New classes of Gram-positive selective antibacterials: Inhibitors of MRSA and surrogates of the causative agents of anthrax and tuberculosis

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ABSTRACT

An antimicrobial phenolic stilbene, (E)-3-hydroxy-5-methoxystilbene, **1** was recently isolated from the leaves of Comptonia peregrina (L.) Coulter and shown to possess inhibitory activity against several Gram-positive bacteria, including isolates of methicillin-resistant Staphylococcus aureus (MRSA), Mycobacterium bovis BCG, and avirulent Bacillus anthracis (Sterne strain), among others. These results prompted the design and synthesis of two new classes of compounds, phenoxystyrenes and phenothiostyrenes, as analogs of the natural antimicrobial stilbene. These and additional stilbenoid analogs were synthesized using new, efficient, copper-mediated coupling strategies. Minimum inhibitory concentration (MIC) antimicrobial assays were performed on all compounds prepared. These preliminary structure–activity relationship studies indicated that both new classes of synthetic analogs, as well as the stilbenes, show promising activity against Gram-positive bacteria when at least one phenolic moiety is present, but not when absent. The potencies of the phenolic phenoxystyrenes and phenothiostyrenes were found to be comparable to those of the phenolic stilbenes tested.

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As part of our ongoing ethnopharmacological efforts to discover new molecules that might be used to combat clinically significant bacterial infections, we identified the phenolic stilbene, (E)-3-hydroxy-5-methoxystilbene, **1** in the leaves of the shrub, Comptonia peregrina (L.) Coulter ('sweet fern').[1] This plant has been used traditionally for its antimicrobial and other biological properties by a number of native North American cultures of the Great Lakes and Maritime regions.[2] Although the cytoxic compound, methyl-p-coumarate had previously been found in this plant,[3] along with numerous other compounds isolated from the essential oil,[4] stilbene **1** had not previously been reported to be present in C. peregrina. Interestingly, compound **1** was identified in the plants, Didymochlaena truncatala (Sw.) J. Sm (Dryopteridaceae)[5] and Alpinia katsumadai Hayata.[6] However, the antimicrobial properties of this natural stilbene **1** were not reported in either of the prior studies.

After the purification of the natural product **1**, a battery of minimum inhibitory concentration (MIC) assays was performed to assess the range of antimicrobial activity.[7] Analysis of those studies indicated that **1** exhibited selective inhibition of the growth of numerous clinically relevant Gram-positive bacteria at reasonably low concentrations.[1] For example, **1** was found to inhibit the growth of methicillin-resistant Staphylococcus aureus (MRSA) with an MIC of 32 μg/mL and of vancomycin-resistant enterococci (VRE) with an MIC of 16 μg/mL (Table 1).[1] Currently, MRSA and VRE are two bacterial species of growing concern to the medical community because of the development of widespread resistance to existing frontline drugs.[8–10] In addition, **1** also showed very good inhibitory activity against the Sterne strain of Bacillus anthracis (MIC 8 μg/mL), the attenuated variant of the causative species of anthrax, as well as promising activity against other related Bacillus species (Table 1). Furthermore, natural product **1** was found to be effective in inhibiting the growth of Mycobacterium bovis with an MIC of 26 μg/mL, which indicated it might serve as a promising lead scaffold in the development of drugs used to combat drug resistant Mycobacterium tuberculosis infections.

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Table 1
Minimum inhibitory concentrations (MIC) of (*E*)-3-hydroxy-5-methoxystilbene **1** purified from *C. peregrina* against several species of bacteria[a]

Organisms	Gram reaction	MIC (µg/mL)
Corynebacterium xerosis	+	16
Corynebacterium diphtheriae Tox-	+	32
Corynebacterium pseudodipthericum	+	16
Staphylococcus aureus ATCC 29213	+	32
Staphylococcus aureus ATCC 25923	+	32
Staphylococcus aureus MRSA MC-1	+	32
Staphylococcus aureus MRSA MC-4	+	32
Enterococcus faecium VRE 1	+	16
Enterococcus faecium VRE 14	+	16
Enterococcus faecalis ATCC 29212	+	16
Listeria monocytogenes	+	32
Streptococcus mitis	+	64
Streptococcus agalactiae	+	32
Streptococcus pyogenes	+	16
Streptococcus pneumoniae ATCC 49619	+	8
Bacillus cereus	+	16
Bacillus megaterium	+	64
Bacillus subtilis	+	16
Bacillus anthracis (Sterne strain)	+	8
Mycobacterium bovis BCG	N/A	26
Escherichia coli	−	>128
Pseudomonas aeruginosa	−	>128

[a] Values represent the mean of three experiments. ATCC, American Type Culture Collection; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci.

Organic and medicinal chemists have found *trans*-stilbene scaffolds to be an intriguing structural class of compounds (e.g., see methoxylated and phenolic stilbenes, Fig. 1).[11,12] These stilbene scaffolds may be considered to be 'privileged structures' because of their wide variety of biological activity.[11,12] Indeed, (*E*)-stilbenoid compounds are widely distributed throughout nature,[13–15] and natural methoxylated and phenolic stilbenes are found in numerous medicinal plants and food products.[11,12] For instance, the natural phenolic stilbene, resveratrol (*trans*-3,5,4′-trihydroxystilbene), has been shown to possess a diverse range of biological and potential therapeutic properties.[11–26] Specifically, resveratrol and its related substituted (*E*)-stilbenes have been forwarded as potential: (i) cancer chemotherapeutic agents that inhibit in vitro growth of human cancer cell lines;[11–15] (ii) anti-inflammatory agents that act through the cyclooxygenase (COX) enzyme inhibition mechanism;[16–18] and (iii) antioxidants,[18] which may serve as chemo-protective agents. Resveratrol and its analogs also may prevent heart disease due to the lipid lowering effects and through the inhibition of lipid peroxidation.[14] Additional properties of resveratrol, such as radical scavenging,[15] neuroprotection,[15] antiviral activity,[15] antibacterial activity,[18–23] inhibition of SARS coronavirus replication,[24] anti-platelet aggregation,[25] anti-carcinogenic activity,[25] anti-HIV activity,[25] and inhibition of human cytochrome P450 1B1[26] have all recently been documented.

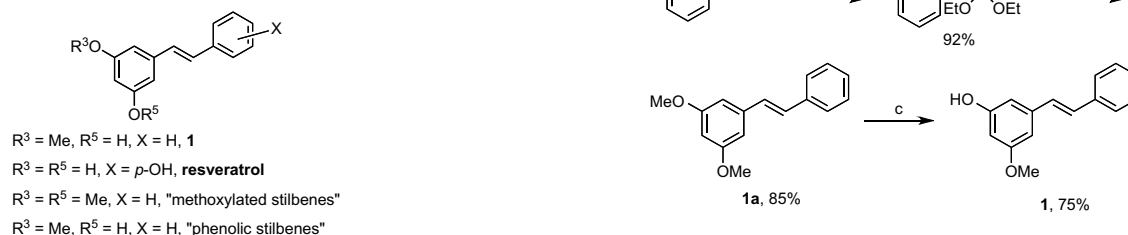On the basis of the many reports on the activity of substituted stilbenes such as resveratrol, as well as the promising antimicrobial studies on the natural stilbene **1**, the design and synthesis of chemically novel analogs of these compounds was undertaken.[1] It was envisioned that functionalized analogs of *E*-phenoxystyrenes and *E*-phenothiostyrenes might fill this role; consequently, new synthetic approaches were developed to create these new agents.[1] In addition, the synthetic methods developed for the preparation of the phenoxystyrenes and phenothiostyrenes was extended to the construction of several substituted *E*-stilbenoid analogs to explore the structure–antimicrobial activity relationships of these classes of compounds related to **1** in greater detail.

Initially, it was decided to prepare gram quantities of the active natural stilbene **1**, and this was accomplished using a Horner–Wadsworth–Emmons process, as shown in Scheme 1.[16] This novel approach to creating the substituted stilbenes was convenient and inexpensive, and the relatively high yields easily produced gram quantities of the desired targets. The condensation of the benzyl bromide-derived phosphorate and 3,5-dimethoxybenzaldehyde, under basic conditions, yielded a mixture of stilbenes **1a** in a *Z:E* ratio of approximately 1:4 (Scheme 1). The pure *E*-3,5-dimethoxystilbene **1a** was obtained from this mixture by crystallization from methanol and water. Subsequent mono-demethylation of (*E*)-**1a** using BBr$_3$ at −78 °C gave the desired phenolic stilbene **1** in good yield.

To begin construction of derivatives of **1** for SAR studies, a practical synthesis was developed using a Negishi cross-coupling process that was directly applied to thiophenes **3** and **4** to give the 2- and 3-styrylthiophenes **5**, **6**, **8**, and **9** (Scheme 2).[27] This approach, gratifyingly, gave only the desired (*E*)-alkene isomers, thereby eliminating the need for the tedious separation of the unwanted (*Z*)-isomer. Importantly, the phenolic 2- and 3-styrylthiophenes **8** and **9**, respectively, were produced efficiently using this one-pot process.[27] Thus, coupling between the TBDPS-protected arylvinyl iodide **7** and the thiophenyl zinc reagent, followed by deprotection using TBAF–THF, afforded the target phenolic styrylthiophenes without the requirement of an extra demethylation step.

It was then felt extension of the vinyl linkage of the stilbenes by addition of either an oxygen or sulfur heteroatom would yield novel, unnatural phenoxystyrene and phenothiostyrene scaffolds that might exhibit enhanced antimicrobial activity. Therefore, the phenolic phenoxystyrene analogs **10**-**15** and the phenolic phenoxythiostyrene analog **16** were prepared employing copper(I)-catalyzed coupling reactions that were recently developed in this laboratory (Scheme 3).[28] This process involved the cross-coupling between the aryl vinyl iodide **7** and the corresponding phenols or thiophenol.[28] In similar fashion to the sequence outlined in Scheme 2, this copper-mediated coupling reaction and the subsequent deprotection could be carried out conveniently in one reaction vessel. Overall, this approach provided an efficient and high-yielding access to these new classes of phenolic stilbene analogs.

Motivated by the positive results obtained from the reactions outlined in Scheme 3, the corresponding oxy-functionalized, dimethoxy phenoxystyrene and phenothiostyrene analogs **17**–**23**, were

R^3 = Me, R^5 = H, X = H, **1**

R^3 = R^5 = H, X = *p*-OH, **resveratrol**

R^3 = R^5 = Me, X = H, "methoxylated stilbenes"

R^3 = Me, R^5 = H, X = H, "phenolic stilbenes"

Figure 1. General structures of methoxylated and phenolic (*E*)-stilbenes.

1a, 85%

1, 75%
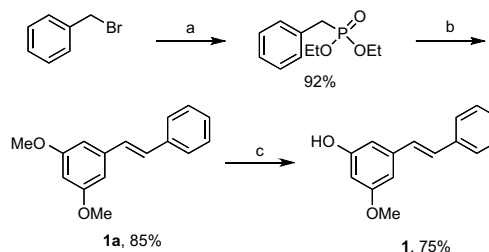
Scheme 1. Reagents and conditions: (a) P(OEt)$_3$, 130 °C, 3 h; (b) NaH, DMF, 0 °C, 3,5-dimethoxybenzaldehyde, rt, 3 h; (c) BBr$_3$, CH$_2$Cl$_2$, −78 °C, 12 h.
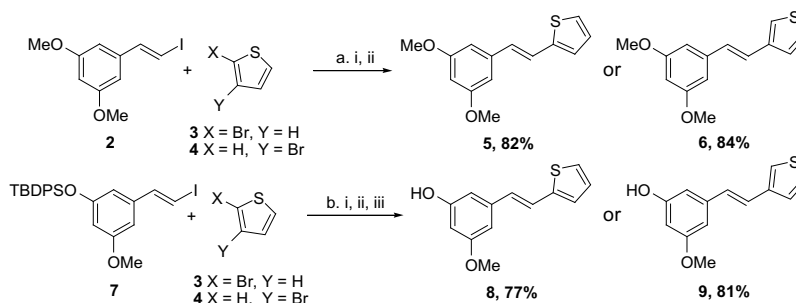
Scheme 2. Reagents and conditions: (a) i—**3** or **4**, *n*-BuLi, −78 °C, 30 min, ZnCl$_2$, 0 °C, 1 h; ii—**2**, Pd$_2$(dba)$_3$, THF, rt, 2 h; (b) i—**3** or **4**, *n*-BuLi, −78 °C, 30 min, ZnCl$_2$, 0 °C, 1 h; ii—**7**, Pd$_2$(dba)$_3$, THF, rt, 2 h; iii—TBAF·THF, rt, 2 h.
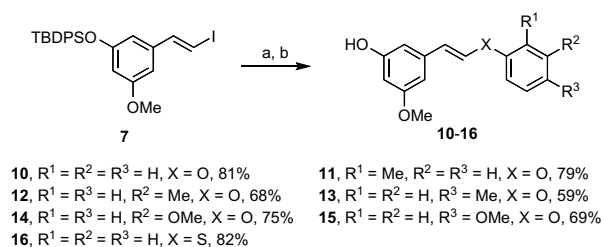
Scheme 3. Reagents and conditions: (a) ArXH, Cu(I)Cl, NMM, Cs$_2$CO$_3$, toluene, reflux, 12–16 h; (b) TBAF·THF, rt, 2 h.
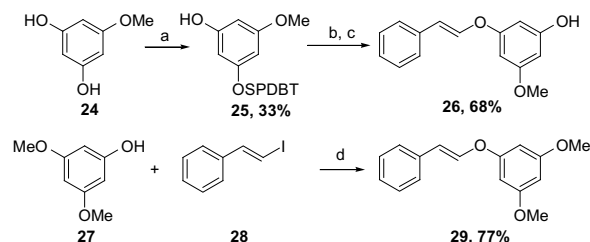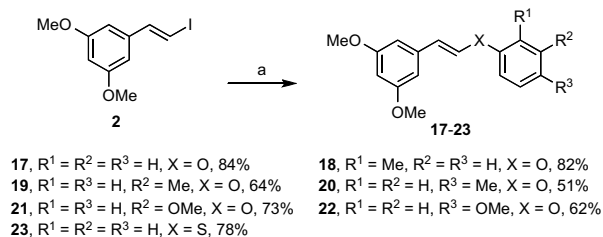

Scheme 5. Reagents and conditions: (a) TBDPSCl, imidazole, DMF, −20 °C, 10 min; (b) **28**, Cu(I)Cl, NMM, Cs$_2$CO$_3$, toluene, reflux, 16 h; (c) TBAF·THF, rt, 2 h; (d) Cu(I)Cl, NMM, Cs$_2$CO$_3$, toluene, reflux, 12 h.

also prepared using the same copper(I) mediated coupling approach (Scheme 4).[28] These cross-coupling reactions were carried out between dimethoxy aryl vinyl iodide **2** and the same phenols, or thiophenol presented in Scheme 3.

The apparent versatility of this coupling method for this class of compounds prompted the modification of the asymmetric phenoxystyrene scaffold by placing the hydroxy/methoxy groups on the phenoxy ring rather than the styryl ring, as represented in compounds **26** and **29** (Scheme 5). As shown in the scheme, the O-protected monophenol **25** gave the 'reversed' phenolic phenoxystyrene **26** after coupling with phenylvinyl iodide **28** and subsequent deprotection. Similarly, the dimethoxy phenoxystyrene **29** was obtained after coupling between **28** and 3,5-dimethoxyphenol **27**.

All compounds prepared in Schemes 2–5 were purified by flash column chromatography using silica gel and an appropriate solvent system. After purification of the phenoxystyrene and phenothiostyrene targets shown in Schemes 3–5, the stability of selected agents was evaluated under acidic (1 M HCl), basic (1 M NaOH), and neutral aqueous media to assess chemical stability. After stirring under these conditions for 36 h, it was found that all of the novel phenoxy and phenothio analogs were stable, and no hydrolysis/degradation products were observed in the solutions (TLC).

Each of the stilbene, phenoxystyrene, and phenothiostyrene target compounds synthesized, as illustrated in Schemes 1–5, was assayed for the ability to inhibit the growth of five selected strains of clinically significant bacteria. The results of these MIC (minimum inhibitory concentration) assays are presented in Table 2. The in vitro MIC determinations for each compound were performed according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines.[7] For safety reasons, Gram-positive microorganisms, *Bacillus cereus* and *Mycobacterium smegmatis* were used as surrogates for the more virulent strains of *B. anthracis* and *M. tuberculosis*, respectively.[29,30] All of the analogs were also tested for their ability to inhibit the growth of *Escherichia coli* as a representative Gram-negative microorganism.

Analysis of the results reported in Table 2 illustrates a number of interesting antimicrobial structure–activity features of the stilbenoid compounds and their extended, heteroatom-containing phenoxystyrene and phenothiostyrene analogs. As expected, none of the compounds exhibited antimicrobial activity against the representative Gram-negative species, *E. coli*. This is in line with the results of preliminary assays performed using the natural stilbene **1** in which no Gram-negative bacteria were affected by this agent (see data in Table 1).

From examination of the data in Table 2, it is clear that the presence of at least one phenolic group is required for activity in this class of compounds. All of the dimethoxylated compounds, **1a**, **5**–**6**, **17**–**23**, and **29**, were devoid of inhibitory activity against the Gram-positive (and Gram-negative) species investigated. However, natural product **1** and all of its analogs that contain a *meta*-hydroxy group (**8**–**16**, **26**) exhibited inhibitory activity against Gram-positive organisms, even when this moiety was located in the phenoxy ring as opposed to the styryl ring, as seen in compound **26**. These results are not surprising because phenolic compounds are viewed as one of the major classes of natural antimicrobial agents.[31]

Perhaps the most exciting finding in this study is that both the phenoxystyrene and phenothiostyrene analogs (**10**–**16**, **26**) of the phenolic stilbenes (**1**, **8**–**9**) are roughly equivalent in their potency in the MIC assays performed. Although none of these vinyl ether or vinyl thioether analogs was significantly better at inhibition of the growth of Gram-positive microorganisms than the original natural

Scheme 4. Reagents and conditions: (a) ArXH, Cu(I)Cl, NMM, Cs$_2$CO$_3$, toluene, reflux, 12–16 h.

Table 2
Minimum inhibitory concentration (MIC μg/mL) values for natural stilbene **1** and the synthetic analogs[a]

Compound	*S. aureus* ATCC 29213	MRSA MC-1	*B. cereus*	*M. smegmatis*	*E. coli*
1	16	16	16	64	>512
1a	>512	>512	>512	>512	>512
5	>512	>512	>512	>512	>512
6	>512	>512	>512	>512	>512
8	16	32	32	128	>512
9	32	32	32	64	>512
10	32	64	32	128	>512
11	16	32	64	>128	>512
12	16	32	32	64	>512
13	16	32	64	>128	>512
14	16	64	64	128	>512
15	16	64	64	128	>512
16	16	32	16	128	>512
17	>512	>512	>512	>512	>512
18	>512	>512	>512	>512	>512
19	>512	>512	>512	>512	>512
20	>512	>512	>512	>512	>512
21	>512	>512	>512	>512	>512
22	>512	>512	>512	>512	>512
23	>512	>512	>512	>512	>512
26	32	32	64	128	>512
29	>512	>512	>512	>512	>512

[a] MIC values represent the mean of three experiments. ATCC, American Type Culture Collection; MRSA, methicillin-resistant *Staphylococcus aureus*.

product **1**, many were approximately equipotent in the assays. For example, the phenolic ether **12** was equipotent to **1** in the inhibition of the growth of both *S. aureus* and *M. smegmatis* (16 μg/mL) and only 2-fold less potent than **1** in the inhibition of the growth of methicillin-resistant *S. aureus* (MRSA) and *B. cereus* (32 μg/mL). Likewise, phenolic thioether **16** had the same MIC value as **1** in the inhibition of the growth of both *S. aureus* and *B. cereus* (16 μg/mL), and was only 2-fold less potent than **1** against MRSA (32 μg/mL) and *M. smegmatis* (128 μg/mL). These initial results are highly encouraging in consideration of the viability of using phenoxystyrene and phenothiostyrene scaffold structural analogs of bioactive antimicrobial stilbenes to treat disease because they are stable at low and high pH values. Indeed, these novel scaffolds may serve as excellent starting points for further chemical optimization and the development of new drugs that are certainly needed to combat threatening MRSA, tuberculosis, and anthrax infections. Furthermore, because *trans*-stilbenes have been shown to possess such a diverse array of biological activity,[11–26] these findings should promote additional SAR studies of stilbenoid compounds for additional therapeutic areas.

In conclusion, the antibacterial activity of a phenolic (*E*)-stilbene 1 isolated from *C. peregrina* has been evaluated and demonstrated to be active against a range of Gram-positive bacteria, including drug resistant *S. aureus* and enterococci spp., but not Gram-negative bacteria. Importantly, **1** was active against *M. bovis* (BCG) and against *B. anthracis*, as well as three related *Bacillus* species. When the *phenolic* stilbene scaffold was modified with other aryl moieties or extended by one heteroatom to create the novel phenoxystyrene and phenothiostyrene derivatives, these agents also demonstrated promising antimicrobial activity, given that at least one phenolic moiety was present, as in compounds **8–16** and **26**. Consequently, further exploration of these novel scaffolds is warranted from both synthetic and microbiological perspectives. Additional SAR studies of these novel homologous stilbenoid compounds and more extensive microbiological characterization of the active agents are currently underway.

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