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Synthesis of zwitterionic *N*-chlorohydantoins for antibacterial applications

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ABSTRACT

Two novel zwitterionic *N*-chlorohydantoin biocides, containing an *N*-chlorohydantoin unit and a sulfobetaine unit or a carboxybetaine unit, were chemically synthesized and characterized. Using the quaternary ammonium *N*-chlorohydantoin as control, the antibacterial activity of synthetic zwitterionic *N*-chlorohydantoins was challenged and the antibacterial data showed that carboxybetaine *N*-chlorohydantoin exhibited distinctively higher biocidal efficacy than QA counterpart, while sulfobetaine *N*-chlorohydantoin displayed slightly inferior antimicrobial efficacy. Our results may inspire further exploration of more zwitterionic *N*-chlorohydantoin analogs for antibacterial application.

Nowadays pathogenic microbial contamination and resultant infections have seriously threatened public healthcare, which has been recognized as one of the greatest global challenges for decades.[1–3] Accordingly, there is a pressing need to develop highly efficacious antimicrobial agents to combat the pathogen contamination and cross infections. In this regard, large amounts of antimicrobial agents have been extensively studied including quaternary ammonium (QA) salts,[4] *N*-Chloramines,[5] quaternary phosphonium salts,[6] chitosans[7] et al. Among these biocides, *N*-Chloramine has well been recognized as one of the most efficacious antibacterial candidates due to its broad antimicrobial spectrum, fast contact killing manner, rechargeability, and low toxicity.[5]

However, *N*-chlorohydantoins, the major *N*-Chloramines in prevalent studies, usually display poor aqueous solubility which is quite unfavorable to its bacterium-killing manner,[8–9] so that the according antimicrobial application was greatly restricted. To this end, typical QA *N*-chlorohydantoins (**1** and **3** in Fig. 1) with substantially improved water solubility were developed.[9–10] By antibacterial tests either in solution (**1**) or on polymer surface (**2**), it was proved that the introduced cationic QA unit significantly enhanced the antimicrobial efficiency of the *N*-Cl moiety. More recently, other ionic analogs were also developed in our group including pyridinium *N*-chlorohydantoin[11] (**4** in Fig. 1) and quaternary phosphonium *N*-chlorohydantoin[12] (**5** in Fig. 1), both of which demonstrated even higher biocidal activity than counterpart **1**. The boosting antimicrobial mechanism was proposed that the cationic center probably facilitate the arrest of cationic biocides on negatively charge bacterial cell via electrostatic attractions, promoting the oxidative chlorine (Cl^+) transfer from *N*-Cl to bacterial cellular receptors.[8] Furthermore, **1a** and the precursor of **4** have been covalently integrated on polyurethane and cotton swatch surface, affording non-leaching antibacterial materials with potential practical applications.[9,13–15] Liang et al[16] also prepared *N*-chlorohydantoin precursor/QA salt siloxane copolymers and grafted onto cotton swatches to give antimicrobial cellulose materials with improved powerful biocidal efficacy.

It has been reported that the active chlorine cover formed on bacteria superficial surface does not impair the bacteria viability if belows a critical concentration: 3.3×10^{-16} mol Cl^+ per CFU (Colony-forming Units).[17] For antibacterial surface that containing QA groups, there exists a positive charge-density (N^+ cm^{-2}) threshold, above which bacterial death process is extremely rapid.[18] Therefore, it is conceivable that the displayed biocidal activity for these ionic *N*-chlorohydantoins may probably be interrelated with the gathering density around bacteria surface. Compared with zwitterionic surfactants, there exists stronger electrostatic repulsion between QA head groups in the QA surfactant micelles[19], so that the QA *N*-chlorohydantoins may be arrested on bacteria cell in a similar manner and the displayed antimicrobial efficacy may thus be highly closely related with surface distribution density.

Betaine derivatives, including sulfobetaines and carboxybetaines, have attracted considerable research interests due to the good biodegradability,[20–21] biocompatibility,[22–23] antifouling[24–25] and antimicrobial property.[21,25,26] As betainemolecules may probably be dispersed on bacteria surface in a relatively higher density than QA

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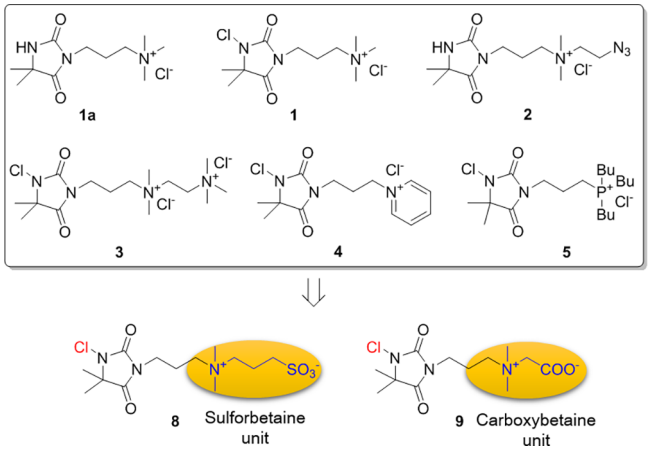


Fig. 1. Structures of reported cationic *N*-chlorohydantoins (precursors) **1–5** and proposed betaine *N*-chlorohydantoins **8–9** in this work.

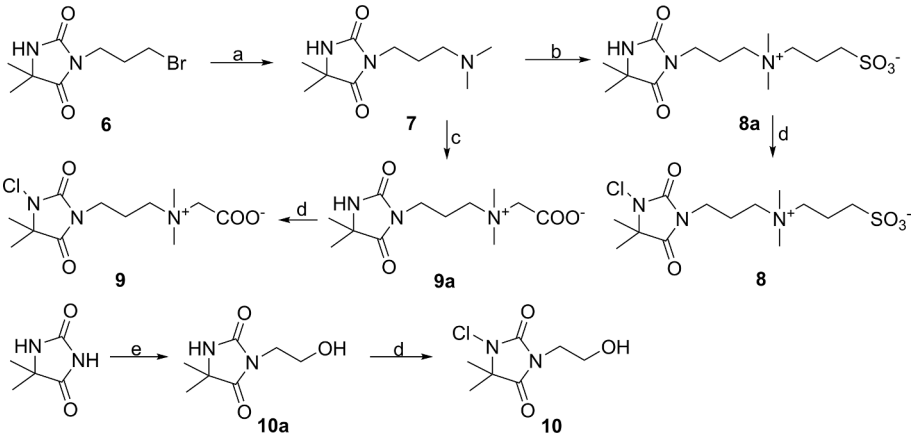
counterparts, a hypothesis was then proposed that a new type "composite" *N*-chlorohydantoin may exert more remarkable antimicrobial efficacy if the QA unit of **1** was replaced with a betaine unit. To this end, we focused on chemical synthesis of two novel zwitterionic *N*-chlorohydantoins: sulforbetaine *N*-chlorohydantoin **8** and carboxybetaine *N*-chlorohydantoin **9**. Structure of **8–9** and precursors **8a-9a** were fully examined by NMR and HRMS spectra analysis. Antimicrobial performance was validated using QA *N*-chlorohydantoin **1** as control, and the relationship between bactericidal activity and the type of hydrophilic head group was discussed.

As depicted in Scheme 1, chemical synthesis of the target zwitterionic compounds **8–9** involved covalent combination an *N*-chlorohydantoin moiety with a sulfobetaine unit or a carboxybetaine unit.[27] First of all, DMH-bromine **6** was prepared via *N*-alkylation reaction of 5, 5-dimethylhydantoin (DMH) according to our previous protocols,[11–12] which was then reacted with excess dimethylamine to produce tertiary amine **7**.[10] Subsequent treatment with the commercial 1,3-propanesultone was performed in acetonitrile under reflux conditions to produce the sulfobetaine *N*-chlorohydantoin precursor **8a,** which could be further purified by recrystallization from MeOH-EtOAc as described in literatures.[28–29] Another carboxybetaine *N*-chlorohydantoin precursor **9a** was also prepared by treatment of tertiary amine **7** with sodium chloroacetate in EtOH-H_2O mixture solvent at 90 °C.[30–31] The by-product of inorganic salt was precipitated in isopropanol and then the product was obtained after filtration, giving **9a** as white solid in 81% yield. To further validate the hypothesis regarding the aggregation mode on microbial cell towards the antimicrobial activity of zwitterionic *N*-chlorohydantoins **8–9**, compound **10a**, a neutral precursor but with pretty good aqueous solubility, was also prepared by *N*-alkylation reaction of DMH with 2-bromoethanol in acetone under reflux conditions.[32] Subsequent chlorination reactions were performed using *t*-butyl hypochlorite as chlorination agent[11–12] at room temperature to produce target *N*-chlorohydantoins **8–10**. After facile removal of excess *t*-butyl hypochlorite and mixed solvent *t*-BuOH-H_2O, the as-prepared *N*-chlorohydantoins were thus obtained in quantitative yield.

All the *N*-chlorohydantoin compounds and precursors were characterized by NMR and HRMS analysis. It is noteworthy that, after chlorination reactions, some noticeable chemical shift change in NMR spectra were observed for the final *N*-chlorohydantoins, which well corresponds with our previous reports.[11–12] For instance, the signal of CH_3- (DMH) and -CH_2- (adjacent to DMH ring) of **8a** shifted downfield distinctly from 1.35 ppm to 1.42 ppm and 3.52 ppm to 3.61 ppm (Fig. 2), respectively, probably due to the electron-withdrawing inductive effect of the newly formed *N*-Cl bond. Similar chemical shift difference was also observed from its ^{13}C NMR data analysis (see Fig. S1 in ESI). The NMR data together with the HRMS data definitely illustrated the successful chlorination from precursors to *N*-chlorohydantoins.

In order to evaluate the bactericidal activity of the synthetic zwitterionic *N*-chlorohydantoins **8–9**, we herein adopted QA *N*-chlorohydantoin **1** as a control and selected *Escherichia coli* ATCC 25,922 and *Staphylococcus aureus* ATCC 25,923 as model microbes. As shown in Table 1, no bacteria reduction was observed for all the precursors (**1a, 8a-10a)**, which coincided with expectations completely. Precursor **1a** have been previously reported to be inactive against bacteria due to lack of long alkyl hydrophobic chain.[10] Wieczorek D. et al. also prepared a series of *n*-alkyl chain sulfobetaines and found that only those sulfobetaines containing longer *n*-alkyl chain (> 10 -(CH_2)- units) displayed noticeable bactericidal activities.[21] Similar report also indicated that the antimicrobial property of carboxybetaines was very poor if the tethered *n*-alkyl chain length is less than 8 -(CH_2)- units.[33] Hence, it is not a surprising finding that neither precursor **8a** nor **9a** displayed antimicrobial activities since there is no structural long alkyl chain exists.

As for the zwitterionic *N*-chlorohydantoins **8–9**, bacteria reduction was achieved to different levels. As shown in Table 1, sulfobetaine *N*-chlorohydantoin **8** overall shared the inferior bacteria reduction in contrast to QA *N*-chlorohydantoin **1** within our duration timeframe except that slight higher bacteria reduction of *S. aureus* was observed within 5 min. On the contrary, carboxybetaine *N*-chlorohydantoin **9** presented noticeable higher bacteria reduction than that of QA *N*-chlorohydantoin **1**. For instance, **9** realized complete inactivation against both bacterial stains within 10 min, while QA *N*-chlorohydantoin **1** and sulfobetaine *N*-chlorohydantoin **8**, at this moment, displayed 2.18 ± 0.10 (*S. aureus*) and 1.00 ± 0.07 (*E. coli*) log reduction, respectively. The observed microbial reduction difference temporarily implied the bactericidal activity in sequence of



Scheme 1. Chemical synthesis of **7–10**: (a) Dimethylamine hydrogen chloride, KOH, EtOH, reflux, overnight, 67%; (b) 1,3-propanesultone, CH_3CN, reflux, 24 h, 80%; (c) Sodium chloroacetate, CH_3CH_2OH-H_2O (1:9, v/v), 90 °C, 8 h, 81%; (d) *t*-butyl hypochlorite, *t*-BuOH: H_2O (4:1, v/v), rt, quantitative yield; (e) $Br(CH_2)_2OH$, K_2CO_3, acetone, reflux, 6 h, 85%.

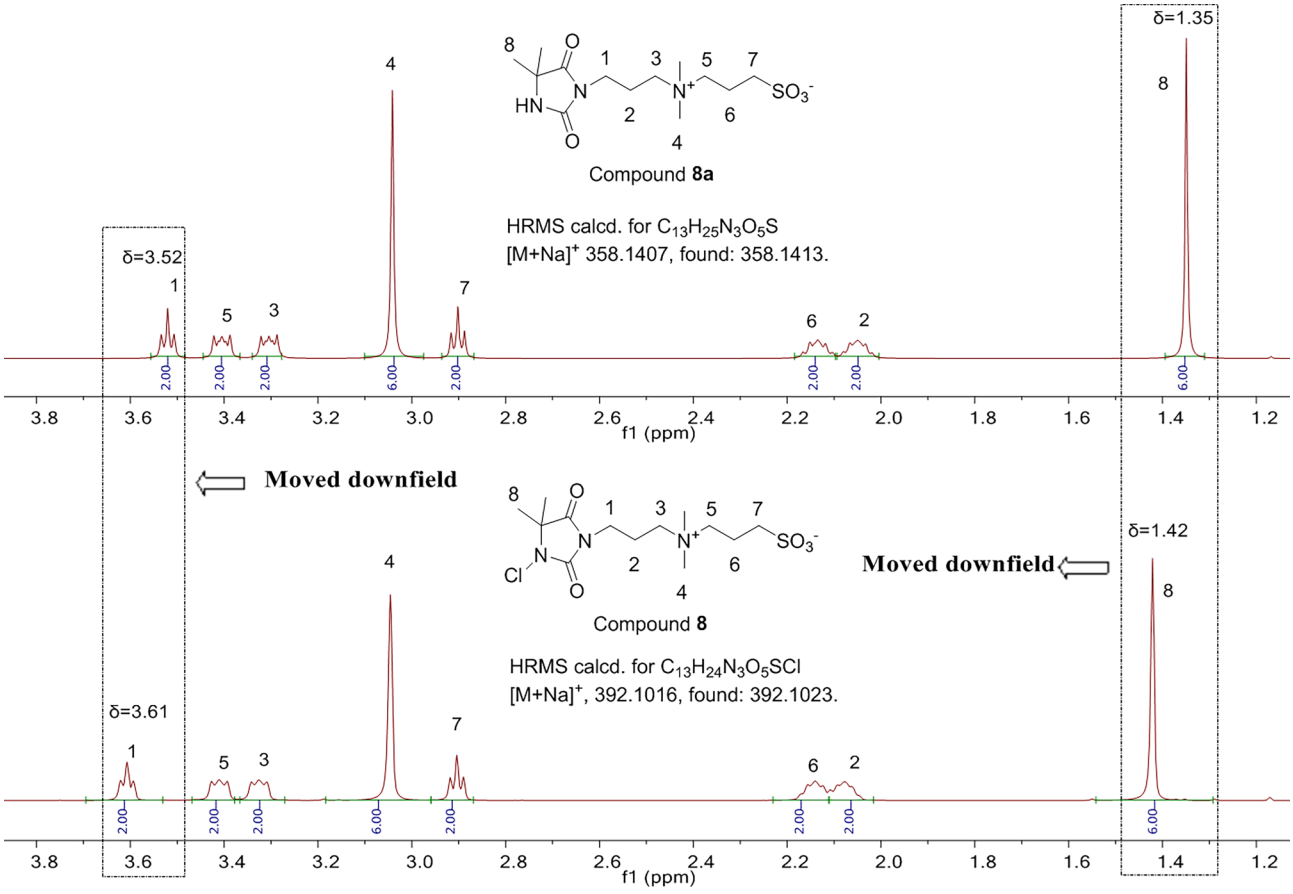


Fig. 2. ^{1}H NMR spectra of compound **8** and its precursor **8a** (In D_2O).

9 > **1** > **8**, which may attribute to their different molecular structure, namely, the different hydrophilic head group of biocides.

It's conceivable that the optimal antimicrobial efficacy for *N*-chlorohydantoins is usually achieved if maximum biocide molecules was distributed on bacteria surface with uniform contact killing actions. The distribution density of biocides on the cell surface may then play an indispensable contribution to the biocidal efficacy. Since there exists weaker electrostatic repulsion (ER)[19] between zwitterionic units in contrast to that among QA units (Fig. 3), biocide **9** is believed to be capable of being more densely distributed on bacteria surface, probably thus leading to a more favorable contact manner and faster active Cl^+ transfer from *N*-chlorohydantoins to bacteria cellular receptors. Although QA *N*-chlorohydantoin **1** may be adsorbed more readily[9] on bacteria surface due to electrostatic attraction between QA moiety and

Table 1
Antibacterial Activity of zwitterionic *N*-chlorohydantoins **8**–**9** against *S. aureus* and *E. coli*.

Bacteria	Synthetic compounds	Active chloramine/ppm	Contact time (min)			
			5		10	
			Percent reduction/%	Log reduction	Percent reduction/%	Log reduction
S. aureus[a]	**8a**	0	0	0	0	0
	8	20	19.8 ± 1.5	0.10 ± 0.01	49.1 ± 1.8	0.29 ± 0.01
	9a	0	0	0	0	0
	9	20	34.3 ± 2.4	0.18 ± 0.02	100	6.83
	10a	0	0	0	0	0
	10	20	100	6.83	100	6.83
	1a	0	0	0	0	0
	1	20	11.8 ± 2.7	0.05 ± 0.01	75.0 ± 0.7	0.60 ± 0.01
E. coli[b]	**8a**	0	0	0	0	0
	8	20	25.1 ± 1.4	0.13 ± 0.01	89.9 ± 1.7	1.00 ± 0.07
	9a	0	0	0	0	0
	9	20	86.7 ± 1.9	0.88 ± 0.06	100	6.63
	10a	0	0	0	0	0
	10	20	100	6.63	100	6.63
	1a	0	0	0	0	0
	1	20	42.2 ± 2.4	0.24 ± 0.02	99.3 ± 0.2	2.18 ± 0.10

[a] Inoculum concentration was 6.8×10^6 CFU/mL.
[b] Inoculum concentration was 4.2×10^6 CFU/mL.

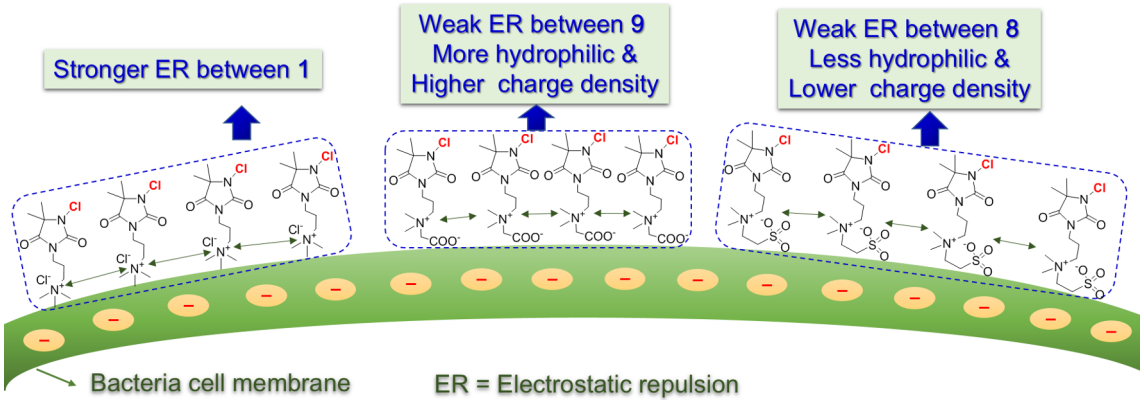


Fig. 3. Schematic illustration of synthetic biocides distribution mode on bacteria cell membrane.

negatively charged bacteria surface, antibacterial activity of betaine molecules has also been extensively studied,[25–26] which indicated that the proposed disperse phase[11] from remote positions to surface around sites may not herein play a leading role for **9** in this contact killing process. The arrested biocide **9** may exert facilitated transportation manner across cell membrane in absence of remarkable electrostatic attraction as **1** probably does for the contact killing.[11] Furthermore, considering the biodegradability and biocompatibility[20–23] of carboxybetaines, contact killing the "composite" *N*-chlorohydantoin **9** may behave in a milder manner and we may discuss this part soon elsewhere.

However, although also containing a zwitterionic unit, sulfobetaine *N*-chlorohydantoin **8** exhibited significantly decreased antibacterial activity compared with carboxybetaine *N*-chlorohydantoin **9**. The inferior antimicrobial efficacy of **8** may be caused by hydrophilicity and space size of its ionic head group. It has been reported that hydrophilicity of zwitterionic structure heavily relies on the type of negatively charged center (SO_3^- or CO_2^-): carboxylic unit shows far outweighed hydrophilicity in contrast to sulfonic unit.[34] The hydrophilicity difference could also be interpreted in terms of the "hard-soft" acid concept because carboxylate radical could be regarded as a hard base while sulfonic radical seems a little more "soft" comparatively. In this regard, aqueous solvation of **9** could be readily realized between the hard base radical and another hard base of water. Shao et al. also reported that the charge density of CO_2^- is higher than that of SO_3^-, resulting in more completely hydration and superior protein-resistant adsorption performance of carboxybetaines.[22,35] Similarly, **9** may be adsorbed on bacteria surface due to the higher charge density, which might contribute its higher bactericidal efficacy in contrast to **8**. In addition, sulfonic radical is bulkier in nature than carboxylic radical which might also cause comparatively lower disperse density on bacteria surface (Fig. 3). The considerations may also partially explain why sulfobetaine *N*-chlorohydantoin **8** produced overall lower antibacterial efficacy than QA *N*-chlorohydantoin **1**. Another point is that the distance between the opposite charges of the synthetic zwitterionic *N*-chlorohydantoins may obviously influence its hydrophilicity, so that analogs of *N*-chlorohydantoin **8** or **9** with various linker length (between the opposite charged centers) are needed to seek the potential relationship between biocidal activity and molecule structures. Such work is being undertaking in our lab and may be delivered later on. In addition, it is worthwhile to mention that the antibacterial potential may not compromised at all according our previous report[9], although different bactericidal efficacy was achieved for synthetic *N*-chlorohydantoins with different aqueous head groups. It is predictable that total kill may be realized over a longer bacterium contact duration even for sulfobetaine *N*-chlorohydantoin **8** which here exerted relatively inferior antibacterial efficacy.

To further discuss the above-mentioned interpretation regarding discrepant antimicrobial efficacies among QA *N*-chlorohydantoin **1** and zwitterionic *N*-chlorohydantoins **8–9**, we here particularly prepared hydroxyethyl-DMH-Cl **10**, a nonionic *N*-chlorohydantoin with pretty good aqueous solubility, and tested its antimicrobial activity. Surprisingly, *N*-chlorohydantoin **10** exerted exceptional biocidal efficacy as shown in Table 1: total kill was achieved for both *S. aureus* (6.83 log reduction) and *E. coli* (6.63 log reduction) within 5 min. To further probe its antibacterial potential, the inoculum concentration of *S. aureus* was once increased to 4.0×10^7 CFU mL^{-1} for antibacterial tests. It was found that biocide **10** achieved 99.8 ± 0.1% bacteria reduction within 5 min, whereas carboxybetaine *N*-chlorohydantoin **9** merely displayed 15.0 ± 2.5% bacteria reduction (data not shown). This outstanding antimicrobial capability may also be caused by its satisfactory hydrophilicity as well as the specific neutral nature. As there lacks ER between molecule **10**, it may be dispersed on bacteria surface in a more crowded manner in contrast to the other three ionic *N*-chlorohydantoins. Furthermore, as a neutral aqueous soluble *N*-chlorohydantoin biocide with comparatively smaller molecule size, **10** may penetrate bacterial cell membrane more smoothly,[32] behaving as other molecules does such as ethanol and glycerol. Of course, such considerations may not completely cover the whole picture of the proposed bacterial mechanism, and more neutral *N*-chlorohydantoin analogs are necessary to pinpoint the principle of the contact killing behavior. Anyway, excellent antibacterial activity of **10** may at least confirm the hypothesis that higher density of *N*-chlorohydantoins covered on bacteria surface is probably indispensable for its fast contact killing action.

In conclusion, novel sulfobetaine *N*-chlorohydantoin **8** and carboxybetaine *N*-chlorohydantoin **9** were herein successfully synthesized. Antimicrobial studies showed that **9** exhibited significantly higher antimicrobial efficacy in contrast to QA *N*-chlorohydantoin **1**, while **8** presented slightly inferior bactericidal efficacy. However, **8** may still be regarded as a pretty good *N*-chlorohydantoin candidate in that its biocidal capability could be exerted over a wide range of pH as well as in presence of hard water. Moreover, hydroxyethyl *N*-chlorohydantoin **10** exhibited the best biocidal activity among all the synthetic *N*-chlorohydantoins, which also supported our interpretation regarding the antibacterial efficacy difference between **8** and **9** and **1**. Our research provided two zwitterionic *N*-chlorohydantoins, which may hold promising applications in medical pathogen-combating areas.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2018.10.034.

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