The antagonistic activity of Padina arborescens extracts on mPRα

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Abstract

The current study attempted to evaluate the antagonistic activity of compounds isolated and purified from the marine algae *Padina arborescens* during cultivation. The compounds were collected on a filter, concentrated on ODS columns and separated by HPLC. Two peaks that showed competitive progesterone binding activity with membrane progesterone receptor α (mPRα) were purified. Their physiological activity was further uncovered by *in vitro* and *in vivo* oocyte maturation and ovulation-inducing assays using zebrafish. The compounds inhibited the induction of oocyte maturation and ovulation. Moreover, the results showed that the compounds have antagonistic activity against mPRα. The purified compounds with antagonistic activity against mPRα would be considered as new pharmaceutical candidate.

Key words: marine algae, *Padina arborescens*, membrane progesterone receptor, steroid binding assay

1. Introduction

Reproductive hormones play vital roles in the reproductive systems of both males and females during pubertal development as well as their growth and sexual characteristics. These reproductive hormones include both steroid and peptide hormones (Manocha et al. 2018). Among them, steroids such as progesterone is key regulators that control reproduction. Progesterone occurs naturally in the body and plays key roles in controlling the meiotic cell division of oocytes and ovulation (Manocha et al. 2018). In addition to the well-established actions of progesterone through the nuclear progesterone receptors (nPRs), a new concept of steroid action through membrane progestin receptors (mPRs) was raised after the discovery of the mPRs (Zhu, Bond, et al. 2003, Zhu, Rice, et al. 2003). mPRs are 7-transmembrane receptors that were renamed as members of the progestin and adipoQ receptor (PAQR) family after a genome-wide phylogenetic study. The first identified mPR, mPRα (also referred to as PAQR7), is the seventh gene in the family and one of the five mPR genes implicated in physiological functions in reproductive tissues (Pang et al. 2013).

A number of researchers have reported that mPRs can be found in a wide range of organisms, and progestin binding activity has been discovered in numerous species, from fish to humans (Ben-Yehoshua et al. 2007, Smith et al. 2008, Thomas 2008, Tokumoto et al. 2006, Tokumoto et al. 2012, Tubbs and Thomas 2009). Various rapid nongenomic actions have been shown to occur through mPRs, such as mammalian sex behavior, breast tumor progression, human T cell attack on fetuses, and the inflammatory response (Chien et al. 2009, Dressing et al. 2012, Frye et al. 2013, Frye et al. 2014, Lu et al. 2015, Vares et al. 2015, Xie et al. 2012). Currently, mPRs have been proven to be potential cellular mediators of a variety of progesterone responses. Consequently, scientists are now extremely interested in identifying new mPR ligands. Org OD 02-0 was previously established in early research (Levina et al. 2021, Polikarpova et al. 2017, Rezanujjaman et al. 2020). Thus, the identification of new hormonal compounds from natural resources that can interact with mPR as novel pharmaceutical candidates would be a great breakthrough in reproductive or hormone research.

Marine algae-derived bioactive compounds have been considered potential pharmaceutical agents due to their various activities, including antifungal, antibacterial, antiviral, antioxidant and cytotoxic properties (Carroll et al. 2021). Currently,

researchers have been attracted to the identification of a relevant group of water-soluble natural product secondary metabolites known to present notable, potent biological activities (Berlinck et al. 2021). Compounds with pharmacological potential and antimicrobial, cytotoxic, cardioprotective, hepatoprotective and immunostimulatory activities have already been reported in *Padina* species (Antony et al. 2022, Antony et al. 2021, Rushdi et al. 2021).

Previously, we detected mPR-interacting compounds secreted from marine organisms in the seawater of coral reefs in Mauritius (Tokumoto et al. 2017). We were interested in the marine algae *Padina*, as these organisms secrete mPR-interacting compounds; additionally, the sampling point is the major habitat of these species. In the present study, we concentrated the secreted compounds from *Padina arborescens* using a fish cultivation filter and followed by compound purification. The mPRα-interacting ability of the purified compounds was then demonstrated.

Results and discussion

Thalli of *Padina arborescens* were collected at the marine field of University of Tsukuba or Shizuoka University in Shizuoka prefecture and were cultured in aquariums (Figure S1). Compounds were collected on polyester filters during the cultivation of *Padina arborescens* in a glass aquarium and then eluted from the filter with ethanol and concentrated on ODS columns. Then, the compounds were separated by 4 HPLC steps. Among the four fractions from the first HPLC separation, the third and fourth fractions showed binding activity. In this study, the fourth fraction was further purified, and two compounds (peak 1 and peak 2) were separated (Figure S2). Both peaks showed mPRα binding activity (Figure S3).

The physiological activity of the purified compounds was further analyzed by *in vitro* and *in vivo* assays using zebrafish. In the *in vitro* assays using oocytes, the activity on mPR was analyzed through the induction of oocyte maturation (Tokumoto et al. 2004). *In vivo*, agonistic or antagonistic activity of the compounds against mPR and nPR was analyzed by oocyte maturation and ovulation (Tokumoto et al. 2011). The compounds

showed no effect on the induction of fish oocyte maturation and ovulation. Thus, we demonstrated antagonistic activity against induction with the natural hormone 17,20β-DHP. Inhibition of oocyte maturation induced by 17,20β-DHP was demonstrated for both compounds in an *in vitro* assay (Figure S4). Additionally, *in vivo*, potent inhibition of ovulation was shown for both compounds. This inhibitory activity was also observed against oocyte maturation after treatment with the compound in peak 1, but not the compound in peak 2. These results suggested that the compounds purified from *Padina arborescens* have antagonistic activity against mPRα.

Only a small number of ligands for newly identified mPRα have been demonstrated thus far, and they are currently under investigation. The mPR-interacting chemicals as ligands acting on mPRα from marine algae would provide new insight into reproductive biology, pharmacology, physiology and the medical sector.

3. Conclusion

In this study, two secreted compounds from *Padina arborescens* were purified. The purified compounds possess mPRα-interacting activity. A physiological assay demonstrated that the compounds act as antagonists against fish oocyte maturation and ovulation. Consequently, the newly purified compounds from *Padina arborescens* would be the novel pharmaceutical candidates as antagonists of mPRα.

Acknowledgments

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Disclosure statement

No potential conflicts of interest are reported by the authors.

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References

Antony T, Chakraborty K, Dhara S. 2022. Sulfated galactofucan from seaweed Padina tetrastromatica attenuates proteolytic enzyme dipeptidyl-peptidase-4: a potential anti-hyperglycemic lead. Nat Prod Res. Jan 5.

Antony T, Chakraborty K, Joy M. 2021. Antioxidative dolabellanes and dolastanes from brown seaweed Padina tetrastromatica as dual inhibitors of starch digestive enzymes. Nat Prod Res. Feb 16;35:614-626.

Ben-Yehoshua LJ, Lewellyn AL, Thomas P, Maller JL. 2007. The role of Xenopus membrane progesterone receptor beta in mediating the effect of progesterone on oocyte maturation. Mol Endocrinol. Mar;21:664-673.

Berlinck RGS, Crnkovic CM, Gubiani JR, Bernardi DI, Ioca LP, Quintana-Bulla JI. 2021. The isolation of water-soluble natural products - challenges, strategies and perspectives. Nat Prod Rep. Oct 14.

Carroll AR, Copp BR, Davis RA, Keyzers RA, Prinsep MR. 2021. Marine natural products. Nat Prod Rep.38:362-413.

Chien CH, Lai JN, Liao CF, Wang OY, Lu LM, Huang MI, Lee WF, Shie MC, Chien EJ. 2009. Mifepristone acts as progesterone antagonist of non-genomic responses but inhibits phytohemagglutinin-induced proliferation in human T cells. Hum Reprod. Aug;24:1968-1975.

Dressing GE, Alyea R, Pang Y, Thomas P. 2012. Membrane progesterone receptors (mPRs) mediate progestin induced antimorbidity in breast cancer cells and are expressed in human breast tumors. Horm Cancer. Jun;3:101-112.

Frye CA, Koonce CJ, Walf AA. 2013. Pregnane Xenobiotic Receptors and Membrane Progestin Receptors: Role in Neurosteroid-Mediated Motivated Behaviours. Journal of Neuroendocrinology. Nov;25:1002-1011.

Frye CA, Waif AA, Kohtz AS, Zhu Y. 2014. Progesterone-facilitated lordosis of estradiol-primed mice is attenuated by knocking down expression of membrane progestin receptors in the midbrain. Steroids. Mar;81:17-25.

Levina IS, Kuznetsov YV, Shchelkunova TA, Zavarzin IV. 2021. Selective ligands of membrane progesterone receptors as a key to studying their biological functions in vitro and in vivo. The Journal of Steroid Biochemistry and Molecular Biology. 2021/03/01/;207:105827.

Lu J, Reese J, Hirsch E. 2015. Progesterone-Induced Activation of Membrane-Bound Progesterone Receptors in Murine Macrophage Cells. Reprod Sci. Mar;22:136a-137a.

Manocha A, Kankra M, Singla P, Sharma A, Ahirwar AK, Bhargava S. 2018. Clinical significance of reproductive hormones. Current Medicine Research and Practice. 2018/05/01/;8:100-108.

Pang YF, Dong J, Thomas P. 2013. Characterization, Neurosteroid Binding and Brain Distribution of Human Membrane Progesterone Receptors delta and epsilon (mPR delta and mPR epsilon) and mPR delta Involvement in Neurosteroid Inhibition of Apoptosis. Endocrinology. Jan;154:283-295.

Polikarpova AV, Maslakova AA, Levina IS, Kulikova LE, Kuznetsov YV, Guseva AA, Shchelkunova TA, Zavarzin IV, Smirnova OV. 2017. Selection of Progesterone Derivatives Specific to Membrane Progesterone Receptors. Biochemistry (Mosc). Feb;82:140-148.

Rezanujjaman M, Tanvir R, Ali MH, Tokumoto T. 2020. An agonist for membrane progestin receptor (mPR) induces oocyte maturation and ovulation in zebrafish in vivo. Biochemical and Biophysical Research Communications. Aug 20;529:347-352.

Rushdi MI, Abdel-Rahman IAM, Saber H, Attia EZ, Madkour HA, Abdelmohsen UR. 2021. A review on the pharmacological potential of the genus Padina. South African Journal of Botany. 2021/09/01/;141:37-48.

Smith JL, Kupchak BR, Garitaonandia I, Hoang LK, Maina AS, Regalla LM, Lyons TJ. 2008. Heterologous expression of human mPRalpha, mPRbeta and mPRgamma in yeast confirms their ability to function as membrane progesterone receptors. Steroids. Oct;73:1160-1173.

Thomas P. 2008. Characteristics of membrane progestin receptor alpha (mPR alpha) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. Front Neuroendocrin. May;29:292-312.

Tokumoto M, Nagahama Y, Thomas P, Tokumoto T. 2006. Cloning and identification of a membrane progestin receptor in goldfish ovaries and evidence it is an intermediary in oocyte meiotic maturation. General and Comparative Endocrinology. Jan 1;145:101-108.

Tokumoto T, Kodani S, Takehiro M, Suzuki Y, Casareto B, Bahorun T, Bhagooli R. 2017. Detecting membrane progestin receptor (mPR)- interacting compounds from coral seawater
in Mauritius. WIO Journal of Marine Science.1:77-84.

Tokumoto T, Tokumoto M, Horiguchi R, Ishikawa K, Nagahama Y. 2004. Diethylstilbestrol induces fish oocyte maturation. Proceedings of the National Academy of Sciences of the United States of America. Mar 9;101:3686-3690.

Tokumoto T, Tokumoto M, Oshima T, Shimizuguchi K, Fukuda T, Sugita E, Suzuki M, Sakae Y, Akiyama Y, Nakayama R, et al. 2012. Characterization of multiple membrane progestin receptor (mPR) subtypes from the goldfish ovary and their roles in the induction of oocyte maturation. General and Comparative Endocrinology. May 15;177:168-176.

Tokumoto T, Yamaguchi T, Ii S, Tokumoto M. 2011. In Vivo Induction of Oocyte Maturation and Ovulation in Zebrafish. Plos One. Sep 28;6.

Tubbs C, Thomas P. 2009. Progestin signaling through an olfactory G protein and membrane progestin receptor-alpha in Atlantic croaker sperm: potential role in induction of sperm hypermotility. Endocrinology. Jan;150:473-484.

Vares G, Sai S, Wang B, Fujimori A, Nenoi M, Nakajima T. 2015. Progesterone generates cancer stem cells through membrane progesterone receptor-triggered signaling in basal-like human mammary cells. Cancer Res. Aug 1;75.

Xie MX, Zhu XZ, Liu ZF, Shrubsole M, Varma V, Mayer IA, Dai Q, Chen Q, You SJ. 2012. Membrane Progesterone Receptor Alpha as a Potential Prognostic Biomarker for Breast Cancer Survival: A Retrospective Study. Plos One. Apr 4;7.

Zhu Y, Bond J, Thomas P. 2003. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci U S A. Mar 4;100:2237-2242.

Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. 2003. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci U S A. Mar 4;100:2231-2236.

SUPPLEMENTARY MATERIALS

The antagonistic activity of *Padina arborescens* extracts on mPRα

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Experimental

Materials

Several hundred branches of the marine algae *Padina arborescens* were collected at the marine field of the Shimoda Marine Research Center, University of Tsukuba, Nabetahama in Shimoda city (34°66'64.87"N, 138°93'89.05"E) or the Mochimune marine field of the Faculty of Agriculture Regional Field Science Education and Research Center, Shizuoka University, Mochimunekaigan in Shizuoka city (34°92'07.51"N, 138°36'82.14"E) in Shizuoka Prefecture, Japan (Figure S1). All samples were transported to the aquarium on the day of sampling.

The algae were planted in gravel stone in 120 cm long aquariums and cultivated for several months in the culture room of the Mochimune marine field, Shizuoka University. The aquariums were set with filtering systems and LED lights.

Genomic DNA extraction and DNA sequencing

Species of collected specimens were identified as *Padina arborescens* by DNA sequencing of mitochondrial cytochrome oxidase subunit 3 (DDBJ code AB358905) (Ni-Ni-Win et al. 2008). Voucher specimen of *Padina arborescens* is deposited in the Herbarium of the Graduate School of Science, Hokkaido University (SAP) (SAP105578).

Sample collection

At 15-20 day intervals, all filters were detached from the system and washed vigorously with ultra-pure water (UPW) to remove the seawater. After removing the water, the filters were soaked with ethanol (1 liter for each filter) to extract the trapped compounds. The ethanol extracts were diluted 10-fold with UPW and applied to an ODS column (50 μm, 1.8×11.4 cm, 7 g, Yamazen Corporation, Japan) with a peristaltic pump. Approximately 10-20 L of sample could be applied to a single column.

Purification by HPLC

The compounds on the first ODS column were fractionated by a linear gradient of acetonitrile (0-100%) under acidic conditions (0.05% TFA). Four fractions with different colors were collected. The fourth fraction with mPRα-interacting activity was further fractionated with silica-based C18 columns (4.6×30 cm) containing octadecyl silica

groups or C18 groups (Tosoh Corporation, Shiba-Koen first Bldg. Minato-ku, Tokyo) by a linear gradient of acetonitrile (0-100%) under acidic conditions (0.05% TFA). Finally, the fraction with mPRα-interacting activity was purified with a TSKgel Phenyl-5PW RP Glass (8 mm × 7.5 cm) column (Tosoh Corporation, Tokyo, Japan). The chromatogram was monitored at 254 nm, and the column oven was maintained at 40 °C for each step. The collected fractions were lyophilized with an FDU-810 freeze-dryer (EYELA, Tokyo, Japan). Dried samples were dissolved in ethanol for further purification and analysis.

Steroid binding assay

Competitive mPRα binding activity was determined as previously described by using culture cell membranes stably transfected with goldfish mPRα and the [^{3}H]-labeled fish maturation-inducing hormone 17,20β-DHP (Tokumoto et al. 2007).

In vitro and in vivo oocyte maturation assay

An *in vitro and in vivo* oocyte maturation and ovulation assay was conducted in zebrafish as described previously (Rezanujjaman et al. 2020).

References

Ni-Ni-Win, Hanyuda T, Arai S, Uchimura M, Abbott IA, Kawai H. 2008. Three new records of Padina in Japan based on morphological and molecular markers. Phycol Res. Dec;56:288-300.

Rezanujjaman M, Tanvir R, Ali MH, Tokumoto T. 2020. An agonist for membrane progestin receptor (mPR) induces oocyte maturation and ovulation in zebrafish in vivo. Biochemical and Biophysical Research Communications. Aug 20;529:347-352.

Tokumoto T, Tokumoto M, Thomas P. 2007. Interactions of diethylstilbestrol (DES) and DES analogs with membrane progestin receptor-alpha and the correlation with their nongenomic progestin activities. Endocrinology. Jul;148:3459-3467.

Figure S1. Collection sites of *Padina arborescens.*
Photographs of collection sites of *Padina arborescens* in the marine field of the Shimoda Marine Research Center, University of Tsukuba (A) and the Mochimune marine field of the Faculty of Agriculture Regional Field Science Education and Research Center, Shizuoka University (C). White arrows indicate collection sites. Thalli of *Padina arborescens* at the collection sites (B: Shimoda, D:Mochimune).

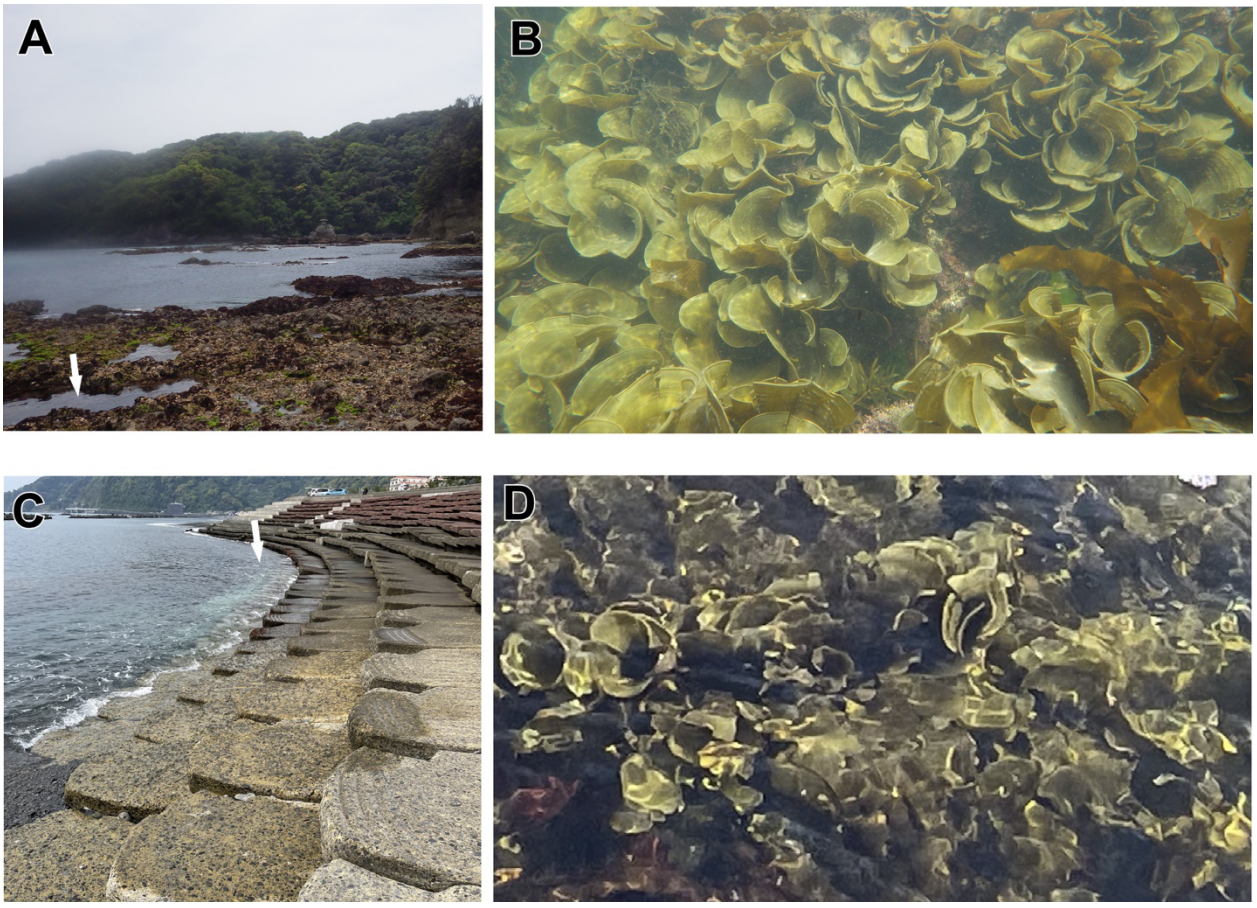

Figure S2. Chromatogram of compound separation with a TSKgel Phenyl-5PW RP column.

The fraction with mPRα-interacting activity from the ODS column was separated by a linear gradient of acetonitrile (0-100%) under acidic conditions (0.05% TFA). Peaks in the flow-through fractions (peak 1) and fractions eluted by acetonitrile (peak 2) were obtained.

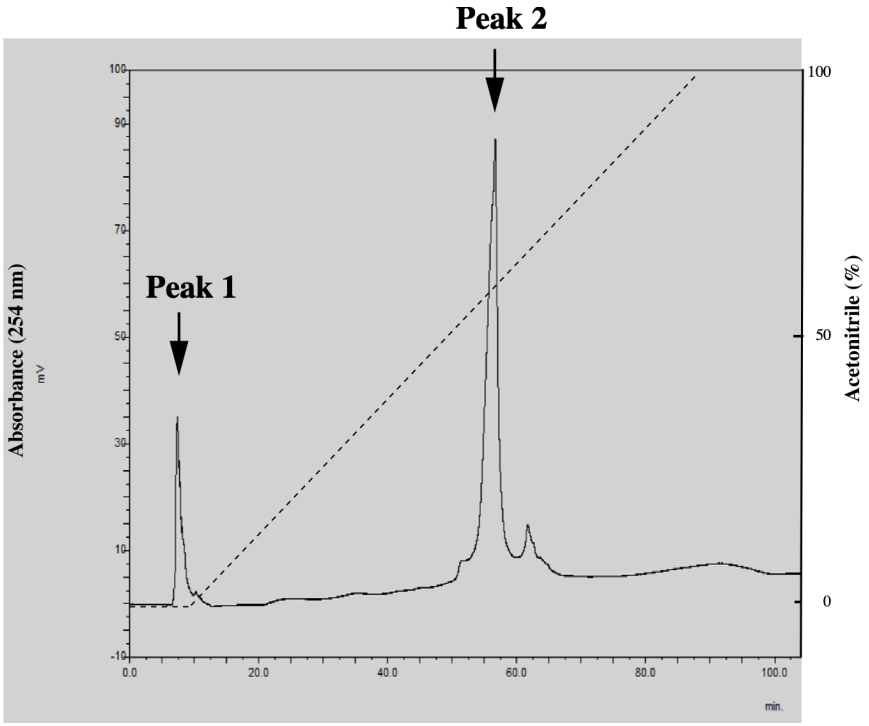

Figure S3. Compounds competing with 17,20β-DHP for binding to mPRα receptors. Samples were incubated with 2 nM [^{3}H]-17,20β-DHP. Competition for [^{3}H]-17,20β-DHP binding is expressed as a percentage of the maximum specific 17,20β-DHP binding. 17,20β-DHP, 17α,20β-dihydroxy-4-pregnen-3-one.

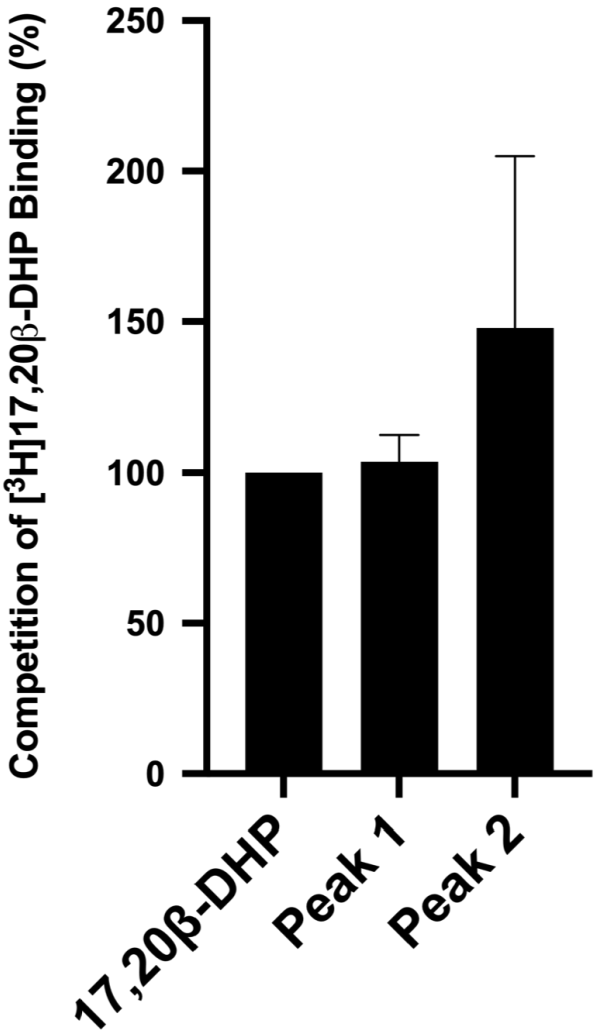

Figure S4. Physiological activity of the purified compounds on oocyte maturation and ovulation in zebrafish and prevention of the induction of oocyte maturation induced by 17,20β-DHP.

The *in vitro* assay results for the compounds in peak 1 (A) and peak 2 (B) are indicated. Each compound was added to the water at a final concentration of 4 μg/mL with 0.1 μM 17,20β-DHP. After two hours of incubation, percentage of germinal vesicle breakdown (%GVBD: indicated by closed column) was determined by scoring the oocytes that became transparent. The *in vivo* assay results for the compounds in peak 1 (C) and peak 2 (D) are indicated. After four hours of incubation, %GVBD (closed column) and %ovulation (open column) were determined by scoring the oocytes that became transparent and formed a fertilization membrane.