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Structure–activity relationship of α hormones, the mating factors of phytopathogen *Phytophthora*

Shylaja D. Molli [a], Jianhua Qi [a,b], Arata Yajima [c], Keisuke Shikai [c], Tadashi Imaoka [c], Tomoo Nukada [c], Goro Yabuta [c], Makoto Ojika [a,*]

[a] *Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*
[b] *College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, PR China*
[c] *Faculty of Applied Bioscience, Tokyo University of Agriculture, Tokyo 156-8502, Japan*

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ABSTRACT

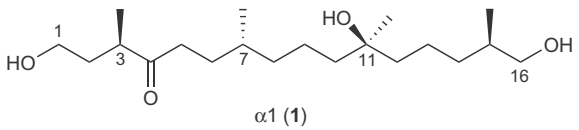
The mating hormones α1 and α2 induce sexual reproduction of the phytopathogenic genus *Phytophthora*. To demonstrate the structural elements responsible to hormonal activity, 17 derivatives of α1 and α2 were synthesized and their hormonal activity (oospore-inducing activity) was evaluated. The terminal ester derivatives of α1 (diacetate and dibenzoate) retained the hormonal activity, whereas a dicarbamate derivative completely suppressed the activity. Even monocarbamates showed weak activities; among them the 1-*O*-carbamate was less active than 16-*O*-carbamate, suggesting that the 1-OH group is a little more important than 16-OH. Dihydro, dehydro, and demethyl derivatives exhibited the minimum level of activity. Surviving activity of 15-*epi*-α1 suggested a less importance of this stereochemistry. Contrary to α1, not only the terminal diacetate derivative but also monoacetates of α2 exhibited no or little activity. Among the monoacetates, 1-*O*-acetyl-α2 exhibited little yet relatively better activity than the others. No activity was observed for mono- and dicarbamoyl derivatives of α2. Dihydro α2 with the saturated double bond lost most of the activity. These findings suggest that both the mating hormones α1 and α2 require most of the functional (hydroxyl, keto, and olefinic) groups they possess in their natural form for inducing the sexual reproduction of *Phytophthora*.

1. Introduction

The species of the plant-damaging genus *Phytophthora* are responsible not only for the enormous loss of agricultural crops world-wide but also for the environmental damage in natural ecosystems due to the use of numerous pesticides. One of the catastrophic moments was the Great Irish Famine in the mid 1840s caused by *Phytophthora infestans*.[1] The sudden oak death, widespread rots found in tomato, potatoes, tobacco and strawberries are the other foremost diseases caused by *Phytophthora*.[2,3]

Generally the heterothallic members of *Phytophthora* require both mating types (A1 and A2) for sexual reproduction.[4] Ashby, in 1929, proposed the occurrence of hormone-like compounds which regulates the sexual reproduction.[5] A hormone moves from one thallus to the other to stimulate the production of oogonia and antheridia; thus each thallus is bisexual.[6] The mating of these two organs results in the formation of sexual spores known as oospores.[7] These hormones were termed as α1 and α2. The α1 hormone secreted by A1 mating type strains induces oospores in the counter mating type A2 and α2 secreted by A2 induces sexual reproduction in the A1 mating type.[8,9] In 2005, Qi et al. isolated and characterized α1 (**1**) from the A1 mating type strain of *Phytophthora nicotianae*.[10] Yajima et al. described the total synthesis and absolute configuration of this hormone in 2008.[11] Recently, α2 (**2**) was also isolated and structurally elucidated.[12]

Mating hormones α1 (**1**) and α2 (**2**) are acyclic oxygenated diterpenes, with four or three stereogenic centers. The present study focuses on the relationship between the hormonal activity, that is, oospore-inducing activity, and chemical structure of the hormones. Nine derivatives of **1** and eight derivatives of **2** were synthesized and tested for their ability to induce sexual reproduction in A2 and A1 strains of *P. nicotianae*. This will help to determine the functional groups responsible for evoking the target biological effect on the organism, and may help to identify the specific receptors for these hormones.



α1 (**1**)

* Corresponding author. Tel./fax: +81 52 789 4284.
E-mail address: ojika@agr.nagoya-u.ac.jp (M. Ojika).

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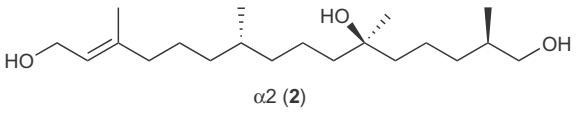


α2 (**2**)

2. Results and discussion

2.1. Preparation of α1 derivatives

Nine α1 derivatives **3**–**11** were prepared in the present study (Fig. 1). Diacetate **3** and dibenzoate **4** were prepared with acylation reagents under the usual conditions. Two monocarbamates, **5** and **6**, and dicarbamate **7** were obtained by the treatment with *p*-bromophenylisocyanate followed by chromatographic separation. Sodium borohydride reduction of the keto group of **1** afforded dihydro-α1 (**8**) as a diastereomeric mixture. Dehydrated derivative **9** was obtained from **3** by a series of reactions (1. $SOCl_2$, pyridine; 2. K_2CO_3, MeOH). The ^1H NMR spectrum indicates that **9** is a mixture of three alkenes in the ratio of 1:1:1. The total synthesis of 15-*epi*-α1 (**10**) was performed as summarized in Scheme 1 (Supplementary data for details). In addition, the total synthesis of 3-demethyl-α1 (**11**) was summarized in Scheme 2 (Supplementary data for details).

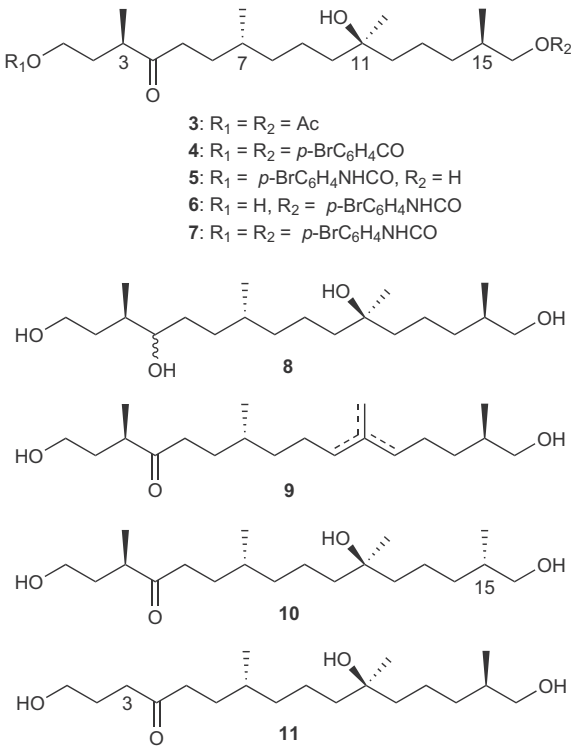


Figure 1. Structures of α1 derivatives.

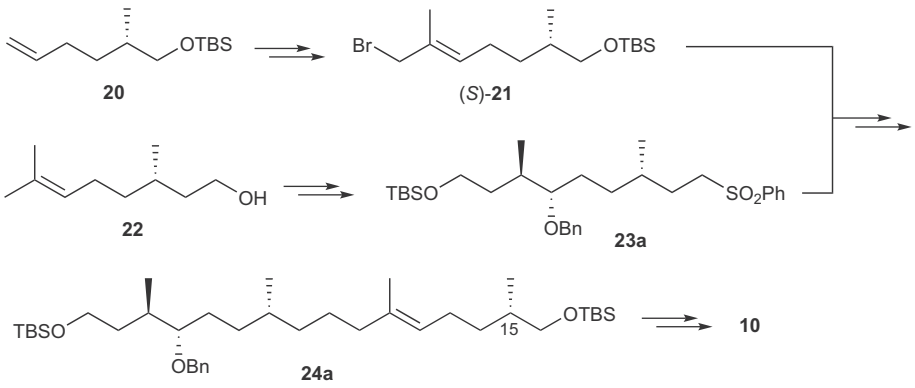
2.2. Preparation of α2 derivatives

Eight α2 derivatives **12**–**19** (Fig. 2) were also prepared to obtain the important structural elements for the hormonal activity against the A1 mating type of *P. nicotianae*. Two monoacetyl-α2s (**12** and **13**) and diacetyl-α2 (**14**) were prepared by partial acetylation followed by chromatographic separation. 11-*O*-acetyl-α2 (**15**) was prepared by the acetylation of disilyl-α2 (**26**)[13] (Scheme 3). Two monocarbamoyl-α2s (**16** and **17**) and dicarbamoyl-α2 (**18**) were also obtained in a similar manner to that for preparing three carbamoyl-α1s (**5**–**7**). Catalytic hydrogenation of α2 (**2**) on Rh-Al_2O_3 afforded dihydro-α2 (**19**).

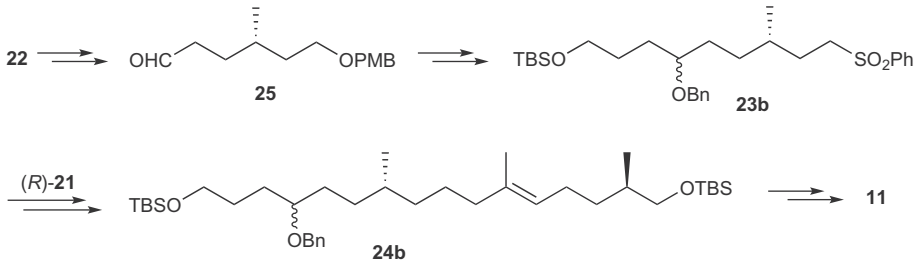
2.3. Structure–activity relationship

The structure–activity relationship of the α1 and α2 derivatives is discussed based on their oospores-inducing competence in A2 and A1 mating type strains of *P. nicotianae*, respectively. Natural α1 (**1**) or α2 (**2**), which induced oospores even at a dose of 3 ng per disk, was used as control in all the experiments. The activity was described in the relative values to control (100%). For instance, 10% activity defines that a 10-fold dosage of the derivative is required to show the same oospore-inducing activity of α1 (**1**) or α2 (**2**).

The relative hormonal activity of the α1 derivatives **3**–**11** is shown in Figure 3a. From the results, it is clear that the substitution of both terminal hydroxyls of **1** with acetyl (**3**) and benzoyl (**4**) groups retained the hormonal (oospore inducing) activity. Compared to all the others assayed, these derivatives exhibited relatively high activities of approximately 50% and 60% for **3** and **4**, respectively. However, no activity was observed when both the terminal hydroxyls of **1** were replaced with the carbamoyl groups (**7**). This result suggests that the ester groups of **3** and **4** were hydrolyzed to α1 (**1**) or to another active form in the bioassay medium. One possibility is the presence of non-specific esterase enzymes secreted by the organism during the bioassay. However, another possibility must be discussed because all acetates (**12**–**15**) of α2 were almost inactive as described later (Fig. 3b). This could be explained by the formation of an active derivative such as 16-*O*-acetyl(or benzoyl)-α1 possessing a free hydroxyl group at C-1 because, among monocarbamates (**5** and **6**), 16-*O*-carbamoyl-α1 (**6**) partially retained an activity of 13% (Fig. 3a). The 1-*O*-acyl group of **3** and **4** might be more susceptible to hydrolysis than the 16-*O*-acyl group due to the participation of



Scheme 1. Outline of the total synthesis of 15-*epi*-α1 (**10**).



Scheme 2. Outline of the total synthesis of 3-demethyl-α1 (**11**).

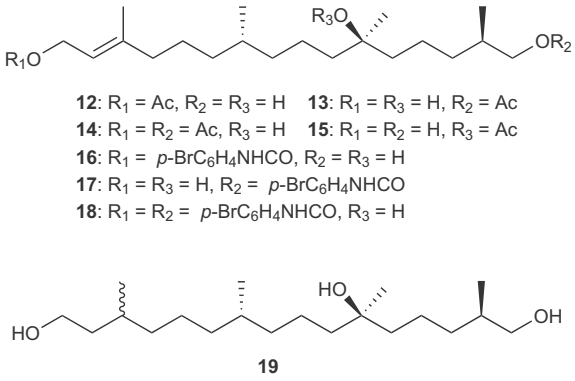
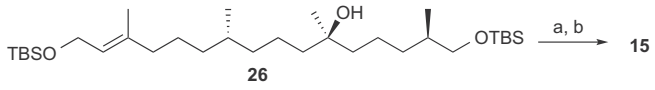


Figure 2. Structures of α2 derivatives.



Scheme 3. Synthesis of 11-*O*-acetyl-α2 (**15**). *Reagents and conditions:* (a) Ac_2O, DMAP, pyridine, rt; (b) TBAF, THF, rt, 7% in two steps.

the neighboring ketone group at C-4 as shown in Scheme 4. A lower activity of **6** possessing a free 1-OH might be due to its lower solubility than that of the hypothetical monoesters possessing a free 1-OH. This mechanism is plausible because (a) the bioassay condition is somewhat acidic (pH 6.0), and (b) α1 (**1**) is actually an equilibrium mixture of the major acyclic form and a trace cyclic hemiacetal as analyzed by ^{1}H NMR; the latter readily became dominant when treated with a weakly acidic condition (Supplementary figure). Therefore, we concluded that the hydroxyl group at C-1 position is more effectual than 16-OH. The derivatives **8**, **9**, and **11** exhibited the minimal level of activity. It is already known that the stereochemistry of 7*R*,11*R* is essential for the hormonal activity,[11] whereas the stereoisomer 15-*epi*-α1 (**10**), retained an activity of 85%, suggesting that the 15*R* configuration is not very important. The 3*R* stereochemistry is also not important because natural α1 (a mixture of 3*R*/*S* isomers) showed a comparable activity to synthetic α1 with the 3*R* stereochemistry.[11] Hence, we conclude that the principle features required for the oospores-inducing activity of α1 (**1**) are: (a) two hydroxyl groups at C-1 and C-11, (b) the ketone group at C-4, and (c) the methyl group at C-3, as well as the stereochemistry at C-7, C-11 positions.[11]

The relative oospore-inducing activity of the eight α2 derivatives **12**–**19** is shown in Figure 3b. Contrary to diacetyl-α1 (**3**), diacetyl-α2 (**14**) showed no activity. Even the monoacetates **12** and **13** were ineffective and induced only a few oospores (3% and 0.2%, respectively). No activity was observed for 11-*O*-acetyl-α2 (**15**), indicating 11-OH group is the most important among the three hydroxyl groups. Neither dicarbamate **18** nor mono carbamates

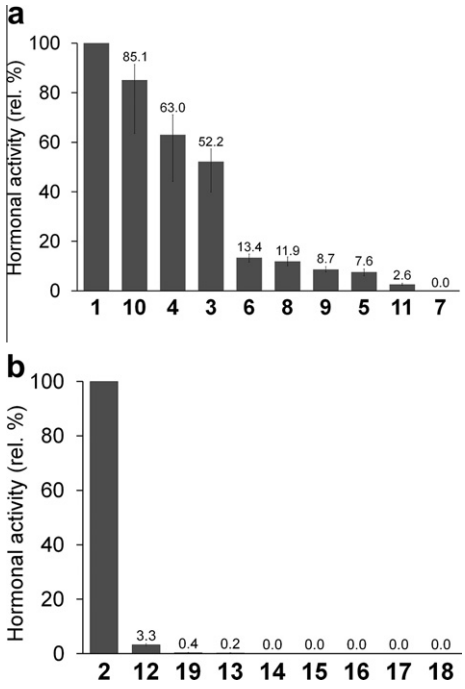
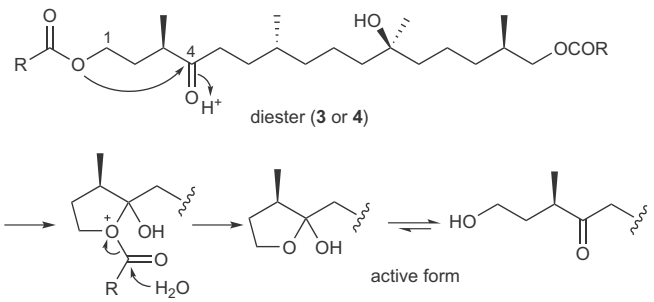


Figure 3. Relative hormonal activity of the derivatives of α hormones. (a) The relative activity of the α1 derivatives (**3**–**11**). The values are mentioned in percentage of reciprocal of the relative doses required for inducing 100 oospores. Relative activity = 100 × [dose of **1** (or **2**) required for inducing 100 oospores]/[dose of a derivative required for inducing 100 oospores]. (b) The relative activity of the α2 derivatives (**12**–**19**).



Scheme 4. A plausible mechanism of the conversion of diacyl-α1 to an active form during the bioassay. The left terminal structure of the product is the same as that of natural α1 (**1**). The equilibration between a hemiacetal and an acyclic structure is also observed in α1 (**1**) itself.

(**16** and **17**) produced any oospores. Very less activity (0.4%) was observed when the double bond at C-2 position (**19**) was saturated. These result indicated that (a) all the three hydroxyl groups of α2 (**2**) play a crucial role in the hormonal activity, and (b) the double bond at C-2 is also essential.

3. Conclusion

Our results about the structure-activity relationship of α hormones clearly demonstrate that all the functional groups of α hormones other than the 3-C and 15-C stereochemistry of α1 (**1**) play a crucial role in the hormonal activity and the modification of the original structures of these hormones did not show any advantage over the natural ones. These results suggest the presence of some specific receptors that recognize the exact chemical structure of α1 (**1**) or α2 (**2**) and are the gate to the signal transduction cascade leading to the *Phytophthora* sexual reproduction. In addition the above findings will provide valuable information for designing efficient molecular probes to identify the receptors.

4. Experimental procedure

4.1. *Phytophthora* strains and hormonal activity evaluation

The A1 (ATCC 38607) and A2 (ATCC 38606) mating type strains of *P. nicotianae* were purchased from the American Type Culture Collection (ATCC, YA, USA). The hormonal activity was evaluated by the method described by Qi et al.[10] and Ojika et al.[12]

4.2. Hormonal activity evaluation

To test α1 (**1**) activity, a loopful of *P. nicotianae* (ATCC 38606, A2 mating type) was inoculated onto a Petri plate (9 cm diameter) containing 20% V-8 juice (Campbell Soup Company, Camden, NJ), 0.3% $CaCO_3$, and 2% agar and pre-incubated at 25 °C, 60% humidity for 10 days in the dark. A piece (3 × 3 × 3 mm) of the colony was then incubated for 4 days on a Petri plate (9 cm) containing 10% V-8 juice, 0.02% $CaCO_3$, and 2% agar. A solution of a test sample in acetonitrile (30 μL) was applied to a paper disc (0.7-mm thick, 8 mm diameter; Advantec, Tokyo, Japan), which was then dried for 30 min in vacuo and placed on the colony at a distance of 1.5 cm from the colony center. After incubating for three additional days, the colony around the paper disc (1.8 cm diameter) was cut out, and the total number of oospores in the whole area (2.5 cm^2) was counted.

To evaluate α2 (**2**) activity, after the pre-incubation of *P. nicotianae* (ATCC 38607, A1 mating type) under the same conditions as those for the A2 strain, a piece of the colony was incubated for 8 days under the same conditions as those for the A1 strain, except for adjusting the medium to pH 8. A solution of a test sample in ethanol (5 μL) was applied to a mixed cellulose ester membrane filter (No. A020A013A, 13 mm diameter, 0.2 μm pore size, White; Advantec, Tokyo, Japan), which was then dried for 30 min and placed on the colony at a distance of 1.5 cm from the colony center. After incubation for 4 days, the colony around the membrane filter (2.1 cm diameter) was cut out, and the total number of oospores formed in the entire area was counted under a microscope. Almost all oospores were formed within the area cut (3.5 cm^2).

Three of four duplicate data for each dose (3, 10, 30, 100, and 300 ng/disk) were used for calculating the relative activity shown in Figure 3. The doses for inducing 100 oospores (as 'D_{100}') were obtained from dose-oospore graphs created by linear approximation method. Relative activity (%) was obtained by the following equation: $100 \times [D_{100}(\mathbf{1} \text{ or } \mathbf{2}) \pm \text{sd}]/[D_{100}(\text{derivative}) \pm \text{sd}]$. The error values (±sd) of the relative activity were calculated in the light of propagation of errors.

4.3. Chemical synthesis

4.3.1. Diacetyl-α1 (3)

Hormone α1 (**1**) (0.1 mg) was treated with acetic anhydride and pyridine at room temperature for 1.5 h. The reaction mixture was dried in vacuo, and the residue was chromatographed on silica gel with hexane-EtOAc (1:1) to afford **3** (0.05 mg): colorless oil, ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 4.05 (2H, m, H-1), 3.96 (1H, dd, *J* = 10.8, 6.0 Hz, H-16b), 3.86 (1H, dd, *J* = 10.8, 7.0 Hz, H-16a), 2.65 (1H, sex, *J* = 7.0 Hz, H-3), 2.51 (1H, m, H-5b), 2.43 (1H, m, H-5a), 2.06 (3H, s, Ac), 2.05 (1H, m, H-2b), 2.03 (3H, s, Ac), 1.80 (1H, m, H-15), 1.55–1.65 (2H, m, H-2a, 6b), 1.25–1.45 (12H, m), 1.10–1.18 (2H, m, H-14a, 8a), 1.16 (3H, s, H-18), 1.11 (3H, d, *J* = 6.6 Hz, H-20), 0.93 (3H, d, *J* = 7.0 Hz, H-17), 0.88 (3H, d, *J* = 6.6 Hz, H-19); HRMS (ESI): calcd for $C_{24}H_{44}O_6Na$ ([M+Na]$^+$) 451.3030, found 451.3018.

4.3.2. Bromobenzoyl-α1 (4)

Hormone α1 (**1**) (0.13 mg) was converted to **4** (0.24 mg) as described in ref 10. **4**: Colorless powder, ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.90 (2H, d, *J* = 8.4 Hz, Ar), 7.87 (2H, d, *J* = 8.4 Hz, Ar), 7.58 (4H, d, *J* = 8.4 Hz, Ar), 4.30 (2H, t, *J* = 6.6 Hz, H-1), 4.21 (1H, dd, *J* = 10.7, 6.6 Hz, H-16b), 4.11 (1H, dd, *J* = 10.7, 6.0 Hz, H-16a), 2.72 (1H, sept, *J* = 6.6 Hz, H-3), 2.49 (1H, m, H-5b), 2.43 (1H, m, H-5a), 2.19 (1H, m, H-2b), 1.95 (1H, m, H-15), 1.76 (1H, m, H-2a), 1.59 (1H, m, H-6b), 1.20–1.60 (13H, m,), 1.16 (3H, d, *J* = 6.6 Hz, H-20), 1.15 (3H, s, H-18), 1.10 (1H, m, H-8a), 1.02 (3H, d, *J* = 6.6 Hz, H-17), 0.83/0.84 (total 3H, d, *J* = 6.6 Hz, H-19); MS (ESI): *m/z* 731.2/733.2/735.2 (1:2:1) ([M+Na]$^+$); HRMS (ESI): calcd for $C_{34}H_{46}{}^{79}Br_2O_6Na$ ([M+Na]$^+$) 731.1553, found 731.1575.

4.3.3. 1-*O*-Carbamoyl-α1 (5), 16-*O*-carbamoyl-α1 (6), and dicarbamoyl-α1 (7)

To a solution of **1** (0.5 mg) in dry pyridine (0.1 mL) was added 4-bromophenyl isocyanate (6 mg). The mixture was stirred at room temperature for 14 h, and then quenched by stirring with a drop of water for 10 min. The mixture was dried and suspended in 70% MeOH. The soluble part was subjected to HPLC [Develosil ODS-UG-5 (10 mm id × 250 mm), 70–100% MeOH, 45 min linear gradient, 3 mL/min, detected at 244 nm] to give **5** (0.06 mg, t_R = 28.2 min) and **6** (0.07 mg, t_R = 30.0 min). **5**: UV (MeOH): λ_{max} = 244 nm (ε 20,000); ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.41 (2H, d, *J* = 8.4 Hz, Ar), 7.27 (2H, m, Ar), 6.76 and 6.81 (total 1H, br s, NH), 4.16 (2H, m, H-1), 3.51 (1H, dd, *J* = 10.6, 5.7 Hz, H-16b), 3.44 (1H, dd, *J* = 10.6, 6.6 Hz, H-16a), 2.69 (1H, sex, *J* = 7.0 Hz, H-3), 2.30–2.53 (2H, m, H-5), 2.09 (1H, m, H-2b), 1.68 (1H, m, H-2a), 1.65 (1H, m, H-15), 1.20–1.65 (15H, m), 1.15 (3H, s, H-18), 1.14 (3H, d, *J* = 7.0 Hz, H-20), 1.13 (2H, m, H-8a, 14a), 0.93 (3H, d, *J* = 6.7 Hz, H-17), 0.86/0.87 (total 3H, d, *J* = 6.2 Hz, H-19); MS (ESI): *m/z* 524.2/526.2 (1:1) ([M-OH]$^+$), 564.2/566.2 (1:1) ([M+Na]$^+$); HRMS (ESI): calcd for $C_{27}H_{44}{}^{79}BrNO_5Na$ ([M+Na]$^+$) 564.2295, found 564.2326.

6: UV (MeOH): λ_{max} = 244 nm (ε 20,000); ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.41 (2H, d, *J* = 8.4 Hz, Ar), 7.29 (2H, br d, *J* = 8.4 Hz, Ar), 6.79 (1H, br s, NH), 4.04 (1H, m, H-16b), 3.98 (1H, m, H-16a), 3.64 (2H, m, H-1), 2.76 (1H, m, H-3), 2.49 (2H, m, H-5), 1.93/2.01 (total 1H, m, H-2b), 1.84 (1H, m, H-15), 1.20–1.70 (16H, m), 1.20 (1H, m, H-14a), 1.16 (3H, s, H-18), 1.12 (3H, d, *J* = 6.6 Hz, H-20), 1.12 (1H, m, H-8a), 0.96 (3H, d, *J* = 6.6 Hz, H-17), 0.87/0.88 (total 3H, d, *J* = 6.6 Hz, H-19); MS (ESI): *m/z* 524.2/526.2 (1:1) ([M−OH]$^+$), 564.2/566.2 (1:1) ([M+Na]$^+$); HRMS (ESI): calcd for $C_{27}H_{44}{}^{79}BrNO_5Na$ ([M+Na]$^+$) 564.2295, found 564.2302.

Another sample of **1** (0.3 mg) was treated under the same conditions to give **7** (0.24 mg, t_R = 40.5 min): UV (MeOH): λ_{max} = 244 nm (ε 40, 000), ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.40 (4H, d, *J* = 8.6 Hz, Ar), 7.29 (2H, br d, *J* = 8.6 Hz, Ar), 7.27 (2H, br d, *J* = 8.6 Hz, Ar), 6.77 (1H, br s, NH), 6.70/6.73 (total 1H, br s, NH), 4.16 (2H, m, H-1), 4.02 (1H, dd, *J* = 10.4, 6.2 Hz, H-16b), 3.98 (1H, dd, *J* = 10.4, 6.5 Hz, H-16a), 2.69 (1H, sex, *J* = 7.0 Hz, H-3), 2.30–2.53 (2H, m, H-5), 2.09 (1H, m, H-2b), 1.84 (1H, m, H-15), 1.67 (1H, m, H-2a), 1.17–1.70 (15H, m), 1.16 (3H, s, H-18), 1.13 (3H, d, *J* = 7.0 Hz, H-20), 1.12 (1H, m, H-8a), 0.96 (3H, d, *J* = 6.6 Hz, H-17), 0.86/0.87 (3H, d, *J* = 6.2 Hz,

H-19); MS (ESI): *m/z* 721.2/723.2/725.2 (1:2:1) ([M-OH]$^+$), 761.2/763.2/765.2 (1:2:1) ([M+Na]$^+$); HRMS (ESI): calcd for $C_{34}H_{48}{}^{79}Br_2N_2O_6Na$ ([M+Na]$^+$) 761.1771, found 761.1794.

4.3.4. Dihydro-α1 (8)

To a solution of **1** (0.1 mg) in MeOH (0.1 mL) was added $NaBH_4$ (1 mg), and the mixture was stirred at room temperature for 14 h. The mixture was diluted with water and extracted with EtOAc. The combined extracts were washed with water and concentrated. The residue was chromatographed on silica gel with $CHCl_3$-MeOH (95:5, 9:1) to give **8** (0.05 mg): ^{1}H NMR (CD_3OD, 600 MHz): δ = 3.64 (1H, m, H-1b), 3.56 (1H, m, H-1a), 3.41 (1H, dd, *J* = 10.8, .5.4 Hz, H-16b), 3.33 (1H, dd, *J* = 10.8, 4.2 Hz, H-16a), 3.34/3.42 (total 1H, m, H-4), 1.69/1.73 (1H, m, H-2b), 1.65 (1H, m, H-3), 1.58 (1H, m, H-15), 1.27–1.55 (15H, m), 1.05–1.17 (2H, m, H-8a, 14a), 1.12 (3H, s, H-18), 0.91 (3H, d, *J* = 6.6 Hz, H-17), 0.90 (3H, m, H-19), 0.88/0.91 (total 3H, m, H-20); HRMS (ESI): calcd for $C_{20}H_{43}O_4$ ([M+H]$^+$) 347.3156, found 347.3141; calcd for $C_{20}H_{42}O_4Na$ ([M+Na]$^+$) 369.2975, found 369.2968.

4.3.5. Dehydrated α1 (9)

To a solution of diacetyl-α1 (**3**) (0.6 mg) in dry pyridine (0.3 mL) was added thionyl chloride (10 μL) at 0 °C, and the mixture was stirred for 30 min at 0 °C. The reaction mixture was diluted with saturated $NaHCO_3$ and extracted with hexane-EtOAc (1:1). The combined extracts were washed with 1 M HCl and then water. The organic layer was concentrated to give crude product (0.4 mg). To a solution of the crude product in MeOH (0.5 mg) was added K_2CO_3 (5 mg), and the mixture was stirred at room temperature for 2 h. The reaction was quenched by adding saturated NH_4Cl (1 mL), and the mixture was extracted with EtOAc. The combined extracts were washed with water and concentrated. The resulting product was purified by HPLC [Develosil ODS-UG-5 (4.6 × 250 mm), 30–70% (40 min) MeCN, 4 mL/min, detected at 205 nm] to give **9** (t_R = 37.1 min, 0.2 mg): ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 5.10 (0.66H, t, *J* = 6.3 Hz, H-10, 12), 4.70 (0.68H, s, H-18), 3.65 (2H, m, H-1), 3.51 (1H, m, H-16b), 3.44 (1H, m, H-16a), 2.75 (1H, sex, *J* = 6.8 Hz, H-3), 2.49 (2H, m, H-5), 1.94 (1H, m, H-2b), 1.92–2.08 (4H, m, H-9b, 10, 12, 13b), 1.63 (1H, m, H-15), 1.59 (1H, m, H-2a), 1.58 (2H, s, H-18), 1.25–1.65 (9.7H, m), 1.15 (1H, m, H-8), 1.13 (1H, m, H-14), 1.12 (3H, d, *J* = 7.0 Hz, H-20), 0.93 (3H, d, *J* = 6.6 Hz, H-17), 0.88 (3H, m, H-19); HRMS (ESI): calcd for $C_{20}H_{37}O_2$ ([M−OH]$^+$) 309.2788, found 309.2777; calcd for $C_{20}H_{38}O_3Na$ ([M+Na]$^+$) 349.2713, found 349.2689.

4.3.6. 15-*Epi*-α1 (10)

Total synthesis of this α1 isomer is described in Supplementary data. **10**: $[\alpha]_D^{23}$ = −12.4 (*c* 0.6, MeOH); IR (film): ν_{max} = 3374 (br), 2935, 1705, 1121, 1049, 939, 917, 737, 618 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): δ = 3.52 (2H, br. t, *J* = 6.6 Hz, H-1), 3.41 (1H, dd, *J* = 6.6, 10.7 Hz, H-16b), 3.33 (1H, m, H-16a), 2.76 (1H, sex, *J* = 6.8 Hz, H-3), 2.53 (2H, t, *J* = 7.1 Hz, H-5), 1.89 (1H, sex, *J* = 6.8 Hz, H-2b), 1.10–1.66 (15H, m), 1.12 (3H, s, H-18), 1.10 (2H, m, H-8a, 14a), 1.07 (3H, d, *J* = 6.8 Hz, H-20), 0.91 (3H, d, *J* = 6.8 Hz, H-17), 0.89 (3H, d, *J* = 6.4 Hz, H-19); ^{13}C NMR (100 MHz, CD_3OD): δ = 16.9 (C-20), 17.1 (C-17), 19.9 (C-19), 22.3 (C-9 or 13), 22.4 (C-13 or 9), 27.0 (C-18), 31.7 (C-6), 33.6 (C-7), 35.0 (C-14), 36.7 (C-2), 36.9 (C-15), 38.6 (C-8), 40.0 (C-5), 42.9 (C-10 or 12), 43.0 (C-12 or 10), 43.9 (C-3), 60.6 (C-1), 68.4 (C-16), 73.4 (C-11), 217.5 (C-4). These NMR spectra are superimposable on those of α1 (**1**). Anal. Calcd for $C_{20}H_{40}O_4$: C, 69.72; H, 11.70. Found: C, 69.92; H, 11.86.

4.3.7. 3-Demethyl α1 (11)

Total synthesis of this derivative is described in Supplementary data. **11**: Colorless oil; $[\alpha]_D^{25}$ = −2.0 (*c* 0.10, MeOH), IR (film): ν_{max} = 3386 (br), 1705, 1037, 699, 666 cm^{-1}; ^{1}H NMR (600 MHz, CD_3OD): δ = 3.52 (2H, t, *J* = 6.6 Hz, H-1), 3.41 (1H, dd, *J* = 10.6, 6.3 Hz, H-16b), 3.32 (1H, dd, *J* = 10.6, 6.6 Hz, H-16a), 2.54 (2H, t, *J* 8 6.6 Hz, H-5), 2.42–2.52 (2H, m, H-3), 1.75 (2H, quint, *J* = 6.6 Hz, H-2), 1.60 (1H, m, H-6b), 1.58 (1H, m, H-15), 1.28–1.46 (12H, m), 1.13 (1H, m, H-8a), 1.12 (3H, s, H-18), 1.08 (1H, m, H-14a), 0.91 (3H, d, *J* = 7.0 Hz, H-17), 0.88 (3H, d, *J* = 6.6 Hz, H-19); ^{13}C NMR (150 MHz, CD_3OD): δ = 17.1 (C-17), 19. (C-19), 22.3 (C-9 or 13), 22.4 (C-13 or 9), 26.9 (C-18), 27.7 (C-2), 31.9 (C-6), 33.6 (C-7), 35.1 (C-14), 36.9 (C-15), 38.6 (C-8), 39.8 (C-3), 41.3 (C-5), 43.0 (C-10 and 12), 62.2 (C-1), 68.5 (C-16), 73.4 (C-11), 213.9 (C-4); HRMS (ESI): calcd for $C_{19}H_{38}O_4Na$ ([M+Na]$^+$) 353.2662, found 353.2638.

4.3.8. 1-*O*-Acetyl-α2 (12), 16-*O*-acetyl-α2 (13), and 1,16-*O*,*O*-diacetyl-α2 (14)

Hormone α2 (**2**) (0.55 mg) was treated with acetic anhydride in pyridine at room temperature for 30 min. TLC analysis [hexane-EtOAc (1:1)] showed that the most of **2** was converted to diacetate **14** with small amounts of monoacetates. Another **2** (0.55 mg) was acetylated under the same conditions except for the reaction time of 5 min, producing additional monoacetates. The crude products from the two reactions were combined and subjected to preparative TLC [20 cm × 10 cm × 0.25 mm thickness, hexane-EtOAc (1:1)] to give **12** (0.3 mg, R_f = 0.3), **13** (0.2 mg, R_f = 0.42), and **14** (0.6 mg, R_f = 0.81). **12**: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 5.33 (1H, t, *J* = 7.0 Hz, H-2), 4.59 (2H, d, *J* = 7.0 Hz, H-1), 3.51 (1H, dd, *J* = 10.2, 6.0 Hz, H-16b), 3.44 (1H, dd, *J* = 10.2, 6.6 Hz, H-16a), 2.05 (3H, s, Ac), 2.00 (2H, m, H-4), 1.69 (3H, s, H-20), 1.65 (1H, m, H-15), 1.20–1.50 (16H, m), 1.07–1.20 (3H, m, H-6a, 8a, and 14a), 1.16 (3H, s, H-18), 0.93 (3H, d, *J* = 6.6 Hz, H-17), 0.87 (3H, d, *J* = 6.6 Hz, H-19)8; HRMS (ESI): calcd for $C_{22}H_{42}O_4Na$ ([M+Na]$^+$) 393.2975, found 393.2997.

13: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 5.41 (1H, t, *J* = 6.6 Hz, H-2), 4.15 (2H, d, *J* = 6.6 Hz, H-1), 3.96 (1H, dd, *J* = 10.5, 5.7 Hz, H-16b), 3.86 (1H, dd, *J* = 10.5, 7.0 Hz, H-16a), 2.05 (3H, s, Ac), 1.99 (2H, m, H-4), 1.80 (1H, m, H-15), 1.67 (3H, s, H-20), 1.20–1.50 (16H, m), 1.16 (3H, s, H-18), 1.05–1.20 (3H, m, H-6a, 8a, and 14a), 0.94 (3H, d, *J* = 6.6 Hz, H-17), 0.87 (3H, d, *J* = 6.6 Hz, H-19); HRMS (ESI): calcd for $C_{22}H_{42}O_4Na$ ([M+Na]$^+$) 393.2975, found 393.2995.

14: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 5.33 (1H, t, *J* = 7.0 Hz, H-2), 4.59 (2H, d, *J* = 7.0 Hz, H-1), 3.96 (1H, dd, *J* = 10.8, 6.0 Hz, H-16b), 3.86 (1H, dd, *J* = 10.8, 7.0 Hz, H-16a), 2.05 (6H, s, Ac), 2.01 (2H, m, H-4), 1.80 (1H, m, H-15), 1.69 (3H, s, H-20), 1.20–1.50 (15H, m), 1.16 (1H, m, H-14a), 1.16 (3H, s, H-18), 1.10 (2H, m, H-6a and 8a), 0.94 (3H, d, *J* = 6.6 Hz, H-17), 0.87 (3H, d, *J* = 6.0 Hz, H-19); HRMS (ESI): calcd for $C_{24}H_{44}O_5Na$ ([M+Na]$^+$) 435.3081, found 435.3079.

4.3.9. 11-*O*-Acetyl-α2 (15)

To a stirred solution of bis-TBS-α2 (**26**) (8 mg, 0.015 mmol) in pyridine (0.5 mL) were added DMAP (15 mg, 0.12 mmol) and acetic anhydride (10 μL, 0.11 mmol), and the mixture was stirred for 84 h at room temperature. The mixture was poured into water. The aqueous mixture was extracted with EtOAc, and the combined organic layers were washed with saturated aqueous $CuSO_4$ solution, water and brine. The organic layer was dried with Na_2SO_4, and concentrated. The residue was purified by silica gel column chromatography with EtOAc-hexane (1:100) to give crude acetate (4 mg). This was dissolved in THF (0.5 mL), and TBAF (1.0 M in THF 14 μL, 14 μmol) was added to the resulting solution at 0 °C. After stirred for 7 h at room temperature, the reaction was quenched with water. The aqueous mixture was extracted with EtOAc, and the combined organic layers were washed with brine, dried with Na_2SO_4, and concentrated. The residue was purified by silica gel column chromatography with EtOAc-hexane (3:2)

and then by silica gel TLC (20 cm × 10 cm × 0.25 mm thickness) with acetone-$CHCl_3$ (1:4) to give **15** (0.4 mg, 7% in two steps): colorless oil, ^{1}H NMR (600 MHz, $CDCl_3$): δ = 5.41 (1H, t, J = 7.0 Hz, H-2), 4.15 (2H, d, J = 7.0 Hz, H-1), 3.50 (1H, dd, J = 10.4, 5.8 Hz, H-16b), 3.42 (1H, dd, J = 10.5, 6.4 Hz, H-16a), 1.99 (2H, m, H-4), 1.97 (3H, s, Ac), 1.83 (1H, dt, J = 4.3, 12.5 Hz, H-12b), 1.81 (1H, dt, J = 4.1, 12.5 Hz, H-10b), 1.67 (3H, s, H-20), 1.67 (2H, m, H-10a, 12a), 1.63 (1H, m, H-15), 1.41 (1H, m, H-7), 1.39 (3H, s, H-18), 1.17–1.50 (11H, m), 1.05–1.15 (3H, m, H-6a, 8a, 14a), 0.92 (3H, d, J = 6.6 Hz, H-17), 0.85 (3H, d, J = 6.6 Hz, H-19); HRMS (ESI): calcd for $C_{22}H_{42}O_4Na$ ([M+Na]$^+$) 393.2975, found 393.2965.

4.3.10. 1-*O*-Carbamoyl-α2 (16), 16-*O*-carbamoyl-α2 (17), and dicarbamoyl-α2 (18)

To a solution of α2 (**2**) (0.86 mg) in dry pyridine (0.2 mL) was added *p*-bromophenyl isocyanate (4 mg), and the mixture was stirred at room temperature for 5 min. The reaction was quenched by adding a drop of water. The mixtures was dryed and suspended in a mixture of hexane-EtOAc (1:1). The insoluble material was removed by filtration, and the filtrate was concentrtaed and subjected to silica gel TLC (20 cm × 10 cm × 0.25 mm thickness) with hexane-EtOAc (1:1) to give **16** (0.3 mg, R_f = 0.31), **17** (0.1 mg, R_f = 0.38), and **18** (0.9 mg, R_f = 0.71). **16**: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.40 (2H, d, J = 8.0 Hz, Ar), 7.29 (2H, d, J = 8.0 Hz, Ar), 6.83 (1H, br s, NH), 5.37 (1H, t, J = 7.0 Hz, H-2), 4.68 (2H, d, J = 7.0 Hz, H-1), 3.51 (1H, dd, J = 10.4, 6.0 Hz, H-16b), 3.44 (1H, dd, J = 10.4, 6.0 Hz, H-16a), 2.04 (2H, t, J = 7.4 Hz, H-4), 1.72 (3H, s, H-20), 1.64 (1H, m, H-15), 1.41 (1H, m, H-7), 1.36–1.50 (9H, m), 1.20–1.36 (4H, m), 1.16 (3H, s, H-18), 1.13 (2H, m, H-8a, 14a), 1.07 (1H, m, H-6), 0.93 (3H, d, J = 6.6 Hz, H-17), 0.86 (3H, d, J = 6.6 Hz, H-19); MS (ESI): *m/z* 548.3/550.3 (1:1) [M+Na]$^+$; HRMS (ESI): calcd for $C_{27}H_{44}{}^{79}BrNO_4Na$ ([M+Na]$^+$) 548.2346, found 548.2373.

17: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.41 (2H, d, J = 8.6 Hz, Ar), 7.29 (2H, d, J = 8.6 Hz, Ar), 6.74 (1H, br s, NH), 5.41 (1H, t, J = 7.0 Hz, H-2), 4.16 (2H, d, J = 7.0 Hz, H-1), 4.04 (1H, dd, J = 10.4, 5.6 Hz, H-16b), 3.98 (1H, dd, J = 10.4, 6.6 Hz, H-16a), 1.99 (1H, m, H-4), 1.85 (1H, m, H-15), 1.66 (3H, s, H-20), 1.16 (3H, s, H-18), 1.15–1.50 (15H, m), 1.05–1.15 (2H, m, H-6a, 8a), 0.97 (3H, d, J = 6.6 Hz, H-17), 0.86 (3H, d, J = 6.6 Hz, H-19); MS (ESI): *m/z* 490.2/492.2 (1:1) ([M–H_2O–OH]$^+$), 548.2/550.2 (1:1) ([M+Na]$^+$); HRMS (ESI): calcd for $C_{27}H_{44}{}^{79}BrNO_4Na$ ([M+Na]$^+$) 548.2346, found 548.2361.

18: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.40 (d, J = 8.6 Hz, 2H, Ar), 7.39 (2H, d, J = 8.6 Hz, Ar), 7.29 (4H, d, J = 8.0 Hz, Ar), 6.74 (1H, br s, NH), 6.83 (1H, br s, NH), 5.37 (1H, t, J = 7.0 Hz, H-2), 4.68 (2H, d, J = 7.0 Hz, H-1), 4.03 (1H, dd, J = 10.4, 6.2 Hz, H-16b), 3.97 (1H, dd, J = 10.4, 6.6 Hz, H-16a), 2.02 (2H, t, J = 7.4 Hz, H-4), 1.72 (3H, s, H-20), 1.83 (1H, m, H-15), 1.41 (1H, m, H-7), 1.20–1.50 (13H, m), 1.19 (1H, m, H-14a), 1.17 (3H, s, H-18), 1.12 (1H, m, H-8a), 1.07 (1H, m, H-6a), 0.96 (3H, d, J = 6.8 Hz, H-17), 0.86 (3H, d, J = 6.6 Hz, H-19); MS (ESI): *m/z* 490.2/492.2 (1:1) ([M–OCONHC$_6$H$_4$Br]$^+$), 745.2/747.2/749.2 (1:2:1) ([M+Na]$^+$); HRMS (ESI): calcd for $C_{34}H_{48}{}^{79}Br_2N_2O_5Na$ ([M+Na]$^+$) 745.1822, found 745.1807.

4.3.11. Dihydro-α2 (19)

To a solution of α2 (**2**) (0.1 mg) in EtOAc (0.5 mL) was added 5% Rh-Al_2O_3 (1.7 mg), and the mixture was stirred under a hydrogen atmosphere at room temperature for 1 h. The catalyst was separated through a cotton plug and washed with EtOAc. The combined filtrates were concentrated to give a crude product, which was purified by TLC (20 cm × 10 cm × 0.25 mm thickness) with hexane-EtOAc (1:9) to give pure **19** (0.1 mg, R_f = 0.47): ^{1}H NMR (600 MHz, $CDCl_3$): δ = 3.63–3.76 (2H, m, H-1), 3.51 (1H, dd, J = 10.4, 6.0 Hz, H-16b), 3.44 (1H, dd, J = 10.4, 6.5 Hz, H-16a), 1.50–1.67 (3H, m), 1.20–1.48 (20H, m), 1.16 (3H, s, H-18), 1.05–1.20 (3H, m), 0.93 (3H, d, J = 6.6 Hz, H-17), 0.90 (3H, d, J = 6.5 Hz, H-20), 0.86 (3H, d, J = 6.6 Hz, H-19); HRMS (ESI): calcd for $C_{20}H_{42}O_3Na$ ([M+Na]$^+$) 353.3026, found 353.3042.

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Supplementary data

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