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A sulfanyl-PEG derivative of relaxin-like peptide utilizable for the conjugation with KLH and the antibody production

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ABSTRACT

A small peptide–keyhole limpet hemocyanin (KLH) conjugate is generally used as an antigen for producing specific antibodies. However, preparation of a disulfide-rich heterodimeric peptide–KLH conjugates is difficult. In this study, we developed a novel method for preparation of the conjugate, and applied it to the production of specific antibodies against the relaxin-like gonad-stimulating peptide (RGP) from the starfish. In this method, a sulfanyl group necessary for the conjugation with KLH was site-specifically introduced to the peptide after regioselective disulfide bond formation reactions. Using the conjugate, we could obtain specific antibodies with a high antibody titer. This method might also be useful for the production of antibodies against other heterodimeric peptides with disulfide cross-linkages, such as vertebrate relaxins.

1. Introduction

Antibodies for peptides/proteins are powerful tools for biochemical and histochemical analyses, and various anti-peptide/protein antibodies are available from commercial resources. Since it is difficult to recognize the peptide by antibodies in immunized animals, the peptide needs to be conjugated to a large protein, such as bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), for general use as an antigen.[1] Since the sulfanyl group easily and specifically reacts with a maleimide moiety to form a covalent bond, this reaction has been widely used for preparing the peptide–KLH conjugates.

Chemical synthesis of various disulfide-rich peptides has been achieved by ordinary solid-phase peptide synthesis (SPPS) and oxidative disulfide bond formation reactions. Chemically, disulfide bonds can be formed regioselectively by repeating the deprotection of Cys-protecting groups and the oxidation to form disulfide bonds. To date, it has been reported that four disulfide bonds could be formed regioselectively using three or four orthogonal protecting groups.[2–4] Heterodimeric disulfide-rich peptides, such as human relaxins[5] and crustacean insulin-like peptides,[3,6] have also been synthesized by this strategy. On the other hand, to use a synthetic heterodimeric peptide with disulfide cross-linkages for preparing a peptide–KLH conjugate, an additional sulfanyl group is needed in the peptide structure. When an additional Cys residue is introduced into the synthetic peptide, the disulfide bond might be randomized by the reducing ability of free sulfanyl group. It is, therefore, difficult to prepare the KLH conjugate of a heterodimeric peptide with the proper disulfide bond arrangement by the ordinary conjugation method, and the preparation method for such a conjugate has not yet been reported.

Relaxin-like gonad-stimulating peptide (RGP) from the starfish *Patiria (Asterina) pectinifera* has a relaxin-like heterodimeric structure that contains one intra-chain and two inter-chain disulfide bonds (Fig. 1), and is an echinodermatous gonadotropic hormone responsible for final gamete maturation.[7] We have tried to obtain antibodies against RGP using the A chain or the B chain conjugated with KLH as an antigen. However, these trials failed and no antibodies that specifically recognized RGP could be obtained (unpublished data).

A cDNA encoding RGP from the crown-of-thorns starfish, *Acanthaster planci*, has recently been cloned.[8] Interestingly, the deduced amino acid sequence of *A. planci* RGP was identical to that of *P. pectinifera* RGP. *A. planci* is the major reef coral predator in the Indo-Pacific region, and it is quite important to investigate the molecular mechanisms of reproduction in order to avoid *A. planci* outbreak. RGP is the key intrinsic factor in the reproduction of starfishes, and therefore anti-RGP antibodies are desired for biochemical and histochemical analyses of RGP. In this study, we tried to prepare KLH conjugated with the whole *P. pectinifera* RGP molecule, and to obtain the specific antibodies against *P. pectinifera* and *A. planci* RGPs.

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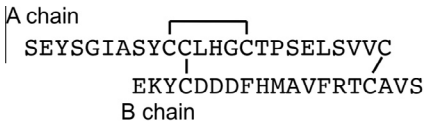


Figure 1. Primary structure of *P. pectinifera* RGP (**1**).

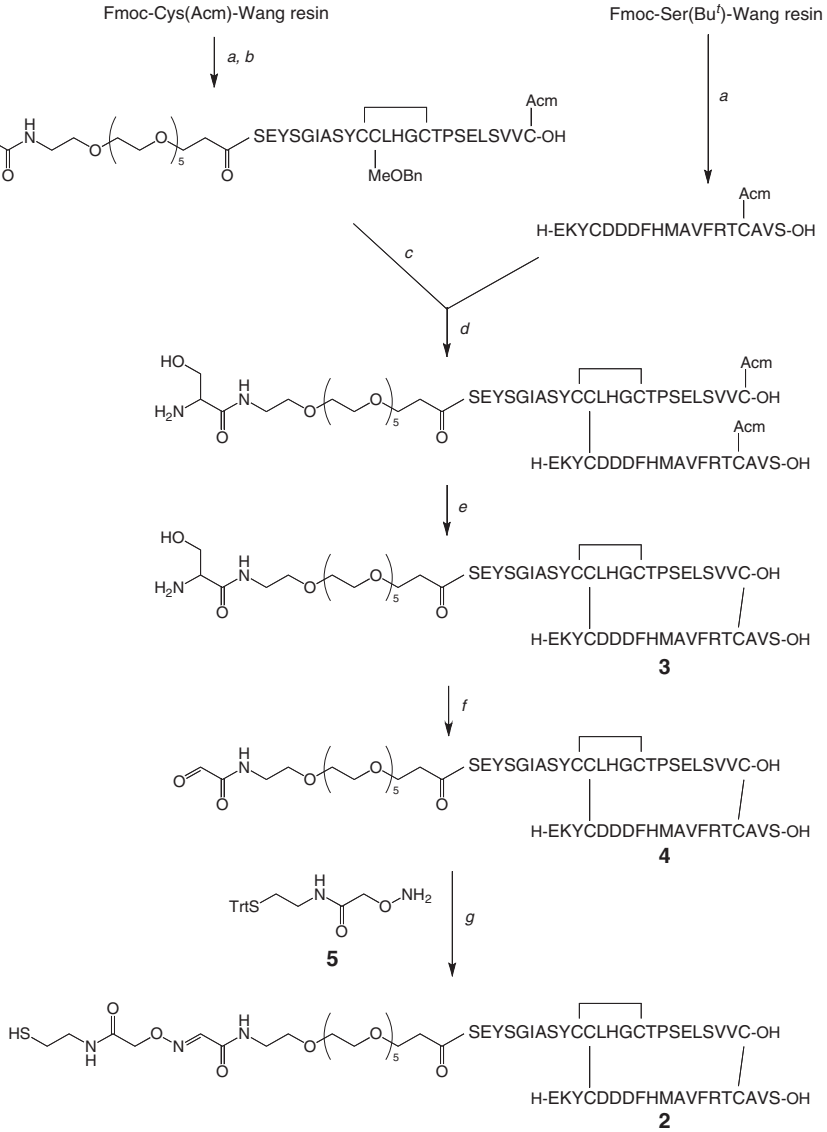
2. Results and discussion

2.1. Chemical synthesis of RGP

In order to obtain *P. pectinifera* RGP (**1**) for use in the comparative studies with RGP derivatives described below, we synthesized it by the ordinary 9-fluorenylmethoxycarbonyl (Fmoc)-based SPPS and regioselective disulfide bond formation reactions, essentially according to the method for the synthesis of crustacean insulin-like peptide described previously.[6] In brief, Cys[11](MeOBn), Cys[24](Acm)-A chain was synthesized by Fmoc-SPPS and dimethyl sulfoxide (DMSO)-mediated intrachain disulfide bond formation reactions. After the *p*-methoxybenzyl (MeOBn) group attached at Cys[11] was replaced with a 2-pyridylsulfenyl group by 2,2′-dipyridyl disulfide (DPDS) treatment under acidic conditions, the heterodimerization was performed by mixing with Cys[16](Acm)-B chain under neutral pH, giving Cys^{A24,B16}(Acm)-RGP. Finally the acetamidomethyl (Acm) groups were oxidatively removed by iodine treatment under acidic pH, giving RGP **1**.

2.2. Chemical synthesis of RGP derivative with a sulfanyl group

To obtain the RGP–KLH conjugate, we first tried to synthesize the RGP derivative, which had an additional Cys residue at the N-terminus of the A chain. Since a free sulfanyl group prevents the regioselective formation of disulfide bonds, the N-terminal Cys residue should be protected by a protecting group that is orthogonal to the other Cys protecting groups used in the RGP synthesis. For this purpose, we used thiazolidinecarboxylic acid as the Cys surrogate. A thiazolidine ring at the peptide N-terminus has been widely used for the protection of N-terminal Cys residue,[9] especially in protein chemical syntheses by the native chemical ligation strategy.[10] Since the thiazolidine ring can be opened by methoxyamine treatment under weakly acidic conditions,[9] it was



Scheme 1. Synthetic procedure of the antigen **2**. Reaction conditions: (a) 1. Fmoc-SPPS, 2. TFA/H_2O/thioanisole/phenol/triisopropylsilane (82.5/5/5/5/2.5), (b) 10% DMSO/6 M guanidine-HCl/50 mM Pi buffer (pH 7.0), (c) 2,2′-dipyridyl disulfide, TfOH/thioanisole/TFA (1/2/20), (d) 40% CH_3CN/50 mM $NaHCO_3$/H_2O (50%), (e) I_2, HCl, H_2O/CH_3OH (91%), (f) $NaIO_4$ (1.3 equiv), 50 mM Pi buffer (pH 7.0) (49%), (g) 1. Probe **5**, 5% AcOH/*N*,*N*-dimethylacetamide, 2. TFA/H_2O/triisopropylsilane (96/2/2) (41% in two steps).

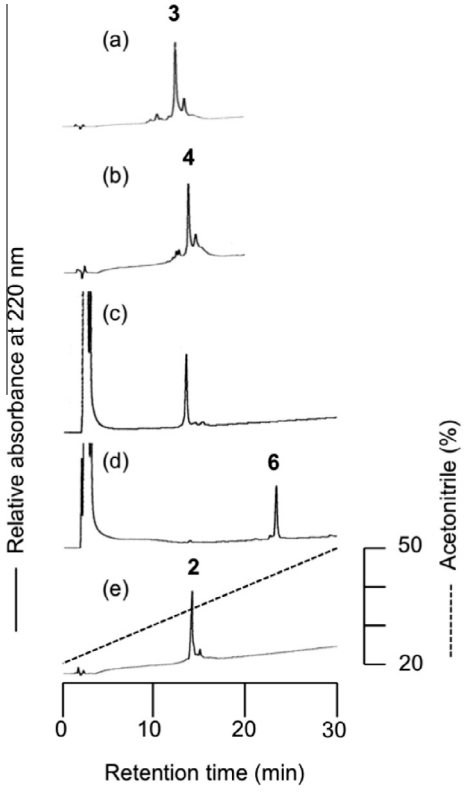
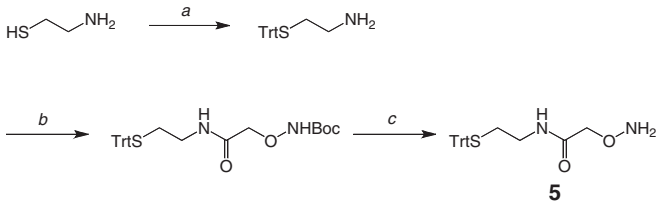


Figure 2. Reversed-phase HPLC elution profiles in the synthesis of peptide **2**. (a) Gel-filtration-purified peptide **3**. (b) The reaction mixture after $NaIO_4$ oxidation. (c) Purified peptide **4**. (d) Reaction mixture after the reaction with thiol probe **5**. (e) Reaction mixture after the deprotection of trityl group. Elution conditions: column, Mightysil RP-18 (4.6ϕ × 150 mm, Kanto Kagaku, Japan) at a flow rate of 1 mL/min.

likely to be orthogonal to the other Cys protecting groups. However, when the thiazolidinecarboxylic acid-attached A chain was treated with DPDS in trifluoroacetic acid (TFA)/trifluoromethanesulfonic acid (TfOH) solution, the thiazolidine ring was opened, and the pyridylsulfenylation of the N-terminal Cys residue was observed. Therefore, it was difficult to use the product in the next reaction. Since it was difficult to find another Cys protecting group that would be orthogonal to the other protecting groups, we changed the strategy in which sulfanyl group used for conjugation with KLH was introduced to the peptide after the disulfide bond formation reactions.

For the introduction of the sulfanyl group to the A chain N-terminus, we used an *O*-alkyloxime formation reaction between a carbonyl group and an alkoxyamine. This oxim-based structure has been used for the preparation of a drug-antibody conjugate, and has also been shown to be stable in animal serum.[11] For preparation of peptides with an N-terminal carbonyl group, two methods have been reported. One is the transamination reaction, in which the N-terminal amino group is specifically converted into a carbonyl



Scheme 2. Synthetic procedure of thiol probe **5**. Reaction conditions: (a) Trityl chloride, TFA, (b) Boc-$NHOCH_2COOH$, WSCD·HCl, HOSu, CH_2Cl_2, (c) 2% H_2O/50% TFA/CH_2Cl_2 (33% in three steps).

group by glyoxylic acid treatment under the existence of nickel ion (Ni^{2+})[12,13] or by pyridoxal 5-phosphate treatment.[14] This method has been used for site-specific modification reactions, such as fluorescent-labeling[15,16] and biotinylation.[17] In our case, however, there were two N-terminal amino groups at the A and B chains, and it was difficult to use this method. The other one is the sodium periodate oxidation method, in which the N-terminal Ser/Thr residue is specifically oxidized to an aldehyde group.[18] Since the N-terminal residue of the B chain is Glu, when a Ser residue is introduced to the A chain N-terminus, only the A chain is allowed to react. Therefore, we selected the sodium periodate oxidation method for the carbonylation of the N-terminus. To take apart the antigen peptide from KLH part, a PEG chain was inserted between RGP and the N-terminal Ser residue, and we designed the antigen **2** as shown in Scheme 1.

RGP carrying seryl-PEG chain (Ser-PEG-RGP, **3**) was synthesized essentially according to the method for the synthesis of RGP **1** as described above (Scheme 1). In the Fmoc-SPPS of the A chain, Fmoc-NH-PEG_5-COOH, which was purchased from a commercial resource, was used as a PEGylation reagent. During SPPS and regioselective disulfide bond formation reactions, the seryl-PEG moiety attached at the A chain N-terminus did not affect the reactions, and the desired product **3** was obtained in yields comparable to those in the case of RGP **1**. The N-terminal Ser residue was converted to a glyoxylyl group by sodium periodate oxidation under neutral pH, giving the N-terminally carbonylated PEG-RGP **4** in 49% yield (Fig. 2).

For introduction of a sulfanyl group into the peptide, a thiol probe **5** was synthesized as shown in Scheme 2. The sulfanyl group of cysteamine was protected by a trityl (Trt) group, and condensed with Boc-$NHOCH_2COOH$. The Boc group was specifically removed in TFA solution without cation scavengers. During the deprotection step, no cleavage of Trt group was observed, and the desired compound **5** was obtained in 33% yield. To introduce the probe into peptide **4**, these were mixed under weakly acidic conditions, giving peptide **6** in 83% yield. The Trt group was finally removed in TFA solution with triisopropylsilane, giving the desired compound **2** in 49% yield. It is well known that the disulfide exchange reaction is generally quite slow under acidic conditions. After the removal of Trt group, acidic conditions were kept until the purified peptide **2** was lyophilized, and no significant disulfide isomerization was observed in the purification step by reversed-phase HPLC (Fig. 2e).

2.3. Evaluation of the synthetic RGP derivatives

In order to confirm biological activity, an in vitro biological assay was performed according to the method described previously.[5] Since antigen **2** had a free sulfanyl group, making it unstable under

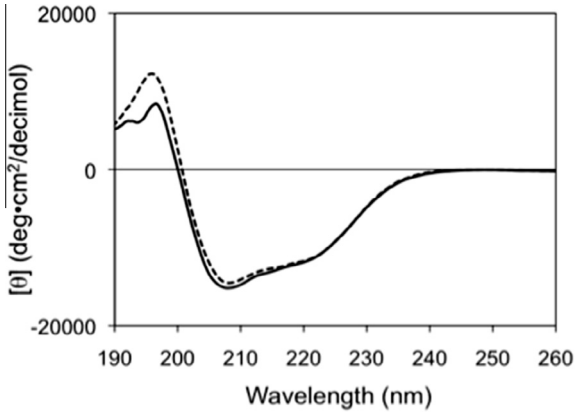


Figure 3. Circular dichroism spectra of the synthetic RGPs. Dotted and solid lines represent the spectra of RGP **1** and Ser-PEG-RGP **3**, respectively.

neutral pH, we used the intermediate, Ser-PEG-RGP **3**, in the assay. As a result, the synthetic RGP **1** and Ser-PEG-RGP **3** showed EC_{50} values of 0.30 ± 0.02 nM and 0.30 ± 0.03 nM (means ± s.e.), respectively, indicating that the PEGylation of the A chain N-terminus had no effect on the biological activity of RGP in vitro. Circular dichroism (CD) spectral analysis of peptides **1** and **3** showed that these spectral patterns were quite similar to each other (Fig. 3), demonstrating that the PEGylation did not affect the RGP folding. Although CD spectrum of the native RGP has not yet been reported, the spectrum of synthetic RGP **1** was similar to those of other insulin/relaxin superfamily peptides.[3,6,19–23] All of these results indicated that the PEGylation had no effect on either biological or chemical aspects of RGP.

2.4. Production and evaluation of anti-RGP antibodies

Using antigen **2** (1.8 mg), the conjugate with KLH was prepared by the maleimide method, which was performed by Eurofins Genomics (Tokyo, Japan). In brief, KLH was modified with excess an amount of *N*-(6-maleimidocaproyloxy)succinimide, and then antigen **2** was conjugated. The conjugation ratio was 54%. The RGP–KLH conjugate was immunized against two rabbits. After four weeks, the sera were collected, and the antibody titers against *P. pectinifera* RGP were measured by ELISA. Both of two anti-sera recognized *P. pectinifera* RGP at the dilution level of 1.3×10^6, and the absorbance reached a maximum at the level of 1.6×10^5, indicating that the antibodies with a high titer (>1:100,000 in ELISA) arose in both rabbits immunized (Fig. 4a). In order to analyze the specificity of antibodies, immunoblotting analysis was performed with one of the anti-sera against RGPs from *P. pectinifera*, *Asterias amurensis*[24] and *Aphelasterias japonica*[25] and against related peptides (Fig. 4b). *P. pectinifera* RGP was recognized by the antibodies, and a clear band was observed on the membrane. On the other hand, the A chain gave only a faint band, and the B chain was not recognized by the antibodies. These results indicated that the antibodies were highly specific to the heterodimeric structure of RGP. The RGPs from other starfishes, bovine insulin and human relaxin-3 also gave no band on the membrane in the immunoblotting analysis. These results clearly indicated that the antibodies obtained in this study were highly specific to *P. pectinifera* RGP. Since the chemical structure of *A. planci* RGP is the same as that of *P. pectinifera* RGP,[8] the antibodies obtained in this study should recognize *A. planci* RGP. Using these anti-sera, various analyses of RGP in *A. planci* and *P. pectinifera*, such as the tissue distribution and the measurement of titers in hemolymph, will be examined.

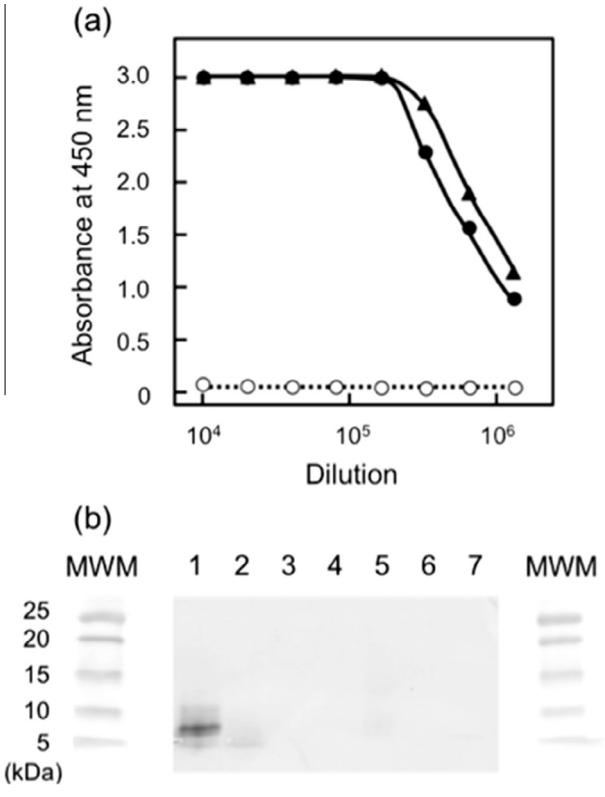


Figure 4. Antibody titers and specificity of the anti-sera. (a) Titer curves of anti-serum in rabbit #1 (●) and #2 (▲) with respect to relaxin-like gonad-stimulating peptide (RGP) of *P. pectinifera*. RGP was used for immobilized antigen at a concentration of 1 mM. Open circles show control preimmune serum. (b) Immunoblotting after SDS–PAGE of RGP and related peptides with anti-RGP anti-serum. 1, *P. pectinifera* RGP; 2, A chain of *P. pectinifera* RGP; 3, B chain of *P. pectinifera* RGP; 4, *Asterias amurensis* RGP; 5, *Aphelasterias japonica* RGP; 6, bovine insulin; 7, human relaxin-3. Each peptide was used at concentration of 0.1 nmol per well. MWM indicates molecular weight marker.

3. Conclusion

We have developed a novel method for the preparation of a disulfide-rich heterodimeric peptide–KLH conjugate, and applied it to the production of antibodies against RGP from the starfish *P. pectinifera*. Specific antibodies with a high titer (>1:100,000 in ELISA) were obtained. Since the antibodies could not be obtained when the A or B chains were immunized as an antigen, it was likely that our novel preparation method for peptide–KLH conjugate successfully produced specific antibodies. To our best knowledge, this is the first report of a preparation method for a heterodimeric peptide–KLH conjugate. This method might be applicable not only to other heterodimeric peptides with disulfide linkages such as a vertebrate relaxin but also to the single-chain disulfide-rich peptides.

4. Experimental procedure

4.1. General

NMR spectra were recorded by a Bruker AVANCE III HD spectrometer (500 MHz in ^{1}H NMR, Bruker, Germany). MALDI-TOF mass spectra were recorded using an Autoflex spectrometer (Brucker). High-resolution ESI mass spectra were measured with The Accu-TOF-plus JMS-T100LP spectrometer (JEOL, Tokyo, Japan). Amino acid composition was determined using a LaChrom amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with a 6 M HCl solution at 150 °C for 2 h in a vacuum-sealed tube. Circular dichroism (CD) spectra were measured with a Jasco J-820 spectropolarimeter (JASCO, Tokyo, Japan) at room temperature with a 2-mm path length cell using a phosphate buffer (50 mM, pH 7.0) as a solvent.

4.2. Thiol probe 5

Cysteamine hydrochloride (570 mg, 5.0 mmol) and trityl chloride (1.4 g, 5.0 mmol) were dissolved in trifluoroacetic acid (TFA, 20 mL), and stirred at room temperature for 10 min. After the removal of TFA with an evaporator, the residue was dissolved in EtOAc, washed with $NaHCO_3$ aqueous solution, H_2O and brine, and dried over Na_2SO_4. After filtration, the solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2 containing Boc-aminooxyacetic acid succinimidyl ester, which was prepared by mixing Boc-aminooxyacetic acid (1.15 g, 6.0 mmol), *N*-hydroxy-succinimide (0.69 g, 6.0 mmol) and water-soluble carbodiimide hydrochloride (1.15 g, 6.0 mmol) in CH_2Cl_2 (15 mL) for 1 h, and stirred at room temperature for 2 h. The reaction mixture was

diluted with EtOAc, washed with H_2O and brine, and dried over Na_2SO_4. After filtration and evaporation, the crude product was dissolved in 2% H_2O/50% TFA/CH_2Cl_2 (50 mL), and stirred at room temperature for 30 min. After the solvent was removed with an evaporator, the residue was chromatographed on silica gel with $CHCl_3/CH_3OH$ (19:1) to give the desired compound **5** (650 mg, 1.66 mmol, 33% in 3 steps) as a colorless solid: mp 106–110 °C (decomp.); R_f 0.55 ($CHCl_3/CH_3OH$, 9/1); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.42–7.20 (m, 15H, Ar), 6.47 (br s, 1H, N*H*), 4.08 (s, 2H, O-C*H*$_2$-CO), 3.12 (q, 2H, J = 6.2 Hz, NH-C*H*$_2$–), 2.43 (t, 2H, J = 6.5 Hz, S-C*H*$_2$–); ^{13}C NMR: δ 169.7 (C=O), 144.6, 129.5, 128.0, 126.9 (Ar), 74.9 (OCH_2), 66.9 (CPh_3), 37.5 ($NHCH_2$), 32.1 (SCH_2); ESI mass, found: m/z 415.154, calcd: 415.150 for $(M+Na)^+$.

4.3. Cys^{16}(Acm)-RGP B chain 7

Fmoc-Ser(Bu^t)-Wang resin (0.31 mmol/g, 0.81 g, 0.25 mmol) was swelled in 1-methyl-2-pyrrolidinone (NMP) for 30 min, and was treated with 20% piperidine/NMP for 5 and 15 min. After washing with NMP, Fmoc-Val-OBt, which was prepared by mixing Fmoc-Val-OH (1.0 mmol), 1 M *N*,*N*′-dicyclohexylcarbodiimide (DCC)/NMP (1.5 mL) and 1 M 1-hydroxybenzotriazole (HOBt)/NMP (1.5 mL) at room temperature for 30 min, was added and the reaction mixture was mixed with vortex at 50 °C for 1 h. The resin was washed with NMP and 50% dichloromethane/methanol, treated with 10% acetic anhydride (Ac_2O)/5% *N*,*N*-diisopropylethylamine (DIEA)/NMP for 5 min, and washed with NMP. The peptide chain was elongated in essentially the same manner as described above, and H-Glu(OBu^t)-Lys(Boc)-Tyr(Bu^t)-Cys(Trt)-Asp(OBu^t)-Asp(OBu^t)-Asp(OBu^t)-Phe-His(Trt)-Met-Ala-Val-Phe-Arg(Pbf)-Thr(Bu^t)-Cys(Acm)-Ala-Val-Ser(Bu^t)-resin (1.57 g) was obtained. A part of the resin (202 mg) was treated with TFA cocktail (TFA/H_2O/thioanisole/phenol/triisopropylsilane, 82.5/5/5/5/2.5, 1.5 mL) at room temperature for 2 h. TFA was removed under an Ar stream and the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitate was dried in vacuo. The crude peptide was purified by RP-HPLC on a Mightysil RP-18 column (Kanto Kagaku, Tokyo, Japan) with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **7** (12.9 μmol, 40% yield). MALDI-TOF mass, found: m/z 2308.7, calcd: 2309.6 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{2.97}Thr_{0.53}Ser_{0.71}Glu_{0.90}Ala_{2.14}Val_{2.01}Met_{0.80}Tyr_1Phe_{2.00}Lys_{1.01}His_{1.01}Arg_{1.02}$.

4.4. Cys^{11}(MeOBn), Cys^{24}(Acm)-RGP A chain 8

Starting from Fmoc-Cys(Acm)-Wang resin (0.64 mmol/g, 0.16 g, 0.10 mmol), the peptide chain corresponding to the A chain of RGP was elongated essentially according to the method for the B chain **7** described above, and the protected peptide resin, H-Ser(Bu^t)Glu(OBu^t)-Tyr(Bu^t)-Ser(Bu^t)-Gly-Ile-Ala-Ser(Bu^t)-Tyr(Bu^t)-Cys(Trt)-Cys(MeOBn)-Leu-His(Trt)-Gly-Cys(Trt)-Thr(Bu^t)-Pro-Ser(Bu^t)-Glu(OBu^t)-Leu-Ser(Bu^t)-Val-Val-Cys(Acm)-resin (0.472 g) was obtained. A part of the resin (101 mg) was treated with TFA cocktail (1.5 mL) at room temperature for 2 h. TFA was removed under an Ar stream and the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitate was dried in vacuo. The crude peptide was dissolved in 6 M guanidine-HCl/50 mM phosphate buffer (pH 7.0, 9 mL), and dimethyl sulfoxide (DMSO, 1 mL) was added. The reaction mixture was gently stirred at room temperature for 2 d, and the crude peptide was purified by RP-HPLC on a Mightysil RP-18 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **8** (3.11 μmol, 15% yield). MALDI-TOF mass, found: m/z 2696.9, calcd: 2697.1 for $(M+H)^+$. Amino acid analysis: $Thr_{0.78}Ser_{3.64}Glu_{1.84}Pro_{0.83}Gly_2Ala_{1.04}Val_{1.78}Ile_{0.97}Leu_{1.99}Tyr_{1.89}His_{0.99}$.

4.5. Cys^{11}(SPy), Cys^{24}(Acm)-RGP A chain 9

The peptide **8** (3.11 μmol) was dissolved in TFA (2.0 mL) and thioanisole (0.2 mL) containing 2,2'-dipyridyl disulfide (DPDS, 22 mg), and cooled to −10 °C. Trifluoromethanesulfonic acid (TfOH, 100 μL) was added to the solution, and the mixture was kept at −10 °C for 5 min. The crude peptide was precipitated with diethyl ether, washed twice with ether, and dried in vacuo. The residue was applied to the gel filtration HPLC using a TSKgel G3000PW$_{XL}$ column (7.8ϕ × 300 mm, Tosoh, Japan) with 0.1% TFA/50% acetonitrile aqueous solution as a solvent at a flow rate of 0.5 mL/min, to give peptide **9** (3.07 μmol, 99% yield). MALDI-TOF mass, found: m/z 2685.9, calcd: 2686.1 for $(M+H)^+$. Amino acid analysis: $Thr_{0.65}Ser_{3.79}Glu_{1.77}Pro_{1.02}Gly_2Ala_{1.09}Val_{1.76}Ile_{0.96}Leu_{1.99}Tyr_{1.88}His_{0.99}$.

4.6. Cys^{A24,B16}(Acm)-RGP 10

Peptides **7** (3.07 μmol) and **9** (3.07 μmol) were dissolved in 40% acetonitrile/50 mM sodium bicarbonate aqueous solution (20 mL) and the solution was gently stirred at room temperature for 1 h. The reaction was quenched by adding acetic acid (400 μL), and the mixture was purified by RP-HPLC on a Mightysil RP-18 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **10** (1.54 μmol, 50% yield). MALDI-TOF mass, found: m/z 4887.9, calcd: 4885.4 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{3.24}Thr_{1.59}Ser_{4.82}Glu_{3.02}Pro_{0.87}$ $Gly_2Ala_{3.13}Val_{3.90}Met_{0.84}Ile_{0.99}$ $Leu_{2.04}Tyr_{3.14}Phe_{2.13}Lys_{1.10}His_{2.11}$ $Arg_{1.03}$.

4.7. RGP 1

Peptide **10** (1.54 μmol) was dissolved in distilled water (5 mL), and the solution was added dropwise to methanol (20 mL) containing 20 mM I_2/methanol (1.2 mL) and conc. HCl (100 μL). The mixture was mixed with vortex at room temperature for 1 h. The reaction was quenched by adding an ascorbic acid aqueous solution until the brownish color was abolished. The mixture was purified by RP-HPLC on a Mightysil RP-18 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **1** (904 nmol, 59% yield). MALDI-TOF mass, found: m/z 4741.8, calcd: 4741.3 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{3.09}Thr_{1.69}Ser_{4.61}Glu_{2.77}Pro_{1.16}Gly_2Ala_{3.23}Val_{3.88}Met_{0.51}Ile_{1.04}$ $Leu_{2.08}Tyr_{2.96}Phe_{2.17}Lys_{1.08}His_{2.07}Arg_{1.04}$.

4.8. Ser-PEG-Cys^{11}(MeOBn), Cys^{24}(Acm)-RGP A chain 11

Starting from Fmoc-Cys(Acm)-Wang resin (0.64 mmol/g, 0.31 g, 0.20 mmol), the peptide chain corresponding to the A chain of RGP was elongated essentially according to the method for peptide **7** described above. After the removal of the Fmoc group attached at the N-terminus by 20% piperidine/NMP treatments for 5 and 15 min, the resin was washed with NMP. Fmoc-NH-PEG$_5$-COOBt, which was prepared by mixing Fmoc-NH-PEG$_5$-COOH (0.40 mmol, Merck, Germany), 1 M DCC/NMP (0.6 mL) and 1 M HOBt/NMP (0.6 mL) at room temperature for 30 min, was added and the reaction mixture was mixed with vortex at 50 °C for 1 h. The resin was washed with NMP and 50% dichloromethane/methanol, treated with 10% Ac_2O/5% DIEA/NMP for 5 min, and washed with NMP. Fmoc-Ser(Bu^t)-OH was then introduced into the resin by the DCC-HOBt method, and H-Ser(Bu^t)-NH-PEG$_5$-CO-Ser(Bu^t)-Glu(OBu^t)-Tyr(Bu^t)-Ser(Bu^t)-Gly-Ile-Ala-Ser(Bu^t)-Tyr(Bu^t)-Cys(Trt)-Cys(MeOBn)-Leu-His(Trt)-Gly-Cys(Trt)-Thr(Bu^t)-Pro-Ser(Bu^t)-Glu(OBu^t)-Leu-Ser(Bu^t)-Val-Val-Cys(Acm)-resin (1.07 g) was obtained. A part of the resin (154 mg) was treated with TFA cocktail (1.8 mL) at room temperature for 2 h. TFA was removed under an Ar stream and the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitate was dried in vacuo. The crude peptide

was dissolved in 6 M guanidine-HCl/50 mM phosphate buffer (pH 7.0, 13.5 mL), and DMSO (1.5 mL) was added. The reaction mixture was gently stirred at room temperature for 2 d, and the crude peptide was purified by RP-HPLC on a Mightysil RP-18 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **11** (4.57 μmol, 16% yield). MALDI-TOF mass, found: *m/z* 3119.4, calcd: 3119.4 for $(M+H)^+$. Amino acid analysis: $Thr_{0.81}Ser_{4.30}Glu_{1.77}Pro_{0.90}Gly_2Ala_{1.02}Val_{1.81}Ile_{0.99}Leu_{2.06}Tyr_{2.01}His_{1.01}$.

4.9. Ser-PEG-Cys^{11}(SPy), Cys^{24}(Acm)-RGP A chain 12

The peptide **11** (4.57 μmol) was dissolved in TFA (2.0 mL) and thioanisole (0.2 mL) containing DPDS (22 mg), and cooled to -10 °C. TfOH (100 μL) was added to the solution, and the mixture was kept at −10 °C for 5 min. The crude peptide was precipitated with diethyl ether, washed twice with ether, and dried in vacuo. The residue was applied to the gel filtration HPLC using a TSKgel G3000PW$_{XL}$ column (7.8ϕ × 300 mm) with 0.1% TFA/50% acetonitrile aqueous solution as a solvent at a flow rate of 0.5 mL/min, to give peptide **12** (3.73 μmol, 82% yield). MALDI-TOF mass, found: *m/z* 3108.2, calcd: 3108.3 for $(M+H)^+$. Amino acid analysis: $Thr_{0.81}Ser_{4.37}Glu_{1.93}Pro_{0.71}Gly_2Ala_{1.03}Val_{1.79}Ile_{1.00}Leu_{2.08}Tyr_{2.03}His_{1.00}$.

4.10. Ser-PEG-Cys^{A24,B16}(Acm)-RGP 13

Peptides **7** (3.73 μmol) and **12** (3.73 μmol) were dissolved in 40% acetonitrile/50 mM sodium bicarbonate aqueous solution (30 mL) and the solution was gently stirred at room temperature for 1 h. The reaction was quenched by adding acetic acid (500 μL), and the mixture was purified by RP-HPLC on a Mightysil RP-18 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **13** (1.86 μmol, 50% yield). MALDI-TOF mass, found: *m/z* 5309.0, calcd: 5307.9 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{3.11}Thr_{1.55}Ser_{5.33}Glu_{2.92}Pro_{0.72}Gly_2Ala_{3.07}Val_{3.90}Met_{0.94}Ile_{0.99}Leu_{2.08}Tyr_{2.98}Phe_{2.22}Lys_{1.06}His_{2.05}Arg_{1.09}$.

4.11. Ser-PEG-RGP 3

Peptide **13** (1.86 μmol) was dissolved in distilled water (8 mL), and the solution was added dropwise to methanol (30 mL) containing iodine (9.2 mg) and conc. HCl (150 μL). The mixture was mixed with vortex at room temperature for 1 h. The reaction was quenched by adding an ascorbic acid aqueous solution until the brownish color was abolished. The mixture was applied to the gel filtration HPLC using a TSKgel G3000PW$_{XL}$ column (7.8ϕ × 300 mm) with 0.1% TFA/50% acetonitrile aqueous solution as a solvent at a flow rate of 0.5 mL/min, to give peptide **3** (1.69 μmol, 91% yield). MALDI-TOF mass, found: *m/z* 5163.0, calcd: 5163.7 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{3.09}Thr_{1.68}Ser_{5.09}Glu_{2.86}Pro_{1.04}Gly_2Ala_{3.11}Val_{3.92}Met_{0.96}Ile_{0.99}Leu_{2.03}Tyr_{2.88}Phe_{2.26}Lys_{1.06}His_{2.04}Arg_{1.09}$.

4.12. Aldehyde-PEG-RGP 4

Peptide **3** (1.69 μmol) was dissolved in 50 mM phosphate buffer (pH 7.0, 1.7 mL) containing 1.3 equiv of sodium periodate (2.2 μmol), and the mixture was mixed with vortex at room temperature for 1 h. Then, the mixture was purified by RP-HPLC on a Mightysil RP-18 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **4** (834 nmol, 49% yield). MALDI-TOF mass, found: *m/z* 5129.3, calcd: 5132.7 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{2.89}Thr_{1.58}Ser_{4.01}Glu_{2.48}Pro_{1.05}Gly_2Ala_{3.05}Val_{3.64}Met_{0.98}Ile_{0.95}Leu_{1.95}Tyr_{2.61}Phe_{2.14}Lys_{1.04}His_{1.94}Arg_{0.98}$.

4.13. Tritylsulfenyl-PEG-RGP 6

Peptide **4** (834 nmol) was dissolved in 5% AcOH/*N*,*N*-dimethylacetamide (830 μL) containing 1% (w/v) of compound **5**, and the solution was mixed with vortex at room temperature for 1 h. The mixture was applied to the gel filtration HPLC using a TSKgel G3000PW$_{XL}$ column (7.8ϕ × 300 mm) with 0.1% TFA/50% acetonitrile aqueous solution as a solvent at a flow rate of 0.5 mL/min, to give peptide **6** (689 nmol, 83% yield). MALDI-TOF mass, found: *m/z* 5507.2, calcd: 5507.2 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{2.96}Thr_{1.66}Ser_{4.39}Glu_{2.70}Pro_{0.85}Gly_2Ala_{2.98}Val_{3.79}Met_{0.95}Ile_{1.03}Leu_{2.08}Tyr_{3.00}Phe_{2.09}Lys_{1.04}His_{1.99}Arg_{1.04}$.

4.14. Sulfanyl-PEG-RGP 2

Peptide **6** (374 nmol) was dissolved in TFA/H_2O/triisopropylsilane (96/2/2, 400 μL), and the solution was mixed with vortex at room temperature for 30 min. The crude peptide was precipitated with diethyl ether, washed twice with ether, and dried in vacuo. The crude peptide was purified by RP-HPLC on a Mightysil RP-18 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **2** (183 nmol, 49% yield). MALDI-TOF mass, found: *m/z* 5264.9, calcd: 5264.9 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{2.97}Thr_{1.63}Ser_{4.40}Glu_{2.71}Pro_{1.09}Gly_2Ala_{3.07}Val_{3.84}Met_{0.94}Ile_{1.01}Leu_{2.12}Tyr_{3.07}Phe_{2.27}Lys_{1.07}His_{2.02}Arg_{1.11}$.

4.15. Biological assay

RGP activity was biologically assayed using ovarian fragments of *Patiria (Asterina) pectinifera* as described previously.[7,26] The ovary of a mature female in *P. pectinifera* was excised and cut into small fragments, each of which containing only a few lobes, using scissors. Ovarian fragments were incubated in artificial seawater containing synthetic RGP **1** or Ser-PEG-RGP **3** at various concentrations for 1 h. The effective dose for inducing gamete spawning in 50% of ovarian fragments was determined. Four separate assays using different animals were performed.

4.16. Production of antibodies

Conjugation with KLH and immunization against two rabbits were committed to Eurofins Genomics (Tokyo, Japan). KLH (Thermo Fisher Scientific, Canada) was dissolved in water at the concentration of 10 mg/mL. *N*-(6-maleimidocaproyloxy)succinimide (EMCS) was dissolved in DMF at a concentration of 10 mg/mL. KLH and EMCS solutions were mixed at a ratio of 10:1, and the mixture was gently stirred at room temperature for 1 h. The solution was applied to a gel-filtration chromatography (PD-10, GE Healthcare UK Ltd., UK), and the elution was monitored with absorbance at 280 nm. The protein fraction was collected and concentrated with Amicon Ultra-15 (Merck-Millipore, Germany) to 5 mg/mL. Peptide **2** (1.8 mg) was dissolved in water (180 μL), and KLH-EMCS solution (180 μL) was added. The mixture was gently stirred at room temperature for 2 h. After the reaction, the mixture was diluted with water at a concentration of 1 mg/mL KLH–peptide conjugate, and used directly for immunization. The reaction ratio was determined using the Ellman's reagent[27] to be 54%. The conjugate was immunized against two rabbits. The sera were collected after four weeks from the immunization.

4.17. Evaluation of antibodies by ELISA

To obtain titer curves of anti-serum against *P. pectinifera* RGP, ELISA was conducted. The wells of PVC microtiter plates were coated with peptide solution (1 μM) in PBS (100 μL) and incubated overnight at 4 °C. After washing and blocking, the plates were dried at 4 °C. The anti-serum diluted in PBST (100 μL) was added to the wells and incubated overnight at 4 °C. After washing, HRP-anti-rabbit goat IgG (Zymed Laboratories Inc., CA) diluted in PBST (100 μL) was added to the wells and incubated for 2 h at room

temperature. After washing, tetramethylbenzine (TMB) was added to the wells and incubated for 30 min at room temperature. The reaction was stopped by adding 2 M sulfuric acid, and absorbance at 450 nm was measured by a microplate reader Model 680 (Bio-Rad Laboratories, Inc., Hercules, CA).

4.18. Immunoblotting analysis

P. pectinifera RGP, *P. pectinifera* RGP-A chain, *P. pectinifera* RGP-B chain, *Asterias amurensis* RGP, *Aphelasterias japonica* RGP, bovine insulin (Sigma), and human relaxin-3 (PeproTech, NJ) were dissolved in gel sample buffer without 2-mercaptoethanol. Aliquots (0.1 nmoles/well) were loaded into the lanes of a sodium dodecylsulfate–polyacrylamide (SDS–PAGE) mini slab (15% gel) (Cosmobio, Tokyo, Japan) and resolved by electrophoresis. Peptides separated by SDS–PAGE were transferred to an Immobilon membrane (Millipore, Billerica, MA, USA) by electro-blotting, as described previously.[28] The membrane was rinsed in Tris-buffered saline (TBS) consisting of 20 mM Tris–HCl (pH 7.5) and 150 mM NaCl, blocked with 5% non-fat dry milk in TBS containing 0.1% Tween-20 (TTBS), and incubated with a 1:5000 dilution of anti-serum against *P. pectinifera* RGP in TTBS overnight at 4 °C. After three washes with TTBS, the membrane was incubated with a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Cosmobio, Tokyo, Japan). After three further washes with TTBS, phosphatase activity was visualized by treating the membrane with 0.2 mM 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and nitroblue tetrazolium in 100 mM diethanolamine buffer (pH 9.5) containing 5 mM $MgCl_2$.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.05.068.

References and notes

1. Adrian, T. E. *Methods Mol. Biol.* **1997**, *73*, 239.
2. Cuthbertson, A.; Indrevoll, B. *Org. Lett.* **2003**, *5*, 2955.
3. Katayama, H.; Hojo, H.; Ohira, T.; Ishii, A.; Nozaki, T.; Goto, K.; Nakahara, Y.; Takahashi, T.; Hasegawa, Y.; Nagasawa, H.; Nakahara, Y. *Biochemistry* **2010**, *49*, 1798.
4. Dekan, Z.; Mobli, M.; Pennington, M. W.; Fung, E.; Nemeth, E.; Alwood, P. F. *Angew. Chem., Int. Ed.* **2014**, *53*, 2931.
5. See recent reviews: (a) Hossain, M. A.; Wade, J. D. *Curr. Opin. Chem. Biol.* **2014**, *22*, 47; (b) Liu, F.; Zaykov, A. N.; Levy, J. J.; DiMarchi, R. D.; Mayer, J. P. *J. Pept. Sci.* **2016**. http://dx.doi.org/10.1002/psc.2863 (in press).
6. Katayama, H.; Kubota, N.; Hojo, H.; Okada, A.; Kotaka, S.; Tsutsui, N.; Ohira, T. *Bioorg. Med. Chem.* **2014**, *22*, 5783.
7. Mita, M.; Yoshikuni, M.; Ohno, K.; Shibata, Y.; Paul-Prasanth, B.; Pitchayawasin, S.; Isobe, M.; Nagahama, Y. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 9507.
8. Mita, M.; Ikeda, N.; Haraguchi, S.; Tsutsui, K.; Nakano, Y.; Nakamura, M. *Invertebrate Reprod. Dev.* **2015**, *59*, 212.
9. Bang, D.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2004**, *43*, 2534.
10. Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776.
11. Thompson, P.; Bezabeh, B.; Fleming, R.; Pruitt, M.; Mao, S.; Strout, P.; Chen, C.; Cho, S.; Zhong, H.; Wu, H.; Gao, C.; Dimasi, N. *Bioconjugate Chem.* **2015**, *26*, 2085.
12. Dixon, H. B. F. *Biochem. J.* **1964**, *92*, 661.
13. Dixon, H. B. F. *J. Protein Chem.* **1984**, *3*, 99.
14. Gilmore, J. M.; Scheck, R. A.; Esser-Kahn, A. P.; Joshi, N. S.; Francis, M. B. *Angew. Chem., Int. Ed.* **2006**, *45*, 5307.
15. Duan, X.; Zhao, Z.; Ye, J.; Ma, H.; Xia, A.; Yang, G.; Wang, C.-C. *Angew. Chem., Int. Ed.* **2004**, *43*, 4216.
16. Dong, S. Y.; Ma, H. M.; Duan, X. J.; Chen, X. Q.; Li, J. *J. Proteome Res.* **2005**, *4*, 161.
17. Sasaki, T.; Kodama, K.; Suzuki, H.; Fukuzawa, S.; Tachibana, K. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4550.
18. Geoghegan, K. F.; Stroh, J. G. *Bioconjugate Chem.* **1992**, *3*, 138.
19. Maruyama, K.; Nagata, K.; Tanaka, M.; Nagasawa, H.; Isogai, A.; Ishizaki, H.; Suzuki, A. *J. Protein Chem.* **1992**, *11*, 1.
20. Hua, Q.-X.; Jia, W.; Frank, B. H.; Phillips, N. F. B.; Weiss, M. A. *Biochemistry* **2002**, *41*, 14700.
21. Hua, Q.-X.; Mayer, J. P.; Jia, W.; Zhang, J.; Weiss, M. A. *J. Biol. Chem.* **2006**, *281*, 28131.
22. Hossain, M. A.; Rosengren, K. J.; Zhang, S.; Bathgate, R. A.; Tregear, G. W.; van Lierop, B. J.; Robinson, A. J.; Wade, J. D. *Org. Biomol. Chem.* **2009**, *7*, 1547.
23. Shabanpoor, F.; Bathgate, R. A.; Belgi, A.; Chan, L. J.; Nair, V. B.; Wade, J. D.; Hossain, M. A. *Biochem. Biophys. Res. Commun.* **2012**, *420*, 253.
24. Mita, M.; Daiya, M.; Haraguchi, S.; Tsutsui, K.; Nagahama, Y. *Gen. Comp. Endocrinol.* **2015**, *222*, 144.
25. Mita, M.; Katayama, H. *Gen. Comp. Endocrinol.* **2016**, *229*, 56.
26. Shirai, H. In *Methods in Cell Biology*; Schroeder, T. E., Ed.; Academic Press: Orland, Florida, 1986; Vol. 27, pp 73–88.
27. Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Methods Enzymol.* **1983**, *91*, 49.
28. Mita, M.; Yamamoto, K.; Nakamura, M.; Takeshige, Y.; Watanebe, M.; Nagahama, Y. *Gen. Comp. Endocrinol.* **2012**, *176*, 432.