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Letter

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Cinnamonitrile Adjuvants Restore Susceptibility to β-Lactams Against Methicillin-Resistant *Staphylococcus aureus*

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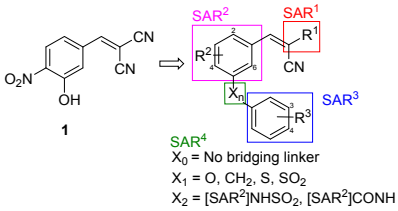
KEYWORDS: MRSA, bacterial resistance, antibiotic adjuvants.

ABSTRACT: β-Lactams are used routinely to treat *Staphylococcus aureus* infections. However, the emergence of methicillin-resistant *S. aureus* (MRSA) renders them clinically precarious. We describe a class of cinnamonitrile adjuvants that restore the activity of oxacillin (a penicillin member of the β-lactams) against MRSA. The lead adjuvants were tested against six important strains of MRSA, one vancomycin-intermediate *S. aureus* (VISA) strain, and one linezolid-resistant *S. aureus* strain. Five compounds out of 84 total compounds showed broad potentiation. At 8 μM (*E*)-3-(5-(3,4-dichlorobenzyl)-2-(trifluoromethoxy)phenyl)-2-(methylsulfonyl)acrylonitrile (**26**) potentiated oxacillin with a >4,000-fold reduction of its MIC (from 256 to 0.06 mg L^{-1}). This class of adjuvants holds promise for reversal of the resistance phenotype of MRSA.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a problematic worldwide pathogen.[1–2] Its key characteristic is resistance to virtually all members of the β-lactam family of antibiotics. MRSA infections confound to the present day, with 11,000 annual fatalities in the US alone.[3] Its resistance mechanisms to β-lactams include a two-component system *vraSR*[4–5] and the two related *mec* and *bla* operons, which detect the presence of the antibiotic in the milieu and transduce the signal to the cytoplasm.[6–12] The signaling unleashes gene derepression that leads to transcription and translation of a class A β-lactamase and/or an additional penicillin-binding protein (PBP), known as PBP2a, as resistance determinants.[13–17] About 80% of MRSA strains express both.[18] A spatiotemporal precision with respect to the mobilization of multiple biochemical steps enables an effective response to the challenge of the antibiotic. This multistep orchestration presents opportunities for medicinal chemical intervention to reverse the MRSA phenotype. In this circumstance the MRSA strain would revert to methicillin-sensitive *S. aureus* (MSSA), which can be treated with existing antibiotics.[19–20]

We describe a class of molecules that emerged from a search for adjuvants[21-23] for β-lactam antibiotics in killing MRSA. Our search identified the known[24] mammalian protein kinase inhibitor **1** as an adjuvant of oxacillin (OXA) against the MRSA252 strain. Potentiation by **1** (at a fixed concentration of 20 μM) was reproducibly two-fold (from 256 to 128 mg·L^{-1}). We embarked on structure optimization of **1**. Our created diversity defined the SAR for **1** with respect to four sites (boxes in Chart 1). The 83 additional compounds so prepared were tested for their potentiation ability. One compound (**26**) potentiated the activity of OXA at 20 μM, by as much as >4,000-fold, against eight MRSA strains.

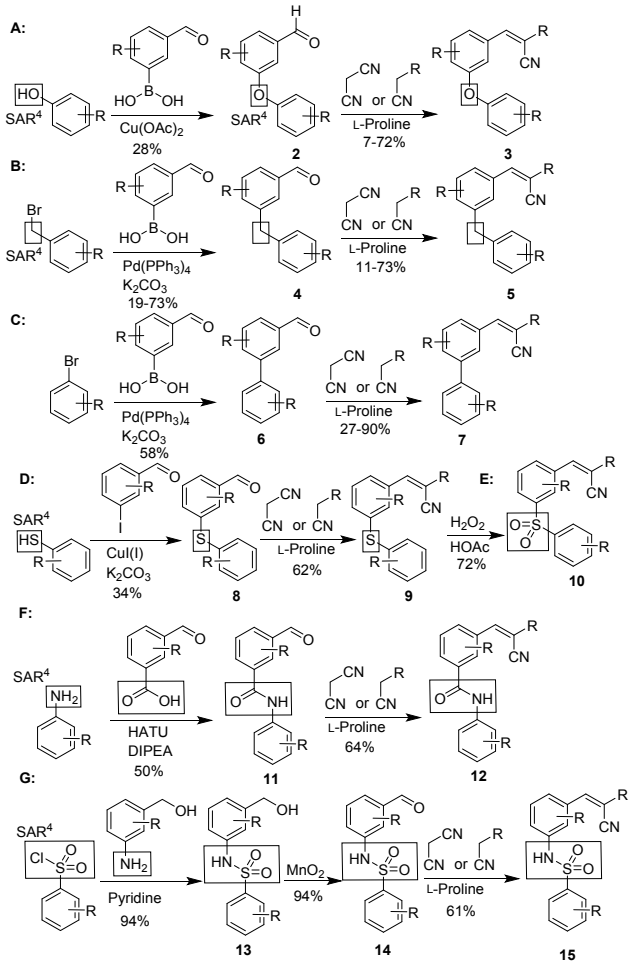
Chart 1. Lead compound **1** and its SAR parsing.



Synthesis. Compound **1** was prepared by Knoevenagel condensation.[24] Four areas for SAR were selected with one of the nitriles as the SAR^1 and the phenyl ring as SAR^2 (Chart 1). We came to an early realization that an additional aromatic ring (blue box, Chart 1) was beneficial. This addition to the template became SAR^3 and that of the bridging linker SAR^4. The choice of the bridging linker in SAR^4 dictated the synthetic route (Scheme 1). The

diarylether derivatives were prepared according to Scheme 1A from the diarylether aldehyde (**2**). Knoevenagel condensation of the aromatic aldehyde with either malononitrile or an appropriate acetonitrile derivative possessing an electron-withdrawing group gave compounds **3**. Preparation of compounds bearing the diarylmethane moiety followed Scheme 1B using Suzuki-Miyaura coupling of a benzyl bromide[25] to give compounds **4**. The transformation of compounds **4** to derivatives **5** was as described. We made a few compounds without the linker (X_0 = no bridging linker, Scheme 1C). Several derivatives were made by the general approaches outlined in Scheme 1D–G. The routes to compounds that do not fit within Scheme 1 are given in the Supporting Information (SI).

Scheme 1. Synthetic routes for the cinnamonitrile family of compounds.



SAR Analysis. The synthetic compounds were screened initially as adjuvants of OXA against the NRS70 MRSA strain. This strain is a USA100-type that is highly pathogenic, and is the most common health care-associated MRSA in the United States.[26-27] The minimal-inhibitory concentrations (MICs) of OXA were evaluated at a fixed concentration (20 μM) of the adjuvants. MIC determinations for OXA (MIC_{OXA}) were done with 2% NaCl unless otherwise specified. Inclusion of NaCl gives a more reliable determination of adjuvant efficacy when using OXA against MRSA strains, as recommended by the Clinical & Laboratory Standards Institute.[28] The MICs of the compounds alone (MIC_{ADJ}) were also determined. To our surprise, some compounds possessed antibacterial activity. We recognized that the initial hit (compound **1**, and other dicyano derivatives) would not pass Pan-Assay Interference Compounds (PAINS) assessment, as implemented with the online service PAINS-Remover.[29-30] This realization guided us towards compounds **16**–**25** (Table 1). Compounds **17**, **18**, and **24** exhibited independently antibacterial activity (MIC_{ADJ} of 1–32 mg·L^{-1}) in the absence of OXA.

The MIC of OXA alone against the strain NRS70 was 64 mg·L^{-1}. When OXA was evaluated in the presence of a fixed concentration (20 μM) of compounds **16** and **17** the MIC_{OXA} was reduced dramatically (from 64 to a range of 1 to ≤0.03 mg·L^{-1}; Table 1). These data supported efforts to modify **1**. Indeed, exceptional potentiation ability was secured by compounds **16** (a ketone) and **17** (a sulfone). Notwithstanding the structural liability of the dicyano moiety, it was used to define the SAR scope. Other substitutions, inclusive of sulfones (as in **17**), were made for the dicyano moiety. Of these substitutions the methylsulfone was especially meritorious. Table 2 identifies the best adjuvants as assessed for the strain NRS70. Several compounds in this table are neither adjuvants nor antibacterials. Several exhibit modest antibacterial activity (MIC_{ADJ} of about 8 mg L^{-1}). Several (**26**–**31** and **53**–**55**) potentiate OXA exceptionally well (≤0.03–1 mg·L^{-1}). The key structural features are summarized. SAR[2] prefers a trifluoromethyl (**26**) or fluoro moiety at positions 2 and/or 6 (**27**–**30**). The best SAR[3] coincides with the 3,4-dichlorophenyl ring. Optimal SAR[4] is seen in the absence of a bridging atom, and with a methylene bridge. The *E*-stereochemistry of **26** (the most potent compound) was verified by nuclear Overhauser effect assignment. Data for additional compounds of less interest (**83–101**) are given in Table S1 (SI).

Table 1. MIC (in mg·L^{-1}) of OXA against strain NRS70 with 20 μM of adjuvant (MIC_{OXA}, column shaded tan), and the adjuvant alone (MIC_{ADJ}).

[a]MIC_{OXA} and MIC_{ADJ} determined in the presence and in the absence of 2% NaCl, respectively. MIC_{OXA} values < 8 mg·L^{-1} are in red. [b] 2-Sulfonylpyridinyl; [c] 2-Benzothiazolyl.

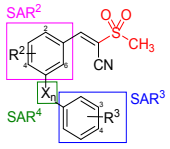
Adjuvant Activity. The five most active adjuvants (**17**, **26**, **27**, **28**, **29**) were tested against seven additional MRSA stains—NRS1 [vancomycin-intermediate *S. aureus* (VISA)], NRS119 [linezolid-resistant *S. aureus* (LRSA)], NRS123, NRS382, NRS383, NRS384, and MRSA252. We further tested these compounds against four MSSA strains—ATCC29213, NRS11, NRS72, NRS77—to explore whether the potentiation was limited to MRSA. All the MRSA strains are resistant to OXA (MIC range of 32 to 256 mg L^{-1}). All MSSA strains (except for NRS77) produce the class A β-lactamase BlaZ by induction of the plasmid-borne *bla* operon.

At 20 μM the adjuvants reduced generally the MIC_{OXA} to values within the range of ≤0.03 to 4 mg·L^{-1} against both the MRSA and MSSA strains (Table 3 and Table S2 in the SI). Strain NRS383 is an exception (Table 3). This strain appeared impervious to the action of four of the adjuvants, barring compound **26**, which exhibited activity against this strain as well. Because of the drastic reduction of MIC_{OXA} against MRSA by five compounds at 20 μM, their potentiation was further investigated at lower concentrations of 16 and 8 μM. The results are tabulated in Table 3. Indeed, substantial potentiation was seen in many cases at lower concentrations of the compounds.

	SAR[1]	SAR[2]	SAR[4]	MIC_{OXA}[a]	MIC_{ADJ}[a]
16	COC_3H_5	2-F	$X_1 = CH_2$	1	≥ 64
17	[b]	2-F	$X_1 = CH_2$	≤ 0.03	16
18	[b]	2-F	X_0	16	32
19	CO(4-MePh)	H	$X_1 = O$	64	≥ 64
20	4-Pyridine	H	$X_1 = O$	64	≥ 64
21	2-Pyridine	H	$X_1 = O$	64	≥ 64
22	2-Thiophene	H	$X_1 = O$	64	≥ 64
23	[c]	H	$X_1 = O$	64	≥ 64
24	$CONH_2$	H	$X_1 = O$	64	8
25	COOH	H	$X_1 = O$	64	≥ 64

Table 2. MIC (in mg·L^{-1}) of OXA against strain NRS70 with 20 μM of adjuvant (MIC_{OXA}, column shaded tan), and the adjuvant alone (MIC_{ADJ}). Panel A has the methylsulfones and panel B has the dicyano derivatives for SAR[1].

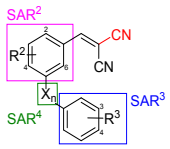
A:



	SAR[2]	SAR[3]	SAR[4]	MIC_{OXA}[a]	MIC_{ADJ}[a]
	Phenyl derivatives				
26	2-OCF_3	3,4-Cl	$X_1 = CH_2$	≤ 0.03	8
27	2-F	3,4-Cl	$X_1 = CH_2$	≤ 0.03	8
28	2,6-F	3,4-Cl	$X_1 = CH_2$	≤ 0.03	8
29	2-F	3,4-Cl	X_0	0.12	16
30	6-F	3,4-Cl	$X_1 = CH_2$	2	8
31	2-OMe	3,4-Cl	$X_1 = CH_2$	4	32
32	6-OMe	3,4-Cl	$X_1 = CH_2$	8	32
33	2,6-OMe	3,4-Cl	$X_1 = CH_2$	64	16
34	2-OEt	3,4-Cl	$X_1 = CH_2$	16	≥ 64
35	H	3,4-Cl	$X_1 = O$	64	16
36	[b]	3,4-Cl	$X_1 = CH_2$	16	≥ 64
37	2-F	4-CF_3	$X_1 = CH_2$	64	32
38	2-F	3-CF_3-4-OMe	$X_1 = CH_2$	64	≥ 64
39	2-F	3-CF_3-4-F	$X_1 = CH_2$	64	≥ 64
40	2-F	3-F-4-CF_3	$X_1 = CH_2$	64	32
41	2-F	4-OCF_3	$X_1 = CH_2$	64	≥ 64
42	2-F	4-SCF_3	$X_1 = CH_2$	64	≥ 64
43	2-OCF_3	3,4-OMe	$X_1 = CH_2$	64	≥ 64
44	2-OCF_3	[c]	$X_1 = CH_2$	64	32
45	2-F	3-CF_3	$X_1 = CH_2$	64	≥ 64
46	2-F	3-OCF_3	$X_1 = CH_2$	64	≥ 64
47	2-F	3,4-F	$X_1 = CH_2$	64	≥ 64
48	2-F	4-CONH(*i*Pr)	$X_1 = CH_2$	64	≥ 64
	Other derivatives in SAR[2]				
49	6-Pyridine	3,4-Cl	X_0	16	32

		Other derivatives in SAR³			
50	2-F	[d]	X_0	64	≥ 64
51	2-F	2-Quinolinyl	X_0	64	≥ 64
52	2-F	4-Quinolinyl	X_0	64	≥ 64

B:



	SAR²	SAR³	SAR⁴	MIC_{OXA}[a]	MIC_{ADJ}[a]
		Phenyl derivatives			
53	2-F	3,4-Cl	$X_1 = CH_2$	4	8
54	6-F	3,4-Cl	$X_1 = CH_2$	2	8
55	2-OMe	3,4-Cl	$X_1 = CH_2$	4	≥ 64
56	H	3,4-Cl	$X_1 = O$	64	32
57	**56** with a reduced double bond			64	32
58	H	3,4-Cl	$X_1 = CH_2$	64	≥ 64
59	**58** with a methyl instead of a hydrogen at the double bond			64	≥ 64
60	4-OMe	3,4-Cl	$X_1 = O$	64	≥ 64
61	H	3,5-Cl	$X_1 = O$	64	16
62	H	3,4-Cl	$X_1 = S$	64	≥ 64
63	H	3,4-Cl	$X_1 = SO_2$	64	16
64	H	3,4-Cl	X_2 = [SAR²]-CONH	16	≥ 64
65	H	4-Me	X_2 = [SAR²]-NHSO²	64	≥ 64
66	H	H	$X_1 = O$	64	32
67	H	4-Cl	$X_1 = O$	64	32
68	**67** with the oxygen in SAR⁴ connected in para			64	32
69	H	3-CF_3	$X_1 = O$	64	32
70	H	4-Me	$X_1 = O$	64	≥ 64
71	H	4-F	$X_1 = O$	64	≥ 64
72	H	3-Cl	$X_1 = CH_2$	64	≥ 64
73	H	2,4,5-F	$X_1 = CH_2$	64	≥ 64
74	H	3-F-4-Cl	$X_1 = CH_2$	64	≥ 64
75	H	3-Cl-4-F	$X_1 = CH_2$	64	≥ 64
76	H	4-OMe	$X_1 = O$	64	≥ 64
77	H	2,5-OMe	$X_1 = CH_2$	64	≥ 64
		Other derivatives			
78	H	[e]	$X_1 = O$	64	≥ 64
79	H	[f]	$X_1 = O$	64	≥ 64

[a]MIC_{OXA} and MIC_{ADJ} determined in the presence and in the absence of 2% NaCl, respectively. MIC_{OXA} values < 8 mg·L^{-1} are in red. [b]*N*-Morpholinyl; [c] 3,4- Methylenedioxy; [d] 2-Quinoxalinyl; [e] 6-Chloropyridazinyl; [f] 4,5-Dichloropyrimidinyl.

As indicated above, some of the adjuvants exhibited modest antibacterial activity. Hence, their synergy with oxacillin could be evaluated. Checkerboard assays[31-33] evaluated the synergy of the adjuvants for oxacillin against NRS1, NRS70, and NRS384. Adjuvants **17**, **27**, **28**, and **29** were synergistic with oxacillin against NRS70 and NRS384. They had an indifferent (additive) effect against the homogeneous MRSA strain NRS1 (Figure S1). Compound **26** showed the best synergy against all three MRSA strains: fractional inhibitory concentration index (FICI) of 0.28, 0.28, and 0.38 for NRS1, NRS70, and NRS384, respectively (Figure S1). The MSSA strains are already exquisitely sensitive to OXA with MIC values of 0.25–0.50 mg·L^{-1}. As a consequence, the potentiation effect is proportionally smaller, ranging between 2- to 10-fold (Table S2).

***In Vitro* Cytotoxicity.** XTT assay with HeLa cells (a human cervical cancer cell line)[34] assessed the *in vitro* potential toxicity of compounds **17**, **26**, **27**, **28**, and **29**. The IC_{50} values were: **17**: 87 ± 7 μM (39 ± 3 mg·L^{-1}); **26**: 73 ± 9 μM (33 ± 4 mg·L^{-1}); **27**: 125 ± 8 μM (48 ± 3 mg·L^{-1}); **28**: 92 ± 5 μM (37 ± 2 mg·L^{-1}); **29**: 130 ± 3 μM (48 ± 1 mg·L^{-1}). In case of **26**, its IC_{50} is about 5-fold higher than the concentration (16 μM) that potentiates OXA against all eight MRSA.

Adjuvant 26 is not a *S. aureus* protein-kinase inhibitor. Several lines of research have reported that inhibitors targeting bacterial protein kinases potentiated the

antibacterial activity of β-lactam antibiotics.[35–38] Ablation of the genes for bacterial serine/threonine kinases substantially increases the bacterial susceptibility to β-lactam drugs.[39–43]

Table 3. Reduced MIC_{OXA} against eight MRSA strains in the presence of adjuvants at 20, 16 and 8 μM.[a]

Adjuvant	MIC_{OXA} (mg·L^{-1})[b]							
	NRS1	NRS70	NRS119	NRS123	NRS382	NRS383	NRS384	MRSA252
None	256	64	256	16	64	128	32	256
17[c]	(16)	(16)	(16)	(16)	(16)	(16)	(16)	(16)
20 μM [8.9 mg·L^{-1}]	≤ 0.03	≤ 0.03	0.12	0.25	0.12	≥ 128	0.06	2
16 μM [7.1 mg·L^{-1}]	0.03	0.03	0.50	0.12	0.25	≥ 128	0.12	128
8 μM [3.6 mg·L^{-1}]	128	4	16	0.50	0.50	≥ 128	0.50	256
26[c]	(32)	(8)	(8)	(≥ 64)	(≥ 64)	(32)	(16)	(8)
20 μM [9.0 mg·L^{-1}]	≤ 0.03	NG[d]	NG[d]	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	NG[d]
16 μM [7.2 mg·L^{-1}]	0.12	≤ 0.03	0.03	0.12	0.06	≤ 0.03	0.03	≤ 0.03
8 μM [3.6 mg·L^{-1}]	8	0.25	0.06	0.50	0.50	16	0.25	2
27[c]	(≥ 64)	(8)	(16)	(≥ 64)	(≥ 64)	(≥ 64)	(32)	(32)
20 μM [7.7 mg·L^{-1}]	1	≤ 0.03	≤ 0.03	≤ 0.03	0.25	≥ 128	0.06	128
16 μM [6.2 mg·L^{-1}]	4	0.50	1	0.50	1	≥ 128	0.25	256
8 μM [3.1 mg·L^{-1}]	8	8	0.50	4	1	≥ 128	2	256
28[c]	(16)	(8)	(16)	(16)	(16)	(16)	(16)	(16)
20 μM [8.0 mg·L^{-1}]	≤ 0.03	NG[d]	≤ 0.03	≤ 0.03	≤ 0.03	≥ 128	≤ 0.03	64
16 μM [6.4 mg·L^{-1}]	4	0.50	1	0.50	1	≥ 128	0.50	256
8 μM [3.2 mg·L^{-1}]	16	64	4	≥ 16	1	≥ 128	32	256
29[c]	(16)	(16)	(16)	(16)	(32)	(16)	(16)	(16)
20 μM [7.4 mg·L^{-1}]	2	0.12	4	2	1	≥ 128	4	128
16 μM [5.9 mg·L^{-1}]	16	2	0.50	0.50	2	≥ 128	8	128
8 μM [3.0 mg·L^{-1}]	128	16	32	1	4	≥ 128	8	256

[a]MIC_{OXA} and MIC_{ADJ} were determined in the presence of 2% NaCl. [b]The MIC_{OXA} values < 8 mg·L^{-1} are in red. [c]The MIC_{ADJ} values are in parentheses. [d]NG, no growth due to adjuvants.

Considering that the progenitor of this entire series of compounds is the mammalian protein-kinase inhibitor **1**,[24] we wondered whether the target for these compounds might be one of the three known *S. aureus* kinases Stk1, Cap5B2, or RsbW.[43-45] Stk1 is a protein kinase involved in regulation of a multitude of cellular processes: metabolism, transcription, cell-cycle progression and bacterial virulence. Cap5B2, a Tyr-kinase, phosphorylates proteins involved in the biosynthesis of extracellular capsules. RsbW, a Ser/Thr-kinase, is an anti-σ^B factor and phosphorylates its substrate RsbV. We used lead compound **26** as representative. Full-length Stk1, the kinase domain of Stk1 (Stk1kd), the chimeric Cap5A1ct/B2, RsbW, and RsbV (the substrate of RsbW) proteins were purified for *in vitro* phosphorylation assays (Figure S2). Myelin basic protein (MBP) was a phosphate acceptor for Stk1, Stk1kd, and Cap5A1ct/B2; and RsbV for RsbW. Compound **26** at a concentration of 100 μM did not inhibit these kinases. Hence, its potentiation mechanism is not inhibition of these kinases of *S. aureus* (Figure S3). Alternative target(s) may be the 16 pairs of two-component systems encoding histidine-kinase sensors and response regulators in *S. aureus*, since their deletion also affects resistance.[4,46] This possibility has not been tested.

Conclusion. As discoveries of new classes of antibiotics has not kept pace with emergence of resistance mechanisms in human bacterial pathogens,[47] it has become evident that the usefulness of existing antibiotics must be extended. This study was undertaken with this objective. Penicillin-based chemotherapy became obsolete with the emergence of MRSA.[48–50] The value of the penicillins can be resurrected by adjuvants such as those described in this report. Based on the mechanistic roles that the two-component systems and the *bla* and *mec* operons play in manifestation of the resistance phenotype to β-lactam antibiotics, we believe that there are ample opportunities for design of small molecules that disrupt these machineries in the resistance response by bacteria. It is likely that **26** (and its related compounds) operate by suppressing these resistance mechanisms. The locus of this interference awaits discovery.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

General synthetic procedures and characterization data, experimental methods, supporting Tables S1–S3 and Figures

S1–S3. ^{1}H and ^{13}C NMR, MS for compounds **17**, **26**, **27**, **28**, and **29**.

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Author Contributions

The manuscript was written through contributions of all authors. All authors approve the manuscript.

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ABBREVIATIONS

MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *S. aureus*; VISA, vancomycin-intermediate *S. aureus*; PBP, penicillin-binding protein; OXA, oxacillin; PAINS, Pan-Assay Interference Compounds; MBP, Myelin basic protein.

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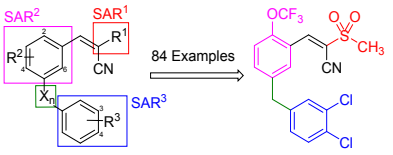

SAR^4
X_0 = No bridging linker
X_1 = O, CH_2, S, SO_2
X_2 = [SAR^2]$NHSO_2$, [SAR^2]CONH

26

Potentiation of oxacillin against NRS119 from 256 mg L^{-1} to 0.06 mg L^{-1} with 3.6 mg L^{-1} of **26**.