The Fungal Sexual Pheromone Sirenin Activates the Human CatSper Channel Complex

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Supporting Information

ABSTRACT: The basal fungus Allomyces macrogynus (A. macrogynus) produces motile male gametes displaying well-studied chemotaxis toward their female counterparts. This chemotaxis is driven by sirenin, a sexual pheromone released by the female gametes. The pheromone evokes a large calcium influx in the motile gametes, which could proceed through the cation channel of sperm (CatSper) complex. Herein, we report the total synthesis of sirenin in 10 steps and 8% overall yield and show that the synthetic pheromone activates the CatSper channel complex, indicated by a concentration-dependent increase in intracellular calcium in human sperm. Sirenin activation of the CatSper channel was confirmed using whole-cell patch clamp electrophysiology with human sperm. Based on this proficient synthetic route and confirmed activation of CatSper, analogues of sirenin can be designed as blockers of the CatSper channel that could provide male contraceptive agents.

Upon deposition into the female reproductive tract, mammalian sperm must undergo a complex process known as capacitation before achieving successful fertilization.1 While the intricate details surrounding capacitation remain largely unsolved, it has long been known that the intracellular concentration of calcium ions ([Ca2+]i) in sperm plays a pivotal role.2−4 In mammalian sperm, [Ca2+]i is predominantly controlled by the cation channel of sperm (CatSper).5 CatSper is expressed exclusively in the principal piece of sperm and is a heterotetrameric channel, comprising four pore-forming subunits (CatSper 1/2/3/4) and at least three auxiliary subunits, β, δ, and γ.6,7 In mice, genetic disruption of any of these four sperm-specific CatSper channel subunits (CatSper1/2/3/4) ablates expression of the entire complex, leading to complete infertility.8

In 2011, it was shown that the sex hormone progesterone activates the human CatSper channel, giving new insight into mammalian sperm chemotaxis.9,10 Although CatSper channels were originally thought to be animal-specific, it has been shown that the CatSper channel complex is present in the basal fungus Allomyces macrogynus (A. macrogynus).11 A. macrogynus produces motile gametes whose chemotaxis is largely driven by the sexual pheromone sirenin (Figure 1).12 It is not known if CatSper channels underlie the molecular mechanisms for sperm cell chemotaxis in A. macrogynus, as a receptor for sirenin has not yet been identified. However, since the mechanism of chemotaxis relies on calcium flux, sirenin may activate fungal...
CatSper to enable gamete fertilization in A. macrognus. Since it is believed that animals and fungi diverged from a common ancestor over 1 billion years ago, a compound that elicits a similar response in the gametes of both species would serve to demonstrate the high degree of conservation thought to govern most reproductive signaling.11,13

Sirenin has been an attractive target for the synthetic chemistry community given its unique properties and complex chemical structure. Sirenin is an oxygenated sesquiterpene [4.1.0] bicyclic ring system bearing two allylic hydroxyl groups. It was first isolated and characterized by Machlis and co-workers in the 1960s14–16 though Machlis had postulated its existence a decade earlier.17 The pheromone displays attraction to male gametes of A. macrognus.16 To date, a number of syntheses have been developed for sirenin;18–27 however, only three of them provided nonracemic sirenin. Rapoport and Plattner,28 Corey et al.,29 and Kitahara et al.30 each used different chemistry to arrive at sirenin. We selected Kitahara’s synthesis, which was derived from the method of Hortman and Ong,31 as an attractive route to sirenin due to its inherent potential for analog synthesis. However, the key cyclopropanation reaction in this report is not efficient and generates significant amounts of previously uncharacterized side-products. Furthermore, the reactions used to extend the alkyl chain proved to be unreliable in our hands and also produced undesired reaction products. We therefore re-examined and then optimized the synthetic steps to develop a more efficient synthetic route for sirenin.

We studied the ability of sirenin to elicit calcium influx in human sperm cells, specifically via the CatSper channel. Sirenin-induced increases in whole cell [Ca2+]i, were monitored in human sperm with a calcium-sensitive dye using a FLIPR Tetra plate reader. Whole-cell patch clamp electrophysiology experiments were conducted to confirm that this rise in [Ca2+]i was a result of direct CatSper channel activation rather than indirectly through activation of another ion channel located on human spermatozoa.32–34

## RESULTS AND DISCUSSION

### Synthesis of Sirenin

Esterification of (S)-perillic acid (1) with Mel/K₂CO₃ followed by epoxidation provided a diastereomeric mixture of methyl ester epoxides 2 in 77% yield (Scheme 1). The subsequent crucial cyclopropanation reaction to generate intermediate 3 provided poor yields and unidentified side products, which have not been isolated and characterized before. An attempt to carry out the NaH-mediated γ-anion generation followed by cyclopropanation with internal epoxide opening of 2 under reported conditions30 provided less than 10% of the desired compound 3 and 3’ (9:1 ratio), along with an unidentified mixture of compounds, rather than the reported 46% yield. Changing reaction conditions such as base equivalents, concentration, and temperatures did not improve the yield of product 3 (Scheme 1).

After extensive investigations toward an improved synthesis of the key intermediate 3, all side products of the reaction were isolated and characterized and found to be heterodimer 4, trimer 5, and homodimer 6 (Scheme 1). These undesired products are the result of competing transesterification reactions at elevated temperature. Heterodimer 4 arises from the transesterification of 3 and 3’ with unreacted starting material 2, and the dimer 4 undergoes transesterification with unreacted starting material 2 to generate trimer 5. Homodimerization of the key intermediate 3 and 3’ by transesterification provided 6. In order to obtain compounds 3 and 3’, a reaction mixture consisting of 4, 5, and 6 was treated with LiOH to hydrolyze the esters followed by re-esterification with Mel/K₂CO₃ to provide an inseparable 9:1 mixture of the diastereomeric cyclopropanols 3 and 3’ (Scheme 1) with an improved overall yield of 55%. In addition, 20% of diol 7 was obtained. The ratio of 3 and 3’ was dependent on reaction time as reported earlier.30 According to the literature,30 the separation of these isomers was quite difficult and required conversion to their corresponding 3,5-dinitrobenzoates, separation by fractional crystallization, and subsequent hydrolysis and re-esterification to provide 3.30 In our synthesis, the diastereoisomers were separated during the subsequent oxidation and Wittig reaction steps (Scheme 2).

Next, we attempted to install the side chain under conditions developed previously as shown in Scheme 2.30 Cyclopropanol 3 was oxidized to its corresponding aldehyde 8 in 50% yield with IBX. Wittig reaction of 8 with 2-hydroxyethyltriphenylphosphonium bromide in the presence of n-BuLi gave a rather disappointing 3:1 mixture of E-allyl alcohol 9 and the undesired regioisomer 9’. Hydrogenation of 9 with PtO₂ provided the required compound 10 in 60% yield (Scheme 2) and the undesired deoxygenated compound 10’ in 18% yield.

Because the reported Wittig/hydrogenation sequence yielded substantial amounts of undesired side products, we explored an

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**Scheme 1. NaH-Mediated Cyclopropanation, Identification of Side Products, and Their Conversion to 3**

![Scheme 1](image-url)

**Scheme 2. Oxidation and Wittig Reaction Steps**

![Scheme 2](image-url)
alternative strategy for the side chain extension (Scheme 3). Elongation of aldehyde 8 by two iterative Wittig reactions with (methoxymethyl)triphenylphosphonium chloride followed by enol ether hydrolysis provided the two carbon chain-extended aldehyde 12 in 75% overall yield. To complete the synthesis of sirenin, a Horner–Wittig reaction of aldehyde 12 with methyl 2-diethylphosphonopropionate in the presence of base provided the diene diester 13 in 75% yield along with 5% Z-isomer. Finally, the reduction of 13 with DIBAL-H yielded sirenin in 60%. The spectroscopic data of sirenin were in agreement with reported data.14,30

Although Kitahara’s synthetic route provided an approach to sirenin, the key cyclopropanation transformation was beset by a low yield and substantial formation of side products. Therefore, we sought an improved procedure for this critical step. However, changes to the reaction conditions did not improve the product yields. We then decided to modify the cyclopropanation substrate by introducing a sterically hindered ester group to prevent transesterification. Hindered ester epoxides such as tert-butyl ester 17, benzyl ester 18, and isopropyl ester 19 were prepared as shown in Scheme 4. These ester epoxides were examined in the NaH-mediated cyclopropanation reaction. The tert-butyl ester 17 and the benzyl ester 18 epoxides provided a complex mixture of products, presumably due to thermal decomposition. However, isopropyl ester epoxide 19 produced the desired cyclopropylcarbinol 20 along with diastereomer 20’ (20:1) in 70% yield. It should be noted that the isopropyl ester diastereomers 20 and 20’ were easily separated by flash column chromatography, unlike the methyl ester isomers 3 and 3’.30 Thus, the use of the isopropyl ester not only improved the yield of cyclopropyl carbinol 20 but also allowed for the facile separation of isomers 20 and 20’.

Having established an efficient synthetic route to cyclopropanation and side chain extensions, intermediate 20 was elaborated to the natural product sirenin (Scheme 5) in the same manner as described above in a five-step sequence beginning with IBX oxidation, iterative Wittig reactions, and the subsequent hydrolysis of enol ethers. Finally, Horner–Wadsworth–Emmons olefination and DIBAL-H reduction yielded l-sirenin in 8% overall yield in 10 steps linear from commercially available (S)-perillic acid. The spectroscopic data, including specific optical rotation, of the synthetic sirenin were in excellent agreement with the reported data for the isolated natural product14,28 and for sirenin prepared by total synthesis.14,28−30 In addition, we prepared the diastereomeric (R)- and (S)-Mosher bis-esters35 of sirenin.17F NMR analysis indicated a diastereomeric ratio of 97:3 and 96:4, respectively.1H NMR analysis of the C7 methyl group of the two diastereoisomers indicated a ratio of 96:4.

**Biological Evaluation.** The efficacy of sirenin acting at the CatSper channel in sperm obtained from healthy human volunteers was measured by two whole-cell methods: a calcium mobilization assay utilizing a calcium-specific dye (FLIPR assay) and patch clamp electrophysiology, which confirmed that this rise in [Ca2+]i, was a result of direct CatSper channel activation rather than indirectly through activation of another ion channel located on human spermatozoa.32−34 In the FLIPR assay, sperm were collected and loaded with the calcium-specific fluorescent dye Fluo-4-AM and the fluorescence of the cells was continuously monitored. Increased fluorescence correlates with increased intracellular calcium levels produced by CatSper activation. It has been shown previously that this increase in calcium-specific fluorescence originates in the tail and propagates toward the sperm head.35 Synthetic sirenin produced a concentration-dependent rise in [Ca2+]i in human sperm with an EC50 of 2.9 ± 0.7 μM (Figure 2A, black traces). As sirenin was reported to increase chemotaxis of A. macrogynus gametes at concentrations as low as 10 μM,33 this fungal pheromone has several orders of magnitude lower potency at the human channel, reflecting the billion years of separation between the two species. The time-course for increase in [Ca2+]i, elicited by sirenin was similar to that of prostaglandin (Figure 2A, red) and prostaglandin E1 (PGE1, Figure 2A, blue), two endogenous openers of the CatSper channel. Sirenin increased calcium fluorescence with the same maximum response as progesterone and PGE1, although substantially higher concentrations of sirenin were required to reach
saturation (Figure 2B). Pretreatment with the known CatSper calcium channel blocker mibefradil10 (30 μM) reduced the maximal sirenin-induced activation by 55% (Figure 2C, gray bar). Mibefradil also reduced the activity of progesterone and PGE1 consistent with previous studies showing that mibefradil and the related T-type calcium channel blocker, NNC 55-0396, reduce progesterone-induced activation of the CatSper channel.9,10 These observations indicate that sirenin increases sperm [Ca2+]i by activation of the CatSper channel.

Interestingly, in the presence of mibefradil, even high concentrations of sirenin failed to elicit a maximal activation, indicating that mibefradil produces an insurmountable inhibition, consistent with noncompetitive blockade of the CatSper channel (not shown).

To confirm that the sirenin-mediated rise in [Ca2+]i, observed in the calcium fluorescence assay was caused by activation of the CatSper channel, rather than by second messenger pathways9 or other ion channels present in sperm,10 whole cell patch clamp electrophysiology experiments were conducted according to established methods.40,41

**MATERIALS AND METHODS**

**Human Sperm Calcium Fluorescence Assay.** Semen from healthy human donors was collected and incubated in a shaker at 37 °C until complete liquefaction was observed. Semen samples were analyzed for motility/cell count/morphology according to updated WHO parameters for semen analysis.42 The sample was diluted to 50 mL in low pH/low K+ (low/low) buffer containing (in mM): 101 NaCl, 4.69 KCl, 0.2 MgSO4, 0.36 KH2PO4, 25 NaHCO3, 0.32 sodium pyruvate, 2.78 glucose, 94 sodium lactate, and 0.2 CaCl2 at a pH of 6.7 adjusted with HCl. The sample was washed by dilution to 50 mL of low/low buffer and centrifugation at 800 × g for 10 min at 10 °C followed by aspiration of the supernatant. The pellet was then resuspended with 10 mL of low/low buffer and centrifugation at 800 × g for 10 min at 10 °C followed by aspiration of the supernatant. The pellet was then resuspended with 10 mL of low/low buffer containing 10 μM Fluo-4 AM (Life Technologies, Grand Island, NY) with 1 μM probenecid (to reduce dye efflux) and incubated for 30 min at ambient temperature. The sample was again diluted to 50 mL in low/low buffer, centrifuged, and the supernatant aspirated to remove extracellular dye; the pellet was resuspended in ca. 10 mL of low/low buffer. The dye-loaded sperm were then plated into black clear-bottom 384-well assay
μsaturating concentration of progesterone (3 μM progesterone, and the activation EC50 and inhibition IC50 values were calculated using Prism (v 6.0, GraphPad). The data for compounds 14 and 15 can be found in the SI.

**General Procedure for the Synthesis of the Esters 14–16.** A solution of perlic acid (1 equiv), 4-(dimethylamino)pyridine (0.1 equiv), and the appropriate alcohol ("BuOH, BnOH, and PrOH (1.1 equiv)) dissolved in CH2Cl2 (10 mL) was cooled with stirring at 0 °C for 10 min. L-ethyl-3-[3-(dimethylamino)propyl]carboxdiimide hydrochloride (EDCI) (1 equiv) was added, and the reaction mixture was stirred at RT for 24 h. After the completion of the reaction (monitored by TLC), the reaction mixture was diluted with CH2Cl2 and washed successively with saturated sodium bicarbonate (10 mL) and water (2 × 15 mL) and brine and then dried over Na2SO4. The solvent was removed in vacuo and the products 14, 15, and 16 were purified by flash column chromatography (silica gel, 0–5% ethyl acetate in hexanes). The data for compounds 14 and 15 can be found in the SI.

**Isopropyl (S)-4-(Prop-1-en-2-yl)cyclohex-1-enecarboxylate (16).** The compound was purified by flash column chromatography (silica gel, 0–5% ethyl acetate in hexanes) to obtain 16 (2.78 g, 74% yield) as a colorless oil. [{16}H NMR (400 MHz, CDCl3): δ 6.96 (m, 2H), 2.54–2.40 (m, 1H), 2.38–2.31 (m, 1H) 2.29–2.01 (m, 3H), 1.95–1.82 (m, 1H), 1.74 (s, 3H), 1.53–1.37 (m, 1H), 1.26 (d, J = 6.3 Hz, 6H). 13C NMR (100 MHz, CDCl3): δ 166.9, 148.9, 138.4, 130.5, 109.1, 67.3, 40.1, 31.0, 27.1, 24.6, 21.9, 20.7. HRMS (ESI-TOF) m/z calculated for C13H20Na1O3 [M + Na]+: 247.1305. Found: 247.1356. 231.1355. Found: 231.1355.

**Isopropyl (S)-4-((R)-2-Methyloxiran-2-yl)cyclohex-1-ene-1-carboxylate and Its 2S Isomer (19).** In a 200 mL round-bottomed flask equipped with a magnetic stir bar was dissolved perillic acid (2.69 g, 12.9 mmol) in CH2Cl2 (110 mL) and cooled to 0 °C. To this solution was added m-chloroperbenzoic acid (m-CPBA; 2.81 g, 16.2 mmol, activity 77%) in four portions over 2 h at 0 °C. After stirring for another 30 min at 0 °C, the precipitated m-chlorobenzoic acid was removed by filtration. The filtrate was washed with saturated Na2SO4 (20 mL) and extracted with CH2Cl2 (4 × 20 mL). The combined organic layers were washed with saturated NaHCO3 (30 mL) and dried over Na2SO4. Solvent removal in vacuo gave a crude oil that was purified by column chromatography (silica gel, 0–10% ethyl acetate in hexanes) to obtain title compound 19 (2.38 g, 82%) as a colorless oil. [19]H NMR (400 MHz, CDCl3): δ 6.93 (dd, J = 8.8, 5.6, 2.5 Hz, 1H), 5.05 (p, J = 6.3 Hz, 1H), 2.65 (dd, J = 6.7, 4.8 Hz, 1H), 2.57 (t, J = 4.8 Hz, 1H), 2.49 (ddt, J = 14.6, 6.0, 5.7 Hz, 1H), 2.39–1.85 (m, 4H), 1.54 (ddt, J = 13.2, 8.2, 2.5 Hz, 1H), 1.46–1.32 (m, 0.5H), 1.29 (s, 3H), 1.26 (d, J = 6.3 Hz, 6.5H). 13C NMR (100 MHz, CDCl3): δ 166.7, 137.8, 137.6, 130.8, 130.7, 67.4, 58.9, 58.7, 52.9, 52.7, 39.1, 39.0, 27.7, 24.5, 24.3, 24.1, 21.8, 18.4, 18.2. HRMS (ESI-TOF) m/z calculated for C13H20Na1O3 [M + Na]+: 247.1305.

**Human Sperm Electrophysiology.** Whole cell electrophysiology was employed as reported in Lishko et al. 31 Gigaohm seals were formed at the cytoplasmic droplet of highly motile human sperm cells in standard high saline (HS) buffer containing (in mM) 130 NaCl, 20 HEPES, 10 sodium lactate, 5 glucose, 5 KCl, 2 CaCl2, 1 MgSO4, and 1 sodium pyruvate at a pH of 7.4 adjusted with NaOH, 320 mOsm/L. The intrapipette solution contained 130 Cs-methanesulfonate (CsMeSO4), 70 HEPES, 3 EGTA, 2 EDTA, and 0.5 TrisHCl at a pH of 7.4 adjusted with CsOH, 330 mOsm/L. Divalent-free bath solution (CsDVF) was used for recording monovalent current through CatSper containing (in mM) 140 CsMeSO4, 40 HEPES, and 1 EDTA at a pH of 7.4 adjusted with CsOH, 320 mOsm/L. The sirenin effect on CatSper was evaluated by diluting stock in DMSO (10 mM) in CsDVF to test concentration. HS solution was used for baseline measurements. All electrophysiology experiments were performed at ambient temperature. Data were analyzed with Origin 9.0 and Clampfit 10.3. Statistical data are presented as the mean ± SEM, where n indicates the number of individual experiments determined using Prism. Electrophysiology experiments used sperm cells from four individual healthy donors.
Figure 3. Sirenin increases intracellular calcium in human sperm through activation of the CatSper channel. (A) Representative monovalent $I_{\text{CatSper}}$ whole-cell recordings from human spermatozoa using divalent free bath solution (DFV) in the absence (control; blue) or presence of test compound. Currents were elicited in response to indicated voltage ramp. Left panel, 50 $\mu$M sirenin (S; green) and 50 $\mu$M mibefradil (M; purple). Right panel, 1 $\mu$M progesterone (P; red). Baseline indicates recordings performed in HS bath solution. (B) Averaged fold amplitude change of $I_{\text{CatSper}}$, recorded from human spermatozoa in the presence of indicated test compound. Potentiation was determined by dividing current amplitudes of $I_{\text{CatSper}}$ at $-80$ mV (negative, inward current) and $+80$ mV (positive, outward current) by the amplitude of $I_{\text{CatSper}}$ in the absence of the corresponding compound from the same cell. (C) Averaged current density of $I_{\text{CatSper}}$ recorded from human spermatozoa in the presence of indicated test compound. Where appropriate, data are represented as mean ± SEM with $n$ indicating the number of individual cells recorded.

Found: 247.1301. The data for compounds 17 and 18 can be found in the SI.

*Isopropyl (1R,6S,7R)-7-(Hydroxymethyl)-7-methylbicyclo[4.1.0]hept-2-ene-3-carboxylate* (20). The solution of epoxy esters mixture 19 in DME was added to the stirred suspension of NaH in DME under N$_2$. The mixture was stirred and heated under reflux for 1 h. The reaction mixture was cooled, and isopropanol was added to destroy the excess NaH. The reaction mixture was poured into ice water and adjusted to pH 6 with AcOH and extracted with EtOAc. The aqueous solution was extracted with EtOAc, and the extracts were washed with saturated NaHCO$_3$ and saturated NaCl solution and dried over Na$_2$SO$_4$. Evaporation of the solvent afforded the crude product. The resulting solution was allowed to stir at RT for 30 min and was filtered over Celite. The crude product, which was purified by flash column chromatography (silica gel, 0–40% ethyl acetate in hexanes) to obtain 20 (0.49 g, 49%) and 20' (0.02 g, 2%). 21 (0.13 g, 13%), and 22 (0.06 g, 6%) in 70% over all yield. Compound 20, colorless oil, $[\alpha]_D^{20}$ +74.6 (c 1.05, CHCl$_3$). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.18 (dd, $J$ = 5.5, 1.6 Hz, 1H), 5.05 (p, $J$ = 6.3 Hz, 1H), 3.46 (d, $J$ = 10.9 Hz, 1H), 3.37 (d, $J$ = 11.0 Hz, 1H), 2.59–2.31 (m, 1H), 2.07–1.69 (m, 3H), 1.51 (bs, 1H), 1.40 (dd, $J$ = 8.2, 5.5 Hz, 1H), 1.26 (d, $J$ = 6.2 Hz, 7H), 1.01 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 166.7, 137.6, 128.8, 72.3, 67.4, 34.3, 21.9, 21.8, 21.6, 20.3, 16.7, 11.4. HRMS (ESI-TOF) m/z calcd for C$_{13}$H$_{27}$Na$_2$O$_4$ [M + Na$^+$]: 247.1305. Found: 247.1304. The crude product from compounds 20, 21, and 22 can be found in the SI.

*Isopropyl (1R,6S,7R)-7-Formyl-7-methylbicyclo[4.1.0]hept-2-ene-3-carboxylate* (23). To a suspension of 2-iodoxybenzoic acid (IBX; 0.93 g, 3.34 mmol) in DMSO (2 mL) was added alcohol 20 (0.50 g, 2.23 mmol) in anhydrous THF (10 mL) at ambient temperature. After stirring for 5 h, the reaction mixture was diluted with ether (40 mL) and filtered over Celite. The filtrate was washed with water, saturated NaHCO$_3$, brine, dried (Na$_2$SO$_4$), and evaporated to form a residue, which was purified by column chromatography (silica gel, 0–60% ethyl acetate in hexanes) to obtain the corresponding enol ether. To a stirred solution of the enol ether in THF (20 mL) was added LiHMDS (1.0 M in THF, 5.04 mmol) at −78 °C. The resulting red solution was stirred at −78 °C for 15 min before being treated with a solution of aldehyde 23 (0.44 g, 2.01 mmol) in THF (5 mL). The reaction mixture was stirred at RT for 1 h, and saturated aqueous NH$_4$Cl was added. The aqueous phase was extracted with EtOAc (3 × 20 mL), and the combined organics were dried over Na$_2$SO$_4$ and evaporated to form a residue, which was purified by column chromatography (silica gel, 0–10% ethyl acetate in hexanes) to obtain the corresponding enol ether. To a stirred solution of the enol ether in THF (10 mL) was added 4 N HCl (0.20 mL) at 0 °C. The resulting solution was allowed to stir at RT for 30 min and was then poured into water (40 mL) and extracted with ether (4 × 30 mL). The combined organic layers were washed with saturated NaHCO$_3$ and brine, dried, and concentrated. Purification of the resulting residue by flash chromatography (silica gel, 0–10% ethyl acetate in hexanes) furnished aldehyde 24 (0.38 g, 80%) as a colorless...
oil, [α]D20 = 42.6 (c 2.95, CHCl3). 1H NMR (400 MHz, CDCl3): δ 7.95 (1H), 7.15 (dt, J = 5.4, 1.6 Hz, 1H), 5.01 (p, J = 6.2 Hz, 1H), 2.40–2.36 (m, 1H), 2.34 (t, J = 2.1 Hz, 2H), 1.97–1.75 (m, 3H), 1.38 (dd, J = 8.2, 5.4 Hz, 1H), 1.36–1.22 (m, 1H), 1.22 (d, J = 6.3 Hz, 6H), 0.94 (s, 3H). 13C NMR (100 MHz, CDCl3): δ 201.7, 166.4, 136.8, 129.0, 20.1, 19.9, 19.4, 18.8, 17.6, 16.3, 13.8, 13.3.

Isosaprophyll (1R,6S,7R)-7-(6-Ethoxy-4-methyl-5-oxopent-3-en-1-yl)-7-methylbicyclo[4.1.0]hept-2-ene-3-carboxylate (25). The title compound, a colorless oil, was prepared similar to compound 24. [α]D20 = 92.7 (c 2.55, CHCl3). 1H NMR (400 MHz, CDCl3): δ 7.95 (1H), 7.15 (dt, J = 5.6, 1.6 Hz, 1H), 5.03 (p, J = 6.3 Hz, 1H), 2.53 (td, J = 7.7, 1.8 Hz, 2H), 2.47–2.26 (m, 1H), 2.01–1.43 (m, 5H), 1.24 (d, J = 6.3 Hz, 7H), 1.13 (td, J = 6.2, 3.1 Hz, 1H), 0.87 (s, 3H). 13C NMR (100 MHz, CDCl3): δ 202.1, 166.7, 137.8, 128.4, 67.3, 41.5, 32.6, 21.9, 21.8, 21.6, 16.9, 12.9. HRMS (ESI-TOF) m/z calcd for C16H22N2O4 [M + Na]+: 273.1461. Found: 273.1462.

Isosaprophyll (1R,6S,7R)-7-(6-Ethoxy-4-methyl-5-oxopent-3-en-1-yl)-7-methylbicyclo[4.1.0]hept-2-ene-3-carboxylate (26). To a stirred suspension of NaH (60% dispersion in mineral oil, 0.06 g, 0.22 mmol) in THF (5 mL) and saturated sodium potassium tartrate (15 mL) and stirred at room temperature. The reaction mixture was quenched with saturated NH4Cl, extracted with EtOAc, and washed with water and brine. The combined organic layers were dried over Na2SO4 and concentrated in vacuo to furnish a mixture of E and Z diene esters. The resulting residue was purified by flash column chromatography (silica gel, 0–10% ethyl acetate in hexanes) to afford E-isomer 26 as a colorless oil (0.24 g, 82% yield) and Z-isomer (0.03 g, 12% yield), [α]D20 = 51.1 (c 2.77, CHCl3). 1H NMR (400 MHz, CDCl3): δ 7.17 (dt, J = 5.6, 1.6 Hz, 1H), 6.73 (tt, J = 7.4, 1.5 Hz, 1H), 5.03 (p, J = 6.3 Hz, 1H), 4.17 (q, J = 7.1 Hz, 2H), 2.45–2.30 (m, 1H), 2.26 (q, J = 7.8 Hz, 2H), 2.02–1.68 (m, 3H), 1.82 (s, 3H), 1.58–1.41 (m, 1H), 1.27 (t, J = 7.1 Hz, 4H), 1.42 (d, J = 6.3 Hz, 7H), 1.13 (td, J = 6.2, 3.0 Hz, 1H), 0.89 (s, 3H). 13C NMR (100 MHz, CDCl3): δ 168.1, 166.8, 161.4, 138.1, 128.1, 127.7, 67.3, 60.5, 41.9, 32.3, 26.8, 24.8, 23.2, 21.9, 21.8, 21.6, 16.9, 14.2, 13.0, 12.2. HRMS (ESI-TOF) m/z calcd for C16H22N2O4 [M + Na]+: 273.1450. Found: 273.1451.

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