Competitive CatSper Activators of Progesterone from Rhynchosia volubilis

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Key words
Leguminosae, Rhynchosia volubilis, CatSper, prenylated isoflavonoids, rhynchones A–E

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ABSTRACT
The root Rhynchosia volubilis was widely used for contraception in folk medicine, although its molecular mechanism on antifertility has not yet been revealed. In human sperm, it was reported that the cation channel of sperm, an indispensable cation channel for the fertilization process, could be regulated by various steroid-like compounds in plants. Interestingly, these nonphysiological ligands would also disturb the activation of the cation channel of sperm induced by progesterone. Therefore, this study aimed to explore whether the compounds in R. volubilis affect the physiological regulation of the cation channel of sperm. The bioguided isolation of the whole herb of R. volubilis has resulted in the novel discovery of five new prenylated isoflavonoids, rhynchones A–E, a new natural product, 5′-O-methylphaseolinisoflavan (6) (1H and 13C NMR data, Supporting Information), together with twelve known compounds (7–18). Their structures were established by extensive spectroscopic analyses and drawing a comparison with literature data, while their absolute configurations were determined by electronic circular dichroism calculations. The experiments of intracellular Ca2+ signals and patch clamping recordings showed that rhynchone A (1) significantly reduced cation channel of sperm activation by competing with progesterone. In conclusion, our findings indicate that rhynchone A might act as a contraceptive compound by impairing the activation of the cation channel of sperm and thus prevent fertilization.
Introduction

The genus *Rhynchosia*, belonging to the family Leguminosae, is composed of about 200 species, distributed in tropical and subtropical regions, but most of them are in Asia and Africa. There are 13 species in China, mainly distributed in the southern provinces of the Yangtze River [1]. The dry roots of *Rhynchosia volubilis* Lour. has shown diverse activities, including dispensing wind and dehumidification, promoting blood circulation, detoxification, detumescence, and relieving pain. It is also known as the king drug of a contraceptive prescription in folk medicine in clinics and has been used by nates in the northwest of Hubei Province, China, for female birth control for a long time [2]. The phytochemical investigations on this genus revealed the presence of flavonoids [3], isoflavonoids [4, 5], favan-3-ols, xanthones [6], biphenyls, simple polyphenols, and sterols [7]. Some of these exhibited antifertility [8], antimicrobial [9], antitumor [10], anti-inflammatory [11], antiproliferative [12], and antihyperlipidemic activities [13, 14].

Calcium signaling in spermatozoa is essential for successful fertilization, which regulates the sperm capacitation, hyperactivation, and acrosome reaction [15, 16]. The vital source of sperm intracellular free Ca²⁺ ([Ca²⁺]ᵢ) is the Ca²⁺ influx, predominantly mediated by the cation channel of sperm (CatSper), a pH-dependent voltage-gated Ca²⁺-selective channel [17, 18]. CatSper is a highly complex multisubunit channel composed of at least ten subunits [19]; four separate pore-forming subunits (CatSper 1–4) and six auxiliary subunits (CatSper β, γ, δ, ε, ζ, and EFCAB9). Mouse knock-out models and genetic screening in infertile men demonstrated that CatSper is essential for male fertility in mice and humans [19]. In human sperm, the steroid hormones, progesterone (P₄), prostaglandin (PG) E₁, and PGE₂, have been noted as potent CatSper agonists [20]. Moreover, structurally diverse endocrine-disrupting chemicals activate the sperm-specific CatSper channel and desensitize sperm for physiological CatSper ligands [21]. Therefore, the CatSper channel is a polymodal chemosensor in human sperm. All these results suggest that the CatSper channel is an ideal target for contraceptive. In order to define whether the compounds from *R. volubilis* disturb the physiological activation of the CatSper channel, we investigated the effects of the phytochemical constituents in the whole plant of *R. volubilis* on the regulation of CatSper.

Results and Discussion

Firstly, given that the CatSper channel mainly dominates Ca²⁺ influx in human sperm, the effect of different extracts from *R. volubilis* on intracellular Ca²⁺ ([Ca²⁺]ᵢ) signals were evaluated. The results showed that the petroleum ether (PE) extracts gave rise to a rapid [Ca²⁺]ᵢ elevation, while ETOAc and n-BuOH extracts failed to reproduce this effect (Fig. 44S, Supporting Information).

The PE and EtOAc fraction from the crude ETOH extract from the whole herb of *R. volubilis* was subjected to repeated chromatography procedures (silica gel, Toyopearl HW-40C, Sephadex LH-20, and semipreparative HPLC), leading to the isolation of five new prenylated isoflavonoids, rhynchones A–E (1–5), the structures of which were characterized by interpretation of their HRMS, 1D and 2D NMR, and electronic circular dichroism (ECD) data. Besides the five new compounds (1–5), a new natural product, 5′-O-methyl-phaseollinosiflavon (6) [22], together with twelve known compounds (7–18) were obtained and identified as tonkinesisolin (7) [23], lipunifolinol (8) [24], cathayanon H (9) [25], cafanone (10) [5], prunetin (11) [26], isowighteone (12) [27], erythrinin B (13) [28], semilicoisoflavon B (14) [29], eriosemaone D (15) [30], formonotenin (16) [31], puerarone (17) [32], and bidwillon C (18) [33] by comparison with literature values (Fig. 1). Hence, the isolation, structure elucidation, and potential CatSper regulation activities of these isolated compounds are described in detail.

To further explore which kind of compound regulated the homeostasis of [Ca²⁺]ᵢ, 18 compounds (1–18) from *R. volubilis* on [Ca²⁺], of human sperm were assessed. Interestingly, only rhynchone A (1) from the PE extracts evoked a transient amplitude of a [Ca²⁺]ᵢ signal (Figs. 44S and 45S, Supporting Information). The results of patch-clamp recordings also manifested that rhynchone A amplified the monovalent current of human sperm, indicating that the elevation of the [Ca²⁺]ᵢ signal caused by rhynchone A resulted from the activation of CatSper (Fig. 2). More importantly, subsequent studies found that the elevation of [Ca²⁺]ᵢ caused by P₄ was suppressed by rhynchone A. The results of patch-clamp recordings on human sperm also manifested that rhynchone A compromised the activation of the CatSper channel elicited by P₄ (Fig. 3). Therefore, these findings suggested that rhynchone A attenuated the physiological activities of P₄ on the CatSper channel, and as a result, affected the function of human sperm. Compared to compound 10, we speculated that the configuration of the B-ring and the substitution of a methoxyl group at C-4′ played a vital role in activating CatSper.

Structure elucidation

Rhychnone A (1), a pale-yellow solid, has a molecular formula of C₂₅H₂₈O₆ based on HR-ESI-TOF-MS data (Fig. 15, Supporting Information) with an m/z ion of 435.1794 for [M – H]⁻ (calcld. 435.1807). The presence of 1H resonances at H-2a (δ₂₂₂, 4.68, 1H, dd, J = 4.8, 11.7 Hz), H-2b (δ₂₂₂, 4.84, 1H, dd, J = 4.1, 11.9 Hz), and H-3 (δ₂₂₂, 3.93, 1H, br t, J = 4.4 Hz), and corresponding oxymethylene and methine signals at δ₂₂₂ 69.3, 44.9 in its ¹H and ¹³C NMR spectra (Tables 1 and 2, Figs. 25 and 35, Supporting Information), respectively, suggested the presence of an isoflavonane skeleton. Signals at δ₂₂₂ 11.94 (1H, s) and 5.93 (1H, s) corresponded to the C-5 hydroxy group and H-8, respectively, which showed an ortho-substitution in the A-ring. The ¹H NMR spectrum of 1 exhibited four methyl groups at δ₁₂₂, 1.42, 1.44 (3H, s, C-2″), 1.66, and 1.71 (3H, s, C-3″), one methoxyl proton at δ 3.77 (3H, s), and three olefinic protons at δ 5.48 (1H, d, J = 10.1 Hz), 6.56 (1H, d, J = 10.1 Hz), and 5.23 (1H, m), which indicated the presence of two isoprenyl groups. ¹H-¹H COSY (Fig. 65, Supporting Information) correlations were observed for H-3″/H-4″ and H-2″/H-3″, indicating the connectivity of C-3″ to C-4″ and C-2″ to C-3″. The HMBC correlations (Fig. 45, Supporting Information) H-3″ to C-2″ and C-6; H-4″ to C-6, C-7, C-2″, and C-3″; H₂-2″ to C-2″, C-3″, and C-10 indicated that C-4″ was attached to C-6, and C-2″ was linked by a ether bond. The aromatic proton signals at δ 6.48 (1H, s, H-3′) and 7.17 (1H, s, H-6′) indicated that the B-ring was 1′, 2′, 4′, 6′-tetrasubstituted. The HMBC correlations from H-3 to C-1′, C-2′; H-2 to C-1′; H₃-4′-OMe to C-4′; H-3′ to
an oxidized aromatic quaternary C-2′; and H2-1″ to C-4′ demonstrated the group substitution model in the B-ring (▶ Fig. 4). In order to determine the absolute configuration of 1, a computational study using the time-dependent density functional theory (TD-DFT) method of ECD spectra at the B3LYP/6-31g (d, p) level was performed with Gaussian 16 B.01 [34]. Additionally, the solvent effects of methanol were taken into consideration with the integral equation formalism polarizable continuum model (IEFPCM) [35] during the calculations. The Boltzmann averaged spectra for all the possible conformers of 1 and their experimental ECD spectra are shown in ▶ Fig. 5a. The experimental ECD spectrum of 1 displayed high similarity to the calculated ECD pattern of 3S-1, which exhibited a calculated ECD spectrum with a distinct positive Cotton effect at 202 nm and a negative Cotton effect at 272 nm (▶ Fig. 5a). Furthermore, a negative Cotton effect at 326 nm (Fig. 8S, Supporting Information) in the ECD spectrum of 1 also suggested the 3S-configuration [36]. Thus, the structure of rhynchone A (1) was determined as 3S-5, 2′, 4′-trihydroxy-2″, 2″-dimethylpyrano[6,7′:5″,6″]-5′-prenyl-isoflavone.

Rhynchone B (2), a yellow oil, was deduced as having the molecular formula C26H28O6 by HR-ESI-TOF-MS [M + H2O − H]− m/z 437.1606 (calcd. 437.1600), indicating one more index of hydrogen deficiency than 1. The NMR spectroscopic data of 2 (▶ Tables 1 and 2) also showed structural similarity with 1. The major difference between these two compounds was found on the B-ring. The substitution at C-4′ and C-5′ was identified as an isopropenyl dihydrofuran group, which was characterized by the following: two endocyclic methylene protons, δH 2.66 (1H, dd, J = 2.0, 14.6 Hz, 3″a) and 2.87 (1H, dd, J = 8.6, 14.9 Hz, 3″b), two exocyclic methylene protons, δH 4.86 (H, m, 5″a) and 4.98 (H, m, 5″b), an oxymethine signal, δH 4.31 (H, t, J = 8.0 Hz), δC 78.49 (C-2″), and a methyl group [δH 1.79 (3H, s, 6″), δC 18.1 (C-6″)]. These were confirmed by 1H-1H COSY correlations (Fig. 14S, Supporting Information) for H-2″/H-3b″, and HMBC correlations (Fig. 13S, Supporting Information) from H-2″ to C-5′/2″/5″/6″ and H-3b″ to C-4′/5′/6′/2′/4″. The S configuration of C-3 was determined based on its circular dichroism spectrum (Fig. 16S, Supporting Information), and showed a negative cotton effect at 325 nm [36]. In the ROESY spectrum (Fig. 15S, Supporting Information), H-2″ (δH 4.31) correlated with H2-3″ (δH 2.66, 2.87), H3-6″ (δH 1.79) correlated with H-2″, and the coupling constants of H-2″ and H-3a″ were different from those in crotadihydrofuran A, which indicated that H-2″ was an β-orientation [37]. Thus, the structure of rhynchone B (2) was identified as 3S,2″R-5,2″-dihydroxy-2′, 2″-dimethylpyrano[6,7′:5″,6″]-2″-allyl furano[4′:5′:4″,5″] isoflavonone.

Rhynchone C (3), a yellow powder, had a molecular formula of C26H26O6 from its HR-ESI-TOF-MS spectra [M + H2O − H]− m/z 451.1807 ([M + H2O − H]− calcd. 451.1757). The NMR data (▶ Table 1) showed structural similarity with 1 and 2. The major difference between these two compounds was found on the B-ring. The substitution at C-4′ and C-5′ was identified as an isopropenyl dihydrofuran group, which was characterized by the following: two endocyclic methylene protons, δH 2.66 (1H, dd, J = 2.0, 14.6 Hz, 3″a) and 2.87 (1H, dd, J = 8.6, 14.9 Hz, 3″b), two exocyclic methylene protons, δH 4.86 (H, m, 5″a) and 4.98 (H, m, 5″b), an oxymethine signal, δH 4.31 (H, t, J = 8.0 Hz), δC 78.49 (C-2″), and a methyl group [δH 1.79 (3H, s, 6″), δC 18.1 (C-6″)]. These were confirmed by 1H-1H COSY correlations (Fig. 14S, Supporting Information) for H-2″/H-3b″, and HMBC correlations (Fig. 13S, Supporting Information) from H-2″ to C-5′/2″/5″/6″ and H-3b″ to C-4′/5′/6′/2′/4″. The S configuration of C-3 was determined based on its circular dichroism spectrum (Fig. 16S, Supporting Information), and showed a negative cotton effect at 325 nm [36]. In the ROESY spectrum (Fig. 15S, Supporting Information), H-2″ (δH 4.31) correlated with H2-3″ (δH 2.66, 2.87), H3-6″ (δH 1.79) correlated with H-2″, and the coupling constants of H-2″ and H-3a″ were different from those in crotadihydrofuran A, which indicated that H-2″ was an β-orientation [37]. Thus, the structure of rhynchone B (2) was identified as 3S,2″R-5,2″-dihydroxy-2′, 2″-dimethylpyrano[6,7′:5″,6″]-2″-allyl furano[4′:5′:4″,5″] isoflavonone.
bales 1 and 2) revealed a methoxy group (δH 3.79, δC 55.5) instead of the C-2′ hydroxyl group in 2. This difference was demonstrated by the HMBC correlation from H3-2′-OMe to C-2′ at δC 158.4 (Fig. 21S, Supporting Information). Thus, 3 was identified as 3S, 2′R-5-hydroxy-2′-methoxyl-2′, 2′-dimethylpyrano[6,7:5′,6′]-2′-allyl furano[4′,5′:4,5] isoflavanone.

Rhynchone D (4), a yellow oily solid, had a molecular formula of C25H24O8 based on its HR-ESI-MS ion at [M + H2O – H]+ m/z 437.1639 (calcd. 437.1600). The NMR spectra (Figs. 26S and 27S, Supporting Information) of 4 exhibited very similar A- and B-ring moieties with those of 1. The C-4′ was substituted by a hydroxyl group in 2 instead of a methoxy group in 1. Additionally, incorporating a furan ring in the flavone system, an extra ring was fused to ring B (C-2-C-3-O-C-2′-C1′). This assertion was supported by the 1H-1H COSY correlations of H-2 (δH 4.72)/H-3 (δH 4.32) (Fig. 30S, Supporting Information) and HMBC correlations from H-2 (δH 4.72) to C-4 (δC 194.8), C-9 (δC 162.4), and C-1′ (δC 115.0) and H-3 (δH 4.32) to C-4 (δC 194.8) and C-1′ (δC 115.0) (Fig. 29S, Supporting Information). According to the coupling constant (J = 11.1/11.4 Hz) between H-2/H-3, we concluded that δC 115.0 (Fig. 29S, Supporting Information) and 1H-1H COSY (Fig. 38S, Supporting Information) analyses. The result of 5 showed that the experimental ECD spectrum exhibited a positive Cotton effect at 206 nm and a negative Cotton effect at 272 nm, which was highly similar to the calculated ECD pattern of 3S-5 (Fig. 5c). So, 5 was identified as 3S,5-dihydroxy-3-((7-hydroxy-2,2-dimethyl-2H-chromen-6-yl)oxy)-8,8-dimethyl-2,3-dihydro-4H,8H-pyrano[2,3-f]chromen-4-one.

**Fig. 2** The effect of different concentrations of rhynchone A (1) on the activation of the CatSper channel of human sperm. a The typical fluorescence traces of [Ca2+]i signals before and after exposure to different concentrations of rhynchone A (1). Arrow indicates the time point of additives in human sperm. b Average amplitudes of Ca2+ response in the presence of different concentrations of rhynchone A (1) are shown. c Representative monovalent current of human CatSper was potentiated by different concentrations of rhynchone A (1). The monovalent CatSper current was recorded in the presence of sodium-based divalent-free solution (NaDVF) by a voltage-clamp ramp protocol (from −100 mV to +100 mV, 1 s). Holding potential (HP) was set to 0 mV. d Average currents of the CatSper channel at −100 mV (negative) and +100 mV (positive) after injecting different concentrations of rhynchone A (1) are shown. Data are expressed as the mean ± SEM; n = 4, *p < 0.05.
## Table 1

$^1$H NMR (600 MHz, $\delta$ in ppm, $J$ in Hz, CDCl$_3$) data for compounds 1–5.

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Materials and Methods

General experiment procedures

Optical rotations were recorded on an AUTOPOL IV-T automatic polarimeter. The ECD spectra were obtained using a JASCO J-810 Circular Dichroism Spectrometer. All NMR data were obtained using a Bruker Avance III 600 MHz NMR spectrometer, and the MS was obtained using a Thermo Fisher Ultimate 3000 HPLC TOF-MS. Toyopearl HW-40C and Sephadex LH-20 were employed for gel permeation. A macroporous adsorption resin (D101) and silica gel (100–200, 200–300 meshes) were employed for column chromatography. HPLC separations were carried out on a WuFeng LC-100 pump that was equipped with an RI2000 refractive index detector using a YMC-Pack ODS-A column (10 × 250 mm, 5 µm) and a YMC-Pack SIL column (10 × 250 mm, 5 µm). The change of hu-
man sperm [Ca$^{2+}$], was measured using the fluorescent Ca$^{2+}$ indicator Fluo-4 AM with the EnSpire Multimode Plate Reader. Pipettes were prepared by a Sutter Micropipette Puller P1000 and Narishige Microforge MF830. The CatSper current was recorded by a patch-clamping system constructed by an Olympus IX71 inverted microscope, a Sutter electric triaxial micromanipulator, Axon Axopatch 200B, and Axon Digidata 1550.

**Plant material**

The whole herb of *R. volubilis* was collected in Zaoyang County by Mr. Rui-Zhong Zhou, a pharmacist from Zaoyang Hospital of Traditional Chinese Medicine, Xiangyang City, Hubei Province. The plant was identified by Dr. Jinbo Fang, who is an Associate Professor from the School of Pharmacy, Tongji Medical College of Huazhong University of Science and Technology (China), where the voucher specimens (NO. RVL 20181 101) were deposited.
Extraction and isolation

The air-dried whole plant of *R. volubilis* (10 kg) was powdered and then extracted three times (24 h each time) with 95% EtOH at room temperature to obtain a crude extract after filtration and evaporation of the combined solution. The crude extract was suspended in H₂O followed by solvent partitions with PE, EtOAc, and n-BuOH, then concentrated in a vacuum to afford extracts weighing 29.1 g, 138.8 g, and 179.2 g, respectively.

PE Fr. (28.1 g) was chromatographed on silica gel (100–200 mesh) (PE-EtOAc 100:1, 99:1, 49:1, 19:1, 14:1, 12:1, 9:1, 4:1, 1:1, v/v) to afford nine fractions (Frs. E0101–E0109). Fr. P0105 (4.2 g) was subjected to Toyopearl HW-40C (CH₂Cl₂-MeOH, 2:1, v/v), resulting in six fractions (Frs. P0701–P0706). Fr. P0705 (2.2 g) was isolated by RP-C18 (MeOH-H₂O, 6:4, 7:3, 8:2, 9:1 to 1:0, v/v) to afford Frs. P0901–P0905. Fr. P0904 (127.4 mg) was purified by RP-HPLC (MeOH-H₂O, 75:25, 1.5 mL/min) to afford compounds 1 (4.6 mg, tᵣ = 86.2 min), 2 (20.7 mg, tᵣ = 91.1 min), and 4 (4.1 mg, tᵣ = 107.6 min).

Fr. P0106 (7.9 g) was subjected to RP-C18 (MeOH-H₂O, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 to 1:0, v/v) to get Frs. P1201–P1208. Fr. P1207 (1.8 g) was subjected to Toyopearl HW-40C (CH₂Cl₂-MeOH, 2:1, v/v) and Sephadex LH-20 (MeOH) to obtain Frs. P1801–P1805. Fr. P1804 (206.9 mg) was isolated by silica gel (300–400 mesh) eluted with PE-acetone (9:1–4:1, v/v), then purified by RP-HPLC and eluted with MeOH-H₂O (86:14, 15 mL/min) followed by PTLC and eluted with PE-acetone (4:1) to afford 6 (3.6 mg).

EtOAc Fr. (138.8 g) was separated using resin HP-20SS (75–150 µm) and eluted with MeOH-H₂O (4:6, 6:4, 8:2, 9:1, 0:10, v/v) to obtain six fractions (Frs. E0101–E0106). Fr. E0103 (54.2 g) was subjected to silica gel [100–200 mesh, (CH₂Cl₂-MeOH, 100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 9:1, 8:1, 6:1, 2:1, 1:1, 0:1, v/v)] to afford 11 fractions (Frs. E0201–E0211). Fr. E0206 (3.8 g) was chromatographed on silica gel (60 µm) and eluted with CH₂Cl₂-MeOH (200:1, 100:1, 50:1, 1:1, 0:1, v/v) to get Frs. E0901–E0905. Fr. E0902 (829.9 mg) was purified by Sephadex LH-20 (MeOH) followed by RP-HPLC eluted with MeOH-H₂O (72:28, 1.5 mL/min) and PTLC eluted with CH₂Cl₂-MeOH (49:1, v/v), to afford compounds 3 (4.3 mg), 5 (22.7 mg), 7 (4.9 mg), and 8 (5.8 mg).

Fr. E0104 (64.7 g) was isolated with silica gel (60 µm) and eluted with n-hexane-EtOAc, 6:1, 5:1, 4:1, 3:1, 2:1, 1:0, v/v) to afford nine fractions (Frs. E1301–E1309). Frs. E1301–E1303 (6.4 g) were chromatographed on silica gel (300–400 mesh) and eluted with PE-EtOAc, 6:5:3, 1:1, v/v) and Sephadex LH-20 (MeOH) and purified by RP-HPLC and eluted with MeOH-H₂O (69:31, 1.5 mL/min) followed by PTLC and eluted with (CH₂Cl₂-MeOH, 49:1, v/v) to afford 14 (6.3 mg), 15 (10.7 mg), 16 (6.1 mg), and 17 (7.7 mg).

Frs. E1304–E1307 (15.2 g) were separated using RP-C18 and eluted with MeOH-H₂O (70:30, 75:25, 80:20, 85:15, 90:10, 100:0, v/v) to obtain seven fractions (Frs. E1801–E1807). Fr. E1804 (6.1 g) was chromatographed on silica gel (300–400 mesh) and eluted with PE-EtOAc (10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 0:1, v/v) to afford 11 fractions (Frs. E1901–E1911). Fr. E1906 (3.1 g) was successively isolated with Sephadex LH-20 (MeOH), RP-HPLC, and eluted with MeOH-H₂O (74:26, 1.5 mL/min) followed by PTLC and eluted with (CH₂Cl₂-MeOH, 50:1, v/v) to afford 10 (15.1 mg), 11 (12.8 mg), 12 (5.2 mg), and 13 (13.6 mg). Fr. E1807 (4.7 g) was chromatographed on silica gel (60 µm) and eluted with PE-EtOAc (10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 0:1, v/v) to afford 11 fractions (Frs. E2201–E2221). Frs. E2204–E2205 (958.2 mg) were successively isolated with Sephadex LH-20 (MeOH), RP-HPLC, and eluted with MeOH-H₂O (76:24, 1.5 mL/min) followed by PTLC and eluted with PE-acetone (4:1) to afford 9 (7.0 mg) and 18 (6.6 mg).

Quantum chemistry calculations

A conformational search of the compounds was implemented in Maestro 10.2 software (Schrodinger, LLC) where conformers with Boltzmann populations >5% were taken into further quantum chemistry calculations. The geometry optimizations, frequency analysis, and TD-DFT calculations of each conformer were subsequently carried out using the B3LYP/6-31g (d, p) level with Gaussian 16 B.01 [34]. The solvent effects of methanol were taken into consideration by using a solvation model of IEFPCM during the calculations [35]. The calculated ECD data were Boltzmann averaged according to Gibbs free energy and their ECD spectra were generated by the SpecDis v1.71 program [38] with a bandwidth (σ) of 0.16 eV. For all calculated spectra, the vertical axes were scaled to fit the experimental spectra. The wavelength shift
of 2, 0, and –35 nm was employed for 1, 4, and 5, respectively (Fig. 41–43S, Supporting Information).

**Measurement of sperm [Ca2+]i**

The change of human sperm [Ca2+]i was measured using the fluorescent Ca2+ indicator Fluoro-4 AM with the EnSpire Multimode Plate Reader as previously described [39]. The action of compounds 1–18 (100 mM stock in DMSO) on [Ca2+]i of human sperm was detected. The final concentration of DMSO was 0.1%. The change of sperm [Ca2+]i was calculated by ΔF/F0 (%), indicating the percent (%) of fluorescent changes (ΔF) normalized to the mean basal fluorescence before the application of any chemicals (F0). ΔF/F0 (%) = (F – F0)/F0 × 100%, where F indicates the fluorescent intensity at each recorded time point.

**Compounds assay – sperm patch-clamp recordings**

The whole-cell patch-clamp technique was applied to record human sperm CatSper as previously described [40]. Seals were formed at the sperm cytoplasmic droplet or the neck region by a 15–30 MΩ pipette. The transition into whole-cell mode was then made by applying short (1 ms) voltage pulses (400–650 mV) combined with light suction. The currents were stimulated by 1 s voltage ramps from –100 to +100 mV from a holding potential of 0 mV. The monovalent current of CatSper and divalent-free (DVF) solution (150 mM NaCl, 20 mM HEPES, and 5 mM EDTA, pH 7.4) was used to record basal CatSper monovalent currents. Then, 1, 10, and 100 µM compounds (1–18), 1 µM progesterone, and 100 µM compounds (1–18) together with 1 µM progesterone in DVF were perfused to record CatSper currents. Data were analyzed with Clampfit version 10.4 software.

**Rhyynchone A (1)**

Pale yellow solid; [α]D 0 = –5.33° (c 0.1, CH3OH); UV (MeOH) λmax nm (log ε): 204 (4.02), 272 (3.97). IR (KBr) νmax 3423, 2970, 2881, 1639, 1560, 1494, 1392, 1187 cm–1; 1H NMR (600 MHz in CDCl3) and 13C NMR (150 MHz in CDCl3), for data, see ➤Tables 1 and 2; HR-ESI-TOF-MS [M+H]+ m/z 437.1639 ([M+H+] calcld. 437.1600).

**Rhyynchone B (2)**

Yellow oil; [α]D 0 = –16.7° (c 0.1, CH3OH); UV (MeOH) λmax nm (log ε): 203 (3.68), 272 (3.59). IR (KBr) νmax 3436, 2982, 2881, 2382, 1624, 1555, 1397, 1165 cm–1; 1H NMR (600 MHz in CDCl3) and 13C NMR (150 MHz in CDCl3), for data, see ➤Tables 1 and 2; HR-ESI-TOF-MS [M+H2O+H]+ m/z 437.1606 ([M+H2O+H+] calcld. 437.1600).

**Rhyynchone C (3)**

Yellow powder; [α]D 0 = –23.1° (c 0.1, CH3OH); UV (MeOH) λmax nm (log ε): 202 (3.66), 272 (3.54). IR (KBr) νmax 3441, 2980, 2882, 1644, 1627, 1392, 1315 cm–1; 1H NMR (600 MHz in CDCl3) and 13C NMR (150 MHz in CDCl3), for data, see ➤Tables 1 and 2; HR-ESI-TOF-MS [M+H]+ m/z 437.1794 ([M+H+] calcld. 437.1807).

**Rhyynchone D (4)**

Yellow oily solid; [α]D 0 = –15.3° (c 0.1, CH3OH); UV (MeOH) λmax nm (log ε): 203 (3.98), 226 (3.56), 273 (3.85). IR (KBr) νmax 3342, 2980, 1630, 1627, 1491, 1376, 1363, 1169, 1130, 1097 cm–1; 1H NMR (600 MHz in CDCl3) and 13C NMR (150 MHz in CDCl3), for data, see ➤Tables 1 and 2; HR-ESI-TOF-MS [M+H2O+H]+ m/z 437.1639 ([M+H2O+H+] calcld. 437.1600).

**Rhynchosia E (5)**

Yellow oily solid; [α]D 0 = –67.1° (c 0.1, CH3OH); UV (MeOH) λmax nm (log ε): 212 (3.90), 276 (3.92), 322 (3.76). IR (KBr) νmax 3440, 2980, 2881, 1647, 1627, 1484, 1381, 1145 cm–1; 1H NMR (600 MHz in CDCl3) and 13C NMR (150 MHz in CDCl3), for data, see ➤Tables 1 and 2; HR-ESI-TOF-MS [M+H]+ m/z 451.1366 ([M+H+] calcld. 451.1392).

**Supporting information**

HR-ESI-MS, NMR spectra, and ECD of compounds 1–5, and effect of extracts and compounds 1–18 on human sperm [Ca2+]i, are available as Supporting Information.

**Contributors’ Statement**


All authors approved the final version of the manuscript.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

**References**


