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Design and synthesis of bile acid-based amino sterols as antimicrobial agents

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ABSTRACT

New bile acid-based amino sterols were synthesized in good yields from C-3β-oxiranes as key intermediates. These derivatives were evaluated for their in vitro antimicrobial properties against human pathogens. These compounds showed better antibacterial activity as compared to antifungal activity. Compounds **21** and **22** showed comparable antibacterial activity to gentamicin against *Staphylococcus aureus* with IC_{50} values of 5.14 and 4.46 μg/mL. This is the first report for the synthesis of C-3β-oxiranes on the steroids having A/B *cis* ring junction and these oxiranes have been used for the synthesis of amino sterols **17**, **18**, **21**, and **22**.

The widespread excessive use of antibacterial agents lead to development of more resistant bacteria to commonly used antibiotics. This has led to intense research for new types of antibiotics. In recent years, a wide variety of compounds having antibacterial activity have been synthesized or isolated from natural resources. Among these natural antibiotics squalamine[1] **1** (Fig. 1) has attracted considerable attention because of its potent antimicrobial activity against a broad spectrum of microorganisms and also its antiangeogenic properties.[2,3] Attempts to obtain large amounts of squalamine from the dogfish shark resulted in the discovery, isolation, and characterization of family of novel aminosterols.[4] The fact that insufficient amount of squalamine was available from natural recourses for mechanistic studies, indicated clear need for the preparation of squalamine and hence several groups undertook the synthesis of squalamine **1**.[5] Regan and co-workers synthesized[6] squalamine analogue **2** (Fig. 1) in which they have attached spermine in the side chain and sulfate group at C-3 position of closely related sterol. This compound showed slightly better activity than squalamine against *Pseudomonas aeruginosa*. Various squalamine analogues **3–8** (Fig. 1) having long and short polyamine linkages at different positions of steroid ring with or without sulfate groups have been reported in the literature and many of them showed moderate biological activities.[7]

A literature survey of antimicrobial steroids revealed that several amino cholesterol derivatives exhibit profound antimicrobial activity.[8] The preparation of various bile acid-based amino sterols was reported with a view to examine their activity as antimicrobial agents.[9] A recent approach to combat against pathogens is to introduce a polycationic chain onto a steroid scaffold that plays key role in the biological systems.[10] Polyamines are of considerable interest due to their advantages of low toxicity, low immunogenicity, controllable synthesis, and defined molecular structure for pharmaceutical characterization.[11] Savage et al. have designed a class of cationic steroid antibiotics (CSA) as steroid–polyamine conjugates,[12] with the intention of mimicking the antibacterial activities of polymyxin B (PMB), while Regen and co-workers have described the utilization of bile acid–polyamine conjugates as synthetic ionophores and extremely useful leads in the process of drug discovery.[13]

Recently we have synthesized amino functionalized novel cholic acid derivatives containing 1, 2 amino alcohol in the C ring of steroidal backbone.[14a] These compounds induced HIV-1 replication and syncytia formation in T cells.

From the literature survey on sterol–polyamine conjugates[14b] it was revealed that, long and rigid hydrophobic unit, a flexible hydrophilic chain linked to hydrophobic unit, pendant polar head groups are required for steroids to be biologically active. The precise structure of the polyamine is not important. The sulfate groups can be replaced by a carboxylate or hydroxyl or even removed altogether. The structure of the rigid hydrophobic unit, that is, steroid can also be varied.

With this in view, novel bile acid-based amino sterols were designed. Bile acids have been chosen because of their natural amphiphatic nature. They are pharmacologically interesting as potential carriers of liver-specific drugs, absorption enhancers, and cholesterol lowering agents.[15] Several cholic acid-derived facial amphiphiles have been reported[16] to improve the permeability of membranes including bacterial cell wall. In continuation[17] of our

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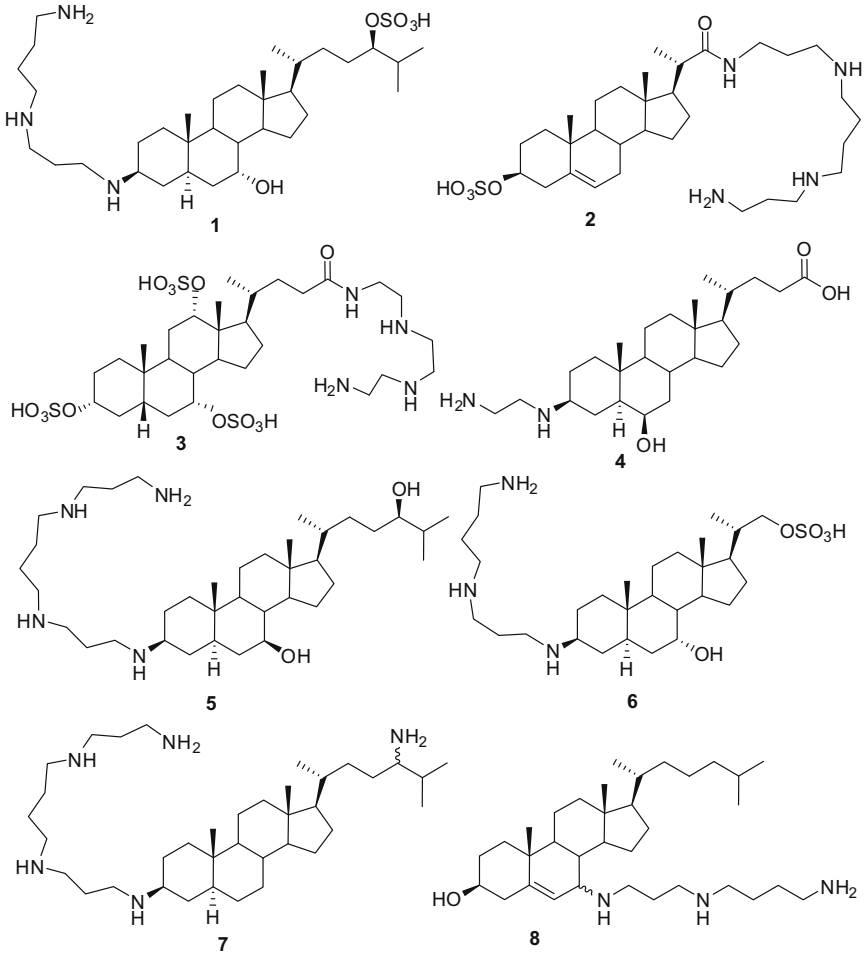


Figure 1. Sterol–polyamine conjugates.

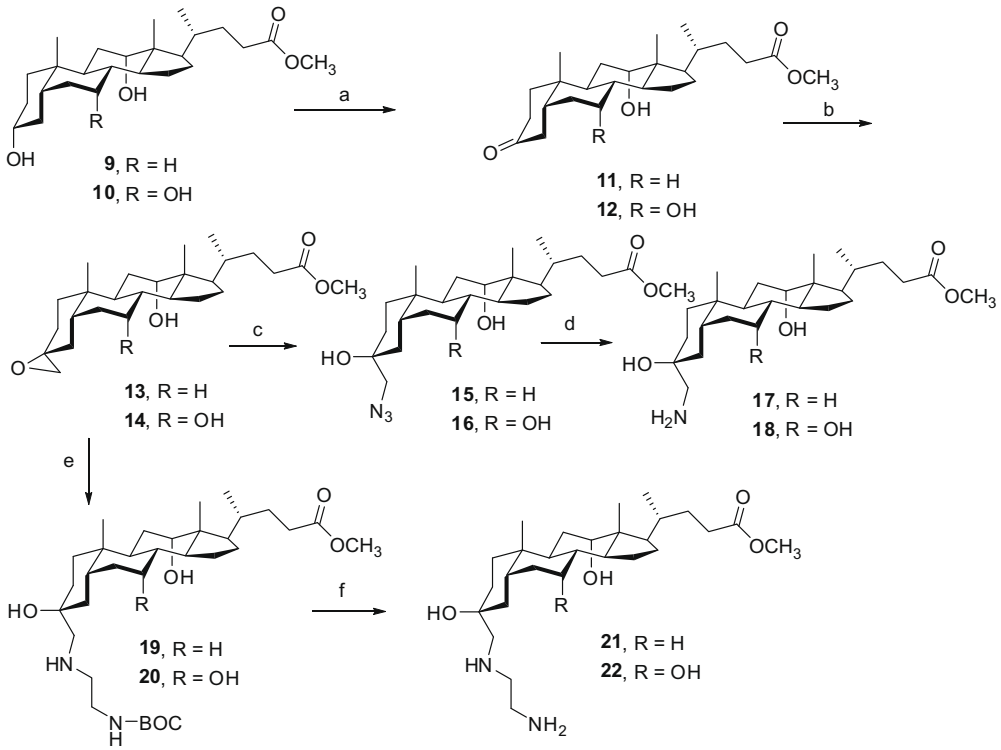
work on bile acids we report here the synthesis and biological evaluation of bile acid–methylamine **17**, **18** and bile acid–ethylenediamine conjugates **21**, **22** in which amino functionality has been introduced at one carbon away from C-3 carbon of steroid unit. These amino sterols **17**, **18**, **21**, and **22** have been synthesized by using C-3β-oxiranes **13** and **14** as key intermediates (Scheme 1).

Chemoselective oxidation of C-3 hydroxy group of methyl deoxycholate **9**[17c] using Ag_2CO_3 on celite[18] afforded compound **11** in 92% yield. In IR spectrum of compound **11** absorbance assigned to C-3-keto carbonyl appeared at 1712 cm^{-1} while ester carbonyl appeared at 1735 cm^{-1}. In ^{13}C NMR compound **11** showed signal at δ 213 ppm, assigned to C-3-keto carbonyl. Reaction of **11** with sulfur ylide (trimethylsulfoxonium iodide/NaH in DMSO–THF) resulted into oxirane **13** as a single isomer in 89% yield. In ^1H NMR compound **13** showed broad singlet at δ 2.64 due to oxirane CH_2. In ^{13}C NMR signal due to C-3 carbonyl at δ 213 ppm disappeared and methylene signal of C-3β-oxirane was observed at δ 53.8.

Formation of C-3β-oxirane from C-3-keto steroids having A/B *trans* ring junction is known in the literature.[19] However, there is no report on synthesis of C-3 oxirane of steroids having A/B *cis* ring junction, in which α-face is more crowded. Hence it was interesting to know the stereochemistry of C-3 oxirane **13**, which was unambiguously confirmed to be C-3β-oxirane by single crystal X-ray (Fig. 2).

Using similar reaction sequence oxirane **14** was synthesized from methyl cholate **10**[17c] in overall 75.6% yield in two steps. Nucleophilic opening of oxirane **13** with azide anion (NaN_3 in DMF) gave azido alcohol **15** (Scheme 1). IR spectrum of **15** showed characteristic bands at 1731 and 2104 cm^{-1} corresponding to the ester carbonyl and azido group, respectively. Its ^1H NMR spectrum showed a characteristic signal at δ 3.26 ppm assigned to C-3α methylene protons (CH_2N_3). Hydrogenation of azido alcohol **15** was carried out using H_2–Pd/C at 45 psi for 3 h to obtain deoxycholic acid-based amino alcohol **17** in 98% yield. In ^1H NMR spectrum of compound **17** a characteristic signal at δ 2.59 ppm was assigned to the C-3α methylene proton (CH_2NH_2). Using similar reaction sequence cholic acid-based amino alcohol **18** was obtained from oxirane **14** in overall 87.3% yield in two steps (Scheme 1). We also synthesized two bile acid–ethylenediamine conjugates **21** and **22**. Nucleophilic opening of oxiranes **13** and **14** with *N*1-(Boc)-1,2-diaminoethane[21] gave compounds **19** and **20**, followed by *N*-Boc deprotection using 50% TFA and purification by column chromatography afforded pure bile acid–ethylenediamine conjugates **21** and **22**, respectively. In ^1H NMR spectrum of **21** and **22** showed a characteristic signal at δ 2.52, 2.72, and 2.81 each for two protons corresponding to methylene protons of ethylenediamine chain. The newly synthesized compounds **15**–**18**, **21**, and **22** were fully characterized by spectroscopic data and were tested for their antifungal as well as antibacterial activity.

Bioevaluation. The synthesized bile acid–amine conjugates **17**, **18**, **21**, and **22** were tested for the in vitro antifungal and antibacterial activity. For comparison purpose antimicrobial activity of bile acid esters **9** and **10**, azido compounds **15** and **16** were also determined. The antifungal activity was evaluated against different fungal strains such as *Candida albicans, Cryptococcus neoformans, Spo-*



Scheme 1. Reagents and conditions: (a) Ag_2CO_3, toluene, reflux, 5 h, **11** (92%), **12** (90%); (b) trimethylsulfoxonium iodide, NaH, DMSO–THF, rt 2 h, **13** (89%), **14** (84%); (c) NaN_3, DMF, 60–65 °C, 12 h, **15** (91%), **16** (90%); (d) H_2, Pd–C, MeOH, 45 psi, 3 h, **17** (98%), **18** (97%); (e) *N*1-(Boc)-1,2-diaminoethane, MeOH reflux 2 h; (f) (i) 50% TFA/CH_2Cl_2; (ii) 50% DIPEA/CH_2Cl_2, **21** (74%), **22** (71%) overall in two steps.

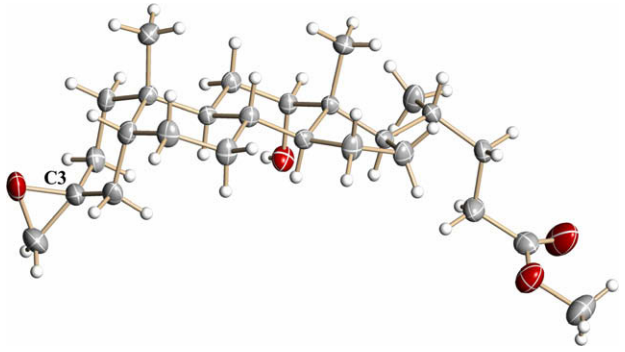


Figure 2. ORTEP view of methyl (3β-oxirane)-12α-hydroxy-5β-cholan-24-oate **13**.[20]

rothrix schenckii, Trichophyton mentagrophytes, Aspergillus fumigatus, and Candida parapsilosis (ATCC 22019). Antibacterial activity was evaluated against different bacterial strains such as *Escherichia coli* (ATCC 9637), *P. aeruginosa* (ATCC BAA-427), *S. aureus* (ATCC 25923), and *Klebsiella pneumoniae* (ATCC 27736). Minimum inhibitory concentration (MIC) and IC_{50} values were determined using standard broth microdilution technique as per NCCLS guidelines.[22] Fluconazole and gentamicin have been used as standard drugs for the comparison of antifungal and antibacterial activity, respectively. The biological data of the tested compounds are depicted in Tables 1 and 2 as MIC and IC_{50} values.

From the biological data, it was observed that bile acid esters **9**, **10** and azido compounds **15**, **16** were almost inactive against all the tested bacterial and fungal strains. The MIC value for all these compounds was >50 μg/mL. As seen in Table 1 bile acid–ethylamine conjugates **17** and **18** and bile acid–ethylenediamine conjugates **21** and **22** showed potent antifungal and antibacterial activity against most of the tested fungal and bacterial strains. The activity of compounds **21** and **22** was higher or comparable to that of gentamicin (IC_{50} 4.46 μg/mL) against *Staphylococcus aureus* (IC_{50} 5.14 for **21** and 4.12 μg/mL for **22**).

The same compounds **21** and **22** also showed good antifungal activity against most of the tested fungal strains but these were found to be less active than fluconazole (Table 2). Compound **21** showed better activity against *Sporothrix schenckii* (IC_{50} 3.36 μg/mL),

Table 1
In vitro antibacterial activities of compounds **17**, **18**, **21**, and **22** and antibacterial drug gentamicin (MIC and IC_{50} in μg/mL)

Compound No.	Inhibitory concentration in μg/mL against bacteria							
	Ec		Pa		Sa		Kp	
	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}
17	>50	>50	>50	>50	25	14.56	50	NA
18	>50	>50	>50	>50	25	18.42	25	18.42
21	50	NA	50	NA	**6.25**	**5.14**	**6.25**	**5.21**
22	25	17.23	50	NA	**6.25**	**4.12**	**6.25**	**4.68**
Gentamicin	0.18	0.11	25	24.64	**6.25**	**4.46**	0.78	0.34

Abbreviations: Ec, *Escherichia coli* (ATCC 9637); Pa, *Pseudomonas aeruginosa* (ATCC BAA-427); Sa, *Staphylococcus aureus* (ATCC 25923); Kp, *Klebsiella pneumoniae* (ATCC 27736).

Table 2
In vitro antifungal activities of compounds **17**, **18**, **21**, and **22** and standard antifungal drug fluconazole (MIC and IC_{50} in μg/mL)

Compound No.	Inhibitory concentration in μg/mL against fungi											
	Ca		Cn		Ss		Tm		Af		Cp	
	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}
17	50	50	50	50	25	12.65	25	24.86	50	50	>50	>50
18	50	>50	>50	>50	50	50	25	21.42	>50	>50	>50	>50
21	>50	25.05	25	17.23	12.5	**3.36**	**6.25**	**6.25**	12.5	**8.14**	25	18.36
22	25	24.05	25	14.56	25	18.32	**6.25**	**6.25**	50	50	25	16.42
Fluconazole	0.5	0.13	1.9	0.46	2.0	1.45	1.0	0.6	2.0	1.06	1.0	0.21

Abbreviations: Ca, *Candida albicans*; Cn, *Cryptococcus neoformans*; Ss, *Sporothrix schenckii*; Tm, *Trichophyton mentagrophytes*; Af, *Aspergillus fumigatus* (AF-27); Cp, *Candida parapsilosis* (ATCC-22019).

Trichophyton mentagrophytes (IC_{50} 6.25 μg/mL), and *Aspergillus fumigatus* (IC_{50} 8.14 μg/mL) while compound **22** was found be active against *Trichophyton mentagrophytes* (IC_{50} 6.25 μg/mL).

In conclusion, new bile acid-based amino sterols were synthesized in good yields from C-3β-oxiranes as key intermediates. In vitro antimicrobial activities of compounds **21** and **22** against human pathogens were found to be comparable to standard antibacterial drug gentamicin against *S. aureus*. Synthesis of steroidal C-3β-oxiranes having A/B *cis* ring junction and amino functionality at one carbon away from C-3 has been reported for the first time. Bile acid–ethylenediamine conjugates **21** and **22** having extra amino groups were found to be more active than bile acid–ethylamine conjugates **17** and **18**. Hence it may be assumed that by increasing the chain length, the activity may be increased. Work on study of structure–activity relationship by increasing the chain length, changing the functional groups for getting more potent lead molecules is in progress in our laboratory.

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Supplementary data

Experimental procedures, ^{1}H and ^{13}C NMR spectra of the synthesized compounds **11**–**18**, **21**, **22**, materials and methods for bioevaluation are provided. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.117.

References and notes

1. (a) Moore, K. S.; Wehrli, S.; Roder, H.; Rogers, M.; Forrest, J. N., Jr.; McCrimmon, D.; Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1354; (b) Wehrli, S. L.; Moore, K. S.; Roder, H.; Durell, S.; Zasloff, M. *Steroids* **1993**, *58*, 370.
2. Stone, R. *Science* **1993**, *259*, 1125.
3. Brunel, J. M.; Salmi, C.; Loncle, C.; Vidal, N.; Letourneux, Y. *Curr. Cancer Drug Targets* **2005**, *5*, 267.
4. Rao, M. N.; Shinnar, A. E.; Noecker, L. A.; Chao, T. L.; Feibush, B.; Snyder, B.; Sharkansky, I.; Sarkhian, A.; Zhang, X.; Jones, S. R.; Kinney, W. A.; Zasloff, M. *J. Nat. Prod.* **2000**, *63*, 631.
5. (a) Pechulis, A. D.; Bellevue, F. H., III; Cioffi, C. L.; Trapp, S. G.; Fojtik, J. P.; McKitty, A. A.; Kinney, W. A.; Frye, L. L. *J. Org. Chem.* **1995**, *60*, 5121; (b) Brunel, J. M.; Letourneux, Y. *Eur. J. Org. Chem.* **2003**, 3897; (c) Okumura, K.; Nakamura, Y.; Takeuchi, S.; Kato, I.; Fugimoto, Y.; Ikekawa, N. *Chem. Pharm. Bull.* **2003**, *51*, 1177; (d) Zhou, X.-D.; Cai, F.; Zhou, W.-S. *Tetrahedron* **2002**, *58*, 10293; (e) Zhang, D.-H.; Cai, F.; Zhou, X.-D.; Zhou, W.-S. *Org. Lett.* **2003**, *5*, 3257.
6. Sadownik, A.; Deng, G.; Janout, V.; Regen, S. L. *J. Am. Chem. Soc.* **1995**, *117*, 6138.
7. (a) Kikuchi, K.; Bernard, E. M.; Sadownik, A.; Regen, S. L.; Armstrong, D. *Antimicrob. Agents Chemother.* **1997**, *41*, 1433; (b) Jones, S. R.; Kinney, W. A.; Zhang, X.; Jones, L. M.; Selinsky, B. S. *Steroids* **1996**, *61*, 565; (c) Shu, Y.; Jones, S. R.; Kinney, W. A.; Selinsky, B. S. *Steroids* **2002**, *67*, 291; (d) Kim, H.-S.; Choi, B.-S.; Kwon, K.-C.; Lee, S.-O.; Kwak, H. J.; Lee, C. H. *Bioorg. Med. Chem.* **2000**, *8*, 2059; (e) Choucair, B.; Dherbomez, M.; Roussakis, C.; Kihel, L. E. *Tetrahedron* **2004**, *60*, 11477.
8. (a) Barnett, J.; Ryman, B. E.; Smith, F. *J. Chem. Soc.* **1946**, 528; (b) Loncle, C.; Brunel, J. M.; Vidal, N.; Dherbomez, M.; Letourneux, Y. *Eur. J. Med. Chem.* **2004**, *39*, 1067.
9. (a) James, S. P.; Smith, F.; Stacy, M.; Webb, M. *J. Chem. Soc.* **1946**, 665; (b) Hilton, M. L.; Jones, A. S.; Westwood, J. R. B. *J. Chem. Soc.* **1955**, 3449; (c) Fini, A.; Fazio, G.; Roda, A.; Bellini, A. M.; Mencini, E.; Guarneri, M. *J. Pharm. Sci.* **1992**, *81*, 726.
10. Blagbrough, I. S.; Carrington, S.; Geall, A. *J. Pharm. Sci.* **1997**, *3*, 223.
11. (a) Behr, J.-P.; Demeneix, B.; Loeffler, J.-P.; Perez-Mutul, J. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6982; (b) Donohue, R.; Mazzaglia, A.; Ravoo, B. J.; Darcy, R. *Chem. Commun.* **2002**, 2864; (c) Byk, G.; Dubertret, C.; Escriou, V.; Frederic, M.; Jaslin, G.; Rangara, R.; Pitard, B.; Crouzet, J.; Wils, P.; Schwartz, B.; Scherman, D. *J. Med. Chem.* **1998**, *41*, 224; (d) Ewert, K.; Ahmed, A.; Evans, H. M.; Schmidt, H.-W.; Safinya, C. R. *J. Med. Chem.* **2002**, *45*, 5023; (e) Nakaramura, E.; Isobe, H.; Tomita, N.; Sawamura, M.; Jinno, S.; Okayama, H. *Angew. Chem., Int. Ed.* **2000**, *39*, 4254; (f) McGeorge, C.; Perrin, C.; Monck, M.; Camilleri, P.; Kirby, A. J. *J. Am. Chem. Soc.* **2001**, *123*, 6215.
12. (a) Savage, P. B.; Li, C. *Exp. Opin. Invest. Drugs* **2000**, *9*, 263; (b) Savage, P. B. *Eur. J. Org. Chem.* **2002**, 759; (c) Savage, P. B.; Li, C.; Taotafa, U.; Ding, B.; Guan, Q. *FEMS Microbiol. Lett.* **2002**, *217*, 1.
13. (a) Deng, G.; Dewa, T.; Regen, S. L. *J. Am. Chem. Soc.* **1996**, *118*, 8975; (b) Merritt, M.; Lanier, M.; Deng, G.; Regen, S. L. *J. Am. Chem. Soc.* **1998**, *120*, 8494; (c) Bandyopadhyay, P.; Janout, V.; Zhang, L.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, *123*, 7691; (d) Zhang, J.; Jing, B.; Regen, S. L. *J. Am. Chem. Soc.* **2003**, *125*, 13984; (e) Chen, W.-H.; Regen, S. L. *J. Am. Chem. Soc.* **2005**, *127*, 6538; (f) Mehiri, M.; Chen, W.-H.; Janout, V.; Regen, S. L. *J. Am. Chem. Soc.* **2009**, *131*, 1338.
14. (a) Salunke, D. B.; Ravi, D. S.; Pore, V. S.; Mitra, D.; Hazra, B. G. *J. Med. Chem.* **2006**, *49*, 2652; (b) Salunke, D. B.; Hazra, B. G.; Pore, V. S. *Curr. Med. Chem.* **2006**, *13*, 813.
15. Enhsen, A.; Kramer, W.; Wess, G. *Drug Discovery Today* **1998**, *3*, 409.
16. Li, C.; Peters, A. S.; Meridith, E. L.; Allman, G. W.; Savage, P. B. *J. Am. Chem. Soc.* **1998**, *120*, 2961.
17. (a) Salunke, D. B.; Hazra, B. G.; Pore, V. S.; Bhat, M. K.; Nahar, P. B.; Deshpande, M. V. *J. Med. Chem.* **2004**, *47*, 1591; (b) Hazra, B. G.; Pore, V. S.; Dey, S. K.; Datta, S.; Darokar, M. P.; Saikia, D.; Khanuja, S. P. S.; Thakur, A. P. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 773; (c) Pore, V. S.; Aher, N. G.; Kumar, M.; Shukla, P. K. *Tetrahedron* **2006**, *62*, 11178; (d) Vatmurge, N. S.; Hazra, B. G.; Pore, V. S.; Shirazi, F.; Deshpande, M. V.; Kadreppa, S.; Chattopadhyay, S. *Org. Biomol. Chem.* **2008**, *6*, 3823; (e) Bavikar, S. N.; Salunke, D. B.; Hazra, B. G.; Pore, V. S.; Dodd, R. H.; Thierry, J.; Shirazi, F.; Deshpande, M. V.; Kadreppa, S.; Chattopadhyay, S. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5512; (f) Aher, N. G.; Pore, V. S.; Shiva Keshava, G. B.; Kumar, A.; Mishra, N. N.; Shukla, P. K.; Sharma, A.; Bhat, M. K. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 759.
18. (a) Rapoport, H.; Reist, H. N. *J. Am. Chem. Soc.* **1955**, *77*, 480; (b) Reagents for Organic Synthesis, Fieser & Fieser, Vol. 2, p 363.
19. (a) Cook, C. E.; Corley, R. C.; Wall, M. E. *Steroids* **1968**, *33*, 2789; (b) Bevins, C. L.; Bantia, S.; Pollack, R. M.; Bounds, P. L.; Kayser, R. H. *J. Am. Chem. Soc.* **1984**, *106*, 4957; (c) Kashino, S.; Katz, H.; Glusker, J. P.; Pollack, R. M.; Bounds, P. L. *J. Am. Chem. Soc.* **1987**, *109*, 6765; (d) Maltais, R.; Tremblay, M. R.; Poirier, D. *J. Comb. Chem.* **2000**, *2*, 604; (e) Maltais, R.; Luu-The, V.; Poirier, D. *J. Med. Chem.* **2002**, *45*, 640.
20. Single-crystal data and CIF file of compound **13** (CCDC # 725608) have been deposited at the Cambridge Crystallographic Data Centre and can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html or from the Cambridge Crystallographic Data Center, 12 Union Road, Cambridge CB21EZ, UK; fax: (+44) 1123 336 033; or email: deposit@ccdc.ac.uk.
21. For the synthesis of *N*1-(Boc)-1,2-diaminoethane see Supplementary data.
22. (a) National Committee for Clinical Laboratory Standard. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast, Approved Standard. Document M27-A; National Committee for Clinical Laboratory Standards: Wayne, PA, USA, 1997; (b) National Committee for Clinical Laboratory Standard. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Conidium Forming Filamentous Fungi: Proposed Standard. Document M38-P; National Committee for Clinical Laboratory Standard: Wayne, PA, USA, 1998; (c) National Committee for Clinical Laboratory Standards. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Approved Standard, 5th ed.; NCCLS: Villanova, PA, 2000; pp M7–A5; (d) Yamamoto, N.; Fujita, J.; Shinzato, T.; Higa, F.; Tateyama, M.; Tohyama, M.; Nakasone, I.; Yamane, N. *Int. J. Antimicrob. Agents* **2006**, *27*, 171.