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Synthesis and olfactory activity of unnatural, sulfated 5β-bile acid derivatives in the sea lamprey (*Petromyzon marinus*)

Aaron C. Burns[a], Peter W. Sorensen[b], Thomas R. Hoye[a,*]

[a] Department of Chemistry, College of Science and Engineering, University of Minnesota, 207 Pleasant St. SE, Minneapolis, MN 55455, United States
[b] Department of Fisheries, Wildlife and Conservation Biology, College of Food, Agriculture, and Natural Science, University of Minnesota, St. Paul, MN 55108, United States

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ABSTRACT

A variety of unnatural bile acid derivatives (**9a**–**9f**) was synthesized and used to examine the specificity with which the sea lamprey (*Petromyzon marinus*) olfactory system detects these compounds. These compounds are analogs of petromyzonol sulfate (PS, **1**), a component of the sea lamprey migratory pheromone. Both the stereochemical configuration at C5 (i.e., 5α vs. 5β) and the extent and sites of oxygenation (hydroxylation or ketonization) of the bile acid derived steroid skeleton were evaluated by screening the compounds for olfactory activity using electro-olfactogram recording. 5β-Petromyzonol sulfate (**9a**) elicited a considerable olfactory response at sub-nanomolar concentration. In addition, less oxygenated systems (i.e., **9b**–**9e**) elicited olfactory responses, albeit with less potency. The sea lamprey sex pheromone mimic **9f** (5β-3-ketopetromyzonol sulfate) was also examined and found to produce a much lower olfactory response. Mixture studies conducted with **9a** and PS (**1**) suggest that stimulation is occurring via similar modes of activation, demonstrating a relative lack of specificity for recognition of the allo-configuration (i.e., 5α) in sea lamprey olfaction. This attribute could facilitate design of pheromone analogs to control this invasive species.

1. Introduction

It is now well established that naturally occurring bile acids and closely related compounds function as potent olfactory stimulants for many species of fish. They also have been postulated to function as pheromones [1–3]. Amongst these species, bile acid function is especially well understood in the ancient sea lamprey (*Petromyzon marinus*) [4–7]. Four behaviorally active bile acid-like compounds have been shown to be produced, released, and detected in this species. In particular, a three-component suite of bile acids [petromyzonol sulfate (PS, **1**), petromyzonamine disulfate (PADS, **2**), and petromyzosterol disulfate (PSDS, **3**); Fig. 1] is known to be produced by larval sea lamprey and to function as a migratory pheromone for adults. 3-Ketopetromyzonol sulfate (**4**), an oxidized analog of **1**, is produced and released by sexually mature male sea lamprey and functions as a sex pheromone that attracts ovulated females [8,9]. Each of these compounds is detected at picomolar thresholds by the lamprey olfactory system with PADS (**2**) being detected at 10^{-13} M. Because the sea lamprey is an invasive species, these novel products are being explored on a commercial basis for application in trapping programs [3]. Because the cost of production is high, there is interest in producing analogs that exhibit similar olfactory potency.

Several studies have explored the specificity with which lamprey olfactory epithelium discerns bile acids using electrophysiological recording. These have provided evidence of multiple receptor types, but the ligand structural features associated with specificity are not yet well understood [4,5,10]. Of special interest have been bile acids with the 'allo' (5α) configuration (cf. **5**, Fig. 2); both lampreys (Family Petromyzontidae) and the minnows and carps (Family Cyprinidae) produce and release several allosteroids and exhibit remarkable sensitivity to them [2,11]. It is important to emphasize the fairly substantial structural difference, largely topological in nature, associated with the allocholate-like (**5**) vs. cholate-like (**6**) topologies (cf., Fig. 2).

Because allo steroids are rare amongst mammals, relatively few have been synthesized. The sea lamprey and related lampreys produce the biologically active allo steroids, allocholic acid (**7**, Fig. 2), PS (**1**), and PADS (**2**). Only for the first of these steroids has the analogous 5β-isomer, namely cholic acid (**10a**, Scheme 1), been tested for olfactory activity in lamprey and other fish [5]. Otherwise, the importance of the more rare allo-configuration to the underlying structure–activity relationship (SAR) has not been studied. Similarly, the carps and other cyprinids, the second largest family of fishes within over 1000 species, produce and detect cyprinol sulfate (**8**, Fig. 2) [12,13]. Other fishes detect **8** with high sensitivity [11]. However, neither the behavioral function nor the importance

* Corresponding author. Tel.: +1 612 625 1891.
E-mail addresses: soren003@umn.edu (P.W. Sorensen), hoye@umn.edu (T.R. Hoye).

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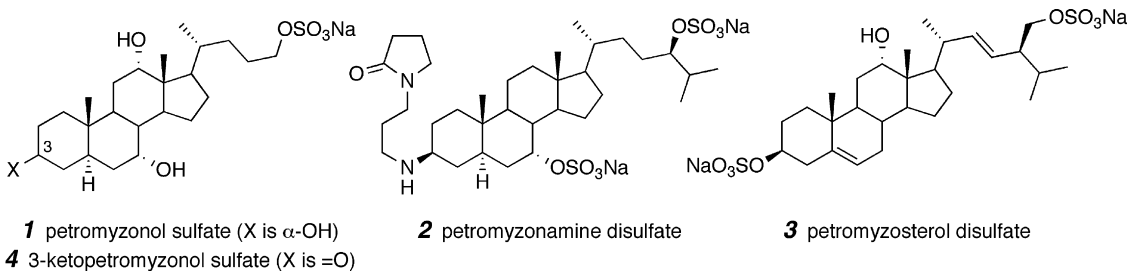


Fig. 1. The three principal components of the sea lamprey migratory pheromone.

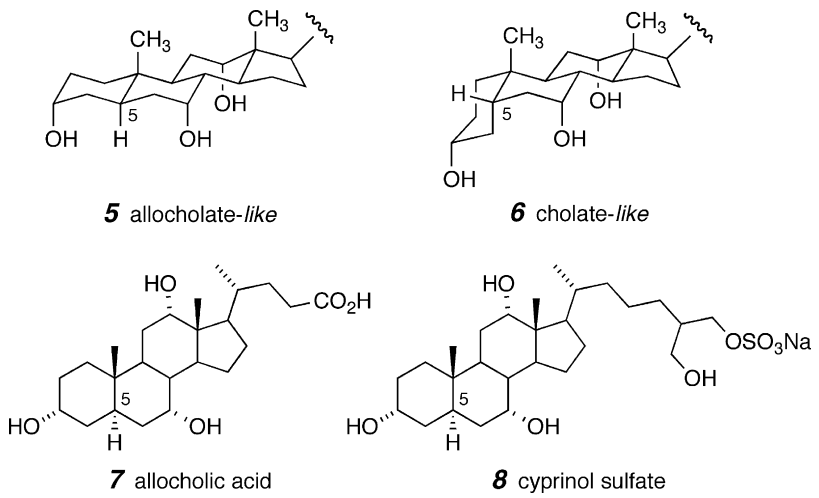
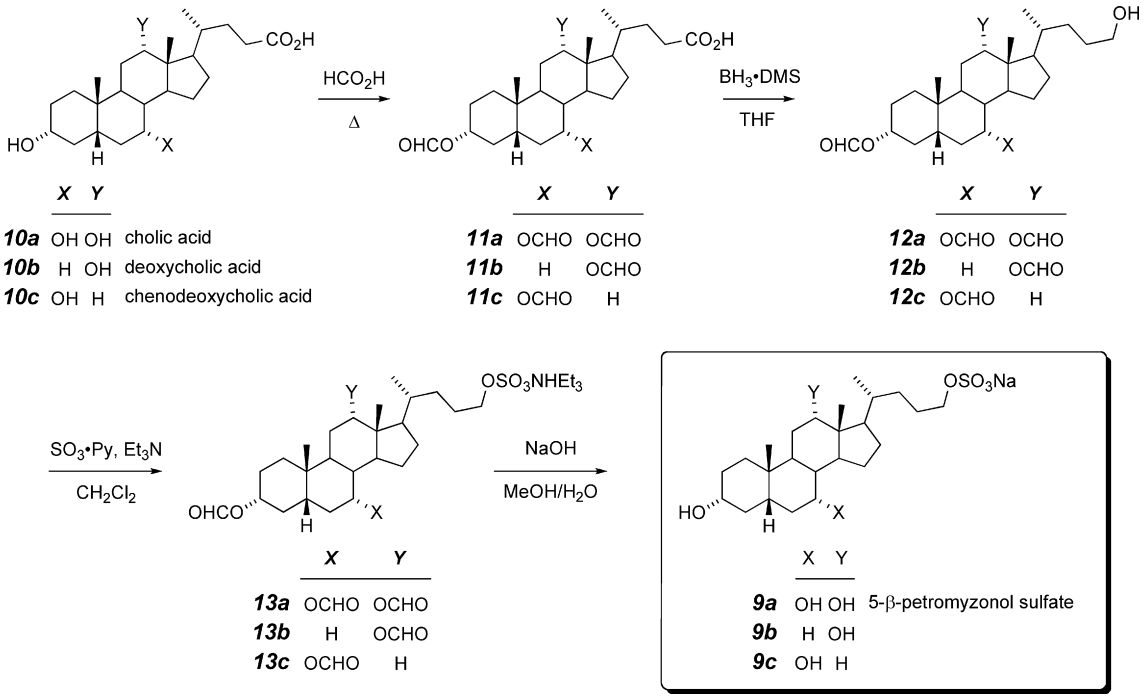


Fig. 2. Topological difference between the allocholate (5, 5α-H) and cholate (6, 5β-H) structural families. Examples (7 and 8) of the allocholate family.

of the allo-configuration in olfactory detection have been explicitly tested. Indeed, the overall importance to the fish olfactory system of most structural features associated with this class of (several hundred Dalton) sulfated allo steroids has not been systematically explored. This is notable because bile acids include the largest olfactory ligands described for a fish (PADS is the largest), so it is reasonable to ask whether the entire structure is discerned.

The study reported here had two related objectives; to synthesize a series of 5β-bile acids and to determine both the sensitivity and specificity with which they are discerned by the sea lamprey



Scheme 1. Synthesis of sulfated bile acid derivatives **9a**–**9c**.

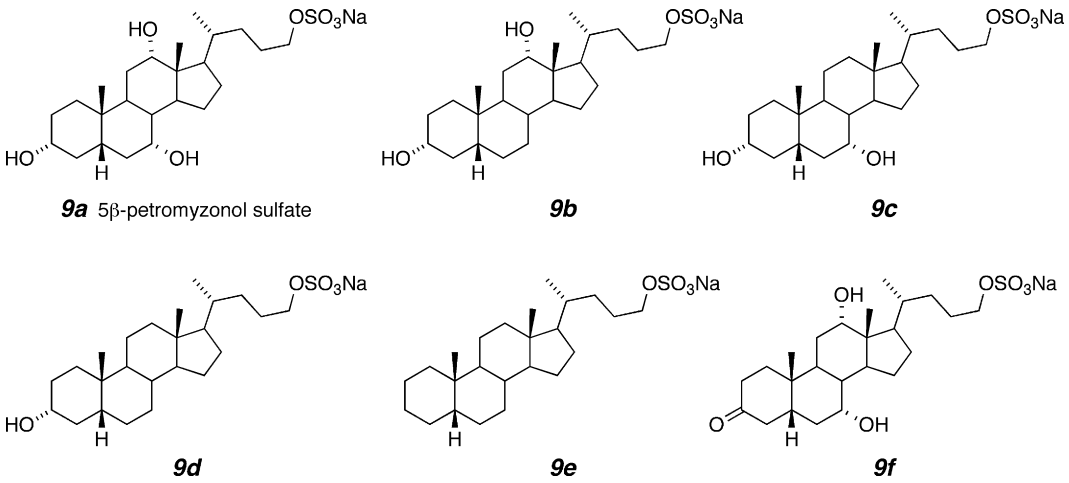


Fig. 3. 5β-Bile acid derivatives tested by the electro-olfactogram method in this study.

olfactory system. Toward that end, we prepared the analogs **9a** [the 5β-epimer of PS (**1**)], **9b–e** (less highly oxygenated analogs of **9a**), and **9f** (the C3-keto analog of **9a**) (Fig. 3) and then tested their olfactory activity using electro-olfactogam recording (EOG).

2. Experimental

2.1. General

Unless noted otherwise, all oxygen and moisture-sensitive reactions were executed in oven-dried glassware sealed under a positive pressure of dry argon or nitrogen. All commercial reagents were used as received. Anhydrous THF and methylene chloride were tapped immediately prior to use after being passed through a column of activated alumina. Flash chromatography was performed using Agela Technologies silica gel 40–60 μm (40 Å); analytical TLC was performed using 0.25 mm EM silica gel 60 F254 plates that were visualized under UV light (254 nm) or by staining with anisaldehyde reagent (450 mL of 95% ethanol, 25 mL concd H_2SO_4, 15 mL acetic acid, and 25 mL anisaldehyde) and heating. Optical rotations were obtained using a Perkin-Elmer-241 polarimeter. IR spectra were recorded using a Prospect 4000 FT-IR spectrophotometer (Midac Corporation). NMR spectra were obtained using an INOVA 500 Varian instrument (^{1}H data recorded at 500 MHz, ^{13}C data recorded at 125 MHz). ^{1}H chemical shifts are reported in delta (δ) units, in parts per million (ppm) relative to tetramethylsilane (0.00 ppm) when $CDCl_3$ was employed or to CD_2HOD (3.31 ppm) when d^4-methanol was used. ^{13}C chemical shifts are reported in delta (δ) units, in parts per million (ppm) relative to $CDCl_3$ (77.23 ppm) or to CD_3OD (49.15 ppm). High-resolution mass spectrometric data were obtained using a Bruker BioTOF II (ESI) mass spectrometer. The activity of these products was studied using electro-olfactogram (EOG) recording as detailed in Section 2.2.

2.1.1. 3α,7α,12α-Triformyloxy-5β-cholan-24-oic acid (*11a*)[14,15]

A 10 mL flask equipped with a magnetic stirring bar was charged with cholic acid (**10a**, 1.0 g, 2.45 mmol) and 4 mL of 88% formic acid. The resultant solution was heated for 24 h at 55 °C, at which time TLC analysis showed consumption of **10a**. The solution was cooled to room temperature and the formic acid was removed in vacuo. The crude material was recrystallized from ethyl acetate/hexane to provide **11a** (785 mg, 65%). White crystalline solid; mp 208–209 °C; R_f = 0.48 (1:1 hexane/ethyl acetate); $[\alpha]_D$ (23 °C): +90.3° (c = 0.050, $CHCl_3$); IR (neat): 3400 (broad), 2943, 1717 (sharp), 1183 cm^{-1}; ^{1}H

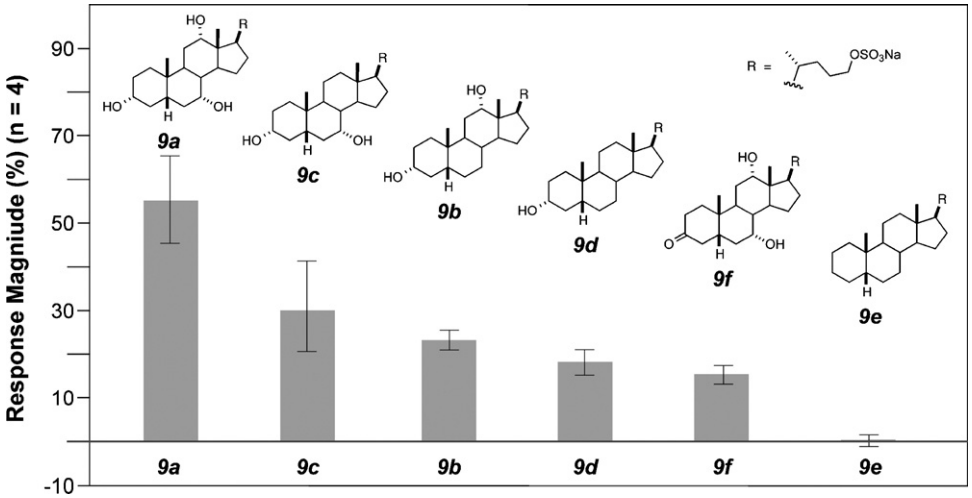


Fig. 4. EOG response magnitudes of 5β-petromyzonol sulfate (**9a**) and analogs **9b–f** tested at 10^{-9} M concentrations. Response magnitudes are presented as a percentage of 10^{-5} M L-arginine. Vertical bars represent one standard error (n = 4 animals). Each of the above responses was deemed to be significantly different from the others (ANOVA, P < 0.001).

NMR (500 MHz, $CDCl_3$): δ 8.17 (s, 1H), 8.11 (s, 1H), 8.03 (s, 1H), 5.27 (dd, *J* = 2.0, 2.0 Hz, 1H, H12), 5.07 (ddd, *J* = 2.5, 2.5, 2.5 Hz, 1H, H7), 4.72 (dddd, *J* = 11.5, 11.5, 4.0, 4.0 Hz, 1H, H3), 2.38 (ddd, *J* = 15.5, 10.0, 5.5 Hz, 1H, H23a), 2.25 (ddd, *J* = 16.0, 9.5, 6.5 Hz, 1H, H23b), 2.17–2.10 (m, 2H), 2.02 (ddd, *J* = 16.0, 5.0, 5.0 Hz, 1H), 1.97–1.85 (m, 2H), 1.82–1.64 (m, 9H), 1.60–1.52 (m, 2H), 1.49–1.39 (m, 2H), 1.37–1.28 (m, 3H), 1.16–1.06 (m, 2H), 0.95 (s, 3H, $CH_3$19), 0.86 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.76 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.0, 160.8 (2C), 160.7, 75.5, 73.9, 70.9, 47.4, 45.2, 43.2, 41.0, 37.9, 34.9, 34.7, 34.6, 34.5, 31.5, 31.0, 30.6, 28.8, 27.3, 26.8, 25.8, 23.0, 22.6, 17.7, 12.4; HRMS (ESI+) calcd for $C_{27}H_{40}O_8Na^+$ (M + Na^+) 515.2615, found 515.2613.

2.1.2. 3α,7α,12α-Triformyloxy-5β-cholan-24-ol (**12a**)[15,16]

A 25 mL flask equipped with a magnetic stir bar was charged with **11a** (500 mg, 1.02 mmol, 1.0 equiv.) and 10 mL of tetrahydrofuran and cooled to −78 °C. To this solution was added 10 M borane–dimethyl sulfide complex (153 μL, 1.53 mmol, 1.5 equiv.) and the resultant solution was warmed to room temperature over 1 h and subsequently allowed to stir for an additional hour. The excess borane was quenched with 200 μL of acetic acid at 0 °C and allowed to stir for 12 h at room temperature. The volatiles were removed in vacuo to give the crude material, which was purified by flash chromatography to provide **12a** (253 mg, 52%). White amorphous solid; mp 86–92 °C; R_f = 0.44 (1:1 hexane/ethyl acetate); $[\alpha]_D$ (23 °C): +60.7° (*c* = 0.018, $CHCl_3$); IR (neat): 3400 (broad), 2941, 1717 (sharp), 1183 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.16 (s, 1H), 8.11 (s, 1H), 8.03 (s, 1H), 5.28 (dd, *J* = 3.0, 3.0 Hz, 1H, H12), 5.07 (ddd, *J* = 2.5, 2.5, 2.5 Hz, 1H, H7), 4.72 (dddd, *J* = 11.5, 11.5, 4.5, 4.5 Hz, 1H, H3), 3.64–3.56 (m, 2H), 2.15–2.09 (m, 2H), 2.02 (ddd, *J* = 15.0, 5.0, 3.0 Hz, 1H), 1.97–1.84 (m, 2H), 1.82–1.52 (m, 10H), 1.48–1.25 (m, 6H), 1.15–1.02 (m, 3H), 0.95 (s, 3H, $CH_3$19), 0.85 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.76 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.8 (2C), 160.7, 75.5, 73.9, 70.9, 63.5, 47.5, 45.1, 43.1, 41.0, 37.9, 35.1, 34.7, 34.6, 34.4, 31.7, 31.5, 29.4, 28.7, 27.4, 26.7, 25.7, 23.0, 22.5, 18.0, 12.3; HRMS (ESI+) calcd for $C_{27}H_{42}O_7Na^+$ (M + Na^+) 501.2823, found 501.2820.

2.1.3. Triethylammonium 3α,7α,12α-triformyloxy-5β-cholan-24-ol sulfate (**13a**)

A 10 mL flask equipped with a magnetic stir bar was charged with **12a** (200 mg, 0.418 mmol, 1.0 equiv.) and 4.2 mL of methylene chloride. To this solution was added triethylamine (233 μL, 1.67 mmol, 4.0 equiv.) and sulfur trioxide–pyridine complex (200 mg, 1.25 mmol, 3.0 equiv.). The reaction mixture was allowed to stir at room temperature for 24 h. The volatiles were removed in vacuo to give the crude material, which was loaded directly onto a silica gel column and purified by flash chromatography (90:9:1 chloroform/methanol/satd aq NH_3) to provide **13a** (183 mg, 66%). White amorphous solid; mp 95.5–99.0 °C; R_f = 0.41 (80:26:2 chloroform/methanol/satd aq NH_3); $[\alpha]_D$ (23 °C): +51.8° (*c* = 0.026, MeOH); IR (neat): 2944, 1717 (sharp), 1180 cm^{-1}; ^{1}H NMR (500 MHz, d^4-methanol): δ 8.22 (s, 1H), 8.15 (s, 1H), 8.05 (d, *J* = 1.0 Hz, 1H), 5.28 (dd, *J* = 5.0, 5.0 Hz, 1H, H12), 5.05 (ddd, *J* = 3.0, 3.0, 3.0 Hz, 1H, H7), 4.68 (dddd, *J* = 11.5, 11.5, 4.5, 4.5 Hz, 1H, H3), 3.98–3.92 (m, 2H), 3.22 (q, *J* = 7.0 Hz, 6H, $NC\underline{H}_2CH_3$), 2.20–2.10 (m, 3H), 2.02 (dd, *J* = 12.5, 7.5 Hz, 1H), 1.93–1.61 (m, 10H), 1.57–1.41 (m, 5H), 1.40–1.28 (m, 10H), 1.22–1.08 (m, 3H), 1.00 (s, 3H, $CH_3$19), 0.88 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.82 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, d^4-methanol) δ 162.8 (2C), 162.7, 77.0, 75.2, 72.4, 69.6, 48.1, 46.4 (3C), 44.5, 42.4, 39.1, 36.3, 36.0, 35.7, 35.5, 33.0, 32.6, 29.9, 28.4, 27.9, 27.1, 26.8, 23.9, 22.8, 18.4, 16.4, 12.6, 9.4 (3C); HRMS (ESI−) calcd for $C_{27}H_{41}SO_{10}^-$ (M − $HNEt_3$) 557.2426, found 557.2428.

2.1.4. Sodium 5β-petromyzonol-24-sulfate (**9a**, **5β-PS**)[17]

A 10 mL flask equipped with a magnetic stir bar was charged with **13a** (150 mg, 0.227 mmol, 1.0 equiv.) and 8.4 mL of a mixture of methanol/water (4:1). To this solution was added sodium hydroxide (90 mg, 2.25 mmol, 10.0 equiv.) and the mixture was heated to 50 °C for 12 h. The solution was cooled to room temperature and the volatiles were removed in vacuo to give the crude material, which was loaded directly onto a silica gel column and purified by flash chromatography (80:26:2 chloroform/methanol/satd aq NH_3) to provide **9a** (104 mg, 92%). White amorphous solid; mp 180.0–182.0 °C; R_f = 0.24 (80:26:2 chloroform/methanol/satd aq NH_3); $[\alpha]_D$ (23 °C): +21.1° (*c* = 0.031, MeOH); IR (neat): 3400 (broad), 2939, 1209 cm^{-1}; ^{1}H NMR (500 MHz, d^4-methanol): δ 3.99–3.94 (m, 3H), 3.79 (ddd, *J* = 3.0, 3.0, 3.0 Hz, 1H, H7), 3.37 (dddd, *J* = 11.0, 11.0, 4.5, 4.5 Hz, 1H, H3), 2.32–2.22 (m, 2H), 2.01–1.71 (m, 8H), 1.67–1.51 (m, 8H), 1.47–1.36 (m, 3H), 1.33–1.25 (m, 1H), 1.19–1.08 (m, 2H), 1.02 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.98 (ddd, *J* = 14.0, 14.0, 3.5 Hz, 1H), 0.92 (s, 3H, $CH_3$19), 0.72 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, d^4-methanol) δ 74.2, 73.0, 69.8, 69.2, 48.4, 47.6, 43.3, 43.1, 41.2, 40.6, 37.0, 36.6, 36.0, 35.9, 33.3, 31.3, 29.7, 28.9, 28.0, 27.4, 24.4, 23.3, 18.1, 13.1; HRMS (ESI−) calcd for $C_{24}H_{41}SO_7^-$ (M − Na^+) 473.2578, found 473.2585.

Compounds **9b**–**13b** and **9c**–**13c** were synthesized by analogous procedures to those detailed above for **9a**–**13a**. Full characterization data for each compound are presented below.

2.1.5. 3α,12α-Diformyloxy-5β-cholan-24-oic acid (**11b**)[15,18]

White crystalline solid; mp 198.0–198.5 °C; R_f = 0.40 (3:1 hexane/ethyl acetate); $[\alpha]_D$ (23 °C): +61.6° (*c* = 0.500, $CHCl_3$); IR (neat): 3400 (broad), 2939, 1698 (sharp), 1179 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.14 (s, 1H), 8.03 (d, *J* = 0.5 Hz, 1H), 5.25 (dd, *J* = 3.0, 3.0 Hz, 1H, H12), 4.84 (dddd, *J* = 11.0, 11.0, 4.5, 4.5 Hz, 1H, H3), 2.39 (ddd, *J* = 15.5, 10.0, 5.5 Hz, 1H, H23), 2.25 (ddd, *J* = 16.0, 9.5, 7.0 Hz, 1H, H23), 2.17–2.10 (m, 2H), 2.02 (ddd, *J* = 15.5, 5.5, 3.5 Hz, 1H), 1.93–1.77 (m, 4H), 1.74–1.57 (m, 8H), 1.51–1.40 (m, 4H), 1.17–1.01 (m, 3H), 0.93 (s, 3H, $CH_3$19), 0.84 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.75 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.2, 160.9, 160.8, 76.2, 74.3, 49.5, 47.6, 45.2, 41.9, 35.8, 35.0, 34.9, 34.4, 34.5, 34.2, 32.3, 31.1, 30.7, 27.6, 27.0, 26.7, 26.1, 26.0, 23.6, 23.1, 17.6, 12.6; HRMS (ESI+) calcd for $C_{26}H_{40}O_6Na^+$ (M + Na^+) 471.2717, found 471.2721.

2.1.6. 3α,12α-Diformyloxy-5β-cholan-24-ol (**12b**)[15]

White amorphous solid; mp 57.0–62.5 °C; R_f = 0.78 (1:1 hexane/ethyl acetate); $[\alpha]_D$ (23 °C): +85.5° (*c* = 0.010, $CHCl_3$); IR (neat): 3400 (broad), 2938, 1721 (sharp), 1178 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.14 (s, 1H), 8.03 (d, *J* = 0.5 Hz, 1H), 5.26 (dd, *J* = 3.0, 3.0 Hz, 1H, H12), 4.84 (dddd, *J* = 11.5, 11.5, 4.5, 4.5 Hz, 1H, H3), 3.65–3.57 (m, 2H), 1.92–1.01 (m, 27H), 0.93 (s, 3H, $CH_3$19), 0.84 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.75 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.9, 160.8, 76.3, 74.3, 63.6, 49.4, 47.7, 45.1, 41.9, 35.8, 35.2, 34.8, 34.4, 34.2, 32.3, 31.8, 29.5, 27.6, 27.0, 26.6, 26.1, 25.9, 23.6, 23.1, 18.0, 12.5; HRMS (ESI+) calcd for $C_{26}H_{42}O_5Na^+$ (M + Na^+) 457.2924, found 457.2932.

2.1.7. Triethylammonium 3α,12α-diformyloxy-5β-cholan-24-ol sulfate (**13b**)

White amorphous solid; mp 153.2–154.0 °C; R_f = 0.25 (80:18:2 chloroform/methanol/satd aq NH_3); $[\alpha]_D$ (23 °C): +65.5° (*c* = 0.025, MeOH); IR (neat): 2943, 1718 (sharp), 1176 cm^{-1}; ^{1}H NMR (500 MHz, d^4-methanol): δ 8.20 (s, 1H), 8.05 (d, *J* = 0.5 Hz, 1H), 5.24 (dd, *J* = 3.0, 3.0 Hz, 1H, H12), 4.80 (dddd, *J* = 11.0, 11.0, 5.0, 5.0 Hz, 1H, H3), 3.99–3.92 (m, 2H), 3.21 (q, *J* = 10.0 Hz, 6H, $NC\underline{H}_2CH_3$), 1.97–1.85 (m, 2H), 1.80–1.30 (m, 27H), 1.20–1.05 (m, 4H), 0.98 (s, 3H, $CH_3$19), 0.87 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.80 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, d^4-methanol) δ 162.8, 162.7, 77.5, 75.4, 69.6, 50.8, 48.1, 46.4 (3C), 43.3, 37.1, 36.4, 35.9, 35.6, 35.3, 33.5, 33.1, 28.6,

28.1, 27.7, 27.3, 27.2, 26.9, 24.7, 23.5, 18.5, 17.9, 12.9, 9.4 (3C); HRMS (ESI−) calcd for $C_{26}H_{41}SO_8^-$ (M − $HNEt_3$) 513.2528, found 513.2533.

*2.1.8. Sodium 3α,12α-dihydroxy-5β-cholan-24-ol sulfate (**9b**)*

White amorphous solid; mp 172.7–176.2 °C; R_f = 0.40 (80:26:2 chloroform/methanol/satd aq NH_3); $[\alpha]_D$ (23 °C): +35.0° (*c* = 0.020, MeOH); IR (neat): 3400 (broad), 2934, 1219 cm^{-1}; ^1H NMR (500 MHz, d^4-methanol): δ 3.98–3.93 (m, 3H), 3.52 (dddd, *J* = 11.0, 11.0, 4.5, 4.5 Hz, 1H, H3), 1.92–1.72 (m, 7H), 1.64–1.38 (m, 13H), 1.32–1.23 (m, 2H), 1.21–1.05 (m, 3H), 1.01 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.98 (ddd, *J* = 14.0, 14.0, 3.0 Hz, 1H), 0.93 (s, 3H, $CH_3$19), 0.71 (s, 3H, $CH_3$21); ^{13}C NMR (125 MHz, d^4-methanol) δ 74.2, 72.7, 69.8, 49.4, 48.5, 47.7, 43.8, 37.6, 37.4, 37.0, 36.6, 35.5, 35.0, 33.2, 31.2, 30.0, 28.9, 28.6, 27.6, 27.3, 25.0, 23.9, 18.0, 13.4; HRMS (ESI−) calcd for $C_{24}H_{41}SO_6^-$ (M − Na^+) 457.2629, found 457.2628.

*2.1.9. 3α,7α-Diformyloxy-5β-cholan-24-oic acid (**11c**)[15]*

White crystalline solid; mp 100.0–102.5 °C; R_f = 0.53 (1:1 hexane/ethyl acetate); $[\alpha]_D$ (23 °C): +32.8° (*c* = 0.017, $CHCl_3$); IR (neat): 3400 (broad), 2948, 1722 (sharp), 1186 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 8.08 (s, 1H), 8.03 (d, *J* = 0.5 Hz, 1H), 5.04 (ddd, *J* = 3.5, 3.5, 3.5 Hz, 1H, H7), 4.73 (dddd, *J* = 11.0, 11.0, 4.5, 4.5 Hz, 1H, H3), 2.40 (ddd, *J* = 15.5, 10.0, 5.0 Hz, 1H, H23a), 2.26 (ddd, *J* = 16.0, 10.0, 6.5 Hz, 1H, H23b), 2.13 (ddd, *J* = 13.0, 13.0, 13.0 Hz, 1H), 2.00 (dddd, *J* = 12.0, 12.0, 4.0, 4.0 Hz), 1.92–1.74 (m, 5H), 1.67–1.62 (m, 3H), 1.54–1.24 (m, 9H), 1.22–1.04 (m, 4H), 0.95 (s, 3H, $CH_3$19), 0.94 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.66 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.3, 161.0 (2C), 74.2, 71.6, 49.5, 55.8, 50.3, 42.9, 41.1, 39.6, 38.0, 35.4, 34.9, 34.7, 34.1, 31.6, 31.1, 30.8, 28.1, 26.9, 23.7, 22.8, 20.8, 18.4, 14.4, 11.9; HRMS (ESI+) calcd for $C_{26}H_{40}O_6Na^+$ (M + Na^+) 471.2717, found 471.2721.

*2.1.10. 3α,7α-Diformyloxy-5β-cholan-24-ol (**12c**) [15]*

White amorphous solid; mp 49.5–52.0; R_f = 0.45 (1:1 hexane/ethyl acetate); $[\alpha]_D$ (23 °C): +11.1° (*c* = 0.114, $CHCl_3$); IR (neat): 3400 (broad), 2937, 1710 (sharp), 1181 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 8.08 (s, 1H), 8.03 (s, 1H), 5.03 (ddd, *J* = 3.0, 3.0, 3.0 Hz, 1H, H7), 4.73 (dddd, *J* = 11.5, 11.5, 4.5, 4.5 Hz, 1H, H3), 3.65–3.57 (m, 2H), 2.14 (ddd, 14.0, 14.0, 14.0 Hz, 1H), 2.04–1.98 (m, 2H), 1.91 (ddd, *J* = 14.5, 3.0, 3.0 Hz, 1H), 1.88–1.79 (m, 2H), 1.78–1.73 (m, 1H), 1.66–1.60 (m, 4H), 1.54–1.03 (m, 16H), 0.95 (s, 3H, $CH_3$19), 0.94 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.66 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.9 (2C), 74.2, 71.6, 63.7, 56.1, 50.3, 42.8, 41.1, 39.6, 38.0, 35.7, 35.0, 34.7, 34.2, 32.0, 31.7, 29.5, 28.3, 26.9, 26.1, 23.7, 22.8, 20.8, 18.8, 11.9; HRMS (ESI+) calcd for $C_{26}H_{42}O_5Na^+$ (M + Na^+) 457.2924, found 457.2926.

*2.1.11. Triethylammonium 3α,7α-diformyloxy-5β-cholan-24-ol sulfate (**13c**)*

White amorphous solid; mp 174.9–176.1 °C; R_f = 0.25 (80:18:2 chloroform/methanol/satd aq NH_3); $[\alpha]_D$ (23 °C): +14.0° (*c* = 0.026, MeOH); IR (neat): 2941, 1718 (sharp), 1187 cm^{-1}; ^1H NMR (500 MHz, d^4-methanol): δ 8.13 (s, 1H), 8.05 (d, *J* = 1.0 Hz, 1H), 5.02 (ddd, *J* = 2.0, 2.0, 2.0 Hz, 1H, H7), 4.69 (dddd, *J* = 11.5, 11.5, 4.5, 4.5 Hz, 1H, H3), 3.99–3.92 (m, 2H), 3.22 (q, *J* = 7.0 Hz, 6H, $NC\underline{H}_2CH_3$), 2.18 (ddd, *J* = 13.5, 13.5, 13.5 Hz, 1H), 2.11 (ddd, *J* = 15.5, 5.0, 3.5 Hz, 1H), 2.05 (ddd, *J* = 12.5, 3.5, 3.5 Hz, 1H), 1.96–1.84 (m, 3H), 1.77–1.70 (m, 3H), 1.65–1.61 (m, 2H), 1.57–1.32 (m, 10H), 1.32 (t, *J* = 7.5 Hz, 9H), 1.26–1.09 (m, 5H), 1.00 (s, 3H, $CH_3$19), 0.97 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.72 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, d^4-methanol) δ 162.9, 162.8, 75.5, 72.9, 69.7, 57.5, 51.6, 48.1 (3C), 43.9, 42.5, 41.0, 39.3, 36.9, 36.1, 36.0, 35.9, 35.5, 33.2, 32.7, 29.2, 28.0, 27.2, 24.6, 23.2, 21.9, 19.2, 12.3, 9.4 (3C); HRMS (ESI−) calcd for $C_{26}H_{41}SO_8^-$ (M − $HNEt_3$) 513.2528, found 513.2530.

*2.1.12. Sodium 3α,7α-dihydroxy-5β-cholan-24-ol sulfate (**9c**)*

White amorphous solid; mp 172.7–176.2 °C; R_f = 0.38 (80:26:2 chloroform/methanol/satd aq NH_3); $[\alpha]_D$ (23 °C): +35.0° (*c* = 0.020, MeOH); IR (neat): 3400 (broad), 2934, 1219 cm^{-1}; ^1H NMR (500 MHz, d^4-methanol): δ 3.98–3.93 (m, 3H), 3.52 (dddd, *J* = 11.0, 11.0, 4.5, 4.5 Hz, 1H, H3), 1.92–1.72 (m, 7H), 1.64–1.38 (m, 13H), 1.29 (dddd, *J* = 4.5, 4.5, 2.0, 2.0 Hz, 1H), 1.26 (ddd, *J* = 4.0, 2.0, 2.0 Hz, 1H), 1.21–1.05 (m, 3H), 1.01 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.98 (ddd, *J* = 14.0, 14.0, 3.0 Hz, 1H), 0.93 (s, 3H, $CH_3$19), 0.71 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, d^4-methanol) δ 73.0, 69.8, 69.2, 57.6, 51.6, 43.8, 43.3, 41.2, 40.9, 40.6, 39.3, 37.0, 36.7, 36.4, 36.0, 34.2, 33.3, 31.5, 29.4, 27.3, 24.8, 23.5, 21.9, 19.3, 12.3; HRMS (ESI−) calcd for $C_{24}H_{41}SO_6^-$ (M − Na^+) 457.2629, found 457.2627.

*2.1.13. 3α-Tetrahydropyranyloxy-5β-cholan-24-ol (**12d**)[19]*

A 100 mL flask equipped with a magnetic stir bar was charged with lithocholic acid (**10d**, 2.0 g, 5.31 mmol, 1.0 equiv.) and 18 mL of methylene chloride. To this solution was added dihydropyran (4.7 mL, 53.1 mmol, 10 equiv.) and pyridinium *p*-toluenesulfonate (133 mg, 0.531 mmol, 0.1 equiv.). The resultant solution was allowed to stir at room temperature for 24 h at which time the volatiles were removed in vacuo to provide crude **11d**. A stir-bar was added to the oily **11d** in this flask, and 53 mL of ethyl ether was added. The solution was cooled with an ice-water bath and lithium aluminum hydride (1.0 g, 26.4 mmol, 5.0 equiv.) was added. The resultant solution was allowed to stir at room temperature for 3 h and quenched at 0 °C with 1 mL of water, 1 mL of 3 N sodium hydroxide, and 3 mL of water. The suspension was filtered and the resultant solution was concentrated in vacuo to give the crude product, which was purified by flash chromatography to provide **12d** (1.43 g, 60%). Colorless oil; R_f = 0.35 (1:1 hexane/ethyl acetate); IR (neat): 3397 (broad), 2915, 2852, 1449, 1025 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 4.73–4.71 (m, 1H), 3.94–3.90 (m, 1H), 3.66–3.58 (m, 3H), 3.51–3.46 (m, 1H), 1.95 (ddd, *J* = 12.5, 2.5, 2.5 Hz, 1H), 1.92–1.76 (m, 4H), 1.75–1.60 (m, 4H), 1.57–1.51 (m, 6H), 1.47–1.17 (m, 13H), 1.15–0.87 (m, 7H), 0.92 (d, *J* = 7.0 Hz, 3H, $CH_3$21), 0.91 (s, 3H, $CH_3$19), 0.64 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 97.0, 96.7, 76.2, 76.0, 63.7, 63.0, 62.9, 56.6, 56.4, 42.9, 42.5, 42.2, 40.4, 40.3, 36.6, 36.0, 35.8, 35.7, 35.5, 34.9, 34.7, 34.5, 32.9, 32.4, 32.0, 31.5, 31.0, 30.7, 29.6, 28.7, 28.5, 27.5, 27.4, 26.9, 25.7, 24.4, 23.6, 23.5, 21.0, 20.9, 20.2, 20.1, 18.8, 12.2; HRMS (ESI+) calcd for $C_{29}H_{50}O_3Na^+$ (M + Na^+) 469.3652, found 469.3660.

*2.1.14. Triethylammonium 3α-tetrahydropyranyloxy-5β-cholan-24-ol sulfate (**13d**)*

Compound **13d** was prepared following the procedure 2.1.3 (for **13a**). White amorphous solid; mp 121.5–128.0 °C; R_f = 0.17 (90:9:1 chloroform/methanol/satd aq NH_3); IR (neat): 2927, 2895, 1441, 1253, 1196, 1023 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 9.77 (bs, 1H), 4.73–4.71 (m, 1H), 4.06–3.98 (m, 2H), 3.94–3.90 (m, 1H), 3.63 (ddd, *J* = 11.0, 11.0, 4.5, 4.5 Hz, 1H, H3), 3.51–3.46 (m, 1H), 3.18 (q, *J* = 7.0 Hz, 6H, $NC\underline{H}_2CH_3$), 1.95 (ddd, *J* = 12.0, 2.5, 2.5 Hz, 1H), 1.89–1.69 (m, 8H), 1.60–1.31 (m, 15H), 1.39 (t, *J* = 7.5 Hz, 9H, $NCH_2C\underline{H}_3$), 1.29–1.17 (m, 3H), 1.12–0.94 (m, 7H), 0.91 (s, 3H, $CH_3$19), 0.90 (d, *J* = 6.0 Hz, 3H, $CH_3$21), 0.63 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 96.9, 76.2, 68.8, 62.9, 56.6, 56.4, 46.6 (3C), 42.8, 42.2, 40.4, 40.3, 36.0, 35.7, 35.6, 34.9, 32.9, 32.0, 31.5, 28.7, 28.4, 27.5, 26.5, 26.2, 25.6, 24.4, 23.5, 20.9, 20.2, 18.7, 12.2, 8.9 (3C); HRMS (ESI−) calcd for $C_{29}H_{49}SO_6^-$ (M − Na^+) 525.3255, found 525.3258.

*2.1.15. Sodium 3α-hydroxy-5β-cholan-24-ol sulfate (**9d**)*

A 5 mL flask equipped with a magnetic stir bar was charged with **13d** (100 mg, 159 μmol, 1.0 equiv.), 1.6 mL of 95% ethanol,

and pyridinium *p*-toluenesulfonate (4 mg, 16 μmol, 0.1 equiv.). The reaction mixture was heated at 50 °C for 3 h at which time 400 μL of aq sodium hydroxide (15% by weight) solution was added and the mixture was allowed to stir for 15 min at this temperature. The mixture was cooled to room temperature and the volatiles were removed in vacuo to give the crude material that was purified by flash chromatography to provide **9d** (46 mg, 62%). White amorphous solid; mp 210.0–212.0 °C; R_f = 0.45 (80:18:2 chloroform/methanol/satd aq NH_3); $[\alpha]_D$ (23 °C): +24.7° (*c* = 0.016, MeOH); IR (neat): 2926, 2855, 1441, 1212 cm^{-1}; ^1H NMR (500 MHz, d^4-methanol): δ 3.99–3.92 (m, 2H, H24), 3.53 (dddd, *J* = 11.0, 11.0, 5.0, 5.0 Hz, 1H, H3), 2.02 (ddd, *J* = 12.5, 2.5, 2.5 Hz, 1H), 1.94–1.70 (m, 5H), 1.64–1.40 (m, 11H), 1.35 (ddd, *J* = 14.5, 3.0, 3.0 Hz, 1H), 1.34–1.24 (m, 3H), 1.19 (dd, *J* = 12.5, 3.5 Hz, 1H), 1.22–1.04 (m, 5H), 1.02–0.96 (m, 4H), 0.95 (s, 3H, $CH_3$19), 0.69 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, d^4-methanol) δ 72.6, 69.8, 58.1, 57.8, 44.0, 43.7, 42.0, 41.7, 37.4, 37.3, 36.9, 36.6, 35.8, 33.2, 31.3, 28.7, 29.4, 28.5, 27.8, 27.3, 25.4, 24.1, 22.1, 19.2, 12.6; HRMS (ESI−) calcd for $C_{24}H_{41}SO_5^-$ ($M - Na^+$) 441.2680, found 441.2680.

2.1.16. (4R)-Methyl 4-((3R,5R,10S,13R)-3-((1H-imidazole-1-carbonothioyl)oxy)-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoate (**11e**)

A 100 mL flask equipped with a magnetic stir bar was charged with methyl lithocholate (**10e**, 2 g, 5.12 mmol, 1.0 equiv.), thiocarbonyldiimidazole (2.7 g, 15.4 mmol, 3.0 equiv.), 4-dimethylaminopyridine (63 mg, 512 μmol, 0.1 equiv.), and 50 mL of methylene chloride. After 5 h the mixture was quenched with 25 mL of saturated aqueous ammonium chloride. The aqueous layer was extracted twice with 50 mL of ethyl acetate. The combined organic layers were combined, washed with 50 mL of saturated aqueous sodium bicarbonate and 50 mL of saturated brine, and dried over anhydrous sodium sulfate. The volatiles were removed in vacuo to give the crude material, which was purified by flash chromatography to provide **11e** (1.62 g, 63%). White amorphous solid; mp 146.0–147.5 °C; R_f = 0.61 (1:1 hexane/ethyl acetate); $[\alpha]_D$ (23 °C): +47.6° (*c* = 0.029, $CHCl_3$); IR (neat): 2949, 2870, 1740 (sharp), 1465, 1387, 1339, 1285, 1233 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 8.35 (dd, *J* = 1.0, 1.0 Hz, 1H, H2), 7.65 (dd, *J* = 2.0, 1.0 Hz, 1H, H5), 7.02 (dd, *J* = 2.0, 1.0 Hz, 1H, H4), 5.45 (dddd, *J* = 11.0, 11.0, 4.5, 4.5 Hz, 1H, H3), 3.67 (s, 3H), 2.35 (ddd, *J* = 15.5, 10.0, 5.0 Hz, 1H, H23a), 2.20 (ddd, *J* = 16.0, 10.0, 6.5 Hz, 1H, H23b), 2.06–1.77 (m, 8H), 1.66–1.53 (m, 3H), 1.45–1.02 (m, 15H), 0.98 (s, 3H, $CH_3$19), 0.92 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.66 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 183.7, 174.9, 137.0, 130.8, 118.1, 84.6, 56.6, 56.2, 51.7, 42.9, 42.1, 40.7, 40.2, 36.0, 35.5, 35.0, 34.8, 31.6, 31.2, 31.1, 28.4, 27.2, 26.5, 26.1, 24.4, 23.4, 21.0, 18.5, 12.2; HRMS (ESI+) calcd for $C_{29}H_{44}N_2SO_3Na^+$ ($M + Na^+$) 523.2965, found 523.2963.

2.1.17. Cholanol (**13e**)[20]

A 10 mL flask equipped with a magnetic stir bar was charged with tri-*n*-butyltin hydride (54 μL, 200 μmol, 4.0 equiv.) and 3.3 mL of toluene. The flask was equipped with a condenser and heated at reflux while a solution of **11e** (25 mg, 50 μmol, 1.0 equiv.) and azobisisobutyronitrile (1.6 mg, 5 μmol, 0.2 equiv.) in 1.7 mL of toluene was added over 1 h. The solution was maintained at reflux for an additional 3 h and subsequently cooled to room temperature, at which time the volatiles were removed in vacuo to provide the crude sample of **12e**, which was used without purification in the subsequent step. ^1H NMR (500 MHz, $CDCl_3$): δ 3.67 (m, 2H), 2.35 (ddd, *J* = 15.5, 10.5, 5.0, 1H, H23a), 2.21 (ddd, *J* = 16.0, 9.5, 6.5, 1H, H23b), 1.95 (ddd, *J* = 6.5, 2.5, 2.5 Hz, 1H), 1.89–1.69 (m, 6H), 1.59–1.51 (m, 2H), 1.43–0.93 (m, 19H), 0.92 (s, 3H, $CH_3$19), 0.90 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 0.64 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.0, 64.5, 56.6, 56.1, 51.7, 42.9, 42.2, 40.5, 40.3, 36.0, 35.6, 35.4, 35.0, 33.0, 31.3, 31.2, 28.4, 27.4, 27.1, 26.5, 24.4, 23.6, 21.0, 18.5, 12.2.

A 5 mL flask equipped with a magnetic stir bar was charged with the crude sample of **12e** and 1 mL of ethyl ether. To this solution lithium aluminum hydride (9.5 mg, 250 μmol, 5.0 equiv.) was added and the solution was allowed to stir for an additional 3 h. The reaction was subsequently quenched 10 μL of water, 10 μL of 3 N sodium hydroxide, and 20 μL of water. The suspension was filtered and the resultant solution was concentrated in vacuo to give the crude alcohol that was purified by flash chromatography to provide cholanol (**13e**) (11.1 mg, 64%). White amorphous solid; mp 129.5–130.5 °C; R_f = 0.43 (3:1 hexane/ethyl acetate); $[\alpha]_D$ (23 °C): +22.2° (*c* = 0.019, $CHCl_3$); IR (neat): 2937, 2864, 1472, 1375, 1054 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 3.65–3.57 (m, 2H), 1.96 (ddd, *J* = 12.0, 3.5, 3.5 Hz, 1H), 1.89–1.53 (m, 7H), 1.45–1.02 (m, 22H), 0.93 (d, *J* = 7.0 Hz, 3H, $CH_3$21), 0.91 (s, 3H, $CH_3$19), 0.65 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 63.8, 56.8, 56.4, 43.9, 42.9, 40.7, 40.5, 37.8, 36.1, 35.8, 35.6, 32.0, 29.6, 28.5, 27.7, 27.4, 27.2, 26.8, 24.5, 24.4, 21.5, 21.0, 18.9, 12.3; LRMS (EI) calcd for $C_{24}H_{42}O$ (M) 346.3, found 346.

2.1.18. Sodium cholan-24-ol sulfate (**9e**)

Compound **9e** was prepared from **13e** by procedure 2.1.3 followed by cation exchange and purification similar to that used in procedure 2.1.4. White amorphous solid; mp 195.5–198.0 °C; R_f = 0.44 (90:9:1 chloroform/methanol/satd aq NH_3); $[\alpha]_D$ (23 °C): +18.3° (*c* = 0.026, MeOH); IR (neat): 2930, 2860, 1449, 1402, 1213 cm^{-1}; ^1H NMR (500 MHz, d^4-methanol): δ 3.99–3.92 (m, 2H, H24), 2.01 (ddd, *J* = 12.5, 3.5, 3.5 Hz, 1H), 1.95–1.71 (m, 6H), 1.61–1.09 (m, 23H), 0.96 (d, *J* = 7.5 Hz, $CH_3$21), 0.95 (s, 3H, $CH_3$19), 0.69 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, d^4-methanol) δ 69.8, 58.1, 57.8, 45.4, 44.0, 42.0, 41.8, 38.9, 37.4, 36.9, 36.6, 33.3, 29.5, 28.8, 28.5, 28.4, 27.9, 27.3, 25.5, 25.0, 22.5, 22.1, 19.3, 12.7; HRMS (ESI−) calcd for $C_{24}H_{41}SO_4^-$ ($M - Na^+$) 425.2731, found 425.2736.

2.1.19. Sodium 3-keto-5β-petromyzonol sulfate (**9f**)

A 25 mL flask equipped with a magnetic stir bar was charged with **9a** (300 mg, 604 μmol, 1.0 equiv.), 400 μL of water, and 1.6 mL of *t*-butanol. To this solution was added potassium bromide (144 mg, 1.21 mmol, 2.0 equiv.), potassium bicarbonate (605 mg, 6.04 mmol, 10.0 equiv.), and 2,2,6,6-tetramethylpiperidine-1-oxyl (104 mg, 664 μmol, 1.1 equiv.). The solution was cooled to 0 °C and 5% sodium hypochlorite (7.2 mL, 4.83 mmol, 8.0 equiv.) was added in 1.8 mL portions at 1-h intervals. After the final addition of sodium hypochlorite, the mixture was allowed to stir for one additional hour (5 h total). The resultant reaction mixture was quenched at 0 °C with 1.2 g sodium thiosulfate pentahydrate in 10 mL of water and allowed to come to room temperature. The aqueous layer was extracted ten times with 10% methanol in chloroform (10 × 40 mL). The volatiles were removed in vacuo to give the crude material, which was purified by flash chromatography (80:18:2–80:26:2 chloroform/methanol/satd aq ammonia) to provide **9f** (188 mg, 63%). White amorphous solid; mp > 230 °C; R_f = 0.43 (80:26:2 chloroform/methanol/satd aq NH_3); $[\alpha]_D$ (23 °C): +23.6° (*c* = 0.035, MeOH); IR (neat): 2946, 2870, 1700 (sharp), 1221 cm^{-1}; ^1H NMR (500 MHz, d^4-methanol): δ 4.02 (dd, *J* = 2.5, 2.5 Hz, 1H, H12), 3.99–3.95 (m, 2H, H24), 3.86 (ddd, *J* = 3.5, 3.5, 3.5 Hz, 1H, H7), 3.54 (dd, *J* = 15.5, 14.0 Hz, 1H, H4α), 2.60 (ddd, *J* = 14.5, 14.5, 5.5 Hz, 1H, H2α), 2.44 (ddd, *J* = 12.5, 12.5, 4.5 Hz, 1H, H2β), 2.15–1.09 (m, 21H), 1.04 (d, *J* = 6.5 Hz, 3H, $CH_3$21), 1.03 (s, 3H, $CH_3$19), 0.76 (s, 3H, $CH_3$18); ^{13}C NMR (125 MHz, d^4-methanol) δ 216.8, 74.0, 69.8, 69.0, 47.7, 45.1, 45.0, 43.1, 41.2, 38.0, 37.8, 37.1, 36.3, 35.2, 35.1, 33.3, 30.0, 28.9, 28.5, 27.4, 24.3, 22.3, 18.1, 13.2; HRMS (ESI−) calcd for $C_{24}H_{39}SO_7^-$ ($M - Na^+$) 471.2422, found 471.2412.

2.2. Biology: olfactory activity and specificity

The olfactory activity of synthesized steroids was assayed using electro-olfactogram (EOG) recording, which is an extracellular, multiunit electrophysiological recording method that measures summated generator potentials from olfactory receptors [2]. This technique had previously been successfully employed to describe the olfactory potency and structure–activity relationships of steroidal odorants in the sea lamprey [4,5,7]. Three types of experiments were performed in a sequential fashion to first identify structures that had significant olfactory activity and then to determine whether and how they bound to the olfactory receptor(s) that recognize native allo-compounds. The last step involved two types of mixture experiments that used both natural (allo-) and unnatural (5β−) compounds to test whether and how different steroids bound with similar receptors.

For EOG recording small (<1 mL) aliquots of test odorants are applied to the fish's nose while electrical responses are measured from the surface of the olfactory tissue that contains the olfactory receptors using established protocols [4,5]. For this study, sea lamprey were anaesthetized with an intramuscular injection of metomidate hydrochloride (Syndel, Vancouver, BC, Canada, 3 mg kg^{-1} body weight), immobilized with an intramuscular injection of Flaxedil (gallamine triethiodide; Sigma, 150 mg kg^{-1} body weight), and placed on a stand where their gills were perfused with 11 °C well water. Their olfactory epithelium was exposed by surgically removing the dorsal portion of their cartilaginous nasal capsules and perfused with flowing well water. Differential EOG responses were recorded using two Ag/AgCl electrodes (type EH-1S; WPI, Sarasota, FL, USA) filled with 3 mol L^{-1} KCl and bridged to saline (8%) gelatin-filled glass capillaries with tip diameters of about 400 μm. The recording electrode was placed between two lamellae and adjusted to maximize responses to the standard (10^{-5} M L-arginine) while minimizing responses to blank water control. A reference electrode was placed on the skin near the naris. The preparation was grounded with a surgical needle that was inserted under the skin and the needle was attached with a wire to the recording cage. Electrical signals were amplified using a DC-preamplifier (Model P16, Grass, Quincy, MA, USA), digitized by a MacLab/4 (Analog Digital Instruments, Castle Hill, Australia), and displayed on an Apple Macintosh computer. Test stimuli were applied in a stream of well water as 5-s pulses using a device that minimized pressure and temperature fluctuations. All stimuli were tested at least twice with 2 min breaks to permit recovery. Controls were performed at the beginning and end of each series of compounds being tested or once an hour (whichever came first). If control responses differed by more than 20% (a rare occurrence), the entire series of measurements was re-run. For analysis, peak responses were measured, blank response subtracted (if present), and the net values expressed relative to the most recent standard. All odorants were tested on at least four lampreys. In the first experiment the sensitivity of the sea lamprey olfactory epithelium was determined to nanomolar concentrations of six analogs of PS **(1)** to gain initial insight into structure–activity relationships and to identity the most stimulatory compounds. Next, dose–response relationships were determined for the most stimulatory agents by testing them at concentrations ranging from 10^{-12} to 10^{-7} M. Last, two types of binary mixture studies were conducted using the test compounds and PADS (**2**) to address whether these compounds stimulate the same (or different) sets of olfactory receptors and, thus, might be discerned from each other by sea lampreys.

The Independent Component Index (ICI) was determined because it provides insight into the extent to which odorants stimulate different (independent) sets of olfactory receptors while the Mixture Discrimination Index (MDI) was determined because it provides insight into the extent to which odorants stimulate the same sets of olfactory receptor(s). We have used both in the past [5]. Briefly, to determine the ICI, EOG responses were measured to equipotent concentrations of each member of an odorant pair (R_a and R_b) and then to a mixture of the two stimuli (R_{ab}). The ratio of the sum of the former to the latter was then calculated (Eq. (1)). An ICI value of 1.0 describes total independence. To determine the MDI, EOG responses were measured to twice the initial concentration of each odorant (R_{2a} or R_{2b}), and these values were summed and averaged. The ratio of this value to the EOG response elicited to the simple mixture of the two (R_{ab}) was calculated using Eq. (2). An MDI value of 1.0 describes a total lack of independence and occurs when odorants are discerned by exactly the same set of receptor(s). Each of these indices is a nonlinear measure and the two need to be interpreted in concert when odorants are discerned by overlapping yet different sets of receptors.

$$\mathrm{ICI} = \frac{R_{ab}}{R_a + R_b} \tag{1}$$

$$\mathrm{MDI} = \frac{R_{ab}}{0.5(R_{2a} + R_{2b})} \tag{2}$$

3. Results and discussion

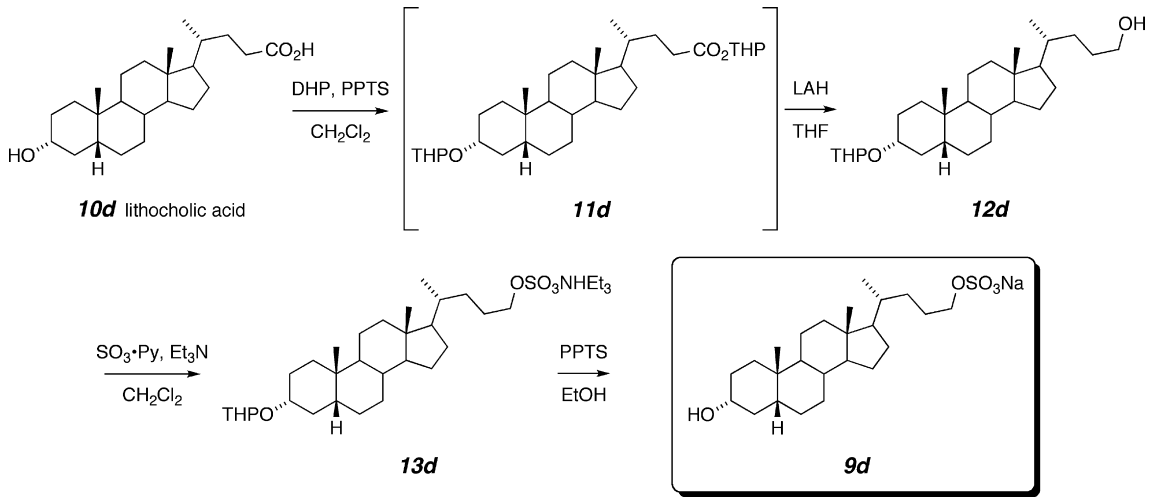
3.1. Chemical synthesis

The synthesis of 5β-petromyzonol sulfate (**9a**) [17] was achieved by protection of cholic acid (**10a**) as the triformate ester **11a** (formic acid, 55 °C) [14] and subsequent chemoselective reduction [15] of the carboxylic acid with borane–dimethylsulfide complex to provide the primary alcohol **12a** (Scheme 1). Sulfation of **12a** was effected by a modified procedure in which a suspension of sulfur trioxide–pyridine complex in methylene chloride was solubilized by the addition of triethylamine (4 equiv.); this permits sulfation to occur at ambient temperature rather than the higher temperatures typically required for sulfation using sulfur–trioxide in pyridine. The triformate **13a** was saponified (aq NaOH, MeOH) with concomitant triethylammonium–sodium exchange to provide **9a**. The analogous compounds **9b** and **9c**, derived from deoxycholic acid (**10b**) and chenodeoxycholic acid (**10c**) respectively, were synthesized using a very similar protocol.

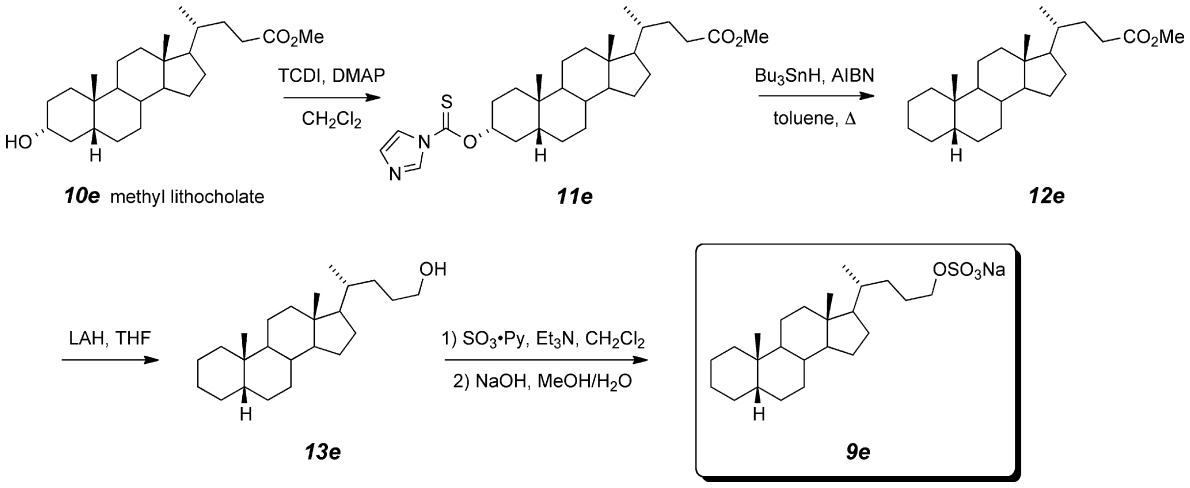
Lithocholic acid (**10d**) was converted to the primary alcohol **12d** using a one-flask/two-reaction sequence (Scheme 2), presumably via the THP-ester **11d** [19]. The primary alcohol **12d** was sulfated (SO_3·Py, Et_3N) to give the THP-ether **13d**, which was converted to the secondary alcohol **9d** (pPTS, EtOH).

For the synthesis of **9e** (Scheme 3), methyl lithocholate (**10e**) was converted to the thiocarmabate **11e** (TCDI, DMAP; Scheme 3) followed by a modified Barton–McCombie deoxygenation [21,22]. The deoxygenation was sensitive to the reaction conditions; formation of a by-product [23] arising from the reduction of the thiocarbamate moiety was observed under the standard Barton–McCombie protocol. This could be overcome by the slow addition (i.e., over 1 h) of the substrate and azobisisobutyronitrile (AIBN) to a dilute solution of an excess of tri-*n*-butyltin hydride (0.06 M, 4.0 equiv.) at toluene reflux [22].

Selective oxidation of 5β-petromyzonol sulfate (**9a**) with TEMPO under aqueous conditions gave 3-keto-5β-petromyzonol sulfate (**9f**, Scheme 4). Under these conditions the regioselective oxidation of the C3 carbinol in the presence of the unprotected C7 and C12 alcohols is noteworthy; the use of a stoichiometric amount of inexpensive TEMPO and the slow addition of the stoichiometric oxidant (5% NaOCl) was important to minimize unselective background oxidation effected by sodium hypochlorite.



Scheme 2. Synthesis of lithocholic acid derivative **9d**.

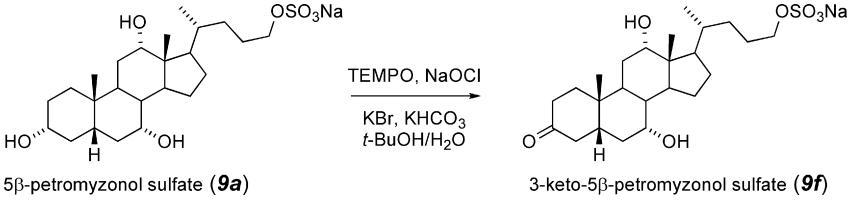


Scheme 3. Synthesis of cholan-24-ol sulfate (**9e**).

3.2. Olfactory activity

All EOG waveforms measured in this study were typical of those we have measured before [5]. They showed a characteristic sharply negative phasic component preceding a peak (typically between 0.2 and 3 mV in magnitude) that decayed back to baseline over a 5–10 s period. The averaged results from EOG tests of 5β-petromyzonol sulfate (**9a**) and five additional analogs (**9b–f**) are summarized in Fig. 4. Although the *tris*-oxygenation present in **9a**, the 5β-analog of natural PS (**1**), was not required to elicit an olfactory response at a concentration of 10^{-9} M, this attribute certainly contributes to olfactory activity because **9a** is the most stimulatory compound in this series. Nevertheless, *bis*-oxygenation at both C3, C7 and C3, C12 (**9c** and **9b**, respectively) elicited a modest olfactory response. The C3 *mono*-oxygenated compound **9d** generated only a small EOG response. Ketone **9f**, a mimic of the natural sex pheromone, 3-ketopetromyzonol sulfate [8–10] produced an olfactory response, but it was small. This may imply that 3-ketopetromyzonol sulfate receptors are less promiscuous than the PS receptors; indeed, earlier cross-adaptation studies [7] demonstrate that receptors for 3-ketopetromyzonol sulfate and PS **(1)** are not completely independent of each other. Finally, the *des*-oxygenated compound **9e** did not elicit an olfactory response, suggesting that some oxygenation is required for binding (although micellization of this detergent like molecule cannot be ruled out as a contributing factor). The entire steroid framework appears to contribute to some extent to binding by the PS receptor.

As shown in Fig. 5, 5β-petromyzonol sulfate (**9a**) elicited relatively large EOG responses from the sea lamprey olfactory epithelium. The dose–response curve for **9a** was shifted to the right



Scheme 4. Selective oxidation of 5β-petromyzonol sulfate.

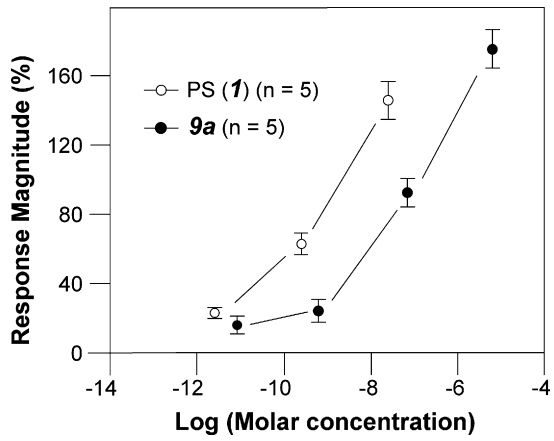


Fig. 5. Semi-logarithmic plots of the dose–response relationship of PS (**1**) and **9a**. Response magnitudes are presented as a percentage of the standard, 10^{-5} M L-arginine. Vertical bars represent one standard error ($n = 5$ animals).

(less responsive) of that of PS (**1**) by only 1–2 orders of magnitude. The latter had a detection threshold of $\sim 10^{-11}$ M and the former $\sim 10^{-10}$ M. This result is analogous to earlier findings with allocholic (**7**) vs. cholic acids (**10a**) [5] where the allo-configured ligand also elicited a stronger response. Moreover, the dose–response curves for **7** vs. **10a** were parallel with the latter shifted to the right of the former (allo isomer) by 1–2 orders of magnitude. Mixture experiments using **7** and **10a** showed an ICI suggesting that they also interact with the same receptor [5]. Together, these results suggest moderate tolerance of 5β-steroids by sea lamprey PS olfactory receptor(s).

Binary mixture experiments using either PS (**1**) or PADS (**2**) with **9a** were performed. The results are shown in Fig. 6 and suggest that **1** and **9a** are detected by the same set of olfactory receptor(s) while **2** and **9a** are not. In the first instance, while the ICI for **1**/**9a** was less than 1.0 (ICI = 0.67; $p = 0.005$ when compared with 1.0 by *t*-test), the MDI was not (MDI = 1.05; $p = 0.789$ when compared with 1.0). This can be taken as additional evidence for recognition by the same binding sites (the PS-receptor), and indicates that the PS-receptor sites are fairly promiscuous with respect to the topology of the steroid nucleus (i.e., *cis*-decalin-like vs. *trans*-decalin-like). In the second instance, while the ICI for the **2**/**9a** mixture was very close to 1.0 (ICI = 0.82), the MDI was far greater than 1.0 (MDI = 1.32, $P = 0.024$)]. These data are in agreement with previous cross-adaptation results [7] that indicate that PS (**1**) and PADS (**2**) are discerned from one another and have different receptor activation structural requirements.

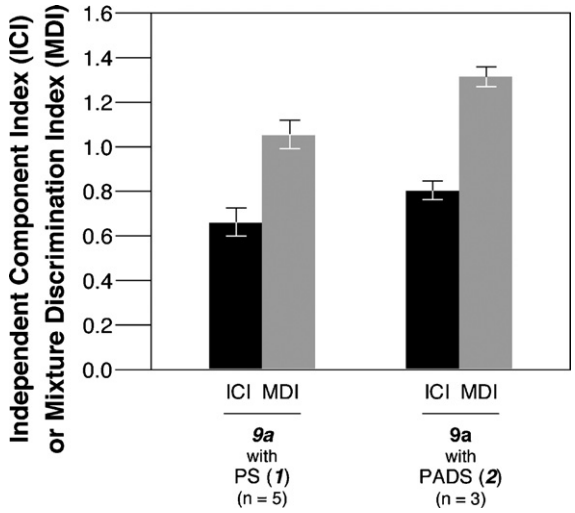


Fig. 6. The independent component index (ICI) and mixture discrimination index (MDI) of binary mixtures of **9a** with PS (**1**) ($n = 5$ animals) or with petromyzonamine disulfate (PADS, **2**) ($n = 3$ animals). Vertical bars represent one standard error.

4. Conclusions

This study has resulted in the synthesis of several new bile acid derivatives. These were used to demonstrate that sea lamprey olfactory receptors for PS (**1**) are relatively insensitive to the allo-configuration at C5 but that structural features elsewhere in the molecules are discerned. 5β-Petromyzonol sulfate (**9a**) elicits EOG responses that are only slightly smaller than those of natural (allo) PS (**1**). Further, mixture studies demonstrated that **9a** and **1** interact with the same receptor (ICI = ca. 1). This result is analogous to previous findings for allocholic (**7**) vs. cholic (**10a**) acid [5]. In the only other example of a steroidal odorant where binding properties have been explored in detail – the goldfish sex pheromone 17,20β-dihydroxy-4-pregenen-3-one – single changes in the structural features decreased binding affinity by 1–2 orders of magnitude [24]. The present work also sheds some insight on possible differences between olfactory receptors for modern teleost (bony) fish and lampreys. In particular, single unit recording from the catfish olfactory bulb suggests that this teleost's olfactory receptors are more sensitive to hydroxylation at the C7 and C12 positions than those of the sea lamprey [25]. In other ways both groups of fishes are similar. They both exhibit specific and remarkable sensitivities for steroids with allo-configurations as well as taurine, glycine, and sulfated conjugates, although the details of these relationships have yet to be fully explored.

The structural influence on olfactory activity in these unnatural 5β-steroidal sulfates was further examined as a function of oxygenation at the C3-, C7-, and C12-positions of the steroid skeleton. Interestingly, the C7-hydroxy substituent appears to be more important to olfactory activity than that of the C12-substituent (cf. **9b** vs. **9c**, Fig. 5). Previous results have shown that the C24-*O*-sulfate is also critical [5]. It appears that the PS (**1**) binding pocket is discerning a large portion, but not the entirety, of each of these structures. Because allo-configured steroids are more difficult and expensive to synthesize than their 5β-counterparts, this information should be useful in the further development of pheromone analogs (e.g., 5β-PADS) for use in controlling the sea lamprey, which is invasive in the Great lakes of North America.

Acknowledgments

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