Competitive CatSper Activators of Progesterone from *Rhynchosia volubilis*

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

**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 (1–5), a new natural product, 5′-O-methylphaseolinosiflavan (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.

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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 dispelling wind and dehumidification, promoting blood circulation, detoxification, detumescence, and relieving pain. It is also known as the king drug for female birth control for a long time [2]. The phytochemical investigation on *Rhynchosia volubilis* has shown diverse activities, including dispelling wind and dehumidification, promoting blood circulation, detoxification, detumescence, and relieving pain. It is also known as the king drug 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 in-}

Results and Discussion

Introduction

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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 Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) is the Ca\(^{2+}\) influx, predominantly mediated by the cation channel of sperm (CatSper), a pH-dependent voltage-gated Ca\(^{2+}\)-selective channel [17, 18]. CatSper is a highly complex multisubunit channel composed of at least ten subunits [19]; four separate pore-forming a 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 polyomodal 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 Ca\(^{2+}\) influx in human sperm, the effect of different extracts from *R. volubilis* on intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) signals were evaluated. The results showed that the petroleum ether (PE) extracts gave rise to a rapid [Ca\(^{2+}\)]\(_{i}\) elevation, while ETOAc and n-BuOH extracts failed to reproduce this effect (Fig. 4GS, 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-phaseolinisoflavon [6] (22), together with twelve known compounds (7–18) were obtained and identified as tonkinesisol (7) [23], lupinofolin (8) [24], cathayanan H (9) [25], cajanone (10) [5], prunetin (11) [26], isowighteone (12) [27], erythrinin B (13) [28], semilicoisoflavone B (14) [29], eriosemaone D (15) [30], formononetin (16) [31], puerarone (17) [32], and bidwillon C (18) [33] by comparison with literature values (Fig. 1). Herein, 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 homestasis of [Ca\(^{2+}\)]\(_{i}\), 18 compounds (1–18) from *R. volubilis* on [Ca\(^{2+}\)]\(_{i}\), of human sperm were assessed. Interestingly, only rhynchone A (1) from the PE extracts evoked a transient amplitude of a [Ca\(^{2+}\)]\(_{i}\) signal caused by rhynchone A resulted from the activation of CatSper (Fig. 2). More importantly, subsequent studies found that the elevation of [Ca\(^{2+}\)]\(_{i}\) 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

Rhynchone A (1), a pale-yellow solid, has a molecular formula of C\(_{32}\)H\(_{22}\)O\(_{4}\), based on HR-ESI-TOF-MS data (Fig. 15S, Supporting Information) with an m/z ion of 435.1794 for [M − H]− (calcd. 435.1807). The presence of 1H resonances at δ(4.68, 1H, dd, J = 4.8, 11.7 Hz), H-2b (δ(4.84, 1H, dd, J = 4.1, 11.9 Hz), and H-3 (δ(3.93, 1H, br t, J = 4.4 Hz), and corresponding oxymethylene and methine signals at δ(69.3, 44.9 in its 1\(^{H}\) and 13\(^{C}\) NMR spectra (Tables 1 and 2, Figs. 25 and 35, Supporting Information), respectively, suggested the presence of an isoflavonane skeleton. Signals at δ(3.77, 1H, s, H-6) and 5.93, 1H, s, H-5) corresponded to the C-5 hydroxy group and H-8, respectively, which showed an ortho-substitution in the A-ring. The 13\(^{C}\) NMR spectrum of 1 exhibited four methyl groups at δ(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. 6S, 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. 45S, Supporting Information) H-3′ to C-2′ and C-6; H-4′ to C-6, C-7, C-2′, and C-3′; H-2′ to C-2′, C-3′, and C-10 indicated that C-4′ was attached to C-6, and C-2′ was linked by an 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′; H-3′ 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. 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 exocyclic 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 = 8.0Hz); δC 78.49 (C-2‴), and a methyl group [δH 1.79 (3H, s, 6‴a), 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′‴/5‴/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 crotaledihydrofuran 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‴] isoflavone.

Rhynchone C (3), a yellow powder, had a molecular formula 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) and 2D-NMR results (Fig. 17S, Supporting Information) showed structural similarity with 1. The major difference was the substitution at C-4‴, which was identified as an isopropenyl dihydrofuran group, which was characterized by the following: two exocyclic 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 = 8.0Hz); δC 78.49 (C-2‴), and a methyl group [δH 1.79 (3H, s, 6‴a), 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′‴/5‴/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 crotaledihydrofuran 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‴] isoflavone.
b) revealed a methoxy group (δ_H 3.79, δ_C 55.5) instead of the C-2′ hydroxy group in 2. This difference was demonstrated by the HMBC correlation from H_2-O to C-2′ at δ_C 158.4 (Fig. 21S, Supporting Information). Thus, 3 was identified as 35, 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 C_{25}H_{24}O_8 based on its HR-ESI-MS ion at [M + H_2O] + 437.1639 (calcld. 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 (δ_H 4.72)/H-3 (δ_H 4.32) (Fig. 30S, Supporting Information) and HMBC correlations from H-2 (δ_H 4.72) to C-4 (δ_C 194.8), C-9 (δ_C 162.4), and C-1′ (δ_C 115.0) and H-3 (δ_H 4.32) to C-4 (δ_C 194.8) and C-1′ (δ_C 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 the two rings were trans-fused. In addition, the absolute configuration of 4 was approximate to 25, 3R-4, which was characteristic of the positive Cotton effects at 212 and 273 nm and the negative Cotton effect at 258 nm (Fig. 5b) in the ECD spectrum. The structure of rhynchone D was deduced as 2S, 3R-5, 4′-dihydroxy-2′, 2′-dimethylpyrano[6,7-5′,6′]-5′-prenyl-furano[2,3-5′,4] flavonone.

Rhynchone E (5), a yellow oily solid, had a molecular formula of C_{25}H_{26}O_7 based on its HR-ESI-MS (Fig. 33S, Supporting Information) and NMR spectra (Figs. 34S and 35S, Supporting Information). The NMR data (Tables 1 and 2) of substitution on its A- and B-rings resembled those of precatorin A, and the main difference between the two compounds was the connection between the B- and C-rings. The molecular mass of rhynchone E (5) was 32 mass units higher than precatorin A [4], indicating that 5 possessed a hemiacetalic carbon (δ_C 105.1, C-3). This was demonstrated by the following changes of carbon chemical shifts compared to precatorin A: C-2 (δ_C 70.2, + 0.5 ppm), C-4 (δ_C 185.1, + 11.6 ppm), C-1′ (δ_C 140.6, + 26.1 ppm), C-2′ (δ_C 146.6, + 9.3 ppm), and C-6′ (δ_C 105.9, + 19.2 ppm) based on HSQC (Fig. 36S, Supporting Information), HMBC (Fig. 37S, 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, 4′-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 [Ca^{2+}]_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 Ca^{2+} 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 ¹H NMR (600 MHz, δ in ppm, J in Hz, CDCl₃) data for compounds 1–5.

| Position | 1 | 2 | 3 | 4 | 5 |
|----------|---|---|---|---|---|
|          | δH (J in Hz) | δH (J in Hz) | δH (J in Hz) | δH (J in Hz) | δH (J in Hz) |
| 2a       | 4.68 (1H, dd, J = 4.8, 11.7 Hz) | 4.62 (1H, dd, J = 4.9, 11.7 Hz) | 4.70 (1H, dd, J = 4.6, 11.9 Hz) | 4.72 (1H, d, J = 11.1 Hz) | 4.52 (2H, s) |
| 2b       | 4.84 (1H, dd, J = 4.1, 11.9 Hz) | 4.78 (1H, dd, J = 5.9, 11.6 Hz) | 4.85 (1H, dd, J = 5.7, 11.3 Hz) | 4.62 (1H, d, J = 11.1 Hz) |  |  |
| 3        | 3.93 (1H, t, J = 4.4 Hz) | 4.03 (1H, t, J = 5.3 Hz) | 3.92 (1H, m) | 4.32 (1H, d, J = 11.4 Hz) |  |
| 4        |  |  |  |  |  |
| 5        |  |  |  |  |  |
| 6        | 5.93 (1H, s) | 5.93 (1H, s) | 5.95 (1H, s) | 5.91 (1H, s) |  |
| 7        |  |  |  |  |  |
| 8        |  |  |  |  |  |
| 9        |  |  |  |  | |
| 10       |  |  |  |  | |
| 1'       |  |  |  |  | |
| 2'       |  |  |  |  | |
| 3'       | 6.48 (1H, s) | 6.48 (1H, s) | 6.52 (1H, s) | 6.42 (1H, s) | 6.42 (1H, s) |
| 4'       |  |  |  |  | |
| 5'       |  |  |  |  | |
| 6'       | 7.17 (1H, s) | 7.00 (1H, s) | 7.22 (1H, s) | 6.64 (1H, s) | 6.51 (1H, s) |
| 2'       |  |  |  |  | |
| 3'       | 5.48 (1H, d, J = 10.1 Hz) | 5.49 (1H, d, J = 10.1 Hz) | 5.48 (1H, d, J = 10.1 Hz) | 5.53 (1H, d, J = 10.0 Hz) | 5.52 (1H, d, J = 10.0 Hz) |
| 4'       | 6.56 (1H, d, J = 10.1 Hz) | 6.58 (1H, d, J = 10.1 Hz) | 6.56 (1H, d, J = 10.1 Hz) | 6.62 (1H, d, J = 10.1 Hz) | 6.59 (1H, d, J = 10.1 Hz) |
| 1''      | 3.20 (2H, m) | 3.13 (2H, d, J = 7.2 Hz) |  |  | |
| 2''      | 5.23 (1H, m) | 4.31 (1H, t, J = 8.0 Hz) | 4.22 (1H, m) | 5.15 (1H, t, J = 7.2 Hz) |  |
| 3''      | 2.87 (1H, dd, J = 8.6, 14.9 Hz) | 2.88 (1H, dd, J = 8.5, 14.2 Hz) | 2.66 (1H, dd, J = 2.0, 14.6 Hz) | 2.68 (1H, dd, J = 2.9, 14.0 Hz) | 5.48 (1H, d, J = 9.8 Hz) |
| 4''      |  |  |  |  | 6.18 (1H, d, J = 9.8 Hz) |
| 5''      | 4.98 (1H, m); 4.86 (1H, m) | 4.91 (1H, m); 4.78 (1H, m) |  |  | |
| 2'-Me    | 1.44 (3H, s) | 1.43 (3H, s) | 1.43 (3H, s) | 1.46 (3H, s) | 1.40 (3H, s) |
| 1.42 (3H, s) | 1.43 (3H, s) | 1.41 (3H, s) | 1.44 (3H, s) | 1.38 (3H, s) |  |
| 2''-Me   |  |  |  |  | 1.45 (3H, s) |
| 3''-Me   | 1.71 (3H, s) | 1.66 (3H, s) |  | 1.67 (3H, s) | 1.45 (3H, s) |
| 6''-Me   | 1.79 (3H, s) | 1.78 (3H, s) |  | 1.60 (3H, s) |  |
| 5-OH     | 11.94 (1H, s) | 12.07 (1H, s) | 11.89 (1H, s) | 11.63 (1H, s) | 11.67 (1H, s) |
| 2'-OMe   |  |  |  |  | 3.79 (3H, s) |
| 4'-OMe   | 3.77 (3H, s) |  |  |  |  |

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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-

Table 2 13C NMR (150 MHz, δ in ppm, CDCl3) data for compounds 1–5.

| Position | 1    | 2    | 3    | 4    | 5    |
|----------|------|------|------|------|------|
| δC Type  | δC Type | δC Type | δC Type | δC Type | δC Type |
| 2        | 69.3 (CH2) | 69.7 (CH2) | 69.2 (CH2) | 74.0 (CH) | 70.2 (CH3) |
| 3        | 44.9 (CH)  | 45.4 (CH)  | 44.7 (CH)  | 72.9 (CH)  | 105.1 (C)  |
| 4        | 196.8 (C) | 197.1 (C) | 196.6 (C) | 194.8 (C) | 185.1 (C) |
| 5        | 159.0 (C) | 159.0 (C) | 159.0 (C) | 158.3 (C) | 161.5 (C) |
| 6        | 103.1 (C) | 103.1 (C) | 103.1 (C) | 103.2 (C) | 96.7 (C)  |
| 7        | 163.0 (C) | 162.8 (C) | 163.2 (C) | 163.5 (C) | 159.3 (C) |
| 8        | 96.0 (CH)  | 96.1 (CH)  | 96.2 (CH)  | 96.6 (CH)  | 103.6 (C) |
| 9        | 162.3 (C) | 162.4 (C) | 162.2 (C) | 162.4 (C) | 163.5 (C) |
| 10       | 101.3 (C) | 101.8 (C) | 101.3 (C) | 101.5 (C) | 101.3 (C) |
| 1′       | 113.5 (C) | 113.9 (C) | 114.0 (C) | 115.0 (C) | 140.6 (C) |
| 2′       | 154.2 (C) | 154.9 (C) | 158.4 (C) | 155.9 (C) | 146.6 (C) |
| 3′       | 100.9 (CH) | 106.3 (CH) | 101.1 (CH) | 105.7 (CH) | 99.3 (CH) |
| 4′       | 158.1 (C) | 156.7 (C) | 155.1 (C) | 155.6 (C) | 148.7 (C) |
| 5′       | 122.8 (C) | 118.5 (C) | 119.5 (C) | 118.4 (C) | 114.8 (C) |
| 6′       | 127.6 (CH) | 130.4 (CH) | 129.4 (CH) | 127.4 (CH) | 109.5 (CH) |
| 2″       | 78.5 (C)  | 78.5 (C)  | 78.6 (C)  | 78.9 (C)  | 78.9 (C)  |
| 3″       | 126.2 (CH) | 126.2 (CH) | 126.2 (CH) | 126.5 (CH) | 126.7 (CH) |
| 4″       | 115.1 (CH) | 115.1 (CH) | 115.0 (CH) | 114.8 (CH) | 114.9 (CH) |
| 1‴       | 27.8 (CH3) | 27.8 (CH3) | 28.7 (CH3) | 28.7 (CH3) | 28.7 (CH3) |
| 2‴       | 122.6 (CH) | 78.4 (CH)  | 75.6 (CH)  | 121.3 (CH) | 76.2 (C)  |
| 3‴       | 132.4 (CH) | 37.4 (CH2) | 36.6 (CH2) | 135.1 (C) | 128.6 (CH) |
| 4‴       | 146.5 (C) | 147.1 (C) | 122.0 (CH) | 122.0 (CH) | 122.0 (CH) |
| 5‴       | 111.3 (CH2) | 110.6 (CH2) | 27.6 (CH3) | 27.4 (CH3) | 27.4 (CH3) |
| 2‴-Me    | 28.4 (CH3) | 28.5 (CH3) | 28.5 (CH3) | 28.5 (CH3) | 28.5 (CH3) |
| 2‴-OMe   | 28.4 (CH3) | 28.5 (CH3) | 28.5 (CH3) | 28.5 (CH3) | 28.5 (CH3) |
| 3‴-Me    | 25.7 (CH3) | 25.5 (CH3) | 17.7 (CH3) | 17.6 (CH3) | 17.6 (CH3) |
| 6‴-Me    | 18.1 (CH3) | 18.1 (CH3) | 18.1 (CH3) | 18.1 (CH3) | 18.1 (CH3) |
| 2‴-OMe   | 55.5 (CH3) | 55.5 (CH3) | 55.5 (CH3) | 55.5 (CH3) | 55.5 (CH3) |
man sperm [Ca^{2+}]_{i} 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 20181101) 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. E0105 (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, 1.5 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, 0:1, v/v) to obtain six fractions (Frs. E0101–E0106). Fr. E0103 (54.2 g) was subjected to silica gel [100–200 mesh, (CH3Cl2-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 CH3Cl2-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 CH3Cl2-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, 1:1, v/v) to afford nine fractions (Frs. E1301–E1309). Frs. E1301–E1303 (6.4 g) were chromatographed on Toyopearl HW-40C (CH3Cl2-MeOH, 2:1, 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 (CH3Cl2-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 (CH3Cl2-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–E2211). 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+]

The change of human sperm [Ca2+]i was measured using the fluorescent Ca2+ indicator Fluo-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 (pH 7.4) was used to record basal CatSper monovalent currents. 0 mV. The monovalent current of CatSper and divalent-free (pH 7.4) was used to record basal CatSper monovalent currents. 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 mono- 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.

Rhynchone A (1)

Pale yellow solid; [α]D20 + 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 Table 1 and 2; HR-ESI-TOF-MS [M+H]+/m/z 435.1794 ([M+H]+ calcd. 435.1807).

Rhynchone B (2)

Yellow oil; [α]D20 + 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 Table 1 and 2; HR-ESI-TOF-MS [M+H+H2O−H]+/m/z 437.1606 ([M+H+H2O−H]+ calcd. 437.1600).

Rhynchone C (3)

Yellow powder; [α]D20 + 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 Table 1 and 2; HR-ESI-TOF-MS [M+H]+/m/z 435.1794 ([M+H]+ calcd. 435.1807).

Rhynchone D (4)

Yellow oily solid; [α]D20 + 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 Table 1 and 2; HR-ESI-TOF-MS [M+H2O−H]+/m/z 437.1639 ([M+H2O−H]+ calcd. 437.1600).

Rhynchone E (5)

Yellow oily solid; [α]D20 − 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 Table 1 and 2; HR-ESI-TOF-MS [M+H]+/m/z 451.1366 ([M+H]+ calcd. 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

J. Xiang: investigation, visualization, and writing – original draft. H. Kang: investigation, visualization, and writing – original draft. H. G. Li: resources and funding acquisition. Y. L. Shi: investigation, visualization, and revision. Y. L. Zhang: investigation. C. L. Ruan: investigation. L. H. Liu: investigation. H. Q. Gao: investigation. T. Luo: resources and funding acquisition. G. S. Hu: investigation. W. L. Zhu: supervision. J. M. Jia: supervision. J. C. Chen: resources. J. B. Fang: writing – review and editing, and funding acquisition. 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.

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