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PAPER

6,6-Spiroimine analogs of (–)-gymnodimine A: synthesis and biological evaluation on nicotinic acetylcholine receptors†

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Simple models of the spiroimine core of (–)-gymnodimine A have been synthesized in racemic and optically active forms. The quaternary carbon of the racemic spiroimines was created by Michael addition of a β-ketoester to acrolein, whereas the asymmetric allylic alkylation of the same β-ketoester was used to access the spiroimines in an enantioselective fashion. Both racemic and enantio-enriched mixtures were tested for their biological activities on *Xenopus* oocytes either expressing (human α4β2) or having incorporated (*Torpedo* $\alpha 1_2\beta\gamma\delta$) nicotinic acetylcholine receptors (nAChRs). These spiroimine analogs of (–)-gymnodimine A inhibited acetylcholine-evoked nicotinic currents, but were less active than the phycotoxin. Our results reveal that the 6,6-spiroimine moiety is important for the blockade of nAChRs and support the hypothesis that it is one of the pharmacophores of this group of toxins.

Introduction

(–)-Gymnodimine A (**1**), first isolated from oysters collected off the coast of New Zealand,[1] is a macrocyclic imine phycotoxin produced by the dinoflagelate *Karenia selliformis* (Fig. 1).[2] Shellfish can concentrate this phycotoxin through filter-feeding on toxic dinoflagellates, acting thereafter as primary vectors for transferring this toxic chemical compound to crabs, fish, birds, marine mammals and ultimately to humans.[3]

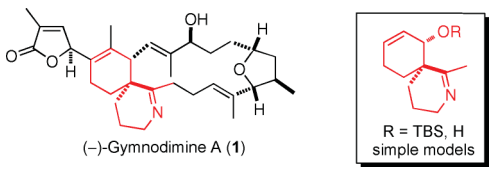

Fig. 1 Structure of (–)-gymnodimine A (**1**) and simple models studied.

(–)-Gymnodimine A (**1**) exhibits acute toxicity in a mouse bioassay[4] by intraperitoneal injection (LD_{50} = 80 μg kg^{-1})[5] or by oral administration (LD_{50} = 755 μg kg^{-1}).[6] Recently, (–)-gymnodimine A (**1**) was shown to bind and block different subset of muscle and neuronal nicotinic acetylcholine receptors (nAChRs).[7] The biological target was then confirmed by the co-crystallization of two representative members of the spiroimine family, (–)-gymnodimine A (**1**) and 13-desmethyl spirolide C, with the acetylcholine binding protein (AChBP), a soluble surrogate for the ligand binding domain of α7 nAChR subtype.[7] This study allowed the identification of the structural determinants in both (–)-gymnodimine A (**1**) and AChBP, which are important for toxin–receptor interaction. Among them, it was shown that the protonated imine nitrogen of the toxin plays a key role in its binding to the acetylcholine binding site of AChBP. These results corroborate the hypothesis that the spiroimine is one of the pharmacophores of (–)-gymnodimine A (**1**) and other members of the family.[8,9]

Given the importance of the spiroimine core as a key structural feature responsible for the toxicity of the phycotoxin (**1**), we report herein racemic and enantioselective syntheses of 6,6-spiroimine analogs of (–)-gymnodimine A (**1**) and their biological activities on nAChRs.

Results

Synthesis of racemic and optically active spiroimines 9 and 11

The synthesis of racemic spiroimines (±)-**9** and (±)-**11** were achieved starting from unsaturated β-ketoester **2** (Scheme 1).[10] Conjugate addition of β-ketoester **2** with acrolein was catalyzed by triethylamine and afforded the aldehyde (±)-**3** in 61% yield. Double reduction of the keto-aldehyde (±)-**3** was carried out in a sequential one-pot operation. Treatment of (±)-**3** with $NaBH_4$ was followed, upon completion of the reduction of the aldehyde, by addition of $CeCl_3 \cdot 7H_2O$ and additional $NaBH_4$. This one-pot

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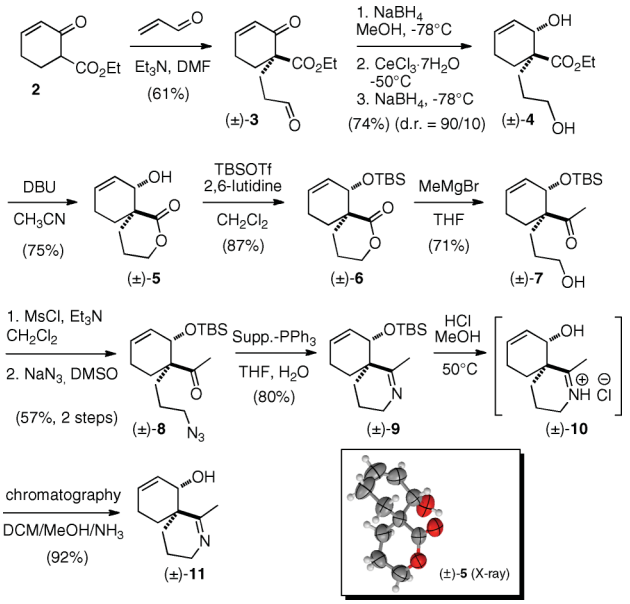
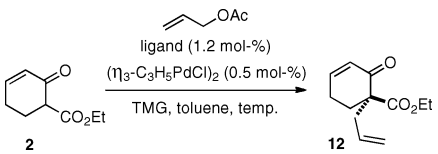


Scheme 1 Racemic syntheses of spiroimines (±)-**9** and (±)-**11**.

Table 1 Ligand screening results

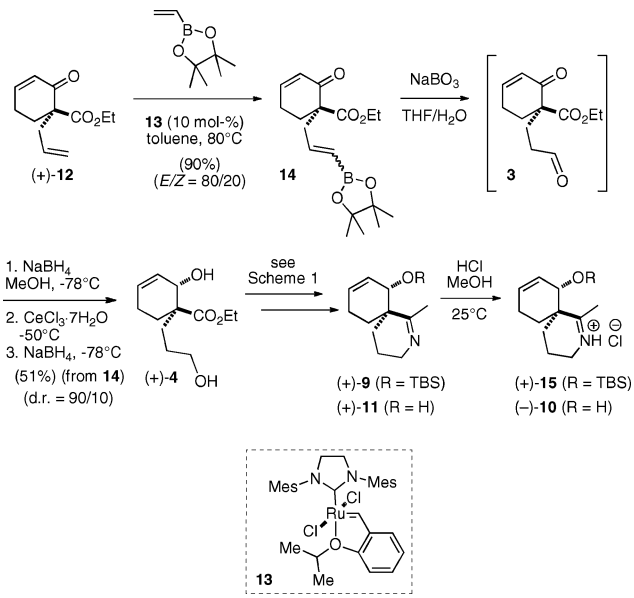


Entry	Ligand	T/°C	Yield (%)	e.r.
1	(*R*)-BINAP	25	35	40 : 50
2	(*R*,*R*)-DUPHOS	25	26	52 : 48
3	(*S*,*S*)-DIOP	25	27	52 : 48
4	(*S*)-*t*BuPHOX	25	41	62 : 38
5	(*R*,*R*)-DACH pyridyl Trost ligand	25	—	—
6	(*R*,*R*)-DACH naphtyl Trost ligand	25	59	14 : 86
7	(*R*,*R*)-ANDEN phenyl Trost ligand	25	30	43 : 57
8	(*S*,*S*)-DACH phenyl Trost ligand	25	40	88 : 12
9	(*S*,*S*)-DACH phenyl Trost ligand	–20	87	91 : 9
10	(*S*,*S*)-DACH phenyl Trost ligand	–78	50	82 : 18

procedure allowed us to obtain diol (±)-**4** in 74% isolated yield (d.r. = 90/10). The major diastereomer was then separated by chromatography and treated with DBU in acetonitrile to afford the key lactone (±)-**5** in 75% yield, with relative configuration secured by X-ray analysis.[11] The allylic alcohol (±)-**5** was protected with a TBS group to give lactone (±)-**6** in 87% yield. Lactone ring opening with methyl magnesium bromide selectively afforded the methyl ketone (±)-**7** in good yield (71%). The primary alcohol of (±)-**7** was then converted into the azide (±)-**8**, which was reduced with supported triphenylphosphine to give spiroimine (±)-**9**. The silyl group was removed under acidic conditions, which proved to be compatible with the imine functionality.[12] The resulting iminium salt (±)-**10** was then purified under basic conditions to afford the imine (±)-**11**. It is worth mentioning that (±)-**9** and (±)-**10** exist in solution as their imine and iminium forms, respectively. By contrast, compound (±)-**11** is in equilibrium with its enamine form in $CDCl_3$ (imine/enamine: 93/7).

With a reliable route to racemic spiroimines, we then focused on an enantioselective synthesis of **9** and **11**. The use of chiral amines derived from *Cinchona* alkaloids in the asymmetric Michael addition of **2** with acrolein resulted in low e.e. The asymmetric syntheses of spiroimines **9** and **11** were thus achieved by using an asymmetric allylic alkylation.[13] To the best of our knowledge, there has been no example of asymmetric allylic alkylation with unsaturated β-ketoesters such as **2**. We thus tested a range of chiral ligands (Table 1) and found that (*S*,*S*)-DACH phenyl Trost ligand gave the best results in terms of yield and enantiomeric ratio at –20 °C (entry 9).[14]

Functionalization of the allyl group was achieved by olefin cross-metathesis of (+)-**12** with vinyl pinacol boronate, catalyzed by Hoveyda–Grubbs second generation catalyst **13**,[15] to give vinyl boronate **14** in 90% yield as a mixture of isomers (*E*/*Z* = 80/20) (Scheme 2).[16] This intermediate underwent oxidation upon treatment with sodium perborate to afford aldehyde **3**, which was directly reduced under previously optimized conditions to give diol (+)-**4** in 51% yield from **14**.[17] The optically active spiroimines (+)-**9** and (+)-**11** (e.r. = 91 : 9) were obtained as described in Scheme 1. Both spiroimines were converted into the hydrochloride salts (+)-**15** and (–)-**10** to evaluate their biological activities.



Scheme 2 Enantioselective syntheses of spiroimines (+)-**9** and (+)-**11** and their corresponding iminiums (+)-**15**, (–)-**10**.

Biological evaluation of spiroimines 9, 11 and their hydrochloride salts 10, 15 on nAChRs

To determine whether the 6,6-spiroimine group plays a key role in the interaction of (–)-gymnodimine A (**1**) with nAChRs, we first evaluated the biological activity of (±)-**9** on *Xenopus* oocytes expressing the human α4β2 nAChR using the voltage-clamp technique[7] (Fig. 2A). Maximal peak amplitude of the acetylcholine (ACh)-induced current (IACh) was reached following 4 s superfusion with 150 μM ACh, after which nAChRs became slowly desensitized (Fig. 2A, a, black superimposed trace). Superfusion of 2 μM (±)-**9** by itself did not elicit nicotinic current responses, but in the presence of the ACh agonist (150 μM), it reduced the peak amplitude and modified the kinetics of the IACh (Fig. 2A, b, red superimposed trace). IACh peak current blockade

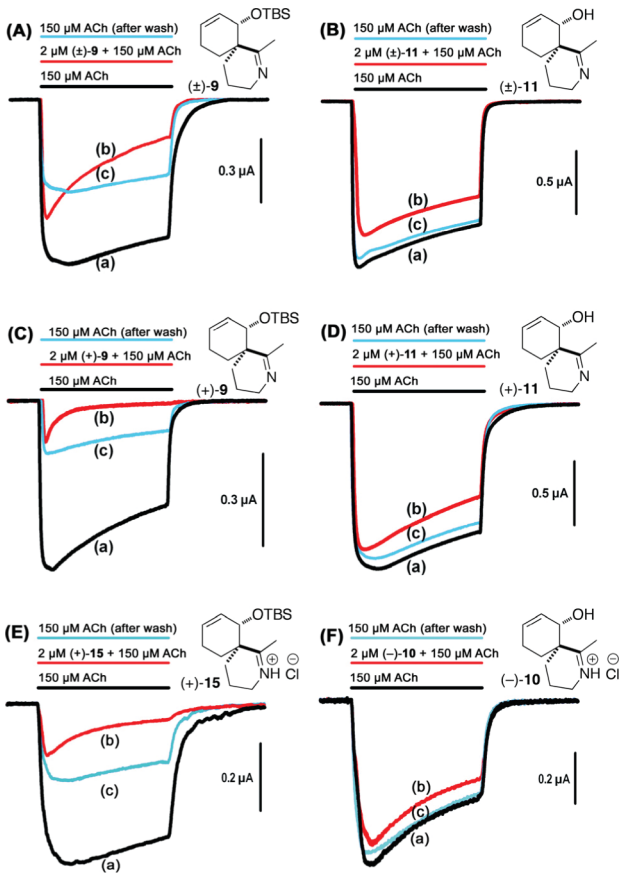


Fig. 2 Examples of the effects of racemic (A,B), optically active (C,D) and hydrochloride salts (E,F) of synthetic spiroimines on human α4β2 nAChRs expressed in *Xenopus* oocytes. (A,B) ACh-evoked current recorded at a holding potential of –60 mV, before (black trace, a), and during the action of 2 μM (±)-**9** with 150 μM ACh (red trace b, in A), or 2 μM (±)-**11** with 150 μM ACh (red trace b in B), followed by 150 s washout of the spiroimine compounds from the medium (blue traces c in A and B). The lines above the current traces indicate the time ACh was perfused, before during or 150 s after the spiroimine compounds. (C,D) show ACh-evoked current recorded before (black trace, a), during the action of 2 μM (+)-**9** with 150 μM ACh (C) and 2 μ M (+)-**11** with 150 μM ACh (D) (red trace b in C and D), and after 150 s washout of the spiroimine molecules from the medium (blue trace c in C and D). (E,F) Data obtained with the hydrochloride salts (+)-**15** and (–)-**10** (2 μM) under the same experimental conditions as in A–D.

by (±)-**9** was not immediately reversible after 150 s wash out of the compound from the medium (Fig. 2A, c, blue superimposed trace). Interestingly, upon 150 s wash out of (±)-**9**, the kinetics of the IACh were similar to the control despite the incomplete recovery of the peak amplitude. The optically active spiroimine (+)-**9** (e.r. = 91/9) showed a similar, but stronger, blocking potency on the human α4β2 nAChR than did the racemic (±)-**9** (Fig. 2C). Thus, (+)-**9**, when tested at a concentration of 2 μM in the presence of 150 μM ACh, blocked 89 ± 4.76% (n = 6) of the IACh peak amplitude (Fig. 2C, b, and Fig. 3) compared to the 31.2 ± 3.02% blockade by (±)-**9** at the same concentration (n = 6) (Fig. 2A, b). Furthermore, the current kinetics was 3.4 ± 0.15 times faster with the enantiomeric (+)-**9** (1.87 ± 0.3 s) than with the racemic compound (±)-**9** (6.37 ± 0.39 s), as evaluated by determining the half decay time of the currents.

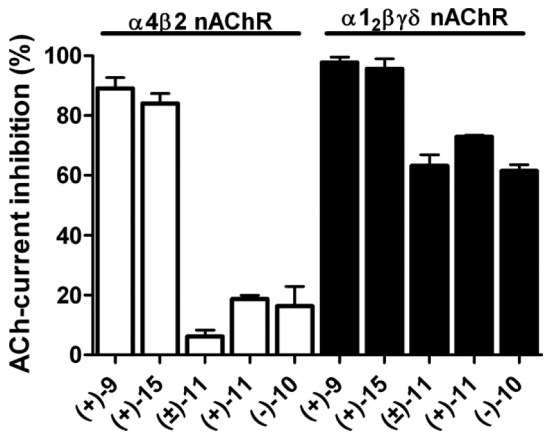


Fig. 3 Inhibition of ACh-induced nicotinic current by synthetic spiroimines in oocytes expressing the human neuronal α4β2 nAChR (white colums), or having incorporated to their membrane the muscle-type *Torpedo* α1$_2$βγδ nAChR (black columns). Oocytes were voltage-clamped at –60 mV and first perfused with 150 μM ACh for 15 s, followed by 150 s washout of ACh with the Ringer solution in order to obtain the control current values. Thereafter, the same oocyte was perfused with 2 μM of one of the synthetic spiroimines for 60 s followed by 15 s perfusion with 150 μM ACh + 2 μM of one of the synthetic spiroimines. Each column represents mean values ± SEM; with n = 5–8 oocytes (from at least 3 different donors per experimental condition). The ACh peak currents in the presence of the spiroimine compounds were normalized to control currents recorded from the same oocyte, to yield fractional (%) response data.

The biological activity of racemic (±)-**11** and the enantiomer (+)-**11** (e.r. = 91/9) compounds, lacking the TBS group, were examined on the human α4β2 nAChR in the same experimental conditions as described above (2 μM (±)-**11** and 2 μM (+)-**11** in the presence of 150 μM ACh). Both spiroimines showed a weak antagonistic activity towards the α4β2 nAChR and did not affect current kinetics, as shown in the representative recordings (Fig. 2B and D). Furthermore, as shown in Fig. 2 E and F, the iminium salts (+)-**15** and (–)-**10** exhibited similar activities regarding both current kinetics and percent blockade of the IACh peak amplitude (84 ± 4.76 and 16.34 ± 8.73, respectively, n = 4) as (+)-**9** and (+)-**11** (Fig. 2, C, D).

In addition, spiroimines (+)-**9**, (±)-**11**, (+)-**11**, (+)-**15** and (–)-**10** were tested on oocytes having the *Torpedo* muscle-type α1$_2$βγδ nAChR incorporated.[7] As shown in Fig. 3, (+)-**9** and (+)-**15** exhibited the highest blocking potency, as compared to equimolecular concentrations of (±)-**11**, (+)-**11** and (–)-**10**, not only towards the muscle-type α1$_2$βγδ nAChR, but also towards the α4β2 nAChR. It should be noted that blockade of nicotinic currents induced by ACh on both *Torpedo* α1$_2$βγδ and human α4β2 nAChRs by (+)-**9** and (+)-**15** was obtained with 40–45 times lower (–)-gymnodimine A concentrations,[7] indicating that the spiroimine analogs are less active than the native toxin.

Discussions

The present work shows for the first time that synthetic spiroimines possess an antagonist activity towards both neuronal- (α4β2) and muscle-type (α1$_2$βγδ) nAChRs. Such activity not only differs in potency between (±)-**9** and (±)-**11** in both α4β2 and α1$_2$βγδ nAChRs as compared to (+)-**9** and (+)-**11** (the racemic compounds

being in general less potent), but also compounds **9** affected the kinetics of the ACh-induced current while compounds **11** did not. A possible explanation for the blocking activity revealed by compounds **9** may arise from the presence of the TBS groups used to protect the allylic alcohol **5**. Since both compounds **9** and **11** were unable, by themselves, to exert an agonist action on nAChRs and to induce nicotinic currents through the nAChR, it is likely that the changes in nicotinic current kinetics induced by **9** may reflect a nAChR channel block triggered once the nAChR channels are being activated by ACh. Alternatively, changes in current kinetics may reflect changes in the desensitization rate of nAChRs. Further work will be needed to clarify these two possibilities. The imines (+)-**9** and (+)-**11** and their corresponding iminium salts (+)-**15** and (−)-**10** showed similar activities. Considering the pK_a of imines (+)-**9** and (+)-**11** (pK_a = 8.8 ± 0.4),[18] they might be protonated in the physiological medium buffered at pH = 7.4. This could explain why both spiroimines (+)-**9**, (+)-**11** and iminiums (+)-**15**, (−)-**10** have a similar activity on nAChRs. Whatever the molecular mechanism involved in the action of compounds **9** and **11**, our results show that the spiroimine constituent of gymnodimines A, B and C is one of the essential groups for interacting with the nAChRs. In contrast, the tetrahydrofuran part of (−)-gymnodimine A did not inhibit the binding of biotinylated α-bungarotoxin to muscle-type ($\alpha1_2\beta\gamma\delta$) nAChRs, as recently reported using a non-radioactive ligand binding assay.[19] These results support the hypothesis that the 6,6-spiroimine core is the, or one of, the main pharmacophore(s) for the gymnodimine family of phycotoxins.

Conclusions

In conclusion, we have developed new synthetic routes to the 6,6-spiroimine skeleton of (−)-gymnodimine A **1**. The quaternary carbon of racemic spiroimines has been created by means of a Michael addition of a cyclic β-ketoester to acrolein. In turn, optically active spiroimines have been synthesized by using for the first time an asymmetric allylic alkylation. Biological evaluations revealed that racemic and optically active spiroimines **9** and **11**, and their corresponding hydrochloride salts **10** and **15**, blocked both neuronal- (α4β2) and muscle-type ($\alpha1_2\beta\gamma\delta$) nAChRs. These results indicate that the 6,6-spiroimine moiety is important for the blockade of nAChRs. A detailed structure–activity relationship study on the 6,6-spiroimine skeleton is currently under investigation, and will be reported in due time.

Experimental

Procedures and characterizations of new compounds

(±)-Ethyl 2-oxo-1-(3-oxopropyl)cyclohex-3-enecarboxylate 3. To a solution of β-ketoester **2** (200 mg, 1.19 mmol, 1eq.) and Et_3N (0.017 mL, 0.120 mmol, 0.1 eq.) in dry DMF (2.4 mL) was added acrolein (0.120 mL, 1.78 mmol, 1.5 eq.). The mixture was stirred overnight at room temperature and then water was added. The aqueous layer was extracted twice with EtOAc and the combined organic layers washed with water, then brine. The organic layer was dried over Na_2SO_4 and the solvent removed *in vacuo*. The crude mixture was purified over silica gel (heptane to heptane/EtOAc 8/2) to afford a yellow oil (*m* = 160.7 mg, 61%). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 9.7 (t, 1H, *J* = 1.5 Hz), 6,88–6.79 (m, 1H), 5.97 (dt, 1H, *J* = 10.0, 2.0 Hz), 4.11 (q, 2H, *J* = 7.1 Hz), 2.70–2.20 (m, 5H), 2.20–1.94 (m, 2H), 1.93–1.80 (m, 1H), 1.17 (t, 3H, *J* = 7.1 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 201.2 (Cq), 196.1 (Cq), 171.4 (Cq), 149.2 (CH), 129.2 (CH), 61.5 (CH_2), 55.9 (Cq), 39.5 (CH_2), 31.0 (CH_2), 25.8 (CH_2), 23.6 (CH_2), 14.1 (CH_3). IR ν (neat): 2929, 2359, 2339, 1723, 1681, 1446, 1388, 1240, 1192, 1094, 1021 (cm^{-1}). MS (ESI) *m/z*: 247.1 [M + Na]+, 279.1 [M + Na + MeOH]+. HRMS calcd for $C_{12}H_{16}O_4Na$+: 247.0946, found: 247.0941.

(±)-Ethyl 2-hydroxy-1-(3-hydroxypropyl)cyclohex-3-enecarboxylate 4. To a solution of ketoaldehyde **3** (70 mg, 0.312 mmol, 1 eq.) in MeOH (3.1 mL) was added $NaBH_4$ (5.9 mg, 0.156 mmol, 0.5 eq.) at −78 °C. The conversion was monitored by TLC. After completion of the reaction, $CeCl_3{\cdot}7H_2O$ (128 mg, 0.343 mmol, 1.1 eq.) was added at −78 °C. The suspension was warmed to −50 °C until all $CeCl_3{\cdot}7H_2O$ was dissolved. The solution was cooled back to −78 °C, then $NaBH_4$ (13 mg, 0.343 mmol, 1 eq.) was added. The solution was stirred for 1 h at −78 °C then saturated NH_4Cl was added dropwise at this temperature. The mixture was warmed to RT and then water was added. The aqueous layer was extracted twice with EtOAc. The combined organic layers were washed with water then brine, and dried over Na_2SO_4. The solvent was removed *in vacuo*, then the crude mixture purified through silica gel (heptane to heptane/EtOAc 5/5) to afford the major isomer as a colorless oil (*m* = 52.9 mg, 74%). ^{1}H NMR: (500 MHz, $CDCl_3$) δ 5.75–5.65 (m, 2H), 4.35 (m, 1H), 4.06 (q, *J* = 7.1 Hz, 2H), 3.59–3.41 (m, 4H), 2.02–1.92 (brm, 2H), 1.83–1.65 (m, 3H), 1.53–1.43 (m, 2H), 1.37 (m, 1H), 1.17 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR: (75 MHz, $CDCl_3$) δ 176.0 (Cq), 129.6 (CH), 128.1 (CH), 67.6 (CH), 63.0 (CH_2), 60.6 (CH_2), 48.9 (Cq), 28.3 (CH_2), 27.3 (CH_2), 25.8 (CH_2), 22.8 (CH_2), 14.2 (CH_3). IR ν (neat): 3343, 2933, 1719, 1018 cm^{-1}. MS: (ESI) *m/z*: 251 (100) [M + Na]. HRMS: calcd for $C_{12}H_{20}O_4Na$: 251.1259. found: 251.1265.

(+)-(6*R*,7*S*)-7-Hydroxy-2-oxaspiro[5.5]undec-8-en-1-one 5. The diol **4** (52.9 mg, 0.232 mmol, 1 eq.) was dissolved in acetonitrile (3 mL) and DBU (41 µL, 0.278 mmol, 1.2 eq.) was added. The solution was stirred at room temperature for 7 h and saturated NH_4Cl was added. The aqueous layer was extracted with EtOAc (×3). The combined organic layers were washed with brine, dried over Na_2SO_4 and the solvent removed *in vacuo*. The crude mixture was purified through silica gel (heptane to heptane/EtOAc: 6/4) to afford a white solid (*m* = 31.6 mg, 75%). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 5.69 (dd, 1H, *J* = 9.8, 2.2 Hz), 5.55 (dd, 1H, *J* = 9.8, 1.6 Hz), 4.92 (s, 1H), 4.36 (m, 2H), 2.15–2.04 (m, 4H), 1.96–1.86 (m, 2H), 1.71–1.66 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 176.8 (Cq), 129.6 (CH), 127.0 (CH), 71.2 (CH), 70.2 (CH_2), 46.8 (Cq), 30.0 (CH_2), 21.4 (×2) (CH_2), 21.1 (CH_2). IR ν (neat): 3419, 1693, 1260, 1164, 1061 (cm^{-1}). MS (ESI, *m/z*): 205.1 [M + Na]+. HMRS (ESI, *m/z*): Calcd for $C_{10}H_{14}O_3Na$+: 205.0841, found: 205.0834. m.p. = 97 °C.

(+)-(6*R*,7*S*)-7-((*tert*-Butyldimethylsilyl)oxy)-2-oxaspiro[5.5]-undec-8-en-1-one 6. To a solution of allylic alcohol **5** (29.1 mg, 0.160 mmol, 1 eq.) in dry dichloromethane (0.53 mL) was added 2,6-lutidine (27.9 µL, 0.240 mmol, 1.5 eq.). The reaction mixture was cooled to 0 °C and TBSOTf (40.3 µL, 0.176 mmol, 1.1 eq.) was added dropwise. The solution was stirred for 1 h at room

temperature and then quenched with a saturated solution of NH_4Cl. The aqueous layer was extracted with dichloromethane (×3). The combined organic extracts were washed with water, brine and dried over $MgSO_4$. The yellow residue was purified by flash chromatography (heptane to heptane/EtOAc 8/2) to give the protected alcohol as a yellow oil (m = 41.2 mg, 87%). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 5.63 (d, 1H, J = 10.5 Hz), 5.44 (d, 1H, J = 10.5 Hz), 4.83 (s, 1H), 4.32 (m, 2H), 2.17 (td, 1H, J = 11.8, 3.9 Hz) 2.04–1.83 (m, 5H), 1.61 (m, 2H), 0.88 (s, 9H), 0.08 (s, 3H), 0.06 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 176.3 (Cq), 130.5 (CH), 126.2 (CH), 72.7 (CH), 70.2 (CH_2), 47.1 (Cq), 30.4 (CH_2), 25.8 (CH_3), 22.0 (CH_2), 21.4 (×2) (CH_2), 17.9 (Cq), –4.6 (CH_3), –5.1 (CH_3). IR ν (neat): 1705, 1255, 833, 774 (cm^{-1}). MS (ESI, m/z): 319.2 [M + Na]+. HMRS (ESI, m/z): Calcd for $C_{16}H_{28}O_3SiNa$+: 319.1705, found: 319.1700. $[\alpha]_D^{25}$ +114.3 (c 0.222, $CHCl_3$, e.r. 91/09).

(+)-1-((1*R*,2*S*)-2-((*tert*-Butyldimethylsilyl)oxy)-1-(3-hydroxypropyl)cyclohex-3-en-1-yl)ethanone 7. To a solution of lactone **6** (31.8 mg, 0.107 mmol, 1 eq.) in dry THF (0.36 mL) at 0 °C was added MeMgBr (229 µL, 0.321 mmol, 3 eq.) and the reaction mixture was allowed to warm to room temperature. After 2 h, a saturated solution of NH_4Cl was added. The aqueous layer was extracted with dichloromethane (×3). The combined organic extracts were washed with water and brine, and dried over $MgSO_4$. The yellow residue was purified by flash chromatography (heptane to heptane/EtOAc 7/3) to give the methyl ketone **6** as a colorless oil (m = 23.7 mg, 71%). ^{1}H NMR (500 MHz, acetone) δ (ppm): 5.77 (m, 1H), 5.66 (m, 1H), 4.45 (d, 1H, J = 4.1 Hz), 3.53 (t, 1H, J = 6.4 Hz), 3.47 (m, 2H), 2.12 (s, 3H), 2.02–1.95 (m, 2H), 1.90–1.78 (m, 3H), 1.60 (td, 1H, J = 12.3, 3.4 Hz), 1.47–1.40 (m, 1H), 1.28–1.20 (m, 1H) 0.90 (s, 9H), 0.11 (s, 6H). ^{13}C NMR (75 MHz, acetone) δ (ppm): 210.9 (Cq), 130.4 (CH), 129.6 (CH), 67.6 (CH), 62.9 (CH_2), 56.2 (Cq), 28.3 (CH_3), 26.7 (CH_2), 26.3 (×3) (CH_3), 26.3 (CH_2), 26.1 (CH_2), 23.9 (CH_2), 18.7 (Cq), –3.3 (CH_3), –4.5 (CH_3). IR ν (neat): 3388, 1697, 1250, 1052, 832, 772 (cm^{-1}). MS (ESI, m/z): 335.2 [M + Na]+. HMRS (ESI, m/z) Calcd for $C_{17}H_{32}O_3SiNa$+: 335.2018, found: 335.2021. $[\alpha]_D^{25}$ +118.2 (c 0.29, $CHCl_3$, e.r. 91/09).

(+)-1-((1*R*,2*S*)-1-(3-Azidopropyl)-2-((*tert*-butyldimethylsilyl)oxy)cyclohex-3-en-1-yl)ethanone 8. To a solution of alcohol **7** (63.3 mg, 0.202 mmol, 1 eq.) in dry dichloromethane (2 mL) was added Et_3N (113 µL, 0.808 mmol, 4 eq.), then methanesulfonyl chloride (62.5 µL, 0.808 mmol, 4 eq.) at 0 °C. The mixture was stirred for 3 h and then a saturated solution of NH_4Cl was added. The aqueous layer was extracted with dichloromethane (×3). The combined organic extracts were washed with water, brine and dried over $MgSO_4$. The crude mixture was dissolved in DMSO (2 mL) and NaN_3 (39.4 mg, 0.606 mmol, 3 eq.) was added at RT. The mixture was stirred overnight at RT then brine was added. The aqueous layer was extracted with dichloromethane (×3). The combined organic extracts were washed with water, brine and dried over Na_2SO_4. The residue was purified by flash chromatography (heptane to heptane/EtOAc 9/1) to give the desired alkyl azide as a colorless oil (m = 38.7 mg, 57%). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 5.78–5.70 (m, 2H), 4.43 (d, 1H, J = 3.8 Hz), 3.32 (m, 1H), 3.20 (m, 1H), 2.14 (s, 3H), 2.08–1.94 (m, 2H), 1.92–1.87 (m, 1H), 1.87–1.84 (m, 2H), 1.59–1.54 (m, 2H), 1.38–1.30 (m, 1H), 0.88 (s, 9H), 0.07 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 211.5 (Cq), 129.2 (CH), 128.9 (CH), 66.6 (CH), 55.5 (Cq), 51.9 (CH_2), 30.1 (CH_2), 26.0 (CH_3), 25.9 (CH_2), 25.8 (CH_3), 23.7 (CH_2), 23.1 (CH_2), 18.0 (Cq), –0.35 (CH_3), –4.8 (CH_3). IR ν (neat): 2093, 1698; 1250, 1054, 834; 774 (cm^{-1}). MS: (ESI, m/z): 360.2 [M + Na]+. HMRS: (ESI, m/z) Calcd for $C_{17}H_{31}N_3O_2SiNa$+: 360.2083, found: 360.2072. $[\alpha]_D^{25}$ +103.8 (c 0.34, $CHCl_3$, e.r. 91/09).

(+)-(6*S*,7*S*)-7-((*tert*-Butyldimethylsilyl)oxy)-1-methyl-2-azaspiro[5.5]undeca-1,8-diene 9. To a solution of azide **8** (38.7 mg, 0.115 mmol, 1 eq.) in a mixture of THF/H_2O (v/v = 9/1) (1 mL) was added supported triphenylphosphine (1.6 mmol g^{-1}) (107.5 mg, 0.172 mmol, 1.5 eq). The mixture was stirred at RT overnight then filtered. The polymer was washed with CH_2Cl_2 (×3) and the solvent removed *in vacuo*. The crude mixture was purified over silica gel (DCM(1%NH_3)/MeOH: 100/0 to 95/5) to afford a colorless oil (m = 27 mg, 80%). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 5.65 (dd, 1H, J = 10.5, 1.9 Hz), 5.48 (dq, 1H, J = 10.5, 1.9 Hz), 4.64 (s, 1H), 3.55 (m, 2H), 2.09 (s, 3H), 2.07–2.01 (m, 3H), 1.85 (m, 2H), 1.56 (m, 2H), 1.47 (m, 1H), 0.87 (s, 9H), 0.08 (s, 3H), 0.06 (s, 3H). ^{13}C NMR (75 Mhz, $CDCl_3$) δ (ppm): 171.9 (Cq), 130.7 (CH), 126.8 (CH), 71.4 (CH), 49.8 (CH_2), 43.3 (Cq), 29.7 (CH_2), 25.7 (CH_3), 22.5 (CH_3), 22.2 (CH_2), 21.6 (CH_2), 19.9 (CH_2), 18.0 (Cq), –3.9 (CH_3), –4.8 (CH_3). IR ν (neat): 2927, 1649, 1253, 1093, 836 (cm^{-1}). MS: (ESI, m/z): 294.2 [M + H]+. HMRS: (ESI, m/z) Calcd for $C_{17}H_{32}NOSi$+: 294.2253, found: 294.2255. $[\alpha]_D^{25}$ +81.0 (c 0.21, $CHCl_3$, e.r. 91/09).

(+)-((6*S*,7*S*)-7-((*tert*-Butyldimethylsilyl)oxy)-1-methyl-2-azaspiro[5.5]undeca-1,8-dien-2-ium chloride 15. To a solution of imine **9** (6 mg, 0.020 mmol) in DCM/MeOH (v/v = 9/1, 2 mL) was added HCl 6 N (0.04 mL) and the mixture was stirred at room temperature for 30 min. The solvent was evaporated *in vacuo* to afford **15** as a colorless oil (m = 6.5 mg, quant.).

^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 5.80 (m, 1H), 5.59 (m, 1H), 4.90 (s, 1H), 3.66 (m, 2H), 2.51 (s, 3H), 2.23–2.13 (m, 4H), 2.08 (m, 1H), 1.88 (m, 1H), 1.79 (m, 1H), 1.73 (m, 1H), 0.90 (s, 9H), 0.17 (s, 3H), 0.11 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm): 197.9 (Cq), 129.8 (CH), 128.8 (CH), 73.1 (CH), 47.4 (Cq), 46.8 (CH_2), 30.7 (Cq), 30.0 (CH_2), 26.2 (CH_3), 22.0 (CH_2), 21.9 (CH_2), 18.6 (CH_2), –3.8 (CH_3), –4.7 (CH_3). IR ν (neat): 3376, 3032–2856, 1681, 1090, 836 (cm^{-1}). MS: (ESI, m/z): 294.2 [M]+. HMRS: (ESI, m/z) Calcd for $C_{17}H_{32}NOSi$+: 294.2253, found: 294.2253. $[\alpha]_D^{25}$ +50 (c 0.26, MeOH, e.r. 91/09).

(+)-(6*S*,7*S*)-1-Methyl-2-azaspiro[5.5]undeca-1,8-dien-7-ol 11. To a solution of imine **9** (30.3 mg, 0.103 mmol, 1 eq.) in MeOH (1 mL) was added concentrated HCl (0.1 mL) and the mixture was stirred at 50 °C for 3 h. The crude mixture was purified over silica gel (DCM(1%NH_3)/MeOH: 100/0 to 90/10) to afford **11** as a white solid (m = 17 mg, 92%). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): imine 5.68 (m, 1H), 5.54 (dd, 1H, J = 10.1, 1.8 Hz), 4.66 (s, 1H), 3.53 (m, 2H), 2.94 (brs, 1H), 2.05 (m, 2H), 2.01 (brs, 3H), 1.90–1.78 (m, 2H), 1.72 (m, 1H), 1.62–1.54 (m, 2H), 1.51 (m, 1H). Enamine 5.85 (m, 1H), 5.75 (m, 1H), 4.05 (d, 1H, J = 4.5 Hz), 3.68 (m, 1H), 2.28 (m, 1H), other signal are masked by the imine form. ^{13}C NMR (75 Mhz, $CDCl_3$) δ (ppm): 172.4 (Cq), 129.7 (CH), 127.7 (CH), 70.6 (CH), 49.6 (CH_2), 43.1 (Cq), 29.3 (CH_2), 22.3 (CH_3), 21.6 (CH_2), 21.6 (CH_2), 19.7 (CH_2). IR ν (neat): 3112, 3022–2742, 1645, 1068 (cm^{-1}). MS: (ESI, m/z): 180.1 [M + H]+. HMRS: (ESI, m/z) Calcd for $C_{11}H_{18}NO$: 180.1388,

found: 180.1380. mp = 110–112 °C. $[\alpha]_D^{25}$ +5.0 (*c* 0.42, $CHCl_3$, e.r. 91/09).

(–)-(6*S*,7*S*)-7-Hydroxy-1-methyl-2-azaspiro[5.5]undeca-1,8-dien-2-ium chloride 10. To a solution of imine **11** (5 mg, 0.028 mmol) in MeOH (0.17 mL) was added concentrated HCl (0.02 mL), and the mixture was stirred at room temperature for 3 h. The solvent was evaporated *in vacuo* to afford **10** as a colorless oil (*m* = 6 mg, quant.). ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 5.78 (m, 1H), 5.59 (m, 1H), 4.74 (s, 1H), 3.67 (m, 2H), 2.51 (s, 3H), 2.19–2.13 (m, 2H), 2.12–1.98 (m, 3H), 1.91 (m, 1H), 1.83–1.71 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm): 196.0 (Cq), 130.2 (CH), 128.3 (CH), 70.7 (CH), 46.9 (CH_2), 46.7 (Cq), 30.2 (CH_2), 22.0 (CH_2), 21.1 (CH_3), 21.0 (CH_2), 18.4 (CH_2). IR ν (neat): 3331, 2996–2652, 1679, 1066 (cm^{-1}). MS: (ESI, *m*/*z*): 180.1 [M]+. HMRS: (ESI, *m*/*z*) Calcd for $C_{11}H_{18}NO$: 180.1388, found: 180.1383. $[\alpha]_D^{25}$ –8.3 (*c* 0.12, MeOH, e.r. 91/09).

(+)-(*R*)-Ethyl 1-allyl-2-oxocyclohex-3-enecarboxylate 12. To a degassed solution of allylpalladium chloride dimer (3.26 mg, 0.0089 mmol, 0.5 mol%) and (*S*,*S*)-DACH-phenyl Trost ligand (14.8 mg, 0.0214 mmol, 1.2 mol%) in toluene (3.7 mL) was added degassed allyl acetate (289 µL, 2.675 mmol, 1.5 eq.). The initial clear orange solution faded and became cloudy. Degassed 1,1,3,3-tetramethylguanidine (269 µL, 2.14 mmol, 1.1 eq.) was added and the mixture returned to a clear orange solution. A solution of ethyl-2-oxocyclohex-3-ene-1-carboxylate **2** (293.3 mg, 1.74 mmol, 1 eq.) in toluene (0.7 mL) was added slowly and the reaction stirred overnight at –20 °C under argon atmosphere. The reaction mixture was quenched with saturated aqueous NH_4Cl and the layers were separated. The aqueous layer was extracted with Et_2O. The combined organic extracts were washed with brine and water, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The resulting oil was purified by flash chromatography (heptane to heptane/EtOAc 90/10) to give the allyl ketone **12** as a colorless oil (317.6 mg, e.r. 91/09. 82%). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.91 (dt, 1H, *J* = 10.1, 4.2 Hz), 6.05 (dt, 1H, *J* = 10.0, 2.0 Hz), 5.85–5.70 (m, 1H), 5.13 (m, 1H), 5.08 (m, 1H), 4.17 (q, 2H, *J* = 7.2 Hz), 2.67 (dt, 1H, *J* = 13.9, 7.3 Hz), 2.54 (dt, 1H, *J* = 13.9, 7.3 Hz), 2.55–2.22 (m, 3H), 2.01–1.91 (m, 1H), 1.24 (t, 3H, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 195.7 (Cq), 171.1 (Cq), 149.6 (CH), 133.3 (CH), 129.0 (CH), 118.8 (CH_2), 61.3 (Cq), 56.6 (CH_2), 38.3 (CH_2), 29.9 (CH_2), 23.6 (CH_2), 14.1 (CH_3). IR ν (neat): 1739, 1726, 1678, 1622, 1190, 919, 670 (cm^{-1}). MS (ESI+) *m*/*z*: 231.1 [M + Na]+. HRMS calcd for $C_{12}H_{16}O_3Na$: 231.0997, found: 231.0994. HPLC: Heptane/*i*PrOH (90/10), column IC 5 µm (4.6 × 250mm), injection volume 10 µL, flow: 0.8 mL min^{-1}: e.r. 91/09; $[\alpha]_D^{25}$ +54.3 (*c* 0.2, $CHCl_3$, e.r. 91/09).

Absolute configuration determination: To a solution of (*R*)-ethyl 1-allyl-2-oxocyclohex-3-enecarboxylate (100.0 mg, 0.48 mmol, 1.0 eq.; e.r. 91/9) in THF (960 µL) at –78 °C, was added L-selectride (480 µL, 0.48 mmol, 1.0 eq.). After 1 h at this temperature, the mixture was quenched with water and extracted with ethyl acetate. The combined organic extracts were washed with brine and water, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The resulting oil was purified by flash chromatography (heptane to heptane/EtOAc 90/10) to give the allyl ketone as a colorless oil (72.0 mg, 71%). All spectroscopic data are in agreement with those reported by Trost *et al*.[13e] HPLC: heptane/*i*PrOH (95/5), column IC 5 µm (4,6 × 250mm), injection volume 10 µL, flow: 0.8 mL min^{-1}: e.r. 91/09. $[\alpha]_D^{25}$ +106.4 (*c* 0.232, $CHCl_3$). Reported for *S* enantiomer e.r. 93/07: $[\alpha]_D^{25}$ –120.7 (*c* 1.0, $CHCl_3$).

(*R*)-Ethyl 2-oxo-1-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)allyl)cyclohex-3-enecarboxylate 14. To a stirred and degassed solution of allyl ketone **12** (300.0 mg, 1.44 mmol, 1.0 eq.) and vinyl boronic acid pinacol ester (1.2 mL, 7.2 mmol, 5.0 equiv.) in toluene (14.5 mL) was added Hoveyda–Grubbs' catalyst II **13** (90.2 mg, 0.14 mmol, 10 mol%). The mixture was heated at 80 °C for 20 h then cooled to ambient temperature. Solvent was removed *in vacuo* and the residue was purified by flash column chromatography (heptane to heptane/EtOAc 90/10). The product **14** was obtained as a slightly pink oil (418.4 mg, 90%) as a mixture of (*E*)- and (*Z*)-boronate (80/20). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): major 6.92 (td, 1H, *J* = 10.0, 3.7 Hz), 6.53 (td, 1H, *J* = 17.8, 7.1 Hz), 6.06 (dt, 1H, *J* = 10.1, 2.0 Hz), 5.54 (d, *J* = 17.7 Hz), 4.19 (q, 2H, *J* = 7.1 Hz), 2.78 (dd, 1H, *J* = 13.9, 7.1 Hz), 2.67 (dd, 1H, *J* = 13.9, 7.1 Hz), 2.58–2.48 (m, 1H), 2.45 (dt, 1H, *J* = 13.6, 4.3 Hz), 2.39–2.30 (m, 1H), 1.97 (ddd, 1H, *J* = 13.7, 9.0, 4.7 Hz), 1.32–1.22 (m, 15H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 195.4 (Cq), 171.0 (Cq), 149.7 (CH), 148.1 (CH), 129.0 (CH), 83.4 (Cq), 83.2 (Cq), 61.4 (CH_2), 56.6 (Cq), 40.2 (CH_2), 29.8 (CH_2), 24.8 (2 × CH_3), 24.7 (2 × CH_3), 23.6 (CH_2), 14.1 (CH_3). IR ν (neat): 2978, 1729, 1686, 1637, 1444, 1359, 1323, 1143, 970, 848 (cm^{-1}). MS (ESI+) *m*/*z*: 357.2 [M + Na]+. HRMS calcd for $C_{18}H_{27}O_5Na_{11}B$: 357.1849, found: 357.1852.

(+)-Ethyl 2-hydroxy-1-(3-hydroxypropyl)cyclohex-3-enecarboxylate 4. To a stirred solution of boronate ester **14** (728.6 mg, 2.18 mmol, 1.0 equiv.) in THF/H_2O (v/v: 1/1) (44 mL) was added $NaBO_3$ (368.9 mg, 2.40 mmol, 1.1 equiv.) and the resulting mixture was stirred at RT for 3 h 30 min. The reaction mixture was diluted with methyl *tert*-butyl ether (MTBE) and water was added. The aqueous layer was extracted with MTBE (×3) and the combined organic layers washed with brine then dried over Na_2SO_4. The solvent was removed *in vacuo*. The crude mixture was dissolved in MeOH (22 mL) and $NaBH_4$ (41.2 mg, 1.09 mmol, 0.5 eq.) at –78 °C was added. The conversion was monitored by TLC. After completion of the reaction, $CeCl_3 \cdot 7H_2O$ (893 mg, 2.40 mmol, 1.1 eq.) was added at –78 °C. The suspension was warmed to –50 °C until all $CeCl_3 \cdot 7H_2O$ was dissolved. The solution was cooled back to –78 °C, then $NaBH_4$ (82.4 mg, 2.18 mmol, 1 eq.) was added. The solution was stirred for 1 h at –78 °C, then saturated NH_4Cl was added dropwise at this temperature. The mixture was warmed to RT, then water was added. The aqueous layer was extracted twice with EtOAc. The combined organic layers were washed with water then brine, and dried over Na_2SO_4. The solvent was removed *in vacuo*, then the crude mixture purified through silica gel to afford the major isomer as a colorless oil (heptane to heptane/EtOAc 5/5) (*m* = 253.8 mg, 51%). $[\alpha]_D^{25}$ +130.9 (*c* 0.202, $CHCl_3$, e.r. 91/09).

Experimental of biological evaluation

Animals and biological materials. Adult female *Xenopus laevis* frogs were obtained from the Centre de Ressources Biologiques Xénopes – CNRS (Université de Rennes 1, France). Live *Torpedo marmorata* fishes were purchased from the *Service Modèles Biologiques* of the Station Biologique de Roscoff (France). Live animals were maintained in the Animal Campus facility of Gif sur

Yvette. Experiments were performed in accordance with European Community guidelines for laboratory animal handling and with the official edict presented by the French Ministry of Agriculture and the recommendations of the Helsinki Declaration. The cDNA coding for human α4β2 nAChR was kindly provided by Pr. J.-P. Changeux and Dr P. J. Corringer (Pasteur Institute, Paris, France)

***Torpedo* membrane preparation and microtransplantation to oocytes.** *Torpedo marmorata* specimens kept in conventional artificial seawater were anaesthetized with tricaine (Sigma-Aldrich.) at a concentration of 0.03% in seawater, before surgical excision of electric organs. The $\alpha1_2\beta\gamma\delta$ nAChR-rich membranes were prepared at 4 °C from freshly dissected and sliced *Torpedo* electric organs using procedures previously described,[20] resuspended in 5 mM glycine, and stored aliquotted at −80 °C until use. Microtransplantation of *Torpedo* nAChR used microinjection into the oocyte cytoplasm of a membrane suspension (50 nL at 3.5 mg mL^{-1} protein) from a Nanoliter2000 Micro4 Controller (World Precision Instruments, Inc., UK) mounted on a microscope.

Expression of human α4β2 nAChR in *Xenopus* oocytes. Oocytes were surgically removed from mature female *Xenopus laevis* frogs under anesthesia using ethyl-3-amino benzoate methanesulfonate salt (Sigma-Aldrich, Saint Quentin Fallavier, France) solution (1.5 g l^{-1}), were treated for 10 min with 1 mg ml^{-1} collagenase type I (Sigma-Aldrich) in calcium-free medium containing (in mM): NaCl 88, KCl 2.5, $MgCl_2$ 1, HEPES 5 (pH 7.6), and were manually defolliculated. Following an extensive washing with this solution, oocytes were transferred to Barth's solution containing (in mM): NaCl 88, KCl 1, $MgSO_4$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $NaHCO_3$ 2.4, HEPES 10 (pH 7.2) supplemented with streptomycin sulphate and 0.01 mg ml^{-1} penicillin-G. Oocytes were maintained at 19 °C for up to 4 days in this solution. Stage V–VI oocytes were selected and microinjected with 50 nL human α4β2 cDNA (1 μg μl^{-1}), as reported previously.[7] Recordings were performed 3 to 4 days after cDNA injection.

Voltage-clamp recording on oocytes. ACh-evoked currents were recorded with a standard two-microelectrode voltage-clamp amplifier (OC-725B, Warner Instrument Corp., Hamden, CT, USA) at a holding potential of −60 mV. Voltage and current micro-electrodes were pulled from borosilicate glass to reach 0.5–1.5 MΩ tip resistance when filled with 3 M KCl. Data were acquired with a pCLAMP-9/Digidata-1322A system (Molecular Devices, Union City, CA, USA). The recording chamber (capacity 300 μL) was superfused (8–12 mL min^{-1}; 20 °C) with a modified Ringer's solution containing (in mM): NaCl 100, KCl 2.8, $BaCl_2$ 0.3, HEPES 5 (pH 7.4), where $BaCl_2$ substitution to $CaCl_2$ prevents secondary activation of a Ca^{2+}-dependent Cl^- current.[21] Oocytes were initially incubated for 3 min with spiroimines, and then ACh was applied for 15 s in oocytes expressing the human α4β2 as well as for the *Torpedo* ($\alpha1_2\beta\gamma\delta$) nAChRs incorporated to the oocyte membrane, using a computer-controlled solution exchange system (VC-6; Warner Instruments). Between successive ACh applications, 4 min perfusion intervals with modified Ringer's solution were maintained to ensure receptor recovery from desensitization.

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