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# Synthesis of fluorescent molecular probes specific for the receptor of blepharismone, a mating-inducing pheromone of the ciliate *Blepharisma japonicum*

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**Abstract**—Blepharismone (gamone 2) is a mating-inducing pheromone of the ciliate *Blepharisma japonicum*. *N*-Pyrenylbutyryl-blepharismone and *N*-biphenylacetyl-blepharismone, which are fluorescent derivatives of blepharismone, were synthesized as molecular probes for the gamone 2 receptor. Further, we proved that they have inhibitory activities against the blepharismone-induced monotypic pairing of *B. japonicum*.
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## 1. Introduction

Sexual reproduction in protozoan ciliates occurs by conjugation via specific interactions between cells of complementary mating types.[1] The ancestral ciliate *Blepharisma japonicum* has two mating types I and II. Substances that play the role of signaling molecules in these extracellular interactions for conjugation are termed gamones.[2] Gamone 1 is a glycoprotein produced by mating type I cells of *B. japonicum*.[3] It is a key factor required to trigger such interactions, and its complete amino acid sequence was recently determined.[4] We showed that transcription of the *gamone 1* gene is strictly regulated by developmental and environmental factors.[5] On the other hand, blepharismone (**1**) that was isolated as gamone 2 from mating type II cells induces conjugation when present in a concentration of 1 ng/mL. The structure of **1** was determined to be that of a calcium salt of (*S*)-4-(2-formylamino-5-hydroxy-phenyl)-2-hydroxy-4-oxo-butyric acid, which is a small molecule presumably derived from tryptophan.[6] Recently, we reported the practical synthesis of both the enantiomers of **1** via the Stille cross-coupling reaction of [4-(*tert*-butyl-dimethyl-silanyloxy)-2-trimethylstannanyl-phenyl]-carbamic acid *tert*-butyl ester with an acid chloride derived from (*S*)- and (*R*)-malic acid; this was a key reaction (Scheme 1). Further, we showed that the mating-inducing activity of synthetic (*S*)-blepharismone was as effective as that of its natural counterpart, while the enantiomer (*R*)-blepharismone showed no mating-inducing activity.[7]

Miyake proposed that preconjugant interactions in *B. japonicum* occur via the following mechanisms. Mating type I cells autonomously secrete gamone 1 (step 1). Then, gamone 1 specifically acts on mating type II cells (step 2) to transform them so that they can undergo conjugation (step 3) and it simultaneously induces or enhances the production and excretion of gamone 2 by these cells (step 4). Next, gamone 2 specifically acts on mating type 1 cells (step 5) to transform them so that they can undergo conjugation (step 6); it also enhances the production and excretion of gamone 1 by these cells; the transformed cells form pairs following contact (step 7). Thus, cells of the two mating types stimulate each other through a positive feedback loop.[2] This indicates that the receptors for gamones are nonself recognized, which is against the self-recognition hypothesis proposed by Luporini and Miceli.[8] To elucidate the molecular mechanism of conjugation in *B. japonicum*, we designed and synthesized fluorescent molecular probes

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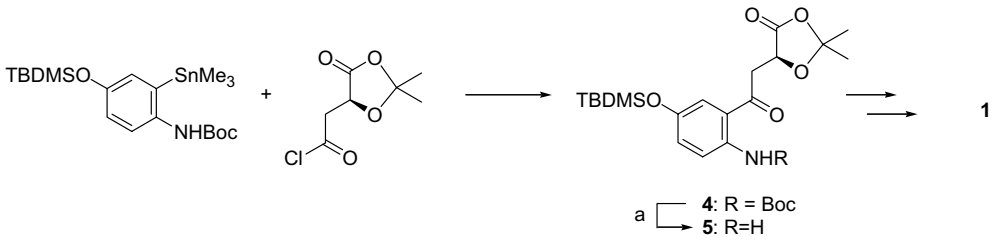


**Scheme 1.** Synthesis of blepharismone (**1**). Reagents and condition: (a) TFA, anisole, acetone, and rt.

specific for the gamone 2 receptor, which would be useful in studying the localization of this receptor. In this paper, we describe the syntheses of fluorescent probes **2** and **3**, and their inhibitory activities against the blepharismone-induced monotypic pairing of *B. japonicum* type I cells (see Fig. 1).

## 2. Results and discussion

### 2.1. Chemistry

Miyake reported that L-tryptophan and 5-hydroxy L-tryptophan competitively inhibit blepharismone; the L-isomers are stronger inhibitors than the D-isomers.[9] Entzeroth and Jaenicke also showed that *N*-formyl-5-hydroxy-L-kynurenine, *N*-formyl-L-kynurenine, and 5-hydroxy-L-kynurenine, which are similar to blepharismone in structure, interfere with the activity of gamone 2 and desensitize the receptor system.[10] None of these inhibitors had gamone 2 activities. These results suggest that gamone 2 acts on type I cells by binding reversibly to the receptor on type I cells.[1b] According to the structure–activity relationship studies on **1**, the 2-hydroxy group of the side chain appears to be essential, and the formylamide group appears to be more important than the 5-OH group on the benzene ring. The activity of 2-deformylamino-blepharismone is approximately 1/1200 that of blepharismone; whereas 2-acetamide-blepharismone has no activity. However at a concentration of 10 μM, 2-acetamide-blepharismone demonstrates inhibitory activity, that is, complete inhibition of blepharismone-induced (concentration = 50 nM) monotypic pairing.[10] Based on this information, we assumed that chemical modification of the 2-amino group afforded inhibition against gamone 2. Fluorescent probes are widely used to track the localization of a receptor.[11] Tryptophan and its congeners are fluorescent compounds, but relatively common substrates for a cell. Hence, we required fluorescent probes with higher specificity to unambiguously track the localization of the receptor for blepharismone. Thus, we designed fluorescent molecular probes **2** and **3** in which the formylamide group of blepharismone was modified by a fluorescent pyrenyl group or a biphenyl group.

Treatment of the synthetic intermediate **4** formed during the synthesis of blepharismone (**1**)[7] with trifluoroacetic

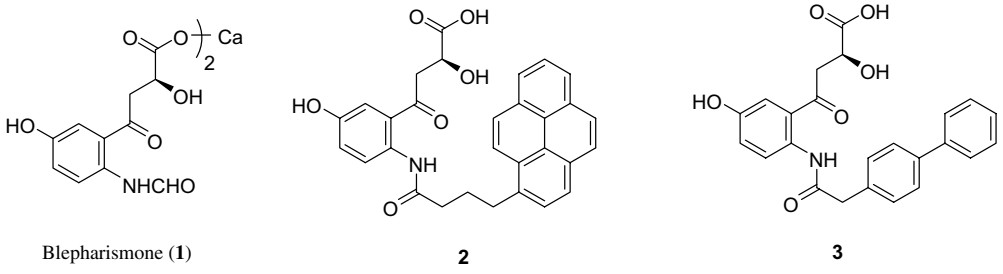


**Figure 1.** Blepharismone and fluorescent probes.

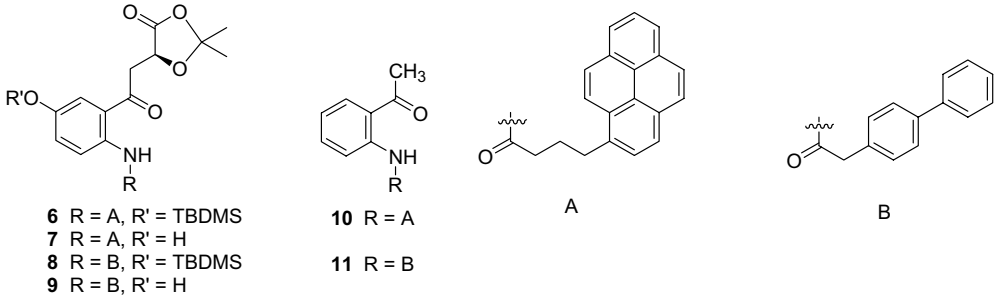


**Figure 2.** Intermediates for the synthesis of fluorescent probes of blepharismone and their control samples.

acid (TFA) in acetone in the presence of anisole[12] achieved selective deprotection of the Boc group to afford amine **5** in 67% yield. Condensation of **5** with 4-pyrene-1-yl-butyric acid by *N*,*N*′-dicyclohexyl-carbodiimide (DCC) was not successful. Consequently, we examined amide formation by using 2-aminoacetophenone, a model compound. The reactivity of the amino group is too low; this is presumably due to the reaction of the vinylogous aromatic amide group with the carboxylic acid. Therefore, we converted the carboxylic acid to its corresponding acid chloride[11a] to enhance reactivity and construct an amide bond.

Treatment of 4-pyren-1-yl-butyric acid or biphenyl-4-yl-acetic acid with oxalyl chloride in dichloromethane in the presence of a catalytic amount of DMF[13] afforded the corresponding acid chlorides. 2-Aminoacetophenone was treated with the acid chlorides in dichloromethane in the presence of pyridine[14] to afford **10** and **11** in 83% and 88% yields, respectively. Similarly, amides **6** and **8** were prepared from amine **5** and acid chlorides in 72% and 79% yields, respectively. Removal of the TBDMS group of intermediates **6** and **8** by HF·Py afforded intermediates **7** and **9** in 71% and 58% yields, respectively. Finally, the acetonide group was deprotected by TFA in THF:$H_2O$ = 4:1 to afford **2** and **3** in 72% and 88% yields, respectively (see Fig. 2).

### 2.2. Biological activity

The mating-inducing activity of compounds **2**, **3**, **10**, and **11**, and their inhibitory activity against blepharismone-induced monotypic pairing of *B. japonicum* were then evaluated. With regard to mating-inducing activity, these compounds were inactive at concentrations of up to 1.0 mM. Inhibitory activities against gamone 2 were examined in the presence of 74 nM of synthetic blepharismone, which was 40-fold (40 U/mL activity) the minimum concentration that induced one pairing between cells. Fluorescent derivatives **2** and **3** of blepharismone effectively induced complete inhibition of blepharismone-induced monotypic pairing of *B. japonicum* at concentrations of 4.0 and 4.8 μM, respectively, while controls **10** and **11** showed no inhibitory activities, as expected. This study did not provide a direct comparison of the inhibitory activities of **2** and **3** with those of tryptophan, 5-hydroxy-tryptophan, and others; however, the result reported by Jaenicke[10c] revealed that the inhibitory activities of **2** and **3** were effective; their inhibitory activities were more than double that of 2-acetamide-blepharismone, which is thus far the most effective known inhibitor against blepharismone-induced monotypic pairing (see Table 1).[1b]

**Table 1.** Inhibitory activities of fluorescent probes **2**, **3**, **10**, and **11** against the blepharismone-induced monotypic pairing of *B. japonicum*

| Compound | Inhibitory activity[a] (μM) |
|---|---|
| **2** | 4.0 |
| **3** | 4.8 |
| **10** (control) | $>1 \times 10^3$ |
| **11** (control) | $>1 \times 10^3$ |

[a] Inhibitory activity indicates the inhibitory concentration at which mating-inducing activity was completely inhibited in the presence of 74 nM of blepharismone.

### 2.3. Fluorescence spectra of probes 2 and 3

Blepharismone (**1**) shows intrinsic relatively weak fluorescence emission at $\lambda_{max}$ 484 nm. Emission from the chromophore of the pyrenyl or biphenyl group is at $\lambda_{max}$ 400 and 310 nm, respectively, and when these chromophores attach to **1**, the intensity of the fluorescence emission of pyrenyl-blepharismone **2** and biphenyl-blepharismone **3** at $\lambda_{max}$ 484 nm, which is attributable to blepharismone chromophore, is increased by a factor of 10 or 6, respectively. This increase in fluorescence intensity at $\lambda_{max}$ 484 nm may be explained by FRET (fluorescence resonance energy transfer)[15] of excited pyrenyl or biphenyl groups to the blepharismone chromophore.

## 3. Conclusion

Fluorescent molecular probes **2** and **3** were successfully synthesized from key intermediate **4** for the synthesis of (*S*)-blepharismone, a mating-inducing pheromone of ciliate *B. japonicum* type II cells. These probes showed no gamone activities; instead, they demonstrated marked inhibitory activities against the blepharismone-induced monotypic pairing of *B. japonicum* type I cells. These probes may be useful for studying and tracking the receptor sites of gamone 2 at the cellular level. Further experiments are currently being performed.

## 4. Experimental

### 4.1. General methods

$^1H$ and $^{13}C$ NMR spectra were recorded at 400 and 100 MHz (JEOL JNM LA400) or 300 and 75 MHz (JEOL JNM LA300), respectively. Chemical shifts relative to $Me_4Si$, $CDCl_3$ with $CHCl_3$ and DMSO-d6 with DMSO-d5 as the internal reference were reported in ppm (7.26 ppm for $^1H$ NMR and 77.0 ppm for $^{13}C$ NMR). Coupling constants were reported in hertz (Hz) and determined directly from the $^1H$ NMR spectra. Spectral splitting patterns were designated as s (singlet), d (doublet), t (triplet), m (multiplet), or br (broad). Mass spectra were obtained using JEOL JMS-700T or JEOL JMS-AX500 spectrometer. Infrared spectra were obtained using a Jasco A-100 spectrometer or a Hitachi 270-30 spectrophotometer. Fluorescence spectra were measured using a Hitachi F-4500 fluorophotometer. Elemental analyses were carried out at the Analytical Center of Osaka City University.

All air- and moisture-sensitive reactions were performed in a flame-dried, argon-flushed, two-necked flask sealed with a rubber septum. Dry solvents and reagents were introduced with a syringe. THF was freshly distilled from sodium benzophenone ketyl. $CH_2Cl_2$ was distilled from $P_2O_5$ and stored over 4-Å molecular sieves. Pyridine was dried over KOH and stored over 4-Å molecular sieves.

TLC was performed on Merck precoated silica gel (#5715) or RP-18 plates (#15685), and the TLC spots were visualized under 254 nm UV light and/or by charring after dropping the plate into vanillin solution in 5% sulfuric acid/methanol. The products were purified via Merck silica gel (#7734 and #9385) flash column chromatography or ODS (Wakogel® 50C18) column chromatography to obtain compounds **2** and **3**. Hexane and ethyl acetate were distilled and used for column chromatography.

### 4.2. (*S*)-{2-[2-Amino-5-(*tert*-butyl-dimethyl-silanyloxy)-phenyl]-2-oxo-ethyl}-2,2-dimethyl-[1,3]dioxolan-4-one (5)

To a solution of (*S*)-{4-(*tert*-butyl-dimethyl-silanyloxy)-2-[2-(2,2-dimethyl-5-oxo-[1,3]dioxolan-4-yl)-acetyl]-phenyl}-carbamic acid *tert*-butyl ester (**4**)[7] (1.82 g, 3.79 mmol) in acetone (31 mL) were added anisole (5.5 mL, 48.5 mmol) and TFA (18.1 mL, 235 mmol), and the mixture was stirred for 9 h at room temperature. To the reaction mixture was added aq $NaHCO_3$ (excess) and the mixture was extracted with EtOAc. The combined organic layers were washed with water and brine, dried over anhydrous $Na_2SO_4$, and solvent was removed in vacuo. The residue was purified by flash column chromatography on silica gel (EtOAc/hexane 1:5 v/v) to give **5** (970 mg) in 67% yield. Oil; $R_f = 0.30$ (EtOAc/hexane 1:5 v/v); IR (neat) $\nu_{max}$ 3470, 3345, 1770, 1735, 1640, 1370, 1170, 1145, 1110 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 400 MHz) $\delta$ 0.17 (s, 6H), 0.98 (s, 9H), 1.61 (s, 3H), 1.65 (s, 3H), 3.33 (dd, 1H, $J = 17.7$, 7.2 Hz), 3.55 (dd, 1H, $J = 17.7$, 2.8 Hz), 4.92 (dd, 1H, $J = 7.2$, 2.8 Hz), 5.98 (br s, 2H), 6.58 (d, 1H, $J = 8.8$ Hz), 6.88 (dd, 1H, $J = 8.8$, 2.9 Hz), 7.08 (d, 1H, $J = 2.9$ Hz); $^{13}C$ NMR ($CDCl_3$, 100 MHz) $\delta$ −4.5 (2C), 18.1, 25.7 (3C), 25.9, 26.9, 40.6, 70.2, 111.1, 117.1, 118.5, 119.9, 128.6, 145.3, 145.6, 173.4, 195.6; HREIMS *m*/*z* 379.1833 (calcd for $C_{19}H_{29}NO_5Si$, 379.1815 $[M]^+$).

### 4.3. General procedure for the synthesis of fluorescent probes

To a stirred solution of 4-pyren-1-yl-butyric acid (167 mg, 0.58 mmol) in $CH_2Cl_2$ (1.0 mL) were added a drop of DMF and oxalyl chloride (60 μL, 0.64 mmol) at 0 °C. After being stirred for 45 min at room temperature, the reaction mixture was concentrated in vacuo to give 4-pyren-1-yl-butyryl chloride. The resulting acid chloride was dissolved in $CH_2Cl_2$ (1.0 mL) and added to a solution of **5** (183 mg, 0.48 mmol) and pyridine (90 μL) in $CH_2Cl_2$ (1.4 mL), and the mixture was stirred at room temperature for 7 h. To the reaction mixture was added $H_2O$ (2 mL), and the resulting mixture was extracted with EtOAc (2× 20 mL). The combined organic layers were washed with brine (5 mL), dried over anhydrous $Na_2SO_4$, and concentrated in vacuo. The residue was purified by flash chromatography (hexane/EtOAc 5:1 v/v) to afford **6** (225 mg, 72%) as a yellow solid. To a solution of **6** (225 mg, 0.35 mmol) in THF (1.75 mL) was added HF·Py (0.39 mL, 0.39 mmol) at 0 °C. Stirring was continued for 1 h at 0 °C and for an additional 1 h at room temperature. To the reaction mixture was added water (3 mL), and the mixture was extracted with EtOAc (2× 20 mL). The combined organic extracts were washed with brine (40 mL), dried over anhydrous $Na_2SO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography (hexane/EtOAc 1:1 v/v) to afford **7** (127 mg, 71%) as a yellow solid. To a stirred solution of **7** (60 mg, 0.11 mmol) in THF (0.44 mL) were added TFA (20 μL) and $H_2O$ (0.11 mL) at 0 °C. After being stirred for 16 h at 0 °C, the reaction mixture was concentrated in vacuo, and the residue was purified with ODS column chromatography ($CH_3OH/H_2O$ 1:1 v/v) to afford **2** (225 mg, 72%) as a yellow solid.

### 4.4. (*S*)-*N*-{4-(*tert*-Butyl-dimethyl-silanyloxy)-2-[2-(2,2-dimethyl-5-oxo-[1,3]dioxolan-4-yl)-acetyl]-phenyl}-4-pyren-1-yl-butyramide (6)

Yellow solid; $R_f = 0.35$ (EtOAc/hexane 1:3 v/v) mp 64–66 °C; IR (nujol) $\nu_{max}$ 3297, 1792, 1515 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 400 MHz) $\delta$ 0.21 (s, 6H), 0.99 (s, 9H), 1.55 (s, 6H), 2.28–2.35 (m, 2H), 2.55 (t, 2H, $J = 7.8$ Hz), 3.37 (dd, 1H, $J = 18.1$, 7.1 Hz), 3.43 (t, 2H, $J = 6.8$ Hz), 3.56 (dd, 1H, $J = 18.1$, 2.7 Hz), 4.80 (dd, 1H, $J = 7.1$, 2.7 Hz), 7.06 (dd, 1H, $J = 9.2$, 2.8 Hz), 7.22 (d, 2H, $J = 2.8$ Hz), 7.90 (d, 1H, $J = 7.8$ Hz), 7.98 (t, 1H, $J = 8.1$ Hz), 8.01 (AB q, 2H), 8.07–8.17 (m, 4H), 8.30 (d, 1H, $J = 9.3$ Hz), 8.63 (d, 1H, $J = 9.2$ Hz), 11.2 (s, 1H); $^{13}C$ NMR ($CDCl_3$, 100 MHz) $\delta$ −4.4 (2C), 25.7 (3C), 25.8, 26.8, 27.2, 30.8, 32.6, 37.7, 41.2, 69.7, 111.1, 120.9, 121.9, 122.4, 123.3, 124.6, 124.8 (2C), 125.0, 125.8, 126.6, 127.33, 127.38, 127.43, 127.49, 128.8, 139.9, 130.8, 131.3, 133.4, 135.2, 135.7, 150.3, 171.8, 172.9, 198.4; HRFABMS *m*/*z* 651.3013 (calcd for $C_{39}H_{45}NO_6Si$, 651.3016 $[M+H]^+$).

### 4.5. (*S*)-*N*-{2-[2-(2,2-Dimethyl-5-oxo-[1,3]dioxolan-4-yl)-acetyl]-4-hydroxy-phenyl}-4-pyren-1-yl-butyramide (7)

Yellow solid; $R_f = 0.25$ (EtOAc/hexane 1:1 v/v); mp 105–106 °C; IR (neat) $\nu_{max}$ 3296, 1789 $cm^{-1}$; FABMS *m*/*z* 536 ($M^+$+H); $^1H$ NMR ($CDCl_3$, 400 MHz) $\delta$ 1.48 (s, 6H), 2.28 (m, 2H), 2.52 (t, 2H, $J = 7.3$ Hz), 3.25 (dd, 1H, $J = 18.3$, 6.5 Hz), 3.41 (br t, 2H, $J = 7.8$ Hz), 3.43 (d, 1H, $J = 18.3$, 2.9 Hz), 4.66 (dd, 1H, $J = 6.5$, 2.9 Hz), 7.02 (dd, 1H, $J = 9.0$, 2.9 Hz), 7.13 (d, 1H, $J = 2.9$ Hz), 7.72 (br s, 1H), 7.81 (d, 1H, $J = 7.7$ Hz), 7.90 (t, 1H, $J = 7.7$ Hz), 7.93 (AB q, 2H), 7.99 (d, 1H, $J = 9.3$ Hz), 8.03 (d, 1H, $J = 7.7$ Hz), 8.07 (d, 2H, $J = 7.7$ Hz), 8.21 (d, 1H, $J = 9.3$ Hz), 8.40 (d, 1H, $J = 9.0$ Hz), 11.1 (s, 1H); $^{13}C$ NMR ($CDCl_3$, 100 MHz) $\delta$ 25.6, 26.6, 27.1, 32.6, 37.9, 40.9, 69.8, 111.4, 116.5, 122.2, 122.6, 122.8, 123.2, 124.7, 124.8 (2C), 124.9, 125.0, 125.7, 126.6, 127.2, 127.3, 127.4, 128.6, 129.8, 130.7, 131.3, 133.2, 135.4, 151.7, 172.4, 173.3, 198.4; HRFABMS *m*/*z* 536.2067 (calcd for $C_{33}H_{30}NO_6$, 536.2073 $[M+H]^+$).

### 4.6. (*S*)-2-Hydroxy-4-[5-hydroxy-2-(4-pyren-1-yl-butyrylamino)-phenyl]-4-oxo-butyric acid (2)

Yellow solid; $R_f$ (ODS) = 0.5 ($CH_3OH/H_2O$ 1:1 v/v); mp 148–149 °C; IR (nujol) $\nu_{max}$ 3000–3600 (br), 2953, 2924, 2854, 1461, 1376 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 400 MHz)

$\delta$ 2.10 (m, 2H), 2.46 (t, 2H, $J$ = 7.1 Hz), 3.19 (dd, 1H, $J$ = 16.3, 7.6 Hz), 3.22 (dd, 1H, $J$ = 16.3, 4.6 Hz), 3.36 (t, 2H, $J$ = 7.6 Hz), 3.57 (br s, 1H), 4.43 (dd, 1H, $J$ = 7.6, 4.6 Hz), 6.96 (dd, 1H, $J$ = 8.8, 2.7 Hz), 7.18 (d, 1H, $J$ = 2.7 Hz), 7.77 (d, 1H, $J$ = 8.8 Hz), 7.96 (d, 1H, $J$ = 7.7 Hz), 8.04 (t, 1H, $J$ = 7.7 Hz), 8.12 (AB q, 2H), 8.20–8.28 (m, 4H), 8.41 (d, 1H, $J$ = 9.3 Hz), 9.63 (br s, 1H), 10.4 (s, 1H); $^{13}$C NMR (DMSO-$d_6$, 100 MHz) $\delta$ 27.2, 32.1, 36.2, 44.5, 66.9, 115.6, 120.1, 123.5, 124.0, 124.1, 124.2, 124.8, 124.9, 125.0, 126.1, 126.5, 127.3, 127.4, 127.6, 128.2, 129.1, 129.32, 129.35, 130.4, 130.9, 136.4, 153.3, 170.9, 174.8, 200.8; HRFABMS *m/z* 496.1759 (calcd for $C_{30}H_{26}NO_6$, 496.1760 $[M+H]^+$).

### 4.7. (*S*)-2-Biphenyl-4-yl-*N*-{4-(*tert*-butyl-dimethyl-silanyloxy)-2-[2-(2,2-dimethyl-5-oxo-[1,3]dioxolan-4-yl)-acetyl]-phenyl}-acetamide (8)

Yellow solid; $R_f$ = 0.20 (EtOAc/hexane 1:3 v/v); mp 176–177 °C; IR (nujol) $\nu_{max}$ 2924, 2854, 1461, 1376 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 0.20 (s, 6H), 0.98 (s, 9H), 1.54 (s, 3H), 1.57 (s, 3H), 3.36 (dd, 1H, $J$ = 18.0, 6.8 Hz), 3.57 (dd, 1H, $J$ = 18.0, 3.1 Hz), 3.76 (s, 2H), 4.82 (dd, 1H, $J$ = 6.8, 3.1 Hz), 7.06 (dd, 1H, $J$ = 9.1, 2.8 Hz), 7.22 (d, 1H, $J$ = 2.8 Hz), 7.34 (br t, 1H, $J$ = 9.3 Hz), 7.43 (m, 4H), 7.59 (m, 4H), 8.64 (d, 1H, $J$ = 9.1 Hz), 11.0 (s, 1H); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ −4.4 (2C), 18.2, 25.6 (3C), 25.7, 26.7, 41.0, 45.3, 69.8, 111.1, 121.0, 122.5, 127.0, 127.2, 127.6, 128.7, 139.9 (2C), 133.4, 135.1, 140.1, 140.8, 150.5, 170.0, 172.8, 198.2; HRFABMS *m/z* 574.2637 (calcd for $C_{33}H_{40}NO_6Si$, 574.2625 $[M+H]^+$).

### 4.8. (*S*)-2-Biphenyl-4-yl-*N*-{2-[2-(2,2-dimethyl-5-oxo-[1,3]-dioxolan-4-yl)-acetyl]-4-hydroxy-phenyl}-acetamide (9)

Yellow solid; $R_f$ = 0.25 (EtOAc/hexane 1:1 v/v); mp 154–155 °C; IR (nujol) $\nu_{max}$ 1647, 1459, 1376 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 1.54 (s, 3H), 1.56 (s, 3H), 3.37 (dd, 1H, $J$ = 18.0, 7.3 Hz), 3.57 (dd, 1H, $J$ = 18.0, 3.1 Hz), 3.77 (s, 2H), 4.79 (dd, 1H, $J$ = 7.3, 3.1 Hz), 5.74 (s, 1H), 7.00 (dd, 1H, $J$ = 9.2, 2.9 Hz), 7.24 (d, 1H, $J$ = 2.9 Hz), 7.34 (br t, 1H, $J$ = 9.3 Hz), 7.43 (m, 4H), 7.59 (m, 4H), 8.57 (d, 1H, $J$ = 9.2 Hz), 11.1 (s, 1H); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$ 25.7, 26.7, 41.0, 45.3, 69.8, 111.4, 116.4, 122.7, 122.8, 127.1 (2C), 127.3, 127.6 (2C), 128.7 (2C), 130.0 (2C), 133.4, 135.1, 140.2, 140.5, 140.8, 150.8, 170.1, 172.8, 198.1; HRFABMS *m/z* 460.1756, (calcd for $C_{27}H_{26}NO_6$, 460.2412 $[M+H]^+$).

### 4.9. (*S*)-4-[2-(2-Biphenyl-4-yl-acetylamino)-5-hydroxy-phenyl]-2-hydroxy-4-oxo-butyric acid (3)

Yellow solid; $R_f$ (ODS) = 0.25 ($CH_3OH/H_2O$ 1:1 v/v); mp 184–185 °C; IR (nujol) $\nu_{max}$ 2924, 2854, 2340, 1462, 1376 cm$^{-1}$; $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 3.15 (dd, 1H, $J$ = 16.3, 7.6 Hz), 3.22 (dd, 1H, $J$ = 16.3, 4.9 Hz), 3.68 (s, 2H), 4.41 (dd, 1H, $J$ = 7.6, 4.9 Hz), 6.95 (dd, 1H, $J$ = 8.8, 2.9 Hz), 7.17 (d, 1H, $J$ = 2.9 Hz), 7.34 (br t, 1H, $J$ = 8.3 Hz), 7.39 (d, 2H, $J$ = 8.3 Hz), 7.45 (t, 2H, $J$ = 8.3 Hz), 7.62 (m, 4H), 7.81 (d, 1H, $J$ = 8.8 Hz), 9.43 (br s, 1H), 10.6 (s, 1H); $^{13}$C NMR (DMSO-$d_6$, 100 MHz) $\delta$ 43.6, 44.8, 67.2, 116.0, 120.6, 124.5, 126.9 (2C), 127.1 (2C), 127.7, 129.3 (2C), 129.4, 129.6, 130.2 (2C), 134.8, 139.0, 140.2, 153.7, 169.7, 175.2, 201.2; HRFABMS *m/z* 420.1444 (calcd for $C_{24}H_{22}NO_6$, 420.1447 $[M+H]^+$).

### 4.10. *N*-(2-Acetyl-phenyl)-4-pyren-2-yl-butyramide (10)

According to the general procedure, 2-aminoacetophenone (200 mg, 1.48 mmol) and 4-pyren-1-yl-butyryl chloride, which was prepared from 4-pyren-1-yl-butyric acid (640 mg, 2.22 mmol), were condensed. Purification by flash column chromatography (hexane/EtOAc 5:1 v/v) afforded **10** (501 mg, 83%) as a yellow solid; $R_f$ = 0.30 (EtOAc/Hexane 1:3 v/v); IR (film) $\nu_{max}$ 3489, 1654, 1605 cm$^{-1}$; $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 2.12 (m, 2H), 2.49 (s, 3H), 2.55 (t, 2H, $J$ = 7.1 Hz), 3.38 (t, 2H, $J$ = 8.1 Hz), 7.18 (t, 1H, $J$ = 8.0 Hz), 7.57 (t, 1H, $J$ = 8.0 Hz), 7.94 (d, 1H, $J$ = 8.0 Hz), 7.96 (d, 1H, $J$ = 7.7 Hz), 8.04 (t, 1H, $J$ = 7.7 Hz), 8.12 (AB q, 2H), 8.18–8.31 (m, 5H), 8.41 (d, 1H, $J$ = 8.0 Hz), 11.2 (s, 1H); $^{13}$C NMR (DMSO-$d_6$, 100 MHz,) $\delta$ 27.1, 28.7, 32.0, 36.8, 120.7, 122.9, 123.5, 124.1, 124.2 (2C), 124.6, 124.8, 124.9 (2), 126.1, 126.5, 127.2, 127.4, 127.5, 129.3, 130.4, 130.9, 131.3, 133.9, 136.2, 138.7, 171.3, 202.5; HRFABMS *m/z* 405.172 (calcd for $C_{28}H_{23}NO_2$, 405.1729 $[M]^+$).

### 4.11. *N*-(2-Acetyl-phenyl)-2-biphenyl-4-yl-acetamide (11)

According to the general procedure, 2-aminoacetophenone (280 mg, 2.07 mmol) and biphenyl-4-yl-acetyl chloride, which was prepared from biphenyl-4-yl-acetic acid (526 mg, 2.48 mmol), were condensed. Purification by flash column chromatography (hexane/EtOAc 5:1 v/v) afforded **11** (602 mg, 88%) as a yellow solid; $R_f$ = 0.30 (EtOAc/hexane 1:3 v/v) mp 128 °C; IR (neat) $\nu_{max}$ 2953, 1732, 1460, 1376 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 2.62 (s, 3H), 3.79 (s, 2H), 7.09 (ddd, 1H, $J$ = 8.1, 7.2, 1.2 Hz), 7.33 (tt, 1H $J$ = 7.1, 1.5 Hz), 7.44–7.49 (m, 4H), 7.53 (ddd, 1H, $J$ = 8.5, 7.2, 1.5 Hz), 7.58–7.61 (m, 4H), 7.86 (dd, 1H, $J$ = 8.1, 1.5 Hz), 8.76 (dd, 1H, $J$ = 8.5, 1.2 Hz), 11.8 (s, 1H); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 28.5, 45.6, 120.7, 121.8, 122.4, 127.1 (2C), 127.2, 127.6 (2C), 128.7 (2C), 129.8 (2C), 131.5, 133.5, 135.1, 140.2, 140.9, 141.0, 170.5, 202.5; Anal. $C_{22}H_{19}NO_2$: C, 80.22; H, 5.81; N, 4.25. Found: C, 80.19; H, 5.75; N, 4.24; HRFABMS *m/z* 330.1486 (calcd for $C_{22}H_{20}NO_2$, 330.1494 $[M+H]^+$).

### 4.12. Cells and cell culture for biological assay

*Blepharisma japonicum* strain R1072 (mating type I) was used to assay for mating-inducing and inhibitory activities. Cells were cultured on *Enterobacter aerogenes* in WGP (Wheat Grass Powder, Pines) medium,[4] concentrated by low-speed centrifugation, washed with physiological balanced solution SMB (synthetic medium for *Blepharisma*),[4] and suspended in SMB at a density of 1500–2000 cells/mL. Cultures were maintained at 25 °C.

### 4.13. Preparation of a solution of blepharismone and fluorescent compounds, and gamone activity assay

Synthetic blepharismone[7] was dissolved in SMB (20 μg/mL) and filtered. One percentage of DMSO in SMB solution did not have any influence in inducing monotypic pairing of *B. japonicum* type I cells. For the biological assay, fluorescent compounds were dissolved in DMSO and diluted using SMB.

Biological gamone activity is represented in units (U). A unit of activity was defined as the smallest amount of gamone activity that could induce at least one face-to-face pair in 750–1000 cells suspended in 1 mL SMB, and the activity was measured by the method described before.[2c,16]

### 4.14. Assay for inhibitory activities against blepharismone-induced monotypic pairing of *B. japonicum*

The mating-inducing activity of gamone 2 with fluorescent samples **2**, **3**, **10**, and **11** was represented by unit and index of pair formation (0–5). The index of pair formation was determined on the basis of the ratio of the tester cell forming hemolytic pairs. Each well contained 500 mL of fluorescent sample and 40 ng of synthetic blepharismone, and 500 mL of tester cells suspension (1500–2000 cell/mL) was added to each well. The ratio of pairs was judged after 3, 5, and 20 h. The index of pair formation (0–5) indicates no pair (0), a few pairs (1), approximately half the cells forming pairs (3), and most cells forming pairs (5). Index (2) and index (4) were between index (1) and (2), index (3) and (5), respectively. The minimum concentration of fluorescent compounds at index (0) is reported as inhibitory activity (μM).

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