301 WLADRSVRYP ISKARPNCGG NLLGVRTVYV HANQTGYPDP SSRYDAICYT GEDFVDIPEN
 361 FFGVGGEEDI TVQTVTWPDM ELPLPRNITE GEARGSVILT VKPIFEVSPS PLEPEEPFTF
 421 APEIGATAFA EVENETGEAT RPWGFPTPGL GPATAFTSED LVVQVTAVPG QPHLPGGVVF
 481 HYRPGPTRYS LTFEEAQQAC PGTGAVIASP EQLQAAYEAG YEQCDAGWLR DQTVRYPIVS
 541 PRTPCVGDKD SSPGVRTYGV RPSTETYDVY CFVDRLEGEV FFATRLEQFT FQEALEFCES
 601 HNATATTGQL YAAWSRGLDK CYAGWLADGS LRYPIVTPRP ACGGDKPGVR TVYLYPNQTG
 661 LPDPLSRHHA FCFRGISAVP SPGEEEGGTP TSPSGVEEWI VTQVVPGVAA VPVEEETTAV
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721 PSGETTAILE FTTEPENQTE WEPAYTPVGT SPLPGILPTW PPTGAETEES TEGPSATEVP
781 SASEEPSPSE VPFPSEEPSP SEEPFPSVRP FPSVELFPSE EPFPSKEPSP SEEPSASEEP
841 YTPSPPEPSW TELPSSGEES GAPDVSGDFT GSGDVSGHLD FSGQLSGDRA SGLPSGDLDS
901 SGLTSTVGSG LTVESGLPSG DEERIEWPST PTVGELPSGA EILEGSASGV GDLSGLPSGE
961 VLETSASGVG DLSGLPSGEV LETTAPGVED ISGLPSGEVL ETTAPGVEDI SGLPSGEVLE
1021 TTAPGVEDIS GLPSGEVLET TAPGVEDISG LPSGEVLETT APGVEDISGL PSGEVLETAA
1081 PGVEDISGLP SGEVLETAAP GVEDISGLPS GEVLETAAPG VEDISGLPSG EVLETAAPGV
1141 EDISGLPSGE VLETAAPGVE DISGLPSGEV LETAAPGVED ISGLPSGEVL ETAAPGVEDI
1201 SGLPSGEVLE TAAPGVEDIS GLPSGEVLET AAPGVEDISG LPSGEVLETA APGVEDISGL
1261 PSGEVLETAA PGVEDISGLP SGEVLETTAP GVEEISGLPS GEVLETTAPG VDEISGLPSG
1321 EVLETTAPGV EEISGLPSGE VLETSTSAVG DLSGLPSGGE VLEISVSGVE DISGLPSGEV
1381 VETSASGIED VSELPSGEGL ETSASGVEDL SRLPSGEEVL EISASGFGDL SGVPSGGEGL
1441 ETSASEVGTD LSGLPSGREG LETSASGAED LSGLPSGKED LVGSASGDLD LGKLPSGTLG
1501 SGQAPETSGL PSGFSGEYSG VDLGSGPPSG LPDFSGLPSG FPTVSLVDST LVEVVTASTA
1561 SELEGRGTIG ISGAGEISGL PSSELDISGR ASGLPSGTEL SGQASGSPDV SGEIPGLFGV
1621 SGQPSGFPDT SGETSGVTEL SGLSSGQPGV SGEASGVLYG TSQPFGITDL SGETSGVPDL
1681 SGQPSGLPGF SGATSGVPDL VSGTTSGSGE SSGITFVDTS LVEVAPTTFK EEEGLGSVEL
1741 SGLPSGEADL SGKSGMVDVS GQFSGTVDSS GFTSQTPEFS GLPSGIAEVS GESSRAEIGS
1801 SLPSGAYYGS GTPSSFPTVS LVDRTLVESV TQAPTAQEAG EGPSGILELS GAHSGAPDMS
1861 GEHSGFLDLS GLQSGLIEPS GEPPGTPYFS GDFASTTNVS GESSVAMGTS GEASGLPEVT
1921 LITSEFVEGV TEPTISQELG QRPPVTHTPQ LFESSGKVST AGDISGATPV LPGSGVEVSS
1981 VPESSSETSA YPEAGFGASA APEASREDSG SPDLSETTSA FHEANLERSS GLGVSGSTLT
2041 FQEGEASAAP EVSGESTTTS DVGTEAPGLP SATPTASGDR TEISGDLSGH TSQLGVVIST
2101 SIPESEWTQQ TQRPAETHLE IESSSLLYSG EETHTVETAT SPTDASIPAS PEWKRESEST
2161 AAAPARSCAE EPCGAGTCKE TEGHVICLCP PGYTGEHCNI DQEVCEEGWN KYQGHCYRHF
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2281 MQFENWRPNQ PDNFFAAGED CVVMIWHEKG EWNDVPCNYH LPFTCKKGTV ACGEPPVVEH
2341 ARTFGQKKDR YEINSLVRYQ CTEGFVQRHM PTIRCQPSGH WEEPRITCTD ATTYKRRLQK
2401 RSSRHPRRSR PSTAH

Claims:

1. The use of a thiopeptolide of the formula

$R\text{-}(Xaa)_n\text{-}Pro\text{-}X\text{-}Gly\text{-}S\text{-}Y\text{-}Z\text{-}Gly\text{-}(Xaa)_m\text{-}R_1$ (I),

where R is H or an N-protective group, preferably a carboxyl group, in particular of C_1-C_5-alkyls, especially of C_1-C_3-alkyls, particularly preferably an acetyl group,

Xaa is any amino acid,

n, m is identically or differently an integer from 0-2415, preferably from 0-35, in particular from 0-14, especially from 0-11 and very particularly preferably equal to 0,

X is Leu, Ile, Phe, Val, Gln, Ala,

Z is Leu, Ile, Phe, Val, Gln, Ala,

R_1 is terminal amide, carboxyl or ester group, preferably of C_1-C_5-alkyls, especially of C_1-C_3-alkyls, in particular an ethyl ester,



S-Y = R_2-CH-COO-,

where R_2 is the side chain of a naturally occurring amino acid, in particular

$-CH_2CH(CH_3)_2$,

$-CH(CH_3)C_2H_5$,

$-CH_2C_6H_5$,

$-CH(CH_3)_2$, or

$-CH_3$,

or a salt thereof,

as a substrate for an ADAM-TS protease.

2. The use as claimed in claim 1, characterized in that
X = Leu or Ala,
Z = Leu, Ala or Phe, and
$R_2 = -CH_2CH(CH_3)_2$.

3. The use as claimed in claim 1 or 2, characterized in that $(Xaa)_n$ and/or $(Xaa)_m$ is the amino acid sequence shown in SEQ ID NO: 2.

4. The use as claimed in claim 1, characterized in that the thiopeptolide has the following structure:

Ac-Pro-Leu-Gly-S-Y-Leu-Gly-OC_2H_5,
in which $R_2 = CH_2CH(CH_3)_2$ and Ac is an acetyl group.

5. The use as claimed in claim 1, characterized in that the thiopeptolide has the following structure:

Ac-Pro-Leu-Gly-S-Y-Phe-Gly-OC_2H_5,
in which $R_2 = CH_2CH(CH_3)_2$ and Ac is an acetyl group.

6. The use as claimed in claim 1, characterized in that the thiopeptolide has the following structure:

Ac-Pro-Ala-Gly-S-Y-Phe-Gly-OC_2H_5,
in which $R_2 = CH_2CH(CH_3)_2$ and Ac is an acetyl group.

7. The use as claimed in claim 1, characterized in that the thiopeptolide has the following structure:

Ac-Pro-Ala-Gly-S-Y-Ala-Gly-OC_2H_5,
in which $R_2 = CH_2CH(CH_3)_2$ and Ac is an acetyl group.

8. The use as claimed in at least one of claims 1-7, characterized in that the ADAM-TS protease is an ADAM-TS protease 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 , 17, 18, 19 and/or 20.

9. The use as claimed in at least one of claims 1-8, characterized in that the ADAM-TS protease is an ADAM-TS protease 1, 4, 5 , 11 and/or 13, preferably an ADAM-TS protease 1, ADAM-TS protease 4 or ADAM-TS protease 5.

10. The use as claimed in at least one of claims 1-9 for determining the activity of an ADAM-TS protease, for purifying an ADAM-TS protease, for functional cloning of a nucleotide sequence coding for an ADAM-TS protease, for finding an ADAM-TS protease modulator, in particular an ADAM-TS protease inhibitor, or for observing the onset or progress of a disease associated with damaged tissue matrix, in particular osteoarthritis, rheumatism, cancer, inflammations, angiogenesis, cell migration, blood clotting and/or blood coagulation, especially osteoarthritis, rheumatism or cancer.

11. A method for determining the activity of an ADAM-TS protease, characterized in that the method comprises the following steps:
(a) incubating an ADAM-TS protease with a thiopeptolide substrate according to formula I as defined in any of claims 1-7, and
(b) carrying out an activity measurement or determination of the ADAM-TS protease.

12. The method as claimed in claim 11, characterized in that the ADAM-TS protease is an ADAM-TS protease 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 , 17, 18, 19 and/or 20.

13. The method as claimed in claim 11 or 12, characterized in that the ADAM-TS protease is an ADAM-TS protease 1, 4, 5 , 11 and/or 13, preferably an ADAM-TS protease 1, ADAM-TS protease 4 or ADAM-TS protease 5.

14. The method as claimed in at least one of claims 11-13, characterized in that the activity of the ADAM-TS protease is measured or determined by spectrophotometry.

15. The method as claimed in claim 14, characterized in that an ADAM-TS protease is measured or determined in the presence of a detection reagent for thiol groups, preferably an iodoacetamide, in particular 5-iodoacetamidofluorescein (5-IAF), a maleimide, in particular fluorescein-5-maleimide, or N,N'-didansyl-L-cystine, 5-(bromomethyl)fluorescein or in particular 4,4'-dithiodipyridine or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).

16. A method for finding an ADAM-TS protease modulator, in particular an ADAM-TS protease inhibitor, characterized in that the method comprises the following steps:
(a) incubating an ADAM-TS protease with a thiopeptolide substrate according to formula I as defined in any of claims 1-7 in the presence of a test compound and
(b) measuring or determining the influence of the test compound on the activity of the ADAM-TS protease.

17. The method as claimed in claim 16, characterized in that the ADAM-TS protease is an ADAM-TS protease 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 , 17, 18, 19 and/or 20.

18. The method as claimed in claim 16 or 17, characterized in that the ADAM-TS protease is an ADAM-TS protease 1, 4, 5 , 11 and/or 13, preferably an ADAM-TS protease 1, ADAM-TS protease 4 or ADAM-TS protease 5.

19. The method as claimed in at least one of claims 16-18, characterized in that the activity of the ADAM-TS protease is measured or determined by spectrophotometry.

20. The method as claimed in claim 19, characterized in that an ADAM-TS protease is measured or determined in the presence of a detection reagent for thiol groups, preferably an iodoacetamide, in particular 5-iodoacetamidofluorescein (5-IAF), a maleimide, in particular fluorescein-5-maleimide, or N,N'-didansyl-L-cystine, 5-(bromomethyl)fluorescein or in particular 4,4'-dithiodipyridine or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).

21. The method as claimed in at least one of claims 16-20, characterized in that the test compound is made available in the form of a chemical compound library.

22. The method as claimed in at least one of claims 16-21, characterized in that the method is carried out on an array.

23. The method as claimed in at least one of claims 16-22, characterized in that the method is carried out by means of a robot.

24. The method as claimed in at least one of claims 16-23, characterized in that the method is carried out with the aid of microfluidic technology.

25. The method as claimed in at least one of claims 16-24, characterized in that the method is a high-throughput screening for an ADAM-TS protease modulator, in particular an ADAM-TS protease inhibitor.

26. A method for manufacturing a medicament for the treatment of a disease which is associated with damaged tissue matrix, in particular osteoarthritis, rheumatism, cancer, inflammations, angiogenesis, cell migration, blood clotting and/or blood coagulation, in particular osteoarthritis, rheumatism or cancer, characterized in that the method comprises the following steps:

(a) carrying out a method as claimed in any of claims 16-25,

(b) isolating a test substance found to be suitable in step (a), and

(c) formulating the test substance isolated in step (b) with one or more pharmaceutically acceptable carriers or adjuvants.

27. A kit comprising a thiopeptolide substrate according to formula I as defined in any of claims 1-7, an ADAM-TS protease and where appropriate one or more buffers.

28. The kit as claimed in claim 27, characterized in that the ADAM-TS protease is an ADAM-TS protease 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 , 17, 18, 19 and/or 20.

29. The kit as claimed in claim 27 or 28, characterized in that the ADAM-TS protease is an ADAM-TS protease 1, 4, 5, 11 and/or 13, preferably an ADAM-TS protease 1, ADAM-TS protease 4 or ADAM-TS protease 5.

30. The kit as claimed in at least one of claims 27-29, characterized in that at least one detection reagent for thiol groups, preferably an iodoacetamide, in particular 5-iodoacetamidofluorescein (5-IAF), a maleimide, in particular fluorescein-5-maleimide, or N,N'-didansyl-L-cystine, 5-(bromomethyl)fluorescein or in particular 4,4'-dithiodipyridine or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), is additionally present.

SEQUENCE LISTING

<110> Sanofi-Aventis Deutschland GmbH

<120> Use of thiopeptolides for determining the activity of ADAM-TS proteases

<130> DE 2005/006

<140>
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<170> PatentIn Ver. 2.1

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<213> Artificial sequence

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<213> Homo sapiens

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35 40 45

Ile Pro Cys Tyr Phe Ile Asp Pro Met His Pro Val Thr Thr Ala Pro
50 55 60

Ser Thr Ala Pro Leu Ala Pro Arg Ile Lys Trp Ser Arg Val Ser Lys
65 70 75 80

Glu Lys Glu Val Val Leu Leu Val Ala Thr Glu Gly Arg Val Arg Val
85 90 95

Asn Ser Ala Tyr Gln Asp Lys Val Ser Leu Pro Asn Tyr Pro Ala Ile
100 105 110

Pro Ser Asp Ala Thr Leu Glu Val Gln Ser Leu Arg Ser Asn Asp Ser
115 120 125

Gly Val Tyr Arg Cys Glu Val Met His Gly Ile Glu Asp Ser Glu Ala
130 135 140

Thr Leu Glu Val Val Val Lys Gly Ile Val Phe His Tyr Arg Ala Ile
145 150 155 160

Ser Thr Arg Tyr Thr Leu Asp Phe Asp Arg Ala Gln Arg Ala Cys Leu
165 170 175

Gln Asn Ser Ala Ile Ile Ala Thr Pro Glu Gln Leu Gln Ala Ala Tyr
180 185 190

Glu Asp Gly Phe His Gln Cys Asp Ala Gly Trp Leu Ala Asp Gln Thr
195 200 205

Val Arg Tyr Pro Ile His Thr Pro Arg Glu Gly Cys Tyr Gly Asp Lys
210 215 220

Asp Glu Phe Pro Gly Val Arg Thr Tyr Gly Ile Arg Asp Thr Asn Glu
225 230 235 240

Thr Tyr Asp Val Tyr Cys Phe Ala Glu Glu Met Glu Gly Glu Val Phe
245 250 255

Tyr Ala Thr Ser Pro Glu Lys Phe Thr Phe Gln Glu Ala Ala Asn Glu
260 265 270

Cys Arg Arg Leu Gly Ala Arg Leu Ala Thr Thr Gly His Val Tyr Leu
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Arg Ser Val Arg Tyr Pro Ile Ser Lys Ala Arg Pro Asn Cys Gly Gly
305 310 315 320

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Asn Leu Leu Gly Val Arg Thr Val Tyr Val His Ala Asn Gln Thr Gly
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            340                 345                 350

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        355                 360                 365

Asp Ile Thr Val Gln Thr Val Thr Trp Pro Asp Met Glu Leu Pro Leu
    370                 375                 380

Pro Arg Asn Ile Thr Glu Gly Glu Ala Arg Gly Ser Val Ile Leu Thr
385                 390                 395                 400

Val Lys Pro Ile Phe Glu Val Ser Pro Ser Pro Leu Glu Pro Glu Glu
                405                 410                 415

Pro Phe Thr Phe Ala Pro Glu Ile Gly Ala Thr Ala Phe Ala Glu Val
            420                 425                 430

Glu Asn Glu Thr Gly Glu Ala Thr Arg Pro Trp Gly Phe Pro Thr Pro
        435                 440                 445

Gly Leu Gly Pro Ala Thr Ala Phe Thr Ser Glu Asp Leu Val Val Gln
    450                 455                 460

Val Thr Ala Val Pro Gly Gln Pro His Leu Pro Gly Gly Val Val Phe
465                 470                 475                 480

His Tyr Arg Pro Gly Pro Thr Arg Tyr Ser Leu Thr Phe Glu Glu Ala
                485                 490                 495

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Trp Leu Ala Asp Gly Ser Leu Arg Tyr Pro Ile Val Thr Pro Arg Pro
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                645                 650                 655

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            660                 665                 670

Phe Arg Gly Ile Ser Ala Val Pro Ser Pro Gly Glu Glu Glu Gly Gly
        675                 680                 685

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    690                 695                 700

Val Pro Gly Val Ala Ala Val Pro Val Glu Glu Glu Thr Thr Ala Val
705                 710                 715                 720

Pro Ser Gly Glu Thr Thr Ala Ile Leu Glu Phe Thr Thr Glu Pro Glu
                725                 730                 735

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Leu Pro Gly Ile Leu Pro Thr Trp Pro Pro Thr Gly Ala Glu Thr Glu
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    770                 775                 780

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785                 790                 795                 800

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865                 870                 875                 880

Phe Ser Gly Gln Leu Ser Gly Asp Arg Ala Ser Gly Leu Pro Ser Gly
                885                 890                 895

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            900                 905                 910

Val Glu Ser Gly Leu Pro Ser Gly Asp Glu Glu Arg Ile Glu Trp Pro
        915                 920                 925

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    930                 935                 940

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