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Structure–Activity Relationships of 2-Aryl-1*H*-indole Inhibitors of the NorA Efflux Pump in *Staphylococcus aureus*

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Abstract

The synthesis of 22 2-aryl-1*H*-indoles, including 12 new compounds, has been achieved via Pd- or Rh- mediated methodologies, or selective electrophilic substitution. All three methods were based on elaborations from simple indole precursors. SAR studies on these indoles and 2-phenyl-1*H*-indole in *S. aureus* as NorA efflux pump inhibitors indicated 5-nitro-2-(3-methoxycarbonyl)phenyl-1*H*-indole was a slightly more potent inhibitor than the lead INF55. A promising new antibacterial lead compound against *S. aureus*, (2-phenyl-1*H*-indol-5-yl)-methanol, was also found.

Keywords

2-Arylindoles; NorA efflux pump inhibitors; Antibacterial

Efflux pumps compromise the efficacy of a wide range of antibiotics by actively extruding them from bacterial cells.[1,2] The pumps can be expressed in many different forms in both Gram-positive[3] and Gram-negative bacteria[4] and for some species a variety of pumps may be present with different or overlapping substrates. For the important community and nosocomially acquired human pathogen *Staphylococcus aureus* a number of pumps have been identified, including NorA, which has been shown to play a role in the development of clinical multidrug resistance (MDR) by this organism.[5] One promising strategy for combating MDR in *S. aureus* is to treat infections with a combination of a NorA efflux pump inhibitor and a conventional antibiotic, with the pump inhibitor serving to restore the antibiotic's potency by reducing its efflux from bacterial cells.[6]

Reported classes of NorA inhibitors include flavones and flavonolignans,[7] pyrroloquinoxalines,[8] 4-arylpyridine-3,5-dicarboxylate esters,[9] N-aryl ureas,[10] and indoles.[11]

From the indole class, 5-nitro-2-phenylindole (**2**, INF55) (Scheme 1) represents a promising lead structure capable of producing a 4-fold increase in *S. aureus* susceptibility to ciprofloxacin when co-administered with the antibiotic at a concentration of 1.5 μg/mL.[11]

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Structure-activity relationships for INF55 are only just starting to emerge. In one theoretical COMFA (3D QSAR) analysis it was suggested that replacement of the indole 5-nitro group with other electron withdrawing substituents should be favorable for activity.[10] A series of 2-arylbenzo[*b*]thiophenes related to INF55 were recently reported as NorA inhibitors[12] suggesting that the indole-NH is not essential for activity. In 2006 we reported preliminary structure-activity data showing that various substituents around the 2-aryl ring of INF55 improved NorA inhibitory potency.[13] For example, 5′-benzyloxy-2′-hydroxymethyl-5-nitroindole was found to potentiate the activity of the mild antibacterial agent berberine (a known substrate of NorA) more than 15-fold against the NorA overexpressing *S. aureus* strain K2361 at a concentration of 0.8 μg/mL (c.f. INF55 potentiates berberine activity to the same degree at the higher concentration of 3.0 μg/mL).

The current work discloses our latest systematic SAR exploration around the 2-aryl ring of INF55 and details the first experimental description of the effects of substituting the indole 5-nitro group. The goal of this study was to obtain a deeper understanding of the SAR of INF55 and analogues with a view to increasing potency against NorA in *S. aureus*. Furthermore, we sought to broaden our knowledge of substituent tolerance around the INF55 nucleus in order to advance our long-term goals of developing potent dual-action antimicrobials,[14] including both non-cleavable hybrid molecules[15] and cleavable "mutual" prodrugs[16] which combine antibacterial agents and NorA-inhibiting moieties into single molecules.

INF55 **2** was synthesized in one step (Scheme 1) from commercially available 2-phenylindole **1** using the known regioselective nitration procedure.[17] Compounds **3–6** were each synthesized in two steps starting from **1** and proceeding via a 5-chlorosulfonyl-2-phenylindole intermediate **1a** (Scheme 2). The intermediate was cleanly accessed using a previously unreported, highly regioselective 5-chlorosulfonation of 2-phenylindole. Briefly, **1** was stirred with neat chlorosulfonic acid at 0 °C and allowed to warm to rt with stirring over 30 mins. The reaction mixture was then poured slowly onto crushed ice and the product filtered, washed with water and dried under vacuum (80%). As **1a** degraded when left at rt over a period of several days, the freshly prepared crude 5-chlorosulfonyl-2-phenylindole (**1a**) was then reacted with appropriate nucleophiles to furnish the 5-sulfonyl-2-phenylindole derivatives **3–6** (Scheme 1) in good yields (**3** = 72%; **4** = 70%; **5** = 57%; **6** = 76%).

We propose that the regioselective 5-chlorosulfonation of 2-phenylindole proceeds via the electrophilic aromatic substitution mechanism shown in Scheme 2. Under strongly acidic conditions (e.g. neat $ClSO_3H$) indoles are known to protonate at the indole C3 position which serves to protect this normally reactive site from electrophiles. C3 protonation of 2-phenylindole would yield a 2-phenylindolinium cation that could potentially react directly with a weak nucleophile like $ClSO_3^-$ to form an indoline-2-chlorosulfonate adduct.

A related indoline-2-sulfonate adduct has been reported as a participant in the regioselective formation of 5-substituted indoles.[18] What is perhaps more likely is that the indoline-2-chlorosulfonate is formed in a concerted process that proceeds via the cyclic 6-membered transition state shown in Scheme 2. The resulting indoline-2-chlorosulfonate, which contains an *ortho*-substituted aniline ring, should direct a $ClSO_2^+$ electrophile to the indole C5 position (i.e. *para* to the aniline nitrogen), with indole **1a** returned after quenching with water.

Compounds **14**, **15**, **18** and **22** were prepared by direct C2 arylation of commercially available indoles **7–9** with the respective aryl iodides **10–13** using the recently reported Rh-catalyzed coupling procedure (Scheme 3).[19] Yields for the couplings were disappointing (14–43%) but nevertheless provided sufficient quantities of pure materials for use in our study. 2D NOESY spectra of compounds **18** and **22** confirmed that indole C2 arylation had occurred in preference

to arylation at C3 (i.e. NOEs were observed between the indole H3 and H4 signals for both **18** and **22**).

The methyl ester **15** was smoothly hydrolyzed to the carboxylic acid **16** under standard conditions using LiOH/THF, and **15** was also reduced without incident to the hydroxymethyl derivative **17** using $LiAlH_4$ (Scheme 3). Esters **18** and **22** were reduced in essentially quantitative yield to their respective hydroxylmethyl derivatives **19** and **23** using $LiBH_4$. The same esters **18** and **22** could be regioselectively nitrated at −20 °C in $NaNO_3/H_2SO_4$ mixtures[17] to provide the 5-nitro-2-arylindole derivatives **20** and **24** in 80% and 81% yields respectively. Compounds **22** and **24** were comprehensively characterized by 2D NMR spectroscopy, with NOESY spectra confirming that mononitration had occurred selectively at the indole C5 position for both compounds (i.e. observed NOEs included NH/H7, H6/H7 and H3/H4). Esters **20** and **24** were subsequently reduced to their respective hydroxymethyl derivatives **23** and **25** in high yields (90% and 79% respectively) using $LiBH_4$ (Scheme 3).

A Pd-mediated intramolecular cyclization approach was used to access the 2′-substituted-2-arylindoles **31–35**. All attempts to access these analogues via Rh-catalyzed 2-arylation of indoles with *ortho*-substituted aryl iodides failed. *N*-benzoylindoles **26** and **27** (Scheme 4) were converted to their respective 6-oxo-6*H*isoindolo[2,1-α]indoles **28** and **29** using the palladium (II) acetate promoted oxidative intramolecular cyclization procedure reported by Itahara.[20] Ring-opening of compounds **28** and **29** by lactam hydrolysis (*t*-BuOK, *t*-BuOH, H_2O)[20] yielded the 2′-carboxylic acids **30**[20] and **31**[13] respectively. Compound **30** was converted to the methyl ester **32** under standard conditions (AcCl/MeOH) and to the hydroxymethyl derivative **33** using $LiAlH_4$. The acid 2-(2′-carboxyphenyl)-5-nitroindole **31** was converted to its methyl ester **34** using AcCl/MeOH and to its hydroxymethyl derivative **35**[13] using BH_3.THF.

The potentiation activities of compounds **1–6** and **14–17** (Table 1) were determined against the wild type S. aureus strain (8325-4), a mutant strain deleted in NorA (K1758) and a NorA overexpressing strain (K2378) with the antibacterial agent berberine using a checkerboard assay which involved co-administration of varying concentrations of the inhibitor and berberine.[21] This assay provides access to combinatorial interactions (synergistic, antagonistic, indifferent, or additive) between the two molecules. The inhibitor 'MIC' is defined as the lowest concentration of inhibitor required to potentiate berberine's MIC by at least a factor of two. The MIC values for berberine (as the chloride salt) alone against *S. aureus* were 269 μM (8325-4) and 67 μM (K1758)), and 2152 μM (K2378). The activities of compounds **18–25** and **32–35** (Table 2) were determined against the same three strains of *S. aureus* but using our previously published methodology[13] (even though not as complete as the full checkerboard assay), in order to enable a better comparison with the prior results on compounds with other 2-aryl substituents. Briefly, for this methodology, cells were grown in the presence of a sub-inhibitory concentration of berberine [81 μM (8325-4); 20 μM (K1758); and 161 μM (K2378)] and varying concentrations of the test compounds. Bacterial growth was monitored by measuring the absorption at 600 nm and MIC values were determined. MICs represent the minimum concentrations of the test compounds (in combination with the fixed concentration of berberine) that completely inhibited cell growth during an 18 h incubation at 37 °C. Differences in the values for INF55 (**2**) in Table 1 and Table 2 reflect the difference in the potentiation assay used, although the different result with the NorA knockout mutant K1758 (Table 1) was not resolved.

The inherent antibacterial activity of each compound in the absence of berberine was > 1000 μM (the testing limit employed in this assay), except for the sulfonate esters **5** and **6** (MIC values of 174 μM and 79.3 μM respectively against all three *S. aureus* strains), and **17**. The alcohol **17** showed an MIC of 14.0 μM against the NorA knockout strain, and 28.0 μM against

both the wild type and NorA overexpressing strains.[22] Interestingly, in further testing, **17** was shown to be inactive as an antibacterial against a panel of other Gram-negative and Gram-positive pathogens.[23]

Compounds **1–6** and **14–17** (Table 1) varied only with respect to substituents at the indole-C5 position allowing for direct conclusions to be drawn regarding the importance of the 5-NO_2 group of INF55. Three of the compounds tested (**5**, **6**, and **17**) showed intrinsic antibacterial activity and thus the potentiation of the MIC value could not be accurately determined for these analogues.

Structure-activity conclusions regarding the importance of the C5 position of INF55 for inhibitory activity against the NorA pump include:

1. Removing the C5 substituent (**1**) is deleterious to activity.
2. Carbonyl based electron-withdrawing groups at C5 (**15**, **16**) abolish all activity.
3. Inhibitors with sulfonic acids/esters/amides at C5 (compounds **3–6**) show no potentiation. Compounds **5** and **6** display mild intrinsic antibacterial activity.
4. Substitution with a nitrile group leads to retention of potency with **14** showing similar potentiation activity to INF55.

Compounds **20**, **21**, **24**, **25**, **34** and **35** (Table 2)[24] systematically explore the effects of methyl ester and hydroxymethyl substituents at the 2′, 3′ and 4′ positions of the 2-aryl ring of INF55. Compounds **18**, **19**, **22**, **23**, **32** and **33** explore these same effects in compounds lacking the 5-NO_2 of INF55. One of the goals of this study was to identify analogues of INF55 that retained or improved NorA inhibitory potency while containing functional groups useful for covalent attachment to antimicrobial agents. We were particularly interested in analogues bearing hydroxymethyl groups since these could be incorporated into dual-action NorA inhibitor-antibacterial "mutual" prodrugs bearing labile ester linkages. Success of this strategy of course requires that the alcohol-bearing INF55 analogue released from such prodrugs is a potent NorA inhibitor. The methyl ester analogues were intermediates in the synthesis of the hydroxymethyl analogues and were tested here to add depth to the SAR. The analogous series lacking the 5-NO_2 group was explored in an attempt to identify inhibitors which might avoid the known toxicity problems of nitroaromatic compounds.

Table 2 shows that all compounds lacking 5-NO_2 groups (i.e. **18**, **19**, **22**, **23**, **32**, **33**) were significantly less potent than INF55. Another trend to emerge was that, apart from **24** with respect to **25**, each of the methyl ester analogues was more potent than its corresponding hydroxymethyl derivative. The 3′-CO_2Me derivative **20** proved to be the most active compound identified in this study, displaying slightly higher potency than INF55 against all three *S. aureus* strains. Another important finding was that analogue **25** bearing a 4′-CH_2OH group was almost equipotent with INF55 against the wild type and NorA overexpressing strain. This suggests that **25** is the best compound to progress into our studies of mutual prodrugs employing labile ester linkages. It should be mentioned that there is a possibility that non-covalent complex formation between berberine and the indole-based inhibitors reported here may play a role in the potentiation observed.[26]

In conclusion, the SAR data for indole-based NorA efflux pump inhibitors has been deepened by this study and three new potent inhibitors, **14**, **20** and **25**, have been uncovered. Inhibitor **25** represents a promising candidate for incorporation into dual action mutual prodrugs targeting the NorA pump. In addition, a new indole derivative **17** was identified with specific antibacterial activity against *S. aureus*. The mode of action of this compound and the reason for its selective activity against this organism remains to be investigated.

Acknowledgments

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21. A checkerboard assay was conducted to specify the degree of potentiation of berberine by the test compounds and to determine the specificity of these compounds for the NorA efflux pump. Serial 2-fold dilutions of berberine and a test compound were mixed in each well of a 96-well microtiter plate so that each row (and column) contained a fixed amount of one agent and increasing amounts of the second agent. The resulting plate presents a pattern in which every well contains a unique combination of concentrations between the two molecules. The concentrations of berberine ranged from 100 to 12.5 μg/mL (*S. aureus* 8325-4) and 200 to 800 μg/mL (*S. aureus* K2378), while inhibitor concentrations ranged from 1.56 to 100 μg/mL. Each plate also contained a row and column in which a serial dilution of each agent was present alone. Cells were added to each well at a final concentration of 5 10^5 CFU/mL, and plates incubated at 37 °C for 20 h. Growth was assayed by absorption at 600nm with a microtiter plate reader (Spectramax PLUS384, Molecular Devices). An OD less than 0.05 was considered to reveal no growth.
22. For the antimicrobial susceptibility assay, cells (10^5 CFU/mL) were inoculated into broth and dispensed at 0.2 mL/well in 96-well microtiter plates. MICs were determined by serial 2-fold dilution of the test compound. The MIC was defined as the concentration of an antimicrobial that completely

inhibited cell growth during an 18–20 h incubation at 37 °C Growth was assayed with a microtiter plate reader (Spectramax PLUS384; Molecular Devices) by monitoring absorption at 600 nm.

23. Compound **17** was tested against the following panel of Gram-negative pathogens with MICs given in brackets in μg/mL; the highest concentration tested was 200 μg/mL: *Escherichia coli* O157:H7, BW25113, and UT189 (all >200); *Salmonella typhimurium* CS132 (>200); *Pseudomonas aeruginosa* PA01 and PA14 (both > 200). This compound was also tested against a number of Gram-positive pathogens as follows with MICs in brackets in μg/mL; the highest concentration tested was 200 μg/mL for all species apart from the *Clostridium* species, where the highest concentration tested was 100 μg/mL: *Enterococcus faecalis* MMH594, V583, and OG1RF (all >200); *Clostridium difficile* VPI10463, CD196, and CD2001 (all>100), and *Clostridium perfringens* (>100).
24. Biological testing of compounds **30** and **31** was not undertaken as the latter compound with an *orthocarboxylic* acid group had been tested previously[13] and showed poor potentiation of berberine, while the former compound was only made as a precursor for the corresponding methyl ester **32** in order to obtain a complete set of methyl esters for SAR purposes.
25. The MIC of this compound with berberine was also determined, for comparison purposes, against *S. aureus* K2361 and found to be 1.56 μg/mL, broadly commensurate with the previously obtained value (3 μg/mL)[13] in this NorA pump overexpressing strain.
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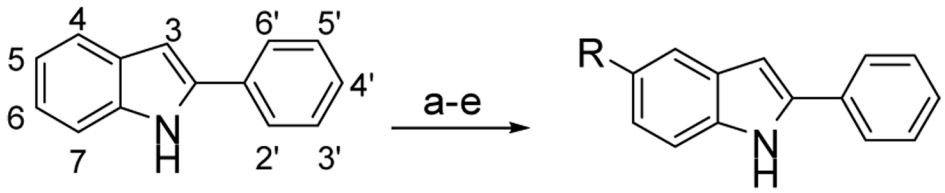
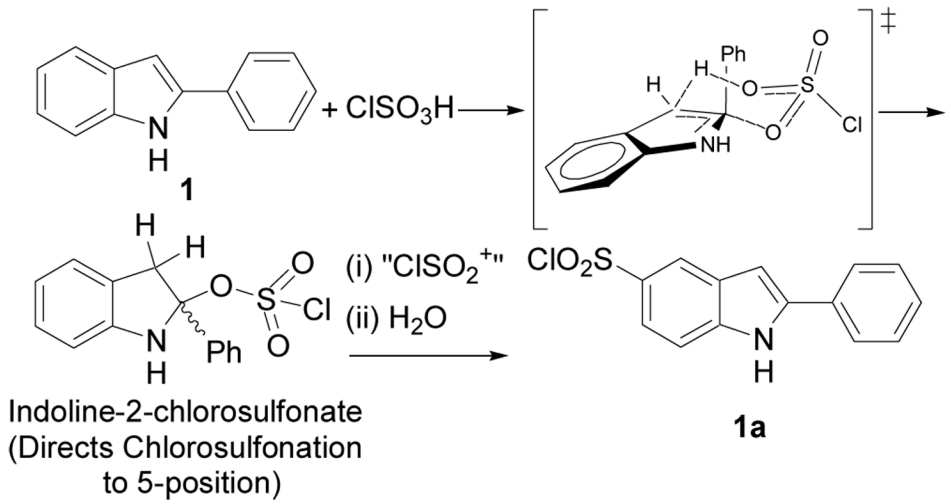


1 **2-6**

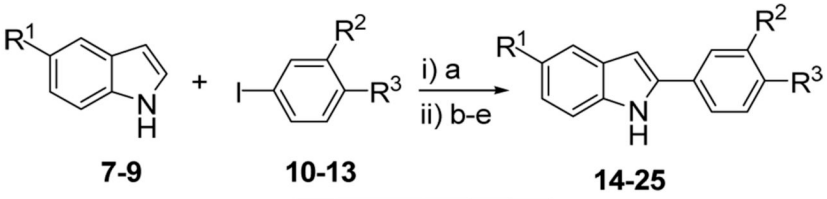
Method	Compound	R
a	**2**	NO_2 (INF55)
b	**3**	SO_3H
c	**4**	SO_2NH_2
d	**5**	SO_2OCH_3
e	**6**	SO_2O*n*-Pr

Scheme 1.

Reagents and conditions: (a) $NaNO_3$, H_2SO_4 (conc.), −20 °C; [17] (b) (i) $ClSO_3H$, 0 °C to rt, 30 mins. (ii) NaOH, H_2O, rt and then 1M HCl; (c) (i) followed by (ii) NH_3/THF, rt; (d) (i) followed by (ii) MeOH, pyridine, rt; (e) (i) followed by (ii) *n*-PrOH, pyridine rt.



Scheme 2.
Proposed mechanism for the regioselective 5-chlorosulfonation of 2-phenylindole in neat chlorosulfonic acid.



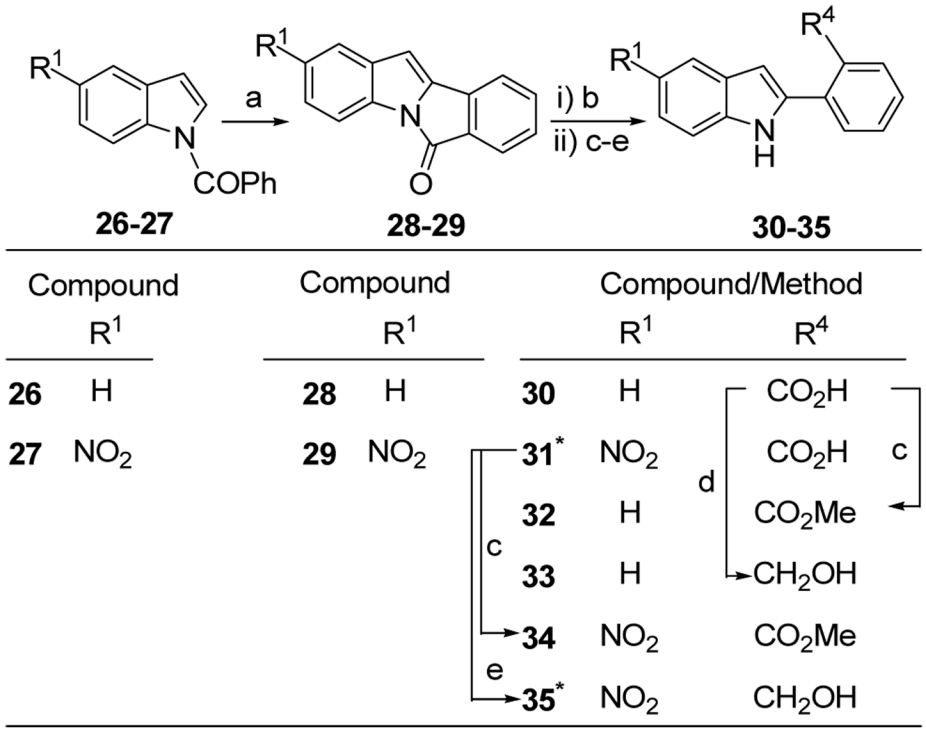
7-9 **10-13** **14-25**

Compound	R^1	Compound	R^2	R^3
7	CN	**10**	H	H
8	CO_2Me	**11**	H	H
9	H	**12**	CO_2Me	H
		13	H	CO_2Me

Compound/Method	R^1	R^2	R^3
14	CN	H	H
b → **15**	CO_2Me	H	H
16 (c)	CO_2H	H	H
17 (c)	CH_2OH	H	H
d → **18**	H	CO_2Me	H
19 (e)	H	CH_2OH	H
d → **20**	NO_2	CO_2Me	H
21	NO_2	CH_2OH	H
d → **22**	H	H	CO_2Me
23 (e)	H	H	CH_2OH
d → **24**	NO_2	H	CO_2Me
25	NO_2	H	CH_2OH

Scheme 3.

Reagents and conditions: (a) $[Rh(coe)_2Cl_2]_2$, $[p\text{-}CF_3\text{-}C_6H_4]_3P$, $Cs(OPiv)_2$, 1,4-dioxane, 120 °C;[19] (b) 1M LiOH, THF, 75 °C; (c) $LiAlH_4$, THF, rt; (d) $LiBH_4$, THF, rt; (e) $NaNO_3$, H_2SO_4 (conc.), −20 °C.[17]



Scheme 4.

Reagents and conditions: (a) $Pd(OAc)_2$, AcOH, 110 °C;[20] (b) tBuOK, tBuOH/H_2O, 82 °C;[20] (c) AcCl, MeOH, reflux; (d) $LiAlH_4$, THF, rt; (e) BH_3-THF, rt.[13] * Denotes compounds synthesized previously.[13]

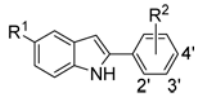
Table 1

Bacterial inhibition and potentiation results for C5-substituted analogues of INF55 (**2**) with berberine in *S. aureus.* NP denotes no antibacterial potentiation; IA denotes intrinsic antibacterial activity. Potentiation values are shown in brackets and are the ratios of berberine MICs with and without inhibitor present.

Compd	R	MIC (μM) of inhibitor		
		Δ *norA* K1758 plus berberine	**WT 8325-4 plus berberine**	**NorA^{++} K2378 plus berberine**
1	H	NP	65 (4)	259.0 (4)
2	NO_2	NP	3.3 (2)	6.5 (2)
3	SO_3H	NP	NP	NP
4	SO_2NH_2	NP	NP	NP
5	SO_2OCH_3	IA	IA	IA
6	SO_2O*n*-Pr	IA	IA	IA
14	CN	NP	3.6 (2)	7.1 (2)
15	CO_2Me	NP	NP	NP
16	CO_2H	NP	NP	NP
17	CH_2OH	IA	IA	IA

Table 2

Bacterial inhibition and potentiation results for 2-aryl analogues of INF55 (**2**) with berberine in *S. aureus*. NP denotes no antibacterial potentiation. The inhibitor concentrations are the minimum for potentiation of berberine MIC by factors of 3.3 (K1758), 3.3 (8325-4), and 13.3 (K2378).

Compd			MIC (μM) of inhibitor		
	R^1	R^2	Δ *norA* K1758 plus berberine	WT 8325-4 plus berberine	NorA^{++} K2378 plus berberine
32	H	2′-CO_2Me	NP	20	20
33	H	2′-CH_2OH	NP	45	45
18	H	3′-CO_2Me	40	5	10
19	H	3′-CH_2OH	NP	45	45
22	H	4′-CO_2Me	NP	20	40
23	H	4′-CH_2OH	NP	22	45
2[25]	NO_2	H	0.63	1.3	1.3
34	NO_2	2′-CO_2Me	NP	34	NP
35[13]	NO_2	2′-CH_2OH	24	47	47
20	NO_2	3′-CO_2Me	0.51	1.0	1.0
21	NO_2	3′-CH_2OH	9	5	9
24	NO_2	4′-CO_2Me	NP	17	NP
25	NO_2	4′-CH_2OH	5	2	2

Bioorg Med Chem Lett. Author manuscript; available in PMC 2009 August 1.