The Synthesis and Antimicrobial Activity of Heterocyclic Derivatives of Totarol

Michelle B. Kim,[†] Terrence E. O'Brien,[†] Jared T. Moore,[†] David E. Anderson,[†] Marie H. Foss,[‡] Douglas B. Weibel,[‡,§] James B. Ames,[†] and Jared T. Shaw*,[†]

[†]Department of Chemistry, University of California, One Shields Avenue, Davis, California 95616, United States

[‡]Department of Biochemistry and [§]Department of Biomedical Engineering, University of Wisconsin, Madison, Wisconsin 53706, United States

S *Supporting Information*

ABSTRACT: The synthesis and antimicrobial activity of heterocyclic analogues of the diterpenoid totarol are described. An advanced synthetic intermediate with a ketone on the A-ring is used to attach fused heterocycles, and a carbon-to-nitrogen atom replacement is made on the B-ring by de novo synthesis. A-ring analogues with an indole attached exhibit, for the first time, enhanced antimicrobial activity relative to the parent natural product. Preliminary experiments demonstrate that the indole analogues do not target the bacterial cell division protein FtsZ as had been hypothesized for totarol.

KEYWORDS: *totarol, antimicrobial activity, small molecule inhibitors, FtsZ, structure−activity relationships and optimization*
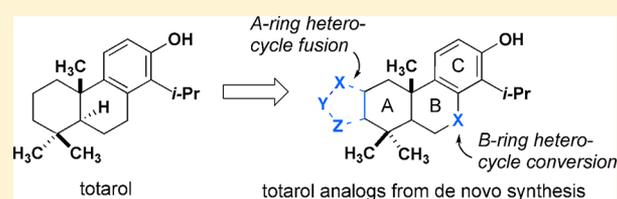
Methicillin-resistant *Staphylococcus aureus* (MRSA) is now the leading cause of bacterial infections affecting numerous hospitalized patients worldwide.[1] Despite the rise in antibiotic resistance, the discovery of new small molecule antibiotics has decreased. The demand for a new antimicrobial agent that targets a new protein function is necessary, but in recent years, there have been few antibiotics based on new structural classes that have been discovered.

The phenolic diterpenoid totarol is a major constituent isolated from the sap of *Podocarpus totara*, a tree that is native to New Zealand.[2] Totarol exhibits antiplasmodial,[3] antifungal,[4] and antimicrobial activity against several organisms, including *Propionibacterium acnes*[5,6] and MRSA.[5,7] The mechanism of action by which totarol derives its antimicrobial activity has not been fully elucidated. That said, it has been speculated that this compound may inhibit bacterial respiratory transport,[8] disrupt phospholipid membranes,[9] or function as an efflux-pump inhibitor.[10] Totarol was recently reported to inhibit bacterial cytokinesis by targeting the protein FtsZ.[11]

Previous structure−activity relationship (SAR) studies have focused on modifications of the B- and C-rings without any observed increase in antimicrobial activity (Figure 1).[12−14] Our interests have been directed toward SAR studies on heterocyclic analogues of totarol, which have not yet been explored. In addition to A-ring analogues that might exhibit enhanced activity (Figure 1C), we envisioned that a more soluble form of totarol would emerge by replacing one of the B-ring carbons with nitrogen. This report describes the synthesis and preliminary evaluation of these two basic modifications to totarol.

The concise, enantioselective synthesis of (+)-totarol developed in our lab enabled us to modify the A-ring through
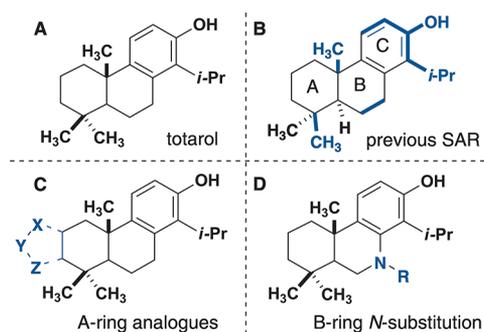

Figure 1. (A) Structure of (+)-totarol (**1**). (B) Previous SAR of (+)-totarol; areas modified in the study are highlighted in blue (refs 12−14). (C) A-ring analogues described in this study. (D) B-ring derivative containing a heterocycle installed de novo.

an advanced intermediate (+)-**2** (Figure 2). Intermediate (+)-**2** was synthesized from two commercially available fragments, dimethoxybenzonitrile **3** and geraniol **4**, through an asymmetric nine-step sequence.[15] In the first series of analogues, methyl totarolone (+)-**2** was converted into structurally diverse analogues at positions C2 and C3. Claisen condensation of **2** with ethyl formate formed an intermediate used in the preparation of several azoles. Substituting the ketone to an oxime allowed us to expand the A-ring to a lactam by Beckmann rearrangement or reduced to the primary amine.
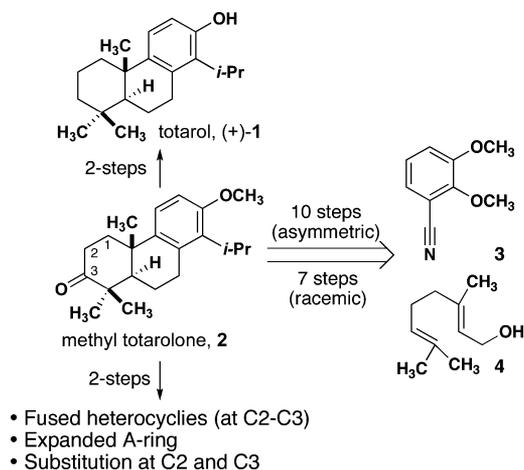
Figure 2. Advanced intermediate (+)-**2**, synthesized from nitrile **3** and geraniol (**4**) in 10 steps, as a key intermediate for A-ring analogues.

Eight enantiomerically enriched derivatives were synthesized by exploiting the A-ring ketone functionality on (+)-**2**. A formate group was installed by a Claisen condensation as a means to synthesize heterocyclic derivatives on the A-ring of (+)-**2** (Scheme 1).[16] Treating (+)-**2** with sodium hydride,

**Scheme 1. Synthesis of Azole-Fused Totarol Analogues**
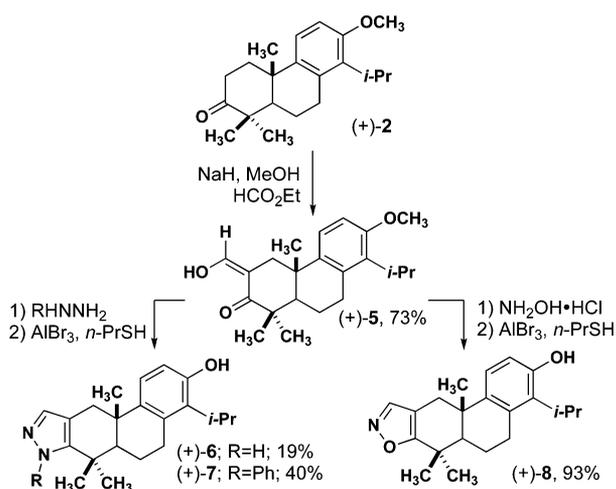
methanol, and ethyl formate produced intermediate (+)-**5** in 73% yield. Azoles (+)-**6** and (+)-**7** were prepared as their methyl ethers (not shown) by treatment of (+)-**5** with hydrazine monohydrate or phenylhydrazine hydrochloride, respectively.[17] Demethylation was achieved using AlBr$_3$ and n-PrSH to give pyrazole (+)-**6** in 19% and phenyl pyrazole (+)-**7** in 40% yield over two steps. Isoxazole (+)-**8** resulted from heating (+)-**5** in the presence of hydroxylamine hydrochloride[18] and subsequent deprotection.

The A-ring ketone of **2** was used in several additional modifications. Acetic acid-mediated Fischer indole conditions with (+)-**2** and phenylhydrazine hydrochloride followed by methyl deprotection yielded "indolototarol" (+)-**9** in 20% yield over two steps.[19] A-ring analogues with hydrogen-bonding capability were also prepared. Methyl totarolone (+)-**2** was converted to methyl oxime (+)-**10** in 83% yield.[20] A ring expansion of the A-ring to the corresponding caprolactam was achieved by Beckmann rearrangement induced by treating

oxime (+)-**10** with thionyl chloride.[21] The resultant lactam was demethylated to (+)-**12** in 46% yield over two steps. Two additional derivatives were obtained sequentially by removing the methyl ether on (+)-**10** with n-PrSH and AlBr$_3$ to oxime (+)-**11** and then reducing oxime (+)-**11** to amine (+)-**13** with LiAlH$_4$ (Scheme 2).[22]

Scheme 2. Synthesis of Diverse Totarol A-ring Analogues from Ketone 2
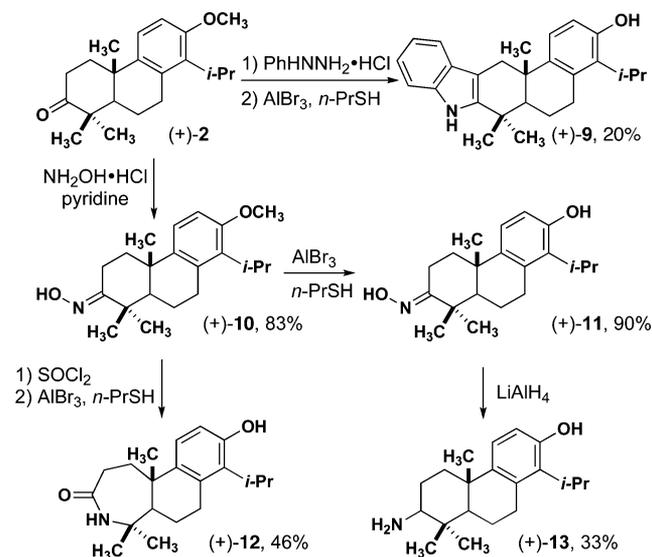
Carbon-to-nitrogen substitution of the B-ring was also investigated as a means to enhance solubility and, potentially, activity toward FtsZ.[23] Although both oxygen- and nitrogen-containing derivatives were initially investigated, preparation of the oxygenated core structure could not be accomplished by a cyclization reaction analogous to what was employed for totarol.[24] The synthesis of nitrogen-containing derivatives proved successful using a catalytic cyclization reaction recently discovered in our laboratory.[25] Azatotarol **16** was synthesized by replacing a benzylic carbon with nitrogen prior to the cyclization. Complex tetrahydroisoquinoline **14** (Scheme 3) resulted from a diastereoselective heteropolycyclization reaction of a geranylated aniline derivative via an episulfonium intermediate.[26] Compound **14** was converted to "azatotarol" (**16**) by removal of both the thioether and the nitrobenzenesulfonyl (Ns, nosyl) groups by reduction with Raney nickel and subsequent deprotection. N-Acetylated azatotarol **17**

Scheme 3. Synthesis of Totarol B-Ring Analogues from Cyclization Product 14
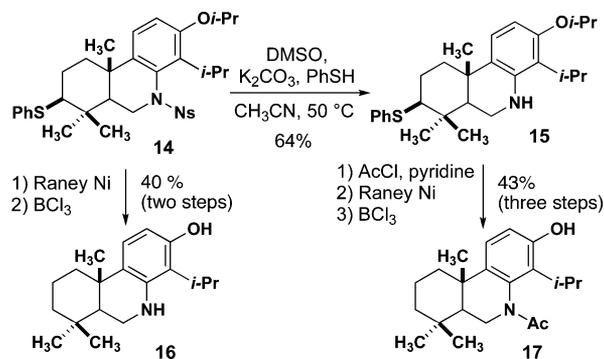
required a different order of events, specifically nosyl group cleavage of intermediate **14** to yield the free amine **15** in 64% yield. This compound was acylated with acetyl chloride and treated with Raney nickel to remove the thiophenyl ether. Final deisopropylation was achieved using BCl₃ to give **17** in 43% yield over the three steps.

The first round of totarol analogues was evaluated for antibacterial activity by measuring minimum inhibitory concentrations (MICs, Table 1) for *B. subtilis* 168. Although

Table 1. Preliminary MIC Measurements for A-Ring Derivatives against *B. subtilis* 168

compound	MIC	compound	MIC
1, totarol	10	**11**	>160
5	>160	**12**	>160
6	>160	**13**	>160
7	>160	**16**	20
8	40	**17**	>256
9	2.5		

most analogues showed little or no inhibition of growth, three compounds showed notable activity. A-ring analogue isoxazole (+)-**8** and indole (+)-**9** were approximately 4-fold less and 4-fold more potent, with MICs of 40 and 2.5 μM, respectively (Table 1). "Azatotarol" analogue **16** showed modest growth inhibition (20 μM), while N-acylated analogue **17** was inactive. These results are consistent with the relatively stark structural changes that were made to the structure of totarol and were successful in identifying one compound (**9**) with enhanced activity. These studies are also notable in demonstrating that the whole of totarol's activity is not simply due to the presence of a free phenolic hydroxyl group.

The promising antimicrobial activity of the indolototarol derivative prompted us to further functionalize the indole with the goal of developing a more potent antimicrobial compound. Unfortunately, the yields of indoles from standard Fischer conditions using hindered ketone **16** varied greatly depending on the electronic nature of the hydrazine. Standard protic and Lewis acidic conditions did not offer a general solution.[27,28] The Fischer indole synthesis on our hindered ketone required us to investigate conditions on a model system. A model system of dimethyl-β-tetralone **18** was employed to find optimal reaction conditions (Table 2). After examining several different conditions, a recently reported Sb₂(SO₄)₃-catalyzed Fischer

Table 2. Optimization of Fischer Indole Synthesis with Ketone 18 as a Model System
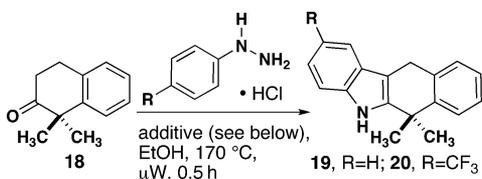
| entry	R	additive	mol %	yield (%)
1	H	Sb₂(SO₄)₃	10	78
2	H	MgSO₄	10	79
3	H	ZnCl₂	10	80
4	H			85
5	CF₃			NR
6	CF₃	Sb₂(SO₄)₃	100	61

indole synthesis provided significant improvements in yield.[29] Optimization reactions were carried out using microwave irradiation, and the initial improvement in the reaction of phenylhydrazine was traced to this more efficient heating method and the use of ethanol as solvent. Use of these optimized conditions with the more problematic *p*-CF₃ phenylhydrazine demonstrated that the addition of Sb₂(SO₄)₃ was crucial to the success of the reaction. (Table 2, entries 5 and 6). Little product formation was observed without the additive, whereas a 61% yield of indole **19** was observed in the presence of 1 equiv of Sb₂(SO₄)₃. Although the role of Sb₂(SO₄)₃ in this reaction is unclear, subsequent Fischer indole reactions were consistently carried out using 1 equiv of this additive to ensure optimal yields.

The second round of A-ring indole analogues was prepared using racemic starting material ($\pm$)-**2** (Table 3). The racemic synthesis of methyl totarolone ($\pm$)-**2** was achieved in a seven-step synthesis that is both more expedient and higher yielding than the enantioselective route. In addition to the ease of synthesis, examination of a racemic mixture can reveal instances where the opposite enantiomer has enhanced activity provided that separate enantiomers are easily accessed once activity is observed. Our optimized indole synthesis involved treating ($\pm$)-**2** with substituted phenylhydrazine salts and heating in a microwave reactor in the presence of 1 equiv of Sb₂(SO₄)₃. The resulting ($\pm$)-indole analogues were taken directly into established demethylation conditions using *n*-PrSH and AlBr₃ to give the desired products over two steps.

Indolototarol derivatives ($\pm$)-**21**−**29** were assessed for antimicrobial activity against *B. subtilis* 168 (Table 4). In this experiment, we observed minor variation from the first round in that totarol's MIC (2.5 μM) was slightly lower than (+)-**9** (4 μM). The *o*-ethyl ($\pm$)-**28** and *p*-ethyl ($\pm$)-**25** indoles were also comparable to totarol with MIC values of 1.0 and 2.5 μM, respectively. Three indoles with electron-withdrawing substituents, that is, *m,m*-dichloro ($\pm$)-**21**, *p*-CF₃ ($\pm$)-**22**, and *p*-CN ($\pm$)-**23**, exhibited the best activity at 0.78 μM. The hydroxyl and sulfonamide groups of ($\pm$)-**29** and ($\pm$)-**26** reduced the activity slightly and showed MICs of 6.25 and 5.5 μM, respectively. Previous studies by Evans et al. demonstrated that O-methylation completely removes all antimicrobial activity.[12] Consistent with this trend, compound ($\pm$)-**30**, the methyl ether precursor of ($\pm$)-**23**, is completely inactive. Totarol[11] and vancomycin[30] were used as positive controls, and their MICs corresponded with literature values.

Indole analogues (+)-**9** and ($\pm$)-**23** were investigated for their ability to inhibit the GTPase activity of FtsZ, one of the putative targets of totarol (Figure 3). An enzyme-coupled assay monitoring the hydrolysis of GTP (guanosine triphosphate) was used to assess the inhibitory concentration resulting in 50% enzyme activity (IC₅₀). Totarol (**1**) exhibited an IC₅₀ of 24 μM, which compares favorably to the reported value. To our surprise, (+)-**9** and ($\pm$)-**23** showed no activity in the assay up to 64 μM. Because of this drastic decrease in activity between totarol and indolototarol analogues in this enzymatic assay, the validity of totarol as an FtsZ inhibitor was brought into question. Studies conducted by Shoichet have demonstrated that small organic molecules can act as nonspecific inhibitors by forming large colloidal aggregates in aqueous media.[31,32] In their investigations, aggregates were disrupted by including 0.01% of a detergent into the enzymatic assay, resulting in a loss of activity against the target protein in the cases of nonspecific inhibition. The addition of 0.01% of triton X-100, a nonionic

Table 3. $Sb_2(SO_4)_3$-Mediated Synthesis of Indoles 21−29
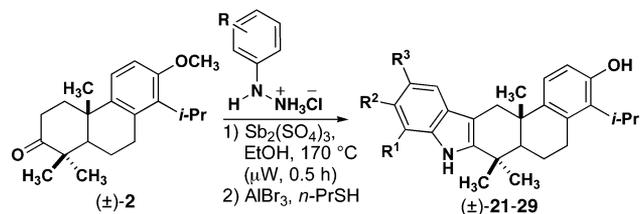
compound	R^1	R^2	R^3	yield[a] (%)	compound	R^1	R^2	R^3	yield[a] (%)
21	Cl	H	Cl	34	26	H	H	SO$_2$NH$_2$	33
22	H	H	CF$_3$	20	27	CH$_3$	CH$_3$	H	66
23	H	H	CN	40	28	Et	H	H	11
24	H	H	F	66	29	OH	H	H	33
25	H	H	Et	41					

[a]Over two steps after purification.

Table 4. MIC of Indolototarol Derivatives against *B. subtilis*
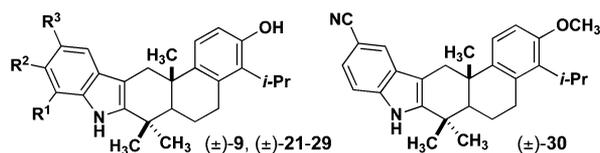
compound	R^1	R^2	R^3	MIC	compound	R^1	R^2	R^3	MIC
9	H	H	H	4.0	27	CH$_3$	CH$_3$	H	11
21	Cl	H	Cl	0.78	28	Et	H	H	1.0
22	H	H	CF$_3$	0.78	29	OH	H	H	6.3
23	H	H	CN	0.78	30				>100
24	H	H	F	80	1, totarol				2.5
25	H	H	Et	2.5	vancomycin				0.63
26	H	H	SO$_2$NH$_2$	5.5					

detergent, into the enzyme-coupled GTPase assay eliminated the inhibitory activity of totarol. This experiment suggests that the activity of totarol is caused by aggregates of the molecule and not inhibition of FtsZ.
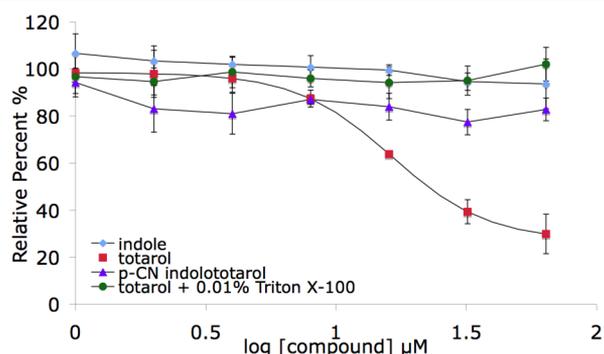

Figure 3. Enzyme-coupled GTPase assay of totarol and analogues for *B. subtilis* FtsZ.

We have described the synthesis of heterocyclic analogues of totarol enabling the discovery, for the first time, of a compound with enhanced antimicrobial activity. By examining structurally diverse scaffolds available from our synthetic intermediate, we were able to identify the indole moiety as a scaffold for further exploration. Three indolototarol derivatives were found to have increased antimicrobial activity as compared to the parent compound totarol. The lack of activity in the enzymatic assay for FtsZ inhibition prompted us to demonstrate that inhibition

by totarol results from unselective inhibition by aggregates that form in solution.[33] Given the compelling antimicrobial activity of (±)-23, current studies are directed at identifying the target of this compound through the selection and sequencing of resistant strains of *B. subtilis*.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic experimental details, analytical data of compounds, and biological assay protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jtshaw@ucdavis.edu.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

■ ABBREVIATIONS

MIC, minimum inhibitory concentration; IC_{50}, inhibitory concentration at 50%; GTP, guanosine triphosphate

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