MedChemComm

RESEARCH ARTICLE

View Article Online
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Cite this: *Med. Chem. Commun.*, 2017, **8**, 942

# Targeting quinolone- and aminocoumarin-resistant bacteria with new gyramide analogs that inhibit DNA gyrase†‡§

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Received 7th January 2017,
Accepted 21st February 2017

DOI: 10.1039/c7md00012j

rsc.li/medchemcomm

Bacterial DNA gyrase is an essential type II topoisomerase that enables cells to overcome topological barriers encountered during replication, transcription, recombination, and repair. This enzyme is ubiquitous in bacteria and represents an important clinical target for antibacterial therapy. In this paper we report the characterization of three exciting new gyramide analogs—from a library of 183 derivatives—that are potent inhibitors of DNA gyrase and are active against clinical strains of Gram-negative bacteria (*Escherichia coli*, *Shigella flexneri*, and *Salmonella enterica*; 3 of 10 wild-type strains tested) and Gram-positive bacteria (*Bacillus* spp., *Enterococcus* spp., *Staphylococcus* spp., and *Streptococcus* spp.; all 9 of the wild-type strains tested). *E. coli* strains resistant to the DNA gyrase inhibitors ciprofloxacin and novobiocin display very little cross-resistance to these new gyramides. *In vitro* studies demonstrate that the new analogs are potent inhibitors of the DNA supercoiling activity of DNA gyrase ($IC_{50}$s of 47–170 nM) but do not alter the enzyme's ATPase activity. Although mutations that confer bacterial cells resistant to these new gyramides map to the genes encoding the subunits of the DNA gyrase (*gyrA* and *gyrB* genes), overexpression of GyrA, GyrB, or GyrA and GyrB together does not suppress the inhibitory effect of the gyramides. These observations support the hypothesis that the gyramides inhibit DNA gyrase using a mechanism that is unique from other known inhibitors.

## Introduction

DNA gyrase is a validated antibiotic target that continues to capture attention in drug discovery.[1] DNA gyrase is a type II topoisomerase consisting of two subunits that combine into a heterotetrameric A2B2 holoenzyme complex and uses the energy from ATP hydrolysis to negatively supercoil double-stranded DNA preceding the replication fork. The GyrA subunit is involved in stabilizing the double-stranded DNA break, passing the second DNA strand through the break, and religating the DNA strands. The GyrB subunit contains the ATP binding site and is involved in ATP hydrolysis.[2,3]

Antibiotics that bind to different regions of DNA gyrase have been reported. Ciprofloxacin (**1**) (Fig. 1A) is a clinical fluoroquinolone antibiotic that binds DNA gyrase between the DNA nucleotides on either side of the double stranded DNA break, prevents DNA religation, and stabilizes double-stranded breaks in the DNA bound to the protein.[4] The formation of linear double stranded DNA triggers the SOS response and inhibits cell division until the DNA is repaired.[5] Novobiocin (**2**) (Fig. 1A) is an aminocoumarin that binds in the ATP pocket of the GyrB subunit and competitively inhibits its ATPase activity.[6,7] In contrast to **1**, **2** does not cause DNA damage or activate the SOS response;[5] however it causes many species of cells to become filamentous, which is a hallmark of the SOS response.[8] Both **1** and **2** also inhibit topoisomerase IV[9–11]—the bacterial topoisomerase involved in decatenation of chromosomes during DNA replication. **1** also binds to the

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† The authors declare no competing interests.
‡ The authors declare no competing financial interest.
§ Electronic supplementary information (ESI) available: Tables, figures, bacterial strain information and compound purity data. See DOI: 10.1039/c7md00012j
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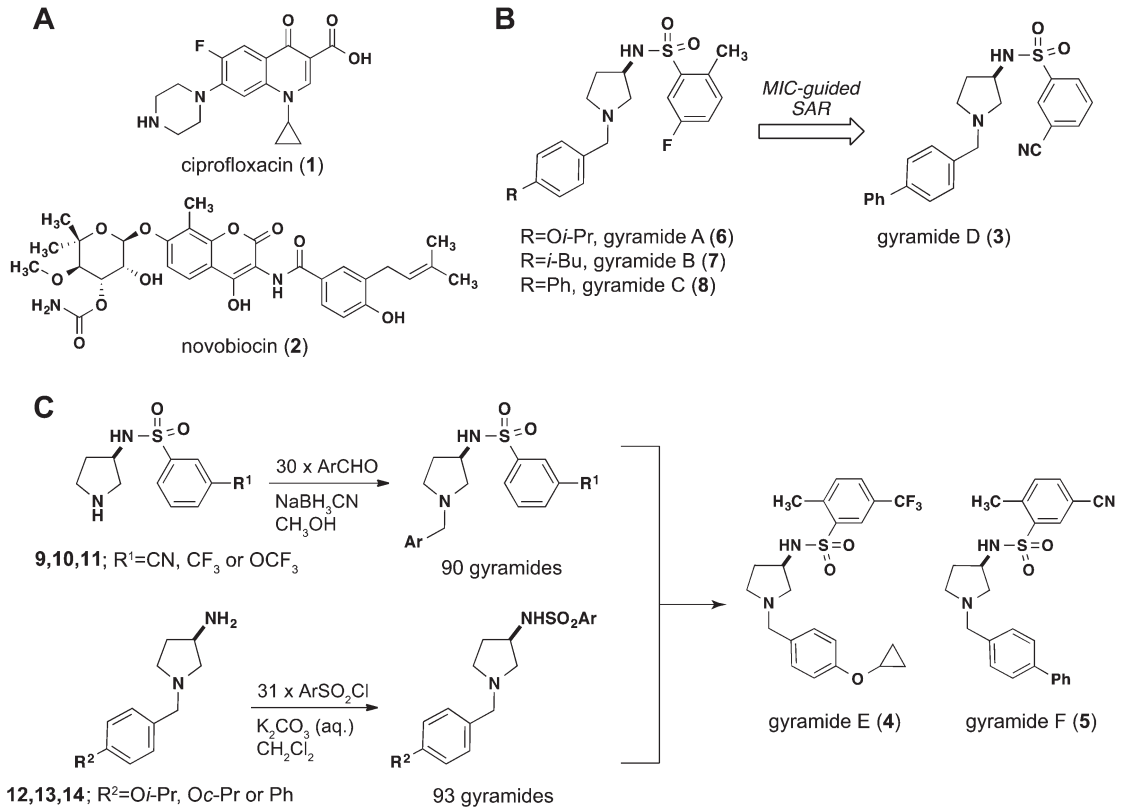


**Fig. 1** A) The chemical structures of ciprofloxacin (**1**) and novobiocin (**2**). B) **3** was created from a MIC-guided SAR study of **6**–**8**. **3** has activity against wild-type *E. coli* and was used as a lead structure for further analog design and synthesis. C) Design of a chemical library of 183 gyramide analogs. Two reactions were performed with 30 aldehydes (and 3 arylsulfonamides) and 31 sulfonyl chlorides (and 3 arylamines) to create 183 gyramide analogs.

eukaryotic homologue topoisomerase II (topo II) causing associated side effects.[12]

The gyramides are a family of synthetic, small molecules that inhibit the type II topoisomerase DNA gyrase;[13,14] our current data supports these compounds binding to a new site of DNA gyrase[14] that differentiates them from the binding site for **1**, **2**, and other families of DNA gyrase inhibitors.[4,15,16] Mapping amino acid mutations that convey gyramide resistance to *Escherichia coli* cells and comparing to related data for **1** and **2** reveals that these positions are spatially distinct.[14] Gyramides are specific inhibitors of DNA gyrase and do not inhibit bacterial topoisomerase IV.[17] We hypothesize that DNA gyrase inhibition by the gyramides stalls replication fork progression, impairs chromosome segregation, and initiates the SOS response through a non-canonical pathway that ultimately blocks cell division.[17] A challenge with this family of compounds has been reducing drug efflux out of cells, which has limited *in vivo* experiments to-date and created a roadblock toward developing antimicrobial agents that are effective against wild-type cells. Using insight gleaned from an analysis of successful antibiotics proved largely ineffective in our design of gyramide analogs.[18]

In this paper we describe a series of new gyramides with potent activity against DNA gyrase and that have an apparent reduction in efflux from different bacterial species. We tested the compounds for antibiotic activity against 9 strains of non-pathogenic (biosafety level 1, BSL-1) bacteria and 13 pathogenic isolates (BSL-2) to identify compounds with reduced drug efflux, improved inhibitory activity, and extended spectrum. We performed further characterization of three new gyramide analogs [gyramide D (**3**), gyramide E (**4**), and gyramide F (**5**)] (Fig. 1B and C) that fit these criteria, and demonstrate that these compounds have several exciting characteristics: 1) they are among the most potent inhibitors of the supercoiling activity of DNA gyrase to-date; 2) they have MICs in the single μg $mL^{-1}$ range against a range of wild-type, clinical Gram-negative and Gram-positive bacteria; 3) they have activity against *E. coli* strains resistant to **1** and **2**; and 4) their activity is not antagonized by the overexpression of DNA gyrase (akin to **1**). Our data supports the hypothesis that the improvement of the antibacterial activity of these compounds arises from reducing efflux, suggests chemical modifications for reducing the efflux of other aryl-containing antibiotics, and moves these compounds one step further along their development as a new family of antibiotics.

## Results and discussion

### Discovery and characterization of the antibacterial activity of 3

We previously altered the substituents of the benzyl group in gyramide A (**6**)—discovered in a high-throughput screen—to

create analogs with limited success, including gyramide B (7) and gyramide C (8).[13] In this paper we introduce different functional groups to the arylsulfonamide ring to change its size and polarity (Fig. 1B). In lieu of a co-crystal structure to enable a rational optimization of the compounds, we expanded the initial set of compounds reported previously by exploring the diversity available within commercially available aldehydes and sulfonyl chlorides, while avoiding functionality on the two aromatic rings with known metabolic liabilities (*e.g.*, nitro groups). We initially prepared 48 analogs (structures not shown) and found that 47 had higher MICs than the parent compounds (**6–8**) against *E. coli* strains. The remaining compound (gyramide D, **3**)—containing a 3-cyano group on the arylsulfonamide ring—was remarkably potent and killed wild-type *E. coli* strains (Fig. 1B). Based on this compound, we performed a thorough and systematic variation of the two core appendages, *i.e.* the *N*-benzyl and *N*-arylsulfonyl substituents. Although we initially envisioned a matrix of four cores containing 50 appendages, the limited availability of different benzaldehydes, sulfonyl chlorides, and hetero-aromatic substrates created a hurdle. After scrutinizing available building blocks, we settled on an expanded set of cores: a set of three 3-amino-1-benzyl pyrrolidines treated with 31 sulfonyl chlorides and a set of three 3-sulfonamido pyrrolidines benzylated with 30 substituted benzaldehydes and heteroaromatic analogs (Fig. S1§); the combination of these two sets of intermediates yielded a library consisting of 183 analogs (Fig. 1C). Each gyramide analog in the library was synthesized on >5 mg scale and purified to >95% purity by HPLC (see ESI§). Screening of these 183 compounds as described below revealed two new gyramides (E and F; **4** and **5**) that we re-synthesized on preparative scale to perform additional experiments (Fig. S2§).

We found that inclusion of a 3-cyano group in **3** significantly improved the MIC of the compounds against a wild-type laboratory strain of *Escherichia coli* (16 μg mL$^{-1}$) compared to previously reported analogs **6–8** (activity of >160 μg mL$^{-1}$ against wild-type *E. coli*);[14] **3** also displayed activity against an *E. coli* UPEC strain (16 μg mL$^{-1}$), *Shigella flexneri* (8 μg mL$^{-1}$), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S. enterica enterica* Typhimurium) (2 μg mL$^{-1}$), and *Edwardsiella tarda* (16 μg mL$^{-1}$) (Table 1).

### 3–5 are active against various strains of Gram-negative and Gram-positive bacteria

The antibacterial activity of **3** against *E. coli*, *S. flexneri*, *S. enterica enterica* Typhimurium, and *E. tarda* encouraged us to explore additional changes to the substituents on the sulfonamide ring. We were interested in whether we could increase the activity against *E. coli*, identify derivatives that were potent and effective against other Gram-negative bacteria, and discover compounds with a preference for Gram-negative or Gram-positive bacteria. After introducing a series of electron-withdrawing groups into the 3-position of the arylsulfonamide ring (not shown), we noticed that a nitrile increased activity and inhibited growth of wild-type *E. coli* cells. Additional modifications eventually produced **4** and **5**, both of which have an MIC of 8 μg mL$^{-1}$ against *E. coli* BW25113 (Fig. 1C and Table 1).

Of the Gram-negative strains we tested, **3–5** were effective against *E. coli*, *Edwardsiella tarda*, *S. enterica enterica*

**Table 1** MICs of **3–5** against Gram-negative and Gram-positive bacterial strains. Italics text highlights the lowest MIC values

| | | Compound (μg mL$^{-1}$) | | |
|---|---|---|---|---|
| Bacterial strains | | **3** | **4** | **5** |
| Gram-negative bacteria | *E. coli* BW25113 | *16* | *8* | *8* |
| | *E. coli* JW5503 | *0.1* | *0.4* | *0.1* |
| | *E. coli* MC4100 | *8* | *16* | *8* |
| | *E. coli* BAS849[b] | *1* | *1* | *0.5* |
| | *E. coli* UPEC[a] | *16* | >16 | >16 |
| | *Acinetobacter baumannii*[a] | >16 | >16 | >16 |
| | *Edwardsiella tarda*[a] | *16* | *8* | *8* |
| | *Enterobacter aerogenes*[a] | >16 | >16 | >16 |
| | *Klebsiella pneumoniae* | >16 | >16 | >16 |
| | *Morganella morganii*[a] | >16 | >16 | >16 |
| | *Pseudomonas aeruginosa* | >16 | >16 | >16 |
| | *Salmonella enterica* | *2* | *4* | *2* |
| | *Shigella flexneri* | *8* | *4* | *4* |
| Gram-positive bacteria | *Bacillus subtilis* | >16 | *8* | >16 |
| | *Bacillus cereus* | >16 | *8* | >16 |
| | *Enterococcus faecalis* | >16 | *16* | >16 |
| | *Enterococcus faecium* | >16 | *8* | >16 |
| | *Staphylococcus aureus*[a] | >16 | *8* | >16 |
| | *Staphylococcus saprophyticus*[a] | >16 | *8* | >16 |
| | *Staphylococcus epidermidis*[a] | >16 | *8* | >16 |
| | *Streptococcus agalactiae* | >16 | *8* | >16 |
| | *Streptococcus pyogenes* | >16 | *4* | >16 |

[a] Denotes clinical isolates. [b] Denotes hyperpermeable strain derivative of MC4100.

 

Typhimurium, and *S. flexneri* with MICs in the range of 2–16 μg mL$^{-1}$ (Table 1). Of the three gyramide analogs, **4** was the only compound that was effective against all 9 of the Gram-positive strains tested (MIC values ranging from 4–16 μg mL$^{-1}$), including *Bacillus subtilis*, *Bacillus cereus*, *Enterococcus faecium*, *Staphylococcus aureus* subsp. *aureus*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Enterococcus faecalis*, and *Streptococcus pyogenes* (Table 1).

### Analogs 3–5 reduce gyramide efflux in bacteria

Drug efflux systems and membrane permeability are two factors that affect the activity of antimicrobials against Gram-negative and Gram-positive bacteria.[19] To gauge the extent of drug efflux, we measured the MIC of **3–5** against an *E. coli* strain lacking the outer membrane component of the AcrAB-TolC efflux pump (TolC): strain JW5503 (derived from *E. coli* BW25113). We used the relative ratio of the MICs of compounds against *E. coli* BW25113 and *E. coli* JW5503 as an indicator of compound efflux, where $MIC_{BW25113}/MIC_{JW5503}$ is 1 in the absence of efflux, and high values indicate more efflux. As a point of reference, **8** [a previously reported analogue structurally related to **3** (ref. 14)] had the same MIC (0.1 μg mL$^{-1}$) as **3** against *E. coli* strain JW5503, yet its MIC against *E. coli* BW25113 was >160 μg mL$^{-1}$ (a ratio of $MIC_{BW25113}/MIC_{JW5503} > 1600$, indicating significant efflux). We found ratios for **3**, **4**, and **5** of 160, 20, and 80, respectively, indicating that the AcrAB-TolC efflux pump complex reduces the potency of the gyramides (Table 1), albeit less so than for previous analogues. Modification of the gyramides to include a cyano group (**3** and **5**) or a trifluoromethyl group (**4**) at the 3-position of the benzenesulfonamide ring reduced efflux significantly; our data suggests that the trifluoromethyl modification has the largest reduction on efflux. To study the role of membrane permeability on the potency of gyramides in *E. coli*, we tested the MIC of **3** against a strain with increased membrane permeability, *E. coli* BAS849.[20] This strain contains multiple mutations, including changes in *lptD* that alters outer membrane permeability to small organic molecules; although this mutation does not change inner membrane structure *per se*, it increases the concentration of antibiotics presented at the inner membrane and thereby increases the permeability of lipophilic molecules across this barrier.[20,21] We found that the MIC of **3** against *E. coli* BAS849 is 1 μg mL$^{-1}$ and is 8-fold lower than the MIC against its parent strain *E. coli* MC4100 (8 μg mL$^{-1}$), indicating that poor membrane permeability also reduces the antibacterial activity of the gyramides.

### *E. coli* strains resistant to 1 and 2 are susceptible to 3–5

We evaluated the cross-resistance of gyramide-resistant (gyr$^R$),[14] ciprofloxacin-resistant (cip$^R$), and novobiocin-resistant (nov$^R$) strains of *E. coli* to **1–5**.[22] We tested multiple mutations conferring resistance to **1**, **2**, or **3–5** (Table 2). MIC values of **3–5** against the five gyr$^R$ strains derived from *E. coli* JW5503 were ~10–100-fold higher than against *E. coli* JW5503. Gyr$^R$ strains remained susceptible to **1** and **2** and the MIC values of these compounds were 1.6–3.2 μg mL$^{-1}$ and 0.8 μg mL$^{-1}$, respectively, and thus similar to *E. coli* JW5503 (Table 2). MIC values of **3–5** against the cip$^R$ strains derived from *E. coli* JW5503 were 2-4-fold higher than against *E. coli* JW5503, and the MIC value of **2** against the cip$^R$ strains was 0.8 μg mL$^{-1}$ for all of the strains, including *E. coli* JW5503 (Table 2). These results demonstrate very minor cross-resistance of gyr$^R$ and cip$^R$ *E. coli* strains to the gyramides.

Compounds **3–5** are effective against nov$^R$ strains in the presence of 100 μM of the efflux pump inhibitor, phenylalanine arginine β-naphthylamide (PAβN).[22] We used PAβN because, unlike the cip$^R$ and gyr$^R$ strains, the nov$^R$ strains contain the TolC protein and therefore are capable of pumping the drug out of the cell.[22,23] MIC values of **3–5** against nov$^R$ strains were 2–4-fold higher than the MIC values against *E. coli* HB101 (the background strain used to make the nov$^R$ strains). These results indicate the absence of cross-resistance between **2** and **3–5** in nov$^R$ strains. We detected some examples of cross-resistance to **1** and **2** in nov$^R$ strains; **1** had MIC values of 13 ng mL$^{-1}$ and 26 ng mL$^{-1}$ against the nov$^R$ strains CC5 and LE316, respectively, which is 8-fold and 16-fold higher than the MIC observed for the background strain *E. coli* HB101 (Table 2). The cross-resistance between **1** and **2** in nov$^R$ strains is in contrast to a previous report.[24] Based on the promising potent antibacterial activity of the gyramides and the lack of cross-resistance between **3–5** and the known DNA gyrase inhibitors **1** and **2**, we characterized the activity of these analogs against *E. coli* DNA gyrase *in vitro*.

### Compounds 3–5 inhibit the supercoiling activity of DNA gyrase *in vitro*

We evaluated the effect of compounds **3–5** on the DNA supercoiling activity of DNA gyrase as described previously.[14] We measured the 50% inhibitory concentration ($IC_{50}$) of compounds using *E. coli* DNA gyrase and relaxed pUC19 plasmid as a substrate. $IC_{50}$ values of **3**, **4**, and **5** were 52 ± 9.2, 170 ± 48, and 47 ± 19 nM, respectively. The $IC_{50}$ value of **1** and **2** was 170 ± 63 nM and 144 ± 20 nM, respectively (Fig. 2 and Fig. S3§). Gyramide $IC_{50}$s are ~4-fold lower than the corresponding MICs, suggesting that efflux systems and membrane permeability are the primary barriers currently limiting the activity of the gyramides.

We attempted to measure $IC_{50}$ values of ATPase activity of the tested compounds; however, we were only reliably able to measure an $IC_{50}$ for **2** (245 ± 42 nM). We measured the inhibition of ATPase activity of **1**, and **3–5** up to a concentration of 100 μM and did not observe a decrease in ATPase activity, which is expected for **1**;[27] compounds **3–5** precipitated at a concentration >100 μM, which invalidates earlier measurements suggesting that these compounds are ATPase inhibitors.[17]

Table 2 MIC of compounds **1**–**5** against *E. coli* and isogenic resistant mutants

| Strains[a] | | **1** (ng mL$^{-1}$) | **2** (μg mL$^{-1}$) | **3** (μg mL$^{-1}$) | **4** (μg mL$^{-1}$) | **5** (μg mL$^{-1}$) |
|---|---|---|---|---|---|---|
| Parent strain: *E. coli* JW5503 | | 1.6 | 0.8 | 0.1 | 0.4 | 0.1 |
| **gyr**$^{R}$ strains | GyrA Pro35Thr | 1.6 | 0.8 | 10 | 11 | 5.4 |
| | GyrA Ser97Pro | 1.6 | 0.8 | >10 | 22 | 22 |
| | GyrA Phe96Leu | 3.2 | 0.8 | 10 | 11 | 2.7 |
| | GyrA His45Tyr | 3.2 | 0.8 | 10 | 22 | 22 |
| | GyrB Thr508Met | 3.2 | 0.8 | 10 | 5.5 | 1.3 |
| **cip**$^{R}$ strains[b] | GyrA Ser83Leu | 6.4 | 0.8 | 0.2 | 0.4 | 0.2 |
| | GyrA Asp87Gly | 16 | 0.8 | 0.4 | 0.8 | 0.2 |
| | GyrA Asp87Tyr | 32 | 0.8 | 0.4 | 0.2 | 0.1 |
| | GyrB Ser464Phe | 16 | 0.8 | 0.2 | 1.6 | 0.4 |
| Parent strain: *E. coli* HB101 | | 1.6 | >100 | >16 | 4 | 1 |
| *E. coli* HB101 + 100 μM PAβN[c] | | 1.6 | 3.2 | 0.1 | 0.1 | 0.1 |
| **nov**$^{R}$ strains + 100 μM PAβN[c] | CC5 (GyrB Arg136His) | 13 | 102 | 0.2 | 0.4 | 0.2 |
| | CC7 (GyrB Arg136Ser) | 3.2 | 6.4 | 0.1 | 0.2 | 0.1 |
| | LE316 (GyrB Arg136Val) | 26 | 12.8 | 0.2 | 0.2 | 0.2 |

[a] All the gyr$^{R}$ and cip$^{R}$ mutants are derived from *E. coli* JW5503 (ref. 14 and this study), while nov$^{R}$ mutants are derived from *E. coli* HB101.[22] [b] We isolated the cip$^{R}$ strains from a spontaneous resistant mutant screen and determined that 3 of the 4 mutations in *gyrA* that conferred resistance to **1** have been confirmed in other studies.[25,26] [c] The MIC determination for nov$^{R}$ strains was performed in LB broth supplemented with 100 μM of the efflux pump inhibitor, phenylalanine arginine β-naphthylamide (PAβN). We used PAβN because these strains contain an intact *tolC* gene.

### Overexpression of GyrA and GyrB does not suppress the biological activity of gyramides

To obtain more insight into the potency of **3** and the region of DNA gyrase to which it binds, we performed a target-multicopy suppression experiment. In this experiment, we tested whether an abundance of DNA gyrase outcompetes the toxicity of an inhibitor and its suppression of cell growth.[28,29]

We used two *E. coli* JW5503 strains for these experiments, each containing a *gyrA* or *gyrB* operational reading frame on a multicopy plasmid vector.[30] Overexpression of *gyrB*—and not *gyrA*—was sufficient to suppress the inhibitory effect of **2**, as cultures of cells harboring pCA24N-*gyrB* grew at concentrations of **2** as high as 51 μg mL$^{-1}$, which corresponds to a >10-fold increase in the MIC observed for *E. coli* JW5503 (Table S1§). The result we observed for **2** is consistent with the model for it binding to DNA gyrase and its inhibition of the ATP hydrolysis activity of the GyrB subunit.[6,7,31] Overexpression of *gyrA* or *gyrB* was not sufficient to suppress the susceptibility of cells to **1** or **3** (Table 3). Although we are unsure of the DNA gyrase subunit to which the gyramides bind, we anticipated that overexpression of GyrA would reduce the susceptibility of cells to **1**.

We hypothesized that the results of the target-multicopy suppression experiment we observed for **1** and **3** may be due to the requirement of an excess of the DNA gyrase holoenzyme to titrate the effect of the drug. To test whether the DNA gyrase complex suppressed the biological activity of **1** and **3**, we cloned *gyrA* and *gyrB* into compatible vectors to simultaneously overexpress GyrA and GyrB in *E. coli* cells. To

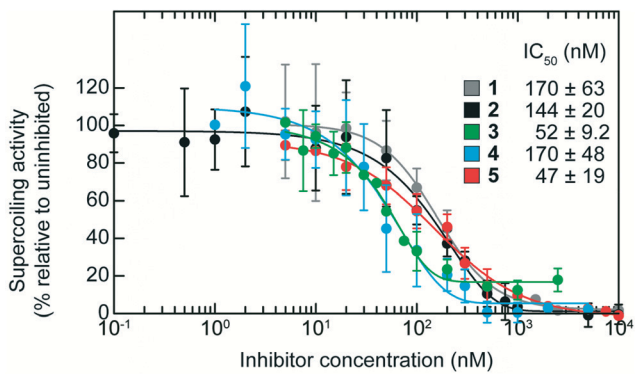


Fig. 2 Dose-response curves for DNA gyrase-dependent supercoiling activity in the presence of **1**, **2**, and **3**–**5**. We performed DNA supercoiling reactions with *E. coli* DNA gyrase, relaxed pUC19 plasmid substrate, and various concentrations of **1**, **2** and **3**–**5**. Reactions were run for 30 min and the DNA was separated on a 0.8% (w/v) agarose gel for 3 h. We labeled the DNA by immersing the gel in a solution of 0.5 mg mL$^{-1}$ ethidium bromide for 1 h. The fluorescence intensity of the supercoiled DNA band was normalized to the DMSO solvent control and plotted for each concentration of compound tested. See the Material and methods section for experimental details. Each reaction was performed in triplicate. Error bars indicate the standard deviation.

Table 3 Target overexpression confers resistance to **2**, but not to **1** or **3**

| Overexpression | | Compound | | |
|---|---|---|---|---|
| (*gyrA*/*gyrB*) | Induction | **1** (ng mL$^{-1}$) | **2** (μg mL$^{-1}$) | **3** (μg mL$^{-1}$) |
| (−/−) | + | 2.1 | 0.4 | 0.2 |
| | — | 2.1 | 0.4 | 0.2 |
| (+/−) | + | 2.1 | 0.8 | 0.1 |
| | — | 2.1 | 0.8 | 0.2 |
| (−/+) | + | 2.1 | 6.3 | 0.1 |
| | — | 2.1 | 0.8 | 0.2 |
| (+/+) | + | 2.1 | 3.2 | 0.1 |
| | — | 2.1 | 0.4 | 0.2 |

 

confirm the overexpression of GyrA and GyrB and the formation of the functional DNA gyrase holoenzyme, we performed controls using *E. coli* mutant strains containing temperature sensitive GyrA or GyrB (Fig. S4§). Simultaneous overexpression of *gyrA* and *gyrB* did not suppress the biological activity of **1** or **3**. The results for **1** are different from recent studies in which overexpression of DNA gyrase increased the susceptibility of *E. coli* cells to **1** from 9 ng $mL^{-1}$ to 2 ng $mL^{-1}$,[32] which is attributed to a drug-bound DNA gyrase creating additional DNA damage rather than conferring drug resistance. Our experiments using a single concentration of IPTG for GyrA and GyrB induction from different plasmids and a different nutrient media—from that reported in[32]—yielded an MIC of 2 ng $mL^{-1}$. Small differences in MICs may be due to differences between the two studies: *e.g.*, growth conditions, strains, and methods for performing measurements. Our results similarly show that overexpression of DNA gyrase subunits in cells did not reduce their susceptibility to the gyramides toxicity. If the gyramides inhibit DNA gyrase partway through its catalytic cycle as demonstrated for **1**,[4,32] overexpression of DNA gyrase subunits may exacerbate cellular toxicity.

## Conclusions

DNA gyrase inhibitors are one of the most successful classes of clinical antibiotics currently used.[33] Nevertheless, the success of these therapeutic agents has been compromised by the development of drug tolerance and resistance among clinical strains of bacteria.[34] Here we report the characterization of three new analogs of the gyramides, a family of synthetic small molecules that inhibit bacterial DNA gyrase. Compounds **3** and **5** both contain a 3-cyano group on the benzenesulfonamide ring (and are identical with the exception of a 6-methyl group on the benzenesulfonamide ring in **5**), display narrow spectrum activity against only Gram-negative bacteria, and are only active against ~30% of the Gram-negative strains that we tested. The effectiveness of **4** against both Gram-negative and Gram-positive bacteria is likely due to the inclusion of the trifluoromethyl group at the 3-position on the benzenesulfonamide ring. It is tempting to point out that **4** also differs from **3** and **5** by incorporation of an arylisopropylether at the other end of the molecule (compared to a phenyl group in **3** and **5**), however this difference has no obvious effect on gyramide efflux. We support this hypothesis with the observation that gyramide A (**6**) is very similar in structure to **4** with the exception of a 3-fluoro group (**6**) compared to a 3-trifluoromethyl group (**4**), and yet the compounds have extremely different efflux profiles from a comparison of their MICs against *E. coli* BW25113 and JW5503. It may also be possible that **4** also targets DNA gyrase from different organisms effectively.

Although we do not yet understand the structural and biochemical basis for the difference in the sensitivity of gyrase from different organisms (*i.e.*, Gram-negative *versus* Gram-positive bacteria), previous studies of quinolones suggested that the extended α4-helix domain of *S. aureus* GyrA is partially responsible for the increased resistance of *S. aureus* gyrase to quinolones compared to *E. coli* gyrase, and the higher concentration of potassium glutamate in the cytoplasm of *S. aureus* modulates this effect.[35] The differences in the activity that we observed for the gyramide analogs against different organisms could similarly connect back to the DNA-gyrase-gyramide ternary complex, the intracellular environment in which these compounds are active, or a combination of these factors.

Many antibiotics are transported out of Gram-negative organisms by a bacterial efflux transporter in the resistance-nodulation-division (RND) protein family that spans the cell envelope;[36,37] specifically the AcrAB-TolC tripartite pump is found in *E. coli*, *Salmonella* spp., and *Shigella* spp. and contributes to multidrug resistance.[37–39] Our observation that **3–5** are most active against strains of the Gram-negative bacteria *E. coli*, *S. enterica*, and *S. flexneri*—and very potent against strains harboring the Δ*tolC* mutation—supports the hypothesis that the cyano and trifluoromethyl modifications in the 3-position of the benzenesulfonamide ring reduce drug efflux against this family of drug pumps.

Five efflux drug pump families [ATP binding cassette (ABC), major facilitator superfamily (MFS), multidrug and toxin extrusion (MATE), RND, and small multidrug resistance (SMR)] pump antibiotics out of Gram-positive cells.[40] Surprisingly, **4**—and not **3** and **5**—is also active against Gram-positive bacteria, suggesting that the trifluoromethyl modification to the benzenesulfonamide ring may play a unique role in reducing its specificity for pumping by these five efflux protein families. Lacking a definitive set of molecular design rules for drug reducing efflux by bacteria, and taking into consideration that many clinical antibiotics contain an arylfluoro group (*e.g.*, the entire family of fluoroquinolones), replacing this group with a trifluoromethyl moiety may reduce efflux further, expand the activity of drugs to Gram-positive organisms, contribute to understanding bacterial efflux mechanisms, and contribute to antibiotic development.

Importantly, the gyramides are still effective against *E. coli* strains resistant to the known gyrase inhibitors **1** and **2**, indicating no cross-resistance when bacteria with altered susceptibility to **1** or **2** are treated with compounds **3–5**. Our results imply that the mutations that confer resistance to **1** and **2** occur in a region of DNA gyrase that does not affect the binding of gyramides to DNA gyrase. Further structural biology data can provide insight into the region of DNA gyrase to which the gyramides bind. We observed that two of the $nov^R$ mutants confer resistance to **1**, which may arise from uncharacterized mutations.

Compounds **3** and **5** are more potent inhibitors of the *in vitro* DNA supercoiling activity of *E. coli* DNA gyrase than **1**, **2**, **4**, and the gyramide analogs that we reported previously.[14] Importantly, we previously demonstrated that the gyramides do not affect the activity of other type II topoisomerases.[17] When testing the inhibition of the ATPase activity of DNA gyrase, we observed **3–5** precipitate at a

concentration >100 μM and therefore obfuscate absorbance measurement, which previously led us to the incorrect conclusion that **6** competitively inhibited the ATPase activity of DNA gyrase at millimolar concentrations;[17] it does not. We do not currently have an explanation for the reduction in the activity of **4** (3-trifluoromethyl modification) against *E. coli* DNA gyrase compared to **3** and **5** (3-cyano modification).

Our investigation of the inhibition of ATPase activity and overexpression studies of different gyrase subunits suggests that **3** does not compete directly for the ATP binding site. Unlike **2**, **3** did not inhibit the ATPase activity of DNA gyrase and overexpression of GyrB did not promote resistance to **3**. Overexpression of both GyrA and GyrB in the same cell did not affect the antibacterial activity of either **1** or **3**. However, it has been recently demonstrated that simultaneous overexpression of GyrA and GyrB exacerbates the antibacterial activity of **1**. This effect could be caused by the covalent attachment of **1** to the GyrA-DNA bound state as the amount of GyrA-DNA bound complexes increases, thus blocking key cellular processes.[32] Compound **3** may also create DNA gyrase-DNA bound complexes that are toxic to cells and overexpression may not reduce susceptibility to the compounds. We have previously shown the mutations that confer resistance to gyramides do not map to the same region as mutations that confer resistance to quinolones.[14]

Our previous and current data support the hypothesis that the gyramides may bind to a new site on DNA gyrase and operate through a new mechanism of inhibiting DNA replication and, consequently, bacterial cell growth. We hypothesize that gyramides inhibit DNA gyrase during its catalytic cycle resulting in stalled DNA-bound DNA gyrase and are currently working on the structural biology of the gyramide/DNA gyrase complex to test this hypothesis.

## Experimental section

### General synthetic chemistry methods and compound characterization

We synthesized a library of 183 gyramide analogs using a previously reported strategy[13] and varying the aldehyde and sulfonyl chloride building blocks (Fig. S1§); all 183 analogs that we tested were >95% pure. After determining activities against bacteria in minimum inhibitory concentration (MIC) experiments, we resynthesized the most potent analogs (compounds **3–5**) and characterized them exhaustively using HPLC, NMR, and MS (ESI§).

Unless otherwise specified, all commercially available reagents were used as received. All reactions using dried solvents were carried out under an atmosphere of argon in flame-dried glassware with magnetic stirring. Purification of reaction products was carried out by flash chromatography using SiliCycle Reagent silica gel F60 (230–400 mesh) and Dynamic Absorbents, Inc. Reagent silica gel. Analytical thin layer chromatography was performed on Dynamic Absorbant Inc. Reagent 0.25 mm silica gel F-254 plates. Visualization was accomplished with UV light or acidic ceric ammonium molybdate (CAM) followed by heating.

$^1$H NMR spectra and proton-decoupled $^{13}$C NMR spectra were obtained on a 400 MHz Bruker NMR spectrometer. Chemical shifts ($\delta$) are reported in parts per million (ppm) relative to residual solvent. Multiplicities are given as: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), td (triplet of doublet), ddd (doublet of doublet of doublets), m (multiplet), br s (broad singlet) and sep (septet).

For AMM analysis, samples were analyzed by flow-injection analysis into a Thermo Fisher Scientific LTQ Orbitrap XL (San Jose, CA) operated in the centroided mode. Samples were injected into a mixture of 50% MeOH and 0.1% formic acid/$H_2O$ at a flow of 200 μl min$^{-1}$. Source parameters were 5 kV spray voltage, capillary temperature of 275 °C and sheath gas setting of 20. Spectral data were acquired at a resolution setting of 100 000 FWHM with the lockmass feature, which typically results in a mass accuracy <2 ppm.

***tert*-Butyl (*R*)-(1-([1,1′-biphenyl]-4-ylmethyl)pyrrolidin-3-yl)-carbamate (15).** To a solution of 4-phenylbenzaldehyde (323 mg, 1.1 equiv.) and *tert*-butyl (*R*)-pyrrolidin-3-ylcarbamate (300 mg, 1 equiv.) in anhydrous THF (16 mL) was added $NaBH(OAc)_3$ (854 mg, 2.5 equiv.) at ambient temperature. After stirring for 24 hours, the solvent was removed *in vacuo*, and the remaining material was partitioned between 1 M HCl and EtOAc. The aqueous portion was quickly neutralized with saturated aqueous $NaHCO_3$, then extracted with EtOAc. The combined organic portions were dried over $Na_2SO_4$ and concentrated *in vacuo*. The crude product mixture was purified by flash chromatography (95 : 5 to 90 : 10 $CH_2Cl_2$ : $CH_3OH$, $R_f$ = 0.36 in 90 : 10 $CH_2Cl_2$ : $CH_3OH$) to produce the title compound as a white amorphous solid (535 mg, 94%). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 7.67–7.57 (m, 4H), 7.50 (d, $J$ = 8.0 Hz, 2H), 7.43 (t, $J$ = 7.7 Hz, 2H), 7.34 (t, $J$ = 7.4 Hz, 1H), 4.19 (td, $J$ = 7.6, 3.9 Hz, 1H), 3.23 (dd, $J$ = 11.1, 7.3 Hz, 1H), 3.19–3.06 (m, 1H), 3.06–2.92 (m, 1H), 2.87 (dd, $J$ = 11.5, 4.8 Hz, 1H), 2.31 (dtd, $J$ = 14.3, 8.2, 6.3 Hz, 1H), 1.96 (s, 2H), 1.83 (dq, $J$ = 13.4, 6.9 Hz, 1H), 1.43 (s, 9H). Carbamate NH not observed. $^{13}$C NMR (101 MHz, $CD_3OD$) $\delta$ 157.8, 142.9, 141.6, 134.2, 131.5, 129.9128.6, 128.4, 128.0, 80.5, 60.1, 59.9, 53.6, 50.4, 31.6, 28.7. AMM (ESI) calculated for $C_{22}H_{29}N_2O_2^+$ $(M + H)^+$ 353.2224, found 353.2228.

**(*R*)-1-([1,1′-Biphenyl]-4-ylmethyl)pyrrolidin-3-amine (14).** To a solution of **15** (120 mg) in $CH_3OH$ (3.7 mL) was added dropwise 4 M HCl in dioxane (3.7 mL). After 17 hours, the mixture was concentrated *in vacuo* to produce the title compound as the hydrochloride salt. The material was used without further purification.

**(*R*)-*N*-(1-([1,1′-Biphenyl]-4-ylmethyl)pyrrolidin-3-yl)-3-cyanobenzenesulfonamide (3).** To a solution of **14** (assumed to be monohydrochloride salt, 65 mg, 1 equiv.) and 3-cyanobenzenesulfonyl chloride (52 mg, 1.15 equiv.) in anhydrous $CH_2Cl_2$ (1.75 mL), $Et_3N$ (95 μL, 3 equiv.) was added. After stirring for 21 hours, the mixture was diluted with $CH_2Cl_2$ (3 mL) and washed with $H_2O$ (7 mL). The

 

aqueous portion was made basic with saturated aqueous $NaHCO_3$, then extracted with $CH_2Cl_2$. The combined organic portions were dried over $Na_2SO_4$ and concentrated *in vacuo*. The crude product mixture was purified by flash chromatography (100 : 0 to 93 : 7 $CH_2Cl_2$ : $CH_3OH$, $R_f$ = 0.36 in 93 : 7 $CH_2Cl_2$ : $CH_3OH$) to produce the title compound as a slightly yellow amorphous solid (64 mg, 68%). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.18 (t, $J$ = 1.8 Hz, 1H), 8.10 (dt, $J$ = 8.0, 1.5 Hz, 1H), 7.94–7.88 (m, 1H), 7.71 (t, $J$ = 7.9 Hz, 1H), 7.64–7.58 (m, 2H), 7.58–7.53 (m, 2H), 7.43 (dd, $J$ = 8.4, 6.9 Hz, 2H), 7.36–7.29 (m, 3H), 3.82 (ddt, $J$ = 9.2, 7.0, 5.3 Hz, 1H), 3.63 (d, $J$ = 12.7 Hz, 1H), 3.55 (d, $J$ = 12.6 Hz, 1H), 2.63 (td, $J$ = 9.1, 8.3, 6.4 Hz, 2H), 2.52 (ddd, $J$ = 9.4, 8.0, 6.0 Hz, 1H), 2.26 (dd, $J$ = 10.0, 5.5 Hz, 1H), 2.18–1.98 (m, 1H), 1.56 (ddt, $J$ = 13.7, 8.5, 5.7 Hz, 1H). Sulfonamide NH not observed. $^{13}$C NMR (101 MHz, $CD_3OD$) $\delta$ 144.5, 142.1, 141.6, 138.2, 136.9, 132.2, 131.61, 131.59, 130.7, 129.9, 128.3, 127.92, 127.90, 118.4, 114.6, 60.9, 60.6, 53.41, 53.36, 32.6. AMM (ESI) calculated for $C_{24}H_{24}N_3O_2S^+$ $(M + H)^+$ 418.1584, found 418.1583.

**4-Cyclopropoxybenzaldehyde (16).** In a sealed microwave vial, 4-hydroxybenzaldehyde (120 mg, 1 equiv.), bromocyclopropane (315 μL, 4 equiv.) and $Cs_2CO_3$ (960 mg, 3 equiv.) were combined in anhydrous DMF (1.6 mL). The mixture was heated using microwave irradiation to 200 °C for 1 hour. The resulting mixture was diluted with EtOAc, filtered, and concentrated *in vacuo.* The crude product mixture was purified by flash chromatography (100 : 0 to 80 : 20 hexanes : EtOAc, $R_f$ = 0.51 in 80 : 20 hexanes : EtOAc) to produce the title compound as a clear oil (145 mg, 91%). A minimal inseparable impurity remained, but did not negatively impact further reactions. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 9.90 (s, 1H), 7.84 (d, $J$ = 8.7 Hz, 2H), 7.16 (d, $J$ = 8.7 Hz, 2H), 3.82 (tt, $J$ = 6.1, 3.2 Hz, 1H), 0.90–0.78 (m, 4H). $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 190.9, 164.2, 131.8, 130.3, 115.4, 51.4, 6.3. AMM (ESI) calculated for $C_{10}H_{11}O_2^+$ $(M + H)^+$ 163.0754, found 163.0751.

***tert*-Butyl (*R*)-(1-(4-cyclopropoxybenzyl)pyrrolidin-3-yl)-carbamate (17).** To a solution of **16** (52 mg, 1.05 equiv.), *tert*-butyl (*R*)-pyrrolidin-3-ylcarbamate (57 mg, 1 equiv.), and NaOAc (25 mg, 1 equiv.) in anhydrous THF (3 mL), AcOH (20 μL, 1.15 equiv.) was added. After stirring at ambient temperature for 1 hour, $NaBH(OAc)_3$ (160 mg, 2.5 equiv.) was added. The mixture was stirred at ambient temperature for 22 hours, then concentrated *in vacuo*. The remaining solids were partitioned between EtOAc and 50 : 50 $H_2O$ : $NaHCO_3$ (sat. aq.). The aqueous portion was extracted with EtOAc, and the combined organics were dried over $Na_2SO_4$ and concentrated *in vacuo*. The crude product mixture was purified by flash chromatography (100 : 0 to 93 : 7 $CH_2Cl_2$ : $CH_3OH$, $R_f$ = 0.29 in 93 : 7 $CH_2Cl_2$ : $CH_3OH$) to produce the title compound as a clear oil (84 mg, 84%). $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.20 (d, $J$ = 8.6 Hz, 2H), 6.99 (d, $J$ = 8.6 Hz, 2H), 4.84 (s, 1H), 4.24–4.07 (m, 1H), 3.79–3.66 (m, 1H), 3.53 (s, 2H), 2.77 (d, $J$ = 11.7 Hz, 1H), 2.59 (dd, $J$ = 9.8, 6.4 Hz, 1H), 2.50 (d, $J$ = 9.8 Hz, 1H), 2.40–2.18 (m, 2H), 1.43 (s, 10H), 0.84–0.69 (m, 4H). Carbamate NH not observed. $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 158.0, 155.4, 131.2, 129.8, 114.7, 79.2, 60.8, 59.5, 52.5, 50.7, 49.8, 32.8, 28.4, 6.2. AMM (ESI) calculated for $C_{19}H_{29}N_2O_3^+$ $(M + H)^+$ 333.2173, found 333.2174.

**(*R*)-1-(4-Cyclopropoxybenzyl)pyrrolidin-3-amine (13).** To a solution of **17** (70 mg, 1 equiv.) in $CH_3OH$ (2.1 mL), 4 M HCl in dioxane (2.1 mL) was added. After stirring for 1 hour, the mixture was concentrated *in vacuo*. The crude product mixture was used without further purification.

**2-Methyl-5-(trifluoromethyl)benzenesulfonyl chloride (18).** To chlorosulfonic acid (1 mL) cooled to 0 °C, 4-methylbenzotrifluoride (160 mg) was added dropwise. The mixture was warmed to ambient temperature, then stirred for 2 hours. The mixture was then carefully added dropwise to 15 g ice, then neutralized with solid $NaHCO_3$. After allowing the ice to melt, the aqueous slurry was extracted with $CHCl_3$. The combined organic portions were dried over $Na_2SO_4$ and concentrated *in vacuo*. The crude product mixture was purified by flash chromatography (100 : 0 to 50 : 50 hexanes : $CH_2Cl_2$, $R_f$ = 0.28 in 90 : 10 hexanes : $CH_2Cl_2$) to produce the title compound as a clear oil (115 mg, 44%). $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.33 (s, 1H), 7.87 (d, $J$ = 8.0 Hz, 1H), 7.59 (d, $J$ = 8.0 Hz, 1H), 2.87 (s, 3H). $^1$H NMR matched previously reported values.

**(*R*)-*N*-(1-(4-Cyclopropoxybenzyl)pyrrolidin-3-yl)-2-methyl-5-(trifluoromethyl)benzenesulfonamide (4).** To a solution of **13** (57 mg, 1 equiv., assumed to be monohydrochloride) and **18** (71 mg, 1.3 equiv.) in anhydrous $CH_2Cl_2$ (1.6 mL), $Et_3N$ (90 μL, 3 equiv.) was added. After stirring at ambient temperature for 14 hours, the mixture was diluted with $CH_2Cl_2$ and washed with 50 : 50 $H_2O$ : $NaHCO_3$ (saturated aq.). The organic portion was extracted with $CH_2Cl_2$, and the combined organics were dried over $Na_2SO_4$ and concentrated *in vacuo*. The crude product mixture was purified by flash chromatography (100 : 0 to 98 : 2 $CH_2Cl_2$ : $CH_3OH$, $R_f$ = 0.56 in 92 : 8 $CH_2Cl_2$ : $CH_3OH$) to produce the title compound as a pale yellow oil (77 mg, 81%). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.14 (d, $J$ = 1.9 Hz, 1H), 7.81–7.74 (m, 1H), 7.55 (d, $J$ = 8.0 Hz, 1H), 7.14 (d, $J$ = 8.6 Hz, 2H), 6.97 (d, $J$ = 8.6 Hz, 2H), 3.81–3.69 (m, 2H), 3.51 (d, $J$ = 12.5 Hz, 1H), 3.44 (d, $J$ = 12.5 Hz, 1H), 2.68 (s, 3H), 2.58 (ddd, $J$ = 13.5, 9.5, 6.6 Hz, 2H), 2.48 (ddd, $J$ = 9.5, 8.0, 6.0 Hz, 1H), 2.20 (dd, $J$ = 10.0, 5.7 Hz, 1H), 2.06 (dddd, $J$ = 13.5, 9.2, 8.0, 5.9 Hz, 1H), 1.60 (ddt, $J$ = 13.9, 8.6, 5.7 Hz, 1H), 0.85–0.72 (m, 2H), 0.72–0.61 (m, 2H). Sulfonamide NH not observed. $^{13}$C NMR (101 MHz, $CD_3OD$) $\delta$ 159.8, 143.2, 141.7, 134.8, 131.3, 130.1, 126.8, 123.7, 115.8, 115.6, 79.5, 60.6, 60.3, 53.18, 53.15, 51.7, 32.4, 20.4, 6.6. AMM (ESI) calculated for $C_{22}H_{26}F_3N_2O_3S^+$ $(M + H)^+$ 455.1611, found 455.1610.

**(*R*)-*N*-(1-([1,1′-Biphenyl]-4-ylmethyl)pyrrolidin-3-yl)-5-cyano-2-methylbenzenesulfonamide (5).** To a solution of **14** (400 mg, 1.2 equiv.) in anhydrous $CH_2Cl_2$ (11 mL), $Et_3N$ (485 mL, equiv.) was added. Then, 5-cyano-2-methylbenzenesulfonyl chloride (250 mg, 1 equiv.) was added. The resulting mixture was stirred for 3 hours at ambient temperature. The mixture was diluted with $CH_2Cl_2$ and washed with $H_2O$. The aqueous portion was back-extracted with $CH_2Cl_2$. The combined organic portions were dried over $Na_2SO_4$ and concentrated *in vacuo*. The crude product mixture was purified by flash

chromatography (100 : 0 to 99.5 : 0.5 $CH_2Cl_2$ : $CH_3OH$, $R_f$ = 0.23 in 97 : 3 $CH_2Cl_2$ : $CH_3OH$) to produce the title compound as a slightly yellow amorphous solid (324 mg, 54%). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.21 (d, $J$ = 1.8 Hz, 1H), 7.77 (dd, $J$ = 7.9, 1.8 Hz, 1H), 7.64–7.60 (m, 2H), 7.56 (d, $J$ = 8.2 Hz, 2H), 7.49 (d, $J$ = 7.9 Hz, 1H), 7.43 (dd, $J$ = 8.5, 7.0 Hz, 2H), 7.37–7.29 (m, 3H), 3.79 (ddt, $J$ = 9.2, 6.9, 5.3 Hz, 1H), 3.64 (d, $J$ = 12.6 Hz, 1H), 3.53 (d, $J$ = 12.6 Hz, 1H), 2.71–2.62 (overlapping s and m, 4H), 2.59–2.52 (m, 1H), 2.52–2.46 (m, 1H), 2.24 (dd, $J$ = 10.0, 5.4 Hz, 1H), 2.10 (dtd, $J$ = 13.6, 8.5, 5.7 Hz, 1H), 1.63 (ddt, $J$ = 13.7, 8.6, 5.9 Hz, 1H). Sulfonamide NH not observed. $^{13}$C NMR (101 MHz, $CD_3OD$) $\delta$ 144.3, 142.2, 142.1, 141.6, 138.3, 136.7, 134.9, 133.6, 130.7, 129.9, 128.4, 127.93, 127.90, 118.6, 111.5, 60.7, 60.6, 53.4, 53.3, 32.5, 20.7. AMM (ESI) calculated for $C_{25}H_{26}N_3O_2S^+$ $(M + H)^+$ 432.1740, found 432.1739.

## Acknowledgements

We acknowledge materials from Dr. Tony Maxwell (*E. coli* HB101, *E. coli* CC5, *E. coli* CC7, and *E. coli* LE316) and Dr. Kenneth Kreuzer (*E. coli* KNK453).

This research was supported by the NIH (through award 1DP2OD008735 to D. B. W.) and the Human Frontiers Science Program (through award RGY0076/2013 to D. B. W.). The research described was partially funded by Venture Catalyst in the UC Davis Office of Research through a Science Translation and Innovative Research (STAIR™) Grant. M. R. F. and Z. W. Y. thank the Arnold and Mabel Beckman Foundation.

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