Published in final edited form as:

Bioorg Med Chem. 2009 June 1; 17(11): 3866–3872. doi:10.1016/j.bmc.2009.04.028.

Antibacterial Activity of Berberine-NorA Pump Inhibitor Hybrids with a Methylene Ether Linking Group

Sirirton Samosorn^{a,*}, Bongkot Tanwirat^a, Nussara Muhamad^a, Gabriele Casadei^b, Danuta Tomkiewicz^b, Kim Lewis^b, Apichart Suksamrarn^c, Therdsak Prammananan^d, Karina C. Gornall^e, Jennifer L. Beck^e, and John B. Bremner^e

^a Department of Chemistry, Faculty of Science, Srinakharinwirot University, Bangkok 10110 Thailand ^b Department of Biology and Antimicrobial Discovery Center, Northeastern University, Boston, Massachusetts 02115 USA ^c Department of Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240 Thailand ^d BIOTEC Central Research Unit, National Center for Genetic Engineering and Biotechnology, Pathumthani 10120 Thailand ^e School of Chemistry, University of Wollongong, NSW 2500 Australia

Abstract

Conjugation of the NorA substrate berberine and the NorA inhibitor 5-nitro-2-phenyl-1*H*-indole via a methylene ether linking group gave the 13-substituted berberine-NorA inhibitor hybrid, **3**. A series of simpler arylmethyl ether hybrid structures were also synthesized. The hybrid **3** showed excellent antibacterial activity (MIC *Staphylococcus aureus*, 1.7 μ M), which was over 382-fold more active than the parent antibacterial berberine, against this bacterium. This compound was also shown to block the NorA efflux pump in *S. aureus*.

Keywords

13-Substituted berberine; Antibacterial; NorA efflux pump inhibitors; Dual action; Hybrid

1. Introduction

The problem of resistance by human pathogenic bacteria to antibacterial agents is a serious and growing health issue worldwide.¹ Bacteria employ a number of resistance mechanisms to counter antibacterial challenge and one of these is the overexpression of transmembrane protein-based efflux pumps which can pump out various antibacterials from within the cells, thus lowering the antibacterial concentration to sub-lethal levels.^{2,3} The efflux pump mechanism is a very significant and widespread one occurring in a range of human pathogenic bacterial hosts including *Staphylococcus aureus*, a common cause of bacteremia, sepsis and wound infections. In *S. aureus*, the multidrug resistance (MDR) pump NorA, for example, can efflux structurally diverse antibacterials like berberine and ciprofloxacin.⁴

*Corresponding author. Tel.: +66 2 6495000 ext. 8216; fax: +66 2 2592097; e-mail: sirirton@swu.ac.th.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

One strategy to attempt to contain the threat of resistant bacteria is to block a major resistance mechanism at the same time as blocking another bacterial biochemical pathway. One way this approach could be realised is by combining two drugs with the two actions proposed in the same molecule,^{5,6,7} as is done with the antibacterial hybrid **SS14 (2)**,⁸ which is a covalently linked combination of berberine (**1**) and 2-phenyl-5-nitro-1*H*-indole, **INF55**, via a methylene linking group at the 13 position in **1** and the *ortho* position of the indolic 2-phenyl ring (Figure 1). Such a hybrid has the potential advantage over two separately administered drugs of synchronous or near-synchronous delivery to the respective biological target sites. Compound **1** is a natural antibacterial agent and a preferred substrate of the NorA pump, while **INF55** is a synthetic NorA inhibitor which potentiates the antibacterial activity of **1** by reducing the efflux of **1** from bacterial cells.⁹ Compound **2** is the first hybrid of an anti-infective/multidrug resistance (MDR) pump inhibitor. It was shown to be a superior antibacterial with over 200-fold greater activity than **1** itself against an *S. aureus* overexpressing NorA strain and is insensitive to MDR efflux.⁸

Some studies have been undertaken on structure-activity relationships for **INF55**. A series of 2-arylbenzo[*b*]thiophenes¹⁰ related to **INF55** were reported as NorA inhibitors suggesting that the indole-NH is not essential for NorA inhibitory activity. Replacement of the nitro group in **INF55** with other electron-withdrawing groups such as the cyano group gave a product which retained potentiation activity similar to **INF55**.¹¹ However, other electron-withdrawing groups, like the sulfonamide group, resulted in a complete loss of activity.

In order to gain a deeper understanding of the dispositional and structural requirements of the efflux pump blocking moiety in berberine-based hybrid compounds, the effect of the methylene ether linking group on antibacterial activity in the analogue **3**, of **2**, was investigated, together with the related derivatives **4–7**. These last four compounds involved simplification of the indolic inhibitor moiety with aryl- or heteroarylmethyl and naphthylmethyl groups to assess aromatic ring criteria in this region. The results are now reported in this paper.

2. Results and discussion

2.1 Synthesis of 3–7

The synthesis of the 13-*O*-arylmethyl-substituted berberine derivatives involved three steps (Scheme 1). Treatment of commercially available berberine (**1**) with acetone and sodium hydroxide gave 8-acetonilydihydroberberine (**8**) and subsequent oxidation with potassium permanganate followed by deprotonation with aqueous sodium hydroxide solution then afforded the key synthetic precursor phenol betaine **9**.¹² Finally *O*-alkylation of the betaine **9** with 2-(2-bromomethylphenyl)-5-nitro-1*H*-indole^{8,13} provided **3** in modest yield. In the same way *O*-alkylation of **9** with the appropriate commercially available arylmethyl bromide gave the salts **4–7** in low to moderate yields; isolated yields were affected by the extensive chromatography required to isolate the pure salts. The structures of all the final substituted berberine salts were substantiated by spectroscopic means, with the low field chemical shift for the H8 berberine proton singlet, the absence of a signal for H13 in the berberine nucleus, and the downfield singlet signal for the methylene ether moiety in the linker group being particularly diagnostic in the ¹H NMR spectra.

2.2 Antibacterial activity and cell uptake studies of 3

The antibacterial activity of **3** was evaluated only against Gram-positive bacteria with three strains of *S. aureus* and three antibiotic resistant strains of *Enterococcus faecalis*, since it was known from our previous studies⁸ that the related hybrid **2** had no antibacterial activity against Gram-negative bacteria (concentration up to 150 μ M against *Escherichia coli*) possibly due to the INF55-insensitive RND pumps present. The results are given in Table 1. **3** was more potent

as an antibacterial than **2** with MIC values up to 1.8-fold greater, as well as being over 382-fold greater in activity than **1** itself against NorA overexpressing *S. aureus* strain K2378. The MIC of **3** was essentially the same, 1.7 μ M, in a mutant strain of *S. aureus* lacking the NorA MDR pump (K1758), in the wild type (8325-4), and in the strain overexpressing the NorA pump (K2378). **3** thus seems to block not only the NorA pump but also the additional MDR pumps^{14,15} in *S. aureus*. Furthermore **3** had excellent antibacterial activity (MIC 3.4 μ M, Table 1) against *E. faecalis* which is known for its high levels of “intrinsic antibiotic resistance”^{16, 17} and was resistant to **1** with an MIC of 650–1300 μ M.

It is also pertinent that **3** can be transported rapidly into the bacterial cells, which can be seen in the uptake assay results (Figure 2). Both hybrids **2** and **3** show a higher level of accumulation than **1** alone or in the presence of the MDR inhibitor **INF55** (Figures 2a–2c). No difference in uptake of both hybrids was observed in norA-deleted (Figure 2b) and overexpressed (Figures 2c) strains of *S. aureus*, which suggests an inhibition of the NorA pump and also is consistent with the antibacterial activity data.

The increase in berberine fluorescence on binding to DNA is used to monitor berberine accumulation in the cell.¹⁸ However, berberine hybrids **2** and **3** contain an additional group which could affect their fluorescence properties and/or binding to DNA. The fluorescence spectra of **2** in the presence or absence of DNA (results not shown) indicated that the fluorescence properties were changed due to the attachment of **INF55**. The maximum fluorescence in the absence of DNA shifted from 560 nm in the case of **1** to 530 nm for **2**¹⁹ and **3**. Interestingly the intrinsic fluorescence of **3** was around 36 times higher than that of **1** at the same concentration. The presence of DNA has a different effect on the hybrids, with **3** showing no effect on the fluorescence spectra, while the fluorescence maximum of **2** increased around 3.5 times. Importantly **2** and **3** showed no significant difference in maximum fluorescence due to different DNA concentrations.

2.3 Pump inhibitory studies

In order to assess the MDR pump inhibitory activity of the studied compounds, the uptake and efflux of ethidium bromide, EtBr, in the presence of the berberine hybrids or NorA inhibitor were examined with the results presented in Figures 3 and 4. EtBr is a known substrate for the major MDR pump, NorA, in *S. aureus*.^{14,20} The increased accumulation of EtBr in the presence of the hybrid suggests an inhibition of MDR transporter(s) (Figure 3a–3c). Hybrid **3** potentiates EtBr uptake around 1.7 times stronger than **2** in all strains. Similar EtBr uptake in the *S. aureus* NorA knockout strain K1758 and wild type 8325-4 suggests that the hybrids effectively inhibit the NorA pump. However **3** and **INF55** show a 2- and 2.6-fold increase respectively in EtBr uptake in the strain with a NorA knockout, which implies that these compounds can also inhibit other MDR transporters in *S. aureus* in addition to NorA.

In order to elucidate the specificity towards the NorA pump, we studied the efflux levels in the overexpressing NorA *S. aureus* strain K2378 (Figure 4). The result shows that the efflux of EtBr from this strain is inhibited by both berberine hybrids, although **3** is a little more potent than **2**.

2.4 Antibacterial activity of 4–7

The simplified hybrid structural series **4–7** do not contain the **INF55** moiety, therefore they were tested against both Gram-positive and Gram-negative bacteria, *S. aureus* and *E. coli*, respectively (Table 1). All compounds in this series had stronger activity than **1**. Compounds **4** and **5** showed strong antibacterial activity whereas **6** and **7** had much lower activity in *S. aureus*. No antimicrobial activity against the wild type *E. coli* or strain overexpressing ArcAB pump was observed. This, together with the high activity against RND deletion strains

(ECM1668 and BW25113) suggests that those compounds, as well as berberine are effluxed via the RND pump AcrAB/TolC. The antibacterial activity of all compounds against the antibiotic resistant strains of *E. faecalis* was not pursued.

The effect on EtBr efflux in the presence of the NorA inhibitor, **INF55**, or **4** and **5** in an overexpressing NorA *S. aureus* K2378 was also studied (Figure 5). Compounds **4** and **5** had an inhibitory effect on the MDR pumps, whereas **6** and **7** did not show significant effects through MDR inhibition, a result also commensurate with the antibacterial trends observed.

2.5 DNA binding of **4**

The antibacterial activity of **1** is believed to be mediated, at least in part, through a DNA binding process.²¹ It is thus possible such an interaction may also have some significance for the antibacterial action of our 13-*O*-arylmethyl berberine derivatives. To obtain further information on this binding, and taking into account compound availability, the DNA binding affinity, selectivity and stoichiometry of the most active compound from the simplified hybrid structure series, **4**, was examined. Binding to DNA was assessed using negative ion electrospray ionization mass spectrometry (ESI-MS). Two different 16-mer double-stranded (ds) DNA sequences (D1 and D2) and a DNA-sequence with a 10 base pair double-stranded region and stretches of contiguous adenines at the ends of each strand to produce a non-H-bonded forked structure (F10), were assessed. In addition, binding to an 8-mer tetrameric G-quadruplex (Q1) and a quadruplex constructed from two 16-mer strands (Q3)²² was assessed. The quadruplex forms were included for selectivity purposes and to enable a comparison with related previous work.²³

The results for binding to dsDNA (Figure 6) showed that **4**, which contains a planar intercalating naphthyl moiety can bind to all DNA types tested (D1, D2 and F10). While a greater number of berberine molecules bound to the dsDNA (e.g. 6–7 for D2), binding of **4** was still significant with one molecule bound to each of the DNA sequences. The percentage of DNA bound to **4** as judged from the ESI mass spectra was approximately 30 %, 20 % and 15 % for D1, D2 and F10, respectively.

Compound **4** had a greater preference for Q1 and Q3 with almost 100 % of Q1 and 67 % of Q3 bound to **4** under the same conditions (not shown). As noted previously,²³ the berberine derivative **2** also showed a clear preference for binding to Q1 DNA over D1 or D2 DNA.

3. Conclusions

In summary, an increase of linker chain length in the hybrid **2** by introduction of an oxygen atom produced hybrid **3** which had stronger antibacterial activity and MDR pump inhibitory potency than **2**. Removal of the indole moiety of **3** produced compounds with lower dual activity. Structural simplification of **3** gave molecules with a loss of some antibacterial activity. Substitution of the aryl ring in the 13-substituent with electron-withdrawing groups (**6** and **7**) was unfavourable for both antibacterial and NorA inhibitory activities. Although the antibacterial activity of **4** and **5** was lower than that of **3**, they still retained NorA pump inhibitory activity. This suggested that the indole nucleus is not necessary for NorA pump inhibition, but is required for high antibacterial activity of the hybrid 13-substituted berberines.

4. Experimental

4.1 Chemistry

Solvents were removed under reduced pressure using a rotary evaporator. Berberine (chloride salt), benzyl chloride, 4-cyanobenzyl bromide, 2-(bromomethyl)pyridine, and 2-(bromomethyl)naphthalene were purchased from Sigma-Aldrich Chemical Co. and were used

as supplied. Melting points were obtained using a Griffin melting point apparatus and are uncorrected. Thin layer chromatography (TLC) on aluminum backed sheets of Merck Silica Gel 60 F₂₅₄ plates were used to follow the progress of chemical reactions. Preparative TLC was performed on 20 × 20 cm plates. Compounds were detected by examination under UV light. Column chromatography was performed under medium pressure on silica gel 60 (230–400 mesh). All solvent proportions were vol/vol. NMR spectra were obtained on a Varian Unity 300 MHz spectrometer, where proton (¹H) and carbon (¹³C) spectra were obtained at 300 MHz and 75 MHz, respectively, or on a Varian Inova 500 spectrometer, where the ¹H and ¹³C were obtained at 500 MHz and 126 MHz, respectively. Spectra were recorded in CDCl₃ (unless otherwise indicated) and were referenced to the residual non-deuterated solvent signal or TMS. Hydrogen and carbon assignments were also made using gradient correlation spectroscopy (gCOSY), gradient heteronuclear single quantum correlation (gHSQC) and gradient heteronuclear multiple bond correlation (gHMBC) spectroscopic techniques. Superscript letters refer to interchangeable chemical shift assignments. Positive ion high resolution electrospray mass spectra, HRMS (ES), were obtained with a Micromass Qtof2 mass spectrometer using a cone voltage of 30V and polyethylene glycol (PEG) as an internal reference. Compounds for testing were > 95 % pure on the basis of TLC and ¹H NMR analysis. Compounds **9**¹² and 2-(2-bromomethyl phenyl)-5-nitro-1*H*-indole⁸ were synthesized according to previous methods.

General procedure for the preparation of 3–7—A solution of the phenolbetaine **9** (1 mmol) and the arylmethylbromide (2–10 mmol) in dry acetonitrile (1–2 mL) was heated at 60 °C for 2–6 h under a nitrogen atmosphere. The reaction mixture was then concentrated by evaporation of the CH₃CN. The residue was chromatographed on silica gel (6–10 % MeOH in DCM), followed by preparative TLC (multiple development, silica gel, 5 % MeOH in DCM) of the main fraction from the column. Subsequently, the polar fraction was precipitated from 2 % MeOH in DCM and then recrystallized from EtOH to give the desired product.

9,10-Dimethoxy-13-[2-(5-nitro-1*H*-indol-2-yl)-benzyloxy]-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium bromide (3)—Compound **9** was treated with 2-(2-bromomethyl phenyl)-5-nitro-1*H*-indole according to the general procedure to give the desired bromide salt (**3**) as a yellow solid, yield 35 %; mp 201 °C (dec.). ¹H NMR (500 MHz, CDCl₃/CD₃OD) δ: 9.56 (s, 1H, H-8), 8.31 (s, 1H, H-4'), 7.97 (d, *J* = 8.5 Hz, 1H, H-6'), 7.68 (d, *J* = 8.5 Hz, 1H, H-7'), 7.64 (s, 1H, H-14), 7.53 (d, *J* = 9.5 Hz, 1H, H-12)^a, 7.37 (d, *J* = 7.0 Hz, 1H, H-6''), 7.25 (d, *J* = 7.0 Hz, 1H, H-5''), 7.14 (d, *J* = 7.6 Hz, 1H, H-3''), 7.08 (d, *J* = 9.5 Hz, 1H, H-11)^a, 6.90 (t, *J* = 7.6 Hz, 1H, H-4''), 6.63 (s, 1H, H-4), 6.21 (s, 1H, H-3'), 6.12 (s, 2H, OCH₂O), 5.18 (s, 2H, CH₂O), 4.75 (br s, 2H, H-6), 4.19 (s, 3H, OCH₃), 3.64 (s, 3H, OCH₃), 2.88 (t, *J* = 5.8 Hz, 2H, H-5). ¹³C NMR (126 MHz, CDCl₃/CD₃OD) δ: 149.7 (C-14a), 149.6 (C-10), 147.4 (C-13), 146.9 (C-3a), 144.5 (C-9), 141.9 (C-8), 141.6 (C-5'), 139.7 (C-7a'), 138.4 (2C, C-13a, C-2'), 133.6 (C-2'')^a, 132.7 (C-3''), 132.6 (C-1'')^a, 132.4 (C-13b), 130.1 (C-5''), 129.6 (C-6''), 128.2 (C-12a), 127.5 (C-4''), 127.0 (C-3a'), 123.9 (C-12)^b, 121.6 (C-8a), 119.3 (C-4a), 117.2 (C-11)^b, 117.1 (C-6'), 116.6 (C-4'), 113.1 (C-7'), 111.3 (C-14), 108.1 (C-4), 104.9 (C-3'), 102.2 (OCH₂O), 74.1 (CH₂O), 62.2 (OCH₃), 57.0 (C-6), 56.1 (OCH₃), 28.0 (C-5). HRMS (ES); *m/z* calcd for C₃₅H₂₈N₃O₇ [M]⁺: 602.1927; found: 602.1910.

9,10-Dimethoxy-13-(2-naphthylmethyleneoxy)-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium bromide (4)—Compound **9** was treated with 2-(bromomethyl)naphthalene according to the general procedure to give the desired product **4** as a yellow solid, yield 34 %; mp 175–177 °C. ¹H NMR (500 MHz, CDCl₃/CD₃OD) δ: 10.11 (s, 1H, H-8), 8.00 (d, *J* = 9.5 Hz, 1H, H-12), 7.92 (s, 1H, H-14), 7.80–7.88 (m, 3H, H-4', H-6', H-8')^a, 7.81 (d, *J* = 9.5 Hz, 1H, H-11), 7.72 (s, 1H, H-1'), 7.48–7.58 (m, 2H, H-5', H-7')^a, 7.36

(d, $J = 8.5$ Hz, 1H, H-3'), 6.72 (s, 1H, H-4), 6.02 (s, 2H, OCH₂O), 6.00 (s, 2H, CH₂O), 5.08 (t, $J = 5.5$ Hz, 2H, H-6), 4.32 (s, 3H, OCH₃), 4.07 (s, 3H, OCH₃), 3.17 (t, $J = 5.5$ Hz, 2H, H-5). ¹³C NMR (126 MHz, CDCl₃/CD₃OD) δ : 151.1 (C-10), 150.0 (C-13), 149.8 (C-3a)^a, 147.2 (C-14a)^a, 145.8 (C-9), 143.0 (C-8), 133.3 (C-4a)^b, 132.9 (C-8a)^b, 132.0 (C-4a)^c, 131.8 (C-2'), 131.6 (C-13a), 129.8 (C-12a), 128.4 (C-1', C-4')^d, 128.0 (C-8'), 127.6 (C-6')^d, 126.7 (C-5')^d, 126.5 (C-7')^d, 126.1 (C-3'), 125.5 (C-11), 122.8 (C-8a), 118.4 (C-13b)^c, 118.0 (C-12), 109.0 (C-14), 108.1 (C-4), 101.9 (OCH₂O), 77.7 (CH₂O), 62.8 (OCH₃), 57.1 (C-6), 56.9 (OCH₃), 27.8 (C-5). HRMS (ES); m/z calcd for C₃₁H₂₆NO₅ [M]⁺: 492.1811; found: 492.1825.

13-Benzoyloxy-9,10-dimethoxy-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium bromide (5)—Compound **9** (1 mmol) was treated with benzyl chloride (10 mmol) and sodium bromide (10 mmol) initially, for bromide exchange purposes, and then the general procedure was used to give the desired product **5** as a yellow solid, yield 29 %; mp 141–143 °C. ¹H NMR (300 MHz, CDCl₃) δ : 10.39 (s, 1H, H-8), 7.95 (s, 1H, H-14), 7.92 (d, $J = 9.3$ Hz, 1H, H-11), 7.79 (d, $J = 9.3$ Hz, 1H, H-12), 7.34–7.37 (m, 3H, H-2', H-4'), 7.27–7.30 (m, 2H, H-3'), 6.83 (s, 1H, H-4), 6.09 (s, 2H, OCH₂O), 5.24 (t, $J = 6.0$ Hz, 2H, H-6), 4.89 (s, 2H, CH₂O), 4.35 (s, 3H, OCH₃), 4.07 (s, 3H, OCH₃), 3.26 (t, $J = 6.0$ Hz, 2H, H-5). ¹³C NMR (75 MHz, CDCl₃) δ : 151.2 (C-10)^a, 149.9 (C-3a)^b, 149.8 (C-13), 147.4 (C-14a)^b, 146.2 (C-9)^a, 143.7 (C-8), 134.4 (C-1'), 132.2 (C-4a)^c, 131.3 (C-13a), 129.7 (C-12a), 129.1 (C-4'), 128.8 (2C, C-2'), 128.7 (2C, C-3'), 125.4 (C-11), 122.9 (C-8a), 118.5 (C-13b)^c, 118.0 (C-12), 108.9 (C-14), 108.3 (C-4), 102.0 (OCH₂O), 77.2 (CH₂O), 63.1 (OCH₃), 57.0 (OCH₃), 56.9 (C-6), 28.1 (C-5). HRMS (ES); m/z calcd for C₂₇H₂₄NO [M]⁺: 442.1654; found: 442.1660.

13-(4-Cyanobenzoyloxy)-9,10-dimethoxy-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium bromide (6)—Compound **9** was treated with 4-cyanobenzyl bromide according to the general procedure to give the desired product **6** as a yellow solid, yield 40 %; mp 132–134 °C. ¹H NMR (500 MHz, CDCl₃/CD₃OD) δ : 10.03 (s, 1H, H-8), 7.88 (d, $J = 9.5$ Hz, 1H, H-12), 7.83 (s, 1H, H-14), 7.81 (d, $J = 9.5$ Hz, 1H, H-11), 7.69 (d, $J = 8$ Hz, 2H, H-3'), 7.52 (d, $J = 8$ Hz, 2H, H-2'), 6.87 (s, 1H, H-4), 6.08 (s, 2H, OCH₂O), 5.05 (t, $J = 6.0$ Hz, 2H, H-6), 4.99 (s, 2H, CH₂O), 4.32 (s, 3H, OCH₃), 4.08 (s, 3H, OCH₃), 3.33 (t, $J = 6.0$ Hz, 2H, H-5). ¹³C NMR (126 MHz, CDCl₃/CD₃OD) δ : 151.2 (C-10), 150.1 (C-3a)^a, 149.7 (C-13), 147.4 (C-14a)^a, 145.9 (C-9), 143.4 (C-8), 140.0 (C-1'), 132.5 (2C, C-3'), 132.4 (C-4a), 131.3 (C-13a), 129.5 (C-12a), 128.8 (2C, C-3'), 125.5 (C-11), 122.8 (C-8a), 118.3 (CN), 118.1 (C-13b), 117.6 (C-12), 112.6 (C-4'), 108.4 (C-4, C-14), 102.0 (OCH₂O), 75.8 (CH₂O), 62.7 (OCH₃), 57.0 (C-6), 56.9 (OCH₃), 27.8 (C-5). HMRS (ES); m/z calcd for C₂₈H₂₃N₂O₅ [M]⁺: 467.1607; found: 467.1618.

9,10-Dimethoxy-13-(2-pyridylmethyleneoxy)-5,6-dihydrobenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium bromide (7)—Compound **9** was treated with 2-(bromomethyl)pyridine according to the general procedure to give the desired product **7** as a yellow solid, yield 8 %; mp 134–136 °C. ¹H NMR (300 MHz, CDCl₃) δ : 10.34 (s, 1H, H-8), 8.59 (dd, $J = 4.8, 0.9$ Hz, 1H, H-3'), 8.07 (d, $J = 9.0$ Hz, 1H, H-12), 7.94 (s, 1H, H-14), 7.81 (d, $J = 9.3$ Hz, 1H, H-11), 7.76 (td, $J = 7.5, 1.8$ Hz, 1H, H-5'), 7.42 (d, $J = 8.1$ Hz, 1H, H-6'), 7.29 (td, $J = 4.8, 0.9$ Hz, 1H, H-4'), 6.82 (s, 1H, H-4), 6.06 (s, 2H, OCH₂O), 5.24 (t, $J = 5.9$ Hz, 2H, H-6), 5.01 (s, 2H, CH₂O), 4.34 (s, 3H, OCH₃), 4.07 (s, 3H, OCH₃), 3.30 (t, $J = 5.9$ Hz, 2H, H-5). ¹³C NMR (75 MHz, CDCl₃) δ : 154.2 (C-1'), 151.2 (C-10), 149.9 (C-3a)^a, 149.8 (C-13), 149.6 (C-3'), 147.5 (C-14a)^a, 146.0 (C-9), 143.7 (C-8), 137.1 (C-5'), 132.1 (C-4a)^b, 131.2 (C-13a), 129.5 (C-12a), 125.5 (C-11), 123.7 (C-4'), 123.1 (C-6'), 122.8 (C-8a), 118.3 (C-13b)^b, 118.2 (C-12), 108.8 (C-14), 108.3 (C-4), 102.0 (OCH₂O), 76.6 (CH₂O), 63.1 (OCH₃), 57.1 (C-6), 57.0 (OCH₃), 28.1 (C-5). HRMS (ES); m/z calcd for C₂₆H₂₃N₂O₅ [M]⁺: 443.1607; found: 443.1626.

4.2. Antibacterial testing

Bacterial strains—The following bacterial strains were used in this study: *S. aureus* 8325-4 (wild-type), K1758 Δ norA (8325-4 Δ norA),²⁴ K2378 NorA++ (K1758/pK374:norA, with norA from *S. aureus* SA1199),²⁵ *E. coli* K12 (wild-type), BW25113 Δ TolC, EMC1668 marR1642 Δ acrAB, EMC1642 marR1642 acrAB+ and *E. faecalis* MMH594, OG1RF and V583.²⁶

4.2.1. Antimicrobial susceptibility: Cells (10^5 mL⁻¹) were inoculated into broth and dispensed at 50 μ L well⁻¹ in 384 well microtitre plates. MICs were determined in triplicate by serial 2-fold dilution of the test compound. The MIC was defined as the concentration of the agent that completely inhibited cell growth during an 18 h incubation at 37 °C. Growth was assayed with a microtitre plate reader (Spectramax PLUS384; Molecular Devices) by monitoring absorption at 600 nm.

4.2.2. Uptake experiments: Experiments were performed essentially as described previously,⁸ in which *S. aureus* cultures were grown at 37 °C until the optical density (OD) reached 1.5 (at 600 nm). Cells were pelleted by centrifugation, washed twice with PBS and resuspended in PBS containing 10 mM dextrose to obtain an OD~0.8. Cells were then incubated for 1 h at 37 °C (with aeration) before being washed twice with PBS (containing 10 mM dextrose) and further diluted to OD~0.3 in PBS. The assay was performed in 96-well flat-bottom white microtiter plates in a final volume of 200 μ L. Compounds were added at a concentration of 3 μ M each. For ethidium bromide experiments, **2** or **3** or **INF55** were added first. Fluorescence was measured using a SpectraMax Gemini XS spectrofluorometer (Molecular Devices). Uptake experiments with **1**, **INF55** and hybrids **2** and **3** were performed at excitation/emission wavelengths of 355/517 nm. Experiments with ethidium bromide were performed at excitation/emission wavelengths of 530/600 nm. Background fluorescence for all compounds in the absence of cells was subtracted from the raw data.

4.2.3. Efflux experiments: The efflux assay was performed essentially as described previously⁸ with minor modifications for the use of ethidium bromide. *S. aureus* NorA overexpressing cells (K2378 NorA++) were grown at 37 °C to an OD ~0.9, pelleted, washed twice with PBS and then resuspended in PBS to an OD~0.8. Cells were then loaded with 3 μ M ethidium bromide and 30 μ g/mL of reserpine and incubated at 37 °C for 20 min. After washing twice with ice-cold PBS, cells were added to a chilled 96-well flat-bottom black microtiter plate containing ice-cold PBS + 10 mM dextrose at an OD of 0.3 in a total volume of 200 μ L. Compounds **2–7** or **INF55** were added before cells to give final concentrations of 3 μ M. As a negative control (i.e. no ethidium efflux), PBS (without dextrose) containing 30 μ g/mL of reserpine was added instead of **3**. Fluorescence was measured with a SpectraMax Gemini XS at excitation/emission wavelengths of 530/600 nm, respectively.

4.3. DNA binding

Materials—MilliQTM water (Millipore, Bedford, USA) was used in all experiments. Ammonium acetate, methanol and acetonitrile were purchased from Ajax Finechem (Seven Hills, Australia).

Oligonucleotides—Complementary strands of two duplex DNA sequences (d(CCTCTCTGGACCTTCC), D1A, M_r 4744.1 Da; d(GGAAGGTCCAGAGAGG), D1B, M_r 5020.3 Da; d(GCTGCCAAATACCTCC), D2A, M_r 4786.2 Da; d(GGAGGTATTTGGCAGC), D2B, M_r 4977.3 Da) and a forked duplex sequence (d(AAAAAAAAAACGTCCGAGCA), F10A₁, M_r 6145.1; d(TGCTCGGACGAAAAAAAAA), F10A₂, M_r 6176.1 Da), and two quadruplex DNA strands (d(TTGGGGGT), Q1, M_r 2496.7 Da and d(GGGGTTTTGGGG), Q3, M_r 3788.5 Da

were purchased from Geneworks (South Australia) as “trityl-off” and purified by chromatography on a Waters C18 Delta Pak radial cartridge high-performance liquid chromatography (HPLC) column. The column was equilibrated with 10 mM ammonium acetate, and the DNA was eluted from the column using a gradient of 0–60% acetonitrile in 10 mM ammonium acetate over 30 min (1 mL min⁻¹). The purified DNA strands were freeze-dried using a Savant Speed-Vac and redissolved in MilliQTM water giving a concentration of 1–2 mM prior to storage at –20 °C. The concentrations of the oligonucleotides were determined by measuring the UV absorbance at 260 nm using molar absorption coefficients for D1A, D1B, D2A, D2B, F10A₁, F10A₂, Q1 and Q3 of 137,620, 194,580, 159,363, 177,368, 135,030, 143,190, 85,250, and 129,680 M⁻¹ cm⁻¹, respectively, obtained from the website “Oligonucleotide Properties Calculator”.

4.3.1. Preparation of duplex and quadruplex DNA: The duplexes D1 (CTCTCTGGACCTTCC, GGAAGGTCCAGAGAGG), D2 (GCTGCCAAATACCTCC, GGAGGTATTTGGCAGC) and the forked sequence F10 (AAAAAAAAAACGTCCGAGCA, TGCTCGGACGAAAAAAAAA) were prepared by annealing the complementary single strands in 0.1 M ammonium acetate as previously described,²⁷ to give a stock concentration of 1 mM. The quadruplexes Q1, d(TTGGGGGT)₄, and Q3,²² d(GGGGTTTTGGGG)₂ were prepared by dissolving an appropriate quantity of freeze-dried Q1 or Q3 in 0.15 M ammonium acetate (NH₄OAc), pH 7. The solutions were heated to 56 °C for 15 min and allowed to cool to room temperature. The resulting tetrameric Q1 (1 mM) and dimeric Q3 (1 mM) were stored at –20 °C.

4.3.2. Preparation of drug/DNA complexes: Stock **4** and **1** solutions (1 mM) were prepared in 0.1 M NH₄OAc, pH 8.5. Appropriate volumes of 0.1 M NH₄OAc, D1, D2, or F10 and each of the compounds **1** and **4**, were mixed to give reaction mixtures containing 10 μM dsDNA and drug in the dsDNA/drug ratios of 1:1, 1:3, 1:6, 1:9 and 1:12. The final volume was 100 μL. The same reaction mixtures were set up for the quadruplex Q1. For these solutions, an appropriate volume of stock drug solution was freeze-dried using a Savant Speed-Vac followed by addition of tetrameric Q1 in 0.15 M NH₄OAc, pH 7, to give Q1/drug ratios of 1:1, 1:3, 1:6, 1:9 and 1:12 in a final volume of 100 μL. The D1, D2, F10 and Q1 and Q3 reaction mixtures were diluted with an equal volume of 0.1 M NH₄OAc (or 0.15 M for Q1) prior to ESI-MS analysis.

4.3.3. Electrospray ionization mass spectrometry (ESI-MS): Negative ion ESI mass spectra of DNA and the drug/DNA complexes were acquired using a Waters extended mass range Q-ToF UltimaTM (Wyntheshawe, UK) mass spectrometer, fitted with a Z-spray ESI source.²⁸ The capillary, RF lens 1 and collision cell were at 2.5 kV, 70 V and 4 V, respectively. The cone voltage was 100 V for experiments involving dsDNA and 150 V for experiments involving Q1 and Q3, and the transport and aperture in all experiments were each at 5 V. The pressure in the ion optics region was 3 × 10⁻⁶ mbar. Thirty acquisitions were combined and the resulting spectrum was baseline subtracted and smoothed using a Savitzky Golay algorithm. The instrument was calibrated using 1 mg/mL cesium iodide.

Acknowledgments

We wish to thank The Thailand Research Fund (MRG 4980158), the Commission on Higher Education, Srinakharinwirot University, Ramkhamhaeng University, Thailand, and the University of Wollongong, Australia, and Northeastern University, USA for supporting this work. The work was also supported by Grant RO1-A1076372-01A2 from the NIH awarded to K. Lewis. The award of an APA scholarship to K. Gornall and an Endeavour Award to S. Samosorn are gratefully acknowledged.

6. References and notes

1. Craft AC, D'Antoni AV, Terzulli SL. *Med Sci Mon* 2007;13:RA103.
2. Poole K. *Ann Med* 2007;39:162. [PubMed: 17457715]
3. Lomovskaya, O.; Zgurskaya, HI.; Bostian, KA.; Lewis, K. Bacterial resistance to antimicrobials. Vol. 2. Wax, RG.; Lewis, K.; Salyers, AA.; Taber, H., editors. CRC Press-Taylor and Francis Group; Florida: 2008. p. 45-69.
4. Ines Borges-Walmsley M, Walmsley AR. *Trends Microbiol* 2001;9:71. [PubMed: 11173246]
5. Bremner JB, Ambrus JI, Samosorn S. *Curr Med Chem* 2007;14:103. [PubMed: 17266571]
6. Barbachyn, MR. *Ann Rep Med Chem*. Macor, JE., editor. Vol. 43. Elsevier Academic Press; San Diego: 2008. p. 281-290.
7. German N, Wei P, Kaatz GW, Kerns RJ. *Eur J Med Chem* 2008;43:2453. [PubMed: 18358571]
8. Ball AR, Casadei G, Samosorn S, Bremner JB, Ausubel FM, Moy TI, Lewis K. *ACS Chem Biol* 2006;1:594. [PubMed: 17168555]
9. Markham PN, Westhaus E, Klyachko K, Johnson ME, Neyfakh AA. *Antimicrob Agents Chemother* 1999;43:2404. [PubMed: 10508015]
10. Fournier dit Chabert J, Marquez B, Neville L, Joucla L, Broussous S, Bouhours P, David E, Pellet-Rostaing S, Marquet B, Moreau N, Lemaire M. *Bioorg Med Chem* 2007;15:4482. [PubMed: 17498961]
11. Ambrus JI, Kelso MJ, Bremner JB, Ball AR, Casadei G, Lewis K. *Bioorg Med Chem Lett* 2008;18:4294. [PubMed: 18632270]
12. Iwasa K, Nanba H, Lee DU, Kang SI. *Planta Med* 1998;64:748. [PubMed: 9933992]
13. Samosorn S, Bremner JB, Ball A, Lewis K. *Bioorg Med Chem* 2006;14:857. [PubMed: 16203150]
14. Hsieh PC, Siegel SA, Rogers B, Davis D, Lewis K. *Proc Natl Acad Sci USA* 1998;95:6602. [PubMed: 9618458]
15. Price CTD, Kaatz GW, Gustafson JE. *Int J Antimicrob Agents* 2002;20:206. [PubMed: 12385700]
16. Lynch C, Courvalin P, Nikaido H. *Antimicrob Agents Chemother* 1997;41:869. [PubMed: 9087510]
17. Gilmore MS, Sahm DF, Huycke MM. *Emerging Infect Dis* 1998;4:239. [PubMed: 9621194]
18. Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA, Lewis K. *Proc Natl Acad Sci USA* 2000;97:1433. [PubMed: 10677479]
19. Data obtained together with that for isomers of the berberine hybrid 2 with the methylene linker group attached to the *meta* or *para* position of the 2-phenyl ring in the INF55 moiety; Kelso, M. J.; Tomkiewicz, D.; Casadei, G.; Bremner, J. B.; Lewis, K., unpublished results.
20. Nelson ML. *Curr Med Chem: Anti-Infective Agents* 2002;1:35.
21. Amin AH, Subbaiah TV, Abbasi KM. *Can J Microbiol* 1969;15:1067. [PubMed: 4906191]
22. Rosu F, Gabelica V, Houssier C, Colson P, De Pauw E. *Rapid Commun Mass Spectrom* 2002;16:1729. [PubMed: 12207360]
23. Gornall KC, Samosorn S, Talib J, Bremner JB, Beck JL. *Rapid Commun Mass Spectrom* 2007;21:1759. [PubMed: 17486674]
24. Price CTD, Kaatz GW, Gustafson JE. *Int J Antimicrob Agents* 2002;20:206. [PubMed: 12385700]
25. Kaatz GW, Seo SM. *Antimicrob Agents Chemother* 1997;41:2733. [PubMed: 9420048]
26. Huycke MM, Spiegel CA, Gilmore MS. *Antimicrob Agents Chemother* 1991;35:1626. [PubMed: 1929336]
27. Kapur A, Beck JL, Sheil MM. *Rapid Commun Mass Spec* 1999;13:2489.
28. Sobott F, Hernandez H, McCammon MG, Tito MA, Robinson CV. *Anal Chem* 2002;74:1402. [PubMed: 11922310]

Figure 1. Structures of antibacterial berberine (**1**), NorA inhibitor **INF55**, dual-action hybrid **SS14 (2)**, and 13-*O*-arylmethyl-substituted berberine derivatives **3–7**.

Figure 2. Accumulation of **1** (◆), **1** in the presence of **INF55** (◇), **2** (△), and **3** (▲) by three strains of *S. aureus*. a) wild-type strain, 8325-4; b) NorA deletion strain, K1758; and c) NorA overexpression strain K2378. Accumulation was measured by an increase in fluorescence at 517 nm and expressed as relative fluorescence units (RFUs). Graphs are representative of at least three independent experiments.

Figure 3.

The uptake of ethidium bromide alone (◆) and in the presence of **INF55** (○), **2** (△), and **3** (▲): a) by *S. aureus* 8325-4, wild-type; b) by *S. aureus* K1758, deleting NorA pump; c) by *S. aureus* K2378, overexpressing NorA pump. Graphs are representative of at least three independent experiments.

Figure 4. The efflux of ethidium bromide by *S. aureus* K2378, overexpressing NorA pump, alone (■) and in the presence of **INF55** (◇), **2** (△) and **3** (▲). Graphs are representative of at least three independent experiments.

Figure 5. The efflux of ethidium bromide by *S. aureus* K2378, overexpressing NorA pump, alone (■) and in the presence of INF55 (◇), and 4 (x), 5 (◆), 6 (△) and 7 (○). Graphs are representative of at least three independent experiments.

Figure 6.

Binding of berberine (**1**) (left) and **4** (right) to the dsDNA sequences D1, D2 and F10. Ratio of **4**: dsDNA was 9:1. The negative ion ESI mass spectra show the -5 and -6 charge states. (●) dsDNA alone; (◇) dsDNA + **1** or **4**. Each ion to the right of the ion for dsDNA alone corresponds to another molecule of **1** bound to the DNA. Only one molecule of **4** was bound to each DNA sequence.

Scheme 1.

Reagents and conditions: (a) acetone, NaOH (s), 50 °C; (b) KMnO₄ (aq), acetone, -20 °C; (c) HCl (conc), MeOH, reflux; (d) 5% NaOH (aq), DCM, rt; (e) RBr, CH₃CN, 60 °C.

Table 1
Minimum inhibitory concentrations in μM of **1–7** against Gram-positive and Gram-negative human pathogenic bacteria.

Strain	1	2 ^a	3	Fold change ^c	4	Fold change ^c	5	Fold change ^c	6	Fold change ^c	7	Fold change ^c
<i>S. aureus</i> K1758 Δ <i>nraA</i>	40 ^a	3.1	1.7	24	5.5	7	12.0	3	91.4	2-fold less active	191.2	5-fold less active
<i>S. aureus</i> 8325-4 Wild-type	325 ^a	3.1	1.7	191	10.9	30	24.0	14	182.8	2	>191.2	<2
<i>S. aureus</i> K2378 NorA++	>650 ^a	3.1	1.7	>382	21.9	>30	95.8	7	>182.8	4	>191.2	>3
<i>E. coli</i> BW25113 Δ <i>TolC</i>	325	>150	nt ^b	-	10.9	30	12.0	27	91.4	4	191.2	2
<i>E. coli</i> ECM1668 marR1642 Δ <i>acrAB</i>	325	>150	nt	-	21.9	15	24.0	14	45.7	7	191.2	2
<i>E. coli</i> K12 Wild-type	>2600	>150	nt	-	>174.8	-	>191.6	-	>182.8	-	>191.2	-
<i>E. coli</i> ECM1642 marR1642 <i>arcAB</i> +	>2600	>150	nt	-	>174.8	-	>191.6	-	>182.8	-	>191.2	-
<i>E. faecalis</i> OG1RF	650 ^a	6.3	3.4	191	nt	-	nt	-	nt	-	nt	-
<i>E. faecalis</i> MMH594	>650 ^a	6.3	3.4	>191	nt	-	nt	-	nt	-	nt	-
<i>E. faecalis</i> V583	>650 ^a	6.3	3.4	>191	nt	-	nt	-	nt	-	nt	-

^a Values are retrieved from Ball *et al.*⁸

^b Not tested.

^c Each compound is compared to **1**.