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BRIEF ARTICLE

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Synthesis of New 3,20-Bispolyaminosteroid Squalamine Analogues and Evaluation of Their Antimicrobial Activities

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Supporting Information

ABSTRACT: 3,20-Amino- and polyaminosteroid analogues of squalamine and trodusquemine were synthesized involving a stereoselective titanium reductive amination reaction in high chemical yields in numerous cases. These derivatives were evaluated for their in vitro antimicrobial properties against references and clinical bacterial strains exhibiting minimum inhibitory concentrations of 2.5−40 μg/mL. The mechanism of action of these derivatives was determined using bioluminescence for ATP efflux measurements and fluorescence methods for membrane depolarization assays.

INTRODUCTION

Since their massive successful use for treatment of numerous bacterial infections in the past century, chemical antibiotics appeared as one of the great health successes, decreasing morbidity and mortality. However, this has led to high levels of unsuitable prescribing, contributing to recent rises in numbers of antibiotic-resistant bacteria. Thus, emergence of multidrug resistant (MDR) microorganisms such as methicillin resistant *S. aureus* and vancomycin resistant *Enterococcus* or *Pseudomonas* has prompted efforts to develop new classes of antibiotics.[1−3] Bacterial membrane structure constitutes an appealing target, since the latter is highly conserved among most species of both Gram-negative and Gram-positive bacteria. Resistance to membrane active antibiotics requires major changes in membrane structure that in turn influences the permeability barrier, increasing susceptibility to hydrophobic antibiotics. Furthermore, the outer membrane of Gram-negative bacteria forms an effective barrier to proteases, lysozymes, and many types of antibiotics.[4] Consequently, numerous molecules that are active against Gram-positive organisms are much less active against Gram-negative bacteria and vice versa. Most of the responses to Gram-negative bacteria could be attributed to lipopolysaccharides (LPS) and their lipid A anchor, which are a primary component of the outer membrane.[5−10] It is widely held that the permeability barrier of the outer membrane is formed via cross-bridging between lipid A molecules and divalent cations (Ca^{2+} and Mg^{2+}).[11] Thus, it is well-known that metal ion chelators such as EDTA, certain cationic peptides,[12] and polyamines, which can attack the binding sites of divalent cations, are able to disrupt the organization of the outer membrane, increasing its permeability and therefore sensitizing bacteria to hydrophobic antibiotics. In this context, an attractive approach for the development of antibacterial agents is the use of compounds targeting membranes of Gram-negative bacteria, since they are not expected to readily induce resistance formation. Among all the numerous antibiotics developed to date, few compounds possessing a cationic steroid structure have been identified and studied. Thus, in 1998 two new cationic pregnane-type steroidal alkaloids, saligcinnamid, pachysamine H, were isolated from the EtOH extracts of the roots and stems of *Sarcococca saligna* and these compounds exhibited good antibacterial activity against several human pathogenic bacteria (Figure 1).[13,14] Among all the envisioned strategies in our laboratory in the search for novel host defense agents, squalamine **3a** and trodusquemine **3b** (Figure 1) were identified as the first natural aminosterols from the dogfish shark, *Squalus acanthias*, exhibiting potent antimicrobial activities against MDR bacterial strains.[15,16] On the basis of such results, we have recently reported the design of new aminosteroid derivatives easily obtained from cheap and available precursors through an original titanium reductive amination reaction.[17,18] It has also clearly appeared that the presence of a polyamino moiety is crucial to encounter high antimicrobial activities.[19,20] In the our work, we report herein the design and antimicrobial activities of a new class of bis(polyamino)steroid derivatives. We will also report a deeper analysis of their mechanism of action against Gram-negative and Gram-positive bacteria, pointing out the original mechanism of action of this class of derivatives.

RESULTS AND DISCUSSION

Using an efficient titanium reductive amination developed in our laboratory, we have envisioned a one step procedure for the preparation of new bis(polyamino)steroid derivatives from progesterone available in large amounts according to the following synthetic pathway (Table 1). First, it clearly appears that isolated yields of **4c** are highly solvent dependent. Thus, the expected amino derivative **4c** was obtained in 63% yield, performing the reaction in MeOH (Table 1, entry 1) whereas only moderate yields of 30%, 19%, and 33% were encountered performing the reaction in CH_2Cl_2, toluene, and THF, respectively (Table 1, entries 3−5). The influence of the nature of the titanium source involved was also investigated, and chemical yield variations of 19−63% were obtained (Table 1, entries 1, 6−8), the best result having been observed using Ti(O-*i*-Pr)$_4$ as titanium source.

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Furthermore, under these best experimental conditions, increasing the reaction temperature from −78 to 0 °C led to a significant decrease of the diastereoselectivity from 95% to 60% de, respectively (Table 1, entries 1 and 2).

A mechanistic rationale, including a nucleophilic attack of the amino group to a carbonyl compound activated by a Lewis acid and a transient imine species which can be subsequently reduced is proposed by rationalizing the stereoinduction in the reaction leading exclusively to the formation of the β-amino or bis-(polyamino) derivative due to steric hydrogen hindrance. Thus, this transition state allows us to justify that the formation of the 3α,20α-parent derivative is unfavored at low temperature.[21−23]

On the other hand, whatever the nature of the considered diamine or polyamine was, the expected products were obtained in chemical overall yields of 18−82% and excellent diastereoselectivities of up to 95% de in all cases (Scheme 1).

Chronic microbial colonization of the respiratory tract, leading to exacerbations of pulmonary infection, constitutes the major cause of disease and death in patients with cystic fibrosis (CF). Typical pathogens in respiratory secretions of CF patients include Gram-positive and Gram-negative such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Inquilinus limosus*, and *Burkholderia cepacia*.[24] In the context of our studies, all of the synthesized compounds were screened for their antimicrobial activities against Gram-positive and Gram-negative bacteria strains and found to possess activities against the microorganisms listed in Table 2. Most of the compounds (typically derivatives **4c**−**l**) present excellent activities against Gram-positive bacteria, exhibiting similar MIC values of 2.5−10 μg/mL against oxacillin sensitive or resistant *Staphylococcus aureus* strains except for **4a** and **4b**, suggesting that the presence of polyamino chains is necessary to lead to biologically active compounds. On the other hand, all the derivatives possess moderate to excellent antimicrobial activities against Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* strains with MIC values of 2.5−40 μg/mL whereas low activities were encountered against *Inquilinus limosus* and *Burkholderia cepacia* strains, suggesting that the structure and the nature of the polyamino groups attached to the steroid moiety play an important role in the mechanism of action. This assumption is supported by the correlation observed between the number of carbons constituting the polyamino chain attached to the steroid core and the antimicrobial activity encountered against *P. aeruginosa* (Figure 2B). Surprisingly, no similar effect was observed against *S. aureus* Gram-positive bacteria, since the antimicrobial activity remained unchanged whatever the derivative used, suggesting that the mechanism of action of these compounds is different depending on the class of bacteria considered (Figure 2A). As shown in Table 3, the addition of Mg^{2+} at 10 mM final concentration increased the MIC of colistin, squalamine, and **4i** against *E. coli* at least 4 times, whereas the MICs of tobramycin were not affected. Moreover, MICs of squalamine, colistin, or tobramycin remain unchanged by Mg^{2+} supplementation in the case of *S. aureus*. Furthermore, **4i** exhibited a complete killing of *P. aeruginosa* reference strains in 2 h (Figure 3A). Moreover, **4i** showed a direct bactericidal effect

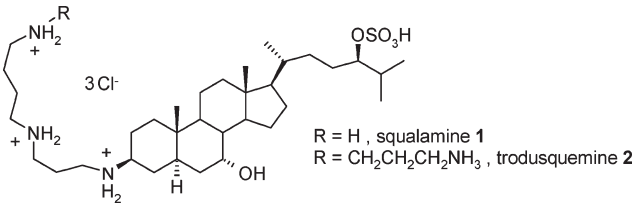
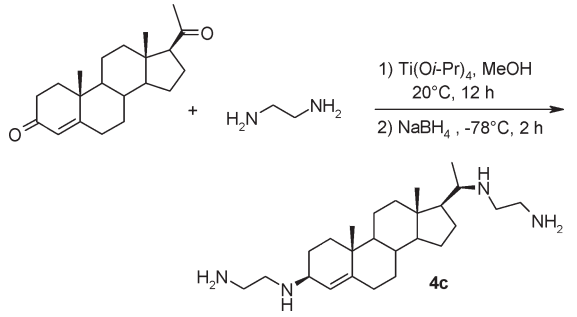


Figure 1. Structures of saligcinnamide **1**, pachysamine-H **2**, squalamine **3a**, and trodusquemine **3b**.

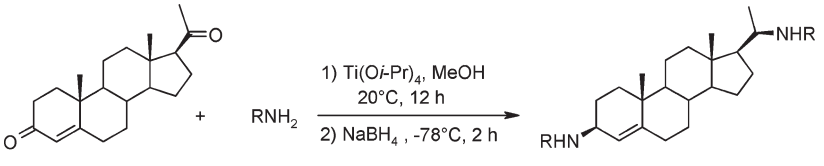
Table 1. Titanium(IV) Reductive Amination Reaction of Progesterone with 1,2-Diaminoethane under Various Experimental Conditions



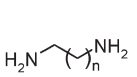
entry	titanium source	solvent	yield (%)[c]	de (%)[d]
1[a]	Ti(O-*i*-Pr)$_4$	MeOH	63	>95
2[b]	Ti(O-*i*-Pr)$_4$	MeOH	72	60
3[a]	Ti(O-*i*-Pr)$_4$	CH_2Cl_2	30	91
4[a]	Ti(O-*i*-Pr)$_4$	toluene	19	92
5[a]	Ti(O-*i*-Pr)$_4$	THF	33	>95
6[a]	Ti(OEt)$_4$	MeOH	41	>95
7[a]	Ti(OBu)$_4$	MeOH	30	>95
8[a]	Ti(O-*t*-Bu)$_4$	MeOH	19	

[a] Reaction performed at −78 °C for 12 h in MeOH on a 0.39 mmol scale of progesterone, Ti(O-*i*-Pr)$_4$ (2.02 mmol), and the amine (6 mmol). [b] Reaction performed at 0 °C. [c] Isolated overall yield. [d] Diastereomeric excess (de) was evaluated by HPLC and ^{1}H and ^{13}C NMR.

Scheme 1. Titanium(IV) Reductive Amination Reaction of Progesterone with Various Diamines



RNH$_2$ = NH$_3$ **4a**; H$_2$N—Me **4b**



4c n = 1 **4h** n = 6
4d n = 2 **4i** n = 7
4e n = 3 **4j** n = 8
4f n = 4 **4k** n = 9
4g n = 5 **4l** n = 11

Table 2. Antimicrobial Activities of 3,20-Bispolyaminosteroid Derivatives 4a−k[a]

	minimum inhibition concentration (MIC (μg/mL))							
	Gram-positive bacteria			Gram-negative bacteria				
	S. aureus			*E. coli*	*P. aeruginosa*	*P. aeruginosa*	*I. limosus*	*B. cepacia*
compd	ref, 2	Oxa S, 10	Oxa R, 6	ref, 1	ref, 2	CS S, 12	CS R, 3	CS R, 2
squalamine	1.25	0.3	1.25	5	2.5	5−20	5	>40
TOB	1.25	1.25		2.5	5	5−20	>40	>40
CS				2.5	5	5−20	>100	>40
4a	40	>40	40	>40	>40	>40	>40	>40
4b	40	20	20	>40	>40	>40	>40	>40
4c	20	20	10−20	20	20	20−40	>40	>40
4d	10	10	5−10	10	10	20−40	20−40	>40
4e	5	10	5−10	10	20	20−30	40−80	>40
4f	5	5−20	10−20	10	10	20−80	40−80	>40
4g	2.5	5−10	5	5	10	20−40	20	>40
4h	2.5	2.5−5	5−10	5	10	10−30	20−40	>40
4i	2.5	2.5	5	5	2.5	2.5−10	10−20	>40
4j	5	5−10	5	20	40	40−80	20−40	>40
4k	10	5−10	5	20	40	40−80	20−40	>40
4l	10	5−10	10	20	40	40−80	20−40	>40

[a] ref: reference strain. CS: colistin. Oxa: oxacillin. S: sensitive. R: resistant;.TOB: tobramycin.

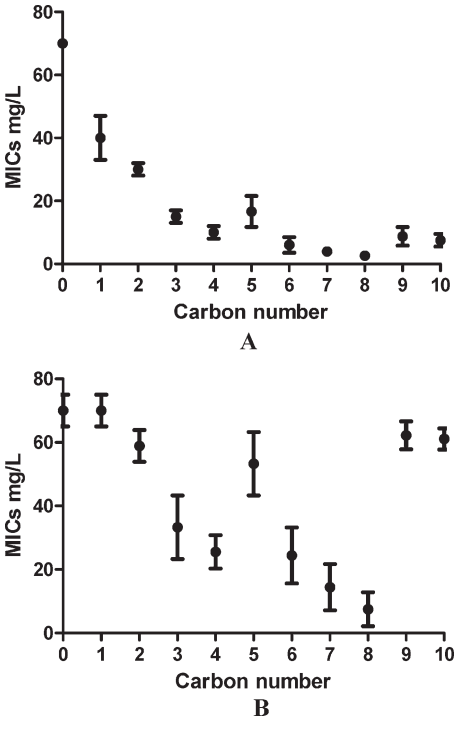


Figure 2. Correlation of the 3,20-bispolyaminosteroid derivatives activities with respect to the length of the polyamino chain against *S. aureus* (A) and *P. aeruginosa* strains (B).

against *S. aureus* reference strain reflect by nearly a 5 log decrease in the counts of this bacteria by 1 h whereas 2 h are necessary using tobramycin (Figure 3B). On the other hand, **4i** and squalamine

Table 3. Effect of Divalent Cation Salt on Antibacterial Activity of Colistin, Tobramycin, Squalamine, and Derivative 4i against *E. coli* and *S. aureus*[a]

	minimum inhibition concentration (MIC (μg/mL))							
	without Mg^{2+}				with Mg^{2+} (10 mM)			
strain	squalamine	CS	TOB	**4i**	squalamine	CS	TOB	**4i**
E. coli ATCC 25922	2,5	0.5	2	2,5	32	16	1	10
S. aureus ATCC 25923	1,25	>128	1	2.5	1.25	>128	2	5

[a] CS: colistin. TOB: tobramycin

effects on bacterial membrane integrity were investigated by measuring intracellular ATP efflux kinetics during 20 min. For *E. coli*, a time-dependent ATP release was observed with less than 35% of maximal efflux (Figure 4A). Conversely, squalamine incited a rapid ATP release from *S. aureus* reaching 100% of maximal efflux after only 5 min (Figure 4B). In this context, treatment with colistin resulted in a slight but significant ATP release ($P < 0.0001$) in the case of *E. coli* leading to 4−5% of maximal efflux after 5 min while no significant effect was noticed in the case of *S. aureus* all along the test time ($P < 0.0001$, Figure 4). Finally, no depolarizing effect was noticed in squalamine-treated *E. coli* (Figure 5A), whereas in the same conditions, squalamine prompted a flagrant depolarization of *S. aureus* bacterial membrane depicted by a rapid and strong increase in relative fluorescence units ΔRFU reaching in less than 3 min 80% of maximal RFU (Figure 5B). On the

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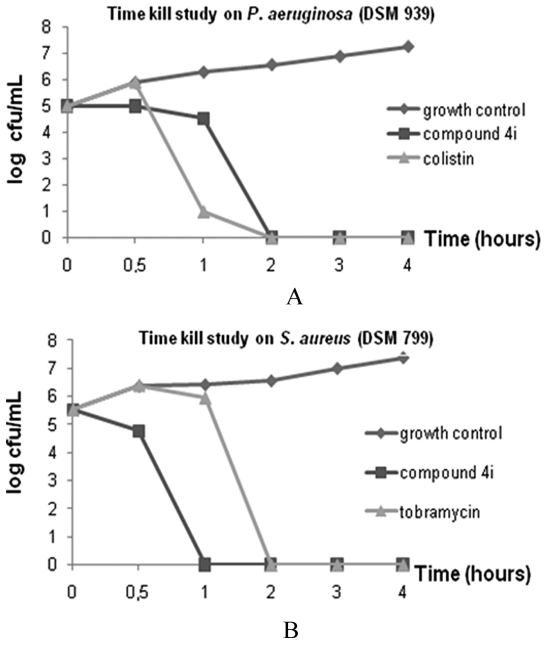

Figure 3. Time-kill curves of derivative **4i** at 1× the MIC over a 4 h period against *P. aeruginosa* (DSM 939) (A) and *S. aureus* (DSM 799) (B) strains.

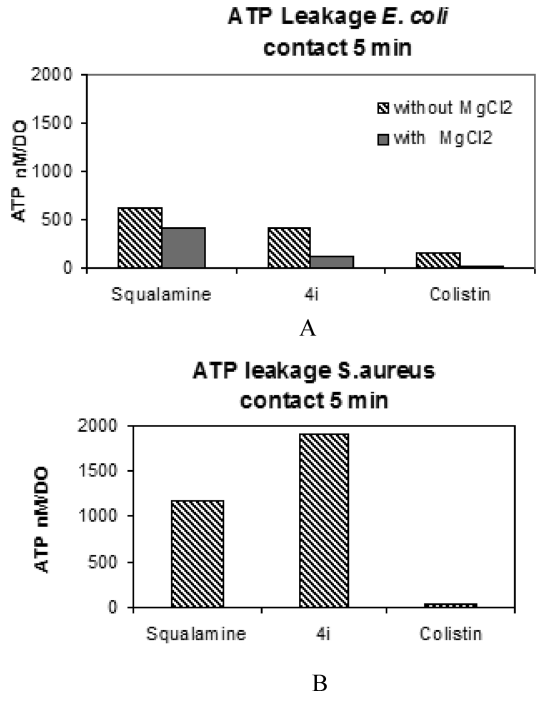

Figure 4. Effect of compound **4i** on ATP efflux for treated *E. coli* (A) and *S. aureus* (B) bacteria.

other hand, **4i** appears to be a 4 times better depolarizing agent than squalamine in both cases because of the presence of a high number of positive charges.

A potent mechanism of action had been previously described with the polymyxin antibiotic colistin that, by using positive amine groups, interacts with the negative phosphate groups of

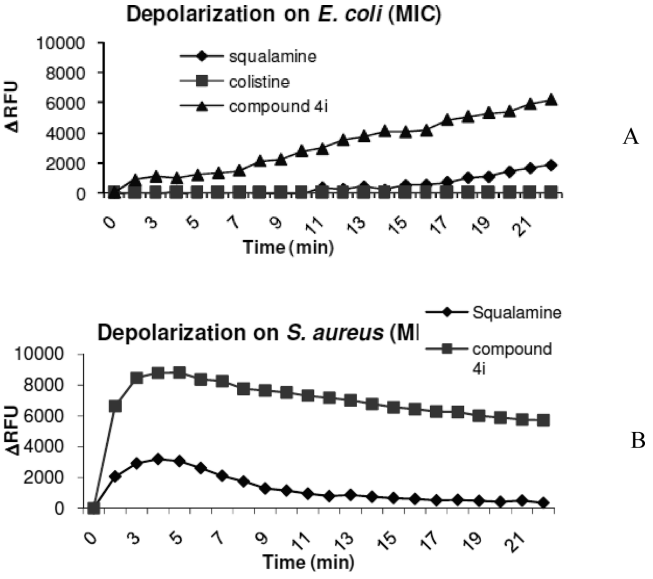

Figure 5. Depolarization of bacterial membrane of *E. coli* (A) and *S. aureus* (B) bacteria in the presence of squalamine and compound **4i**.

LPS displacing divalent cations such as Ca^{2+} and Mg^{2+}.[10,11] Accordingly, an inhibitory effect of Ca^{2+} and Mg^{2+} on colistin but also on bispolyaminosteroid derivatives activities against *E. coli* was found indicating that the interaction with the negatively charged phosphate groups in LPS structure is mandatory for both agents to be active. Interestingly, **4i** showed markedly faster killing rate than colistin against Gram-negative bacteria, suggesting that both compounds might interact differently with bacterial membranes. Accordingly, though the availability of negatively charged phosphate groups represents a common requirement for the activity of **4i** and colistin, both agents probably exhibit different series of action soon after interaction with LPS. Indeed, by use of intracellular ATP efflux as an indicator of membrane lesions, treatment of *E. coli* with **4i** resulted in significantly higher and faster ATP efflux compared to colistin and squalamine, suggesting that LPS damage induced by derivative **4i** is clearly greater and faster than that caused by colistin and squalamine. However, this would not be valid for Gram-positive bacteria that are devoid of LPSs. Thus, it was not surprising that the divalent cations had no effect on **4i**, squalamine, colistin, or tobramycin activities against the Gram-positive bacterium *S. aureus*. Remarkably, derivative **4i** demonstrated a faster killing rate against S. *aureus* than that noted with Gram-negative bacteria, signifying that this compound may possess a rapid and direct bactericidal effect against Gram-positive bacteria. Moreover, **4i** produced an instantaneous intracellular ATP efflux in the case of *S. aureus*, indicating that a rapid phenomenon might specifically be involved in **4i** mode of action against Gram-positive bacteria. Indeed, **4i** led to a strong depolarization of *S. aureus* membranes while a weak depolarization was observed for tested Gram-negative bacteria. Thus, **4i** and by extension other related amino steroids have a particular mode of action mediated by bacterial membrane disruption reducing the possibility of resistance emergence. Altogether and without excluding other intra- or extracellular targets of the action of **4i**, our results indicate that this family of compound acts by disrupting the outer membrane of Gram-negative bacteria in a detergent-like mechanism of action and by depolarizing bacterial membrane of Gram-positive bacteria.

CONCLUSION

Because of the unique mechanism of action, current studies are now underway to evaluate the potentiality of such derivatives by determining their cytotoxicity and their potent use mainly as disinfectant, detergents, or topical antimicrobial agents.

EXPERIMENTAL SECTION

The purity of the compounds was checked by analytical HPLC (C18 column, MeOH/CH_3CN) with PDA detector spanning from 210 to 310 nm. All compounds were >95% pure, as determined by analytical HPLC-PDA at 214 and 254 nm.

General Procedure for the Titanium-Mediated Reductive Amination Reaction of 4c. A mixture of progesterone (123 mg, 0.39 mmol), titanium(IV) isopropoxide (573 μL, 2.02 mmol), and 1,8-diaminooctane (6 mmol) in absolute methanol (5 mL) was stirred under argon at room temperature for 12 h. Sodium borohydride (38 mg, 1 mmol) was then added at −78 °C, and the resulting mixture was stirred for an additional 2 h. The reaction was then quenched by adding water (1 mL), and stirring was maintained at room temperature for 20 min. The resulting inorganic precipitate was filtered off over a pad of Celite and washed with methanol and ethyl acetate. The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo to afford the expected crude amino derivative which was purified by flash chromatography, affording the expected amino derivative. Purification by column chromatography (silica gel, CH_2Cl_2/MeOH/NH_4OH (32%), 7:3:1) afforded a pale yellow pasty solid in 56% yield. ^{1}H NMR (300 MHz, CD_3OD): δ 5.22 (s, 1H), 3.44−3.22 (m, 4H), 2.29−1.02 (m, 60H), 0.82−0.76 (m, 5H). ^{13}C NMR (75 MHz, CD_3OD): δ 148.49, 128.22, 59.83, 57.59, 56.24, 48.03, 47.98, 44.17, 42.92, 41.21, 38.55, 34.06, 31.84, 30.97, 28.94, 28.39, 27.32, 25.99, 22.62, 18.95, 13.20. $C_{37}H_{70}N_4$ MS (ESI$^+$) m/z 571.5634 (100%, $[M + H]^+$).

ASSOCIATED CONTENT

Supporting Information. Details for synthetic preparations, analytical data of all compounds, and biological test systems. This material is available free of charge via the Internet at http://pubs.acs.org.

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