DNA Gyrase Inhibitory and Antimicrobial Activities of Some Diphenic Acid Monohydroxamides

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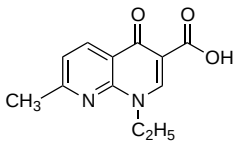
The synthesis and inhibitory activity against DNA gyrase of a series of diphenic acid monohydroxamides **4a**−**f** are described. A protocol of two biological assays showed conclusively that inhibition occurs specifically at the DNA−DNA gyrase complex and is not attributable to nonspecific inhibition. In the enzyme assays, **4c** was as potent as the prototypical quinolone, nalidixic acid (**1**), with an IC_{50} value of 58.3 μg/mL compared to 52 μg/mL for **1**. MIC activity against bacterial strains showed a systematic drop for all compounds relative to **1**. For compounds **4c**−**e**, the addition of PMBN produced dramatic increases in MIC activity indicating that activity is likely to be related to membrane transport. Molecular modeling of **4a** indicates that the diphenic acid monohydroxamides can bind to the DNA−DNA gyrase complex in a similar fashion as that hypothesized for the quinolone series according to the hypothesis suggested by Shen et al. but may not self-associate by $\pi-\pi$ stacking. In contrast to the quinolone series, as the diphenic acid monohydroxamides are shown by molecular mechanics minimizations to be nonplanar, they may present novel approaches for chemotherapeutic intervention with a potential for decreased side effects.

Introduction

The quinolones have become one of the most clinically useful classes of broad spectrum antibacterial drugs available today.[1,2] Nalidixic acid (**1**), the first prototypic "quinolone", exhibited activity against Gram-negative bacteria but lacked substantial Gram-positive activity.[3] Recently newer broad-spectrum agents, for example, ofloxacin (**2**), have been discovered as a result of extensive variations of the ring system and substituents of **1**.[4] In general, the clinically useful members of this class of antibacterials contain a β-keto acid moiety. Because the two carbonyl groups of the β-keto acid are part of a rigid aromatic ring system, they are conformationally restricted to a coplanar orientation which has been postulated to be optimally positioned to form critical hydrogen bonds with the DNA−DNA gyrase complex.[5] The extended aromatic ring system, however, may itself be related to the side effects on the central nervous system (CNS) and the articular cartilage which has been reported for members of the quinolone series.[6−9]



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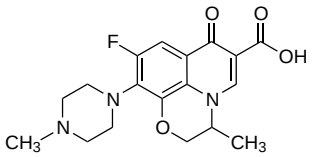

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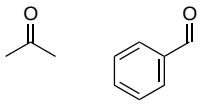
It has therefore been our aim to identify novel inhibitors of DNA gyrase that retain the hydrogen-accepting capabilities of the quinolones but lack the planar ring system.[10] We have employed database-searching techniques using fragments of known inhibitors (**3a,b**, together in a single search query) against our proprietary compound library to identify alternative templates upon which inhibitors of DNA gyrase could be based.[11]



3a **3b**

The results of our searches generated a list of ~200 potential inhibitors. These were subsequently screened for DNA gyrase and antibacterial activity. Among the active compounds in this list were the diphenic acid monohydroxamide series exemplified by **4a**. This class bears an obvious structural similarity to the diphenic acid monoamides, which were reported as having DNA gyrase and antibacterial activity by Kiely and co-workers.[12] We have pursued this series and report herein the synthesis, molecular modeling studies, DNA gyrase inhibitory activities, and antibacterial activities of compound **4a** and its analogs **4b**−**f** (Chart 1).

Chemistry

The synthesis of compounds **4a**−**f** is shown in Scheme 1. The hydroxylamine **7c** was synthesized as published earlier.[13] The ketone **5f** was synthesized from 4-fluoroacetophenone and phenylpiperizine (Scheme 2); **5a**−**e** were obtained from commercial sources. The ketones were converted to the corresponding oximes with hydroxylamine hydrochloride and NaOH, followed by reduction with sodium cyanoborohydride to give the hydroxylamines **7a**−**f**.[14] The final products (**4a**−**c**,**e**) were obtained by reaction of the respective hydroxylamine with diphenic anhydride (**9**) in THF. Compounds **4d,f** were obtained by reacting the hydroxylamines with the half-ester **8** to give derivative **10** which was selectively hydrolyzed with LiOH to give the final products.

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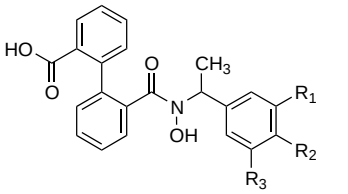
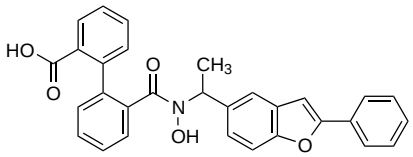
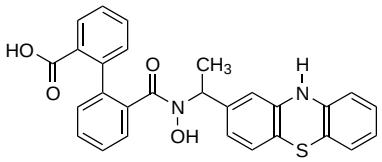
Chart 1



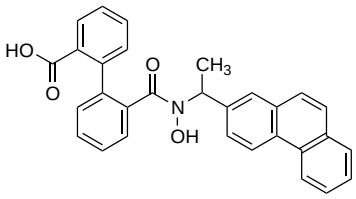
	R_1	R_2	R_3
4a	H	H	H
4b	OCH_3	OCH_3	OCH_3



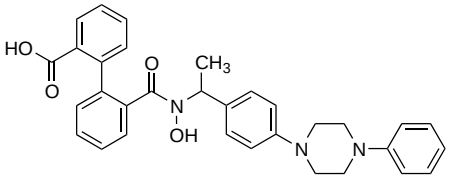
4c



4d

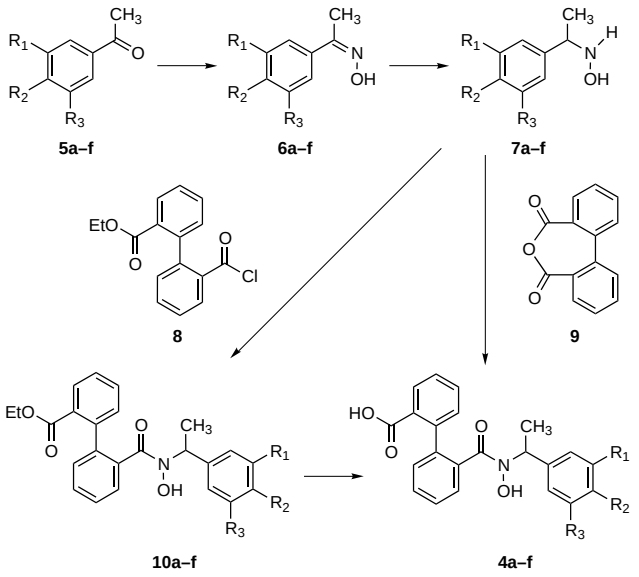


4e



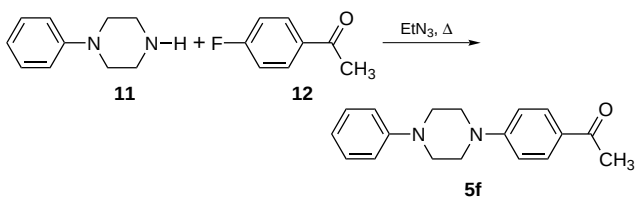
4f

Scheme 1. Synthesis of Compounds **4a**−**f**



5a–f **6a–f** **7a–f** **8** **9** **10a–f** **4a–f**

Scheme 2. Synthesis of compound **5f**



11 **12** **5f**

Results and Discussion

The inhibitory activities of the six diphenic acid monohydroxamides against the *Escherichia coli* DNA gyrase supercoiling activity and their ability to facilitate the "cleavable complex" are shown in Table 1. Also shown in Table 1 are the comparative DNA gyrase inhibitory activities of nalidixic acid. The initial compound **4a** did not have appreciable activity against the enzyme, but as larger hydrophobic substituents were introduced, inhibitory activity was observed. Three of the compounds inhibited the DNA gyrase catalytic activity.

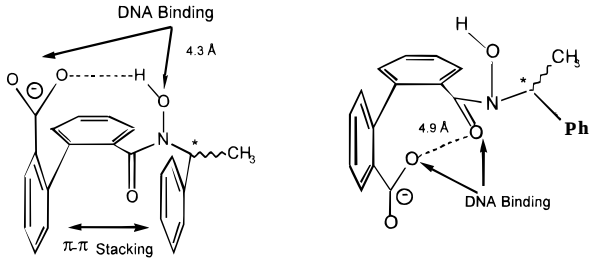
Table 1. Inhibition of DNA Gyrase Activity

compd	supercoiling inhibition $SCIC_{50}$ (μg/mL)	cleavable complex CC_{50} (μg/mL)[a]
4a	>500	NT
4b	>500	NT
4c	58.3	62.5
4d	658	750
4e	95	97.7
4f	>500	NT
1	52	41

[a] NT = not tested if catalytic activity > 500 μg/mL.

The use of the DNA gyrase supercoiling inhibition assay has been the classical approach in identifying and quantitating the inhibition of DNA gyrase by quinolones.[15] This assay can detect both GyrA and GyrB subunit inhibitors, since the reaction exposes the holoenzyme to the potential inhibitor. Unfortunately, in catalytic assays, reaction conditions such as pH, ionic strength, intercalation, chelation, and other nonspecific effects can arise and be mistakenly read as specific inhibition. Since diphenic acid monohydroxamides may inhibit by nonspecific mechanisms, it cannot be concluded based on the catalytic activity that they are bonafide inhibitors of the enzyme. A more specific variation of the supercoiling inhibition assay is the DNA gyrase "cleavable complex" assay.[16,17] A major difference in this assay is that the generation of the linearized DNA fragment requires the catalytic activity of the holoenzyme to be retained (i.e., free from nonspecific inhibition) in order for the "cleavable complex" intermediate of DNA gyrase−DNA−drug to be formed. Any activity that prevents the binding or catalytic activity of DNA gyrase in a non-mechanism-based manner would preclude "cleavable complex" formation. Thus, any gyrase inhibitor that specifically targets the GyrA subunit in a mechanism-based manner, such as the quinolones, would generate a "cleavable complex" and

Chart 2



	Conformer **1**		Conformer 2	
R:	-74.154 kcal/mol	4.25 Å	-64.926 kcal/mol	4.94 Å
S:	-73.558 kcal/mol	4.73 Å	-63.561 kcal/mol	4.97 Å

would be reported as a bonafide active inhibitor. However, nonspecific inhibition that would normally be detected as a "false positive" in the DNA gyrase supercoiling inhibition assay would not appear as active in the DNA "cleavable complex" assay.

As seen in Table 1, all of the diphenic acid monohydroxamides that inhibited the DNA gyrase catalytic activity also facilitated the cleavable complex. We therefore concluded that the activity of all diphenic acid monohydroxamides with supercoiling inhibitory activities is in fact against the GyrA subunit of DNA gyrase.

The potencies of the most active diphenic acid monohydroxamides at the enzyme level were in the range of the first-generation 4-quinolone, nalidixic acid (IC_{50} = 52 μg/mL; **4c**, IC_{50} = 58.3 μg/mL). To characterize the structural features of this series as related to the quinolone series, a molecular modeling study of the initial lead structure **4a** was conducted. The carbonyl and carboxylic acid interatomic distance, which is a prominent feature in the nalidixic acid and ofloxacin series and constitutes an obvious pharmacophore element that should be reproduced in novel DNA gyrase inhibitors, was used as the basis for the initial substructure searches.[18] The optimal distance between the ring carbonyl oxygen and a carboxylate oxygen of nalidixic acid was determined from the X-ray structure to be ~3.9 Å.[19] Additionally, in accord with the cooperative drug−DNA binding model proposed by Shen et al.,[5] mimics of quinolone inhibitors must also possess the ability to self-assemble via intermolecular $\pi-\pi$ interactions and form a tight stacking arrangement within the active site. In the present series, the *ortho*-substituted acid prohibits the biphenyl rings from adopting a coplanar geometry suitable for $\pi-\pi$ stacking analogous to that postulated in the quinolone binding model.

Molecular mechanics and molecular orbital optimizations of **4a** confirm the existence of two prominent local minima (conformers 1 and 2, shown in Chart 2). The preferred conformer (1) is lower in energy by ~10 kcal/mol and is dominated by an internal hydrogen bond between the *N*-hydroxyl hydrogen and carboxylate group. Additionally, the phenyl ring bearing the carboxylic acid and the terminal phenyl ring form a hydrophobically collapsed $\pi-\pi$ interaction.[20] Effectively, the carboxylic acid and carbonyl groups, comparable to those of the quinolone series, are in a transoid configuration and would not be capable of binding in a similar fashion as a β-keto acid to a common DNA strand. However, the *N*-hydroxyl group may act as a hydrogen bond acceptor in place of the carbonyl oxygen.[21] While the higher energy conformer 2 possesses this feature, the interatomic distance between the two hydrogen bond acceptors is slightly increased compared to a β-keto acid. The *R* and *S* stereoisomers were evaluated in both conformations, but the stereogenic center appears sufficiently removed from the pharmacophore center and renders only a minimal effect on the conformational energies and interatomic distances. Both conformations are capable of satisfying the distance requirements as defined by the nalidixic acid and ofloxacin series and can form multiple hydrogen bond interactions with a strand of "relaxed" DNA.

The antibacterial activities of the compounds are shown in Table 2. Antibacterial activity was only observed with the enzyme-active diphenic acid monohydroxamides against Gram-positive organisms. Gram-negative antibacterial activity was not observed with any of the compounds and was presumed to be related to their poor penetration into the bacterial cell. Therefore, we studied antibacterial activity in a modified MIC assay by using polymyxin B nonapeptide (PMBN), which semipermeabilizes Gram-negative cells by forming selective membrane channels.[22] In the presence of PMBN, compounds exhibited Gram-negative antibacterial activity with MICs as low as 2 μg/mL (Table 2).

We have identified the diphenic acid monohydroxamides as a class of antibacterials which exhibit bonafide DNA gyrase inhibition. The potencies of this series are comparable to the first-generation quinolone antibacterials, and the compounds lack a planar structure. This lack of planarity may come at the expense of decreased $\pi-\pi$ stacking, which has been postulated to be a driving force of the observed quinolone drug cooperativity in the drug−DNA−DNA gyrase complex.[5] Design modifications aimed at increasing the potencies of this series are currently underway.

Experimental Section

General Methods. Melting points were determined on a Meltemp II melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on silica gel plates with a fluorescent indicator and visualized with light at 254 nm. ^{1}H NMR spectra were recorded on GE-300 spectrometers; signals are reported in ppm from tetramethylsilane. MS data were collected on a double-focusing magnetic sector Finnigan MAT 8230 mass spectrometer. Combustion analyses were obtained with a Perkin-Elmer Model 2400 elemental analyzer and are reported within 0.4% of the theoretical values unless otherwise indicated.

General Procedure for the Preparation of Oximes 6a−f. The following procedure for the preparation of ***N*-hydroxy-*N*-[2-(3,4,5-trimethoxyphenyl)-2-ethyl]imine (6b)** is representative. To a solution of 3′,4′,5′-trimethoxyacetophenone (44 g, 0.21 mol) and hydroxylamine hydrochloride (50 g, 0.72 mol) in 90% EtOH (700 mL) was added powdered NaOH (75 g, 1.88 mol) in small portions. The mixture was allowed to stir at 25 °C for 30 min and then refluxed for another 30 min. The reaction mixture was then cooled to 25 °C and poured into a mixture of concentrated HCl (80 mL) and water (320 mL). The resulting precipitate was filtered, washed with water, and dried to give 46 g (98% yield) of product. The oximes were taken directly to the next step without purification and characterization.

General Procedure for the Preparation of Hydroxylamines 7a−f. The following procedure for the preparation of ***N*-hydroxy-*N*-[2-(3,4,5-trimethoxyphenyl)-2-ethyl]amine (7b)** is representative. To a solution of *N*-hydroxy-*N*-[2-(3,4,5-trimethoxyphenyl)-2-ethyl]imine **(6b)** (46 g, 0.20 mol) in MeOH (500 mL) were added $NaBH_3CN$ (15 g, 0.24 mol) and a trace of methyl orange; 12 N HCl was added dropwise until the color remained pink. After 1 h at 25 °C, TLC showed starting materials, and more $NaBH_3CN$ (9.23 g, 146.9 mmol) was

Table 2. MIC (μg/mL) of Compounds **4a**−**f** and Nalidixic Acid on Gram-Positive and Gram-Negative (±PMBN) Bacteria

	E.coli$_{ss}$		*E. coli* KL-16		*S. epidermidis*	*S. aureus*
compd	PMBN (0 μg/mL)	PMBN (10 μg/mL)	PMBN (0 μg/mL)	PMBN (10 μg/mL)	strain OC2603	strain OC6538
4a	1000	>1000	>1000	>1000	1000	1000
4b	>1000	>1000	>1000	>1000	1000	1000
4c	>1000	15.6	>1000	31.2	15.6	15.6
4d	>1000	32.5	>1000	125	62.5	62.5
4e	31.2	2.0	>1000	31.25	31.25	31.25
4f	>1000	500	>1000	>1000	500	500
1	1		8		250	500
PMBN		500		>500		

added. Additional 12 N HCl was added dropwise to retain an acidic mixture. The reaction mixture was allowed to stir at 25 °C for 3.5 h. The reaction mixture was reduced in vacuo, and 6 N NaOH was added until the pH was ∼9. The product was extracted with CH_2Cl_2 (3 × 250 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated. The product was purified by column chromatography on silica gel with 1:4 EtOAc:hexane to give 45 g (97% yield) of product: mp 84−85 °C; ^{1}H NMR ($CDCl_3$) δ 6.54 (s, 2 H), 4.00 (q, 1 H), 3.85 (s, 9 H), 3.84 (d, 3 H), 1.35 (d, 1 H); MS *m*/*z* 228 (M + 1). Anal. ($C_{11}H_{17}NO_4$) C, H, N.

General Procedure for the Preparation of the Hydroxamic Acids (4a−c,e). The following procedure for the preparation of ***N*-hydroxy-*N*-[2-(3,4,5-trimethoxyphenyl)-ethyl]-2-(2-carboxyphenyl)benzamide (4b)** is representative. A mixture of *N*-hydroxy-*N*-[2-(3,4,5-trimethoxyphenyl)-2-ethyl]amine **(7b)** (2.23 g, 9.81 mmol) in THF (100 mL) and diphenic anhydride (2.23 g, 9.95 mmol) was refluxed for 5 h. The solvent was removed in vacuo, and the residue was put on a silica gel column and eluted with 5% MeOH/CH_2Cl_2 to give 3 g (68% yield) of product: mp 55−57 °C; ^{1}H NMR ($CDCl_3$) δ 7.95 (m, 2 H), 7.51−7.13 (m, 6 H), 6.44 (d, 2 H), 3.92 (q, 1 H), 3.83 (d, 3 H), 3.78 (s, 9 H); MS *m*/*z* 228 (M + 1). Anal. ($C_{25}H_{25}NO_7$) C, H, N.

4′-(1-Phenylpiperazine)acetophenone (5f). To a solution of 1-phenylpiperazine (10.0 g, 62 mmol) in DMSO (150 mL) were added 4′-fluoroacetophenone (8.5 g, 62 mmol) and Et_3N (10 mL). The solution was refluxed for 23 h and then poured into ice water (500 mL). The product was collected by vacuum filtration to give 16.7 g (97% yield) of product. The ketone was recrystallized from MeOH: mp 179.9−181.5 °C; ^{1}H NMR ($CDCl_3$) δ 7.17 (d, 2 H), 7.33−6.91 (m, 7 H), 3.44 (d, 8 H), 2.54 (s, 3 H); MS *m*/*z* 281 (M + 1). Anal. ($C_{18}H_{20}NO_2$) C, H, N.

***N*-[1-[4-(4-Phenylpiperazin-4-yl)phenyl]ethyl]-*N*-hydroxylamine (7f).** To a solution of 4′-(1-phenylpiperazine)-acetophenone **(5f)** (16 g, 57 mmol) and hydroxylamine hydrochloride (13.8 g, 0.20 mol) was added powdered NaOH (20.2 g, 0.50 mol) in 90% EtOH (200 mL) in small portions. The mixture was allowed to stir at 25 °C for 30 min and then refluxed for another 30 min. The reaction mixture was then cooled to 25 °C and poured into a mixture of concentrated HCl (80 mL) and water (320 mL). The resulting precipitate was filtered, washed with water, and dried. The oxime was dissolved in MeOH (500 mL), and to this solution were added $NaBH_3CN$ (15 g, 0.24 mol) and a trace of methyl orange; 12 N HCl was added dropwise to retain an acidic media. The reaction mixture was allowed to stir at 25 °C for 3.5 h. The reaction mixture was reduced in vacuo, and 6 N NaOH was added until the pH was ∼9. The product was extracted with CH_2Cl_2 (3 × 250 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated. The product was purified by column chromatography on silica gel with 1:4 EtOAc:hexane to give hydroxylamine **7b**: 9.8 g (58% yield overall); mp 149.5−151.7 °C; ^{1}H NMR ($CDCl_3$) δ 7.32−6.89 (m, 9 H), 4.05 (q, 1 H), 3.33 (d, 8 H), 1.38 (d, 3 H); MS *m*/*z* 298 (M + 1). Anal. ($C_{18}H_{23}N_3O$) C, H, N.

General Procedure for the Preparation of the Hydroxamic Acids 10d,f. The following procedure for the preparation of **ethyl 2-[2-[*N*-hydroxy-*N*-[1-[4-(4-phenylpiperazin-1-yl)phenyl]ethyl]formamido]phenyl]benzoate (10f)** is representative**.** *N*-[1-[4-(4-Phenylpiperazin-4-yl)phenyl]ethyl]-*N*-hydroxylamine **(7f)** (2.0 g, 6.72 mmol) in THF (50 mL) and Et_3N (6 mL) and ethyl-2-(2-carbonylphenyl)benzoyl chloride (1.94 g, 6.73 mmol) was allowed to stir at 25 °C for 2 h. The solvent was removed in vacuo, and water was added. The pH of the aqueous solution was adjusted to 7 with 6 N HCl, and then the mixture was extracted with EtOAc. The product was extracted with EtOAc, dried over anhydrous $MgSO_4$, filtered, and concentrated. The product was purified by column chromatography on silica gel (230−400 mesh) eluted with 1−2% MeOH/CH_2Cl_2 to give 1.8 g (49% yield): mp 133.5−135.1 °C; ^{1}H NMR (DMSO-d_6) δ 7.83 (bs, 2 H), 7.45−6.78 (m, 15 H), 4.03 (bs, 1 H), 3.33 (s, 3 H), 3.25 (bs, 8 H), 1.16 (q, 2 H), 0.87 (t, 3 H); MS *m*/*z* 550 (M + 1). Anal. ($C_{34}H_{35}N_3O_4$) C, H, N.

General Procedure for the Preparation of the Hydroxamic Acids 4d,f. The following procedure for the preparation of **1-[4-[(*N*-hydroxy-2′-carboxy-2-biphenylcarboxamido)ethylidene]phenyl]-4-phenylpiperazine (4f)** is representative**.** To a solution of ethyl 2-[2-[*N*-hydroxy-*N*-[1-[4-(4-phenylpiperazin-1-yl)phenyl]ethyl]formamido]phenyl]-benzoate (1.6 g, 2.91 mmol) in 2-propanol (70 mL) and H_2O (5.7 mL) was added LiOH (1.13 g, 47.18 mmol). The mixture was allowed to reflux for 12 h. The solution was then made acidic by adding 12 N HCl. The product was extracted with CH_2Cl_2 (3 × 250 mL), dried over $MgSO_4$, and concentrated in vacuo. Purification by column chromatography on silica gel (230−400 mesh) eluted with 1−2% MeOH/CH_2Cl_2 gave 1.2 g (79% yield) of product: mp 177.8−178.4 °C; ^{1}H NMR (DMSO-d_6) δ 7.83−6.74 (m, 17 H), 5.28 (bs, 1 H), 3.33 (s, 3 H), 3.24 (d, 8 H); MS *m*/*z* 550 (M + 1). Anal. ($C_{34}H_{35}N_3O_4$) C, H, N.

Molecular Modeling. All molecular modeling was performed with the use of the software package SYBYL[23] running on a Silicon Graphics Indigo 2 workstation. Geometry optimizations were first performed with the molecular mechanics SYBYL/Tripos force field[24] using Gasteiger−Huckel charges. The resulting structures were then geometry optimized using the AM1 Hamiltonian[25] in the molecular orbital package SPARTAN.[26] Database searching was accomplished using ISIS 2.01[18] with both **3a**,**b** present in the same search query.

DNA Gyrase Cleavable Complex Assay.[17] Supercoiled BR322 DNA (0.4 μg) was added to a reaction mixture composed of 0.2 mM ATP, 5 mM spermidine, 5 nM DTT, 0.2 mM Na_2EDTA, 1.0% glycerol, 10 mM $MgCl_2$, 100 μg/mL *E. coli* tRNA (Sigma type XXI), 50 μg/mL bovine serum albumin (ultrapure-BRL), and 35 mM Tris-HCl, pH 7.5, in 30 μL reaction mixtures. To this reaction was added drug from DMSO-solubilized stocks (such that the final concentration of DMSO ≤ 3.5%) followed by 3 units of gyrase holoenzyme. The DNA gyrase subunits A and B were purified to homogeneity from *E. coli*-overproducing strains (provided by M. Gellert, NIH) and purified by the procedure of Mizuuchi et al.[27] The individual subunits were combined in 1:1 molar ratios and reconstituted as the holoenzyme for use in the supercoiling and cleavable complex assay. The reaction mixture was incubated for 45 min at 25 °C. The reaction was stopped by the addition of SDS (to 0.2%) followed by the addition of proteinase K (to 90 μg/mL final concentration) to digest the enzyme−DNA complex and incubation at 37 °C for 30 min. This denaturation of the gyrase−DNA complex, followed by proteolytic digestion of the protein portion of the complex, releases the linear DNA fragments from the complex which can then be separated from open circular and supercoiled DNA by agarose gel electrophoresis. One microliter tracking dye (50% glycerol, 0.125% bromophenol blue) was added to the reaction mixture, and the mixture was loaded onto a 0.7% agarose gel for electrophoretic separation. The reaction

mixture was electrophoresed in a horizontal submarine gel through a 0.7% Tris−acetate−EDTA agarose gel to separate different DNA topoisomers and linear DNA fragments from the denatured gyrase−DNA−compound complex. Linearized DNA was detected as a single band between open circular DNA and supercoiled DNA after separation of the products by electrophoresis. The gel was stained with ethidium bromide, visualized by UV fluorescence at 300 nm, and documented by Polaroid film 665 photography. Percent supercoiling was determined by the densitometric tracing (area determination using Collage Image Analysis software, FOTODYNE, New Berlin, WI) of the linearized DNA bands. The cleavable complex CC_{50} was determined by measuring the amount of drug required to induce 50% cleavage of the maximal amount of linear DNA from supercoiled DNA. Under these conditions, the linear range of quantitation of DNA is up to 1.2 μg (data not shown).

DNA Gyrase Supercoiling Inhibition Assay.[16] A Portion of 0.25−0.40 μg of pBR322 DNA (previously relaxed with topoisomerase I) was added to a reaction mixture composed of 1.4 mM ATP, 1.8 mM spermidine, 5 mM DTT, 0.14 mM Na_2EDTA, 6.5% glycerol, 24 mM KCl, 4 mM $MgCl_2$, 0.36 μg/mL bovine serum albumin (molecular biology grade), and 35 mM Tris-HCl, pH 7.5. To this reaction was added drug from DMSO-solubilized stocks (such that the final concentration of DMSO $\leq$ 3.5%), followed by 1 unit of gyrase holoenzyme (reconstituted GyrA and GyrB subunits).[27] Each reaction mixture was incubated for 30 min at 37 °C, and reactions were stopped by the addition of SDS (to 0.5%), Na_2EDTA (to 6 mM), and 5.35% glycerol containing 0.013% bromophenol blue (as tracking dye). The total reaction mix was loaded onto either a 1% TAE or TBE agarose gel and was electrophoresed in a horizontal submarine apparatus to separate different DNA topoisomers. Gels were stained with EtBr and visualized by Polaroid film 667 photography of fluoresced gels. The percent supercoiling was determined by the densitometric tracing (Collage, Image Dynamics Corp.) of supercoiling versus relaxed DNA, normalized against no-drug control lanes.

Minimum Inhibitory Concentration Assay. The MIC of the test compounds was determined in either Mueller Hinton broth for Gram-negatives or Luria broth for Gram-positives in a microtiter well dilution series; 2-fold serial dilutions (range 0.015−1000 μg/mL) of compound in broth were inoculated with adjusted suspensions of test organisms to approximately 5×10^5 CFU/mL. Microtiter plates were incubated overnight at 35 °C. MIC is defined as the well concentration with no visible growth. PMBN was used in conjunction with test compounds being screened at doses (at levels of PMBN exhibiting no antibacterial activity in itself) lower than the MIC and compared to PMBN-free controls.

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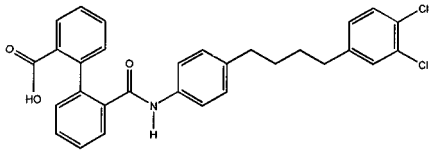

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