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Design, Synthesis and In Vitro Evaluation of Pyridinium Ion Based Cyclase Inhibitors and Antifungal Agents

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Abstract—The design, synthesis and in vitro biological evaluation of pyridinium ion based inhibitors of oxidosqualene cyclase enzymes are reported. *N*-Alkyl- and *N*-prenylpyridinium ions have been found to be potent and specific inhibitors of *Candida albicans* oxidosqualene–lanosterol cyclase and to exhibit antifungal activity. The ability of pyridinium ions to inhibit the *C. albicans* cyclase increases with increasing structural resemblance to a putative monocyclized species formed during the course of the cyclization process. The *N*-(4E,8E)-5,9,13-trimethyl-4,8,12-tetradecatrien-1-ylpyridinium cation **1** inhibits the *C. albicans* enzyme at concentrations more than 100-fold lower than does the directly analogous piperidinium derivative **4**.

Introduction

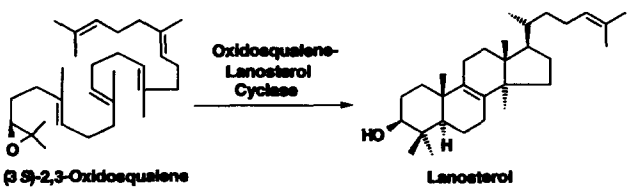
Squalene and oxidosqualene cyclase enzymes catalyze remarkably diverse and complex cyclization and cyclization/rearrangement reactions during the course of sterol and triterpenoid biosynthesis.[1] We have recently determined the DNA sequences of genes encoding cyclase enzymes from the yeasts *Candida albicans* and *Saccharomyces cerevisiae* which, like vertebrates, convert oxidosqualene to lanosterol (Scheme 1).[2,3] The predicted amino acid sequences of these enzymes and other members of the triterpene cyclase family[4–8] are unusually rich in tryptophan and tyrosine residues, many of which are found in conserved positions. These findings and consideration of the electrophilic nature of polyene cyclization/rearrangement reactions led us to formulate an *aromatic hypothesis* for cyclase active site structure.[2,3b,9,10] In this hypothesis, electron-rich indole and phenol side chains from tryptophan and tyrosine residues are essential features of cyclase active sites. Through cation-π interactions,[11,12] these residues stabilize specific, positively charged transition states and high-energy intermediates formed during the course of the cyclization process.[13–16] Thus, tryptophan and tyrosine side chains may consititute the "axial negative point charges" envisioned by Johnson to lower the transition state barriers and control the regio- and stereochemistry of cyclization.[15]

Several groups have shown that acyclic and alicyclic ammonium ions, designed to mimic non- or partially cyclized transition states and high-energy intermediates, can be potent inhibitors of oxidosqualene cyclase enzymes.[17–23] The aromatic hypothesis suggests that electron-poor aromatic species may be good cyclase inhibitors, as they would be able to form favorable packing and charge-transfer interactions with tryptophan and/or tyrosine side chains present at the active site. By virtue of their cyclic nature, aromatic compounds also mimic partially cyclized transition states and high-energy intermediates. The cyclase inhibitory properties of *N*-dodecylimidazole and the *N*-dodecylpyridinium cation have been examined.[19a,20] While *N*-dodecylimidazole is a potent inhibitor of plant cyclase enzymes and both agents inhibit the cyclase from rat liver, they are inactive against the yeast cyclase. Here we report that (1) pyridinium ions, designed to mimic a monocyclized species (Fig. 1) are potent inhibitors of the *C. albicans* cyclase, (2) inhibitory potency varies with the identity of the group attached to pyridinium nitrogen, (3) pyridinium ion cyclase inhibitors inhibit the growth of fungal cells, and (4) compound **1** is substantially more potent as a cyclase inhibitor and antifungal agent than the directly analogous alicyclic piperidine analog **4**, which should be protonated at neutral pH.

Results and Discussion

Synthesis

Compounds **1–4** were prepared by *N*-alkylation of pyridine, picoline, 4-*N*,*N*-dimethylaminopyridine and



Scheme 1.

Dedicated to the memory of Professor William S. Johnson.

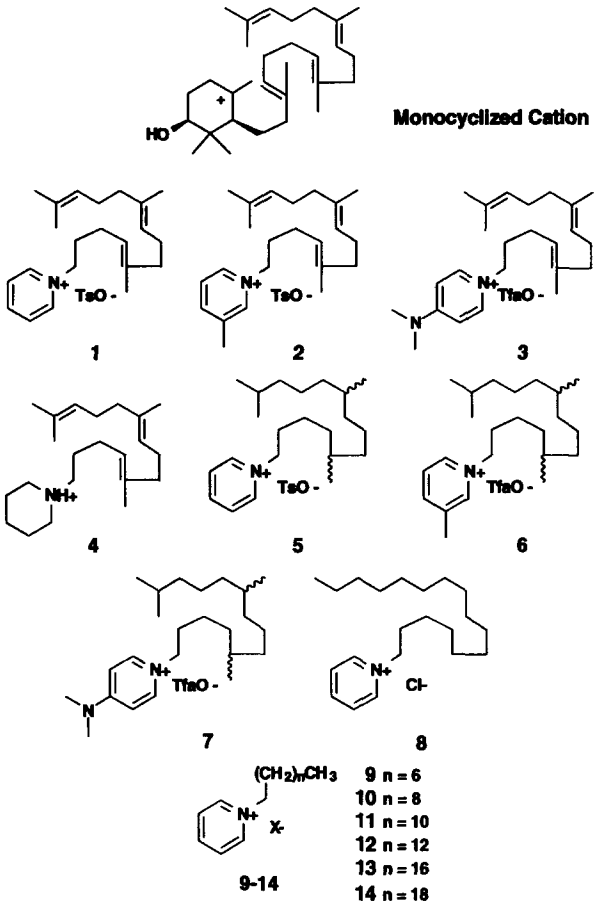
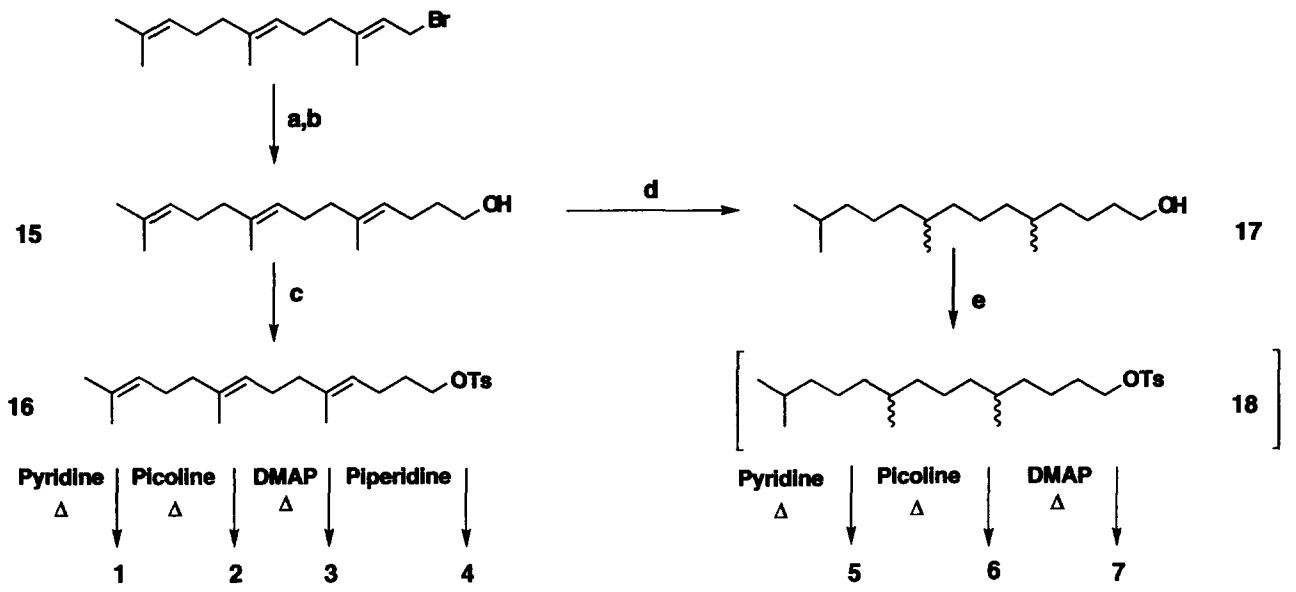


Figure 1. Compounds 1–14 which mimic a monocyclized cationic species (transition state or high-energy intermediate). TsO-: *p*-Toluenesulfonate; TfaO-: Trifluoroacetate.

piperidine, respectively, with the tosylate (**16**) of (4E,8E)-5,9,13-trimethyl-4,8,12-tetradecatrien-1-ol (**15**). Compound **15** was derived by two carbon homologation of farnesyl bromide. Compounds **5–7** were prepared as mixtures of stereoisomers by *N*-alkylation of pyridine, picoline, and 4-*N*,*N*-dimethylaminopyridine with tosylate **18**. Compound **18** was generated in situ from 5,9,13-trimethyl-4,8,12-tetradecan-1-ol (**17**), which was obtained by catalytic hydrogenation of **15** (Scheme 2). Compounds **1-7** exhibited ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectra that were consistent with the assigned structures. The *N*-alkylpyridinium ions **8-14** are well-known compounds; when not commercially available, derivatives in this series were prepared by *N*-alkylation of pyridine with the appropriate *n*-alkyl chlorides (**9**, **10**, **12**, **13**) or bromides (**14**).

Inhibition of in vitro sterol biosynthesis

Compounds **1–8**, **16** and pyridinium *p*-toluenesulfonate (**19**) were tested for their ability to inhibit sterol biosynthesis in a cell free lysate of the opportunistic pathogen *C. albicans*. Compounds **16** and **19** had little effect on sterol biosynthesis in the cell-free system. Compounds **1–8**, on the other hand, were found to inhibit the formation of cyclized products (lanosterol, mono-methylsterols, and ergosterol) in a concentration-dependent fashion. The observed decreases in these products were accompanied by an accumulation of oxidosqualene and an unidentified compound which exhibits TLC mobility similar to 2,3,22,23-dioxidosqualene. Representative data (for compound **1**) are shown in Figure 2. Here it may be seen that oxidosqualene accounts for 22% of total non-saponifiable lipids (NSL) in the control reaction, but 36% of NSL in the presence of 5 μM of **1**. At this concentration, the



Scheme 2. Synthesis of compounds 1–7. (a) $C_2H_6O_2CCH_2Cu(I)$, THF, −30 °C; (b) $LiAlH_4$, Et_2O; (c) TsCl, Et_3N, CH_2Cl_2; (d) H_2, Pd/C; (e) TsCl with the pyridine derivatives as solvent.

combination of oxidosqualene and the unidentified compound account for 62% of NSL.

Inhibitory potency, as indicated by decreasing concentrations required to inhibit the formation of cyclized products by 50% (IC_{50}), increases in the order **4**≪**8**<**7**≈**2**≈**5**<**6**<**1**<**3** (Table 1). All of the pyridinium ions tested are more potent cyclase inhibitors than is **4** and strikingly, *the IC_{50} value for pyridinium ion* **1** *is more than two orders of magnitude lower than that for the directly analogous piperidinium ion* **4**. The IC_{50} data suggest that the aromatic species **1** has substantially greater affinity for the *C. albicans* cyclase active site than does the direct alicyclic analogue **4**; however, more detailed kinetic analysis will be required to determine whether these compounds are competitive inhibitors of oxidosqualene cyclase. With regard to the effects of side chain structure, inhibitory potency increases with increasing structural similarity to that of a putative monocyclized, cationic transition state or high-energy intermediate. Compounds **1-3**, which bear an isoprenoid chain that closely mimics the polyene chain of a monocyclized species, are more potent inhibitors than **5–7**, which bear a saturated branched chain. Compounds **5–7** are in turn more potent than **8**, which bears a linear hexadecyl chain two carbons longer than the longest straight chains found in **1–7**. Modifications in the pyridine ring have quantitatively smaller effects. Addition of a methyl substituent at the 3-position increases IC_{50} by a factor of less than two for **2** relative to **1**. Addition of the basic dimethylamino group at the 4-position, where it could potentially form a salt bridge with a cyclization-initiating general acid group at the active site, increases in vitro inhibitory potency. This is indicated by the factor of four decrease in IC_{50} for **3** relative to **1**.

Inhibition of fungal cell growth

1–14, **16** and **19** were also tested for their effects on the growth of *S. cerevisiae*, *C. albicans*, *Aspergillus niger* and *Cryptococcus albidus* in liquid culture. Compounds **1–8** and **10–14** inhibited fungal cell growth, **9** was active only against *A. niger* at 100 μg mL^{-1} (440 μM) and **16** and **19** were inactive at the highest concentrations examined (MIC>100 μg mL^{-1} (400 μM), Table 1). In a few cases, the potency of pyridinium ions as antifungal agents surpassed that of amphotericin B. The observed trends in antifungal potencies for the different families of pyridinium ions were generally opposite the trends in cyclase inhibition potencies by these compounds. Compound **8** is more potent than **5–7**, which are in turn more potent than **1–3**. MICs for compound **8** are lower than the measured IC_{50} value ($MIC/IC_{50}=0.3–1$). This indicates that cyclase inhibition is probably not the primary mode of antifungal action by this compound. Compound **8** is known to exhibit toxicity towards mammalian,[24] bacterial[25] and fungal cells,[14,26,27] which has generally been attributed to membrane disruption, though recent studies suggest that interactions between **8** and unidentified intracellular components can make a significant contribution to its antifungal effects.[26] For compounds **1–4**, inhibitory potency increases in the order **4**<**1**<**2**<**3**, which is similar but not identical to the ordering of the respective IC_{50} values for this series of derivatives. MICs for compounds **1–3** are substantially higher than their IC_{50} values, which suggests that inhibition of sterol biosynthesis could form the basis for the

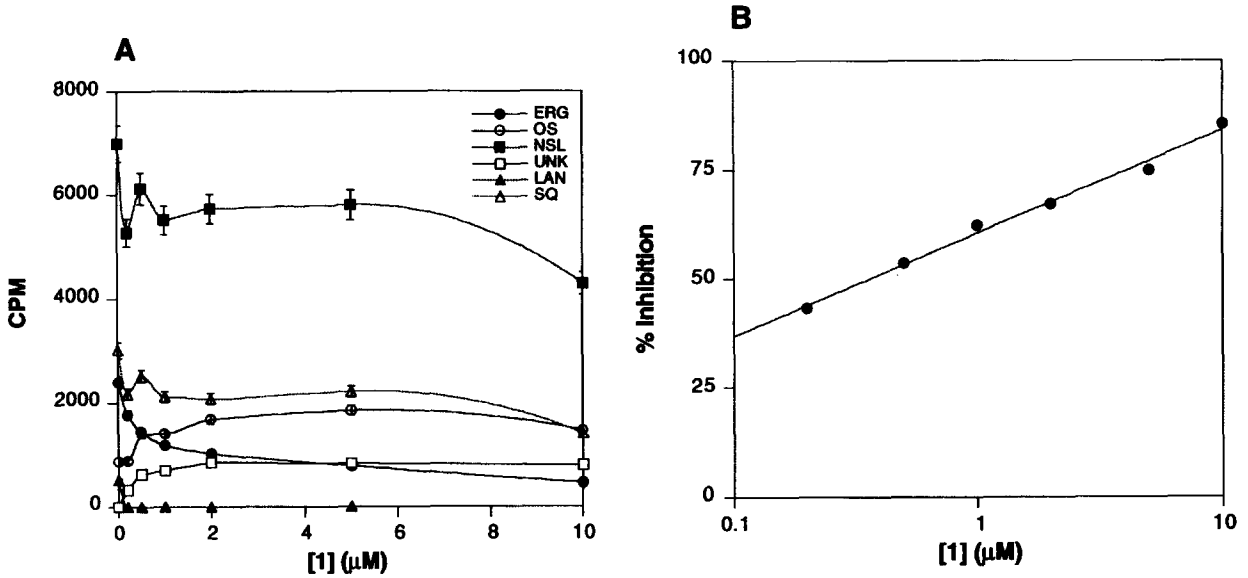


Figure 2. Inhibition of ergosterol synthesis in a cell-free extract from *C. albicans* strain CCH442 by compound **1**. The lysate was treated with increasing concentrations of **1** and NSL were extracted and quantitatively analyzed in triplicate after the synthesis incubation. A: NSL as a function of [**1**]. Filled squares: total NSL; open triangles: squalene; open circles: oxidosqualene; open squares: unidentified compound; filled triangles: lanosterol; filled circles: ergosterol. B: Inhibition of sterol (lanosterol + ergosterol) formation as a function of [**1**].

observed antifungal activity of these compounds. This notion is supported by work to be published separately in which we have found that compounds **1** and **2** inhibit sterol biosynthesis at the cyclization step in intact *C. albicans* cells and that they are fungicidal towards *C. albicans* but do not cause direct membrane damage.[28]

In summary, we have found the aromatic hypothesis to be a useful paradigm for the design of new and potent oxidosqualene cyclase inhibitors. We have shown that pyridinium ions are much more active against the *C. albicans* cyclase in vitro than is the piperidinium ion **4**. While our results do not provide direct evidence for the presence of electron-rich aromatic groups at the active site of fungal oxidosqualene cyclase enzymes, they are consistent with the aromatic hypothesis and prompt further investigation of electron-poor aromatic species as active site probes and potential antifungal agents. We note that potent cyclase inhibitors bearing multiple aromatic rings, including electron-deficient benzophenone units, have recently been reported.[23b] The aromatic groups of these molecules were chosen as rigid spacers to link an ammonium ion and a ketone functionality and as modulators of ketone electrophilicity, but our model and results suggest that these groups may also form specific, stabilizing interactions with electron-rich aromatic side chains at the active site.

Experimental

General

Chemical reagents were used as received unless otherwise noted. Reactions were carried out under inert atmospheres. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ solvent at 400 and 100 MHz, respectively, on a Varian XL-400 instrument. Chemical shifts are reported in parts per million downfield of tetramethylsilane internal standard.

(4E,8E)-5,9,13-Trimethyl-4,8,12-tetradecatrien-1-ol (15). Compound **15** was prepared according to the reported literature procedure.[29]

(4E,8E)-5,9,13-Trimethyl-4,8,12-tetradecatrien-1-yl *p*-toluenesulfonate (16). Compound **15** (2.07 g, 8.3 mmol) and triethylamine (0.88 mL, 12 mmol) were dissolved in methylene chloride (40 mL) and cooled to 0 °C. A solution of *p*-toluenesulfonyl chloride (2.32 g, 12.2 mmol, Aldrich) in methylene chloride (20 mL) was added over the course of 5 min. After stirring for 20 min, the product was extracted with 2×30 mL water. The organic phase was dried over magnesium sulfate, and the solvent was removed under reduced pressure to afford 3.1 g (7.9 mmol, 95%) of **6** as a pale yellow oil. ^1H NMR: δ 1.53 (s, 3H), 1.58 (s, 3H), 1.59 (s, 2H), 1.67 (s, 6H), 1.95 (m, 4H), 2.01 (m, 6H), 2.44 (s, 3H), 4.00 (t, 2H), 4.97 (t, 1H), 5.06 (m, 2H), 7.33 (d, 2H), 7.78 (d, 2H). ^{13}C NMR: δ 15.9, 17.6, 21.6, 23.6, 25.6, 26.5, 26.7, 28.9, 39.6, 39.7, 70.0, 122.1, 124.0, 124.3, 127.8, 129.7, 131.2, 133.2, 135.0, 136.8, 144.6. HR-FABMS, calcd for $C_{25}H_{35}SO3$ ($[M-H]^+$): 403.2307; obsd 403.2300.

5,9,13-Trimethyltetradecan-1-ol (17). Compound **15** (300 mg, 1.2 mmol) was dissolved in 25 mL methanol, treated with 65 mg 10% palladium on carbon (Aldrich) and hydrogenated at 3.75 atm for 6 days in a Paar

Table 1. Inhibition of squalene cyclization and fungal cell growth

Inhibitor	IC_{50}[a] (μM)	MIC μg mL^{-1} (μM)[b]			
		S. cerevisiae GSI-36	*C. albicans* CCH 442	*A. niger* ATCC 16404	*C. albidus* ATCC 34140
Amphotericin B		1.6 (1.7)	1.6 (1.7)	1.6 (1.7)	1.6 (1.7)
1	0.36	3.1 (6.4)	12 (26)	6.2 (13)	12 (26)
2	0.61	1.6 (3.2)	6.2 (13)	3.1 (6.3)	6.2 (13)
3	0.09	0.78 (1.7)	1.6 (3.3)	1.6 (3.3)	3.1 (6.7)
4	>50	12 (39)	50 (160)	25 (79)	12 (39)
5	0.60	0.78 (1.6)	3.1 (6.4)	6.2 (13)	3.1 (6.4)
6	0.47	0.39 (0.88)	3.1 (7.0)	6.2 (14)	1.6 (3.5)
7	0.66	0.78 (1.6)	1.6 (3.3)	6.2 (13)	1.6 (3.3)
8	3.7	0.39 (0.97)	0.78 (1.9)	1.6 (3.9)	0.39 (0.97)
9	ND[c]	>100 (>440)	>100 (>440)	100 (440)	>100 (440)
10	ND	12 (49)	50 (200)	25 (98)	12 (49)
11	ND	0.39 (1.4)	6.2 (22)	12 (44)	1.6 (5.5)
12	ND	0.39 (1.2)	3.1 (10)	3.1 (10)	1.6 (5.0)
13	ND	0.78 (2.1)	1.6 (4.2)	3.1 (8.5)	1.6 (4.2)
14	ND	1.6 (3.4)	1.6 (3.4)	3.1 (6.8)	3.1 (6.8)
16	>20[d]	>100 (>400)	>100 (>400)	>100 (>400)	>100 (>400)
19	>200	>100 (>400)	>100 (>400)	>100 (>400)	>100 (>400)

[a]Concentration required to inhibit formation of cyclized products by 50% in the in vitro assay using *C. albicans* strain CCh 442.
[b]Minimal concentration required to inhibit fungal growth in a microbroth dilution assay using yeast nitrogen base media (Difco) containing glucose in pH 7.0 phosphate buffer.
[c]ND = Not determined.
[d]No effect on ergosterol biosynthesis was observed at a concentration of 20 μM.

apparatus. The reaction mixture was then warmed, filtered and the residue washed with additional methanol. After removal of solvent, 290 mg (1.13 mmol, 94%) of **15** was obtained as a pale yellow oil. ^{1}H NMR: δ 0.85 (m, 12H), 1.12 (m, 6H), 1.24 (m, 12H), 1.51 (m, 3H), 3.63 (t, 2H).

***N*-(4E,8E)-5,9,13-Trimethyl-4,8,12-tetradecatrienylpyridinium *p*-toluenesulfonate (1).** A solution of **16** (0.023 g, 0.057 mmol) in dry pyridine (4 mL, distilled from barium oxide) was stirred at reflux for 1 h. Excess pyridine was removed by distillation, affording a yellow-brown solid. The product was recrystallized from hot ethyl acetate to give 0.026 g (0.054 mmol, 95%) of **1** as flaky white, hygroscopic crystals. ^{1}H NMR: δ 1.51 (s, 3H), 1.58 (s, 6H), 1.66 (s, 3H), 1.69 (s, 2H), 1.92–1.96 (m, 4H), 2.00–2.02 (m, 6H), 2.32 (s, 3H), 4.81 (t, 2H), 4.99 (broad t, 1H), 5.06 (m, 2H), 7.13 (d, 2H), 7.77 (d, 2H), 8.03 (t, 2H), 8.40 (t, 1H), 9.16 (d, 2H). ^{13}C NMR: δ 16.0, 16.2, 17.7, 21.3, 24.3, 25.7, 26.5, 26.8, 31.6, 39.6, 39.7, 61.9, 121.4, 123.9, 124.3, 125.9, 128.3, 128.6, 131.4, 135.3, 137.8, 139.2, 143.9, 144.9, 145.3. HR-FABMS: calcd for $C_{22}H_{34}N$ ($[M]^+$): 312.2691; obsd 312.2693.

***N*-(4E,8E)-5,9,13-Trimethyl-4,8,12-tetradecatrienylpicolinium *p*-toluenesulfonate (2).** A solution of **16** (0.023 g, 0.057 mmol) in 3-picoline (4 mL, Aldrich) was stirred at 100 °C under nitrogen for 6 h. Excess picoline was removed by vacuum distillation at 40 °C, yielding a brown oil. The product was recrystallized from hot ethyl acetate to give 0.027 g (0.054 mmol, 95%) of **2** as flaky white, hygroscopic crystals. ^{1}H NMR: δ 1.52 (s, 3H), 1.58 (s, 6H), 1 66 (s, 3H), 1.92–1.97 (m, 4H), 1.99–2.07 (m, 8H), 2.32 (s, 3H), 2.56 (s, 3H), 4.78 (t, 2H), 4.99 (broad t, 1H), 5.06 (q, 2H), 7.12 (d, 2H), 7.77 (d, 2H), 7.89 (t, 1H), 8.13 (d, 1H), 8.94 (d, 1H), 9.03 (s, 1H). ^{13}C NMR: δ 16.0, 16.2, 17.7, 18.6, 21.2, 24.3, 25.7, 26.5, 26.7, 31.6, 39.6, 39.7, 61.5, 121.5, 123.9, 124.3, 125.9, 127.7, 128.5, 131.3, 135.2, 137.5, 139.0, 139.6, 142.4, 144.0, 144.8, 145.2. HR-FABMS: calcd for $C_{23}H_{36}N$: 326.2848; obsd 326.2860.

***N*-(4E,8E)-5,9,13-Trimethyl-4,8,12-tetradecatrienyl-4′-*N*′,*N*′-dimethylaminopyridinium *p*-toluenesulfonate (3).** A mixture of **16** (155 mg, 0.40 mmol) and 4-*N*,*N*-dimethylaminopyridine (41 mg, 3.3 mmol, Aldrich) was heated at 120 °C for 12 h. The reaction mixture was then fractionated by reversed-phase HPLC (RP-HPLC) using a Rainin Dynamax C18 column and a gradient of ethyl acetate in water containing 0.1% TFA. Fractions containing the desired product were combined and lyopholized to afford 88 mg (0.24 mmol, 60%) of **3** as a colorless oil. ^{1}H NMR: δ 1.56 (s, 3H), 1.58 (s, 6H) 1.66 (s, 3H), 1.90 (m, 2H), 1.97 (m, 2H) 2.04 (m, 8H), 3.24 (s, 6H), 4.14 (t, 2H), 5.06 (m, 3H), 6.87 (d, 2H), 8.03 (d, 2H). ^{13}C NMR: δ 16.0, 16.1, 17.7, 24.1, 25.7, 26.5, 26.7, 30.7, 39.6, 39.7, 40.2, 57.8, 108.2, 121.5, 123.9, 124.2, 131.4, 135.3, 137.8, 141.9, 156.3. HR-FABMS: calcd for $C_{24}H_{39}N_2$: 355.3113; obsd 355.3112.

***N*-(4E,8E)-5,9,13-Trimethyl-4,8,12-tetradecatrienylpiperidine (4).** A solution of **16** (0.10 g, 0.25 mmol) in piperidine (1.0 mL) was stirred under nitrogen at rt for 10 h. Excess piperidine was evaporated, and the product was purified first by chromatography on 20 cm^3 silica gel (1% concentrated aqueous ammonia in methanol eluent, R_f 0.7) and then by dissolution in 10 mL ether and extraction with 5 × 10 mL saturated aqueous sodium bicarbonate. The organic layer was dried over sodium sulfate and evaporated to afford 0.040 g (0.13 mmol, 51%) of **4** as a pale brown oil. ^{1}H NMR: δ 1.43 (m, 2H), 1.5–1.6 (m, 15H), 1 68 (s, 3H), 1.98 (m, 6H), 2.06 (m, 4H), 2.28 (m, 2H), 2.42 (br s, 4H), 5.11 (m, 3H). ^{13}C NMR: δ 16.0, 17.7, 24.5, 25.7, 26.0, 26.5, 26.7, 27.1, 39.7, 54.6, 59.1, 124.2, 124.3, 131.2, 134.9, 135.2. HR-EIMS: calcd for $C_{22}H_{39}N$: 317.3082; obsd 317.3097.

***N*-5,9,13-Trimethyltetradecan-1-ylpyridinium *p*-toluenesulfonate (5).** A solution of **17** (20 mg, 0.078 mmol) in 1 mL dry pyridine was added to a solution of *p*-toluenesulfonyl chloride (42 mg, 0.23 mmol) in 2 mL dry pyridine. This mixture was heated to reflux for 2 h, cooled to rt, treated with 1.5 mL water, and then extracted with 3 × 5 mL diethyl ether. The aqueous phase was lyopholized to afford 33 mg (0.067 mmol, 86%) of **5** as a yellow oil. ^{1}H NMR: δ 0.78 (d, 3H), 0.84 (d, 3H), 0.86 (d, 6H), 1.04 (m, 4H), 1.14 (m, 2H), 1.26 (m, 9H), 1.44 (t, 1H), 1.52 (sep, 2H), 1.89 (m, 2H), 2.34 (s, 3H), 4.79 (t, 2H), 7.14 (d, 2H), 7.76 (d, 2H), 8.03 (t, 2H), 8.39 (t, 1H), 9.18 (d, 2H). ^{13}C NMR: δ 19.3, 19.4, 19.6, 19.7, 21.3, 22.6, 22.7, 23.6, 24.5, 24.8, 27.9, 32.1, 32.6, 32.8, 36.4, 36.5, 37.3, 37.4, 39.3, 62.2, 125.9, 128.3, 128.6, 139.3, 139.2, 143.7, 144.8, 145.2. HR-FABMS: calcd for $C_{22}H_{40}N$ ($[M]^+$): 318.3160; obsd 318.3168.

***N*-5,9,13-Trimethyltetradecan-1-ylpicolinium trifluoroacetate (6).** A solution of **17** (30 mg, 0.12 mmol) in 1 mL 3-picoline was added to a solution of *p*-toluenesulfonyl chloride (22 mg, 0.12 mmol) in 2 mL 3-picoline. This mixture was heated to 100 °C for 1.5 h. After cooling to rt, the resulting solid was dissolved in water and fractionated by RP-HPLC as described for **3**, affording 40 mg (0.090 mmol, 75%) of **6** as a pale yellow oil. ^{1}H NMR: δ 0.82 (d, 3H), 0.83 (d, 3H), 0.86 (d, 6H), 1.05 (m, 3H), 1.12 (m, 3H), 1.24 (m, 6H), 1.34 (m, 6H), 1.52 (sep, 1H), 1.98 (m, 2H), 4.70 (t, 2H), 7.93 (t, 1H), 8.21 (d, 1H), 8.85 (d, 1H), 8.92 (s, 1H). ^{13}C NMR: δ 18.7, 19.4 (2), 19.7 (2), 22.6, 22.7, 23.7, 24.5, 24.8, 28.0, 32.1, 32.6, 32.8, 36.3, 36.4, 37.3 (2), 37.4 (2), 39.4, 62.4, 127.8, 140.1, 141.9, 144.4, 145.6. HR-FABMS: calcd for $C_{23}H_{42}N$ ($[M]^+$): 332.3317; obsd 332.3318.

***N*-5,9,13-Trimethyltetradecan-1-yl-4′-*N*′,*N*′-dimethylaminopyridinium trifluoroacetate (7).** A mixture of 4-dimethylaminopyridine (41 mg, 0.33 mmol), *p*-toluene-

sulfonyl chloride (115 mg, 0.66 mmol) and **17** (155 mg, 0.61 mmol) was heated to 120 °C for 20 h. After cooling to rt, the resulting solid was dissolved in water and fractionated by RP-HPLC as described for **3**, affording 149 mg (0.31 mmol, 52%) of **7** as a colorless oil. ^{1}H NMR: δ 0.82 (s, 3H), 0.83 (d, 3H), 0.85 (s, 3H), 0.87 (s, 3H), 1.06 (m, 2H), 1.14 (m, 2H), 1.24 (m, 6H), 1.32 (m, 8H), 1.52 (sep, 1H), 1.82 (m, 2H), 3.25 (s, 6H), 4.16 (t, 2H), 6.90 (d, 2H), 8.10 (d, 2H). 13C NMR: δ 19.4, 19.5, 19.6, 19.7, 22.6, 22.7, 23.6, 24.4, 27.9, 31.3, 32.6, 32.8, 36.3, 36.4, 37.3, 37.4, 39.4, 40.3, 58.5, 108.2, 141.9, 156.3. HR-FABMS: calcd for $C_{24}H_{45}N_2$ ([M]$^+$): 361.3583; obsd 361.3589.

Cell-free sterol biosynthesis assays[30]

Candida albicans strain CCH442, a clinical isolate, was grown in Sabouraud broth at 30 °C to OD 600 nm = 2. Cells were collected by centrifugation, washed once with 25 mM sodium/potassium phosphate buffer, pH 7.0, collected again by centrifugation (8000 × *g* for 15 min) and then resuspended in buffer at 1 g wet wt mL^{-1}. Two volumes of glass beads were added, the sample was chilled on ice, and then ground 4 × 10 s in a bead beater with 5 min cooling between treatments. The lysate was centrifuged at 1000 × *g* for 10 min and the supernatant was collected and stored at −70 °C following assay of protein concentration (Pierce BCA protein assay). The incorporation of [^{14}C] mevalonic acid lactone into sterols was as follows: cell extract (2 mg protein) was preincubated with aerated phosphate buffer and test compound or solvent at 30 °C for 10 min. Cofactors (NADP, NAD, glucose-6-phosphate, ATP, and reduced glutathione at 1, 1, 3, 5, and 2 mM final concentrations, respectively)[31] were added followed by 1.0 μCi of [^{14}C] mevalonic acid lactone (0.5 mM, 4 mCi mmol^{-1}, Amersham) for a final volume of 0.5 mL. Incubation was continued at 30 °C for 2 h. Reactions were terminated by addition of 0.5 mL saponification reagent (15% KOH (wt/vol) in 90% ethanol (vol/vol)) and then heated under nitrogen at 90 ° for 30 min. After cooling, 2 mL distilled water, 5 μL extraction standard (5-α-dihydro[4-^{14}C]-testosterone, 2.9 mCi mmol^{-1}), and 1 mL methanol were added to each sample. Samples were then extracted with 3 × 4 mL petroleum ether and the pooled extracts were concentrated to ca. 2 mL using a stream of nitrogen. These were washed with 2 mL of 0.1 N NaOH followed by 2 mL of H_2O and then dried over sodium sulfate. The extracts were evaporated to dryness using a stream of nitrogen and the residues were redissolved in 50 μL cyclohexane. Samples (10 μL) were applied to silica 60 TLC plates (20 × 20 cm) and developed with chloroform. Radiolabeled materials were located and quantitated using a radioisotope TLC scanner (Radiomatic). Samples were analyzed in triplicate and standard deviations calculated. From these data, the extent of inhibition of formation of cyclized products could be determined as a function of inhibitor concentration. IC_{50} values were obtained from linear regression analysis of plots of percentage inhibition versus log [inhibitor].

Assay of antifungal activity

Antifungal activity was tested in Yeast Nitrogen Base (Difco) containing 0.5% glucose and buffered with 0.10 M sodium phosphate, pH 7.0. Cells were inoculated into microtiter wells (1000 CFU/well), containing two-fold serial dilutions of drugs. MIC values were determined after 48 h incubation at 37 °C.

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