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Synthesis and in vitro biological evaluation of ring B abeo-sterols as novel inhibitors of *Mycobacterium tuberculosis*

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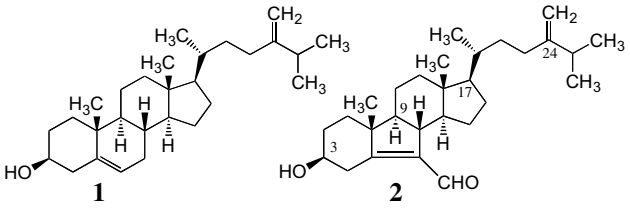
ABSTRACT

A series of 3β-hydroxy steroid analogues possessing a contracted cyclopentane B-ring were prepared based on the initial activity screening of a recently reported naturally occurring marine 5(6 → 7)abeo-sterol against *Mycobacterium tuberculosis*. All of the novel ring B abeo-sterols synthesized showed good inhibitory activity, whereas none of the starting steroids based on the common 3β-hydroxy-Δ^5-cholestane nucleus, proved to be active. Therefore, the 5(6 → 7)abeo-sterol nucleus present in compounds **3**, **5**, **7**, **9**, and **11** represents a novel scaffold for the development of new antitubercular agents.

Tuberculosis is a common and deadly infectious disease caused by mycobacteria, mainly *Mycobacterium tuberculosis*. Tuberculosis most commonly attacks the lungs (as pulmonary TB) but can also affect the central nervous system, the lymphatic system, the circulatory system, the genitourinary system, bones, joints and even the skin. Over one-third of the world's population has been exposed to the TB bacterium, and new infections occur at a rate of one per second.[1] In 2004, mortality and morbidity statistics included 14.6 million chronic active TB cases, 8.9 million new cases, and 1.6 million deaths, mostly in developing countries.[2] In addition, a rising number of people in the developed world are contracting tuberculosis because their immune systems are compromised by immunosuppressive drugs, substance abuse, or HIV/AIDS. The rise in HIV infections and the neglect of TB control programs have enabled a resurgence of tuberculosis.[3] The emergence of drug-resistant strains has also contributed to this new epidemic with, from 2000 to 2004, 20% of TB cases being resistant to standard treatments and 2% resistant to second-line drugs.[4]

During a recent investigation, while searching for novel highly-active antitubercular natural products from the Caribbean Sea sponge *Svenzea zeai*, our research group isolated a complex mixture of sterols the structures of which, after careful purification, were confidently assigned by combined spectroscopic methods.[5,6] Thus, the known sterol **1** was obtained as the main isolate (2.2% yield) from the same hexane-soluble fractions as minor sterol **2**, which was isolated only in 0.007% yield. The MIC values for antitubercular activity of compounds **1** and **2** were determined as 120.1 and 7.8 μg/mL, respectively. Since compound **1**, displaying only marginal activity against *M. tuberculosis* $H_{37}Rv$, is a most plausible biosynthetic precursor to sterol **2**, this finding suggested that in active steroids, maximum antimycobacterial activity could be attained upon contracting the cyclohexane B-ring, most likely as a result of increasing both the hydrophilic impact and rigidity of the steroidal backbone.



On the basis of reasonable activity shown by sterol **2**, and the fact that no prior work has been conducted to explore the potential of 6-5–6-5 fused rings sterols as antitubercular agents, we prepared a small 5(6 → 7)abeo-sterol library for antimycobacterial screening by the Institute for Tuberculosis Research of the University of Illinois at Chicago. The significantly enhanced antitubercular activity suggested the importance of having a contracted cyclopentane B-ring in the steroid series described herein. Additionally, branching in the side chain at C_{24} in combination with the 5(6 → 7)abeo-steroidal nucleus, appear to maximize the sterol's ability to be effective against *M. tuberculosis* (Fig. 1).

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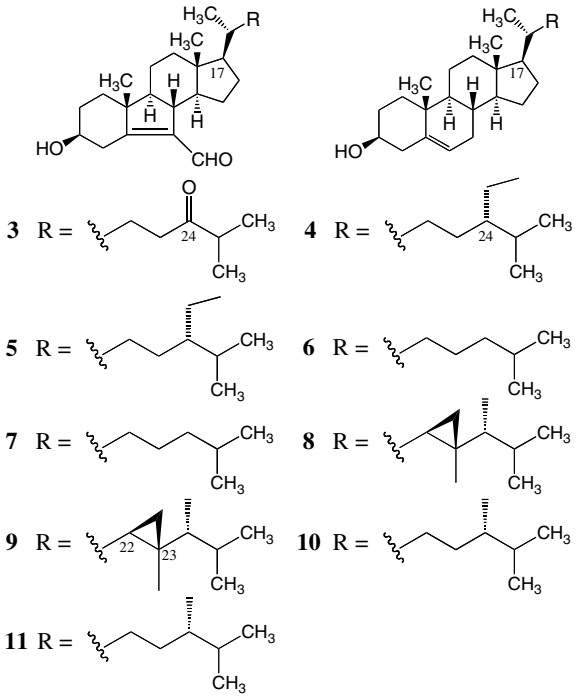

Figure 1. Molecular structures of the sterol analogues submitted for in vitro antimycobacterial and cytotoxicity screenings.

We carried out the synthesis of five structurally diverse 5(6 → 7)abeo-sterols for screening against *M. tuberculosis* $H_{37}Rv$ (Mtb). Whilst all of the sterol analogues synthesized (**3**, **5**, **7**, **9**, and **11**) possess the same tetracyclic 6-5-6–5 steroidal backbone, each analogue has a distinct alkyl side chain at C17. For instance, compound **2**, the only naturally occurring steroid in this series, has a double bond in the side chain at C24, whereas semi-synthetic sterols **3**, **5**, **9**, and **11** possess either a carbonyl, a methyl or ethyl moiety at C24. Analogue **9**, on the other hand, displays further alkyl substitution at C22 and C23 of the side chain, whereas abeo-steroid **7** [prepared as a reference analogue from commercially available cholesterol (**6**)] has no branching in the side chain at C24. Interestingly, except for the latter analogue, all of the abeo-sterols synthesized have shown ⩾80% inhibitory activity against Mtb at a concentration of 8 μg/mL (Table 1). On the other hand, none of the starting Δ^5-unsaturated steroids (**1**, **4**, **6**, **8**, and **10**) displays meaningful activity at ⩽64 μg/mL.

The abeo-sterol analogues herein described were prepared in one-pot by the reactions of the corresponding 3β-hydroxy-Δ^5-cholestanes[7] with ozone at −78 °C in either Et_2O or CH_2Cl_2 solution after reductive work-up with dimethylsulfide under mild conditions according to the literature procedures.[8–10] Intramolecular aldol condensation of the intermediate ε-keto aldehydes (not isolated) under slightly basic conditions led to the desired abeo-sterols in a relatively low isolated yield, between 30% and 50%, depending on the purity of the starting sterols used. Final purification was subsequently achieved by silica gel flash column chromatography and the desired products thus obtained were used as such for rigorous structure characterization studies as well as antimycobacterial screening. The complete structural assignment of all of the synthetic abeo-steroid analogues described in this work was accomplished on the basis of comprehensive 1D and 2D NMR experiments involving ^1H–^1H COSY, DEPT, NOESY, ^1H–^{13}C COSY (HMQC), and HMBC spectra, in addition to IR, UV, and HR-MS measurements.[11] In most cases, the 2D NMR spectra provided both the structure and the complete and unambiguous proton and carbon atom assignments.

Each of the starting 3β-hydroxy-Δ^5-cholestanes (**1**, **4**, **6**, **8**, and **10**) and all of the synthesized 5(6 → 7)abeo-sterols (**3**, **5**, **7**, **9**, and **11**) were screened for their antimycobacterial activity. The activity results of steroids **1**–**11** are presented in Table 1. The primary screen was conducted at 128, 64, 32, 16, 8, 4, and 2 μg/mL against Mtb $H_{37}Rv$ (ATCC 27294) in BACTEC 12B medium using the Microplate Alamar Blue Assay (MABA) as previously described.[12] Because all of the synthesized abeo-sterols demonstrated 100% inhibition at ⩾64 μg/mL, they were retested to determine the MIC, defined as the lowest concentration inhibiting growth by ⩾90%. Rifampin (RMP) was used as a positive control during all the antituberculosis assays.

Concurrent with the determination of MICs, compounds **1**–**11** were tested for cytotoxicity (IC_{50}) in VERO cells in order to investigate the effect of contracting the cyclohexane B-ring on cytotoxic activity.[13] The results described herein (Table 1) show that all of the starting steroids (**1**, **4**, **6**, **8**, and **10**) lacked significant cytotoxicity (IC_{50}'s > 128 μg/mL). However, upon derivatization, analogues **3**, **5**, **9**, and **11** showed some cytotoxicity (IC_{50}'s 26.6–54.7 μg/mL). Remarkably, the absence of appreciable toxicity in analogue **7** (IC_{50} > 128 μg/mL) against mammalian cells in the VERO cell assay suggests that functionalization (branching or oxidation) at C24 of the flexible alkyl side chain alone might explain the mild toxicity displayed by most abeo-sterols.

In conclusion, during a MABA bioassay against *M. tuberculosis* ($H_{37}Rv$), all of the abeo-sterol analogues synthesized showed min-

Table 1
In vitro *M. tuberculosis* growth inhibition by sterols **1–11**[a]

Compound	% Inhibition (μg/mL)							MIC[b]	Cytotoxicity[c]
	128	64	32	16	8	4	2	μg/mL	IC_{50}, μg/mL (SI)
1	97	40	43	33	—	—	—	120.1	>128 (n.d.)
2	99	99	99	105	91	69	52	7.8	51 (6.5)
3	99	100	96	92	83	67	52	13.6	43.8 (3.2)
4	45	48	31	32	7	8	−1	>128	>128 (n.d.)
5	99	99	99	98	99	97	29	3.8	26.6 (7)
6	57	24	13	33	—	—	—	>128	>128 (n.d.)
7	99	99	99	96	51	34	10	15	>128 (>9)
8	38	18	13	10	—	—	—	>128	>128 (n.d.)
9	99	99	98	96	81	33	17	12.7	54.7 (4.3)
10	58	41	31	21	23	14	15	>128	>128 (n.d.)
11	99	100	99	99	97	94	39	3.9	42.3 (10.8)
RMP[d]	100	100	100	99	94	73	49	0.06	89.3 (1488)

[a] Values are means of three experiments.
[b] Lowest drug concentration that effected an inhibition of ⩾90% relative to untreated cultures.
[c] Cytotoxicity against VERO cells (ATCC CCL-81). Selectivity index (SI) = IC_{50}/MIC. 'n.d.' indicates not determined.
[d] Rifampin was used as a positive control.

imum inhibitory concentrations of 3.8–15 μg/mL, while the starting 3β-hydroxy-Δ^5-cholestanes had MIC values >128 μg/mL, respectively (Table 1). The present data suggest that the 5(6 → 7)abeo-steroidal nucleus inherently enhances antimycobacterial activity and thus represents a novel scaffold for the development of clinically useful agents for tuberculosis. Further correlations of structural features and the MICs of the six abeo-sterols scrutinized during this study suggest that, in addition to the 5(6 → 7)abeo-steroidal moiety, the presence of a methyl or ethyl residue at C24 is required for superior activity. The fact that modifications in the side chain can affect the antitubercular activity can be related to the permeability of the compounds through the Mtb membrane. Noteworthy is the activity of compound **3**, which has two carbonyl groups and shows the worse selectivity index. Cytotoxicity results for a mammalian cell line indicate that in general strongly antitubercular abeo-sterols are also mildly cytotoxic. Although cytotoxicity of mammalian cells is low relative to toxicity to Mtb, it is not clear yet whether the observed activity of abeo-sterols is unique to mycobacterial targets. This study has demonstrated the future potential for development of B-ring abeo-sterol analogues as antimycobacterial agents and suggests new directions for the rational design of additional steroids that are active against the tubercle bacillus. In addition, the finding that abeo-sterol derivatives are inhibitors of Mtb is potentially very significant, as interest has been building recently in the metabolism of cholesterol by Mtb and the importance of such metabolism for intracellular survival in vivo.[14]

Acknowledgment

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7. The starting sterols 24-methylene-cholesterol (**1**) and 24-α-ethyl-cholesterol (**4**) were extracted, respectively, from the Caribbean marine sponges *S. zeai* and *Myrmekioderma styx*. Commercial cholesterol (**6**) was purchased from Aldrich Chemical Company. Gorgosterol (**8**) and 24-α-methyl-cholesterol (**10**) were extracted from the Caribbean gorgonian octocoral *Gorgonia mariae*. Abeo-sterol **3** was prepared from precursor **1** as described.
8. A stream of dry oxygen containing about 1–2% ozone was bubbled for 15 min into a solution of starting 3β-hydroxy-Δ^5-unsaturated sterol (50.0 mg) in Et_2O or CH_2Cl_2 (25 mL) kept at −78 °C. After allowing the temperature to warm up to 25 °C, the solvent was evaporated and the oily residue obtained was stirred with a mixture of dimethylsulfide (0.5 mL) and Et_2O (5 mL) for 24 h at 25 °C to afford the ozonolyzed product. Upon removal of excess reagent under reduced pressure, 1.0 M KOH in MeOH (5 mL) was added and the resulting mixture stirred for 5 min at 25 °C. At this point, routine monitoring by TLC typically revealed no starting material left and the presence of a strongly UV active product. After quenching with saturated aqueous NH_4Cl followed by extraction with Et_2O (3× 10 mL), the combined ether layers were washed with brine, dried ($MgSO_4$), and concentrated in vacuo. Purification by silica gel flash column chromatography (hexane/EtOAc 90:10 or hexane/acetone 90:10) afforded the desired 5(6 → 7)abeo-sterol derivatives (15.5–26.0 mg; 30–50% isolated yield) as colorless oils with 97–98% purity.
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11. (a) *Compound **3**:* Colorless oil; α_D^{25} −13.0 (*c* 0.8, $CHCl_3$); IR (film) 3407, 2926, 2868, 1712, 1675, 1598, 1465, 1380, 1262, 1072, 802 cm^{-1}; UV (MeOH) λ_{max} 256 (ε 7400) nm; ^1H NMR (500 MHz, $CDCl_3$) δ 9.97 (s, 1H), 3.70 (m, 1H), 3.47 (m, 1H), 2.70–2.35 (br m, 3H), 2.10 (br m, 2H), 2.05–1.12 (broad envelope, 19H), 1.09 (d, *J* = 5.0 Hz, 6H), 0.93 (s, 3H), 0.92 (d, *J* = 5.0 Hz, 3H), 0.73 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 215.4 (C), 189.6 (CH), 168.9 (C), 139.3 (C), 70.9 (CH), 60.1 (CH), 55.2 (CH), 54.4 (CH), 46.3 (C), 46.1 (CH), 45.2 (C), 40.8 (CH), 39.8 (CH_2), 37.4 (CH_2), 36.2 (CH_2), 35.2 (CH), 33.9 (CH_2), 31.3 (CH_2), 29.9 (CH_2), 28.5 (CH_2), 26.5 (CH_2), 20.7 (CH_2), 18.7 (CH_3), 18.4 (CH_3), 18.3 (CH_3), 15.6 (CH_3), 12.5 (CH_3); HREI-MS *m*/*z* $[M]^+$ Calcd for $C_{27}H_{42}O_3$ 414.3134, found 414.3135; EI-MS *m*/*z* $[M]^+$ 414 (57), 396 (25), 367 (26), 353 (6), 329 (12), 287 (9), 269 (15), 229 (9), 161 (18), 129 (28), 95 (49), 83 (73), 55 (100).
(b) *Compound **5**:* Colorless oil; α_D^{25} −18.6 (*c* 0.9, $CHCl_3$); IR (film) 3376, 2957, 2869, 1675, 1464, 1381, 1072 cm^{-1}; UV (MeOH) λ_{max} 256 (ε 8600) nm; ^1H NMR (500 MHz, $CDCl_3$) δ 9.97 (s, 1H), 3.70 (m, 1H), 3.47 (br d, *J* = 15.0 Hz, 1H), 2.55 (dt, *J* = 4.0, 11.0 Hz, 1H), 2.08 (br m, 3H), 2.00–0.99 (broad envelope, 23H), 0.93 (s, 3H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.85 (t, *J* = 5.0 Hz, 3H), 0.82 (d, *J* = 6.5 Hz, 3H), 0.80 (d, *J* = 6.5 Hz, 3H), 0.73 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.7 (CH), 168.9 (C), 139.3 (C), 70.9 (CH), 60.2 (CH), 55.3 (CH), 54.5 (CH), 46.3 (C), 46.2 (CH), 46.1 (CH), 45.2 (C), 39.8 (CH_2), 36.2 (CH_2), 36.1 (CH), 33.9 (CH_2), 33.8 (CH_2), 31.3 (CH_2), 28.9 (CH), 28.5 (CH_2), 26.6 (CH_2), 26.5 (CH_2), 23.0 (CH_2), 20.7 (CH_2), 19.6 (CH_3), 19.0 (CH_3), 18.9 (CH_3), 15.6 (CH_3), 12.5 (CH_3), 12.3 (CH_3); HREI-MS *m*/*z* $[M]^+$ Calcd for $C_{29}H_{48}O_2$ 428.3654, found 428.3654; EI-MS *m*/*z* $[M]^+$ 428 (61), 410 (34), 381 (46), 353 (9), 337 (10), 269 (15), 145 (27), 107 (30), 91 (37), 85 (100).
(c) *Compound **7**:* Colorless oil; α_D^{25} −42.5 (*c* 0.8, $CHCl_3$); IR (film) 3410, 2951, 2867, 1676, 1465, 1381, 1169, 1073 cm^{-1}; UV (MeOH) λ_{max} 256 (ε 14,200) nm; ^1H NMR (500 MHz, $CDCl_3$) δ 9.97 (s, 1H), 3.71 (m, 1H), 3.46 (ddd, *J* = 2.0, 4.5, 14.5 Hz, 1H), 2.55 (dt, *J* = 4.0, 11.0 Hz, 1H), 2.08 (br m, 3H), 1.96–0.95 (broad envelope, 22H), 0.93 (s, 3H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.86 (d, *J* = 6.5 Hz, 3H), 0.85 (d, *J* = 6.5 Hz, 3H), 0.73 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.7 (CH), 168.9 (C), 139.3 (C), 70.9 (CH), 60.2 (CH), 55.4 (CH), 54.5 (CH), 46.3 (C), 46.2 (CH), 45.2 (C), 39.8 (CH_2), 39.5 (CH_2), 36.2 (CH_2), 36.1 (CH_2), 35.6 (CH), 33.9 (CH_2), 31.3 (CH_2), 28.5 (CH_2), 28.0 (CH), 26.6 (CH_2), 23.9 (CH_2), 22.8 (CH_3), 22.5 (CH_3), 20.7 (CH_2), 18.9 (CH_3), 15.6 (CH_3), 12.5 (CH_3); HREI-MS *m*/*z* $[M]^+$ Calcd for $C_{27}H_{44}O_2$ 400.3341, found 400.3343; EI-MS *m*/*z* $[M]^+$ 400 (22), 382 (9), 353 (12), 149 (10), 145 (10), 120 (25), 118 (27), 95 (26), 87 (100).
(d) *Compound **9**:* Colorless oil; α_D^{25} +1.8 (*c* 1.0, $CHCl_3$); IR (film) 3391, 2956, 2870, 1677, 1460, 1382, 1160, 1072 cm^{-1}; UV (MeOH) λ_{max} 256 (ε 3000) nm; ^1H NMR (500 MHz, $CDCl_3$) δ 9.97 (s, 1H), 3.70 (m, 1H), 3.47 (br d, *J* = 15.0 Hz, 1H), 2.54 (m, 1H), 2.10–1.05 (broad envelope, 19H), 1.01 (d, *J* = 5.0 Hz, 3H), 0.94 (d, *J* = 5.0 Hz, 3H), 0.93 (s, 3H), 0.90 (s, 3H), 0.86 (d, *J* = 5.0 Hz, 3H), 0.78 (d, *J* = 5.0 Hz, 3H), 0.72 (s, 3H), 0.45 (dd, *J* = 5.0, 10.0 Hz, 1H), 0.20 (br m, 2H), –0.13 (dd, *J* = 5.0, 10.0 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.7 (CH), 168.8 (C), 139.3 (C), 70.9 (CH), 60.2 (CH), 57.1 (CH), 54.3 (CH), 50.8 (CH), 46.3 (C), 46.2 (CH), 45.6 (C), 39.9 (CH_2), 36.2 (CH_2), 35.0 (CH), 33.9 (CH_2), 32.1 (CH), 32.0 (CH), 31.3 (CH_2), 28.7 (CH_2), 26.8 (CH_2), 25.9 (C), 22.2 (CH_3), 21.5 (CH_3), 21.4 (CH_3), 21.3 (CH_2), 20.7 (CH_2), 15.6 (CH_3), 15.3 (CH_3), 14.3 (CH_3), 12.6 (CH_3); HREI-MS *m*/*z* $[M]^+$ Calcd for $C_{30}H_{48}O_2$ 440.3654, found 440.3653; EI-MS *m*/*z* $[M]^+$ 440 (10), 422 (7), 414 (21), 400 (27), 367 (13), 353 (13), 287 (22), 269 (22), 107 (39), 95 (56), 83 (59), 55 (100).
(e) *Compound **11**:* Colorless oil, α_D^{25} −2.0 (*c* 1.0, $CHCl_3$); IR (film) 3415, 2953, 2869, 1675, 1463, 1381, 1261, 1166, 1074 cm^{-1}; UV (MeOH) λ_{max} 256 (ε 5000) nm; ^1H NMR (500 MHz, $CDCl_3$) δ 9.96 (s, 1H), 3.71 (br m, 1H), 3.46 (dd, *J* = 5.0, 15.0 Hz, 1H), 2.53 (dt, *J* = 5.0, 10.0 Hz, 1H), 2.08 (m, 3H), 2.05–0.94 (broad envelope, 21H), 0.93 (s, 3H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.85 (br t, *J* = 5.0 Hz, 6H), 0.78 (d, *J* = 5.0 Hz, 3H), 0.72 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.7 (CH), 168.9 (C), 139.3 (C), 70.9 (CH), 60.1 (CH), 55.2 (CH), 54.4 (CH), 46.3 (C), 46.1 (CH), 45.2 (C), 39.8 (CH_2), 39.1 (CH), 36.1 (CH_2), 36.0 (CH), 33.9 (CH_2), 33.7 (CH_2), 31.4 (CH_2), 31.2 (CH), 30.7 (CH_2), 28.7 (CH_2), 26.6 (CH_2), 20.7 (CH_2), 20.5 (CH_3), 19.1 (CH_3), 17.6 (CH_3), 15.6 (CH_3), 15.4 (CH_3), 12.5 (CH_3); HREI-MS *m*/*z* $[M]^+$ Calcd for $C_{28}H_{46}O_2$ 414.3498, found 414.3500; EI-MS *m*/*z* $[M]^+$ 414 (47), 396 (15), 367 (24), 353 (16), 287 (8), 269 (11), 133 (20), 120 (63), 118 (64), 95 (47), 87 (100).
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