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Synthesis of linear and cyclic guazatine derivatives endowed with antibacterial activity

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ABSTRACT

Antibiotic resistance has reached alarming levels in many clinically-relevant human pathogens, and there is an increasing clinical need for new antibiotics active on drug-resistant Gram-negative pathogens who rapidly evolve towards pandrug resistance phenotypes. Here, we report on two related classes of guanidinic compounds endowed with antibacterial activity. The two best compounds (**9a** and **13d**) exhibited the most potent antibacterial activity with MIC values ranging 0.12–8 μg/ml with most tested pathogens, including both Gram-positive and Gram-negative bacteria. Interestingly, MIC values were not affected (1–8 μg/ml) when measured using recent clinical isolates with various antibiotic resistance determinants. The results reported herein identify guazatine derivatives as an interesting starting point for the optimization of a potentially novel class of antibacterial agents.

The treatment of bacterial infections caused by multi-drug, extensively-drug or even pan-drug resistant strains is becoming challenging for health care practitioners. The prevalence of drug-resistant isolates has reached alarming levels in many significant human pathogens, among both Gram-negative and Gram-positive pathogens. These are often referred to as 'ESKAPE' organisms, which include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.[1–5] This term emphasizes that these organisms have the potential to escape most of the available antibacterial therapies. It has been reported that, in U.S.A. hospitals, more people currently die of methicillin-resistant *S. aureus* than of HIV/AIDS and tuberculosis combined.[6–9] Moreover, pandrug-resistant strains are frequently reported emerging in clinically-relevant Gram-negative pathogens, such as *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*, and may represent a dramatic pace forward the return to the pre-antibiotic era.[5,10–12]

Due to both the lack of investment in antibiotic R&D and the increased spread of resistant strains, the therapeutic options are diminishing and might be limited, in some cases, to suboptimal drugs, such as colistin, often burdened by a significant toxicity and severe side effects. As illustrated by the growing concern expressed by many public health agencies and authorities, the need for new therapies and drugs active against resistant strains should be urgently addressed to eventually overcome severe consequences for global Human Health.

Our research group has been involved for years into in-depth studies on linear and cyclic derivatives of guazatine, some of which showed broad-spectrum antifungal properties.[13–18] The potential antibacterial properties of a series of such compounds was evaluated with a panel of different bacteria, including both type strains and clinical isolates (showing various antibiotic susceptibility profile), allowing the identification of some active molecules. A quite large number of compounds bearing guanidine moieties have been reported in the literature as broadly active agents against microbial pathogens, in particular against parasitic fungi.[14,15,19–24] The antibacterial activity of such compounds was not, at our best knowledge, previously evaluated. In this work, compounds from two series were tested on 13 different bacterial species, including some ESKAPE organisms.

Described compounds (Table 1) can be divided in two classes of guazatine derivatives, linear (Scaffold A) and cyclic (Scaffold B). Although the syntheses of these compounds involved some

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common steps, they differs remarkably due to the different reactivity of the intermediates.

The guanylating agents used for each synthesis have been obtained through Mitsunobu reaction between the desired alcohol and the di-Boc-pyrazole-1-carboximidamide (Scheme 1).[25] Compounds **9a–d** and **12a–e** have been described elsewhere.[14,22,24]

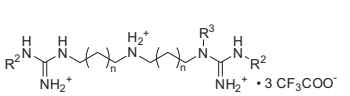
Linear compounds **5a–c** have been obtained following the straightforward protocol described in Scheme 2. Starting material bis(hexamethylene)triamine was subsequently guanylated with the appropriate guanylating agent furnishing, after Boc cleavage, the desired derivatives **5a–c**.

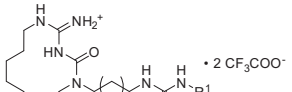
The synthesis of compounds **9a–h**, bearing longer alkyl chains, is reported in Scheme 3 starting from the common intermediates **6a–c** already described in a previous work.[14,21,24]

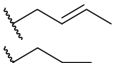
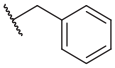
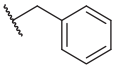
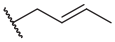
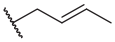
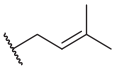
After Cbz deprotection the primary amines **7a–c** were guanylated and Boc-deprotected leading to the desired diguanidines **9a–h**.

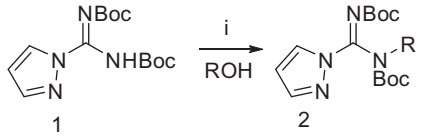
On the other hand, cyclization was obtained as reported in Scheme 4, by refluxing the common intermediate **6** in THF leading

Table 1
Structures of the synthesized compounds

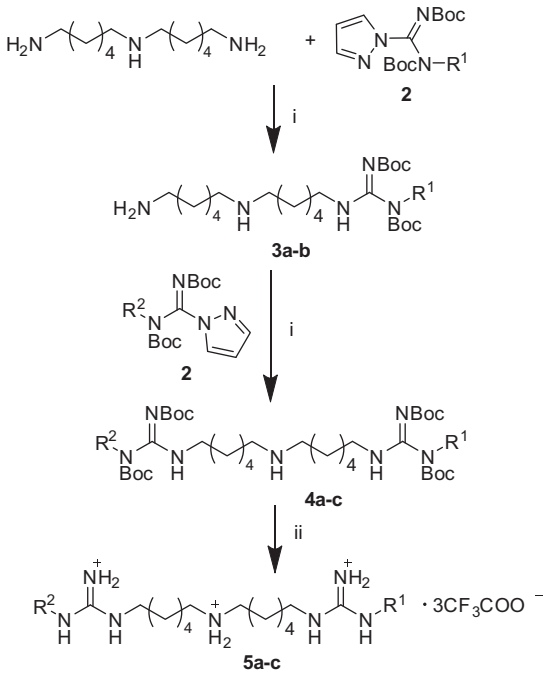




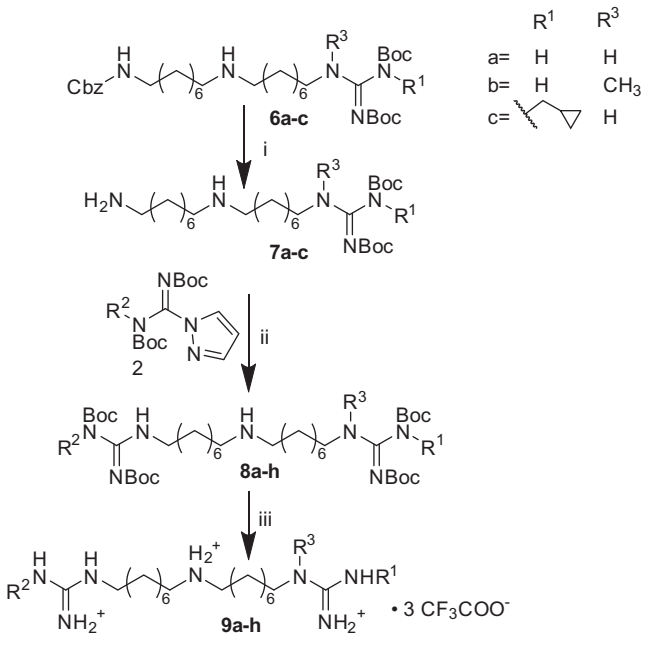
Compd	Scaffold	*n*	R^1	R^2	R^3
5a	A	4		H	H
5b	A	4			H
5c	A	4		H	H
9a[14,22]	A	6		H	H
9b[22]	A	6		H	H
9c[22]	A	6		H	H
9d[22]	A	6		H	H
9e	A	6			H
9f	A	6		H	CH_3
9g	A	6		H	CH_3
9h	A	6		H	CH_3
13a[24]	B	6		H	H
13b[14]	B	6		H	H
13c	B	6		H	H
13d[14]	B	6		H	H
13e[24]	B	6		H	H
13f	B	4		H	H
13g	B	4		H	H
13h	B	4		H	H
13i	B	4		H	H
13j	B	4		H	H



Scheme 1. Synthesis of guanylating compounds **2**. Reagent and conditions: DIAD, PPh_3, THF dry, 0 °C to reflux, 12 h.



Scheme 2. Synthesis of compounds **5a–c**. Reagents and conditions: (i) DIPEA, CH_3CN/MeOH, 50 °C, 12 h, (ii) TFA, DCM, rt, 8 h.

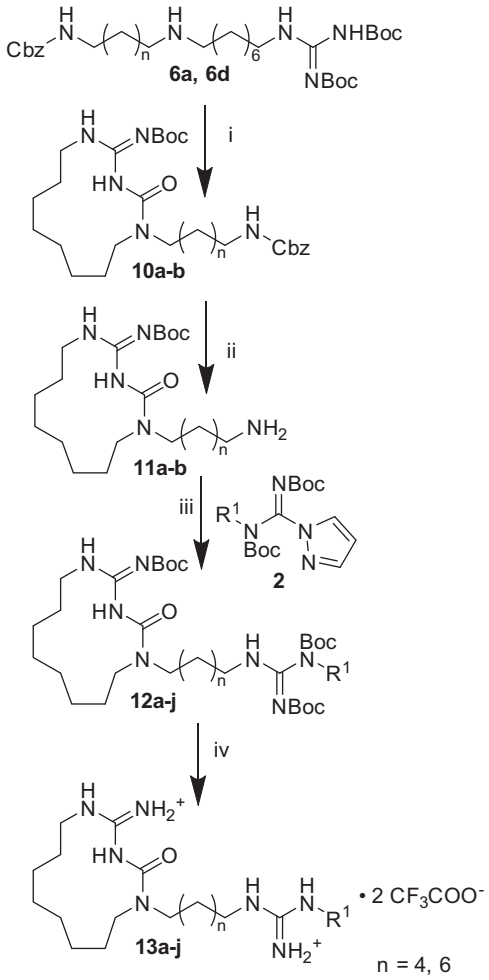


Scheme 3. Synthesis of linear compounds **9a–h**. Reagent and conditions: (i) H_2 Pd/C, 2-propanol, HCl, rt, 5 h; (ii) DIPEA, CH_3CN, 50 °C, 12 h, (iii) TFA, DCM, rt, 8 h.

to the formation of the desired macrocycles with good yield. The Cbz protecting group was then cleaved by hydrogenation affording the corresponding primary amine **11a–b**. This latter compound was then guanylated with the appropriate N-substituted pyrazole-1-carboximidamide leading, after Boc cleavage, to the desired derivatives **13a–j**. The purity of the compounds was assessed by means of HPLC as described in Supplementary information.

Aminooctanoic acid **14** was selected as building block for the synthesis of compounds **9f–h** (Scheme 5). The starting material **14** was reacted with acyl chloride in dry MeOH affording **15** which free amino group was protected using benzyl chloroformate. The ester moiety of **16** was then reduced with DIBAL-H in DCM to give the intermediate **17**. The latter was reacted with *p*-toluenesulfonyl chloride, DMAP and Et_3N in DCM to give **18**. The methyl amino moiety was introduced as described by Sharpless and Gao by reacting compound **18**, previously dissolved in THF, with 40% aqueous methylamine.[26]

Compound **20** was obtained in quantitative yield by reacting **19** with 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine in DCM and Et_3N.[26] The deprotection of the primary amine of **20** was done by hydrogenolysis using 10% Pd/C and H_2 atmosphere. Since the intermediate **21** is unstable as free amine, during the hydrogenolysis we used 36% HCl in order to block the amino group in a hydrochloride salt form. To obtain the linear intermediate **6b**, the aldehyde **22** was reacted with **21** in a reductive amination reaction using triacetoxyborohydride as mild reductive agent in dry DCE.[27] The subsequent functionalization of compound **6b** was accomplished as described in Scheme 3.



Scheme 4. Synthesis of cyclic compounds **13a–j**. Reagents and conditions: (i) THF, reflux, 12 h; (ii) H_2 Pd/C, 2-propanol, HCl, rt, 5 h; (iii) DIPEA, CH_3CN, 50 °C, 12 h, (vi) TFA, DCM, rt 8 h.

All compounds were obtained as trifluoroacetic salts and have been tested in this form. Totally, we synthesized twenty-one guazatine derivatives as reported in Table 1, eleven belonging to the linear family and ten to the macrocyclic one. Among the latters, five have an eight carbons side chain (**13a–e**) and five a six carbons side chain (**13f–j**). Various groups have been attached to the guanidine moiety looking for a structure activity relationship (see Table 2).

Bacterial strains, including representatives of both Gram-positive and Gram-negative bacteria, as well as clinical isolates showing various level of antibiotic resistance, were obtained from the ATCC or CCUG culture collections or present in the authors' collection of clinical isolates.[28–31] Compounds were resuspended in dimethyl sulfoxide (DMSO) at a final concentration of 100 mg/ml and subsequently diluted in the culture medium. The minimum inhibitory concentrations (MICs) of the compounds were determined using the micro-dilution broth method using Mueller–Hinton broth as recommended by the Clinical Laboratory Standards Institute (CLSI).[32] Bacterial inoculum was 5×10^4 CFU/well. MICs were recorded after 16–18 h incubation at 35–37 °C.

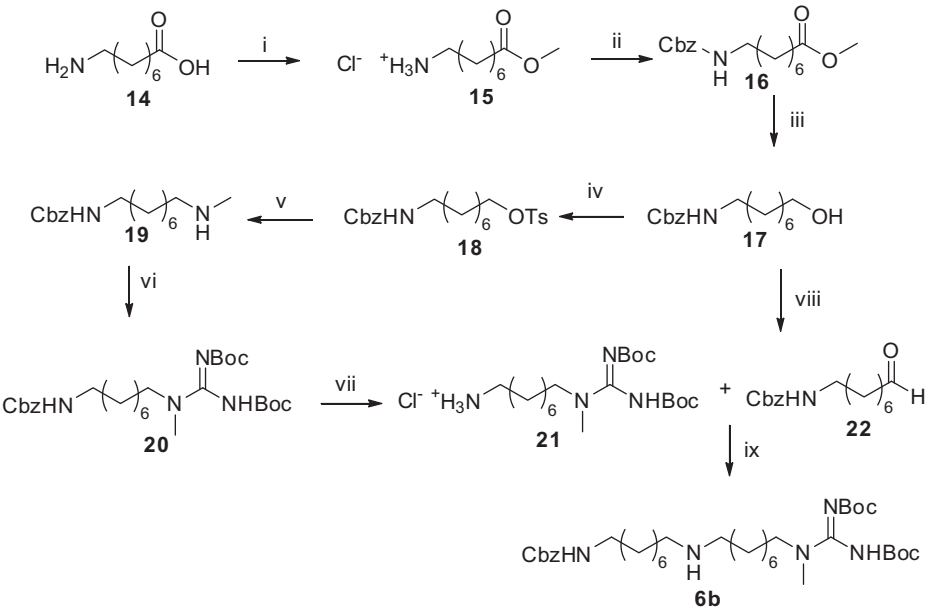
Compounds **9a** and **13d** showed potent and broad-spectrum antibacterial activity, being active against representatives of both Gram-positive and Gram-negative bacteria. Furthermore, the macrocyclic compound bearing the cyclopropylmethyl group, compound **13a**, is only moderately active against Gram-positive bacteria and almost inactive against Gram-negatives. Similarly, compound **9b**, which can be considered the linear analogue of compound **13d**, is also completely inactive against both Gram-negative and Gram-positive organisms.[1–5]

Linear compound **9e** bearing symmetric substitutions on the two guanidinic moieties also was also found to be inactive. Similar results were obtained for linear compound **5a**, bearing shorter chains linking the central nitrogen and the guanidinic moieties. Overall, these data suggest that the nature of the substituents and the distance between the guanidinic moieties control the activity of the linear compounds. This hypothesis is further supported by analyzing compounds **9f–h**, which showed lower antibacterial activity, as compared to their demethylated counterparts.

Macrocyclic compounds **13f–j** characterized by a shorter side chain were found completely inactive on Gram-negative bacteria, and only moderately active on Gram-positives. This finding suggests that, in these macrocyclic compounds, the distance between the two guanidinic moieties might be determinant for their activity, and this information might be important to allow the design of optimized compounds.

Interestingly, the antibacterial potency of compounds **9a** and **13d** was poorly affected when MIC values were determined with recent multi-drug resistant clinical isolates, such as carbapenemase-producing Gram-negative pathogens and vancomycin-resistant staphylococci (see Table 3). The apparent absence of cross-resistance with existing mechanisms is interesting and further could support the potential of such compounds to address antibiotic resistance in relevant pathogens, although their mechanism of action has not been elucidated yet.

In vitro ADME properties (apparent permeability in gastrointestinal model, water solubility, microsomal stability) for the most active compound **9a** were evaluated (see Table 4).[33,34] The parallel artificial membrane permeability assays (PAMPA) reveal a low value of apparent permeability (P_{app}) at physiological pH, while, in duodenal alkaline condition (pH 10) an higher P_{app} was found, as expected for a positively ionizable compound.[35] Noteworthy, compound **9a** shows a solubility of 0.966 ± 0.022 g/L in pure water and metabolic stability, measured by means of human liver microsomal proteins, higher than 99%. Moreover, compound **9a** binds



Scheme 5. Synthesis of intermediate **6b**. Reagent and conditions: (i) acetyl chloride, dry MeOH, reflux, 12 h; (ii) CbzCl, $NaHCO_3$, H_2O/THF 1:1, −7 °C–rt, 12 h; (iii) 1 M DIBAL-H, DCM, 0 °C–rt, 12 h; (iv) 4-toluenesulfonyl chloride, TEA, DMAP, DCM, 0 °C–rt, 12 h; (v) 40% CH_3NH_2, THF, 65 °C 5 h; (vi) 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine, TEA, DCM, 12 h; (vii) H_2 1 atm, Pd/C 10%, HCl 36%, 2-propanol, 4 h; (viii) DMP, $NaHCO_3$, DCM, rt, 5 h (ix) $NaBH(OAc)_3$, dry DCE, 16 h.

Table 2
Antibacterial activity of tested compounds against representative strains of Gram-positive and Gram-negative bacteria

Bacterial strain	MIC (μg/ml)																		
	9a	**9b**	**9c**	**9d**	**9e**	**5a**	**13a**	**13b**	**13c**	**13d**	**13e**	**13f**	**13g**	**13h**	**13i**	**13j**	**9f**	**9g**	**9h**
Acinetobacter baumannii ATCC 17978	4	>256	64	128	>256	>256	64	32	32	8	64	256	256	>256	>256	256	256	256	128
Aeromonas hydrophila ATCC 7966	8	>256	64	256	>256	>256	n.d.	64	64	4	n.d.	>256	>256	>256	>256	>256	>256	>256	256
Elizabethkingia meningoseptica CCUG 4310	32	>256	n.d.	>256	>256	>256	n.d.	128	256	32	n.d.	256	>256	>256	>256	>256	32	64	64
Escherichia coli CCUGT	0.5	128	8	16	>256	>256	n.d.	64	64	0.5	n.d.	256	256	>256	>256	256	>256	>256	>256
Klebsiella pneumoniae ATCC 13833	1	256	16	32	>256	256	128	32	32	1	128	>256	>256	>256	>256	>256	8	16	32
Pseudomonas aeruginosa ATCC 27853	8	>256	64	256	>256	>256	>256	256	>256	8	>256	>256	>256	>256	>256	>256	8	32	32
Bacillus subtilis ATCC 6633	0.5	64	4	4	>256	>256	4	4	4	0.5	1	16	16	64	64	64	256	256	>256
Enterococcus faecalis ATCC 19433	1	256	16	16	>256	16	32	8	8	1	128	32	64	128	128	16	4	8	16
Staphylococcus aureus ATCC 25923	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Staphylococcus epidermidis ATCC 14990	0.5	64	4	4	>256	256	8	4	4	0.5	16	16	16	32	32	16	8	16	32
Streptococcus pyogenes ATCC 12344	<0.125	16	1	2	>256	32	32	2	2	0.5	4	32	8	16	16	8	8	16	16

n.d.: Not determined.

Table 3
Antibacterial activity of compounds **9a** and **13d** on a panel of Gram-negative and Gram-positive clinical isolates, showing various antimicrobial susceptibility profiles

Bacterial strains	Resistance profile	MIC (μg/ml)	
		9a	**13d**
Acinetobacter baumannii AC-54/97	PEN, ES-CEPH, CARB, AZT, AG, FQ, FOS, SXT	4	4
Achromobacter xylosoxidans AX 22	PEN, ES-CEPH, CARB, AG	4	8
Alcaligenes faecalis 424/98	ES-CEPH, CARB, MON	4	4
Enterobacter cloacae VA-417/02	PEN, CEPH, CARB, AG, FQ	2	1
Klebsiella pneumoniae 7023	PEN, ES-CEPH, CARB, AG, FQ, SXT	2	1
Pseudomonas aeruginosa 101/1477	PEN, ES-CEPH, CARB, AG	8	8
Pseudomonas aeruginosa VR-143/97	PEN, ES-CEPH, CARB, MON, AG, FQ	8	8
Stenotrophomonas maltophilia 634/08	PEN, ES-CEPH, CARB, MON, AG, SXT, FOS	4	4
Staphylococcus aureus ATCC 43300 (MRSA)	PEN	4	n.d.
Staphylococcus aureus ATCC 700699 (VanA)	GLY	4	n.d.
Staphylococcus haemolyticus SI-6/2011	AG	2	n.d.
Staphylococcus warneri SI-5/2011	PEN, AG	2	n.d.
Staphylococcus hominis SI-7/2011	LNZ	2	n.d.

The class of drugs for which the isolate was resistant to is indicated in column 2 (PEN, penicillins; ES-CEPH, expanded-spectrum cephalosporins; CARB, carbapenems; AZT; aztreonam [a monobactam]; AG, aminoglycosides; FQ, fluoroquinolones; SXT, trimethoprim/sulfamethoxazole; FOS, fosfomycin; GLY, glycopeptides; LNZ, linezolid). Strains were from either ATCC, our strain collection or described in Pereira et al., 2007 and references therein, Rossolini et al., 2000 and Cagnacci et al., 2008.[29–31]
n.d.: Not determined.

Table 4
PAMPA assay results for compound **9a** rifaximin and chloramphenicol

	P_{app} (nm/s)		% membrane retention	
	pH 7.4	pH 10.0	pH 7.4	pH 10.0
9a	9.5 ± 6.0	166.5 ± 7.0	8.8 ± 0.5	60.6 ± 0.4
Rifamixin	0.06 ± 0.01	0.12 ± 0.09	0.0	0.0
Chloramphenicol	0.30 ± 0.5	0.57 ± 0.10	0.0	0.0

strongly to human serum albumins with a K_d of 26.52 ± 7.2 μM, the value is comparable to that of known bioactive compounds such as Diazepam.[36]

To better assess the potential of compound **9a**, which shows the most potent antibacterial activity, its cytotoxicity was investigated. The CC_{50} values against HeLa cells after 24 h and 48 h of incubation were 92.6 ± 0.7 μM and 62.0 ± 0.3 μM, respectively. Although the cytotoxicity would ideally be improved with the future series, it is encouraging that the observed CC_{50} values are ⩾70-fold higher than the MIC value observed for *E. coli* and *K. pneumoniae*.

Presently, it was neither possible to assess whether cyclic and linear compounds had a similar mechanism of action, nor to identify the target at the molecular level. Nevertheless, these compounds do not show a lytic effect at concentrations up to 10× MIC on *E. coli*, suggesting that they would not act through an aspecific membrane permeabilization mechanism. It is reasonable to think that the mode of action of our compounds can be specific, as suggested by the loss of activity due to small changes in the substituent attached to the guanidinic moiety.

The broad spectrum activity of **9a** and **13d**, which also covers Gram-negative bacteria (including MDR clinical isolates of pathogens currently evolving towards XDR and PDR resistance phenotypes) make them promising candidates for future studies. Accordingly, more chemical modifications of the linker, such as introduction of less flexible groups and new functionalizations of the guanidinic group will be explored to further optimize these starting hits. Moreover, a larger effort will be made to elucidate the mode of action of both linear and cyclic guazatine derivatives reported herein. In conclusion, we have identified original guanidinic compounds showing a promising potent and broad-spectrum in vitro antibacterial activity, which might represent a valuable starting point to obtain optimized analogues with enhanced drug-like properties.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.09.081.

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