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Structural modification of sanguinarine and chelerythrine and their antibacterial activity

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In this study, five derivatives of sanguinarine (**1**) and chelerythrine (**2**) were prepared, with **1** and **2** as starting materials, by reduction, oxidation and nucleophilic addition to the iminium bond C=N$^+$. The structures of all compounds were elucidated on account of their MS, ^{1}H-NMR and ^{13}C-NMR data. The antibacterial activities of all compounds were screened, using *Staphylococcus aureus*, *Escherichia coli*, *Aeromonas hydrophila* and *Pasteurella multocida* as test bacteria. The minimum bacteriostatic concentration and minimum bactericidal concentration of the active compounds were determined by the turbidity method. The structure–activity relationships of **1** and **2** were discussed. The results showed that **1**, **2** and their pseudoalcoholates were found to be potent inhibitors to *S. aureus*, *E. coli* and *A. hydrophila*, while the other derivatives were found to be inactive. The pseudoalcoholates might be the prodrugs of **1** and **2**. The iminium bond in the molecules of **1** or **2** was the determinant for antibacterial activity, and the substituents at the 7 and 8 positions influenced the antibacterial activities of **1** and **2** against different bacteria.

Keywords: sanguinarine; chelerythrine; antibacterial activity; structure–activity relationship

1. Introduction

Sanguinarine (**1**) and chelerythrine (**2**) (Figure 1) belong to the quaternary benzo[*c*]phenanthridine alkaloids (QBAs), which are widely distributed in the high plant families, Papaveraceae, Fumariaceae and Rutaceae (Krane, Fagbule, Shamma, & Gözler, 1984). The main botanical sources of QBAs are *Chelidonium majus* L., *Sanguinaria canadensis* L., *Dicranostigma lactucoides* Hook. f. et Thoms., *Macleaya* and *Bocconia* species from the Papaveraceae family, and some members of *Zanthoxylum* (Rutaceae). Although the QBAs are a relatively small class of plant products, they have attracted much attention from researchers because of their bioactivities: anti-inflammatory (Lenfeld et al., 1981), antimicrobial (Mitscher et al., 1978; Navarro, Villarreal, Rojas, & Lozoya, 1996; Odebiyi & Sofowora, 1979),

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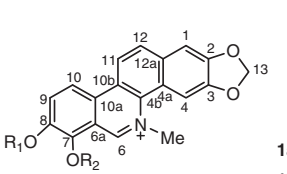
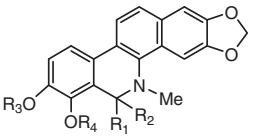


1 $R_1 + R_2 = CH_2$
2 $R_1 = R_2 = Me$



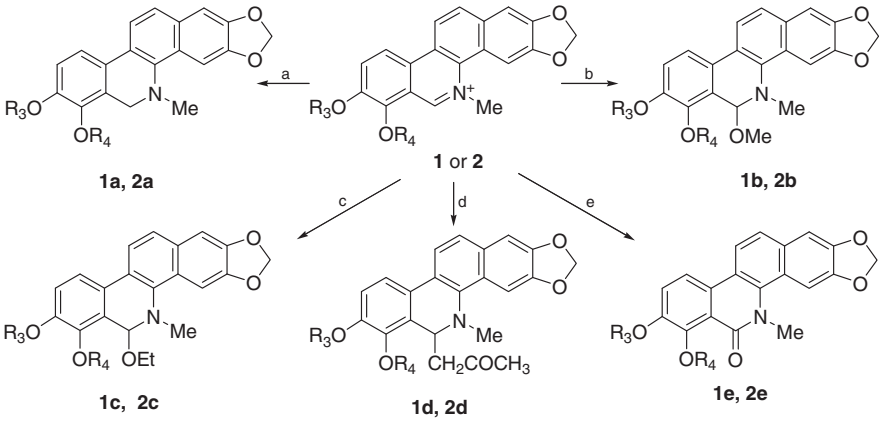
1a R_1=R_2=H, $R_3 + R_4 = CH_2$
1b R_1=H, R_2=OCH_3, $R_3 + R_4 = CH_2$
1c R_1=H, R_2=OEt, $R_3 + R_4 = CH_2$
1d R_1=H, R= CH_2COCH_3, $R_3 + R_4 = CH_2$
1e $R_1 + R_2$ =O, $R_3 + R_4 = CH_2$

2a R_1=R_2=H, R_3=R_4= Me
2b R_1=H, R_2=OCH_3, R_3=R_4= Me
2c R_1=H, R_2=OEt, R_3=R_4= Me
2d R_1=H, R_2= CH_2COCH_3, R_3=R_4= Me
2e $R_1 + R_2$ =O, R_3=R_4= Me

Figure 1. Compounds **1**, **2** and their derivates **1a**–**2e**.

antitumour (Nakanishi, Suzuki, Mashiba, Ishikawa, & Yokotsuka, 1998; Stermitz, Larson, & Kim, 1973; Stermitz et al., 1975; Tin-Wa, Bell, Bevelle, Fong, & Farnsworth, 1974; Zee-Cheng & Cheng, 1975), antiviral (Sethi, 1979), cytotoxic (Cordell & Farnsworth, 1976) and anti-liver mitochondrial monoamine oxidase (Iagodina, Nikol'skaia, & Faddeeva, 2003). With respect to the antimicrobial activities of QBAs, previous research has mainly focused on the total alkaloids containing QBAs from the plant, while only a few studies on **1** and **2** have been reported.

QBAs are highly reactive to reductants such as $NaBH_4$ and nicotinamide adenine dinucleotide (NADH), a biological reducing agent, and nucleophiles such as OH^-, CN^-, NH_3, ROH and acetone, and are easily converted to their corresponding derivatives, such as dihydrosanguinarine, 6-hydroxydihydrosanguinarine (pseudobase or alkanolamine), aminal, bimolecular aminoacetals and 6-alkoxysanguinarine (pseudoalcoholate) (Eldin & Jencks, 1995; Nakanishi et al., 2000; Parenty, Smith, Pickering, Long, & Cronin, 2004; Shimizu, Itou, & Miura, 2005; Yoshida et al., 1999). On the other hand, the pseudobase, aminal and pseudoalcoholate of QBAs may be converted to their corresponding iminium ion form in an acidic condition. Therefore, QBAs have different forms depending on the different solvent conditions, and these different existing forms will have different effects on the biological activity of QBAs. It is this reason that led to the conclusion that the results of the previous research on the biological activities of **1** and **2** were not consistent with each other, but contradictory. Lenfeld et al. (1981) reported that **2** exhibited significant antimicrobial effects upon Gram-positive bacteria and *Candida albicans*, but was ineffective upon the Gram-negative strains *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Navarro et al., 1996). But Odebiyi and Sofowora (1979) proved that **2** was highly active to the Gram-negative strains *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Proteus* sp. Zhao, Yu, Zhou, Meng and Wu (2005) reported that **1** exhibited antibacterial activities against *Staphyloccus aureus*, *E. coli*, *Tetracoccus*, *Bacillus cereus* and *Bacillus subtilis*. Navarro & Delgado (1999) reported that dihydrosanguinarine (**1a**) and dihydrochelerythrine (**2a**) (see Figure 1) displayed significant antimicrobial activities against *S. aureus*, *Streptococcus faecalis*, *E. coli*, *P. aeruginosa*, *Proteus mirabilis* and *C. albicans*. However, more recently Zuo et al. (2008) found that **1a** and **2a** were almost inactive to *S. aureus* and that 8-hydroxydihydrosanguinarine and 8-hydroxydihydrochelerythrine were more active against clinical strains of methicillin-resistant *Staphylococcus aureus* (MRSA), evidencing bacteriostatic and bactericidal effects. In addition, Mitscher



Scheme 1. (a) $NaBH_4$, MeOH, room temperature; (b) MeONa, reflux; (c) EtONa, EtOH, reflux; (d) acetone, Na_2CO_3, reflux; (e) $K_3Fe(CN)_6$, 1% HCL/H_2O, 90°C.

et al. (1978) reported that the pseudoalcoholates of **1** and **2** also exhibited antimicrobial activities. In order to clearly understand which forms of **1** and **2** act as the principal active compounds as well as the structural requirement for antibacterial activity, in this research, we prepared a series of derivatives of **1** and **2** and examined their antibacterial activities. As far as we are aware, this is the first report of the structure–antibacterial activity relationship between **1** and **2**.

2. Results and discussion

2.1. *Chemistry*

The compounds **1**, **2** and their derivatives (compounds **1a**–**1e** and **2a**–**2e**) prepared in this study for bioactivity investigations are shown in Figure 1. Structural modifications included introduction of alkoxyl and acetonyl at C-6 in **1** and **2** by nucleophilic addition, and the reduction and oxidation of the C=N double bond at C-6 in **1** and **2**. The synthesis routes of the derivatives are shown in Scheme 1. The structures of all of the compounds were elucidated on the basis of spectroscopic evidence, including MS, ^{1}H-NMR and ^{13}C-NMR (Table 1 and Section 2).

2.2. *Bioactivity and discussion*

Our previous research showed that the total alkaloids of *M. microcarpa* exhibited antibacterial activities against *S. aureus*, *Streptococcus agalactiae*, *E. coli*, *P. multocida*, *B. cereus*, *A. hydrophila*, *Aeromonas punctata f. intestinalis* and *Vibrio anguillarum* (Hu et al., 2009). Therefore, in this research, *S. aureus*, *E. coli*, *A. hydrophila* and *P. multocida* were first selected as the indicator organisms with which to determine the *in vitro* antibacterial activities of **1**, **2** and **1a**–**2e**. The bioassay results are summarised in Table 1.

Both **1** and **2** exhibited antibacterial activities against *S. aureus*, *E. coli* and *A. hydrophila* to different extents at 1.0 mg mL^{-1}, whereas, with respect to

Table 1. Antibacterial activity of **1**, **2** and their derivates at 1.0 mg mL^{-1}.

Compound	Inhibition zone (mm)			
	S. aureus	*E. coli*	*A. hydrophila*	*P. multocida*
1	17.7	15.3	9.7	8.7
1a	11.7	–	–	–
1b	19.3	14.3	8.7	–
1c	18.0	14.7	10.3	–
1d	–	–	–	–
1e	–	–	–	–
2	20.7	11.7	6.7	–
2a	–	–	–	–
2b	17.7	10.7	7.3	–
2c	20.0	12.3	7.3	–
2d	–	–	–	–
2e	–	–	–	–
Penicillin sodium	36.7	14.0	–	–
Ceftriaxone sodium	13.3	17.3	18.7	21.7
Control	–	–	–	–

Note: –, no activity.

P. multocida, only **1** showed a moderate activity among the compounds tested. Therefore, **1** exhibited a broader antibacterial spectrum than **2**.

By comparing the activities of **1** with those of its derivates (**1a**, **1d** and **1e**), it can clearly be seen that the activity reduced or disappeared when the double bond of $C{=}N^+$ in the molecule of **1** was reduced to a tertiary amine (**1a**) or oxidised to an amide (**1e**), or had acetone added to form a 6-acetonyl-substituted derivate (**1d**). Similar results were also obtained in the comparison of the activities of **2** with those of its derivates (**2a**, **2d** and **2e**). The above results strongly suggest that the double bond of $C{=}N^+$ in the molecules of **1** or **2** is the determinant for their antibacterial activity. On the other hand, it is worthwhile noting that the 6-methyoxyl or 6-ethyloxyl-substituted derivates (**1b**, **1c**, **2b**, **2c**) showed good activities, comparable to those of their corresponding parent compound (**1** or **2**), despite there being no occurrence of a $C{=}N^+$ bond in the molecules.

Based on the above results, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of **1**, **1b**, **1c**, **2**, **2b** and **2c** were further determined for *S. aureus*, *E. coli* and *A. hydrophila*. The results are listed in Table 2.

All the tested compounds showed antibacterial activities against *S. aureus*, *E. coli* and *A. hydrophila* with 12.5–100 μg mL^{-1} of MIC and bactericidal activities against *S. aureus* and *E. coli* with 25–100 μg mL^{-1} of MBC. Among all the tested compounds, only **1** exhibited bactericidal activity against *A. hydrophila* at 100 μg mL^{-1}. For *S. aureus*, **2** (MIC 12.5 μg mL^{-1}, MBC 25 μg mL^{-1}) displayed higher bacteriostatic activity than **1** (MIC 25 μg mL^{-1}) and the same level of bactericidal activity as **1**. For *E. coli*, **1** showed the same level of bacteriostatic activity as **2**, with 25 μg mL^{-1} of MIC value, and higher bactericidal activity (MBC 25 μg mL^{-1}) than **2** (MBC 50 μg mL^{-1}). The above results demonstrated that **1** and **2** are effective upon both Gram-positive bacteria and Gram-negative bacteria, which is

Table 2. MIC and MBC values ($\mu g\,mL^{-1}$) of **1**, **2** and their derivates.

	S. aureus		*E. coli*		*A. hydrophila*	
Compound	MIC	MBC	MIC	MBC	MIC	MBC
1	25	25	25	25	25	100
1b	12.5	25	50	100	25	>200
1c	25	25	25	100	50	>200
2	12.5	25	25	50	50	>200
2b	25	25	50	100	100	>200
2c	50	50	25	100	100	>200
Penicillin sodium	3.2	6.3	>200	>200	>200	>200
Ceftriaxone sodium	100	>200	12.5	25	6.3	12.5

a different result from that reported by Lenfeld et al. (1981). On the other hand, the activity difference between **1** and **2** suggested that the substituents at the 7 and 8 positions influenced the antibacterial susceptibility of **1** and **2** to some extent. Furthermore, it is worth noting that the 6-alkoxy-substituted derivatives (**1b**, **1c**, **2b** and **2c**) showed similar MIC and MBC values to those of their corresponding parent compounds (**1** or **2**), in agreement with the results seen in Table 1.

QBAs, unlike other alkaloids, undergo a dynamic equilibrium between the pseudobase (base form) and iminium ion form in any protic solvent due to the sensitivity of the polar bond ($C{=}N^+$) to the attack of nucleophiles (Harkrader & Jones, 1991; Jones, Harkrader, & Southard, 1986). The pseudobase has higher lipophilicity than its corresponding iminium ion form. Walterova et al. (1995) reported that sanguinarine and chelerythrine were able to penetrate mammalian cell membranes at pH 7.2–7.4 as hydrophobic pseudobases, where they were then able to accumulate to high levels in the acidic environment of the lysosome and convert back to the iminium ion. Similarly, the pseudoalcoholates of QBAs could also be easily converted back to the corresponding iminium form in acidic medium (Dostál & Slavík, 2002). Therefore, we suggest that the pseudoalcoholates (**1b**, **1c**, **2b** and **2c**), like the pseudobases, might be prodrugs of **1** and **2**, and that the real active compounds should be **1** and **2**, not the pseudoalcoholates. The viewpoint given above is further confirmed by the comparison of the activities of the pseudoalcoholates and those of **1a**, **2a**, **1d**, **2d**, **1e** and **2e**. Although **1a**, **2a**, **1d**, **2d**, **1e** and **2e** had similar molecular structures to the pseudoalcoholates (**1b**, **1c**, **2b** and **2c**), the former were inactive or less active because they cannot be converted back to **1** and **2** under physiological conditions. Zuo et al. (2008) also reported that **1a** and **2a** did not possess antibacterial activity. Obviously, the double bond of $C{=}N^+$ in the molecules of **1** and **2** was the determinant not only for their antitumour properties (Nakanishi, Suzuki, Saimoto, & Kabasawa, 1999), but also for their antibacterial activities.

3. Experimental

3.1. *General experimental procedures*

Melting points were taken on a XT-4 microscopic melting point apparatus. NMR spectra were recorded on a Bruker AM-500 FT-NMR spectrometer using TMS as

the internal standard at 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR, respectively. ESI–MS data were obtained with a Trace DSQ spectrometer. TLC was performed on silica gel GF_{254} (0–40 μm, Qingdao Haiyang Chemical Group Co., Qingdao, China) activated at 110°C for 1 h. The compounds were visualised with UV light.

3.2. *Plant material*

Plants of *Macleaya microcarpa* (Maxim) Fedde were collected from Qingling Mountain, Shaanxi Province, China, in August 2006, and were identified by Vice Professor Fang Miao, one of the authors. A voucher specimen is deposited at the Botanic Specimen Centre of Northwest A&F University, Yangling, China.

3.3. *Isolation of 1 and 2*

The entire plant of *M. microcarpa* (Maxim) Fedde (500 g, dry weight) was extracted with 0.1 mol L^{-1} aqueous hydrochloric acid at 35°C under an ultrasound-assisted flow through a column packed with D101 macroporous resin. The column adsorbing total alkaloids was eluted with water, 50% ethanol in water and finally acetone. After the solvent was removed at reduced pressure, acetone eluent provided 9.0 g of fraction B. Fraction B was repeatedly chromatographed on silica gel with petroleum ether–ethyl acetate (5 : 1) to yield crude **1** and **2**, which were subjected to recrystallisation in 0.1 mol L^{-1} aqueous hydrochloric acid to provide **1** (520 mg) and **2** (610 mg), respectively.

3.4. *Syntheses of 1a–2e*

Compounds **1** and **2** were used as starting materials to synthesise **1a**–**1e** and **2a**–**2e**, respectively. Compounds **1a** and **2a** were synthesised by the literatural method (Slavik & Slavikova, 1977). Compounds **1b**, **1c**, **2b** and **2c** were prepared according to the method of Stermitz et al. (1973). Compounds **1d** and **2d** were prepared by previously described methods (Maclean, Gracey, & Saunders, 1969). Compounds **1e** and **2e** were obtained by the oxidation of **1** and **2** with $K_3Fe(CN)_6$ (Masayuki, Kaoru, Kyoko, Naofumi, & Teruo, 1970). The structures of all of the above compounds were elucidated on the basis of spectroscopic evidence, including MS, ^{1}H-NMR and ^{13}C-NMR.

3.4.1. *Syntheses of **1a** and **2a***

To the solution of **1** or **2** (0.33 mmol) in ca 50 mL of MeOH, 0.5 mmol (20 mg) of $NaBH_4$ was added. The reaction solution was stirred for 30 min at room temperature. The solvent was evaporated to dryness under reduced pressure. The residue was subjected to column chromatography over silica gel using petroleum ether–ethyl acetate (20 : 1) as eluent, and recrystallised from petroleum ether–ethyl acetate (20 : 1) to yield **1a** or **2a**.

3.4.2. *Syntheses of **1b**, **1c**, **2b** and **2c***

To the solution of **1** or **2** (0.33 mmol) in ca 40 mL of MeOH, ca 4 mL of the solution of CH_3ONa in MeOH was added for **1b** or **2b** (or EtONa in EtOH for **1c** or **2c**) to pH 10, and refluxed for 40 min. Fifteen millilitres of H_2O were added to the reaction solution, and extracted with $CHCl_3$. The solvent was evaporated to dryness under reduced pressure, and the residue was recrystallised in MeOH to provide **1b** or **2b** (or **1c** or **2c**).

3.4.3. *Syntheses of **1d** and **2d***

To the solution of **1** or **2** (0.33 mmol) in ca 40 mL of acetone, ca 2 mL of the solution of 20% Na_2CO_3 in water was added. The resulting solution was refluxed for 7 h, and the solvent was then evaporated to dryness under reduced pressure. The residue was subjected to column chromatography over silica gel using petroleum ether–ethyl acetate (8 : 1) as eluent, and then recrystallised in a solution of petroleum ether–ethyl acetate (8 : 1) to yield **1d** or **2d**.

3.4.4. *Syntheses of **1e** and **2e***

To a hot solution (90°C) of sanguinarine (**1**) or chelerythrine (**2**) (0.33 mmol) in 50 mL of 0.2% HCl in water was added a hot solution (80°C) of $K_3Fe(CN)_6$ (0.8 g) in H_2O (10 mL) with stirring. Stirring was continued for 3 h at 90°C. During the reaction, 5 mL of 3% KOH in water was added every 30 min. After cooling, the precipitate was collected. The solid was dissolved in $CHCl_3$, washed with water and dried over Na_2SO_4. The residue was recrystallised from $CHCl_3$–acetone to yield **1e** or **2e**.

3.4.5. *Sanguinarine (**1**)*

Orange–red needle crystal; m.p. 244–245°C (H_2O–MeOH); ^{13}C-NMR (125 MHz, CD_3OD) $\delta = 107.2$(C-1), 150.6(C-2), 150.6(C-3), 105.2(C-4), 121.8(C-4a), 133.0(C-4b), 150.7(C-6), 111.1(C-6a), 148.1(C-7), 149.3(C-8), 121.4(C-9), 118.4(C-10), 128.9(C-10a), 127.4(C-10b), 119.8(C-11), 133.0(C-12), 133.9(C-12a), 104.3(2,3-OCH_2O), 106.5(7,8-OCH_2O) and 53.2(N–Me). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 9.95$(1H, s, H-6), 8.57(1H, d, $J = 8.9$ Hz, H-11), 8.48(1H, d, $J = 8.8$ Hz, H-10), 8.19(1H, d, $J = 8.9$ Hz, H-12), 8.13(1H, s, H-4), 7.95(1H, d, $J = 8.8$ Hz, H-9), 7.55(1H, s, H-1), 6.54(2H, s, 1,2-OCH_2O), 6.30(2H, s, 7,8-OCH_2O) and 4.49(3H, s, NCH_3); ESI–MS (positive mode) $m/z = 332[M]^+$.

3.4.6. *Chelerythrine (**2**)*

Yellow needle crystal; m.p. 199–200°C (H_2O–MeOH); ^{13}C-NMR (125 MHz, CD_3OD) $\delta = 107.3$(C-1), 151.0(C-2), 150.9(C-3), 105.3(C-4), 121.8(C-4a), 133.5(C-4b), 152.1(C-6), 120.1(C-6a), 147.6(C-7), 151.8(C-8), 127.6(C-9), 120.9(C-10), 130.1(C-10a), 127.2(C-10b), 119.7(C-11), 63.2(7-OMe), 57.8(8-OMe), 132.8(C-12), 134.3(C-12a), 104.5(2,3-OCH_2O) and 53.2(N–Me). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 9.92$(1H, s, H-6), 8.60(1H, d, $J = 9.0$ Hz, H-10), 8.56(1H, d, $J = 9.0$ Hz, H-11), 8.10(1H, d, $J = 9.0$ Hz, H-12), 8.08(1H, s, H-4), 8.10(1H, d, $J = 9.0$ Hz, H-9),

7.49(1H, s, H-1), 6.26(2H, s, OCH_2O), 4.97 (3H, s, NCH_3), 4.27(3H, s, 7-OCH_3) and 4.12(3H, s, 8-OCH_3); ESI–MS (positive mode) $m/z = 348[M]^+$.

3.4.7. *Dihydrosanguinarine (**1a**)*

Yield: 93 mg (92.5%), red–white granule crystal; m.p. 186–187°C (petroleum ether–ethyl acetate); ^{13}C-NMR (125 MHz, CD_3OD) $\delta = 104.3$(C-1), 148.1(C-2), 147.5(C-3), 100.7(C-4), 126.5(C-4a), 142.5(C-4b), 48.4(C-6), 113.6(C-6a), 144.6(C-7), 147.0(C-8), 107.1(C-9), 116.1(C-10), 127.2(C-10a), 124.3(C-10b), 120.3(C-11), 123.9(C-12), 130.8(C-12a), 101.0(2,3-OCH_2O), 101.3(7,8-OCH_2O), 41.5(N–Me). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 7.67$(1H, d, $J = 8.6$ Hz, H-11), 7.66(1H, s, H-4), 7.45(1H, d, $J = 8.6$ Hz, H-12), 7.28(1H, d, $J = 8.1$ Hz, H-10), 7.09(1H, s, H-1), 6.83(1H, d, $J = 8.1$ Hz, H-9), 6.02(2H, s, OCH_2O), 6.01(2H, s, OCH_2O), 4.17(2H, s, H-6) and 2.62(3H, s, NCH_3); ESI–MS (positive mode) $m/z = 333[M]^+$.

3.4.8. *6-Methoxysanguinarine (**1b**)*

Yield: 61 mg (50.9%), white crystal; m.p. 194–194.5°C (MeOH); ^{13}C-NMR (125 MHz, CD_3OD) $\delta = 104.6$(C-1), 148.1(C-2), 147.4(C-3), 100.6(C-4), 126.9(C-4a), 138.2(C-4b), 85.9(C-6), 108.8(C-6a), 145.3(C-7), 147.2(C-8), 113.2(C-9), 116.4(C-10), 125.8(C-10a), 122.8(C-10b), 120.1(C-11), 123.7(C-12), 131.1(C-12a), 101.1(2,3-OCH_2O), 101.7(7,8-OCH_2O), 40.9(N–Me) and 54.1(6-OMe). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 7.76$(1H, d, $J = 8.6$ Hz, H-11), 7.69(1H, s, H-4), 7.48(1H, d, $J = 8.6$ Hz, H-12), 7.20(1H, d, $J = 8.2$ Hz, H-10), 7.12(1H, s, H-1), 6.93(1H, d, $J = 8.2$ Hz, H-9), 6.11(2H, s, OCH_2O), 6.04(2H, s, OCH_2O), 5.37(1H, s, H-6), 3.46(3H, s, OCH_3) and 2.79(3H, s, NCH_3); ESI–MS (positive mode) $m/z = 363[M]^+$.

3.4.9. *6-Ethoxysanguinarine (**1c**)*

Yield: 53 mg (42.6%), white crystal; m.p. 210–212°C (EtOH); ^{13}C-NMR (125 MHz, CD_3OD) $\delta = 104.6$(C-1), 148.0(C-2), 147.4(C-3), 100.7(C-4), 126.9(C-4a), 138.5(C-4b), 84.2(C-6), 108.7(C-6a), 145.2(C-7), 147.3(C-8), 113.4(C-9), 116.4(C-10), 125.8(C-10a), 122.9(C-10b), 120.3(C-11), 123.6(C-12), 131.0(C-12a), 101.0(2,3-OCH_2O), 101.7(7,8-OCH_2O), 40.9(N–Me), 61.6(OCH_2CH_3) and 15.0(OCH_2CH_3). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 7.76$(1H, d, $J = 8.6$ Hz, H-11), 7.66(1H, s, H-4), 7.48(1H, d, $J = 8.6$ Hz, H-12), 7.40(1H, d, $J = 8.2$ Hz, H-10), 7.12(1H, s, H-1), 6.92(1H, d, $J = 8.2$ Hz, H-9), 6.11(2H, s, OCH_2O), 6.08(2H, s, OCH_2O), 5.48(1H, s, H-6), 3.90–3.93(1H, m, OCH_2CH_3), 3.65(1H, q, $J = 7.0$ Hz, OCH_2CH_3), 2.76(3H, s, NCH_3) and 1.08(3H, t, $J = 7.0$ Hz, OCH_2CH_3); ESI–MS (positive mode) $m/z = 377[M]^+$.

3.4.10. *6-Acetonylsanguinarine (**1d**)*

Yield: 62 mg (48.3%), yellow–white crystal; m.p. 189–190°C (petroleum ether–ethyl acetate); $R_f = 0.37$ (petroleum ether–acetone, 5 : 1), $R_f = 0.70$ (chloroform–acetone, 19 : 1); ^{13}C-NMR (125 MHz, CD_3OD) $\delta = 104.3$(C-1), 148.2(C-2), 147.6(C-3), 100.5(C-4), 127.4(C-4a), 139.2(C-4b), 54.4(C-6), 123.4(C-6a), 144.2(C-7), 147.1(C-8), 107.5(C-9), 116.4(C-10), 125.6(C-10a), 116.0(C-10b), 120.0(C-11), 124.0(C-12), 131.0(C-12a), 101.1(2,3-OCH_2O), 101.5(7,8-OCH_2O), 43.0(N–Me),

46.5(CH_2COCH_3), 207.3(CH_2COCH_3) and 31.7(CH_2COCH_3). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 7.69$(1H, d, $J = 8.4$ Hz, H-11), 7.52(1H, s, H-4), 7.48(1H, d, $J = 8.4$ Hz, H-12), 7.33(1H, d, $J = 8.4$ Hz, H-10), 7.10(1H, s, H-1), 6.86(1H, d, $J = 8.4$ Hz, H-9), 6.04(2H, t, $J = 1.2$ Hz, OCH_2O), 6.03(2H, t, $J = 1.2$ Hz, OCH_2O), 4.87(1H, dd, $J = 4.4$, 9.6 Hz, H-6), 2.60–2.66(1H, m, CH_2COCH_3), 2.62(3H, s, NCH_3), 2.30(1H, dd, $J = 3.6$, 15.2 Hz, CH_2COCH_3) and 2.06(3H, s, CH_2COCH_3); ESI–MS (positive mode) $m/z = 389[M]^+$.

3.4.11. *Oxysanguinarine (**1e**)*

Yield: 74 mg (64.6%), grey amorphous powder; m.p. > 350°C (chloroform–acetone); ^{13}C-NMR (125 MHz, CD_3OD) $\delta = 104.7$(C-1), 147.6(C-2), 147.1(C-3), 102.5(C-4), 121.2(C-4a), 136.7(C-4b), 162.7(C-6), 118.7(C-6a), 147.8(C-7), 147.7(C-8), 115.4(C-9), 118.7(C-10), 128.8(C-10a), 113.2(C-10b), 123.6(C-11), 117.3(C-12), 131.9(C-12a), 101.5(2,3-OCH_2O), 102.9(7,8-OCH_2O) and 40.8(N–Me). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 7.97$(1H, d, $J = 8.8$ Hz, H-11), 7.76(1H, d, $J = 8.7$ Hz, H-9), 7.57(1H, d, $J = 8.8$ Hz, H-12), 7.53(1H, d, $J = 8.7$ Hz, H-10), 7.23(1H, s, H-4), 7.16(1H, s, H-1), 6.27(2H, s, 7,8-OCH_2O), 6.09(2H, s, 2,3-OCH_2O) and 3.91 (3H, s, NCH_3); ESI–MS (positive mode) $m/z = 347[M]^+$.

3.4.12. *Dihydrochelerythrine (**2a**)*

Yield: 75 mg (65.1%), yellow–white needle crystal; m.p. 163–164°C (petroleum ether–ethyl acetate); $R_f = 0.34$ (petroleum ether–ethyl acetate, 17 : 3), $R_f = 0.70$ (chloroform–acetone, 20 : 1); ^{13}C-NMR(125 MHz, CD_3OD) $\delta = 104.3$(C-1), 148.0(C-2), 147.4(C-3), 100.6(C-4), 126.3(C-4a), 142.6(C-4b), 48.7(C-6), 126.2(C-6a), 146.0(C-7), 152.2(C-8), 110.8(C-9), 118.6(C-10), 126.2(C-10a), 124.2(C-10b), 120.1(C-11), 61.1(7-OMe), 55.7(8-OMe), 123.7(C-12), 130.7(C-12a) and 101.0(2,3-OCH_2O), 40.7(N–Me). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 7.70$(1H, d, $J = 8.4$ Hz, H-11), 7.67(1H, s, H-4), 7.50(1H, d, $J = 8.4$ Hz, H-10), 7.48(1H, d, $J = 8.4$ Hz, H-12), 7.11(1H, s, H-1), 6.93(1H, d, $J = 8.4$ Hz, H-9), 6.05(2H, s, OCH_2O), 3.94(3H, s, 8-OCH_3), 3.87(3H, s, 7-OCH_3), 4.30(2H, s, H-6) and 2.59(3H, s, NCH_3); ESI–MS (positive mode) $m/z = 349[M]^+$.

3.4.13. *6-Methoxychelerythrine (**2b**)*

Yield: 57 mg (45.6%), yellow–white prism; m.p. 199–200°C (MeOH); ^{13}C-NMR (125 MHz, CD_3OD) $\delta = 104.7$(C-1), 148.0(C-2), 147.4(C-3), 100.7(C-4), 126.8(C-4a), 138.4(C-4b), 86.1(C-6), 125.7(C-6a), 146.7(C-7), 152.1(C-8), 113.0(C-9), 118.9(C-10), 124.9(C-10a), 122.6(C-10b), 120.1(C-11), 61.7(7-OMe), 56.0(8-OMe), 123.5(C-12), 131.0(C-12a), 101.0(2,3-OCH_2O), 40.6(N–Me) and 54.0(6-OMe). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 7.77$(1H, d, $J = 8.5$ Hz, H-11), 7.69(1H, s, H-4), 7.62(1H, d, $J = 8.6$ Hz, H-10), 7.47(1H, d, $J = 8.5$ Hz, H-12), 7.12(1H, s, H-1), 7.04(1H, d, $J = 8.6$ Hz, H-9), 6.05(2H, s, OCH_2O), 5.54(1H, s, H-6), 3.96(3H, s, 8-OCH_3), 3.92 (3H, s, 7-OCH_3), 3.45(3H, s, 6-OCH_3) and 2.60(3H, s, NCH_3); ESI–MS (positive mode) $m/z = 379[M]^+$.

3.4.14. *6-Ethoxychelerythrine (**2c**)*

Yield: 76 mg (61.1%), yellow–white prism; m.p. 206–206.5°C (EtOH); ^{13}C-NMR (125 MHz, CD_3OD) $\delta = 104.6$(C-1), 147.8(C-2), 147.3(C-3), 100.7(C-4), 126.8(C-4a), 138.7(C-4b), 84.5(C-6), 126.0(C-6a), 146.6(C-7), 152.2(C-8), 112.9(C-9), 119.0(C-10), 124.9(C-10a), 122.7(C-10b), 120.1(C-11), 61.7(7-OMe), 56.0(8-OMe), 123.3(C-12), 131.0(C-12a), 101.0(2,3-OCH_2O), 40.7(N–Me), 61.6(OCH_2CH_3) and 15.2(OCH_2CH_3). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 7.76$(1H, d, $J = 8.5$ Hz, H-11), 7.66(1H, s, H-4), 7.62(1H, d, $J = 8.6$ Hz, H-10), 7.45(1H, d, $J = 8.5$ Hz, H-12), 7.12(1H, s, H-1), 7.02(1H, d, $J = 8.6$ Hz, H-9), 6.04 (2H, s, OCH_2O), 5.67(1H, s, H-6), 3.96(3H, s, 8-OCH_3), 3.92(3H, s, 7-OCH_3), 3.72(2H, q, $J = 7.0$ Hz, OCH_2CH_3), 2.74(3H, s, NCH_3) and 1.09(3H, t, $J = 7.0$ Hz, OCH_2CH_3); ESI–MS (positive mode) $m/z = 393[M]^+$.

3.4.15. *6-Acetonylchelerythrine (**2d**)*

Yield: 89 mg (66.6%), yellow–white crystal; m.p. 196–197°C (petroleum ether–ethyl acetate); ^{13}C-NMR (125 MHz, CD_3OD) $\delta = 104.3$(C-1), 148.2(C-2), 147.6(C-3), 100.6(C-4), 128.2(C-4a), 139.3(C-4b), 54.9(C-6), 127.4(C-6a), 145.5(C-7), 152.1(C-8), 111.6(C-9), 118.8(C-10), 124.8(C-10a), 123.3(C-10b), 119.8(C-11), 61.0(7-OMe), 55.8(8-OMe), 123.8(C-12), 131.1(C-12a), 101.0(2,3-OCH_2O), 42.8(N–Me), 46.9(CH_2COCH_3), 207.5(CH_2COCH_3) and 31.1(CH_2COCH_3). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 7.70$(1H, d, $J = 8.6$ Hz, H-11), 7.53(1H, d, $J = 8.4$ Hz, H-10), 7.51(1H, s, H-4), 7.47(1H, d, $J = 8.6$ Hz, H-12), 7.09(1H, s, H-1), 6.94(1H, d, $J = 8.6$ Hz, H-9), 6.02(2H, t, $J = 1.2$ Hz, OCH_2O), 5.04(1H, dd, $J = 3.6$, 11.1 Hz, H-6), 3.96(3H, s, 8-OCH_3), 3.92(3H, s, 7-OCH_3), 2.64(3H, s, NCH_3), 2.57(1H, t, $J = 3.6$ Hz, CH_2COCH_3), 2.25(1H, dd, $J = 3.6$, 11.1 Hz, CH_2COCH_3) and 2.05(3H, s, CH_2COCH_3); ESI–MS (positive mode) $m/z = 405[M]^+$.

3.4.16. *Oxychelerythrine (**2e**)*

Yield: 37 mg (30.9%), silver grey amorphous powder; m.p. 198–199°C (chloroform–acetone); ^{13}C-NMR (125 MHz, CD_3OD) $\delta = 104.7$(C-1), 152.7(C-2), 150.2(C-3), 102.5(C-4), 121.0(C-4a), 135.6(C-4b), 162.6(C-6), 119.8(C-6a), 147.5(C-7), 147.1(C-8), 117.9(C-9), 118.5(C-10), 129.0(C-10a), 117.2(C-10b), 123.2(C-11), 61.7(7-OMe), 56.6(8-OMe), 117.8(C-12), 131.7(C-12a), 101.5(2,3-OCH_2O) and 40.8(N–Me). ^{1}H-NMR ($CDOD_3$, TMS) $\delta = 7.97$(1H, d, $J = 8.5$ Hz, H-11), 7.97(1H, d, $J = 8.5$ Hz, H-9), 7.51(1H, d, $J = 8.5$ Hz, H-12), 7.37(1H, d, $J = 8.5$ Hz, H-10), 7.15(1H, s, H-4), 7.13(1H, s, H-1), 6.08(2H, s, OCH_2O), 4.08(3H, s, 8-OCH_3), 3.98(3H, s, 7-OCH_3) and 3.89(3H, s, NCH_3); ESI–MS (positive mode) $m/z = 363[M]^+$.

3.5. *Bioactivity assay*

3.5.1. *Screening of antibacterial activity of **1**, **2** and their derivatives (**1a–1e**, **2a–2e**)*

Compounds **1**, **2** and their derivatives were screened for their antibacterial activity using the paper disc diffusion method (Iwasal, Kamigauchi, Ueki, & Taniguchi, 1996), with slight modification. *S. aureus*, *E. coli*, *Aeromonas hydrophila* and *Pasteurella*

multocida were used as the test bacteria. The test compound was dissolved in MeOH at a concentration of 1 mg mL^{-1} and a paper disc ($d = 6$ mm) was immersed in the sample solution for 3 h, and then dried in an oven at 40°C and placed on an agar plate seeded with the 20–24 h cultured fresh bacteria at 28°C. After incubation for 24 h at 37°C, the diameter of the inhibitory zone around the disc was measured. A paper disc saturated with MeOH was used, after drying, as a negative control. Penicillin sodium and ceftriaxone sodium were used as positive controls.

3.5.2. *Determination of MIC and MBC*

MIC and MBC values of **1**, **2**, **1b**, **1c**, **2b** and **2c** were determined by the turbidity method (Urzúa et al., 2006) with slight modification, using *S. aureus*, *E. coli* and *A. hydrophila* as the test bacteria.

A Baird–Parker agar (BPA) broth consisting of beef extract (0.3%), peptone (1%) and NaCl (0.5%) was used for culturing the test bacteria. A 20–24 h cultured fresh broth (*E. coli* and *S. aureus* at 37°C for 20 h and *A. hydrophila* at 28°C for 24 h) was diluted by the same broth to 2.2×10^7 colony forming units (CFU) for *S. aureus*, 3.6×10^7 CFU for *E. coli* and 2.3×10^8 CFU for *A. hydrophila* to serve as inocula. The test sample was dissolved in methanol, and 20 µL of the resulting solution was added to a first tube containing 980 µL of BPA broth. Two-fold serial dilutions were made by adding the same BPA broth to obtain concentrations of 400–3.12 µg mL^{-1}. A bacterial suspension (0.5 mL) was added to each tube and incubated for 24 h at 37°C for *S. aureus* and *E. coli*, and for 24 h at 28°C for *A. hydrophila*. The MIC was determined by visually judging the bacterial growth in the series of test tubes, which was defined as the lowest concentration at which no bacterial growth was observed after incubation. The MBCs were determined by inoculating the surfaces of Mueller–Hinton agar (MHA) plates with 25 µL of the samples taken from the clear tubes of the MIC determination. After the bacterial suspensions had fully absorbed into the agar, the plates were further incubated at 28°C for 24 h and were examined for growth in daylight. The MBC was defined as the concentration at which no colony was observed after incubation. All experiments were performed in triplicate.

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