

DESCRIPTION

Title of the Invention: ANTI-CHYMASE APTAMER AND USE FOR SAME
[Technical Field]
[0001]

5 The present invention relates to an aptamer against
chymase, a method of utilizing the same and the like.
[Background Art]
[0002]

Human chymase (EC.3.4.21.39), a chymotrypsin-like serine
10 protease, is stored in mast cell secretory granules. Upon
external stimulation, mast cells undergo degranulation,
resulting in the release of human chymase, along with a wide
variety of inflammation mediators, outside the cells. The
released human chymase specifically recognizes aromatic amino
15 acids contained in substrate proteins and peptides, such as
phenylalanine and tyrosine, and cleaves the peptide bonds
adjoining to the amino acids. A representative substrate for
human chymase is angiotensin I (AngI). Human chymase cleaves
AngI to produce angiotensin II (AngII), a vasoconstricting
20 factor.

Mammalian chymases are phylogenetically classified under
two subfamilies: α and β . Primates, including humans, express
only one kind of chymase, which belongs to the α family.
Meanwhile, rodents express both the α and β families of chymase.
25 In mice, there are a plurality of kinds of chymases, of which
mouse mast cell protease-4 (mMCP-4), which belongs to the β
family, is considered to be most closely related to human
chymase, judging from its substrate specificity and mode of
expression in tissue. In hamsters, hamster chymase-1, also a
30 member of the β family, corresponds to human chymase.
Meanwhile, mMCP-5 and hamster chymase-2, which belong to the α
family as with human chymase, possess elastase-like activity
and differ from human chymase in terms of substrate specificity.

Chymase is profoundly associated with the activation of
35 transforming growth factor β (TGF- β). TGF- β exists in a latent

form (latent-TGF- β) in extracellular matrices around epithelial cells and endothelial cells, and is retained in extracellular matrices via large latent TGF- β binding protein (LTBP). TGF- β is released from extracellular matrices as required and
5 activated, and the activated TGF- β is a cytokine of paramount importance to living organisms reportedly involved in cell proliferation and differentiation and tissue repair and regeneration after tissue injury. Collapse of its signal leads to the onset and progression of a wide variety of diseases. It
10 is thought that in this process, chymase is involved in the release of latent TGF- β from extracellular matrices and the conversion of latent TGF- β to active TGF- β .

Chymase is known to be associated with a broad range of diseases, including fibrosis, cardiovascular diseases,
15 inflammation, allergic diseases and organ adhesion. Fibrosis is an illness characterized by abnormal metabolism of extracellular substrates in the lung, heart, liver, kidney, skin and the like, resulting in excess deposition of connective tissue proteins. In pulmonary fibrosis, for example,
20 connective tissue proteins such as collagen deposit in excess in the lung, resulting in hard shrinkage of pulmonary alveoli and ensuing respiratory distress. Lung fibrosis has been shown to result from pneumoconiosis, which is caused by exposure to a large amount of dust, drug-induced pneumonia, which is caused
25 by use of drugs such as anticancer agents, allergic pneumonia, pulmonary tuberculosis, autoimmune diseases such as collagen disease, and the like. However, there are not a few cases in which the cause is unknown.

The mechanism of onset of fibrosis at the molecular level
30 has not been elucidated well. Generally, in normal states, the proliferation and functions of fibroblasts are well controlled. In case of serious or persistent inflammation or injury, however, the tissue repair mechanism works in excess, resulting in abnormal proliferation of fibroblasts and overproduction of
35 connective tissue proteins. TGF- β is known as a factor that

causes these phenomena. As evidence suggestive of its involvement, it has been reported that administration of an anti-TGF- β neutralizing antibody to an animal model of fibrosis causes decreased collagen expression and significantly
5 suppressed fibrosis. In patients with idiopathic pulmonary fibrosis, increased levels of TGF- β and elevated counts of chymase-positive mast cells are observed.

Meanwhile, association of chymase in fibrosis has been demonstrated by experiments using animal models. In a hamster
10 model of bleomycin-induced pulmonary fibrosis, facilitated chymase activity, increased expression of collagen III mRNA, tissue fibrosis and other phenomena are significantly reduced by chymase inhibitors. The same effects have been observed for a mouse model of bleomycin-induced pulmonary fibrosis;
15 administration of chymase inhibitors suppressed chymase activity and reduced hydroxyproline content.

With these features, chymase inhibitors can be used as prophylactic or therapeutic drugs for diseases related to chymase, such as fibrosis. Chymase inhibitors that have been
20 developed include small molecular compounds such as TPC-806, SUN-13834, SUN-C8257, SUN-C8077, and JNJ-10311795 (Patent document 1).

[0003]

In recent years, applications of RNA aptamers to
25 therapeutic drugs, diagnostic reagents, and test reagents have been drawing attention; some RNA aptamers have already been in clinical study stage or in practical use. In December 2004, the world's first RNA aptamer drug, Macugen, was approved as a therapeutic drug for age-related macular degeneration in the US.
30 An RNA aptamer refers to an RNA that binds specifically to a target molecule such as a protein, and can be prepared using the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) method (Patent documents 2-4). In the SELEX method, an RNA that binds specifically to a target molecule is selected
35 from an RNA pool with about 10^{14} different nucleotide sequences.

The RNA used has a random sequence of about 40 nucleotides, which is flanked by primer sequences. This RNA pool is mixed with a target molecule, and only the RNA that has bound to the target molecule is separated using a filter and the like. The
5 RNA separated is amplified by RT-PCR, and this is used as a template for the next round. By repeating this operation about 10 times, an RNA aptamer that binds specifically to the target molecule can be acquired.

Aptamer drugs, like antibody drugs, can target
10 extracellular proteins. With reference to many scientific papers and other reference materials in the public domain, aptamer drugs are judged to potentially surpass antibody drugs in some aspects. For example, aptamers often exhibit higher affinity and specificity for target molecules than do
15 antibodies. Aptamers are unlikely to undergo immune elimination, and adverse reactions characteristic of antibodies, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), are reportedly unlikely to occur with the use of aptamers. From the viewpoint
20 of drug delivery, aptamers are likely to migrate to tissues because of their molecular size of about one-tenth that of antibodies, enabling easier drug delivery to target sites. Because aptamers are produced by chemical synthesis, they permit site-selective chemical modifications, and enable cost
25 reduction by mass-production. Other advantages of aptamers include long-term storage stability, heat resistance and solvent resistance. Meanwhile, the blood half-lives of aptamers are generally shorter than those of antibodies; however, this property is sometimes advantageous in view of
30 toxicity. These facts lead to the conclusion that even when the same molecule is targeted, aptamer drugs potentially surpass antibody drugs.

As an aptamer that binds to chymase to inhibit a chymase activity, the present inventors produced an aptamer containing
35 a nucleotide sequence represented by $X_1GAUAGAN_1N_2UAAX_2$ (each of

X₁ and X₂, whether identical or not, is A or G, and each of N₁ and N₂, whether identical or not, is A, G, C, U or T) (patent document 5 (the sequence is shown by SEQ ID NO: 21 in patent document 5)).

5 [Document List]

[Patent documents]

[0004]

patent document 1: US-B-6500835

patent document 2: WO 91/19813

10 patent document 3: WO 94/08050

patent document 4: WO 95/07364

patent document 5: WO 2010/143714

[SUMMARY OF THE INVENTION]

[Problems to be Solved by the Invention]

15 [0005]

The present invention is directed to providing an aptamer for chymase and a method for utilizing the same, and the like.

[Means of Solving the Problems]

[0006]

20 The present inventors have conducted intensive studies in an attempt to solve the aforementioned problems and succeeded in preparing an aptamer that has good quality for chymase, has a sequence completely different from that of the chymase aptamer described in patent document 5, and is characterized by
25 markedly high activity, which resulted in the completion of the present invention.

[0007]

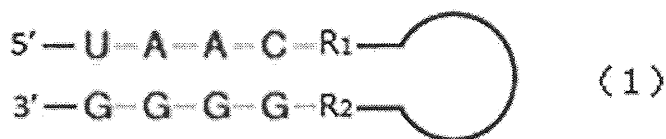
Accordingly, the present invention provides the following:

30 [1] An aptamer that binds to chymase, and comprises a common sequence represented by UAACR₁N₁R₂G GGG wherein R₁ and R₂ are each any one base, and N₁ shows 3 to 30 bases (uracil is optionally thymine).

[2] An aptamer that binds to chymase, and comprises a potential
35 secondary structure represented by the formula (1) (uracil is

optionally thymine):

[0008]



[0009]

5 wherein

[0010]



[0011]

in the formula (1) is a stem-loop partial structure, and R₁ and
10 R₂ are each any one base.

[3] The aptamer of [1] or [2] that inhibits the activity of
chymase.

[4] The aptamer of any of [1] to [3], wherein a combination of
R₁ and R₂ is A/U, C/G, A/C or G/U (uracil is optionally
15 thymine).

[5] The aptamer of any of [1] to [4], wherein at least one of
pyrimidine nucleotides is modified or altered.

[6] The aptamer of any of [1] to [5], wherein U and/or C in the
sequence UAACR₁ are/is modified or altered.

20 [7] The aptamer of any of [2] to [6], wherein the stem-loop
part of (1') has a base length of 3 - 21.

[8] The aptamer of any of [1] to [7], wherein a sequence of N₁
or the stem-loop partial structure represented by

[0012]



25

[0013]

is UUGU, CUGG or AAUU (uracil is optionally thymine).

[0014]

[9] The aptamer of [1] or [2], comprising any of the nucleotide sequences of the following (a), (b) and (c):

(a) a nucleotide sequence selected from any of SEQ ID NOs: 1 -
5 11 and 14 - 33 (uracil is optionally thymine);

(b) a nucleotide sequence selected from any of SEQ ID NOs: 1 -
11 and 14 - 33 (uracil is optionally thymine) wherein one to
several nucleotides are substituted, deleted, inserted or
added; and

10 (c) a nucleotide sequence having identity of not less than 70%
with a nucleotide sequence selected from any of SEQ ID NOs: 1 -
11 and 14 - 33 (uracil is optionally thymine).

[10] The aptamer of [9], wherein at least one nucleotide
contained in the aptamer is modified or altered.

15 [11] The aptamer of [1] or [2], wherein a hydroxyl group at the
2'-position of ribose of each pyrimidine nucleotide contained
in the aptamer is the same or different and unsubstituted or
substituted by an atom or a group selected from the group
consisting of a hydrogen atom, a fluorine atom and a methoxy
20 group.

[12] The aptamer of [1] or [2], wherein the hydroxyl group at
the 2'-position of ribose of each purine nucleotides contained
in the aptamer is the same or different and unsubstituted or
substituted by an atom or a group selected from the group
25 consisting of a hydrogen atom, a fluorine atom and a methoxy
group.

[13] A complex comprising the aptamer and the functional
substance of any of [1] to [12].

[14] A medicament comprising the aptamer of any of [1] to [12]
30 or the complex of [13].

[15] The medicament of [14] that is a therapeutic drug for a
disease involving organ or tissue fibrosis, or a circulatory
disease.

[16] The medicament of [14] that is a therapeutic drug for
35 fibrosis.

[17] A method for detecting chymase, comprising using the aptamer of any of [1] to [12] or the complex of [13].

[18] A method for treating a disease involving organ or tissue fibrosis, or a circulatory disease, comprising administering
5 the aptamer of any of [1] to [12], the complex of [13], or the medicament of [14] to a target.

[19] The aptamer of any of [1] to [12], the complex of [13], or the medicament of [14] for use in the treatment of a disease involving organ or tissue fibrosis, or a circulatory disease.

10 [20] Use of the aptamer of any of [1] to [12] or the complex of [13] for the production of a medicament for a disease involving organ or tissue fibrosis, or a circulatory disease.

[Effect of the Invention]

[0015]

15 The aptamer or the complex of the present invention can be useful as a pharmaceutical or a diagnostic reagent or a reagent for various diseases caused by chymase, such as fibrosis and cardiovascular diseases. The aptamer or the complex of the present invention can also be useful in
20 purifying and concentrating chymase, and detecting and quantifying chymase.

[Brief Description of the Drawings]

[0016]

Fig. 1 shows the predicted secondary structures of the
25 aptamers of the sequences shown by SEQ ID NO: 1 - 6.

Fig. 2 shows the predicted secondary structures of the aptamers of the sequences shown by SEQ ID NO: 7 - 8.

Fig. 3 shows the predicted secondary structures of the aptamers of the sequences shown by SEQ ID NO: 9 - 10.

30 Fig. 4 shows the predicted secondary structures of the aptamers of the sequences shown by SEQ ID NO: 14 - 18, 20.

Fig. 5 shows random sequence parts of DNA templates in optimized SELEX.

Fig. 6 shows the predicted secondary structures of the
35 aptamers of the sequences shown by SEQ ID NO: 26 - 31.

Fig. 7 shows the predicted secondary structures of the aptamers of the sequences shown by SEQ ID NO: 32 - 33.

[Description of Embodiments]

[0017]

5 The present invention provides an aptamer possessing a binding activity for chymase. The aptamers of the present invention are capable of inhibiting activities of chymase.

[0018]

10 An aptamer refers to a nucleic acid molecule having a binding affinity for a particular target molecule. The aptamer can also inhibit the activity of a particular target molecule by binding to the particular target molecule. The aptamer of the present invention possesses binding activity for chymase, and is capable of inhibiting a chymase activity. The aptamer
15 of the present invention can be an RNA, a DNA, a modified nucleic acid or a mixture thereof. The aptamer of the present invention can also be in a linear or circular form.

[0019]

20 Chymase, a publicly known serine protease, is stored in mast cell secretory granules. Chymase is profoundly involved in a wide variety of biological reactions mediated by mast cells, including, for example, bioactive peptide production and degradation, extracellular matrix remodeling, networks with cytokines, and immunity. The aptamer of the present invention
25 can exhibit inhibitory activity against chymase derived from any mammals. Such mammals include primates (e.g., humans, monkeys), rodents (e.g., mice, rats, guinea pigs, hamsters), and companion animals, domesticated animals and work animals (e.g., dogs, cats, horses, bovines, goat, sheep, pigs), with
30 preference given to humans. The amino acid sequence of human chymase is shown by Accession Number AAB26828, and may be a sequence having one to several mutated residues, a domain moiety thereof, or a peptide moiety thereof. The structure of human chymase may be not only a monomer, but also a dimer or
35 polymer.

[0020]

The aptamer of the present invention binds to chymase in physiological buffer solutions. Although there is no limitation on the choice of buffer solution, preference is given to buffer solutions having a pH of about 5.0-10.0. Such buffer solutions include, for example, the solution A and solution C described below (see Examples 1 and 2). The aptamer of the present invention binds to chymase at strength detectable by any one of the tests described below.

10 Binding strength is measured using Biacore T100 (manufactured by GE Healthcare). In a method of measurement, the aptamer is first immobilized onto a sensor chip, the amount immobilized being about 1000 RU. 20 μ L of a chymase solution for analyte, prepared at 0.2 μ M, is injected, and the binding of chymase to the aptamer is detected. An RNA comprising a random nucleotide sequence of 30 or 40 nucleotides is used as a negative control. If the chymase binds to the aptamer equivalently or significantly more potently compared with the control RNA, the aptamer is judged to have the capability of binding to chymase.

In another method, chymase is first immobilized onto a sensor chip, the amount immobilized being about 4000 RU. 20 μ L of an aptamer solution for analyte, prepared at 0.01 μ g/ μ L, is injected, and the binding of the aptamer to chymase is detected. An RNA containing a random nucleotide sequence of 30 or 40 nucleotides is used as a negative control. If the chymase binds to the aptamer equivalently or significantly more potently compared with the control RNA, the aptamer is judged to have the capability of binding to chymase.

30 [0021]

An inhibitory activity against chymase means an inhibitory potential against any activities possessed by chymase. For example, enzyme activity to hydrolyze and cleave peptide chains, which is one of the functions of chymase, is inhibited. Acceptable substrates for enzyme activity are not

limited to proteins and bioactive peptides present in living organisms (e.g., AngI, latent TGF- β and the like), but include peptides, containing partial amino acid sequences of the foregoing peptides, conjugated with chromogenic substance or
5 fluorescent substance. Chromogenic substances and fluorescent substances are known to those skilled in the art. Phenomena that occur via protein or bioactive peptide degradation reactions include increased expression of collagen I/III, increased hydroxyproline content, increased expression of IgE
10 and the like; suppressive effects thereon are also included in the inhibitory activities against chymase. In addition, inhibitory activities against the migration of neutrophils, monocytes, and eosinophils to chymase is also included in inhibitory activity against chymase. Furthermore, suppressive
15 effects against chymase-induced histamine release promotion, mast cell count elevation, increased vascular permeability, tissue fibrosis, inflammation, angiogenesis, vascular intimal thickening and the like are also included in the inhibitory activities against chymase.

20 [0022]

A substrate for chymase means a peptide, protein or the like that undergoes hydrolytic cleavage by chymase. Substrates for chymase known to exist in living organisms include peptides and proteins such as AngI, latent TGF- β , stem cell factor (SCF),
25 procollagen, procollagenase, fibronectin, promatrix metalloprotease-1 (pro-MMP-1), pro-MMP-9, tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), apolipoprotein A-I (apoA-I), apoE, phospholipid transfer protein, IL-1 β precursor, big-endothelin-1 (big-ET-1), big-ET-2, connective tissue-activating
30 peptide III, IL-18 precursor, substance P, vasoactive intestinal peptide (VIP), kallidin, bradykinin, and C3a. Herein, chymase substrates are not limited to them, but include artificially designed model peptides comprising amino acid residues specifically recognized by chymase, such as Phe and
35 Tyr, as well as these peptides conjugated with chromogenic

substance or fluorescent substance.

[0023]

Whether an aptamer inhibits the enzyme activity of chymase can be determined by, for example, the two testing
5 methods described below.

[0024]

A first method employs a synthetic substrate. A useful chymase substrate is Suc-Ala-Ala-Pro-Phe-MCA (4-methylcoumaryl-7-amide group) (manufactured by Peptide Institute, Inc.), which
10 contains the 4-amino acid peptide "Ala-Ala-Pro-Phe" (SEQ ID NO: 41), a standard substrate for chymotrypsin-like proteases.

[0025]

The assay is performed using a 96-well plate (F16 Black Maxisorp Fluoronunc, manufactured by Nunc), with a reaction
15 mixture volume of 100 μ L, in a buffer solution (solution C; see Example 2 below). First, each nucleic acid is prepared in solution C to obtain 50 μ L solutions. After 10 μ L of the 1 mM substrate prepared in solution C is added, the plate is set to the microplate reader SpectraMax190 (manufactured by Molecular
20 Devices Corporation), and incubated at 37°C for 5 minutes. Separately, 0.05 μ g of chymase (recombinant, manufactured by SIGMA) is diluted in solution C, and 40 μ L of this dilution is incubated at 37°C for 5 minutes. The chymase solution is added to the mixture of the nucleic acid and substrate to initiate an
25 enzyme reaction. The plate containing the reaction mixture is set to the microplate reader SpectraMax190 (manufactured by Molecular Devices Corporation), and examined for time-dependent changes in the fluorescence intensity at 37°C for 5 minutes (excitation wavelength 380 nm, detection wavelength 460 nm). A
30 linear approximation of the increase in the fluorescence of the AMC (7-amino-4-methylcoumarine) released from the substrate by chymase activity is generated, and its slope is taken as the initial reaction velocity. For control, samples are treated and analyzed in the same manner in two cases: use of a nucleic
35 acid pool containing a random sequence of 30 or 40 nucleotides

(negative control), and use of chymostatin, a known chymotrypsin-like serine protease inhibitor (positive control). Taking the initial reaction velocity without the nucleic acid and inhibitor as a 100% enzyme activity, the inhibitory rate
5 for each test substance is calculated, and the inhibitor concentration required to cause a 50% inhibition of the enzyme activity (IC_{50}) is determined. An aptamer exhibiting a lower IC_{50} value than that of chymostatin, a known inhibitor, is judged to possess excellent inhibitory activity.

10 [0026]

A second method of evaluation employs a native substrate. A useful native substrate for chymase is angiotensin I. Here, His-Leu, a peptide fragment released upon degradation of angiotensin I, is fluorescently derivatized, and the
15 fluorescence intensity therefrom is quantified.

[0027]

In the assay, an enzyme reaction is carried out in 50 μ L of solution C. First, 0.3-0.75 ng of chymase (recombinant, manufactured by SIGMA; or native, manufactured by Calbiochem)
20 is diluted in solution C to obtain 5 μ L chymase solution. Each nucleic acid is prepared in solution C to obtain 25 μ L solutions. 5 μ L of each chymase dilution and 25 μ L of each nucleic acid solution are mixed, and then the mixture is incubated at 37°C for 5 minutes. Separately, 20 μ L of 125 μ M
25 angiotensin I (manufactured by Peptide Institute, Inc.) is prepared in solution C and incubated at 37°C for 5 minutes. The angiotensin I solution is added to the mixture of chymase and nucleic acid, and the enzyme reaction is initiated. After the reaction was allowed to proceed at 37°C for 90 minutes, 25
30 μ L of ice-cooled 30% trichloroacetic acid solution is added to terminate the reaction. The entire mixture is centrifuged at 4°C, 14000 rpm for 10 minutes, and 30 μ L of the resulting supernatant is used for the subsequent reaction for fluorescent derivatization.

35 [0028]

After 30 μ L of the supernatant is added to a 96-well plate (Black, manufactured by Costar), 15 μ L of 2% o-phthalaldehyde (manufactured by SIGMA) solution in methanol and 170 μ L of 0.3M NaOH solution are mixed in each well, and the plate is allowed to stand at room temperature for 10 minutes. Subsequently, 25 μ L of 3M HCl solution is added to terminate the reaction. The plate is set to the microplate reader SpectraMax190 (manufactured by Molecular Devices Corporation), and the fluorescence intensity is determined at an excitation wavelength of 355 nm and a fluorescence wavelength of 460 nm. [0029]

For control, samples are treated and analyzed in the same manner in two cases: use of SEQ ID NO: 58 (negative control) and use of chymostatin, a known chymotrypsin-like serine protease inhibitor (positive control). In both cases, fluorescence intensity obtained at 0 minute reaction time serves as a blank determination. Taking the fluorescence intensity detected with the addition of an equal volume of solution C, in place of each nucleic acid, in the chymase enzyme reaction as 100%, the inhibitory rate for each test substance is calculated, and the inhibitor concentration required to cause a 50% inhibition of the enzyme activity (IC_{50}) is determined. An aptamer exhibiting a lower IC_{50} value than that of chymostatin, a known inhibitor, is judged to possess excellent inhibitory activity. [0030]

As shown in detail in the below-mentioned Examples, the aptamer of the present invention has about 10 to 100 times higher inhibitory activity against chymase, as compared to the aptamer against chymase that has already been reported in WO 2010/143714. To be specific, while the aptamer against chymase disclosed in WO 2010/143714 is considered a superior aptamer since it shows an IC_{50} value of not more than 0.1 μ M (see paragraph [0105] and the like), many of the aptamers of the present invention showed an IC_{50} value of not more than 1 nM as

shown in Example 3 (Table 4).

Therefore, the inventors did not expect when the present invention was completed that such an aptamer that has good quality for chymase was obtained. From the above, it can be
5 said that the aptamer of the present invention affords advantageous effects that cannot be easily envisaged from known documents even by those of ordinary skill in the art.
[0031]

There is no limitation on the aptamer of the present
10 invention, as far as it is capable of binding to any portions of chymase to inhibit the activity thereof.
[0032]

The length of the aptamer of the present invention is not limited, and can usually be about 25 to about 200 nucleotides,
15 and can be, for example, not more than about 100 nucleotides, preferably not more than about 50 nucleotides, more preferably not more than about 40 nucleotides, most preferably not more than about 35 nucleotides. When the total number of nucleotides is smaller, chemical synthesis and mass-production
20 will be easier, and there is a major advantage in terms of cost. It is also thought that chemical modification is easy, stability in the body is high, and toxicity is low. On the other hand, the length of the aptamer of the present invention is generally not less than about 25 nucleotides, preferably
25 about not less than 28 nucleotides. When the total number of nucleotides is too small, the common sequence explained below cannot be contained, and the potential secondary structure becomes unstable and the activity may be lost in some cases.
[0033]

30 Each of the nucleotides contained in the aptamer of the present invention, whether identical or different, can be a nucleotide comprising a hydroxyl group at the 2' position of ribose (e.g., ribose of pyrimidine nucleotide, ribose of purine nucleotide) (i.e., an unsubstituted nucleotide) or a nucleotide
35 substituted by any atom or group at the 2' position of ribose.

As examples of such any atom or group, a nucleotide substituted by a hydrogen atom, a fluorine atom or an -O-alkyl group (e.g., -O-Me group), an -O-acyl group (e.g., -O-COMe group), or an amino group (e.g., -NH₂ group) can be mentioned.

5 [0034]

The aptamer of the present invention contains the common sequence represented by UAACR₁N₁R₂GGGG (hereinafter sometimes to be referred to as the common sequence of the present invention). An aptamer having this sequence inhibits the activity of
10 chymase by strongly binding to chymase. That is, an aptamer known to inhibit the activity of chymase is clearly an aptamer that binds to chymase even without confirmation of the binding to chymase.

[0035]

15 In the above-mentioned common sequence, R₁ and R₂ are each any one base. The combination of R₁ and R₂ is not particularly limited as long as the aptamer of the present invention binds to chymase. For example, the combination of R₁ and R₂ is A/U, C/G, A/C or G/U (uracil may be thymine), more
20 preferably a combination that forms Watson-Crick base pairs by R₁ and R₂ (A/U or C/G).

[0036]

The above-mentioned combination of R₁ and R₂ is not particularly limited as long as the aptamer of the present
25 invention binds to chymase, and R₁ and R₂ may be interchanged. That is, when the combination of R₁ and R₂ is indicated as A/U, R₁ is A and R₂ is U, or R₁ is U and R₂ is A. The same applies to C/G, A/C and G/U.

[0037]

30 The sequence of the N₁ part of the common sequence UAACR₁N₁R₂GGGG is not particularly limited as long as the aptamer of the present invention binds to chymase and may be any sequence. The base length of the part is not particularly limited as long as the aptamer of the present invention binds
35 to chymase and is typically 3 - 30, preferably 3 - 21, more

[0045]



[0046]

indicates a stem-loop partial structure. The base length of
5 the partial structure is not particularly limited as long as
the aptamer of the present invention binds to chymase and is
typically 3 - 30, preferably 3 - 21, more preferably 3 - 19.
When the base length of the partial structure is less than 3,
the loop structure cannot be formed and when it exceeds 30, the
10 influence of the base in the partial structure becomes
remarkable and the activity of the aptamer may decrease in some
cases. Particularly, when the base length is not more than 21,
stable aptamer activity is found. In the stem-loop partial
structure, the stem structure may be present or absent and the
15 whole may be a loop structure. That is, for the aptamer of the
present invention to have binding activity to chymase, it is
important that the structure of the following formula (1)

[0047]



20 [0048]

take a stem-loop structure as a whole. Since a stem structure
is already formed between UAACR₁ and R₂GGGG, whether a stem
structure is present or absent in the partial structure

[0049]



25

[0050]

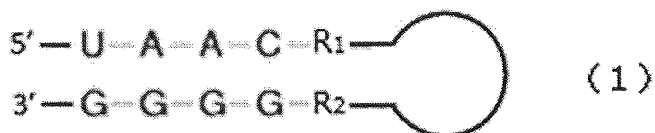
in the formula (1) is not important. On the other hand, when a

stem structure is present, the stem structure may have a bulge structure.

[0051]

As mentioned above, in the aptamer of the present invention, it is highly important for the expression of the activity as an aptamer against chymase that the structure of the formula (1)

[0052]



[0053]

has a stem-loop structure as a whole. That is, in the present invention, the sequence of the stem-loop partial structure

[0054]



[0055]

in the formula (1) is not particularly limited as long as the aptamer has the structure of the above-mentioned formula (1) and has binding activity to chymase (or chymase inhibitory activity). As actually shown in the below-mentioned Examples, the sequence of the above-mentioned stem-loop partial structure is not limited to a particular one, and various sequences may have a similar stem-loop partial structure and have binding activity to chymase.

A preferable sequence of the above-mentioned formula (1') is UUGU, UUGC, CUGG or AAUU (uracil may be thymine), more preferably UUGU.

[0056]

Furthermore, the sequence of the both ends of the structure shown by the above-mentioned formula (1), namely, the 5'-terminal side and the 3'-terminal side, is not limited to a

particular one, and various sequences may have similar binding activity to chymase (or chymase inhibitory activity).

[0057]

The aptamer of the present invention can also be the
5 nucleotide wherein at least one kind (e.g., 1, 2, 3 or 4 kinds)
of nucleotide comprises a hydroxyl group, or the above-
described any atom or group, for example, at least two kinds
(e.g., 2, 3 or 4 kinds) of groups selected from the group
consisting of a hydrogen atom, a fluorine atom, a hydroxyl
10 group and a methoxy group, at the 2' position of ribose.
Particularly, U and/or C in the common sequence and the
structure represented by the formula (1) may be modified by a
group selected from the group consisting of a hydrogen atom, a
fluorine atom, and a methoxy group, preferably at the 2'-
15 position of ribose.

[0058]

In the aptamer of the present invention, all pyrimidine
nucleotides can be nucleotides substituted by a fluorine atom,
or nucleotides substituted by any atom or group mentioned above,
20 preferably an atom or group selected from the atom or group
consisting of a hydrogen atom, a hydroxyl group and a methoxy
group whether identical or not, at the 2' position of ribose.

[0059]

In the aptamers of the present invention, all purine
25 nucleotides can be nucleotides comprising a hydroxyl group, or
nucleotide substituted by any atom or group mentioned above,
preferably an atom or a group selected from the group
consisting of a hydrogen atom, a methoxy group, and a fluorine
atom, whether identical or not, at the 2'-position of ribose.

30 [0060]

The aptamer of the present invention can also be one
wherein all nucleotides identically comprise a hydroxyl group,
or any atom or group mentioned above, for example, the
identical group selected by the group consisting of a hydrogen
35 atom, a fluorine atom, a hydroxyl group and a methoxy group, at

the 2' position of ribose.

[0061]

Herein, in this specification, the nucleotides constituting the aptamer are assumed to be RNAs (i.e., the sugar groups are assumed to be ribose) in describing how the sugar groups are modified in the nucleotides. However, this does not mean that DNA is exempted from the aptamer-constituting nucleotides, and a modification of RNA should read as a modification of DNA as appropriate. When the nucleotide constituting the aptamer is DNA, for example, substitution of the hydroxyl group at the 2' position of ribose by X should read as a substitution of one hydrogen atom at the 2' position of deoxyribose by X.

[0062]

The aptamer of the present invention can also be:

- (a) an aptamer comprising a nucleotide sequence selected from among SEQ ID NO:1 - 11 and 14 - 33 (with the provision that the uracil may be thymine);
- (b) an aptamer comprising a nucleotide sequence selected from among SEQ ID NO:1 - 11 and 14 - 33 (with the provision that the uracil may be thymine), wherein 1 or several nucleotides are substituted, deleted, inserted or added; or
- (c) an aptamer comprising a nucleotide sequence having an identity of 70% or more (preferably 80% or more, more preferably 90% or more, most preferably 95% or more) to a nucleotide sequence selected from among SEQ ID NO: 1 - 11 and 14 - 33 (with the provision that the uracil may be thymine).

The aptamer of the present invention also includes:

- (d) a conjugate selected from the group consisting of a conjugate of a plurality of aptamers (a) above, a conjugate of a plurality of aptamers (b) above, a conjugate of a plurality of aptamers (c) above, and a conjugate of a plurality of aptamers (a), (b) and (c) above.

[0063]

Not only the aptamer (a) above, but also the aptamers (b)

to (d) are capable of binding to chymase and/or inhibiting a chymase activity (chymase enzyme activity and the like).

[0064]

The aptamer of the present invention can also be:

5 (a') an aptamer comprising a nucleotide sequence selected from among SEQ ID NO: 17(1) - 17(25) and 33(1) - 33(18) (with the provision that the uracil may be thymine);

(b') an aptamer comprising a nucleotide sequence selected from among SEQ ID NO: 17(1) - 17(25) and 33(1) - 33(18) (with the
10 provision that the uracil may be thymine), wherein 1 to 5 nucleotides are substituted, deleted, inserted or added; or

(c') an aptamer comprising a nucleotide sequence having an identity of 70% or more to a nucleotide sequence selected from among SEQ ID NO: 17(1) - 17(25) and 33(1) - 33(18) (with the
15 provision that the uracil may be thymine). The aptamer of the present invention also includes:

(d') a conjugate selected from the group consisting of a conjugate of a plurality of aptamers (a') above, a conjugate of a plurality of aptamers (b') above, a conjugate of a plurality
20 of aptamers (c') above, and a conjugate of a plurality of aptamers (a'), (b') and (c') above. Furthermore, the aptamer of the present invention also includes:

(e) a conjugate consisting of one or more aptamers selected from the group consisting of (a), (b) and (c) above, and one or
25 more aptamers selected from the group consisting of (a'), (b') and (c').

[0065]

The aptamers (a')-(d') and (e) above are also capable of binding to chymase and/or inhibiting a chymase activity
30 (chymase enzyme activity and the like).

[0066]

In (b) and (b') above, there is no limitation on the number of nucleotides substituted, deleted, inserted or added, as far as the aptamer is capable of binding to chymase and/or
35 inhibiting a chymase activity (chymase enzyme activity and the

like). The number of nucleotides can be, for example, not more than about 30, preferably not more than about 20, more preferably not more than about 10, still more preferably not more than 5, most preferably 4, 3, 2 or 1.

5 [0067]

With respect to (c) and (c') above, "an identity" means a ratio (%) of identical nucleotide residues to all overlapping nucleotide residues in the optimal alignment where two nucleotide sequences are aligned using a mathematical algorithm known in the technical field (preferably, the algorithm considers introduction of gaps on one or both of the sequences for the best alignment).

[0068]

Nucleotide sequence identity in the present description can be calculated by, for example, aligning the two nucleotide sequences using the homology calculation algorithm NCBI BLAST-2 (National Center for Biotechnology Information Basic Local Alignment Search Tool) under the following conditions (gap open=5 penalties; gap extension=2 penalties; x_dropoff=50; expectancy=10; filtering=ON).

[0069]

In (d), (d') and (e) above, conjugation can be achieved by tandem binding. In the conjugation, a linker may be utilized. As the linker, nucleotide chains (e.g., 1 to about 20 nucleotides) and non-nucleotide chains (e.g., $-(CH_2)_n$ - linker, $-(CH_2CH_2O)_n$ - linker, hexaethylene glycol linker, TEG linker, peptide-containing linker, -S-S- bond-containing linker, -CONH- bond-containing linker, $-OPO_3$ - bond-containing linker) can be mentioned. The plurality as mentioned in the above-described plurality of conjugates is not particularly limited, as long as it is two or more, and the plurality can be, for example, 2, 3 or 4. Each of the nucleotides in (a) to (d), (a') to (d') and (e) above, whether identical or different, can be a nucleotide comprising a hydroxyl group at the 2' position of ribose or a nucleotide substituted by any groups (e.g.,

hydrogen atom, fluorine atom or -O-Me group) at the 2' position of ribose (e.g., ribose of pyrimidine nucleotide).

[0070]

The aptamer of the present invention may be one wherein a sugar residue (e.g., ribose) of each nucleotide has been modified to increase the chymase binding activity, stability, drug deliverability and the like. As examples of the site to be modified in a sugar residue, one having the oxygen atom at the 2'-position, 3'-position and/or 4'-position of the sugar residue replaced with another atom, and the like can be mentioned. As examples of the modification, fluorination, O-alkylation (e.g., O-methylation, O-ethylation), O-arylation, S-alkylation (e.g., S-methylation, S-ethylation), S-arylation, and amination (e.g., -NH₂) can be mentioned. Such alterations in the sugar residue can be performed by a method known per se (see, for example, Sproat et al., (1991) Nucle. Acid. Res. 19, 733-738; Cotton et al., (1991) Nucl. Acid. Res. 19, 2629-2635; Hobbs et al., (1973) Biochemistry 12, 5138-5145).

[0071]

In the aptamer of the present invention, the common sequence or the structure represented by the formula (1)

[0072]



[0073]

of the present invention may be modified as mentioned above. However, the second G of the GGGG part in UAACR₁N₁R₂GGGG is desirably free of O-methylation (modification with O-methyl) at the 2'-position of the sugar residue. Therefore, the aptamer of the present invention may be an aptamer in which the second G of the GGGG part in UAACR₁N₁R₂GGGG and the structure of the formula (1)

[0074]



[0075]

is free of O-methylation (modification with O-methyl). In this case, the second G of the GGGG part is preferably modified with
 5 a fluorine atom (fluorinated).

[0076]

The aptamer of the present invention may also have a nucleic acid base (e.g., purine or pyrimidine) altered (e.g., chemical substitution) to increase the chymase binding activity
 10 and the like. As examples of such alterations, 5-position pyrimidine alteration, 6- and/or 8-position purine alteration, alteration with an extracyclic amine, substitution with 4-thiouridine, and substitution with 5-bromo or 5-iodo-uracil, and substitution with 8-(propyl)phenyl-rA (n-bz)-2'-tBDMS
 15 amidite can be mentioned.

[0077]

The phosphate group contained in the aptamer of the present invention may be altered to confer resistance to nuclease and hydrolysis. For example, the P(O)O group may be
 20 substituted with P(O)S (thioate), P(S)S (dithioate), P(O)NR₂ (amidate), P(O)CH₃, P(O)BH₃, P(O)R, R(O)OR', CO or CH₂ (formacetal) or 3'-amine (-NH-CH₂-CH₂-) [wherein each unit of R or R' is independently H or a substituted or unsubstituted alkyl (e.g., methyl, ethyl)].

25 [0078]

The joining group is, for example, -O-, -N- or -S-, and nucleotides can bind to an adjoining nucleotide via these joining groups.

The alterations may also include alterations such as
 30 capping at 3' and 5'.

[0079]

An alteration can further be performed by adding to an end a polyethyleneglycol, amino acid, peptide, inverted dT,

nucleic acid, nucleosides, Myristoyl, Lithocolic-oleyl, Docosanyl, Lauroyl, Stearoyl, Palmitoyl, Oleoyl, Linoleoyl, other lipids, steroids, cholesterol, caffeine, vitamins, pigments, fluorescent substances, anticancer agent, toxin,
5 enzymes, radioactive substance, biotin and the like. For such alterations, see, for example, US Patents 5,660,985 and 5,756,703.

[0080]

The aptamer of the present invention can be synthesized
10 as disclosed herein and by a method known per se in the art. A method of synthesis employs RNA polymerase. A DNA having a desired sequence and a promoter sequence of RNA polymerase is chemically synthesized, which, as a template, is transcribed by a publicly known method to obtain the desired RNA. The aptamer
15 of the present invention can also be synthesized using DNA polymerase. A DNA having a desired sequence is chemically synthesized, which, as a template, is amplified by a method of public knowledge known as the polymerase chain reaction (PCR). This is rendered single-stranded by a publicly known method of
20 polyacrylamide electrophoresis or enzyme treatment. When synthesizing a modified aptamer, elongation reaction efficiency can be increased by using a polymerase mutated at a particular site. The aptamer thus obtained can easily be purified by a publicly known method.

25 [0081]

An aptamer can be synthesized in large amounts by chemical synthetic methods such as the amidite method and the phosphoramidite method. These synthetic methods are well known, as described in Nucleic Acid (Vol.2) [1] Synthesis and Analysis
30 of Nucleic Acid (edited by Yukio Sugiura, published by Hirokawa Publishing Company) and the like. Practically, a synthesizer such as OligoPilot100 or OligoProcess (manufactured by GE Healthcare Bioscience) is used. The aptamer thus synthesized can be purified by a method known per se such as
35 chromatography.

[0082]

Provided that an active group such as an amino group is introduced to an aptamer during the process of chemical synthesis by the phosphoramidite method or the like, a functional substance can be added after the synthesis. For example, by introducing an amino group to an end of the aptamer, it is possible to condense a polyethylene glycol chain incorporating a carboxyl group.

[0083]

10 An aptamer binds to the target molecule in a wide variety of binding modes, such as ionic bonds based on the negative charge of the phosphate group, hydrophobic bonds and hydrogen bonds based on ribose, and hydrogen bonds and stacking interaction based on nucleic acid bases. In particular, ionic bonds based on the negative charge of the phosphate group, which are present in the same number as the number of constituent nucleotides, are strong, and bind to lysine and arginine being present on the surface of the positive charge of protein. For this reason, nucleic acid bases not involved in the direct binding to the target molecule can be substituted. In particular, because the region of stem structure has already formed base pairs and faces the inside of the double helical structure, nucleic acid bases are unlikely to bind directly to the target molecule. Therefore, even when a base pair is replaced with another base pair, the activity of the aptamer often does not decrease. In structures wherein no base pairs are formed, such as loop structures, provided that the nucleic acid base is not involved in the direct binding to the target molecule, base substitution is possible. Regarding modifications of the 2'-position of ribose, the functional group at the 2'-position of ribose infrequently interacts directly with the target molecule, but in many cases, it is of no relevance, and can be substituted by another modified molecule. Hence, an aptamer, unless the functional group involved in the direct binding to the target molecule is

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substituted or deleted, often retains the activity thereof. It is also important that the overall three-dimensional structure does not change widely.

[0084]

5 An aptamer can be prepared by utilizing the SELEX method or an improved version thereof (for example, Ellington et al., (1990) Nature, 346, 818-822; Tuerk et al., (1990) Science, 249, 505-510). In the SELEX method, by rendering the selection criteria more rigorous by increasing the number of rounds or
10 using a competing substance, an aptamer exhibiting a stronger binding potential for the target molecule is concentrated and selected. Hence, by adjusting the number of rounds of SELEX and/or changing the competitive condition, aptamers with different binding forces, aptamers with different binding modes,
15 and aptamers with the same binding force or binding mode but different base sequences can be obtained in some cases. The SELEX method comprises a process of amplification by PCR; by causing a mutation by using manganese ions and the like in the process, it is possible to perform SELEX with higher diversity.

20 [0085]

 The aptamers obtained by SELEX are nucleic acids that exhibit high affinity for the target molecule, but this does not mean inhibiting a bioactivity of the target molecule. Chymase is a basic protein to which nucleic acids are thought
25 to be likely to bind non-specifically, but aptamers other than those that bind strongly to a particular site of chymase do not influence the activity of the target molecule. In fact, the RNA comprising a random sequence used as a negative control did not inhibit the enzyme activity of chymase, although it bound
30 to chymase weakly.

[0086]

 Based on an active aptamer thus selected, SELEX can be performed to acquire an aptamer possessing higher activity. Specifically, after preparing a template wherein an aptamer
35 with a determined sequence is partially randomized or a

template doped with about 10 to 30% of random sequences, SELEX is performed again.

[0087]

An aptamer obtained by SELEX has a length of about 80
5 nucleotides, and this is difficult to prepare as a
pharmaceutical as it is. Hence, it is necessary to repeat try-
and-error efforts to shorten the aptamer to a length of about
50 nucleotides or less enabling easy chemical synthesis.
Depending on the primer design for an aptamer obtained by SELEX,
10 the ease of the subsequent minimization operation changes.
Unless the primer is designed successfully, subsequent
development will be impossible even if an aptamer with activity
is selected by SELEX. In the present invention, an aptamer
retaining activity even with 28 nucleotides was obtained.

15 [0088]

Aptamers are easily modifiable because they permit
chemical synthesis. For aptamers, by predicting the secondary
structure using the MFOLD program, or by predicting the steric
structure by X-ray analysis or NMR analysis, it is possible to
20 predict to some extent which nucleotide can be substituted or
deleted, and where to insert a new nucleotide. A predicted
aptamer with the new sequence can easily be chemically
synthesized, and it can be determined whether or not the
aptamer retains the activity using an existing assay system.

25 [0089]

If a region important to the binding of the aptamer
obtained with the target molecule is identified by repeated
try-and-error efforts as described above, the activity remains
unchanged in many cases even when a new sequence is added to
30 both ends of the sequence. The length of the new sequence is
not particularly limited.

[0090]

Those of ordinary skill in the art can make a wide range
of design or alterations of modifications, like sequences.

35 [0091]

As stated above, aptamers permit a wide range of design or alterations of modifications. The present invention also provides a production method of aptamer that enables a wide range of design or alteration of an aptamer comprising a specified sequence (e.g., a sequence corresponding to a portion selected from among stem regions, internal loop regions, bulge regions, hairpin loop regions and single-strand regions: hereinafter, abbreviated as fixed sequence as required).

[0092]

For example, the production method of such aptamer includes production of an aptamer comprising a fixed sequence by using a single kind of nucleic acid molecule or a plurality of kinds of nucleic acid molecules (e.g., a library of nucleic acid molecules with different numbers for "a" or "b") consisting of a nucleotide sequence shown by the formula:

[0093]

Primer sequence (i) - (N)a-fixed sequence-(N)b- Primer sequence (ii)

[0094]

[wherein (N)a represents a nucleotide chain consisting of "a" units of N; (N)b represents a nucleotide chain consisting of "b" units of N; each of the units of N, whether identical or different, is a nucleotide selected from the group consisting of A, G, C, U and T (preferably, A, G, C and U). Each of "a" and "b", whether identical or different, can be any numbers, and can be, for example, 1 to about 100, preferably 1 to about 50, more preferably 1 to about 30, still more preferably 1 to about 20 or 1 to about 10], and primer pairs corresponding to the primer sequences (i) and (ii), respectively.

[0095]

The present invention also provides a complex comprising the aptamer of the present invention and a functional substance bound thereto. The bond between the aptamer and the functional substance in the complex of the present invention can be a covalent bond or a non-covalent bond. The complex of the

present invention can be one wherein the aptamer of the present invention and one or more (e.g., 2 or 3) of functional substances of the same kind or different kinds are bound together. The functional substance is not particularly limited, as far as it newly confers a certain function to an aptamer of the present invention, or is capable of changing (e.g., improving) a certain characteristic which an aptamer of the present invention can possess. As examples of the functional substance, proteins, peptides, amino acids, lipids, sugars, monosaccharides, polynucleotides, and nucleotides can be mentioned. As examples of the functional substance, affinity substances (e.g., biotin, streptavidin, polynucleotides possessing affinity for target complementary sequence, antibodies, glutathione Sepharose, histidine), substances for labeling (e.g., fluorescent substances, luminescent substances, radioisotopes), enzymes (e.g., horseradish peroxidase, alkaline phosphatase), drug delivery vehicles (e.g., liposome, microspheres, peptides, polyethyleneglycols), drugs (e.g., those used in missile therapy such as calicheamycin and duocarmycin; nitrogen mustard analogues such as cyclophosphamide, melphalan, ifosfamide or trofosfamide; ethylenimines such as thiotepa; nitrosoureas such as carmustine; alkylating agents such as temozolomide or dacarbazine; folate-like metabolic antagonists such as methotrexate or raltitrexed; purine analogues such as thioguanine, cladribine or fludarabine; pyrimidine analogues such as fluorouracil, tegafur or gemcitabine; vinca alkaloids such as vinblastine, vincristine or vinorelbine and analogues thereof; podophyllotoxin derivatives such as etoposide, taxans, docetaxel or paclitaxel; anthracyclines such as doxorubicin, epirubicin, idarubicin and mitoxantrone, and analogues thereof; other cytotoxic antibiotics such as bleomycin and mitomycin; platinum compounds such as cisplatin, carboplatin and oxaliplatin; pentostatin, miltefosine, estramustine, topotecan, irinotecan and bicalutamide), and toxins (e.g., ricin toxin,

liatoxin and Vero toxin) can be mentioned. These functional molecules are finally removed in some cases. Furthermore, the molecules may be peptides that can be recognized and cleaved by enzymes such as thrombin, matrix metalloproteinase (MMP), and
5 Factor X, and may be polynucleotides that can be cleaved by nucleases or restriction endonuclease.

[0096]

The aptamer or the complex of the present invention can be used as, for example, a pharmaceutical or a diagnostic
10 reagent, a test reagent or a reagents.

[0097]

The aptamer and complex of the present invention can possess an activity of inhibiting a function of chymase. As stated above, chymase is profoundly associated with fibrosis
15 and cardiovascular diseases. Therefore, the aptamer and complex of the present invention are useful as pharmaceuticals for treating or preventing diseases accompanied by fibrosis or cardiovascular disorders, as well as fibrosis and cardiovascular diseases.

20 [0098]

The aptamer and complex of the present invention are capable of binding specifically to chymase. Therefore, the aptamer and complex of the present invention are useful as probes for chymase detection. The probes are useful in in vivo
25 imaging of chymase, measurements of blood concentrations of chymase, tissue staining, ELISA and the like. The probes are also useful as diagnostic reagents, testing reagents, analytical reagents and the like for diseases involved by chymase (fibrosis, cardiovascular disorders, diseases
30 accompanied by fibrosis or cardiovascular disorders, and the like).

[0099]

Based on their specific binding to chymase, the aptamer and complex of the present invention can be used as ligands for
35 purification of chymase.

[0100]

The aptamer and complex of the present invention can be used as drug delivery vehicles.

[0101]

5 Fibrosis is a disease caused by the progression of fibrosis of an organ or tissue for some reason, resulting in a decrease in the function of the organ or tissue. Therefore, fibrosis in a broad sense can be said a disease involving fibrosis of an organ or tissue.

10 Diseases involved by organ or tissue fibrosis include pulmonary fibrosis, prostatic hyperplasia, myocardial fibrosis, musculoskeletal fibrosis, myelofibrosis, hysteryoma, scleroderma, adhesion after surgical operations, postoperative scars, burn scars, hypertrophic scars, keloid, atopic
15 dermatitis, peritoneal sclerosis, asthma, liver cirrhosis, chronic pancreatitis, scirrhous gastric cancer, liver fibrosis, renal fibrosis, fibrous vascular diseases, retinitis due to fibrous microvasculitis as a diabetic complication, neurosis, nephropathies, glomerulonephritis, tubulointerstitial nephritis,
20 hereditary renal diseases, arteriosclerotic peripheral arteritis and the like. In addition, colitis, osteoporosis, allergic conjunctivitis, fulminant hepatitis, skin vasculitis, urticaria pigmentosa, pruritus, dermatitis herpetiformis, bullous pemphigoid, psoriasis, esophagitis, achalasia, airway
25 inflammation, atherosclerosis, varicose vein and the like can also be mentioned.

[0102]

Cardiovascular diseases include angiopathies, aortic aneurysms, renal insufficiency, hypertension, arteriosclerosis,
30 myocardial infarction, cardiac hypertrophy, heart failure, restenosis after angiopathies due to percutaneous transluminal coronary angioplasty and the like, diabetic and non-diabetic nephropathies, peripheral circulatory disorders and the like. In addition, pulmonary hypertension, aortic stenosis and the
35 like can also be mentioned.

[0103]

Chymase possesses enzyme activity and cleaves bioactive substances that can serve as substrates. Examples of substrates known to date include AngI, latent TGF- β , SCF, 5 procollagen, procollagenase, pro-MMP-9, IL-1 β precursor and the like. Chymase exhibits biological actions, via reactions for production or degradation of these bioactive peptides, including extracellular matrix remodeling, networks with cytokines, immunity, and vasoconstriction. Meanwhile, chymase 10 itself acts to activate mast cells and to promote histamine release, and is closely associated with inflammation. Therefore, the aptamer and complex of the present invention are not limited to the above-described substrates, and can be used as pharmaceuticals or diagnostic reagents, testing reagents, 15 and analytical reagents for diseases related to biological functions mediated by substrates accepted by chymase and diseases involved by chymase itself.

[0104]

The pharmaceutical of the present invention can be one 20 formulated with a pharmaceutically acceptable carrier. As examples of the pharmaceutically acceptable carrier, excipients such as sucrose, starch, mannitol, sorbitol, lactose, glucose, cellulose, talc, calcium phosphate, and calcium carbonate; binders such as cellulose, methylcellulose, 25 hydroxylpropylcellulose, polypropylpyrrolidone, gelatin, gum arabic, polyethylene glycol, sucrose, and starch; disintegrants such as starch, carboxymethylcellulose, hydroxylpropylstarch, sodium-glycol-starch, sodium hydrogen carbonate, calcium phosphate, and calcium citrate; lubricants such as magnesium 30 stearate, Aerosil, talc, and sodium lauryl sulfate; flavoring agents such as citric acid, menthol, glycyrrhizin-ammonium salt, glycine, and orange powder; preservatives such as sodium benzoate, sodium hydrogen sulfite, methylparaben, and propylparaben; stabilizers such as citric acid, sodium citrate, 35 and acetic acid; suspending agents such as methylcellulose,

polyvinylpyrrolidone, and aluminum stearate; dispersing agents such as surfactants; diluents such as water, physiological saline, and orange juice; base waxes such as cacao butter, polyethylene glycol, and kerosene; and the like can be
5 mentioned, but these are not limitative.

[0105]

There is no limitation on the route of administration of the pharmaceutical of the present invention, which can be administered by, for example, oral administration and
10 parenteral administration.

Preparations suitable for oral administration are a solution prepared by dissolving an effective amount of ligand in a diluent such as water, physiological saline, or orange juice; capsules, sachets or tablets comprising an effective
15 amount of ligand in solid or granular form; a suspension prepared by suspending an effective amount of active ingredient in an appropriate dispersant; an emulsion prepared by dispersing and emulsifying a solution of an effective amount of active ingredient in an appropriate dispersant; C10, which
20 promotes the absorption of water-soluble substances, and the like.

[0106]

The pharmaceutical of the present invention can be coated by a method known per se for the purpose of taste masking,
25 enteric dissolution, sustained release and the like as required. As examples of coating agents used for the coating, hydroxypropylmethylcellulose, ethylcellulose, hydroxymethylcellulose, hydroxypropylcellulose, polyoxyethylene glycol, Tween 80, Pluronic F68, cellulose acetate phthalate,
30 hydroxypropylmethylcellulose phthalate, hydroxymethylcellulose acetate succinate, Eudragit (manufactured by Rohm, Germany, methacrylic acid/acrylic acid copolymer), pigments (e.g., red iron oxide, titanium dioxide and the like) and the like are used. The pharmaceutical may be a rapid-release preparation or
35 sustained-release preparation.

[0107]

As preparations suitable for parenteral administration (for example, intravenous administration, subcutaneous administration, intramuscular administration, topical administration, intraperitoneal administration, intranasal administration and the like), aqueous and non-aqueous isotonic sterile injectable liquids are available, which may comprise an antioxidant, a buffer solution, a bacteriostatic agent, an isotonizing agent and the like. Aqueous and non-aqueous sterile suspensions can also be mentioned, which may comprise a suspending agent, a solubilizer, a thickener, a stabilizer, an antiseptic and the like. The preparation can be included in a container such as an ampoule or a vial in a unit dosage volume or in several divided doses. An active ingredient and a pharmaceutically acceptable carrier can also be freeze-dried and stored in a state that may be dissolved or suspended in an appropriate sterile vehicle just before use.

[0108]

Sustained-release preparations are also suitable preparations. Dosage forms of sustained-release preparations include sustained release from carriers or containers embedded in the body, such as artificial bones, biodegradable bases or non-biodegradable sponges, bags and the like. Devices for continuous or intermittent, systemic or topical delivery from outside the body, such as drug pumps and osmotic pressure pumps, are also included in the scope of sustained-release preparations. Biodegradable bases include liposome, cationic liposome, Poly(lactic-co-glycolic) acid (PLGA), atelocollagen, gelatin, hydroxyapatite, polysaccharide chitosan.

[0109]

In addition to liquid injections, suspensions and sustained-release preparations, inhalants suitable for transpulmonary administration, ointments suitable for percutaneous administration, and the like are acceptable.

[0110]

In the case of an inhalant, an active ingredient in a freeze-dried state is micronized and administered by inhalation using an appropriate inhalation device. An inhalant can be formulated as appropriate with a conventionally used surfactant,
5 oil, seasoning, cyclodextrin or derivative thereof and the like as required. An inhalant can be produced according to a conventional method. Specifically, an inhalant can be produced by powdering or liquefying the aptamer or complex of the present invention, blending it in an inhalation propellant
10 and/or carrier, and filling it in an appropriate inhalation vessel. When the above-described aptamer or complex of the present invention is a powder, an ordinary mechanical powder inhalator can be used; in the case of a liquid, an inhalator such as a nebulizer can be used. Here, as the propellant,
15 conventionally known one can be widely used; chlorofluorocarbon-series compounds such as chlorofluorocarbon-11, chlorofluorocarbon-12, chlorofluorocarbon-21, chlorofluorocarbon-22, chlorofluorocarbon-113, chlorofluorocarbon-114, chlorofluorocarbon-123,
20 chlorofluorocarbon-142c, chlorofluorocarbon-134a, chlorofluorocarbon-227, chlorofluorocarbon-C318, and 1,1,1,2-tetrafluoroethane, hydrocarbons such as propane, isobutane, and n-butane, ethers such as diethyl ether, compressed gases such as gaseous nitrogen and gaseous carbon dioxide and the like can
25 be mentioned.

[0111]

Here, as examples of the surfactant, oleic acid, lecithin, diethyleneglycol dioleate, tetrahydroflurfuryl oleate, ethyl oleate, isopropyl myristate, glyceryl trioleate, glyceryl
30 monolaurate, glyceryl monooleate, glyceryl monostearate, glyceryl monolysinoate, cetyl alcohol, stearyl alcohol, polyethyleneglycol 400, cetylpyridinium chloride, sorbitan trioleate (trade name Span 85), sorbitan monooleate (trade name Span 80), sorbitan monolaurate (trade name Span 20),
35 polyoxyethylene hardened castor oil (trade name HCO-60),

polyoxyethylene (20) sorbitan monolaurate (trade name Tween 20),
polyoxyethylene (20) sorbitan monooleate (trade name Tween 80),
lecithin of natural resource origin (trade name EPICLON),
oleylpolyoxyethylene (2) ether (trade name Brij 92), stearyl
5 polyoxyethylene (2) ether (trade name Brij 72), lauryl
polyoxyethylene (4) ether (trade name Brij 30),
oleylpolyoxyethylene (2) ether (trade name Genapol 0-020),
block copolymer of oxyethylene and oxypropylene (trade name
Synperonic) and the like can be mentioned. As examples of the
10 oil, corn oil, olive oil, cottonseed oil, sunflower oil and the
like can be mentioned. In the case of an ointment, an
appropriate pharmaceutically acceptable base (yellow petrolatum,
white petrolatum, paraffin, plastibase, silicone, white
ointment, beeswax, lard, vegetable oils, hydrophilic ointment,
15 hydrophilic petrolatum, purified lanolin, hydrolyzed lanolin,
water-absorbing ointment, hydrophilic plastibase, macrogol
ointment and the like) is blended with an aptamer of the
present invention, which is the active ingredient, and used as
a preparation.

20 [0112]

The dosage of the pharmaceutical of the present invention
varies depending on the kind and activity of active ingredient,
seriousness of disease, animal species being the subject of
administration, drug tolerability of the subject of
25 administration, body weight, age and the like, and the usual
dosage, based on the amount of active ingredient per day for an
adult, can be about 0.0001 to about 100 mg/kg, for example,
about 0.0001 to about 10 mg/kg, preferably about 0.005 to about
1 mg/kg.

30 [0113]

The present invention also provides a solid phase carrier
having the aptamer and/or the complex of the present invention
immobilized thereon. As examples of the solid phase carrier, a
substrate, a resin, a plate (e.g., multiwell plate), a filter,
35 a cartridge, a column, and a porous material can be mentioned.

The substrate can be one used in DNA chips, protein chips and the like; for example, nickel-PTFE (polytetrafluoroethylene) substrates, glass substrates, apatite substrates, silicon substrates, alumina substrates and the like, and substrates prepared by coating these substrates with a polymer and the like can be mentioned. As examples of the resin, agarose particles, silica particles, a copolymer of acrylamide and N,N'-methylenebisacrylamide, polystyrene-crosslinked divinylbenzene particles, particles of dextran crosslinked with epichlorohydrin, cellulose fiber, crosslinked polymers of aryl dextran and N,N'-methylenebisacrylamide, monodispersed synthetic polymers, monodispersed hydrophilic polymers, Sepharose, Toyopearl and the like can be mentioned, and also resins prepared by binding various functional groups to these resins were included. The solid phase carrier of the present invention can be useful in, for example, purifying, detecting and quantifying chymase.

[0114]

The aptamer and/or the complex of the present invention can be immobilized onto a solid phase carrier by a method known per se. For example, a method that introduces an affinity substance (e.g., those described above) or a predetermined functional group into the aptamer and/or the complex of the present invention, and then immobilizing the aptamer or complex onto a solid phase carrier via the affinity substance or predetermined functional group can be mentioned. The present invention also provides such methods. The predetermined functional group can be a functional group that can be subjected to a coupling reaction; for example, an amino group, a thiol group, a hydroxyl group, and a carboxyl group can be mentioned. The present invention also provides an aptamer having such a functional group introduced thereto.

[0115]

The present invention also provides a method of purifying and concentrating chymase. In particular, the present

invention makes it possible to separate chymase from the proteins of other family proteins. The method of purification and concentration of the present invention can comprise adsorbing chymase to the solid phase carrier of the present invention, and eluting the adsorbed chymase with an eluent. Adsorption of chymase to the solid phase carrier of the present invention can be achieved by a method known per se. For example, a chymase-containing sample (e.g., bacterial or cell culture or culture supernatant, blood) is introduced into the solid phase carrier of the present invention or a composition containing the same. Chymase can be eluted using an eluent such as a neutral solution. There is no limitation on the neutral eluent, which can have a pH of, for example, about 6 to about 9, preferably about 6.5 to about 8.5, and more preferably about 7 to about 8. The neutral solution can also comprise, for example, urea, a chelating agent (e.g., EDTA), a sodium salt (e.g., NaCl), a potassium salt (e.g., KCl), a magnesium salt (e.g., $MgCl_2$), a surfactant (e.g., Tween 20, Triton, NP40), and glycerin. The method of purification and concentration of the present invention can further comprise washing the solid phase carrier using a washing solution after chymase adsorption. Examples of the washing solution include those containing urea, a chelating agent (e.g., EDTA), Tris, an acid, an alkali, Transfer RNA, DNA, surfactants such as Tween 20, salts such as NaCl and the like. The method of purification and concentration of the present invention can still further comprise heating the solid phase carrier. This step enables the regeneration and sterilization of the solid phase carrier.

[0116]

The present invention also provides a method of detecting and quantifying chymase. In particular, the present invention makes it possible to detect and quantify chymase separately from the proteins of other family proteins. The method of detection and quantitation of the present invention can comprise measuring chymase by utilizing the aptamer of the

present invention (e.g., by the use of the complex and solid phase carrier of the present invention). The method of detecting and quantifying chymase can be performed in the same manner as an immunological method, except that the aptamer of the present invention is used in place of an antibody. Therefore, by using the aptamer of the present invention as a probe in place of an antibody, in the same manner as such methods as enzymeimmunoassay (EIA) (e.g., direct competitive ELISA, indirect competitive ELISA, sandwich ELISA), radioimmunoassay (RIA), fluorescent immunoassay (FIA), Western blot technique, immunohistochemical staining method, and cell sorting method, detection and quantitation can be performed. The aptamer of the present invention can also be used as a molecular probe for PET and the like. These methods can be useful in, for example, measuring chymase contents in living organisms or biological samples, and in diagnosing a disease associated with chymase.

[0117]

The disclosures in all publications mentioned herein, including patents and patent application specifications, are incorporated by reference herein in the present invention to the extent that all of them have been given expressly.

[0118]

The present invention is hereinafter described in more detail by means of the following Examples, which, however, never limit the scope of the invention.

Examples

[Example]

[0119]

Example 1: Preparation of RNA aptamers that bind specifically to chymase (1)

RNA aptamers that bind specifically to chymase were prepared using the SELEX method. SELEX was performed with improvements of the method of Ellington et al. (Ellington and Szostak, Nature 346, 818-822, 1990) and the method of Tuerk et

round was theoretically 10^{14} .

[0123]

The RNA pool was added to the chymase-immobilized carrier, and allowed to stand at room temperature for 30 minutes. Then, to remove the RNA not bound to chymase, the resin was washed with solution A. Here, the solution A was a mixed solution of 145 mM sodium chloride, 5.4 mM potassium chloride, 1.8 mM calcium chloride, 0.8 mM magnesium chloride, and 20 mM Tris. The RNA bound to chymase was heated at 95°C for 10 minutes with the addition of solution B as an eluent, and recovered from the supernatant. Here, the solution B was a mixture of 7M Urea, 3 mM EDTA, and 100 mM Tris-HCl (pH 6.6). The recovered RNA was amplified by RT-PCR and transcribed using the DuraScribe™ T7 Transcription Kit, and this was used as the pool for the next round. With this procedure taken as 1 round, the same operation was performed plural times. After completion of SELEX, the PCR product was cloned into pGEM-T Easy vector (manufactured by Promega), and the Escherichia coli strain DH5α (manufactured by Toyobo) was transformed therewith. After the plasmid was extracted from a single colony, the base sequences of clones were determined using a DNA sequencer (3130xl Genetic Analyzer, manufactured by ABI).

[0124]

After 9 rounds of SELEX, the sequences of 56 clones were sequenced; sequence convergence was seen. The sequences of some of the clones are shown by SEQ ID NO: 1-6. These sequences contain the common sequence shown below:

[0125]

UAACR₁N₁R₂GGGG

[0126]

[0127]

The aforementioned common sequence was present in 7 out of 56 clones. The secondary structures of these sequences were predicted using the MFOLD program (M. Zuker, Nucleic Acids Res. 31(13), 3406-3415, 2003). As a result, the part formed by the

aforementioned common sequences UAACR₁ and R₂GGGG was a very similar stem structure, and the part formed by N₁ sandwiched thereby was a very similar loop structure. The predicted secondary structures of the aptamers of the sequences shown by
 5 SEQ ID NO: 1 - 6 are shown in Fig. 1. It is clear that the aforementioned common sequence takes a stem-loop structure represented by the following formula (1)
 [0128]



10 [0129]

The stem-loop structure is surrounded by a square (□).
 [0130]

Given below are the respective nucleotide sequences.

Unless otherwise stated, the following individual sequences are
 15 shown in the direction from 5' to 3', with the purine bases (A and G) being in the 2'-OH form, and the pyrimidine bases (U and C) in the 2'-fluoro-modified form.

[0131]

SEQ ID NO: 1

20 GGGAGCAGGAGAGAGGUCAGAUGAGCAUGC UUUUUGGUAACCGAUAAUGGGGGCCUAUGCGUG
 CUAGUGUGA

[0132]

SEQ ID NO: 2

GGGAGCAGGAGAGAGGUCAGAUGAUCGGACAUAACAUUGUUGGGGUGUCAAGGCCUAUGCGUG
 25 CUAGUGUGA

[0133]

SEQ ID NO: 3

GGGAGCAGGAGAGAGGUCAGAUGAUAACAGUUGGGGGGUCAAUUAUGGGACCUAUGCGUG
 CUAGUGUGA

30 [0134]

SEQ ID NO: 4

GGGAGCAGGAGAGAGGUCAGAUGUAACUCUAUUGAGGGGCAUCAGCACAGUAGCCUAUGCGUG

CUAGUGUGA

[0135]

SEQ ID NO: 5

GGGAGCAGGAGAGAGAGGUCAGAUGAUGACCGAUUAUAGGUAACCACUUAGGGGGCCUAUGCGUG

5 CUAGUGUGA

[0136]

SEQ ID NO: 6

GGGAGCAGGAGAGAGAGGUCAGAUGUCAUGACUUAUAGGUAACCGAUAAUGGGGGCCUAUGCGUG

CUAGUGUGA

10 [0137]

[Common sequence] UAACR₁N₁R₂GGGG

[0138]

The above-mentioned SELEX performed up to 9 rounds was continued under similar conditions, and the sequences of 8 clones at 10 rounds, 47 clones at 11 rounds, total 55 clones, were examined. The sequences of a part of the clones are shown in SEQ ID NO: 7 - 8. These sequences contained common sequences similar to that at 9 rounds. The common sequences were contained in 3 clones in 55 clones. The predicted secondary structures of the aptamers of the sequences shown by SEQ ID NO: 7 - 8 are shown in Fig. 2. The stem-loop structure is surrounded by a square (□). They all have a characteristic stem-loop structure as in Fig. 1.

[0139]

25 Given below are respective nucleotide sequences. Unless otherwise stated, the following individual sequences are shown in the direction from 5' to 3', with the purine bases (A and G) being in the 2'-OH form, and the pyrimidine bases (U and C) in the 2'-fluoro-modified form.

30 [0140]

SEQ ID NO: 7

GGGAGCAGGAGAGAGAGGUCAGAUGUUUGGUAGUAACUGGAAUAGGGGCUACAGGCCUAUGCGUG

CUAGUGUGA

[0141]

35 SEQ ID NO: 8

GGGAGCAGGAGAGAGGUCAGAUGAUUGACGACUAUAGGUAACCUUUACGGGGGCCUAUGCGUG
CUAGUGUGA

[0142]

Whether the nucleic acids shown by SEQ ID NO: 1-8 inhibit
5 the enzyme activity of chymase was determined as described
below. The chymase substrate used was Suc-Ala-Ala-Pro-Phe-MCA
(manufactured by Peptide Institute, Inc.), which contains the
4-amino-acid peptide Ala-Ala-Pro-Phe, a standard substrate for
chymotrypsin-like proteases. Here, Suc is a protecting
10 succinyl group, and MCA is a 4-methylcoumaryl-7-amide group;
upon cleavage of the C-terminal side of phenylalanine, AMC (7-
amino-4-methylcoumarine) is released. By detecting the
fluorescence of this AMC, the enzyme activity of chymase can be
determined. The assay was performed using a 96-well plate (F16
15 Black Maxisorp Fluoronunc, manufactured by Nunc), with a
reaction mixture volume of 100 μ L, in solution C as a buffer
solution. Here, the solution C is a mixed solution of 145 mM
sodium chloride, 5.4 mM potassium chloride, 1.8 mM calcium
chloride, 0.8 mM magnesium chloride, 20 mM Tris (pH 7.6), and
20 0.05% Tween20. First, nucleic acid was serially diluted in
solution C to obtain 50 μ L solutions. After 10 μ L of the 1 mM
substrate prepared in solution C was added thereto, the plate
was set to the microplate reader SpectraMax190 (manufactured by
Molecular Devices Corporation), and incubated at 37°C for 5
25 minutes. Separately, 0.05 μ g of chymase (recombinant,
manufactured by SIGMA) was diluted in solution C to obtain a 40
 μ L chymase solution, and incubated at 37°C for 5 minutes. The
chymase solution was added to the mixture of the nucleic acid
and substrate to initiate an enzyme reaction. The final
30 chymase concentration in the reaction mixture was 16.7 nM, the
final substrate concentration being 100 μ M. The plate
containing the reaction mixture was set to the microplate
reader SpectraMax190 (manufactured by Molecular Devices
Corporation), and examined for time-dependent changes in the
35 fluorescence intensity at 37°C for 5 minutes (or 30 minutes)

(excitation wavelength 380 nm, detection wavelength 460 nm). A linear approximation of the increase in the fluorescence of the AMC released from the substrate by chymase activity was generated, and its slope was taken as the initial velocity ($V_{\max}$). For control, samples were treated and analyzed in the same manner in two cases: use of a 30N (a nucleotide of 30 consecutive bases represented by N; N is A, G, C or T) nucleic acid pool (negative control), and use of chymostatin, a known chymotrypsin-like serine protease inhibitor (positive control). Taking the initial reaction velocity without the nucleic acid and inhibitor (V_0) as a 100% enzyme activity, the inhibitory rate of each test substance was calculated using the following equation:

[0143]

Inhibitory rate (%) = $(1 - V_{\max}/V_0) \times 100$

[0144]

The inhibitor concentration required to cause a 50% inhibition of the enzyme activity (IC_{50}) was determined. The results are shown in Table 1. ">0.5" indicates that no inhibitory activity was observed in the concentration range up to 0.5 μM . Each IC_{50} value is a mean of 2 or 3 measurements.

[0145]

[Table 1]

SEQ ID NO:	length	IC_{50} [μM]
1	72	0.0410
2	72	0.0310
3	72	0.0260
4	72	0.0108
5	72	0.0070
6	72	0.0055
7	72	0.0520
8	72	0.0350

[0146]

The negative control 30N did not exhibit inhibitory activity ($IC_{50} > 0.5 \mu\text{M}$). The positive control chymostatin

to remove the RNA not bound to chymase, the resin was washed with solution A. Here, the solution A was a mixed solution of 145 mM sodium chloride, 5.4 mM potassium chloride, 1.8 mM calcium chloride, 0.8 mM magnesium chloride, and 20 mM Tris.

5 The RNA bound to chymase was heated at 95°C for 10 minutes with the addition of solution B as an eluent, and recovered from the supernatant. Here, the solution B was a mixture of 7M Urea, 3 mM EDTA, and 100 mM Tris-HCl (pH 6.6). The recovered RNA was amplified by RT-PCR and transcribed using the DuraScribe™ T7
10 Transcription Kit, and this was used as the pool for the next round. With this procedure taken as 1 round, the same operation was performed plural times. After completion of SELEX, the PCR product was cloned into pGEM-T Easy vector (manufactured by Promega), and the Escherichia coli strain DH5α
15 (manufactured by Toyobo) was transformed therewith. After the plasmid was extracted from a single colony, the base sequences of clones were determined using a DNA sequencer (3130xl Genetic Analyzer, manufactured by ABI).

[0151]

20 After 8 rounds of SELEX, the sequences of 38 clones were sequenced; sequence convergence was seen. The sequences of some of the clones are shown by SEQ ID NO: 9-10. Among them, two sequences were present that are shown by SEQ ID NO: 10. The common sequence was present in 3 out of the 38 clones. The
25 secondary structures of these sequences were predicted using the MFOLD program (M. Zuker, Nucleic Acids Res. 31(13), 3406-3415, 2003); the portions formed by the common sequence portion had a similar loop structure. The predicted secondary structures of the aptamers of the sequences shown by SEQ ID NO:
30 9 - 10 are shown in Fig. 3. The stem-loop structure is surrounded by a square (□).

[0152]

Given below are the respective nucleotide sequences. Unless otherwise stated, the following individual sequences are
35 shown in the direction from 5' to 3', with the purine bases (A

and G) being in the 2'-OH form, and the pyrimidine bases (U and C) in the 2'-fluoro-modified form.

[0153]

SEQ ID NO: 9

5 GGGAACGAAGCGACAGACGUUCCAGCGUCUAAUACGUGAAUAACCUGAUCGUAGGGGGUUCAA
GUACUGAGGACAGA

[0154]

SEQ ID NO: 10

GGGAACGAAGCGACAGACCGAAUCAGAAGUUCAACAUGGACAUACAUCGAUGGGGUGUCAAG
10 UACUGACGACAGA

[0155]

Whether the nucleic acids shown by SEQ ID NO: 9 and 10 inhibit the enzyme activity of chymase was determined by the following method. Angiotensin I is converted by chymase to
15 angiotensin II, during which a peptide fragment His-Leu is released. Since the peptide His-Leu is fluorescently derivatized by o-phthalaldehyde, its fluorescence intensity can be quantitatively measured.

[0156]

20 The total volume of solution for an enzyme reaction in the assay was set to 50 μ L, and the reaction was performed in solution C buffer. At first, native (manufactured by Calbiochem) was diluted with solution C to give 5 μ L thereof. Here, the native is chymase purified from human skin mast cell.
25 The nucleic acid was serially diluted with solution C at a concentration of 0.0027-2 μ M to give 25 μ L thereof. The chymase solution (5 μ L) and the nucleic acid solution (25 μ L) were mixed, and the mixture was incubated at 37°C for 5 min. On the other hand, 125 mM angiotensin I (manufactured by
30 PEPTIDE INSTITUTE, INC.) was prepared in solution C to give 20 μ L thereof, which was incubated at 37°C for 5 min. The angiotensin I solution was added to a mixture of chymase and nucleic acid to start an enzyme reaction. The final chymase concentration of the reaction solution was 0.5 nM, and the
35 final substrate concentration was 50 μ M. After reaction at

37°C for 90 min, ice-cooled 30% trichloroacetic acid solution (25 µL) was added to quench the reaction. The whole mixture was centrifuged at 4°C, 14000 rpm for 10 min, and the supernatant (30 µL) was used for the next fluorescence
5 induction reaction.

[0157]

The above-mentioned supernatant (30 µL) was added to a 96 well plate (black, manufactured by Costar), a solution (15 µL) of 2% o-phthalaldehyde (manufactured by SIGMA) in methanol and
10 0.3M NaOH solution (170 µL) were added to each well, and the mixture was incubated at room temperature for 10 min. Then, 3M HCl solution (25 µL) was added to quench the reaction. The plate was set on a microplate reader SpectraMax190 (manufactured by Molecular device) and the fluorescence
15 intensity was measured at an excitation wavelength of 355 nm and a fluorescence wavelength of 460 nm.

[0158]

The fluorescence intensity at reaction time 0 min under each condition was used as a blank. The fluorescence intensity
20 detected by the addition of the same amount of solution C instead of the nucleic acid in the chymase enzyme reaction was taken as 100%, and the inhibitory rate of each test substance was calculated by the following formula.

[0159]

25 Inhibitory rate (%) = $[1 - \{(\text{fluorescence intensity with test substance} - \text{fluorescence intensity of blank with test substance}) / (\text{fluorescence intensity without test substance} - \text{fluorescence intensity of blank without test substance})\}] \times 100$

[0160]

30 The concentration of the inhibitor necessary for 50% inhibition of the enzyme activity (IC₅₀) was determined. The results are shown in Table 2. In the Table, the IC₅₀ value is a mean of 2 or 3 measurements.

[0161]

[Table 2]

SEQ ID NO:	length	IC50 [μ M]
9	77	0.00022
10	76	0.00043

[0162]

5 The IC₅₀ value of the chymostatin used as a positive control is 0.35 - 0.5 μ M, and any nucleic acid contained in Table 2 is expected as a drug for the prophylaxis and/or treatment of various diseases involving angiotensin, since it shows a strong chymase inhibitory activity even when
10 angiotensin I, a native substrate, is used.

[0163]

Example 2: Strand shortening of aptamer

 The aptamer shown by SEQ ID NO: 2 was shortened. The aptamer shown by SEQ ID NO: 2 contains the common sequence.
15 The shortened sequences are shown in SEQ ID NO: 11 - 20. The predicted secondary structures of a part of the aptamers shown by SEQ ID NO: 11 - 20 are shown in Fig. 4. In Fig. 4, the common sequence is surrounded by a square ($\square$).

[0164]

20 Given below are the nucleotide sequences shown by SEQ ID NO: 11-20, respectively. Unless otherwise stated, the following individual sequences are shown in the direction from 5' to 3', with the purine bases (A and G) being in the 2'-OH form, and the pyrimidine bases (U and C) in the 2'-fluoro-
25 modified form.

[0165]

SEQ ID NO: 11

(sequence obtained by shortening sequence shown by SEQ ID NO: 2 to length of 43 nucleotides containing common sequence)

30 GGGUCAGAUGAUCGGACAUAACAUUGUUGGGGUGUCAAGGCC

[0166]

SEQ ID NO: 12

- (sequence obtained by shortening sequence shown by SEQ ID NO: 2
to length of 22 nucleotides containing common sequence)
GACAUACAUGUUGGGGUGUC
[0167]
- 5 SEQ ID NO: 13
(sequence obtained by shortening sequence shown by SEQ ID NO: 2
to length of 27 nucleotides containing common sequence)
GGACAUACAUGUUGGGGUGUCAAGG
[0168]
- 10 SEQ ID NO: 14
(sequence obtained by shortening sequence shown by SEQ ID NO: 2
to length of 39 nucleotides containing common sequence)
GGCAGAUGAUCGGACAUAACAUGUUGGGGUGUCAAGCC
[0169]
- 15 SEQ ID NO: 15
(sequence obtained by shortening sequence shown by SEQ ID NO: 2
to length of 35 nucleotides containing common sequence)
GAUGAUCGGACAUAACAUGUUGGGGUGUCAAGCC
[0170]
- 20 SEQ ID NO: 16
(sequence obtained by shortening sequence shown by SEQ ID NO: 2
to length of 36 nucleotides containing common sequence)
GGCAGAUGAUCGGACAUAACAUGUUGGGGUGUCAA
[0171]
- 25 SEQ ID NO: 17
(sequence obtained by shortening sequence shown by SEQ ID NO: 2
to length of 32 nucleotides containing common sequence)
GAUGAUCGGACAUAACAUGUUGGGGUGUCAA
[0172]
- 30 SEQ ID NO: 18
(sequence obtained by shortening sequence shown by SEQ ID NO: 2
to length of 30 nucleotides containing common sequence)
UGAUCGGACAUAACAUGUUGGGGUGUCAA
[0173]
- 35 SEQ ID NO: 19

(sequence obtained by shortening sequence shown by SEQ ID NO: 2 to length of 28 nucleotides containing common sequence)

AUCGGACAUAACAUAUUGUUGGGGUGUCA

[0174]

5 SEQ ID NO: 20

(sequence obtained by shortening sequence shown by SEQ ID NO: 2 to length of 31 nucleotides containing common sequence)

GAUGAUCGGACAUAACAUAUUGUUGGGGUGUCA

The nucleic acids of SEQ ID NO: 11 – 20 were all produced
10 by chemical synthesis. In the evaluation of SEQ ID NO: 11 – 20, a method similar to the evaluation method of inhibitory activity against chymase shown in Example 1 by using angiotensin I as a substrate was used for measurement. The measurement results are shown in Table 3. In the Table, ">1"
15 indicates that no inhibitory activity was observed in the concentration range up to 1 μ M. The IC₅₀ value is a mean of 2 or 3 measurements.

[0175]

[Table 3]

SEQ ID NO:	length	IC50 [μ M]
2	72	0.0100
11	43	0.0547
12	22	>1
13	27	>1
14	39	0.0068
15	35	0.0014
16	36	0.0020
17	32	0.0014
18	30	0.0021
19	28	0.0144
20	31	0.0018

20

[0176]

Many of the shortened forms of SEQ ID NO: 2 described in Table 3 exhibited inhibitory activity against chymase. The aptamers exhibiting IC₅₀ values of 0.01 μ M or less, in
25 particular, can be judged to have an excellent inhibitory

effect. From the results of SEQ ID NO: 18, it was found that an aptamer shortened to 30 nucleotides containing the common sequence also maintained the activity. This demonstrates that the common sequence is critical to the binding and inhibitory
5 activity against chymase.

[0177]

Example 3: Alteration of shortened aptamer

To enhance nuclease resistance of the aptamer shown by SEQ ID NO: 17, an altered aptamer with terminal modification,
10 an altered aptamer wherein the 2'-position of ribose of purine base in the sequence is modified with O-methyl group or F, and an altered aptamer wherein phosphorothioate is introduced were prepared. The sequences are shown by SEQ ID NO: 17(1) - 17(25).
[0178]

15 Given below are the respective nucleotide sequences. Unless otherwise stated, the following individual sequences are shown in the direction from 5' to 3', modification at the 2'-position of ribose is shown in the parenthesis, F is a fluorine atom, M is an O-methyl group, and PHE is 8-(propyl)phenyl-rA(n-
20 bz)-2'-tBDMS amidite. In respective sequence terminals, idT shows modification with inverted-dT, and S shows modification with thiol group. X shows a peptide sequence by a peptide bond of the C-terminal of phenylalanine and the N-terminal of cysteine, and Y shows a peptide sequence by a peptide bond of
25 the N-terminal of phenylalanine and the C-terminal of cysteine. In the sequences, s shows phosphorothioation of a phosphate group between the nucleotides. Z shows TC6F amidite.

[0179]

SEQ ID NO: 17(1)

30 sequence obtained by introducing 2'-O-methyl modification into 5'-terminal and 3'-terminal, introducing 2'-O-methyl modification into the first G and the second G of the common sequence GGGG of the sequence shown by SEQ ID NO: 17

GAU (F) GAU (F) C (F) GGAC (F) AU (F) AAC (F) AU (F) U (F) GU (F) U (F) G (M) G (M) GGU
35 (F) GU (F) C (F) AA

[0180]

SEQ ID NO: 17(2)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
5 modification into 12 positions in the sequence other than the
common sequence, and introducing 2'-O-methyl modification into
G other than the second G in the common sequence GGGG, of the
sequence shown by SEQ ID NO: 17

idT-

10 G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)AU(F)
U(F)G(M)U(F)U(F)G(M)GG(M)G(M)U(F)G(M)U(F)C(F)A(M)A(M)-idT

[0181]

SEQ ID NO: 17(3)

sequence obtained by introducing idT modification into
15 5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 12 positions in the sequence other than the
common sequence, and introducing 2'-O-methyl modification into
G other than the first G in the common sequence GGGG, of the
sequence shown by SEQ ID NO: 17

20 idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)AU(F)
U(F)G(M)U(F)U(F)GG(M)G(M)G(M)U(F)G(M)U(F)C(F)A(M)A(M)-idT

[0182]

SEQ ID NO: 17(4)

25 sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 12 positions in the sequence other than the
common sequence, and introducing 2'-O-methyl modification into
G other than the second G in the common sequence GGGG, of the
30 sequence shown by SEQ ID NO: 17

idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)ATTG(
M)U(F)U(F)G(M)GG(M)G(M)U(F)G(M)U(F)C(F)A(M)A(M)-idT

[0183]

35 SEQ ID NO: 17(5)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 12 positions in the sequence other than the
common sequence, and introducing 2'-O-methyl modification into
5 G other than the second G in the common sequence GGGG, of the
sequence shown by SEQ ID NO: 17

idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)ATTG(
M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT

10 [0184]

SEQ ID NO: 17(6)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 13 positions in the sequence other than the
15 common sequence, and introducing 2'-O-methyl modification into
G other than the second G in the common sequence GGGG, of the
sequence shown by SEQ ID NO: 17

idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)A(M)T
20 TG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT

[0185]

SEQ ID NO: 17(7)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
25 modification into 13 positions in the sequence other than the
common sequence, and introducing 2'-O-methyl modification into
G other than the second G in the common sequence GGGG, of the
sequence shown by SEQ ID NO: 17

idT-

30 G(M)A(M)TG(M)A(M)U(F)C(F)G(M)G(M)A(M)CA(M)U(F)AAC(F)A(M)TTG(M)U
(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT

[0186]

SEQ ID NO: 17(8)

sequence obtained by introducing idT modification into
35 5'-terminal and 3'-terminal, introducing 2'-O-methyl

modification into 13 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into U in
the common sequence UAAC, and introducing 2'-O-methyl
modification into G other than the second G in the common
5 sequence GGGG, of the sequence shown by SEQ ID NO: 17

idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(M)AAC(F)A(M)T
TG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT

[0187]

10 SEQ ID NO: 17(9)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 13 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into C in
15 the common sequence UAAC, and introducing 2'-O-methyl
modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 17

idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(M)A(M)T
20 TG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT

[0188]

SEQ ID NO: 17(10)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
25 modification into 14 positions in the sequence other than the
common sequence, and introducing 2'-O-methyl modification into
G other than the second G in the common sequence GGGG, of the
sequence shown by SEQ ID NO: 17

idT-

30 G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)A(M)T
TG(M)U(M)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT

[0189]

SEQ ID NO: 17(11)

sequence obtained by introducing idT modification into
35 5'-terminal and 3'-terminal, introducing 2'-O-methyl

modification into 14 positions in the sequence other than the common sequence, and introducing 2'-O-methyl modification into G other than the second G in the common sequence GGGG, of the sequence shown by SEQ ID NO: 17

5 idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)A(M)T
TG(M)U(F)U(M)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT
[0190]

SEQ ID NO: 17(12)

10 sequence obtained by introducing modification of peptide sequence by peptide binding of C-terminal of phenylalanine and N-terminal of cysteine into 5'-terminal by thiol group, introducing idT modification into 3'-terminal, introducing 2'-O-methyl modification into 13 positions in the sequence other
15 than the common sequence, and introducing 2'-O-methyl modification into G other than the second G in the common sequence GGGG, of the sequence shown by SEQ ID NO: 17

X-S-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)A(M)T
20 TG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT
[0191]

SEQ ID NO: 17(13)

sequence obtained by introducing modification of peptide sequence by peptide binding of N-terminal of phenylalanine and
25 C-terminal of cysteine into 5'-terminal by thiol group, introducing idT modification into 3'-terminal, introducing 2'-O-methyl modification into 13 positions in the sequence other than the common sequence, and introducing 2'-O-methyl modification into G other than the second G in the common
30 sequence GGGG, of the sequence shown by SEQ ID NO: 17

Y-S-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)A(M)T
TG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT
[0192]

35 SEQ ID NO: 17(14)

sequence obtained by introducing idT modification into 5'-terminal, introducing modification of peptide sequence by peptide binding of N-terminal of phenylalanine and C-terminal of cysteine into 3'-terminal by thiol group, introducing 2'-O-methyl modification into 13 positions in the sequence other than the common sequence, and introducing 2'-O-methyl modification into G other than the second G in the common sequence GGGG, of the sequence shown by SEQ ID NO: 17

idT-

10 G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)A(M)T
TG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-S-Y
[0193]

SEQ ID NO: 17(15)

sequence obtained by introducing idT modification into 5'-terminal and 3'-terminal, introducing 2'-O-methyl modification into 12 positions and 8-(propyl)phenyl-rA(n-bz)-2'-tBDMS amidite modification into one position in the sequence other than the common sequence, and introducing 2'-O-methyl modification into G other than the second G in the common sequence GGGG, of the sequence shown by SEQ ID NO: 17

idT-

G(M)A(PHE)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)A(M)
)TTG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT
[0194]

25 SEQ ID NO: 17(16)

sequence obtained by introducing idT modification into 5'-terminal and 3'-terminal, introducing 2'-O-methyl modification into 12 positions and 8-(propyl)phenyl-rA(n-bz)-2'-tBDMS amidite modification into one position in the sequence other than the common sequence, and introducing 2'-O-methyl modification into G other than the second G in the common sequence GGGG, of the sequence shown by SEQ ID NO: 17

idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)A(M)T
35 TG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(PHE)A(M)-idT

[0195]

SEQ ID NO: 17(17)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
5 modification into 12 positions and 8-(propyl)phenyl-rA(n-bz)-
2'-tBDMS amidite modification into one position in the sequence
other than the common sequence, and introducing 2'-O-methyl
modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 17

10 idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)A(M)T
TG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(PHE)-idT

[0196]

SEQ ID NO: 17(18)

15 sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 13 positions in the sequence other than the
common sequence, phosphorothioating phosphoric acid group
binding U nucleotides in the common sequence UAAC, and
20 introducing 2'-O-methyl modification into G other than the
second G in the common sequence GGGG, of the sequence shown by
SEQ ID NO: 17

idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)sAAC(F)A(M)
25 TTG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT

[0197]

SEQ ID NO: 17(19)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
30 modification into 13 positions in the sequence other than the
common sequence, phosphorothioating phosphoric acid group
binding the first A nucleotides in the common sequence UAAC,
and introducing 2'-O-methyl modification into G other than the
second G in the common sequence GGGG, of the sequence shown by

35 SEQ ID NO: 17

idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AsAC(F)A(M)
TTG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT
[0198]

5 SEQ ID NO: 17(20)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 13 positions in the sequence other than the
common sequence, phosphorothioating phosphoric acid group
10 binding the second A nucleotides in the common sequence UAAC,
and introducing 2'-O-methyl modification into G other than the
second G in the common sequence GGGG, of the sequence shown by
SEQ ID NO: 17

idT-

15 G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAAsC(F)A(M)
TTG(M)U(F)U(F)G(M)GG(M)G(M)TG(M)TCA(M)A(M)-idT
[0199]

SEQ ID NO: 17(21)

sequence obtained by introducing idT modification into
20 5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 13 positions in the sequence other than the
common sequence, phosphorothioating phosphoric acid group
binding the second A nucleotides in the common sequence UAAC,
introducing 2'-O-methyl modification into G other than the
25 second G in the common sequence GGGG, and phosphorothioating
phosphoric acid group binding the second G nucleotides, of the
sequence shown by SEQ ID NO: 17

idT-

G(M)A(M)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)A(M)T
30 TG(M)U(F)U(F)G(M)GsG(M)G(M)TG(M)TCA(M)A(M)-idT
[0200]

SEQ ID NO: 17(22)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
35 modification into 19 positions and 8-(propyl)phenyl-rA(n-bz)-

2'-tBDMS amidite modification into one position in the sequence other than the common sequence, introducing 2'-O-methyl modification into U and C of the common sequence UAAC, and introducing 2'-O-methyl modification into G other than the second G in the common sequence GGGG, of the sequence shown by
5 SEQ ID NO: 17

idT-

A(PHE)A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)A(M)
)U(M)U(M)G(M)U(M)U(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT

10 [0201]

SEQ ID NO: 17(23)

sequence obtained by introducing idT modification into 5'-terminal and 3'-terminal, introducing 2'-O-methyl modification into 11 positions and 8-(propyl)phenyl-rA(n-bz)-
15 2'-tBDMS amidite modification into one position in the sequence other than the common sequence, and introducing 2'-O-methyl modification into G other than the second G in the common sequence GGGG, of the sequence shown by SEQ ID NO: 17

idT-

20 G(M)A(PHE)U(F)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(F)AAC(F)AU(F)U(F)G(M)U(F)U(F)G(M)GG(M)G(M)U(F)G(M)U(F)C(F)A(M)A(M)-idT

[0202]

SEQ ID NO: 17(24)

sequence obtained by introducing TC6F amidite modification into 5'-terminal, introducing idT modification into 3'-terminal, introducing 2'-O-methyl modification into 20 positions in the sequence other than the common sequence, introducing 2'-O-methyl modification into U and C of the common sequence UAAC, and introducing 2'-O-methyl modification into G
30 other than the second G in the common sequence GGGG, of the sequence shown by SEQ ID NO: 17

idT-

ZA(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)A(M)U(M)
U(M)G(M)U(M)U(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT

35 [0203]

SEQ ID NO: 17(25)

sequence obtained by introducing TC6F amidite
modification into 5'-terminal, introducing idT modification
into 3'-terminal, introducing 2'-O-methyl modification into 20
5 positions in the sequence other than the common sequence,
introducing 2'-O-methyl modification into U and C of the common
sequence UAAC, and introducing 2'-O-methyl modification into G
other than the second G in the common sequence GGGG, of the
sequence shown by SEQ ID NO: 17

10 idT-

ZA(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)A(M)U(M)
U(M)G(M)U(M)U(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT
[0204]

All nucleic acids of SEQ ID NO: 17(1) - 17(25) were
15 prepared by chemical synthesis. In the evaluation of SEQ ID
NO: 17(1) - 17(25), a method similar to the evaluation method
of inhibitory activity against chymase shown in Example 1 by
using angiotensin I as a substrate was used for measurement.
The measurement results are shown in Table 4. In the Table,
20 the IC₅₀ value is a mean of 2 or 3 measurements.

[0205]

[Table 4]

SEQ ID NO:	length	IC ₅₀ [μM]
2	72	0.01000
17	32	0.00140
17(1)	32	0.04436
17(2)	32	0.00021
17(3)	32	0.00566
17(4)	32	0.00020
17(5)	32	0.00017
17(6)	32	0.00014
17(7)	32	0.00017
17(8)	32	0.00013
17(9)	32	0.00016
17(10)	32	0.00013
17(11)	32	0.00014
17(12)	32	0.00019
17(13)	32	0.00016
17(14)	32	0.00019
17(15)	32	0.00013
17(16)	32	0.00018
17(17)	32	0.00019
17(18)	32	0.00015
17(19)	32	0.00019
17(20)	32	0.00018
17(21)	32	0.00019
17(22)	32	0.00017
17(23)	32	0.00016
17(24)	32	0.00019
17(25)	32	0.00020

[0206]

5 Many of the altered forms of SEQ ID NO: 17 described in Table 4 exhibited inhibitory activity against chymase. The aptamers exhibiting IC₅₀ values of 0.001 μM or less, in particular, can be judged to have an excellent inhibitory effect. From the results of SEQ ID NO: 17(11) - SEQ ID NO:
10 17(14), it was shown that the terminal modification does not influence the activity.

[0207]

As for the nucleotides contained in the common sequence,

the 2'-O-methyl modification of the second G in GGGG in the above-mentioned common sequence as in SEQ ID NO: 17(1) and SEQ ID NO: 17(3) resulted in a decrease in the activity even though the activity was sufficient as that of an aptamer, and it was
5 shown that changes in the modification embodiment of this part affects the activity. On the other hand, it was shown that the 2'-O-methyl modification of G other than the second G in GGGG in the above-mentioned common sequence as in SEQ ID NO: 17(2) does not influence the activity.

10 [0208]

From the results of the aptamers shown by SEQ ID NO: 17(1) - SEQ ID NO: 17(25), modification of sequence other than the second G of GGGG in the above-mentioned common sequence improves stability. Thus, it was found that at least one
15 nucleotide of the aptamer of the present invention may be modified as long as the activity is maintained. As the modification of nucleotide, for example, 2'-amino modification and the like can be mentioned in addition to 2'-O-methyl modification.

20 [0209]

From the above, SEQ ID NO: 17 altered by chemical modification has a strong chymase-inhibitory activity and was suggested to be usable as a chymase inhibitor.

[0210]

25 Example 4: Effect of base insertion on loop structure of aptamer

A base was inserted into the loop structure of the aptamer shown by SEQ ID NO: 2, and an influence on the binding activity and inhibitory activity was examined. The sequences
30 are shown in SEQ ID NO: 21 - SEQ ID NO: 25.

[0211]

All nucleic acids of SEQ ID NO: 21 - 25 were prepared by chemical synthesis. The nucleotide sequences of respective aptamers shown by the following SEQ ID NO: 21 - 25 are shown
35 below. Unless otherwise specified, the respective sequences

recited below are in the direction of from 5' to 3',
modification at the 2'-position of ribose is shown in the
parenthesis (for example, U(F) shows modification of the 2'-
position of ribose of uracil with F), and F is a fluorine atom.

5 [0212]

SEQ ID NO: 21

sequence obtained by inserting 17 nucleic acid bases into
loop structure of sequence shown by SEQ ID NO: 2

GGCAGAUGAUCGGACAUAACAGGUUAGAUAGAGUUAAAAACCUGGGGUGUCAAA

10 [0213]

SEQ ID NO: 22

sequence obtained by inserting 15 nucleic acid bases into
loop structure of sequence shown by SEQ ID NO: 2

GGCAGAUGAUCGGACAUAACAGUUAGAUAGAGUUAAAAACUGGGGUGUCAAA

15 [0214]

SEQ ID NO: 23

sequence obtained by inserting 13 nucleic acid bases into
loop structure of sequence shown by SEQ ID NO: 2

GGCAGAUGAUCGGACAUAACAGUAGAUAGAGUUAAAACUGGGGUGUCAAA

20 [0215]

SEQ ID NO: 24

sequence obtained by inserting 11 nucleic acid bases into
loop structure of sequence shown by SEQ ID NO: 2

GGCAGAUGAUCGGACAUAACAUAAGAUAGAGUUAAAUGGGGUGUCAAA

25 [0216]

SEQ ID NO: 25

sequence obtained by inserting 9 nucleic acid bases into
loop structure of sequence shown by SEQ ID NO: 2

GGCAGAUGAUCGGACAUAACAAGAUAGAGUUAAAUGGGGUGUCAAA

30 [0217]

All nucleic acids of SEQ ID NO: 21 - 25 were prepared by
chemical synthesis.

[0218]

In the evaluation of SEQ ID NO: 21 - 25, a method similar
35 to the evaluation method of inhibitory activity against chymase

shown in Example 1 by using MCA as a substrate except that the amount chymase was changed from 0.05 μg to 0.005 μg was used for measurement. The measurement results are shown in Table 5. In the Table, the value of IC_{50} is the value of one measurement.

5 [0219]

[Table 5]

SEQ ID NO:	number inserted into loop structure	IC_{50} [μM]
21	17	0.0057
22	15	0.0050
23	13	0.0078
24	11	0.0037
25	9	0.0058

[0220]

The sequences of Table 5 showed an inhibitory activity against chymase. All showed an IC_{50} value of not more than 0.01 μM and an inhibitory effect. It was found that the insertion of up to 17 bases into the loop structure of SEQ ID NO: 2 resulted in the maintenance of activity.

[0221]

15 Example 5: Production of RNA aptamer by optimized SELEX

Optimized SELEX was performed to obtain aptamers that specifically bind to chymase and have higher activity. The optimized SELEX was the same as the SELEX described in Example 1 except for the DNA template and primers described below.

20 [0222]

The DNA templates used were 4 kinds of DNA templates prepared by chemical synthesis based on SEQ ID NO: 17 obtained in Example 2, by forming a random sequence as a part of the sequence. The primers used were SEQ ID NO: 35 and SEQ ID NO: 36 used in Example 1. Fig. 5 shows random sequence parts (surrounded by square) of the DNA templates.

[0223]

DNA template: 5'-

TCACACTAGCACGCATAGGCCTTGACACCCCAACAATGTTATGTCCGATCATCTGACCTCTCT
30 CCTGCTCCC-3' (SEQ ID NO: 40)

DNA template 1: template in which a part surrounded by square of SEQ ID NO: 17 is doped with 9% random sequence (A, G, C or T in any combination of nucleotides)

5 DNA template 2: template in which a part surrounded by square of SEQ ID NO: 17 is doped with 9% random sequence (A, G, C or T in any combination of nucleotides)

DNA template 3: template in which a part surrounded by square of SEQ ID NO: 17 is doped with 15% random sequence (A, G, C or T in any combination of nucleotides)

10 DNA template 4: template in which a part surrounded by square of SEQ ID NO: 17 is random sequence (A, G, C or T in any combination of nucleotides)

[0224]

After 6 rounds of SELEX using DNA template 1 and DNA
15 template 2, the sequences of 48 clones were examined to find sequence convergence. Similarly, after 3 rounds of SELEX using DNA template 3 and DNA template 4, the sequences of 32 clones were examined to find sequence convergence. The sequences of some of the clones are shown by SEQ ID NO: 26 - 31. The
20 predicted secondary structures of the aptamers of the sequences shown by SEQ ID NO: 26 - 31 are shown in Fig. 6. The stem-loop structure represented by the formula (1) is surrounded by a square (□).

[0225]

25 Given below are the respective nucleotide sequences.

Unless otherwise stated, the following individual sequences are shown in the direction from 5' to 3', with the purine bases (A and G) being in the 2'-OH form, and the pyrimidine bases (U and C) in the 2'-fluoro-modified form.

30 [0226]

SEQ ID NO: 26

GGGAGCAGGAGAGAGGUCAGAUUUUCGGGCAUAACAUUGUUGGGGUGUAACGACCUAUGCGUG
CUAGUGUGA

[0227]

35 SEQ ID NO: 27

GGGAGCAGGAGAGAGGUCAGAUGAACGGACAUAACAUAUUGUUGGGGUGUCAAGGCCUAUGCGUG
CUAGUGUGA

[0228]

SEQ ID NO: 28

5 GGGAGCAGGAGAGAGGUCAGAUGAUCGGACAUAACUCUGGAGGGGUGUCAAGGCCUAUGCGUG
CUAGUGUGA

[0229]

SEQ ID NO: 29

GGGAGCAGGAGAGAGGUCAGAUGAUCGGGCAUAACAUAUUGUUGGGGUGUCAAGGCCUAUGCGUG
10 CUAGUGUGA

[0230]

SEQ ID NO: 30

GGGAGCAGGAGAGAGGUCAGAUGAUCGGACAUAACUAAUUAGGGGUGUCAAGGCCUAUGCGUG
CUAGUGUGA

15 [0231]

SEQ ID NO: 31

GGGAGCAGGAGAGAGGUCAGAUGAUCGGACAUAACAUAUUGCUGGGGUGUCAAGGCCUAUGCGUG
CUAGUGUGA

[0232]

20 All nucleic acids of SEQ ID NO: 26 - 31 were prepared by
chemical synthesis. The aptamers shown by these sequences
exhibited activity similar to that of the aptamers shown in
Example 2.

[0233]

25 Example 6: Shortening of aptamer obtained by optimized SELEX

The aptamer shown by SEQ ID NO: 30 was shortened. The
aptamer shown by SEQ ID NO: 30 contains the common sequence.
The shortened sequences are shown in SEQ ID NO: 32 - 33. The
predicted secondary structures of the aptamers shown by SEQ ID
30 NO: 32 - 33 are shown in Fig. 7. In Fig. 7, the stem-loop
structure represented by the formula (1) is surrounded by a
square (□).

[0234]

Respective nucleotide sequences shown in SEQ ID NOs: 32 -
35 33 are presented below. Unless particularly indicated, the

sequences shown below are in the 5' to 3' direction, purine bases (A and G) are 2'-OH forms and pyrimidine bases (U and C) are 2'-fluoro modified forms.

[0235]

5 SEQ ID NO: 32

sequence obtained by shortening sequence shown by SEQ ID NO: 30 to length of 32 nucleotides containing common sequence GAUGAUCGGACAUAACUAAUUAGGGGUGUCAA

[0236]

10 SEQ ID NO: 33

sequence obtained by shortening sequence shown by SEQ ID NO: 30 to length of 31 nucleotides containing common sequence AUGAUCGGACAUAACUAAUUAGGGGUGUCAA

[0237]

15 All nucleic acids of SEQ ID NO: 32 - 33 were prepared by chemical synthesis. The aptamers shown by these sequences exhibited activity similar to that of the aptamers shown in Example 2.

[0238]

20 Example 7: Alteration of aptamer obtained by optimized SELEX and shortening

To enhance nuclease resistance of the aptamer shown by SEQ ID NO: 33, an altered aptamer with terminal modification, an altered aptamer wherein the 2'-position of ribose of purine base in the sequence is modified with O-methyl group or F, and an altered aptamer wherein phosphorothioate is introduced were prepared. The sequences are shown in SEQ ID NO: 33(1) - 33(18).

[0239]

The respective nucleotide sequences are shown below.

30 Unless otherwise specified, the respective sequences recited below are in the direction of from 5' to 3', modification at the 2'-position of ribose is shown in the parenthesis, F is a fluorine atom, M is an O-methyl group, and PHE is 8-(Propyl)phenyl-rA(n-bz)-2'-tBDMS amidite. In respective
35 sequence terminals, idT shows modification with inverted-dT,

PEG shows modification with 40 kDa branched polyethylene glycol, and S shows modification with a thiol group.

[0240]

SEQ ID NO: 33(1)

5 sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 15 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into U
and C in the common sequence UAAC, and introducing 2'-O-methyl
10 modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 33

idT-

A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)U(F)AAU(F)
)U(M)AG(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT

15 [0241]

SEQ ID NO: 33(2)

 sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 13 positions in the sequence other than the
20 common sequence, introducing 2'-O-methyl modification into U
and C in the common sequence UAAC, and introducing 2'-O-methyl
modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 33

idT-

25 A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(M)AAC(M)U(F)AAU(F)
)U(M)AG(M)GG(M)G(M)TG(M)TC(M)A(M)A(M)-idT

[0242]

SEQ ID NO: 33(3)

 sequence obtained by introducing idT modification into
30 5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 17 positions and 8-(propyl)phenyl-rA(n-bz)-
2'-tBDMS amidite modification into one position in the sequence
other than the common sequence, introducing 2'-O-methyl
modification into U and C in the common sequence UAAC, and
35 introducing 2'-O-methyl modification into G other than the

second G in the common sequence GGGG, of the sequence shown by
SEQ ID NO: 33

idT-

A(PHE)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)U(F)A(M)
5)A(M)U(F)U(M)A(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT
[0243]

SEQ ID NO: 33(4)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
10 modification into 15 positions and 8-(propyl)phenyl-rA(n-bz)-
2'-tBDMS amidite modification into one position in the sequence
other than the common sequence, introducing 2'-O-methyl
modification into U and C in the common sequence UAAC, and
introducing 2'-O-methyl modification into G other than the
15 second G in the common sequence GGGG, of the sequence shown by
SEQ ID NO: 33

idT-

A(PHE)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(M)AAC(M)U(F)A(M)
)A(M)U(F)U(M)A(M)G(M)GG(M)G(M)TG(M)TC(M)A(M)A(M)-idT
20 [0244]

SEQ ID NO: 33(5)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 16 positions and 8-(propyl)phenyl-rA(n-bz)-
25 2'-tBDMS amidite modification into one position in the sequence
other than the common sequence, introducing 2'-O-methyl
modification into U and C in the common sequence UAAC, and
introducing 2'-O-methyl modification into G other than the
second G in the common sequence GGGG, of the sequence shown by
30 SEQ ID NO: 33

idT-

A(PHE)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(M)AAC(M)U(F)A(M)
)A(M)U(F)U(M)A(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT
[0245]

35 SEQ ID NO: 33(6)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 18 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into U
5 and C in the common sequence UAAC, and introducing 2'-O-methyl
modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 33

idT-

A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(F)A(M)U(M)AAC(M)U(F)A(M)A
10 (M)U(M)U(M)A(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT
[0246]

SEQ ID NO: 33(7)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
15 modification into 19 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into
nucleotides other than the second A of the common sequence UAAC,
and introducing 2'-O-methyl modification into G other than the
second G in the common sequence GGGG, of the sequence shown by
20 SEQ ID NO: 33

idT-

A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)A(M)AC(M)U(F)A(
M)A(M)U(M)U(M)A(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT
[0247]

25 SEQ ID NO: 33(8)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 19 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into
30 nucleotides other than the first A of the common sequence UAAC,
and introducing 2'-O-methyl modification into G other than the
second G in the common sequence GGGG, of the sequence shown by
SEQ ID NO: 33

idT-

35 A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AA(M)C(M)U(F)A(

M)A(M)U(M)U(M)A(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT
[0248]

SEQ ID NO: 33(9)

sequence obtained by introducing idT modification into
5 5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 19 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into U
and C in the common sequence UAAC, and introducing 2'-O-methyl
modification into common sequence GGGG, of the sequence shown
10 by SEQ ID NO: 33
idT-

A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)U(F)A(M)A
(M)U(M)U(M)A(M)G(M)G(M)G(M)G(M)tG(M)U(M)C(M)A(M)A(M)-idT
[0249]

15 SEQ ID NO: 33(10)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 19 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into U
20 and C in the common sequence UAAC, and introducing 2'-O-methyl
modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 33
idT-

A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)TA(M)A(M)
25 U(M)U(M)A(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT
[0250]

SEQ ID NO: 33(11)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
30 modification into 18 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into U
and C in the common sequence UAAC, and introducing 2'-O-methyl
modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 33
35 idT-

A (M) U (M) G (M) A (M) U (F) C (F) G (M) G (M) A (M) C (M) A (M) U (M) AAC (M) U (F) A (M) A
(M) TU (M) A (M) G (M) GG (M) G (M) TG (M) U (M) C (M) A (M) A (M) -idT
[0251]

SEQ ID NO: 33(12)

5 sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 18 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into U
and C in the common sequence UAAC, and introducing 2'-O-methyl
10 modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 33
idT-

A (M) U (M) G (M) A (M) U (F) C (F) G (M) G (M) A (M) C (M) A (M) U (M) AAC (M) U (F) A (M) A
(M) U (M) TA (M) G (M) GG (M) G (M) TG (M) U (M) C (M) A (M) A (M) -idT

15 [0252]

SEQ ID NO: 33(13)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 20 positions in the sequence other than the
20 common sequence, introducing 2'-O-methyl modification into U
and C in the common sequence UAAC, and introducing 2'-O-methyl
modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 33
idT-

25 A (M) U (M) G (M) A (M) U (F) C (F) G (M) G (M) A (M) C (M) A (M) U (M) AAC (M) T (M) A (M) A
(M) U (M) U (M) A (M) G (M) GG (M) G (M) TG (M) U (M) C (M) A (M) A (M) -idT

[0253]

SEQ ID NO: 33(14)

sequence obtained by introducing idT modification into
30 5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 19 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into U
and C in the common sequence UAAC, and introducing 2'-O-methyl
modification into G other than the second G in the common
35 sequence GGGG, of the sequence shown by SEQ ID NO: 33

idT-

A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)U(F)A(M)A
(M)T(M)U(M)A(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT
[0254]

5 SEQ ID NO: 33(15)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 19 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into U
10 and C in the common sequence UAAC, and introducing 2'-O-methyl
modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 33

idT-

A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)U(F)A(M)A
15 (M)U(M)T(M)A(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT
[0255]

SEQ ID NO: 33(16)

sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
20 modification into 20 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into U
and C in the common sequence UAAC, and introducing 2'-O-methyl
modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 33

25 idT-

A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)U(F)A(M)A
(M)U(M)U(M)A(M)G(M)GG(M)G(M)T(M)G(M)U(M)C(M)A(M)A(M)-idT
[0256]

SEQ ID NO: 33(17)

30 sequence obtained by introducing idT modification into
5'-terminal and 3'-terminal, introducing 2'-O-methyl
modification into 18 positions and 8-(propyl)phenyl-rA(n-bz)-
2'-tBDMS amidite modification into one position in the sequence
other than the common sequence, introducing 2'-O-methyl
35 modification into U and C in the common sequence UAAC, and

introducing 2'-O-methyl modification into G other than the second G in the common sequence GGGG, of the sequence shown by SEQ ID NO: 33

idT-

5 A(PHE)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)U(F)A(M)
)A(M)U(M)U(M)A(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT
[0257]

SEQ ID NO: 33(18)

sequence obtained by introducing 40 kDa branched
10 polyethylene glycol modification into 5'-terminal, introducing
idT modification into 3'-terminal, introducing 2'-O-methyl
modification into 18 positions in the sequence other than the
common sequence, introducing 2'-O-methyl modification into U
and C in the common sequence UAAC, and introducing 2'-O-methyl
15 modification into G other than the second G in the common
sequence GGGG, of the sequence shown by SEQ ID NO: 33

PEG-

A(M)U(M)G(M)A(M)U(F)C(F)G(M)G(M)A(M)C(M)A(M)U(M)AAC(M)U(F)A(M)A
(M)U(M)TA(M)G(M)GG(M)G(M)TG(M)U(M)C(M)A(M)A(M)-idT
20 [0258]

All nucleic acids of SEQ ID NO: 33(1) - 33(18) were
prepared by chemical synthesis. The aptamers shown by these
sequences were measured by a method similar to the inhibitory
activity evaluation method for chymase inhibitory activity
25 shown in Example 1 by using angiotensin I as a substrate. The
results are shown in Table 6. In the Table, the IC₅₀ value is
a mean of 2 or 3 measurements.

[0259]

[Table 6]

SEQ ID NO:	length	IC50 [μ M]
2	72	0.010000
17	32	0.001400
33	32	0.000210
33(1)	31	0.000074
33(2)	31	0.000078
33(3)	31	0.000071
33(4)	31	0.000072
33(5)	31	0.000076
33(6)	31	0.000053
33(7)	31	0.000071
33(8)	31	0.000061
33(9)	31	0.001355
33(10)	31	0.000071
33(11)	31	0.000051
33(12)	31	0.000048
33(13)	31	0.000069
33(14)	31	0.000075
33(15)	31	0.000072
33(16)	31	0.000063
33(17)	31	0.000038
33(18)	31	0.000109

[0260]

5 As shown in Table 6, many of the altered forms exhibited inhibitory activity against chymase. The aptamers exhibiting IC₅₀ values of 0.0001 μ M or less, in particular, can be judged to have a very superior inhibitory effect. From the above, it was found that at least one nucleotide of the aptamer may be
10 modified to increase the stability of the aptamer of the sequence shown by SEQ ID NO: 33 with various modifications applied thereon as long as its activity is ensured. As the modification of nucleotide, for example, 2'-amino modification and the like can be mentioned in addition to 2'-O-methyl
15 modification.

[0261]

As for the nucleotides contained in the common sequence, the 2'-O-methyl modification of the second G in the common

sequence GGGG as in SEQ ID NO: 33(9) resulted in a decrease in the activity as compared to other modified aptamer having sequence SEQ ID NO: 33, even though the activity was sufficient as that of an aptamer. On the other hand, modification as in
5 SEQ ID NO: 33(12) did not influence the activity.

[0262]

From the results of SEQ ID NO: 33(18), a certain level of the activity of PEG-bound aptamer was maintained as compared to the aptamer not bound with PEG (SEQ ID NO: 33(12)). Since
10 binding with PEG markedly improves in vivo pharmacokinetics, an in vivo effect is expected from the results.

[0263]

From the above results, any nucleic acid contained in Table 6 other than nucleic acid in which the 2nd G of GGGG in
15 the common sequence is modified with 2'-O-methyl is expected as a drug for the prophylaxis and/or treatment of various diseases involving angiotensin, since it shows an extremely strong chymase inhibitory activity even when angiotensin I, a native substrate, is used.

20 [Industrial Applicability]

[0264]

The aptamer of the present invention can be useful as a pharmaceutical for preventing and/or treating various diseases involving activation of TGF- β such as fibrosis and the like, or
25 a diagnostic reagent or a labeling agent.

[0265]

This application is based on a patent application No. 2017-230503 filed in Japan (filing date: November 30, 2017), the contents of which are incorporated in full herein.

SEQUENCE LISTING

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 <151> 2017-11-30
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<220>
<223> Substrate for Chymotrypsin

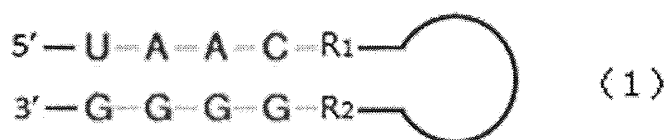
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Ala Ala Pro Phe
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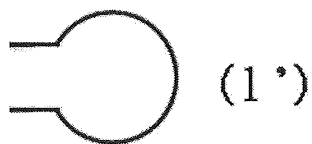
Claims

1. An aptamer that binds to chymase, and comprises a common sequence represented by UAACR₁N₁R₂GGGG wherein R₁ and R₂ are
 5 each any one base, and N₁ shows 3 to 30 bases (uracil is optionally thymine).

2. An aptamer that binds to chymase, and comprises a potential secondary structure represented by the formula (1) (uracil is
 10 optionally thymine):



wherein



in the formula (1) is a stem-loop partial structure, and R₁ and
 15 R₂ are each any one base.

3. The aptamer according to claim 1 or 2 that inhibits the activity of chymase.

20 4. The aptamer according to any one of claims 1 to 3, wherein a combination of R₁ and R₂ is A/U, C/G, A/C or G/U (uracil is optionally thymine).

5. The aptamer according to any one of claims 1 to 4, wherein
 25 at least one of pyrimidine nucleotides is modified or altered.

6. The aptamer according to any one of claims 1 to 5, wherein U and/or C in the sequence UAACR₁ are/is modified or altered.

30 7. The aptamer according to any one of claims 2 to 6, wherein

the stem-loop part of (1') has a base length of 3 - 21.

8. The aptamer according to any one of claims 1 to 7, wherein a
sequence of N₁ or the stem-loop partial structure represented
5 by



is UUGU, CUGG or AAUU (uracil is optionally thymine).

9. The aptamer according to claim 1 or 2, comprising any of the
10 nucleotide sequences of the following (a), (b) and (c):

(a) a nucleotide sequence selected from any of SEQ ID NOs: 1 -
11 and 14 - 33 (uracil is optionally thymine);

(b) a nucleotide sequence selected from any of SEQ ID NOs: 1 -
11 and 14 - 33 (uracil is optionally thymine) wherein one to
15 several nucleotides are substituted, deleted, inserted or
added; and

(c) a nucleotide sequence having identity of not less than 70%
with a nucleotide sequence selected from any of SEQ ID NOs: 1 -
11 and 14 - 33 (uracil is optionally thymine).

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10. The aptamer according to claim 9, wherein at least one
nucleotide contained in the aptamer is modified or altered.

11. The aptamer according to claim 1 or 2, wherein a hydroxyl
25 group at the 2'-position of ribose of each pyrimidine
nucleotide contained in the aptamer is the same or different
and unsubstituted or substituted by an atom or a group selected
from the group consisting of a hydrogen atom, a fluorine atom
and a methoxy group.

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12. The aptamer according to claim 1 or 2, wherein the hydroxyl
group at the 2'-position of ribose of each purine nucleotides

contained in the aptamer is the same or different and unsubstituted or substituted by an atom or a group selected from the group consisting of a hydrogen atom, a fluorine atom and a methoxy group.

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13. A complex comprising the aptamer and the functional substance according to any one of claims 1 to 12.

14. A medicament comprising the aptamer according to any one of
10 claims 1 to 12 or the complex according to claim 13.

15. The medicament according to claim 14 that is a therapeutic drug for a disease involving organ or tissue fibrosis, or a circulatory disease.

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16. The medicament according to claim 14 that is a therapeutic drug for fibrosis.

17. A method for detecting chymase, comprising using the
20 aptamer according to any one of claims 1 to 12 or the complex according to claim 13.