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Discovery and structure–activity relationships of pyrazolodiazepine derivatives as the first small molecule agonists of the *Drosophila* sex peptide receptor

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ABSTRACT

In behavioral research, the sex peptide receptor in *Drosophila melanogaster* (*Drm*SPR) is the most interesting G protein-coupled receptor (GPCR) and is involved in post-mating responses such as increased egg-laying and decreased receptivity of the female; during these responses, the receptors are activated by a specific natural peptide agonist (sex peptide, SP). To discover small molecule agonists for *Drm*SPR, a compound library based on a pyrazolodiazepine scaffold, which was previously reported as a potential privileged structure, was screened. Structure–activity relationship (SAR) studies of the hit compounds, which exhibited weak agonistic effects (69–72% activation at 100 μM), were explored through the synthesis of various analogs with substituents at the R_1, R_2, R_3 and R_4 positions of the pyrazolodiazepine skeleton. As a result, compounds **21** and **31** of the 6-benzyl pyrazolodiazepine derivative series were found to be small molecule agonists for *Drm*SPR with EC_{50} values of 3–4 μM.

1. Introduction

The *Drosophila melanogaster* sex peptide receptor (*Drm*SPR) is the most important target protein of type 1 G protein-coupled receptor (GPCR) in the field of insect reproductive behavior in the *Drosophila* model system, especially with respect to the reproductive behavior of females.[1–5] Sex peptide (SP) is known to function as a specific natural agonist for *Drm*SPR and is found in seminal fluid. SP is transferred to the female from the male during copulation and activates the SPR expressed in the uterine sensory neurons in the female reproductive tract.[6–8] Consequently, SPR-mediated post-mating responses (PMR), which are characterized by the suppression of mating receptivity and the initiation of egg laying, are triggered in the female.[9–14]

*Drm*SPR is broadly expressed in the reproductive tract of females and in the central nervous system (CNS) in both males and females; however, SPR expressed in the CNS of males is not likely related to behavioral responses because of the absence of SP in the CNS of males.[4,7,8] Although genes encoding SP-like peptides exist in a few closely related *Drosophila* species, SPR is detected in many species, including lophotrochozoa and ecdysozoa.[4] Thus, the SPR has been posited to be activated by other ligands or to be involved in other functions. For example, SPRs from other taxa, including a mosquito, *Aedes aegypti*; a moth, *Bombyx mori*; and a sea slug, *Aplysia californica*, do not respond to SP. Instead, those receptors, along with *Drm*SPR, are highly sensitive to myoinhibitory peptides (MIPs), a group of neuropeptides that are distinct from SP. These findings indicate that SPR is also a receptor for MIPs, which function as alternative peptide agonists and may be ancestral ligands.[15–17] However, the binding of MIPs to SPR in the reproductive tract of females does not evoke SPR-mediated behavioral responses.[15] Additionally, while *Drosophila* SP activates only *Drm*SPR, MIPs are potent ligands that act on SPRs in various species.[15,18–22]

Abbreviations: SPR, sex peptide receptor; SP, sex peptide; MIPs, myoinhibitory-peptides; *Drm*, *Drosophila melanogaster*; TEA, triethyl amine; TFA, trifluoroacetic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMF, dimethylformamide; DCM, dichloromethane; HOBt, *N*-hydroxylbenzotriazole; DIBAL-H, diisobutylaluminium hydride; EC_{50}, half-maximal effective concentration; HPLC, high-pressure liquid chromatography; ESI, electrospray ionization; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; SAR, structure–activity relationship.

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SP consists of 36 amino acids, including a cyclic sequence at the C-terminal, that is, responsible for the activation of SPR and the consequent behavioral responses.[12,14,23] In the sequence of SP, two tryptophan residues (Trp^{23} and Trp^{32}) have been reported to be crucial for its agonistic activity. Similarly, in the case of MIPs, the representative amino acid sequence contains a W(X_6)W amide motif, and the two tryptophan residues were also observed to be important for activation of the SPR.[15–17]

The differences in receptor selectivity, expression pattern and effect on post-mating responses between SP and MIPs indicate that the SPR may bind in different modes or activate different signaling pathways depending on the ligand. To investigate these potential molecular functions of SPR in detail, the binding mode of the ligands is crucial. Because large peptide molecules such as SP or MIPs cannot be effectively used as probes for in silico molecular docking studies to predict the binding mode, the development of small molecule ligands for SPR is necessary. In addition, small molecule ligands could be utilized for in vivo functional studies of SPR to elucidate the novel mechanisms of action related to SPR.

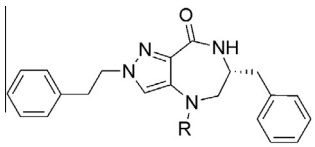
Recently, the reported NMR structure of SP indicated the presence of a type 1 beta turn conformation (Gly^{29}-Trp^{32}) and a hydrophobic patch related to two tryptophan residues (Trp^{23} and Trp^{32}) detected at the cyclic C terminal region; these two moieties were identified as key pharmacophores for peptide activity.[15–17,23] In the previous study, we reported the generation of a compound library using a pyrazolodiazepine skeleton, a privileged structure, that is, commonly utilized for the development of peptide mimetics.[24,25] In this article, we report the discovery of small molecule agonists, for the first time, for *Drm*SPR from hit compounds (**1**, **2** and **3** in Fig. 1) from the screening of pyrazolodiazepine derivatives. The hit compounds were further optimized through the synthesis and SAR analysis of various analogs with different substituents at four positions of the pyrazolodiazepine skeleton, as shown in Figure 2.

2. Results and discussion

2.1. Chemistry

The chemical synthesis of compound **5** from commercially available starting materials and the generation of its derivatives with phenethyl, 2-naphthyl methyl and 1-Boc-4-piperidine ethyl groups at the R_1 position were reported in a previous study.[24] Novel compounds with various substituents at the R_3 position (**28**–**41**) and indole-substituted derivatives at the R_2 and R_5 positions (**45**–**57**) were synthesized using the strategies shown in Schemes 1 and 2, respectively.

As shown in Scheme 1, for the synthesis of the compounds substituted with an indole moiety at the R_2 position, the ester group of **4C** was hydrolyzed using 1 N aqueous NaOH in methanol, followed by subsequent coupling with D-Trp-methyl ester in the presence of general coupling reagents. The aryl nitro group of **5Cc** was reduced



1 R = $COCH{=}C(CH_3)_2$
2 R = $COCH_2C(CH_3)_3$
3 R = $CO(CH_2)_2(C_5H_9)$

Figure 1. Hit compounds as agonists of *Drosophila* sex peptide receptor hit compounds showed weak agonistic effect (**1** = 69%, **2** = 69% and **3** = 72%) at 100 μM concentration.

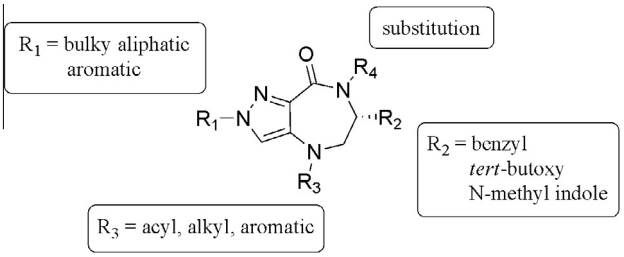


Figure 2. Strategy for optimization of hit compounds.

by hydrogenation in the presence of 10% Pd/C in methanol to yield **6Cc**. The aldehyde derivative **7**, which could interconvert reversibly into the imine **8**, was synthesized by reduction of the ester to an aldehyde with DIBAL-H in toluene at −78 °C. Reductive amination of imine **8** using $NaBH(OAc)_3$ and 1% acetic acid in dichloromethane afforded the 7-membered ring system of the pyrazolodiazepine-8-one derivative **9**. Finally, compound **9** was reacted with various acid chlorides, alkyl halides or aromatic acyl groups to yield the novel compounds **10**–**18** and **25**–**41** to study the SAR of the R_3 position.

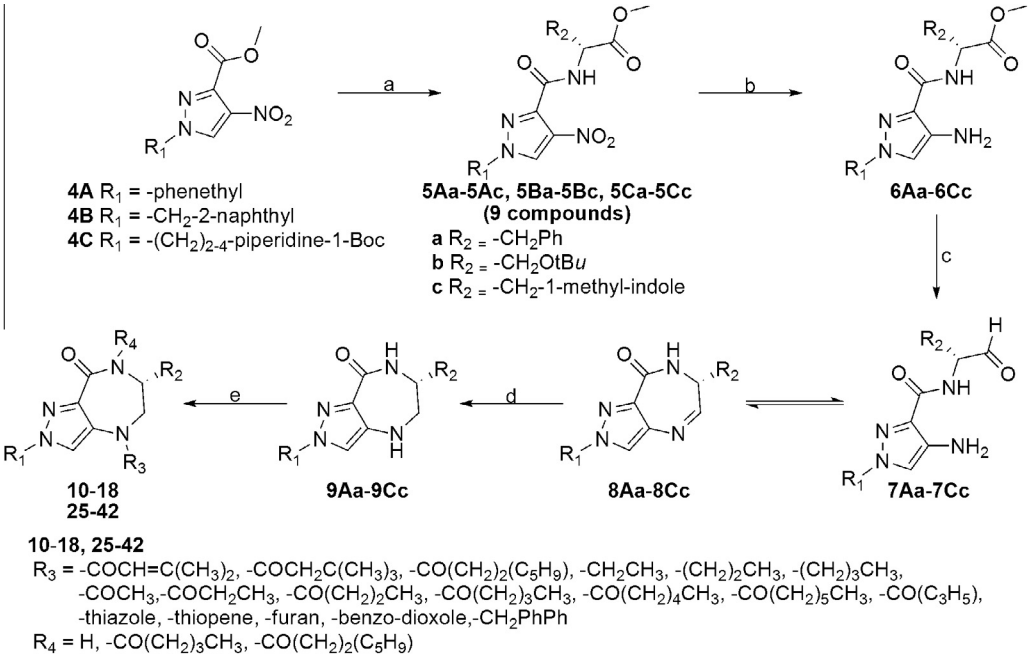
Because the two tryptophan residues of SP and MIPs are key pharmacophores, we replaced the Boc group with an aliphatic or indole moiety at the R_5 position, as shown in Scheme 2. Briefly, the Boc groups of compounds **31** and **42** were removed to yield **43** and **44**, which were subjected to coupling reactions with various building blocks to yield compounds substituted in the R_5 position with bulky aliphatic (**45**–**49**), indole groups (**50**–**56**).

2.2. Compound library screening and structure–activity relationship study

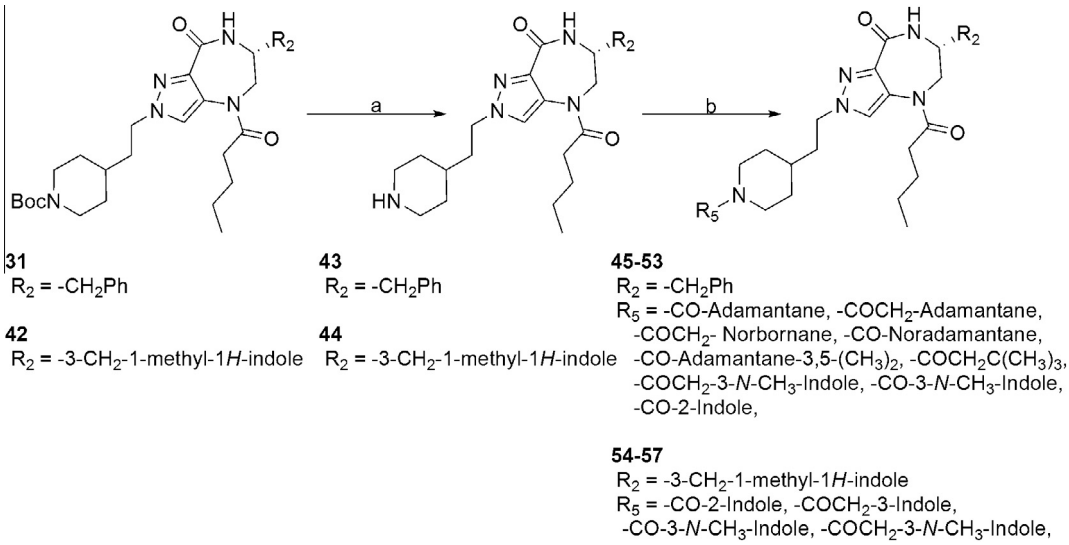
The initial hit compounds exhibited weak agonist effects at 100 μM (Fig. 1 and 1 = 72%, **2** = 69%, **3** = 69%). All three hit molecules were pyrazolodiazepine analogs and had phenethyl, benzyl and acyl chain moieties at the R_1, R_2 and R_3 positions, respectively. The hit compounds were further optimized through the synthesis and SAR analysis of various analogs with differing substituents at four positions of the pyrazolodiazepine skeleton, as shown in Figure 2. These substitutions included aromatic groups or bulky aliphatic groups at the R_1 position; *tert*-butoxy or aromatic groups at the R_2 position; and various acyl, alkyl or aromatic groups at the R_3 position to replace the acyl groups of the hit compounds.

The initial SAR studies of the three hit compounds, **1**–**3**, were designed to investigate the effect of various substituents at the R_2 position (compounds **10**–**15**) on SPR agonist activity. As shown in Table 1, altering the benzyl moiety at the R_2 position of the hit compounds to *tert*-butoxymethylene (**10**–**12**) and hydroxymethylene (**13**–**15**) resulted in significant loss of agonistic activity, from 69% to 72% to less than 20%. Thus, the next SAR study was performed with a benzyl group fixed at the R_2 position.

For the SAR studies at the R_1 position, compounds with 2-naphthylmethylene, 1-Boc-piperidine-4-ethyl and piperidine-4-ethyl moieties were compared, as shown in Table 2. The presence of bulkier aromatic groups such as the 2-naphthyl moiety at the R_1 position (**16**–**18**) resulted in the total loss of agonistic activity. Encouragingly, compounds **19**–**21** with a 1-Boc-piperidine moiety instead of the benzyl groups of the hit compounds, showed much higher activity, with three- to four-fold increases in the agonistic activity (e.g., **19** = 207%, **20** = 228%, **21** = 295%, at 100 μM). However, removal of the Boc groups from the piperidine moiety of compounds **19**–**21** to provide **22**–**24** resulted in activities less than those of the hit compounds with a phenethyl moiety. Interestingly, among the series of compounds in Table 2, the



Scheme 1. Synthesis of pyrazolodiazepine derivatives. Reagents and conditions: (a) (i) 1 N NaOH in MeOH, rt, 1 h, 98–99%, (ii) D-Phe-methyl ester or D-Trp-methyl ester, EDC, HOBt, TEA, DCM, rt, 8 h, 69–78%; (b) Pd/C, H_2, MeOH, rt, 4 h, 96–98%; (c) DIBAL-H, toluene, −78 °C, 3 h, 60–70%; (d) $NaBH(OAc)_3$, 1% AcOH, DCM, rt, 1 h, 55–60%; (e) (i) RCOCl, TEA, DCM, rt, 1 h, 80–90%; in case of **13**–**15**, (ii) 50% TFA, anisole, DCM, 95–97%; **22**–**24**, (ii) 20% TFA, DCM, rt, 30 min, 93–97%.

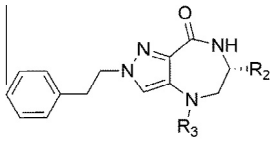


Scheme 2. Synthesis of pyrazolodiazepine derivatives. Reagents and conditions: (a) 20% TFA, DCM, rt, 30 min, 95–97%; (b) RCOOH, EDC, TEA, DCM, rt, 8 h, 49–78%.

agonist activity showed dependence on the R_3 substituents. Thus, the agonistic activity increased in the following order: $-CO(CH_2)_2(C_5H_9) > -COCH_2C(CH_3)_3 > -COCH{=}C(CH_3)_2$ for moieties at the R_3 position, respectively. These results suggest that aliphatic moieties at the R_1 and R_3 positions may be important for the agonistic activity of the compounds.

To further optimize the activity, various acyl, alkyl and aromatic groups were substituted at the R_3 position while the 1-Boc-piperidine group at the R_1 position and benzyl group at the R_2 position were fixed (Table 3). The compounds with acyl groups at the R_3 position (**28**–**30**) showed approximately two-fold higher activity than the corresponding compounds with alkyl groups (**25**–**27**). For example, the acetyl, propionyl and butanoyl moieties (**28** = 22%, **29** = 73%, **30** = 207%, respectively) exhibited better activity than the ethyl, propyl and butyl moieties (**25** = 2%, **26** = 46%, **27** = 120%, respectively). This result indicates that the carbonyl group might serve as an important pharmacophore for agonistic activity of SPR. In addition, when this carbon chain was further elongated up to seven carbons to provide the valeryl group-substituted compound **31**, the greatest agonistic effect (321%, at 100 μM) among the series of acyl-substituted compounds was observed. The activity was slightly enhanced compared with **21**, which was one of the most potent agonists in the earlier series of compounds in Table 2. However, compounds **32**–**34**, which contained longer alkyl chains than **31** or a cyclopropyl acyl group at the R_3 position, were less effective agonists. In the case of **35**–**38**, which were substituted with various heteroaromatic groups at the R_3 position, the agonistic activities were higher than those

Table 1
Agonistic effects of synthesized compounds (**10–15**) at *Drosophila* sex peptide receptors

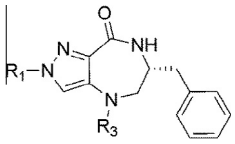


10-15

Compounds	R_2	R_3	% Act (100 μM)[a]
1	$-CH_2Ph$	$-COCH{=}C(CH_3)_2$	69 ± 17
2	$-CH_2Ph$	$-COCH_2C(CH_3)_3$	69 ± 13
3	$-CH_2Ph$	$-CO(CH_2)_2(C_5H_9)$	72 ± 23
10	$-CH_2O(^tBu)$	$-COCH{=}C(CH_3)_2$	N.A.[b]
11	$-CH_2O(^tBu)$	$-COCH_2C(CH_3)_3$	N.A.[b]
12	$-CH_2O(^tBu)$	$-CO(CH_2)_2(C_5H_9)$	14 ± 4
13	$-CH_2OH$	$-COCH{=}C(CH_3)_2$	N.A.[b]
14	$-CH_2OH$	$-COCH_2C(CH_3)_3$	12 ± 3
15	$-CH_2OH$	$-CO(CH_2)_2(C_5H_9)$	16 ± 4

[a] 100% receptor activation was determined by 50 μM ATP activation at the *Drm*SPR expressed in CHO-K1 cell, and the percentage of activation by 100 μM compounds was measured for *Drm*SPR, respectively (mean ± SEM, $n \geqslant 3$).
[b] Not active.

Table 2
Agonistic effects of synthesized compounds (**16–24**) at *Drosophila* sex peptide receptors



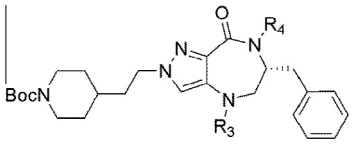
16-24

Compounds	R_1	R_3	% Act (100 μM)[a]
1	$-(CH_2)_2Ph$	$-COCH{=}C(CH_3)_2$	69 ± 17
2	$-(CH_2)_2Ph$	$-COCH_2C(CH_3)_3$	69 ± 13
3	$-(CH_2)_2Ph$	$-CO(CH_2)_2(C_5H_9)$	72 ± 23
16	$-CH_2$naphthyl	$-COCH{=}C(CH_3)_2$	N.A.[b]
17	$-CH_2$naphthyl	$-COCH_2C(CH_3)_3$	N.A.[b]
18	$-CH_2$naphthyl	$-CO(CH_2)_2(C_5H_9)$	13 ± 6
19	$-(CH_2)_2$piperidine-1-Boc	$-COCH{=}C(CH_3)_2$	207 ± 19
20	$-(CH_2)_2$piperidine-1-Boc	$-COCH_2C(CH_3)_3$	228 ± 37
21	$-(CH_2)_2$piperidine-1-Boc	$-CO(CH_2)_2(C_5H_9)$	295 ± 59
22	$-(CH_2)_2$-4-piperidine	$-COCH{=}C(CH_3)_2$	40 ± 12
23	$-(CH_2)_2$-4-piperidine	$-COCH_2C(CH_3)_3$	44 ± 18
24	$-(CH_2)_2$-4-piperidine	$-CO(CH_2)_2(C_5H_9)$	75 ± 13

[a] 100% receptor activation was determined by 50 μM ATP activation at the *Drm*SPR expressed in CHO-K1 cell, and the percentage of activation by 100 μM compounds was measured for *Drm*SPR, respectively (mean ± SEM, $n \geqslant 3$).
[b] Not active.

for alkyl-substituted compounds (**25–27**) but lower than those for acyl-substituted compounds. Interestingly, a non-aliphatic derivative, compound **39**, was nearly as potent (261%) as the optimized analogs such as compounds **21** and **31**. Compounds **40** and **41**, which contain additional substituents, valeryl and 3-cyclopentylpropane carbonyl moieties, respectively, at the R_4 position showed two- to three-fold decreases in the agonistic effect compared with the corresponding R_3 monosubstituted compounds **21** and **31**. Compounds **21** and **31** showed EC_{50} values of 3.2 μM and 3.9 μM, respectively, for *Drm*SPR agonist activity, as shown in Figure 3. Based on the above results, the valeryl moiety at the R_3 position

Table 3
Agonistic effects of synthesized compounds (**25–41**) at *Drosophila* sex peptide receptors



25-41

Compounds	R_3	R_4	% Act (100 μM)[a]
19	$-COCH{=}C(CH_3)_2$	–H	207 ± 19
20	$-CO(CH_2)C(CH_3)_3$	–H	228 ± 37
21	$-CO(CH_2)_2(C_5H_9)$	–H	295 ± 59
25	$-CH_2CH_3$	–H	N.A.[b]
26	$-(CH_2)_2CH_3$	–H	46 ± 12
27	$-(CH_2)_3CH_3$	–H	120 ± 23
28	$-COCH_3$	–H	22 ± 10
29	$-COCH_2CH_3$	–H	73 ± 16
30	$-CO(CH_2)_2CH_3$	–H	207 ± 37
31	$-CO(CH_2)_3CH_3$	–H	321 ± 40
32	$-CO(CH_2)_4CH_3$	–H	275 ± 51
33	$-CO(CH_2)_5CH_3$	–H	214 ± 34
34	$-CO(C_3H_5)$	–H	129 ± 3
35	–Thiazole-2-carbonyl	–H	145 ± 14
36	–Thiopene-2-carbonyl	–H	75 ± 13
37	–Furan-2-carbonyl	–H	83 ± 10
38	–4-Phenyl benzyl	–H	14 ± 5
39	–Benzo-[1,3]dioxole-5-carbonyl	–H	261 ± 26
40	$-CO(CH_2)_2(C_5H_9)$	$-CO(CH_2)_2(C_5H_9)$	147 ± 26
41	$-CO(CH_2)_3CH_3$	$-CO(CH_2)_3CH_3$	106 ± 19

[a] 100% receptor activation was determined by 50 μM ATP activation at the *Drm*SPR expressed in CHO-K1 cell, and the percentage of activation by 100 μM compounds was measured for *Drm*SPR, respectively (mean ± SEM, $n \geqslant 3$).
[b] Not active.

without substitution at the R_4 position was selected for the next series of SAR studies.

To potentially improve the agonistic activity further, the Boc group at the R_1 position of compound **21** was replaced with various bulky polycycloalkane moieties at the R_5 position (Table 4) because the Boc moiety appeared to be an important pharmacophore, as shown in Table 2. Unfortunately, replacement of the Boc group at the R_5 position with an adamantane carbonyl group resulted in a two-fold decrease in agonistic activity (**45**, 164% at 100 μM), and compound **46**, which was one carbon longer than **45**, showed a further decrease in activity (96%). Other compounds (**47–49**) also showed significantly decreased agonist activity. In the case of substitution of 3,3-dimethylbutanoyl group instead of Boc group at R_5 position, which was an exchange of the oxygen of Boc group by methylene moiety, slightly decreased agonistic effect (**50**, 208% at 100 μM) was observed. These result indicated that Boc group was most important for the agonistic activity and oxygen of Boc group is considered to be influential pharmacophore at R_5 position.

Another modification was the incorporation of indole moieties at the R_2 and R_5 positions according to the previously published studies that have indicated that the two tryptophan residues of SP and MIPs were critical for agonistic effects at the SPR. In the first attempt, various indole moieties were substituted on the R_5 position instead of the Boc group (**51–53**), as shown in Table 5. Unexpectedly, the agonist effect of these compounds was markedly decreased, similar to the results for substitution with bulky aliphatic moieties (**45–49**). In the second attempt, the agonistic effects of the indole groups at both the R_2 and R_5 positions (**54–57**) were investigated. However, those compounds also showed negligible

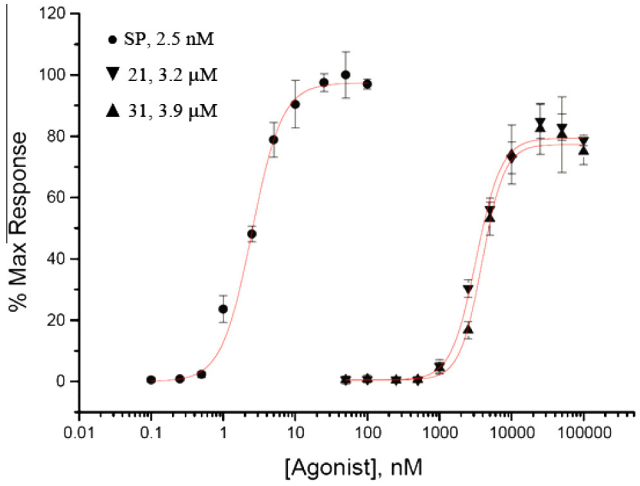
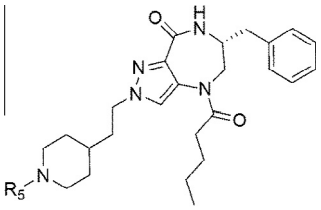


Figure 3. EC_{50} values of SP and synthesized compounds **21**, **31** of *Drosophila* sex peptide receptors. Dose–response curves of CHO cells expressing SPR, aequorin, and a chimeric G protein, $G\alpha_{qo}$, and treated with compounds **21** (▼), **31** (▲) and SP (●). Each data point is mean ± SEM ($n \geqslant 3$).

Table 4
Agonistic effects of synthesized compounds (**45**–**50**) at *Drosophila* sex peptide receptors



45-50

Compounds	R_5	% Act (100 μM)[a]
45	–CO-adamantane	164 ± 62
46	$-COCH_2$-adamantane	96 ± 25
47	$-COCH_2$-norbornane	72 ± 15
48	–CO-noradamantane	127 ± 12
49	–CO-adamantane-3,5-$(CH_3)_2$	N.A.[b]
50	$-COCH_2C(CH_3)_3$	208 ± 36

[a] 100% receptor activation was determined by 50 μM ATP activation at the *Drm*SPR expressed in CHO-K1 cell, and the percentage of activation by 100 μM compounds was measured for *Drm*SPR, respectively (mean ± SEM, $n \geqslant 3$).
[b] Not active.

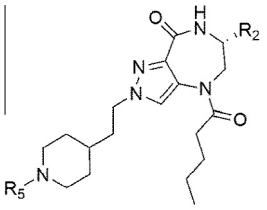
agonist activities for the activation of SPR (<25% at 100 μM). The poor agonist effects of this series of compounds might be due to differences in the binding mechanism of our small compound and those of SP and MIPs.

The first small molecule agonists for *Drm*SPR discovered in this study could be utilized to elucidate the structure and function of the SPR, which is a highly important target receptor related to post-mating responses in *Drosophila*.

3. Conclusion

In summary, we report the discovery of the first small molecule agonists for *Drm*SPR from the synthesis and SAR studies of various pyrazolodiazepine derivatives. The optimized compounds for SPR, **21** and **31**, showed EC_{50} values of 3–4 μM. SAR analysis of the pyrazolodiazepine derivatives as SPR agonist indicated that the following conditions are required for agonism of *Drm*SPR: (1) a benzyl moiety at the R_2 position, (2) a 1-Boc-piperidine ethyl moiety at

Table 5
Agonistic effects of synthesized compounds (**51**–**57**) at *Drosophila* sex peptide receptors



51-57

Compounds	R_2	R_5	% Act (100 μM)[a]
51	$-CH_2Ph$	$-COCH_2$-3-N-CH_3-indole	118 ± 15
52	$-CH_2Ph$	–CO-3-N-CH_3-indole	27 ± 2
53	$-CH_2Ph$	–CO-2-indole	N.A.[b]
54	$-CH_2$-N-CH_3-indole	–CO-2-indole	N.A.[b]
55	$-CH_2$-N-CH_3-indole	$-COCH_2$-3-indole	23 ± 4
56	$-CH_2$-N-CH_3-indole	–CO-3-N-CH_3–indole	17 ± 3
57	$-CH_2$-N-CH_3-indole	$-COCH_2$-3-N-CH_3-indole	15 ± 2

[a] 100% receptor activation was determined by 50 μM ATP activation at the *Drm*SPR expressed in CHO-K1 cell, and the percentage of activation by 100 μM compounds was measured for *Drm*SPR, respectively (mean ± SEM, $n \geqslant 3$).
[b] Not active.

the R_1 and R_5 positions, (3) an aliphatic acyl group (optimum carbon chain length of (4) at the R_3 position, and (4) no substitution at the R_4 position. Our discovery of the first small molecule agonists for *Drm*SPR could assist in further research in this field, including in vivo functional and in silico structural studies of *Drm*SPR.

4. Experimental section

4.1. Chemistry

Starting materials, solvents and reagents were obtained from commercial suppliers and used as received unless otherwise noted. All products reported showed ^1H NMR and mass spectra in agreement with the assigned structures. ^1H NMR data were collected on a JEOL JNM-ECX 400P spectrometer at 400 MHz and were recorded in parts per million (ppm) values, relative to tetramethylsilane as the internal standard. Spectra were taken in $CDCl_3$ or CD_3OD. Data are reported as follows: chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad; app, apparent), and coupling constants. ^{13}C NMR data was obtained from the Korea Basic Science Institute (Gwangju) using 500 MHz FT-NMR spectrometer. Mass spectroscopy was carried out on ESI and MALDI-TOF instruments. High-resolution mass spectra (m/z) were recorded on a FAB (JEOL: mass range 2600 au, 10 kV acceleration) at Korea Basic Science Institute (Daegu). Compounds **21** and **31** were prepared according to literature procedures which we previously reported.[24]

4.1.1. (*R*)-*tert*-Butyl 4-(2-(3-(1-methoxy-3-(1-methyl-1*H*-indol-3-yl)-1-oxopropan-2-ylcarbamoyl)-4-nitro-1*H*-pyrazol-1-yl)ethyl)piperidine-1-carboxylate (5Cc)

4C (4.5 g, 11.77 mmol) was dissolved in 1 N NaOH in MeOH (300 mL) and stirred for 30 min. Subsequently, the reaction mixture was neutralized with 1 N HCl aqueous solution and concentrated under reduced pressure. The residue was dissolved in DCM

and washed with 1 N HCl aqueous solution. The organic layer was concentrated under reduced pressure to get the desired product as white solid. Without further purification, it was used for next reaction. The carboxylic acid was dissolved in DCM (300 mL) and HOBt (3.2 g, 23.54 mmol) and EDC (4.5 g, 23.54 mmol) were added. Subsequently, D-Trp-OMe HCl (6.0 g, 23.54 mmol) and TEA (2.4 mL, 23.54 mmol) were added to the solution and stirred at room temperature for 16 h. The reaction mixture was diluted with chloroform, washed with saturated aqueous NH_4Cl, dried with Na_2SO_4, and concentrated. The residue was purified by column chromatography on silica using hexanes/ethyl acetate = 3:2 to give the amide **5Cc** (5.3 g, 85%) as colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.13 (1H, s, CH), 8.03–8.01 (1H, m, indole), 7.56–7.54 (1H, m, indole), 7.27–7.25(1H, m, indole), 7.04 (1H, t, J = 7.6 Hz, indole), 6.94 (1H, s, indole), 5.13–5.11 (1H, m, CH), 4.18–4.15 (2H, t, J = 7.2 Hz, CH_2), 4.10–4.09 (2H, m, CH_2), 3.73 (3H, s, CH_3), 3.71 (3H, s, CH_3), 3.45–3.41 (2H, m, CH_2), 2.70–2.67 (2H, m, CH_2), 1.94–1.90 (2H, m, CH_2), 1.68–1.65 (2H, m, CH_2), 1.46 (9H, s, Boc), 1.46–1.44 (1H, m, CH), 1.20–1.15 (2H, m, CH_2), MS (ESI): m/z = 582.9 [M+H].

4.1.2. (*R*)-*tert*-Butyl 4-(2-(4-amino-3-(1-methoxy-3-(1-methyl-1*H*-indol-3-yl)-1-oxopropan-2-ylcarbamoyl)-1*H*-pyrazol-1-yl)ethyl)piperidine-1-carboxylate (6Cc)

A solution of **5Cc** (5.0 g, 8.6 mmol) in MeOH was added 10% Pd/C (300 mg, 5 mmol) and stirred under hydrogen gas atmosphere for 2 h at room temperature. The product was filtered through Celite filter agent pad and the filtrate was concentrated in vacuum. The residue was purified by flash column chromatography on silica using hexanes/ethyl acetate = 1:1 to afford amine **6Cc** (4.6 g, 97%) as yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.02–8.00 (1H, m, indole), 7.52–7.50 (1H, m, indole), 7.23–7.21(1H, m, indole), 7.01 (1H, t, J = 7.6 Hz, indole), 6.94 (1H, s, indole), 6.90 (1H, s, CH), 5.09–5.07 (1H, m, CH), 4.13–4.11 (2H, t, J = 7.2 Hz, CH_2), 4.03–4.01 (2H, m, CH_2), 3.70 (3H, s, CH_3), 3.68 (3H, s, CH_3), 2.67–2.64 (2H, m, CH_2), 1.82–1.78 (2H, m, CH_2), 1.68–1.65 (2H, m, CH_2), 1.46 (9H, s, Boc), 1.46–1.44 (1H, m, CH), 1.18–1.13 (2H, m, CH_2), MS (ESI): m/z = 552.9 [M+H].

4.1.3. (*R*)-*tert*-Butyl 4-(2-(6-((1-methyl-1*H*-indol-3-yl)methyl)-8-oxo-5,6,7,8-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-2(4*H*)-yl)ethyl)piperidine-1-carboxylate (9Cc)

To the solution of ester **6Cc** (4.5 g, 8.1 mmol) in toluene at −78 °C, 1.0 M DIBAL-H in toluene (16.3 mL, 16.3 mol) was added by syringe pump over 30 min under nitrogen atmosphere and stirred for 3 h at −78 °C. The reaction was quenched with MeOH, washed with saturated aqueous potassium sodium tartrate solution, and extracted with DCM. The combined organic layer was dried with Na_2SO_4. The crude product was concentrated and afforded the aldehyde functional compound **7**, which converted reversibly into imine **8**. Without purification, crude **7** and **8** mixtures were dissolved in 1% acetic acid in DCM and stirred for 1 h. $NaBH(OAc)_3$ (3.5 g, 16.3 mmol) was added and stirred for overnight. The reaction mixture was washed with saturated aqueous NH_4Cl and extracted with DCM. The organic extracts were dried with $MgSO_4$ and purified by silica gel chromatography using chloroform/methanol = 40:1 to give the pyrazolodiazepine-8-one (2.0 g, 48%) as yellow sticky solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.58–7.56 (1H, m, indole), 7.38–7.36 (1H, m, indole), 7.23–7.21 (1H, m, indole), 7.10 (1H, t, J = 7.6 Hz, indole), 6.95 (1H, s, CH), 5.94 (1H, s, indole), 4.13 (2H, t, J = 7.2 Hz, CH_2), 4.10–3.98 (2H, m, CH_2), 3.85–3.81 (1H, m, CH_2), 3.78 (3H, s, CH_3), 3.45–3.43 (1H, m, CH), 3.29–3.23 (1H, m, CH), 3.08–2.84 (2H, m, CH_2), 2.63 (2H, t, J = 9.2 Hz, CH_2), 2.17 (1H, s, CH), 1.82–1.78 (2H, m, CH_2), 1.64–1.60 (2H, m, CH_2), 1.60–1.58 (2H, m, CH_2), 1.43 (9H, s, Boc), 1.47–1.38 (2H, m, CH_2), 1.18–1.04 (2H, m, CH_2), MS (ESI): m/z = 508.1 [M+H].

4.1.4. (*R*)-*tert*-Butyl 4-(2-(6-benzyl-4-(3-cyclopentylpropanoyl)-8-oxo-5,6,7,8-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-2(4*H*)-yl)ethyl)piperidine-1-carboxylate (21)

Compound **21** was reported.[24] ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.33 (1H, s, NH), 7.37–7.28 (5H, m, phenyl), 7.20 (1H, s, CH), 4.23–4.19 (2H, m, CH_2), 4.08–4.05 (2H, m, CH_2), 3.83–3.63 (2H, m, CH_2), 3.03–2.79 (2H, m, CH_2), 2.67–2.64 (2H, m, CH_2), 1.89–1.84 (4H, m, CH_2, CH_2), 1.68–1.58 (5H, m, CH, CH_2, CH_2), 1.43 (9H, s, Boc), 1.15–0.86 (11H, m, CH_2, cyclopentyl), ^{13}C NMR ($CDCl_3$, 500 MHz) δ 170.89, 163.51, 154.74, 135.91, 134.15, 129.20 (2C), 129.15 (2C), 127.51, 124.12, 123.95, 79.31, 54.31, 51.78, 51.07, 39.32, 36.57, 33.48 (2C), 33.14, 32.49, 32.39 (2C), 31.73, 30.88, 28.41 (3C), 25.02, 24.97 (3C), MS (ESI): m/z = 578.2 [M+H]. HRMS (FAB) ($C_{33}H_{47}N_5O_4$): calcd 578.7586, found 578.7591.

4.1.5. (*R*)-*tert*-Butyl 4-(2-(6-benzyl-8-oxo-4-pentanoyl-5,6,7,8-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-2(4*H*)-yl)ethyl)piperidine-1-carboxylate (31)

Compound **31** was reported.[24] ^{1}H NMR ($CDCl_3$, 400 MHz) 8.34 (1H, s, NH), 7.36–7.28 (5H, m, phenyl), 7.20 (1H, s, CH), 4.23–4.19 (2H, m, CH_2), 4.08–4.05 (2H, m, CH_2), 3.83–3.63 (2H, m, CH_2), 3.03–2.79 (2H, m, CH_2), 2.67–2.64 (2H, m, CH_2), 1.89–1.84 (4H, m, CH_2, CH_2), 1.68–1.53 (7H, m, CH, CH_2, CH_2, CH_2), 1.43 (9H, s, Boc), 1.14–1.12 (2H, m, CH_2), 0.92 (3H, t, J = 7.6 Hz, CH_3), ^{13}C NMR ($CDCl_3$, 500 MHz) δ 172.48, 163.60, 153.82, 140.50, 138.04, 129.35, 129.09 (2C), 128.52, 128.34 (2C), 126.49, 79.18, 54.20, 52.94, 49.98, 39.37, 36.96, 32.79, 32.72 (2C), 28.09 (3C), 26.79, 21.64 (2C), 13.67, MS (ESI): m/z = 538.2 [M+H]. δ HRMS (FAB) ($C_{30}H_{43}N_5O_4$): calcd 538.6946, found 538.6950.

4.1.6. General procedure for the acylation to the N-1 of tetrahydro-1,4-pyrazolodiazepin-8-one (28–38)

Various starting compounds of scaffold **9** (1.0 equiv) and various acid chlorides (1.0 equiv) in DCM was treated with TEA (1.2 equiv) by dropwise to the mixture for a period of 5 min. After this mixture was stirred for 0.5–1 h at room temperature, it was neutralized with saturated aqueous $NaHCO_3$ and extracted with DCM 2 times. The combined organic layer was dried with anhydrous Na_2SO_4. After Na_2SO_4 was filtered, the solvent was removed in vacuum. The residue was purified by silica gel column chromatography with *n*-hexanes/ethyl acetate = 4:1 to afford acyl compounds as solid.

4.1.7. (*R*)-*tert*-Butyl 4-(2-(4-acetyl-6-benzyl-8-oxo-5,6,7,8-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-2(4*H*)-yl)ethyl)piperidine-1-carboxylate (28)

Following the general procedure for the synthesis of (**28**–**38**), acylation reaction of 9Ca (30 mg, 0.07 mmol) using acetyl chloride (5 μL, 0.07 mmol) afforded 28 (28 mg, 85%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.33 (1H, s, NH), 7.36–7.20 (5H, m, phenyl), 6.98 (1H, s, CH), 4.23–4.19 (2H, m, CH_2), 4.05–4.02 (2H, m, CH_2), 3.84–3.63 (2H, m, CH_2), 3.03–2.79 (2H, m, CH_2), 2.77–2.64 (2H, m, CH_2), 1.88–1.84(2H, m, CH_2), 1.74(3H, s, CH_3), 1.70–1.61 (3H, m, CH, CH_2), 1.43 (9H, s, Boc), 1.14–1.12 (2H, m, CH_2), MS (ESI): m/z = 496.1 [M+H].

4.1.8. (*R*)-*tert*-Butyl 4-(2-(6-benzyl-8-oxo-4-propionyl-5,6,7,8-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-2(4*H*)-yl)ethyl)piperidine-1-carboxylate (29)

Following the general procedure for the synthesis of (**28**–**38**), acylation reaction of **9Ca** (30 mg, 0.07 mmol) using propionyl chloride (6 μL, 0.07 mmol) afforded **29** (25 mg, 75%). ^{1}H NMR ($CDCl_3$,

400 MHz) δ 8.34 (1H, s, NH), 7.36–7.28 (5H, m, phenyl), 7.20 (1H, s, CH), 4.23–4.19 (2H, m, CH_2), 4.08–4.05 (2H, m, CH_2), 3.83–3.63 (2H, m, CH_2), 3.03–2.79 (2H, m, CH_2), 2.67–2.64 (2H, m, CH_2), 1.89–1.84 (4H, m, CH_2, CH_2), 1.66–1.59 (3H, m, CH, CH_2), 1.43 (9H, s, Boc), 1.14–1.12 (2H, m, CH_2), 0.98 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 510.1 [M+H].

4.1.9. (*R*)-*tert*-Butyl 4-(2-(6-benzyl-4-butyryl-8-oxo-5,6,7,8-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-2(4*H*)-yl)ethyl)piperidine-1-carboxylate (30)

Following the general procedure for the synthesis of (**28**–**38**), acylation reaction of **9Ca** (30 mg, 0.07 mmol) using butyryl chloride (7 μL, 0.07 mmol) afforded **30** (28 mg, 76%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.34 (1H, s, NH), 7.36–7.28 (5H, m, phenyl), 7.20 (1H, s, CH), 4.23–4.19 (2H, m, CH_2), 4.08–4.05 (2H, m, CH_2), 3.83–3.63 (2H, m, CH_2), 3.03–2.79 (2H, m, CH_2), 2.67–2.64 (2H, m, CH_2), 1.89–1.84 (4H, m, CH_2, CH_2), 1.68–1.58 (5H, m, CH, CH_2, CH_2), 1.43 (9H, s, Boc), 1.14–1.12 (2H, m, CH_2), 0.95 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 524.1 [M+H].

4.1.10. (*R*)-*tert*-Butyl 4-(2-(6-benzyl-4-hexanoyl-8-oxo-5,6,7,8-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-2(4*H*)-yl)ethyl)piperidine-1-carboxylate (32)

Following the general procedure for the synthesis of (**28**–**38**), acylation reaction of **9Ca** (30 mg, 0.07 mmol) using hexanoyl chloride (9 μL, 0.07 mmol) afforded **32** (15 mg, 41%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.34 (1H, s, NH), 7.36–7.28 (5H, m, phenyl), 7.20 (1H, s, CH), 4.23–4.19 (2H, m, CH_2), 4.08–4.05 (2H, m, CH_2), 3.83–3.63 (2H, m, CH_2), 3.03–2.79 (2H, m, CH_2), 2.67–2.64 (2H, m, CH_2), 1.89–1.84 (4H, m, CH_2, CH_2), 1.68–1.58 (5H, m, CH, CH_2, CH_2), 1.43 (9H, s, Boc), 1.21–1.18 (2H, m, CH_2), 1.14–1.10 (4H, m, CH_2, CH_2), 0.95 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 552.5 [M+H].

4.1.11. (*R*)-*tert*-Butyl 4-(2-(6-benzyl-4-heptanoyl-8-oxo-5,6,7,8-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-2(4*H*)-yl)ethyl)piperidine-1-carboxylate (33)

Following the general procedure for the synthesis of (**28**–**38**), acylation reaction of **9Ca** (30 mg, 0.07 mmol) using heptanoyl chloride (10 μL, 0.07 mmol) afforded **33** (28 mg, 76%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.34 (1H, s, NH), 7.36–7.28 (5H, m, phenyl), 7.20 (1H, s, CH), 4.23–4.19 (2H, m, CH_2), 4.08–4.05 (2H, m, CH_2), 3.83–3.63 (2H, m, CH_2), 3.03–2.79 (2H, m, CH_2), 2.67–2.64 (2H, m, CH_2), 1.89–1.84 (4H, m, CH_2, CH_2), 1.68–1.58 (5H, m, CH, CH_2, CH_2), 1.43 (9H, s, Boc), 1.21–1.18 (2H, m, CH_2), 1.14–1.10 (6H, m, CH_2, CH_2), 0.95 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 566.1[M+H].

4.1.12. General procedure for the acylation to the N-1 of tetrahydro-1,4-pyrazolodiazepin-8-one (45–49)

To a solution of **43** in DCM was added the various carboxylic acid (1.2 equiv) and TEA (1.2 equiv) by dropwise for a period of 5 min. After this mixture was stirred for 0.5–1 h at room temperature, it was neutralized with saturated aqueous $NaHCO_3$ and extracted with DCM 2 times. The combined organic layer was dried with anhydrous Na_2SO_4. After Na_2SO_4 was filtered, the solvent was removed in vacuum. The residue was purified by silica gel column chromatography with chloroform/methanol = 20:1 to afford acyl compounds as solid.

4.1.13. (*R*)-6-Benzyl-2-(2-(1-(1-adamantanecarbonyl)piperidin-4-yl)ethyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (45)

Following the general procedure for the synthesis of (**45**–**49**), coupling reaction of **43** (10 mg, 0.02 mmol) and adamantane carboxylic acid (4 mg, 0.02 mmol) afforded **45** (73%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.37 (1H, s, NH), 7.38–7.26 (5H, m, phenyl), 7.22 (1H, s, CH), 4.60–4.48 (2H, m, CH_2), 4.28–4.22 (2H, m, CH_2), 4.15–3.92 (2H, m, CH_2), 3.82–3.81 (2H, m, CH_2), 3.12–2.91 (2H, m, CH_2), 2.79–2.78 (2H, m, CH_2), 2.75–2.72 (2H, m, CH_2), 2.33–2.27 (4H, dd, J = 7.2 Hz, J = 8.0 Hz, CH_2, CH_2), 2.14–1.03 (22H, m, CH, CH_2), 0.86 (3H, t, J = 7.6 Hz, CH_3), ^{13}C NMR ($CDCl_3$, 500 MHz) δ 173.56, 170.74, 163.37, 135.77, 134.14, 129.24 (2C), 129.20 (2C), 127.62, 124.18, 123.98, 54.51, 51.82, 51.04, 45.37, 43.58, 41.68, 93.05 (3C), 36.64 (3C), 33.60, 32.30 (2C), 29.68, 28.50 (3C), 27.91 (2C), 26.80, 22.17, MS (ESI): m/z = 600.2 [M+H].

4.1.14. (*R*)-6-Benzyl-2-(2-(1-(1-adamantylacetyl)piperidin-4-yl)ethyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (46)

Following the general procedure for the synthesis of (**45**–**49**), coupling reaction of **43** (10 mg, 0.02 mmol) and 1-adamantane acetic acid (4 mg, 0.02 mmol) afforded **46** (7 mg, 49%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.37 (1H, s, NH), 7.38–7.26 (5H, m, phenyl), 7.22 (1H, s, CH), 4.60–4.48 (2H, m, CH_2), 4.28–4.22 (2H, m, CH_2), 4.15–3.92 (2H, m, CH_2), 3.82–3.81 (2H, m, CH_2), 3.12–2.91 (2H, m, CH_2), 2.79–2.78 (2H, m, CH_2), 2.75–2.72 (2H, m, CH_2), 2.33–2.27 (4H, dd, J = 7.2 Hz, J = 8.0 Hz, CH_2, CH_2), 2.16–1.02 (24H, m, CH, CH_2), 0.85 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 614.2 [M+H].

4.1.15. (*R*)-6-Benzyl-2-(2-(1-(2-(bicyclo[2.2.1]heptan-2-yl)acetyl)piperidin-4-yl)ethyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (47)

Following the general procedure for the synthesis of (**45**–**49**), coupling reaction of **43** (10 mg, 0.02 mmol) and 2-norbormane acetic acid (3 μL, 0.02 mmol) afforded **47** (7 mg, 61%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.37 (1H, s, NH), 7.38–7.26 (5H, m, phenyl), 7.22 (1H, s, CH), 4.60–4.48 (2H, m, CH_2), 4.28–4.22 (2H, m, CH_2), 4.15–3.92 (2H, m, CH_2), 3.82–3.81 (2H, m, CH_2), 3.12–2.91 (2H, m, CH_2), 2.79–2.78 (2H, m, CH_2), 2.75–2.72 (2H, m, CH_2), 2.33–2.27 (4H, dd, J = 7.2 Hz, J = 8.0 Hz, CH_2, CH_2), 2.14–1.03 (20H, m, CH, CH_2), 0.86 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 574.2 [M+H].

4.1.16. (*R*)-6-Benzyl-2-(2-(1-(3-noradamantanecarbonyl)piperidin-4-yl)ethyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (48)

Following the general procedure for the synthesis of (**45**–**49**), coupling reaction of **43** (10 mg, 0.02 mmol) and 3-noradamantane carboxylic acid (5 mg, 0.02 mmol) afforded **48** (8 mg, 67%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.34 (1H, s, NH), 7.35–7.19 (5H, m, phenyl), 7.27 (1H, s, CH), 4.63–4.43 (2H, m, CH_2), 4.24–4.20 (2H, m, CH_2), 4.12–4.08 (2H, m, CH_2), 3.83–3.82 (2H, m, CH_2), 3.65–3.58 (2H, m, CH_2), 2.80–2.78 (2H, m, CH_2), 2.75–2.72 (2H, m, CH_2), 2.33–2.27 (4H, dd, J = 7.2 Hz, J = 8.0 Hz, CH_2, CH_2), 2.18–1.06 (20H, m, CH, CH_2), 0.86 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 586.2 [M+H].

4.1.17. (*R*)-6-Benzyl-2-(2-(1-(3,5-dimethyl-1-adamantanecarbonyl)piperidin-4-yl)ethyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (49)

Following the general procedure for the synthesis of (**45**–**49**), coupling reaction of **43** (10 mg, 0.02 mmol) and 3,5-dimethyl-adamantane-1-carboxylic acid (5 mg, 0.02 mmol) afforded **49** (7 mg, 56%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.37 (1H, s, NH), 7.38–7.26 (5H, m, phenyl), 7.22 (1H, s, CH), 4.60–4.48 (2H, m, CH_2), 4.28–4.22 (2H, m, CH_2), 4.15–3.92 (2H, m, CH_2), 3.82–3.81 (2H, m, CH_2), 3.12–2.91 (2H, m, CH_2), 2.79–2.78 (2H, m, CH_2), 2.75–2.72 (2H, m, CH_2), 2.33–2.27 (4H, dd, J = 7.2 Hz, J = 8.0 Hz, CH_2, CH_2), 2.16–1.08 (20H, m, CH, CH_2), 1.01 (6H, s, CH_3, CH_3), 0.85 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 628.1 [M+H].

4.1.18. (R)-6-Benzyl-2-(2-(1-(3,3-dimethylbutanoyl)piperidin-4-yl)ethyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (50)

The secondary amine **43** (10 mg, 0.02 mmol) was dissolved in DCM (1 mL). 3,3dimethyl-butyryl chloride (2 μL, 0.02 mmol) and

TEA was added at 0 °C. The reaction mixture was stirred for 1 h at room temperature. After reaction was done, the solution was diluted with NH_4Cl saturated water and extracted with DCM. The combined organic layer was dried with anhydrous Na_2SO_4. After Na_2SO_4 was filtered, the solvent was removed in vacuum. The residue was purified by silica gel column chromatography with chloroform/methanol = 30:1 to afford **50** (9 mg, 73%). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.32 (1H, s, NH), 7.37–7.28 (5H, m, phenyl), 7.19 (1H, s, CH), 4.24–4.19 (2H, m, CH_2), 4.08–4.04 (2H, m, CH_2), 3.83–3.64 (2H, m, CH_2), 3.02–2.79 (2H, m, CH_2), 2.67–2.63 (2H, m, CH_2), 2.17–2.04 (2H, m, CH_2), 1.88–1.82 (4H, m, CH_2, CH_2), 1.67–1.51 (7H, m, CH, CH_2, CH_2, CH_2), 1.15–1.12 (2H, m, CH_2), 0.97 (9H, s, CH_3, CH_3, CH_3), 0.91 (3H, t, J = 7.6 Hz, CH_3), ^{13}C NMR ($CDCl_3$, 500 MHz) δ 170.22, 163.39, 157.19, 135.84, 130.84, 129.24 (2C), 239.20 (2C), 128.76, 127.59, 124.14, 68.11, 54.47, 51.80, 51.03, 46.69, 44.71, 41.44, 33.55 (2C), 32.43, 31.68, 31.41, 30.04 (3C), 26.79, 22.15 (2C), 13.83, MS (ESI): m/z = 536.63 [M+H].

4.1.19. General procedure for the acylation to the N-1 of tetrahydro-1,4-pyrazolodiazepin-8-one (51–57)

To the compound **43** or **44** (1.0 equiv) in DCM was added various carboxylic acid (1.2 equiv) and TEA (1.2 equiv) by dropwise for a period of 5 min. After this mixture was stirred for 0.5–1 h at room temperature, it was neutralized with saturated aqueous $NaHCO_3$ and extracted with DCM 2 times. The combined organic layer was dried with anhydrous Na_2SO_4. After Na_2SO_4 was filtered, solvent was removed in vacuum. The residue was purified by silica gel column chromatography with chloroform/methanol = 20:1 to afford acyl compounds as solid.

4.1.20. (*R*)-6-Benzyl-2-(2-(1-(2-(1-methyl-1*H*-indol-3-yl)acetyl)piperidin-4-yl)ethyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (51)

Following the general procedure for the synthesis of (**51**–**57**), coupling reaction of **43** (10 mg, 0.02 mmol) and 1-methyl-3-indole acetic acid (5 mg, 0.02 mmol) afforded **51** (7 mg, 59%).^1H NMR ($CDCl_3$, 400 MHz) δ 8.32 (1H, s, NH), 7.51–7.49 (1H, m, indole), 7.36–7.25 (7H, m, phenyl, indole), 7.12–7.09 (1H, m, indole), 6.94 (1H, s, CH), 6.20 (1H, s, CH), 4.25–4.20 (2H, m, CH_2), 4.18–4.07 (2H, m, CH_2), 3.75 (2H, s, CH_2), 3.71 (3H, s, CH_3), 3.49–3.47 (2H, m, CH_2), 2.55–2.48 (2H, m, CH_2), 2.23–2.18 (2H, m, CH_2), 1.83–1.80 (2H,m, CH_2), 1.80–1.78 (2H, m, CH_2), 1.60–1.58 (2H, m, CH_2), 1.46–1.44 (2H, m, CH_2), 1.27–1.25 (2H, m, CH_2), 1.18–1.15 (2H, m, CH_2), 0.85 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 609.2 [M+H].

4.1.21. (*R*)-6-Benzyl-2-(2-(1-(1-methyl-1*H*-indole-3-carbonyl)piperidin-4-yl)ethyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (52)

Following the general procedure for the synthesis of (**51**–**57**), coupling reaction of **43** (10 mg, 0.02 mmol) and 1-methyl-indole-3-carboxylic acid (4 mg, 0.02 mmol) afforded **52** (5 mg, 38%). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.36 (1H, s, NH), 7.89 (1H, s, CH), 7.51–7.49 (1H, m, indole), 7.36–7.25 (7H, m, phenyl, indole), 7.12–7.09 (1H, m, indole), 6.94 (1H, s, CH), 4.25–4.21 (2H, m, CH_2), 4.18–4.07 (2H, m, CH_2), 3.71 (3H, s, CH_3), 3.49–3.46 (2H, m, CH_2), 2.55–2.48 (2H, m, CH_2), 2.23–2.18 (2H, m, CH_2), 1.83–1.80 (2H,m, CH_2), 1.80–1.78 (2H, m, CH_2), 1.60–1.58 (2H, m, CH_2), 1.46–1.44 (2H, m, CH_2), 1.27–1.25 (2H, m, CH_2), 1.18–1.15 (2H, m, CH_2), 0.85 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 596.1 [M+H].

4.1.22. (*R*)-2-(2-(1-(1*H*-Indole-2-carbonyl)piperidin-4-yl)ethyl)-6-benzyl-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (53)

Following the general procedure for the synthesis of (**51**–**57**), coupling reaction of **43** (10 mg, 0.02 mmol) and indole-2-carboxylic acid (4 mg, 0.02 mmol) afforded **53** (8 mg, 64%). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.36 (1H, s, NH), 7.56–7.51 (1H, m, indole), 7.38 (1H, s, CH), 7.40–7.25 (7H, m, phenyl, indole), 7.12–7.09 (1H, m, indole), 6.94 (1H, s, CH), 4.25–4.20 (2H, m, CH_2), 4.18–4.07 (2H, m, CH_2), 3.71 (3H, s, CH_3), 3.49–3.47 (2H, m, CH_2), 2.55–2.48 (2H, m, CH_2), 2.23– 2.18 (2H, m, CH_2), 1.83–1.80 (2H,m, CH_2), 1.80–1.78 (2H, m, CH_2), 1.60–1.58 (2H, m, CH_2), 1.46–1.44 (2H, m, CH_2), 1.27–1.25 (2H, m, CH_2), 1.18–1.15 (2H, m, CH_2), 0.85 (3H, t, J = 7.6 Hz, CH_3), MS (MALDI-TOF): m/z = 581.2 [M+H].

4.1.23. (*R*)-2-(2-(1-(1*H*-Indole-2-carbonyl)piperidin-4-yl)ethyl)-6-((1-methyl-1*H*-indol-3-yl)methyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (54)

Following the general procedure for the synthesis of (**51**–**57**), coupling reaction of **44** (10 mg, 0.02 mmol) and indole-2-carboxylic acid (3 mg, 0.02 mmol) afforded **53** (8 mg, 61%). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.37 (1H, s, NH), 7.69–7.72 (1H, m, indole), 7.54–7.48 (3H, m, indole), 7.34–7.23 (2H, m, indole), 7.21 (1H, s, CH), 7.18–7.12 (3H, m, indole), 6.98 (1H, s, CH), 6.20 (1H, s, CH), 4.32–4.28 (3H, m, CH, CH_2), 4.27–4.20 (2H, m, CH_2), 3.98–3.90 (2H, m, CH_2), 3.75 (3H, s, CH_3), 3.62–3.57 (2H, m, CH), 3.05–3.03 (2H, m, CH_2), 2.70 (2H, t, J = 9.2 Hz, CH_2), 2.02–2.01 (2H, m, CH_2), 1.86–1.85 (3H, m, CH, CH_2), 1.66–1.62 (2H, m, CH_2), 1.27–1.24 (2H, m, CH_2), 1.15–1.07 (2H, m, CH_2), 0.85 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 634.3 [M+H].

4.1.24. (*R*)-2-(2-(1-(2-(1*H*-Indol-3-yl)acetyl)piperidin-4-yl)ethyl)-6-((1-methyl-1*H*-indol-3-yl)methyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (55)

Following the general procedure for the synthesis of (**51**–**57**), coupling reaction of **44** (10 mg, 0.02 mmol) and indole-3-acetic acid (4 mg, 0.02 mmol) afforded **55** (7 mg, 58%). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.37 (1H, s, NH), 7.56–7.48 (3H, m, indole), 7.32–7.22 (2H, m, indole), 7.12–7.04 (4H, m, indole), 6.98 (1H, s, CH), 6.18 (1H, s, CH), 4.35–4.30 (3H, m, CH, CH_2), 4.28–4.20 (4H, m, CH_2, CH_2), 3.90–3.88 (2H, m, CH_2), 3.78 (2H, s, CH_2), 3.75 (3H, s, CH_3), 3.60–3.56 (2H, m, CH), 3.03–3.01 (2H, m, CH_2), 2.67–2.60 (2H, t, J = 9.2 Hz, CH_2), 2.02–2.01 (2H, m, CH_2), 1.86–1.84 (3H, m, CH, CH_2), 1.66–1.62 (2H, m, CH_2), 1.27–1.24 (2H, m, CH_2), 1.16–1.07 (2H, m, CH_2), 0.85 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 648.2 [M+H].

4.1.25. (*R*)-6-((1-Methyl-1*H*-indol-3-yl)methyl)-2-(2-(1-(1-methyl-1*H*-indole-3-carbonyl)piperidin-4-yl)ethyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (56)

Following the general procedure for the synthesis of (**51**–**57**), coupling reaction of **44** (10 mg, 0.02 mmol) and 1-methyl indole-3-carboxylic acid (4 mg, 0.02 mmol) afforded **56** (8 mg, 62%). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.32 (1H, s, NH), 7.87 (1H, s, CH), 7.32–7.28 (3H, m, indole), 7.21–7.18 (3H, m, indole), 6.98 (1H, s, CH), 6.14 (1H, s, indole), 4.45–4.40 (3H, m, CH, CH_2), 4.28–4.20 (2H, m, CH_2), 3.92–3.90 (2H, m, CH_2), 3.75 (6H, s, CH_3), 3.61–3.56 (2H, m, CH_2), 3.05–3.03 (2H, m, CH_2), 2.72–2.67 (2H, t, J = 9.2 Hz, CH_2), 2.02–2.01 (2H, m, CH_2), 1.91–1.86 (3H, m, CH, CH_2), 1.66–1.62 (2H, m, CH_2), 1.27–1.24 (2H, m, CH_2), 1.16–1.07 (2H, m, CH_2), 0.85 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 648.1 [M+H].

4.1.26. (*R*)-2-(2-(1-(2-(1-Methyl-1*H*-indol-3-yl)acetyl)piperidin-4-yl)ethyl)-6-((1-methyl-1*H*-indol-3-yl)methyl)-4-pentanoyl-4,5,6,7-tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one (57)

Following the general procedure for the synthesis of (**51**–**57**), coupling reaction of **44** (10 mg, 0.02 mmol) and 1-methyl indole-3-acetic acid (4 mg, 0.02 mmol) afforded **57** (7 mg, 54%). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.32 (1H, s, NH), 7.54–7.49 (4H, m, indole), 7.34–7.32 (2H, m, indole), 7.13–7.11 (2H, m, indole), 6.99 (1H, s,

CH), 6.13 (2H, s, indole), 4.45–4.40 (3H, m, CH, CH_2), 4.28–4.20 (2H, m, CH_2), 3.92–3.90 (2H, m, CH_2), 3.77 (2H, s, CH_2), 3.75 (6H, s, CH_3), 3.61–3.56 (4H, m, CH_2, CH_2), 3.05–3.03 (2H, m, CH_2), 2.72–2.67 (2H, t, J = 9.2 Hz, CH_2), 2.02–2.01 (2H, m, CH_2), 1.91–1.86 (3H, m, CH, CH_2), 1.66–1.62 (2H, m, CH_2), 1.27–1.24 (2H, m, CH_2), 1.16–1.07 (2H, m, CH_2), 0.85 (3H, t, J = 7.6 Hz, CH_3), MS (ESI): m/z = 662.2 [M+H].

4.2. Biological methods

4.2.1. CHO-K1 cell culture and aequorin/coelenterazine luminescence Assay at *Drosophila* sex peptide receptors

CHO-K1 cells were incubated in DMEM/F-12 (Welgene, Korea) supplemented with 10% fetal bovine serum and 1% Penicillin/streptomycin (Gibco). Cells were suspended as a 1×10^6 cells/mL. After 24 h, CHO-K1 cells were transfected with 3 pcDNA3.1(+) vectors which contain *Drosophila melanogaster* sex peptide receptor (FlyBase: CG16752), $G\alpha_{qo}$ chimeric G-protein, aequorin by Fugene6 (Promega) as previously described.[4] 24 h later, coelenterazine was treated, which makes luminescence with aequorin. Cell suspensions were added to 96 well plates containing the *Drm*SPR agonists. Luminescent signals were measured for 20 s after treatment of cells with Centro XS^3 LB 960 Microplate Luminometer (Berthold technologies). The percentage of receptor activation by agonist was measured and normalized against 50 μM ATP activation. Data are presented as mean ± SEM ($n \geqslant 3$).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.02.035.

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