


Cite this: *RSC Adv.*, 2023, 13, 27391

Amidinoquinoxaline *N*-oxides: synthesis and activity against anaerobic bacteria†

Nadia Gruber,^a Liliana Fernández-Canigia,^{*b} Natalia B. Kilimciler,^a Pierluigi Stipa,^c Juan A. Bisceglia,^a María B. García,^a Daniel H. Gonzalez Maglio,^d Mariela L. Paz^d and Liliana R. Orelli^{*a}

We present herein an in-depth study on the activity of amidinoquinoxaline *N*-oxides **1** against Gram-positive and Gram-negative anaerobic bacteria. Based on 5-phenyl-2,3-dihydropyrimidoquinoxaline *N*-oxide **1a**, the selected structural variations included in our study comprise the substituents α - to the *N*-oxide function, the benzofused ring, substitution and quaternization of the amidine moiety, and the amidine ring size. Compounds **1** showed good to excellent antianaerobic activity, evaluated as the corresponding CIM₅₀ and CIM₉₀ values, and an antimicrobial spectrum similar to metronidazole. Six out of 13 compounds **1** had CIM₉₀ values significantly lower than the reference drug. Among them, imidazoline derivatives **1i–l** were the most active structures. Such compounds were synthesized by base-promoted ring closure of the corresponding amidines. The *N*-oxides under study showed no significant cytotoxicity against RAW 264.7 cells, with high selectivity indexes. Their calculated ADME properties indicate that the compounds are potentially good oral drug candidates. The antianaerobic activity correlated satisfactorily with the electron affinity of the compounds, suggesting that they may undergo bioreductive activation before exerting their antibacterial activity.

Received 22nd February 2023
Accepted 20th August 2023

DOI: 10.1039/d3ra01184d

rsc.li/rsc-advances

1 Introduction

Anaerobic bacteria are the main component of the bacterial microbiota of normal human skin and mucous membranes and can behave as opportunistic pathogens under an appropriate environment. Infections have commonly an endogenous origin and, comparatively, a small number of diseases are associated with exogenous anaerobic pathogens. These bacteria can lead to a broad spectrum of infections, which in some cases are life-threatening: septicemia, endocarditis, brain, lung and liver abscesses and aspiration pneumonias, among others.^{1,2}

Among the antimicrobials that can be chosen according to the infection localization and the anaerobic species, metronidazole continues to be one of the drugs of choice³ showing generally low resistance levels among pathogenic anaerobes.⁴

However, resistant strains have been reported over the past decades^{5–9} and decreased *in vitro* susceptibility has been observed in recent years.^{10,11} In addition, even if metronidazole resistance was first reported limited to *Bacteroides* spp, it now includes Gram-positive cocci and other bacilli.¹² Several mechanisms of metronidazole resistance in anaerobic bacteria have been proposed,^{13–16} including specific genes (*nim*), that encode an alternative reductase that can convert 4- or 5-nitroimidazoles to a non-bactericidal derivative by reduction of the nitro group to an amino function.^{17,18} These genes have been isolated in Gram-positive and Gram-negative anaerobic bacteria.^{19–22} Thus, as resistance to metronidazole emerges, the development of new specific anti-anaerobe agents becomes necessary.

Among anaerobic bacteria, *Clostridioides difficile*, an anaerobe responsible for intestinal infections associated with life-threatening severe diarrhea, abdominal pain and fever, is currently a topic of concern, given that virulent strains are causing nosocomial outbreaks in North America, Canada and Europe.²³ It is also an important agent of diarrheal illness in outpatients. Infections with *C. difficile* have been classified as an urgent public health threat because of the number of infections and deaths directly attributable to it.²⁴ The first line treatments for this pathogen are metronidazole or oral vancomycin. However, a recent review reports 22.4% and 14.2% treatment failure and 27.1% and 24.0% recurrence after treatment with metronidazole and vancomycin, respectively.²⁵ Metronidazole, vancomycin, and fidaxomicin drug resistance in *C. difficile* is

^aUniversidad de Buenos Aires, CONICET, Química Orgánica II, Departamento de Ciencias Químicas, Facultad de Farmacia y Bioquímica, Junín 956, (1113) Buenos Aires, Argentina. E-mail: lorelli@ffyb.uba.ar

^bLaboratorio de Microbiología, Hospital Alemán, Av. Pueyrredón 1640, (1118) Buenos Aires, Argentina

^cSIMAU Departament – Chemistry Division, Università Politecnica delle Marche, Via Brecce Bianche 12, Ancona (I-60131), Italy

^dUniversidad de Buenos Aires, Instituto de Estudios de la Inmunidad Humoral (IDEHU), Cátedra de Inmunología, Facultad de Farmacia y Bioquímica, Junín 956, (1113) Buenos Aires, Argentina

† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d3ra01184d>



fortunately not widespread at this time,²⁶ but given the increasing prevalence of *C. difficile* infections over the past decade, the requirement for new antimicrobials effective against *C. difficile* is an important preventive measure.²⁷

Several research groups have sought for alternatives to treat anaerobic infections, including traditional antimicrobials and other biological therapeutics. In recent years, metronidazole derivatives were reported, which in many cases involve modification in the hydroxyethyl chain such as the replacement of the hydroxyl group by an *N*-piperazino carbamoyl,²⁸ a triazole²⁹ or a triazolylthio group.³⁰ Other modifications of this chain include the preparation of ester and ether derivatives using terpenes.³¹ Nitroheterocycles including secnidazole derivatives and their copper(II) complexes,³² substituted nitroimidazoles,^{31,33} nitazoxanide analogs^{34–36} and nitrofuranylsemicarbazones³⁷ were also tested on anaerobes. Since most of the developed compounds are modifications of existing antimicrobials, they are only short term solutions that cannot usually overcome multiple resistance mechanisms.³⁸

Amidinoquinoxaline *N*-oxides represent a heterocyclic core of interest due to their pharmacological properties. Some suitably substituted derivatives possess antineoplastic activity,^{39–41} in particular against hypoxic tumors, while others behave as antiamoebic agents.⁴² The activity of some related compounds against a small number of anaerobic bacterial strains is also described in the literature.^{43–46} In addition, recent results of our group show that these nitrones behave as antioxidants⁴⁷ due to their ability to act as spin traps^{48,49} as well as to undergo single electron transfer reactions. The pharmacological interest of this heterocyclic core, the versatility of the *N*-oxide function and the results of our recent research, prompted us to study the activity of amidinoquinoxaline *N*-oxides **1** (Fig. 1) against anaerobic bacteria as an alternative to metronidazole. Since the molecular size and functional groups present in our compounds are different from those of metronidazole, it could be expected that they would circumvent the action of reductases encoded by *nim* genes. As a substantial difference, the functional group to be reduced in metronidazole is the nitro function, while in the nitrones the bioreducible functionality is the *N*-oxide.

In this work we have evaluated the activity of the nitrones under study against anaerobic bacteria and analyzed the structural features that influence their bioactivity. We also present the results of cytotoxicity assays and ADME predictions as well as an insight to a general mechanism proposal. The synthetic approach previously reported by our group⁵⁰ included a spontaneous cyclodehydration step which was too slow for some derivatives, leading in those cases to byproducts and affording low yields. We present herein an improved synthetic procedure that circumvents these problems.

2 Materials and methods

2.1 Synthesis

2.1.1. General information. Melting points were determined with a Büchi capillary apparatus and are uncorrected. ¹H and ¹³C NMR spectra were recorded on a Bruker Bio Spin Avance III 600 MHz spectrometer or a Bruker Avance II 500 MHz

spectrometer. Chemical shifts are reported in ppm (δ) relative to tetramethylsilane. D₂O was employed to confirm exchangeable protons (ex). Splitting multiplicities are reported as singlet (s), broad signal (bs), doublet (d), double doublet (dd), triplet (t), triplet of doublets (td), quartet (q) and multiplet (m). Diamond ATR-FTIR (attenuated total reflectance Fourier-transform infrared spectroscopy) spectra were acquired using a Nicolet iS50 Advanced Spectrometer (Thermo Scientific); the signal intensity is indicated as strong (s) or medium (m). High-resolution mass spectrometry (HRMS) was performed with a Xevo G2S Q-TOF (Waters Corp). Elemental analyses were determined in a Carlo Erba EA 1108 Analyzer. Reagents, solvents, and starting materials were purchased from standard sources and purified according to literature procedures.

2.1.2. Representative procedures for syntheses. Aminoamides **3a–m** were prepared according to the method developed by our group.⁴⁵

N-Oxides **1a–h,m** were prepared by cyclodehydration of aminoamides **3a–h,m** (Scheme 1).⁴⁵ A mixture of the aminoamide (1 mmol) and ethyl polyphosphate (PPE, 1 mL/0.05 g) was refluxed for 5 h in an oil bath. After reaching room temperature, the resulting solution was extracted with water (5 × 6 mL). The aqueous phases were pooled, filtered and made alkaline with 10% aqueous NaOH. The mixture was extracted with chloroform (3 × 15 mL). The organic phases were washed with water, dried over sodium sulphate and filtered. The crude chloroformic solution of amidines **4a–h,m** was left at r.t. until no further conversion to compounds **1a–h,m** was evidenced by TLC (silica gel, chloroform : methanol 9 : 1). The solvent was then removed *in vacuo* and the crude product was purified by column chromatography (silica gel, chloroform : methanol 10 : 0–9 : 1).

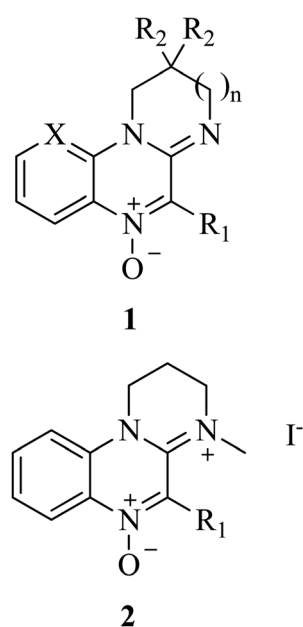
Amidinium salts **2a–c** were prepared from the corresponding *N*-oxides **1a–c** according to the method developed by our group (Scheme 2).⁴⁶

2.1.2.1 Base-promoted synthesis of 4-aryl-1,2-dihydroimidazo[1,2-*a*]quinoxaline 5-oxides **1i–l.** Imidazolines **4i–l** were prepared in the same way as the analogous tetrahydropyrimidines and tetrahydrodiazepines **4a–h,m**. The crude imidazolines **4i–l** were stirred with 4% KOH in absolute ethanol (10 mL/100 mg of compound) in an ice bath. After 10 min the cold bath was removed and the mixture was stirred at room temperature for 50 min. The mixture was then concentrated *in vacuo*, diluted with water (5 mL) and extracted with methylene chloride (3 × 15 mL). The organic phases were pooled, dried with anhydrous sodium sulphate, filtered and the solvent was removed *in vacuo*. The crude compounds were purified by crystallization (EtOH) or column chromatography (Silica gel, chloroform : methanol 10 : 0–9 : 1).

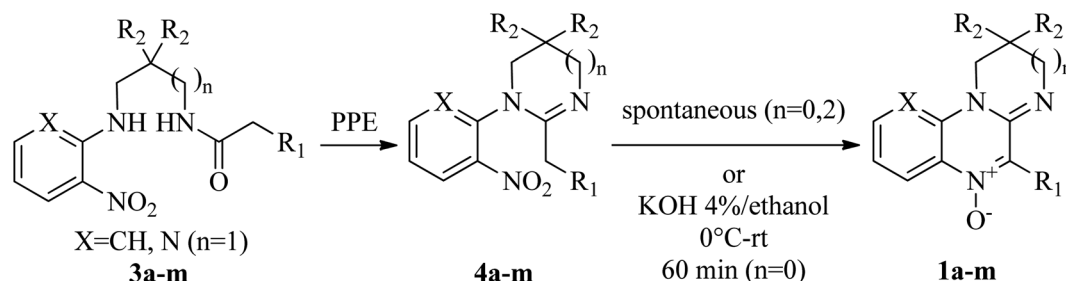
Compounds **1a–d,h,m**,⁴⁵ **1e**,⁵¹ **2a–c**,⁴⁶ **1f,g**,⁴⁸ **1i** (ref. 51) and **1l** (ref. 49) were described in the literature. Yields and analytical data of nitrones **1j,k**, aminoamides **3j,k** and amidines **4j–l** are as follows.

2.1.2.1.1 4-(4-Chlorophenyl)-1,2-dihydroimidazo[1,2-*a*]quinoxaline 5-oxide (1j**).** This compound was obtained as an orange hygroscopic solid (244 mg, 82% yield), mp = 181–182 °C (from EtOH). Anal. calc. for C₁₆H₁₂ClN₃O: C, 64.5; H, 4.1; N, 14.1.





- 1a:** $n=1$, $X=CH$, $R_1=C_6H_5$, $R_2=H$
1b: $n=1$, $X=CH$, $R_1=4-ClC_6H_4$, $R_2=H$
1c: $n=1$, $X=CH$, $R_1=4-NO_2C_6H_4$, $R_2=H$
1d: $n=1$, $X=CH$, $R_1=4-OCH_3C_6H_4$, $R_2=H$
1e: $n=1$, $X=CH$, $R_1=4-OCH_3C_6H_4$, $R_2=CH_3$
1f: $n=1$, $X=N$, $R_1=C_6H_5$, $R_2=H$
1g: $n=1$, $X=CH$, $R_1=3-C_4H_3S$, $R_2=H$
1h: $n=1$, $X=CH$, $R_1=CH_2C_6H_5$, $R_2=H$
1i: $n=0$, $X=CH$, $R_1=C_6H_5$, $R_2=H$
1j: $n=0$, $X=CH$, $R_1=4-ClC_6H_4$, $R_2=H$
1k: $n=0$, $X=CH$, $R_1=4-NO_2C_6H_4$, $R_2=H$
1l: $n=0$, $X=CH$, $R_1=4-OCH_3C_6H_4$, $R_2=H$
1m: $n=2$, $X=CH$, $R_1=4-ClC_6H_4$, $R_2=H$
2a: $R_1=C_6H_5$
2b: $R_1=4-ClC_6H_4$
2c: $R_1=4-NO_2C_6H_4$

Fig. 1 Amidinoquinoxaline *N*-oxides **1** and their quaternary salts **2**.Scheme 1 Synthesis of amidinoquinoxaline *N*-oxides **1**.

Found: C, 64.8; H, 4.1; N, 14.1%. IR (neat) $\bar{\nu}/cm^{-1}$: 1618m, 1578s, 1505s, 1467m, 1373m, 1282s, 1087s, 819s, 548m, 437s. 1H NMR (600 MHz, $CDCl_3$, 25 °C, TMS): δ = 8.27 (1H, dd, J = 8.3, 1.3 Hz), 7.91 (2H, d, J = 8.6 Hz), 7.48–7.52 (1H, m), 7.46 (2H, d, J = 8.6 Hz), 7.10–7.15 (1H, m), 6.82 (1H, dd, J = 8.1, 1.1 Hz), 4.17–4.22 (2H, m), 4.05–4.10 (2H, m). ^{13}C NMR (151 MHz, $CDCl_3$, 25 °C) δ = 152.7, 136.1, 134.8, 133.3, 132.3, 131.7, 131.0, 128.2, 126.8, 121.4, 121.3, 111.9, 54.2, 46.5. HRMS (ESI) m/z : $[M + H]^+$ calcd for $C_{16}H_{13}ClN_3O$: 298.0747. Found: 298.0743.

2.1.2.1.2 4-(4-Nitrophenyl)-1,2-dihydroimidazo[1,2-a]quinoxaline 5-oxide (1k). This compound was obtained as a red hygroscopic solid (246 mg, 80%), mp = 211–212 °C (from EtOH). Anal. calc. for $C_{16}H_{12}N_4O_3$: C, 62.3; H, 3.9; N, 18.2. Found: C, 61.4; H, 3.9; N, 17.7%. IR (neat) $\bar{\nu}/cm^{-1}$: 1607m, 1599s, 1577s, 1503s, 1343s, 1282s. 1H NMR (600 MHz, $CDCl_3$, 25 °C, TMS): δ = 8.33 (2H, d, J = 8.9 Hz), 8.26 (1H, dd, J = 8.1, 1.2 Hz), 8.16 (2H, d, J = 8.9 Hz), 7.51–7.56 (1H, m), 7.12–7.17 (1H, m), 6.85 (1H, dd, J = 8.1, 1.0 Hz), 4.18–4.23 (2H, m), 4.07–4.13 (2H, m). ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 152.3, 148.1, 134.9, 133.7, 133.5, 132.9, 131.6, 131.0, 123.0, 121.51, 121.48,

112.1, 54.2, 46.5. HRMS (ESI) calcd for $C_{16}H_{13}N_4O_3$: 309.0988. Found: 309.0985.

2.1.2.1.3 2-(4-Chlorophenyl)-N-(2-(2-nitrophenylamino)ethyl)acetamide (3j). This compound was obtained as an orange solid (237 mg, 71% yield), mp = 132–134 °C (from hexane/chloroform). 1H NMR (600 MHz, $CDCl_3$, 25 °C, TMS): δ = 8.14 (1H, dd, J = 8.6, 1.0 Hz), 8.08 (1H, bs ex), 7.40–7.44 (1H, m), 7.28 (2H, d, J = 8.3 Hz), 7.15 (2H, d, J = 8.3 Hz), 6.91 (1H, d, J = 8.6 Hz), 6.64–6.69 (1H, m), 5.88 (1H, bs ex), 3.53 (2H, s), 3.46–3.52 (4H, m). ^{13}C NMR (151 MHz, $CDCl_3$, 25 °C) δ = 171.2, 145.3,

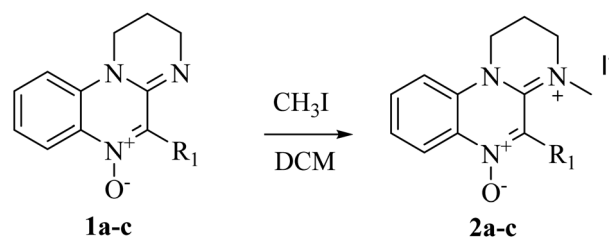
Scheme 2 Synthesis of amidinium salts **2a–c**.

Table 1 Yields obtained for 1i–1l by spontaneous cyclization (I) and base promotion (II)

Comp	Yield 3 → 1 (%) (I)	Yield 3 → 1 (%) (II)
1i	64	90
1j	59	82
1k	17	80
1l	71	82

136.4, 133.4, 132.9, 132.2, 130.7, 129.1, 126.9, 115.8, 113.7, 42.8, 41.9, 39.0. HRMS (ESI) m/z : $[M + H]^+$ calcd for $C_{16}H_{17}ClN_3O_3$: 334.0959. Found: 334.0957.

2.1.2.1.4 2-(4-Nitrophenyl)-N-(2-((2-nitrophenyl)amino)ethyl)acetamide (3k). This compound was obtained as an orange solid (151 mg, 44% yield), mp = 190–192 °C (from hexane/chloroform). 1H NMR (500 MHz, DMSO- d_6 , 25 °C, TMS): δ = 8.41 (1H, bs), 8.18 (1H, bs), 8.13 (2H, d, J = 8.7 Hz), 8.04 (1H, dd, J = 8.7, 1.6 Hz), 7.48–7.53 (3H, m), 7.11 (1H, dd, J = 8.7, 0.9 Hz), 6.65–6.70 (1H, m), 3.58 (2H, s), 3.44 (2H, c, J = 6.1 Hz), 3.30–3.34 (2H, m). ^{13}C NMR (125 MHz, DMSO- d_6 , 25 °C): δ = 169.7, 146.3, 145.2, 144.3, 136.6, 131.1, 130.4, 126.3, 123.3, 115.3, 114.4, 41.9, 41.8, 37.8. HRMS (ESI) calcd for $C_{16}H_{17}N_4O_5$: 345.1199. Found: 345.1204.

2.1.2.1.5 2-(4-Chlorobenzyl)-1-(2-nitrophenyl)-4,5-dihydro-1H-imidazole (4j). This compound was obtained as an orange oil (246 mg, 78%). 1H NMR (600 MHz, $CDCl_3$, 25 °C, TMS): δ = 7.87 (1H, dd, J = 8.1, 1.5 Hz), 7.54 (1H, td, J = 7.7, 1.5 Hz), 7.41–7.44 (1H, m), 7.10–7.14 (3H, m), 6.89 (2H, d, J = 8.5 Hz), 3.98 (2H, t, J = 9.5 Hz), 3.77 (2H, t, J = 9.5 Hz), 3.39 (2H, s). ^{13}C NMR (151 MHz, $CDCl_3$, 25 °C): δ = 162.9, 147.8, 133.7, 133.6, 132.7, 132.2, 130.9, 130.0, 128.5, 128.2, 125.2, 54.0, 53.3, 34.0.

2.1.2.1.6 2-(4-Nitrobenzyl)-1-(2-nitrophenyl)-4,5-dihydro-1H-imidazole (4k). This compound was obtained as an orange oil (231 mg, 71%). 1H NMR (600 MHz, $CDCl_3$, 25 °C, TMS): δ = 8.05 (2H, d, J = 8.5 Hz), 7.88 (1H, dd, J = 8.1, 1.6 Hz), 7.58 (1H, td, J = 7.8, 1.6 Hz), 7.44–7.48 (1H, m), 7.20 (1H, dd, J = 7.8, 1.4 Hz), 7.17 (2H, d, J = 8.5 Hz), 3.98 (2H, t, J = 9.6 Hz), 3.78 (2H, t, J = 9.6 Hz), 3.52 (2H, s). ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 162.0, 147.9, 146.9, 142.8, 134.8, 133.9, 130.7, 129.7, 128.5, 125.4, 123.6, 54.1, 53.6, 34.4.

2.1.2.1.7 2-(4-Methoxybenzyl)-1-(2-nitrophenyl)-4,5-dihydro-1H-imidazole (4l). This compound was obtained as a yellow oil (255 mg, 82%). 1H NMR (600 MHz, $CDCl_3$, 25 °C, TMS): δ = 7.88 (1H, dd, J = 8.2, 1.5 Hz), 7.55 (1H, td, J = 7.6, 1.5 Hz), 7.40–7.46 (1H, m), 7.17 (1H, dd, J = 7.6, 1.3 Hz), 6.81 (2H, d, J = 8.6 Hz), 6.68 (2H, d, J = 8.6 Hz), 3.99 (2H, t, J = 9.7 Hz), 3.81 (2H, t, J =

9.7 Hz), 3.74 (3H, s), 3.43 (2H, s). ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 164.3, 158.5, 147.5, 134.7, 133.8, 131.98, 131.0, 129.7, 128.3, 125.3, 113.8, 55.2, 54.0, 52.3, 33.6.

2.2 Clinical isolates

A total of 100 bacterial isolates, including ATCC strains and clinical isolates of Gram-positive and Gram-negative anaerobic bacteria were tested. A first panel composed of 84 strains of special clinical relevance was further subdivided into six groups, which include the same or related species (Table 2). Groups 1 to 4, corresponding to Gram-negative bacilli, were assigned to *B. fragilis*, *Bacteroides* spp. and *Parabacteroides distasonis*, *Prevotella* spp. and *Fusobacterium nucleatum*, respectively. The species of genus *Bacteroides* are some of the most frequently isolated anaerobes in clinical laboratories and are also typically more virulent and resistant. Among them, *B. fragilis* has the greatest clinical relevance and was therefore considered as a separate group. Groups 5 and 6 were assigned to *Clostridium perfringens* and *C. difficile* as Gram-positive spore-forming bacilli (Table 2).

The remaining isolates were included in Table 3 as representative examples of less common genera and/or species that, together with the previous results, contribute to outline the spectrum of action of this nitron family.

2.3 Minimal inhibitory concentration (MIC) determinations

MIC determinations were performed using the Agar Dilution Procedure according to CLSI guidelines.^{52,53} This technique is used to quantitatively measure the *in vitro* antimicrobial activity and is considered the standard method for anaerobic bacteria. MIC is defined as the minimum antimicrobial concentration that prevents the visible development of a microorganism in a susceptibility test by dilution in broth or agar.⁵⁴ The culture medium, Brucella agar, was prepared fresh and supplemented with vitamin K (1 $\mu g mL^{-1}$), hemin (5 $\mu g mL^{-1}$) and laked sheep blood (5% V/V). Clinical isolates from the Microbiology Laboratory of the Hospital Aleman, stored at –70 °C, were subcultured twice or until normal growth rate. The isolate purity was controlled and identification, if needed, was confirmed with a MALDI-TOF BD mass spectrometer.

Stock solutions were prepared with 50% DMSO in sterile distilled water and two-fold serial dilutions of the nitrones were made. Compound concentration in the culture plate ranged typically from 64 to 0.06 $\mu g mL^{-1}$ standardized bacterial inoculums of $\sim 1.5 \times 10^8$ CFU mL^{-1} were prepared in BHI broth (0.5 of the McFarland standard). Agar dilution test plates were inoculated with 1 μL (approximately 1.5×10^5 CFU per spot) using a Steers multipoint replicator. Plates were incubated at 37 °C for

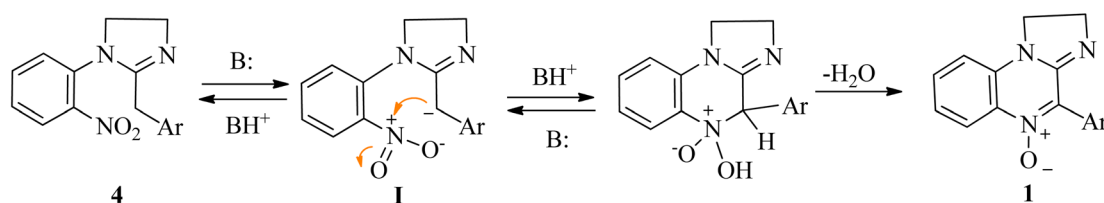
**Scheme 3** Probable base-promoted cyclodehydration mechanism.



Table 2 *In vitro* activity of amidinoquinoline N-oxides **1**, **2** against ATCC strains (informed as MIC) and clinical isolates (informed as MIC₅₀ and MIC₉₀) of relevant anaerobic species^{d,e}

Organism and compounds	MIC ₅₀ (μg mL ⁻¹)/MIC ₉₀ (μg mL ⁻¹)/(n° of isolates)																
	1a	1b	1c	1d	1e	1f	1g	1h	1i	1j	1k	1l	1m	2a	2b	2c	Mtz
Gram negative bacilli																	
<i>Bacteroides fragilis</i> (ATCC 25285)	1	2	≤0.06	2	0.5	0.125	0.5	1	0.125	0.125	≤0.06	≤0.06	2	4	4	1	0.25
<i>Bacteroides fragilis</i>	0.5	1	≤0.06	1	0.25	≤0.06	0.25	1	≤0.06	≤0.06	≤0.06	≤0.06	1	2	4	0.5	0.5
	1 (19)	2 (19)	≤0.06 (19)	2 (19)	0.5 (16)	≤0.06 (16)	0.5 (16)	0.4 (16)	≤0.06 (16)	0.125 (16)	≤0.06 (16)	≤0.06 (16)	2 (19)	2 (16)	8 (16)	1 (16)	1 (16)
<i>Bacteroides thetaiotaomicron</i> (ATCC 29148)	0.5	1	0.125	1	0.5	≤0.06	0.5	2	≤0.06	0.125	≤0.06	≤0.06	1	2	4	0.5	1
<i>Bacteroides ovatus</i> (ATCC 84834)	ND	ND	ND	ND	0.5	0.125	1	4	≤0.06	0.125	≤0.06	≤0.06	ND	4	8	0.5	1
Other <i>Bacteroides</i> spp.	0.5	1	≤0.06	1	0.25	≤0.06	0.25	1	≤0.06	≤0.06	≤0.06	≤0.06	1	2	4	0.25	1
and <i>Parabacteroides</i> ^a	1 (13)	1 (13)	≤0.06 (13)	2 (13)	0.5 (13)	≤0.06 (13)	0.5 (13)	4 (13)	≤0.06 (13)	0.125 (13)	≤0.06 (13)	≤0.06 (13)	2 (13)	4 (13)	8 (13)	1 (13)	1 (13)
<i>Prevotella intermedia/nigrescens</i> (ATCC 25611)	0.5	1	≤0.06	1	0.5	0.125	0.25	1	≤0.06	≤0.06	≤0.06	≤0.06	0.5	8	16	2	1
<i>Prevotella</i> spp.	0.25	0.5	≤0.06	0.5	0.25	≤0.06	0.125	1	≤0.06	≤0.06	≤0.06	≤0.06	0.5	4	4	0.5	0.5
	1 (21) ^b	1 (21) ^b	0.125 (21) ^b	1 (21) ^b	0.5 (14) ^c	0.125 (14) ^c	0.5 (14) ^c	4 (14) ^c	≤0.06 (14) ^c	≤0.06 (14) ^c	≤0.06 (14) ^c	≤0.06 (14) ^c	0.5 (21) ^b	8 (14) ^c	8 (14) ^c	1 (14) ^c	1 (21) ^b
<i>Fusobacterium nucleatum</i> (ATCC 25586)	0.125	0.25	≤0.06	0.5	0.25	0.125	0.125	0.5	≤0.06	≤0.06	≤0.06	≤0.06	0.5	2	4	0.25	0.125
<i>Fusobacterium nucleatum</i>	0.25	0.25	≤0.06	0.5	0.25	0.125	0.25	1	≤0.06	≤0.06	≤0.06	≤0.06	0.5	4	4	0.25	≤0.06
	1 (7)	1 (7)	≤0.06 (7)	2 (7)	0.25 (7)	0.5 (7)	0.5 (7)	4 (7)	0.125 (7)	0.125 (7)	≤0.06 (7)	≤0.06 (7)	0.5 (7)	4 (7)	8 (7)	1 (7)	0.25 (7)
Gram positive bacilli																	
<i>Clostridium difficile</i> (ATCC 43255)	4	8	≤0.06	8	4	0.5	4	32	1	0.25	≤0.06	0.5	4	32	16	0.5	0.25
<i>Clostridium difficile</i>	2	8	0.25	4	2	0.5	2	16	0.5	0.25	≤0.06	0.25	2	16	32	0.25	0.25
	4 (9)	16 (9)	0.5 (9)	8 (9)	8 (14)	0.5 (14)	2 (14)	16 (14)	0.5 (14)	0.25 (14)	≤0.06 (14)	0.5 (14)	4 (9)	32 (14)	32 (14)	0.5 (14)	0.25 (14)
<i>Clostridium perfringens</i>	8	32	0.5	16	4	0.5	4	<32	0.5	0.5	≤0.06	0.5	16	32	32	0.25	0.5
	16 (10)	64 (10)	2 (10)	32 (10)	8 (10)	1 (10)	8 (10)	<32 (10)	1 (10)	1 (10)	0.125 (10)	1 (10)	32 (10)	32 (10)	32 (10)	1 (10)	1 (10)

^a Includes 3 isolates of *Parabacteroides distasonis* and 10 corresponding to species of *Bacteroides*: 6 *Bacteroides thetaiotaomicron/ovatus*, 2 *Bacteroides uniformis*, 1 *Bacteroides vulgatus* and 1 *Bacteroides caccae*. ^b Includes 16 isolates of *Prevotella intermedia/nigrescens*, 2 of *Prevotella oralis* group, 2 of *Prevotella buccae* and 1 of *Prevotella bivia*. ^c Includes 5 isolates of *Prevotella intermedia/nigrescens*, 1 of *Prevotella corporis*, 1 of *Prevotella oralis* group, 1 of *Prevotella oris*, 1 of *Prevotella buccae*, 2 of *Prevotella baroniae*, 2 of *Prevotella buccae*, 2 of *Prevotella bivia* and 1 of *Prevotella dentatis*. ^d MTZ = metronidazole. ^e ND: not determined.

Table 3 *In vitro* activity of *N*-oxides 1, 2 against ATCC strains and other clinical isolates (CI)^{a,b}

	MIC (µg mL ⁻¹)																
Organism	1a	1b	1c	1d	1e	1f	1g	1h	1i	1j	1k	1l	1m	2a	2b	2c	Mtz
Gram negative bacilli																	
<i>Porphyromonas gingivalis</i> (ATCC 33277)	≤0.06	0.125	≤0.06	≤0.06	ND	ND	ND	ND	ND	≤0.06	ND	ND	0.25	ND	ND	ND	≤0.06
<i>Porphyromonas asaccharolytica</i> (CI)	ND	ND	ND	ND	0.25	≤0.06	0.125	2	≤0.06	≤0.06	≤0.06	≤0.06	ND	4	4	0.25	0.25
<i>Porphyromonas</i> sp. (CI)	0.125	0.5	≤0.06	0.5	0.25	≤0.06	0.125	1	≤0.06	≤0.06	≤0.06	≤0.06	0.25	4	4	0.5	0.125
<i>Fusobacterium mortiferum</i> (CI 1)	ND	ND	ND	ND	0.5	0.125	0.25	2	≤0.06	≤0.06	≤0.06	≤0.06	ND	4	8	1	0.25
<i>Fusobacterium mortiferum</i> (CI 2)	ND	ND	ND	ND	2	0.25	1	8	0.25	0.25	0.125	0.25	ND	8	16	2	0.25
Gram positive bacilli																	
<i>Spore-forming</i>																	
<i>Clostridium sporogenes</i> (ATCC 3584)	0.5	2	≤0.06	1	1	0.25	1	8	0.25	0.25	≤0.06	0.125	1	8	8	0.125	0.25
<i>Eggerthella lenta</i> (ATCC 43055)	0.5	0.5	≤0.06	0.5	0.5	≤0.06	0.25	2	≤0.06	≤0.06	≤0.06	≤0.06	1	4	8	0.5	0.25
<i>Clostridium sordellii</i> (CI)	16	64	0.5	32	ND	ND	ND	ND	ND	4	ND	ND	64	ND	ND	ND	1
<i>Clostridium butyricum</i> (CI)	ND	ND	ND	ND	0.25	≤0.06	0.25	8	≤0.06	≤0.06	≤0.06	≤0.06	ND	8	4	≤0.06	≤0.06
<i>Non-spore-forming</i>																	
<i>Actinomyces odontolyticus</i> (CI)	32	32	>64	64	ND	ND	ND	ND	ND	64	ND	ND	>64	ND	ND	ND	64
<i>Cutibacterium acnes</i> (CI 1)	64	64	>64	>64	ND	ND	ND	ND	ND	64	ND	ND	>64	ND	ND	ND	>64
<i>Cutibacterium acnes</i> (CI 2)	ND	ND	ND	ND	16	16	32	>32	16	8	32	8	ND	16	8	>16	>64
<i>Cutibacterium acnes</i> (CI 3)	>64	>64	>64	>64	ND	ND	ND	ND	ND	32	ND	ND	>64	ND	ND	ND	>64
Gram positive cocci																	
<i>Parvimonas micra</i> (ATCC 3870)	0.125	0.25	≤0.06	0.25	≤0.06	≤0.06	≤0.06	0.125	≤0.06	≤0.06	≤0.06	≤0.06	0.25	0.5	1	≤0.06	0.125
<i>Finegoldia magna</i> (CI)	2	2	1	1	4	0.5	0.25	8	0.125	0.25	1	0.25	0.25	32	>16	1	0.125
<i>Peptostreptococcus anaerobius</i> (CI)	ND	ND	ND	ND	1	0.25	1	8	0.5	0.25	≤0.06	0.25	ND	16	16	0.125	0.125

^a ND: not determined. ^b Mtz: metronidazole.

48 h in anaerobic conditions using anaerobic atmosphere generation bags (Anaero-Pack, Key Scientific, Mitsubishi).

Positive growth controls were performed at different times of the assay to ensure anaerobes viability. Viability controls with DMSO 5% and 2.5% were also included. Contamination with aerobic bacteria was controlled at the beginning and the end of each assay by culturing the bacterial suspensions in Chocolate Agar under aerobic conditions. Reproducibility was controlled by testing *B. fragilis* ATCC 25285 strain and metronidazole as inter-assay controls, following CLSI recommendations.⁵⁸

The results have been reported using population parameters: MIC ranges, MIC₅₀ and MIC₉₀ (minimum concentrations able to inhibit 50 and 90% of the tested isolates, respectively).

2.4 Cell viability assay

Murine macrophage cell line RAW 264.7 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U mL⁻¹ penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin, and 0.29 mg mL⁻¹ L-glutamine. The cells were grown in plastic flasks in a 5% CO₂ humidified atmosphere at 37 °C. Medium was changed every 2–3 days until subconfluence was achieved. Then, cells were harvested with an EDTA 0.05% solution and placed thereafter in 96-well plates to perform all assays.

The 50% cytotoxic concentration (CC₅₀), defined as the drug concentration that reduces cell viability by 50% when compared to untreated controls, was evaluated using these cells. A preliminary study was conducted to determine the optimal number of cells per well and their tolerance to DMSO, briefly:

amounts of 2.5×10^4 , 1×10^5 and 4×10^5 cells per well were seeded in duplicate and cultured in DMEM containing serial two-fold dilutions of DMSO. The cells were cultured for 21 h and the cell viability was determined using the Alamar Blue reagent in a fluorometer as described below. In this assay, the optimal amount of cells per well was determined as 4×10^5 and 1% DMSO was tolerated without affecting cell growth.

Afterwards, serial dilutions 1/5 of each nitroene and metronidazole were tested in duplicate, ranging from 0.03 to 100 $\mu\text{g mL}^{-1}$. Plates were seeded with 4×10^5 cells per well and 100 μL of DMEM supplemented medium with the corresponding nitroene dilution was added. Cells were cultured for 21 h. After that, 20 μL of the Alamar Blue Reagent (Resazurin) were added to each well and incubated for 3 h. The fluorescence of each well was measured using a micro-plate reader (Victor3, PerkinElmer) with excitation/emission 560/590 nm. The resulting data were presented as survival percentage.⁵⁵

Cell viability controls were performed in every assay in triplicate by culturing the cells with DMEM supplemented medium with and without 1% DMSO. Cell death controls were performed in triplicate with DMSO 10%. Basal fluorescence of each nitroene in a 100 $\mu\text{g mL}^{-1}$ dilution was measured in duplicate. Metronidazole was used for comparison and as inter-assay control.

2.5 Electron affinity calculations

Density Functional Theory⁵⁶ calculations were carried out with GAUSSIAN 09.⁵⁷ Nitroene conformations were systematically screened by means of appropriate relaxed (*i.e.*, with optimization



Table 4 Calculated electron affinity

Compounds	EA _{calc} – Δ <i>H</i> (kcal mol ^{–1})
1a	24.5
1b	29.2
1c	43.2
1d	22.7
1e	23.8
1f	28.4
1g	25.6
1h	21.7
1i	28.1
1j	32.2
1k	46.9
1l	26.0
1m	29.0
2a	117.6
2b	120.2
2c	126.1

at each point) Potential Energy Surfaces (PES). Scans were performed at the B3LYP/6-31G(d) level to find global minimum energy structures. Imaginary (negative) values were never found in frequency calculations, confirming that the computed geometries were always referred to a minimum. Thermodynamic parameters were computed at 298 K by means of frequency calculations employing the B3LYP functional in conjunction with the 6-31+G(d,p) basis set. Geometries of the corresponding radical anions and their thermodynamic parameters were calculated at the same level. The electron affinity (EA) was calculated as the energy difference (measured as Δ*H*) between the nitron and its radical anion in their ground states (Table 4). The EA thus calculated is known as adiabatic electron affinity and is a good general estimation of the experimental EA.⁵⁸

2.6 ADME calculations

Qikprop program, version 3.0 (Schrödinger, LLC., New York) was used for this *in silico* study. Geometry optimization of the input structures was calculated with the Hyperchem program, using the MM+ force field. Since Qikprop is not able to calculate charged species, amidinium salts **2** were excluded of this study, even if numerous works have shown that ionic compounds are capable of crossing lipidic membranes.⁵⁹

3 Results and discussion

3.1 Synthesis

In our previously developed synthetic approach,⁴⁵ the last reaction step (Scheme 1) is a spontaneous heterocyclization that leads from the cyclic amidine **4** to the corresponding *N*-oxide **1**. This reaction was achieved within 1–24 h with high yields for most tetrahydropyrimidines. Imidazolines, in contrast, required significantly longer reaction times (7–15 days) to afford the corresponding *N*-oxides. As a consequence, these nitrones were isolated in low yields and accompanied by collateral products. Literature on the synthesis of related compounds suggested that this step could be base promoted.⁶⁰ After screening a few basic

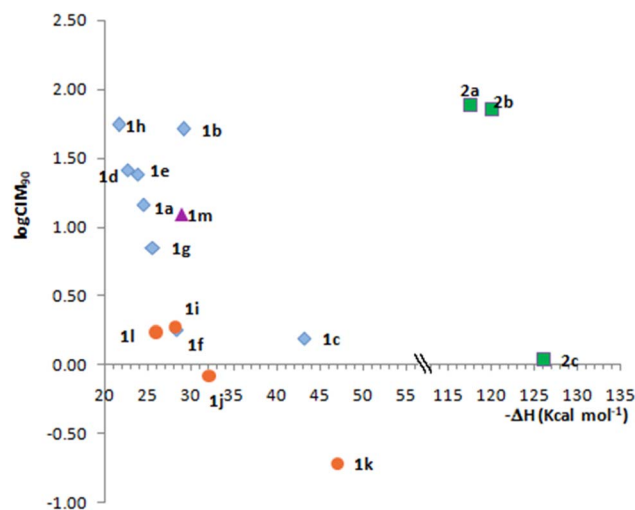


Fig. 2 Relationship between calculated electron affinity and log CIM₉₀ for *C. difficile*. Six-membered ring amidines are shown in blue, five-membered analogues in orange and the 7-membered ring nitron in violet; quaternary salts are in green.

media (Et₃N/DCM; Et₃N/MeCN; 10% aq. NaOH/EtOH; 4% KOH/abs. ethanol) we found that treatment of imidazolines **3i–l** with an ethanolic solution of KOH 4% resulted in a drastic reduction of the reaction times (from more than one week to one our) and a significant improve in the yields (Table 1).

In the absence of additional base, the reaction would be autocatalyzed by the amidine moiety with a p*K*_a dependent rate. The striking p*K*_a difference between different cyclic amidines homologues (p*K*_a = 10.51 for 1-phenyl-2-4-nitrophenyl-1,4,5,6-tetrahydropyrimidine vs. 7.65 for its imidazoline homologue)⁶¹ would account for the different conversion times. A plausible reaction mechanism involves the semistabilized carbanion **I** as the intermediate (Scheme 3).

3.1.1. Selected amidinoquinoxaline *N*-oxides. In order to individualize the structural features more favourable to the biological activity of the compounds under study, we explored representative structural variations on compound **1a**. Firstly, we analyzed the influence of electron withdrawing/donating groups in the aryl substituent α– to the *N*-oxide moiety (H, Cl, NO₂, OCH₃, compounds **1a–d**). The replacement of the phenyl substituent by a bioisosteric heterocycle (3-thienyl substituent) (**1g**) or by a benzyl group (**1h**) was also studied. Additional structural variations comprised quaternization of the amidine nitrogen (compounds **2a–c**), the presence of a lipophilicity enhancing gem-dimethyl group on the methylene chain (**1e**) and the effect of an electron withdrawing (pyridine) nitrogen atom in the fused ring (**1f**). Taking into account the fact that the amidine ring size significantly modifies its basicity,⁶¹ we included in our study a seven membered amidinoquinoxaline derivative (**1m**) as well as imidazoquinoxalines with electron withdrawing and donating groups in the α– aryl substituent (compounds **1j–l**).

3.2 Antimicrobial activity

Results, expressed as MIC₅₀ and MIC₉₀ are shown in Tables 2 and 3 (a complete version including the MIC ranges is included in the



Table 5 Cell viability determined at 100 $\mu\text{g mL}^{-1}$ *N*-oxides **1**, **2** and selectivity indexes^{a,b,c}

Comp.	Cell viability (%)	Selectivity index ($>100 \mu\text{g mL}^{-1}/\text{MIC}_{90}$)					
		<i>B. fragilis</i>	Other <i>Bacteroides</i> spp. and <i>Parabacteroides</i>	<i>Prevotella</i> spp.	<i>Fusobacterium nucleatum</i>	<i>C. difficile</i>	<i>C. perfringens</i>
1a	98	>100	>100	>100	>100	>25	>6
1b	94	>50	>100	>100	>100	>6	>2
1c	74	>1667	>1667	>800	>1667	>200	>50
1d	94	>50	>50	>100	>50	>13	>3
1e	94	>200	>200	>200	>400	>13	>13
1f	83	>1667	>1667	>800	>800	>200	>100
1g	88	>200	>200	>200	>200	>50	>8
1h	61	>25	>25	>25	>25	>6	ND
1i	91	>1667	>1667	>1667	>800	>200	>100
1j	75	>800	>800	>1667	>800	>400	>100
1k	63	>1667	>1667	>1667	>1667	>1667	>800
1l	59	>1667	>1667	>1667	>1667	>200	>100
2a	98	>50	>25	>12.5	>25	>3	>3
2b	67	>13	>13	>13	>13	>3	>3
2c	59	>100	>100	>100	>100	>200	>100

^a Mtz = metronidazole. ^b ND: not determined. ^c Compound **1m** was not tested in this study.

ESI, Table S1†). The *N*-oxides under study showed great efficiency against anaerobic bacteria. Among them, compounds **1c,f,i-l** exhibited significantly lower MIC_{90} than those determined for metronidazole.† Exceptions to the high activity of these heterocycles are non-spore-forming Gram-positive bacilli, against which these compounds, as well as metronidazole, were not active.

Several nitrones had MIC_{50} and MIC_{90} values against *B. fragilis* 8 and 16 fold lower than MTZ and related compounds,³⁵ and their activity also compared favourably to other reference drugs like clindamycin and amoxicillin-clavulanate.³⁸ MICs of **1c,k,l** were lower than those reported for these compounds also for *F. nucleatum*.^{35,38}

Regarding clostridia, compound **1k** had MIC values significantly lower than MTZ and some analogues as well as other reference drugs for *C. difficile*.^{36–39} It is striking that most compounds show a lower MIC_{90} against *C. difficile* than that observed with *C. perfringens*. This is an interesting feature, since *C. difficile* has high levels of resistance^{62–64} to many antibacterial drugs (β -lactams including carbapenems, quinolones, clindamycin and rifampicin, among others) except for vancomycin and metronidazole, while *C. perfringens* remains susceptible to a large number of antimicrobials, including penicillin.

The activity of each nitrone did not vary significantly against *C. difficile* isolates. This is important since *C. difficile* has a highly mobile, mosaic genome and there is wide strain diversity,⁶⁵ resulting sometimes in activity variations among new drug candidates.²⁹

3.3 Structure–activity relationship (SAR)

General trends are presented in this section. Non-sporulated Gram-positive bacilli, against which this family of compounds was only slightly active, were excluded from the analysis.

† Since metronidazole has a molecular weight of 171 g mol^{-1} and compounds **1** and **2** range between $263\text{--}464 \text{ g mol}^{-1}$, this difference is accentuated if the results are expressed in molar concentration instead of $\mu\text{g mL}^{-1}$ units.

In order to analyze the influence of some model electronic variations in the aryl moiety in position α – to the *N*-oxide function we compared compounds **1a–d**, **1i–1l**, **2a–c**. Derivatives with a 4-nitro group were considerably more active, while 4-H, 4-Cl, and 4- OCH_3 substitution did not lead to significant differences, although 4-H compounds were generally slightly more active. Additionally, replacement of the phenyl moiety in **1a** by a thienyl ring (**1g**) subtly enhanced the activity, while a benzyl substituent (**1h**) had a negative influence. In summary, besides *p*-nitrophenyl substitution, the remaining electronic variations did not significantly improve the antimicrobial activity.

Introduction of a *gem*-dimethyl group in the methylene chain of a pyrimido derivative (**1e**) resulted in a slightly more active compound than its counterpart **1d**. On the contrary, quaternization of the amidine nitrogen was generally not favourable for antianaerobic activity since amidinium salts **2a–c** were in almost all cases considerably less active than the corresponding *N*-oxides **1a–c**. Concerning the fused ring, replacement of the phenyl ring in compound **1a** by a pyridine core (**1f**) improved the activity. This suggests that EWGs in the fused ring enhance the antianaerobic activity of these heterocycles.

When comparing compounds **1b**, **1j** and **1m**, no significant differences in the activity between 6- and 7-membered homologues was observed, although **1m** was slightly more active against *C. difficile* and *C. perfringens* than **1b**. On the other hand, imidazoquinoxaline **1j** was notoriously more active than its six- and seven-membered homologues. The trend was confirmed when comparing the remaining derivatives **1i,k–l** with their counterparts **1a,c–d**: in every case, the 5-membered derivatives were more active than their higher homologues. Among imidazoquinoxalines **1i–l**, the 4-nitrophenyl derivative (**1k**) was again the most active compound.

From the previous analysis it turns out that the most significant structural variations are the presence of a 5-membered amidine ring, a fused pyridine core and a *p*-



nitrophenyl substituent α - to the *N*-oxide function. Compound **1k**, containing two out of the three favourable structural features, namely the five-membered amidine ring and the *p*-nitrophenyl substituent, was the most active in the series. Although the nitro group is generally not sought for during drug discovery due to safety issues, there are many therapeutic agents that include it in their composition such as antibacterials and antiparasitics, among others.⁶⁶

3.4 Analysis of the probable mechanism of action

The activity spectrum of the new molecules is analogous to that of metronidazole. Aerobic bacteria and non-sporulated Gram positive bacilli show resistance both to metronidazole and amidinoquinoxaline *N*-oxides, which would in principle suggest a similar mechanism of action. Metronidazole enters the cell as a prodrug by passive diffusion and is reduced to the corresponding nitro radical anion by electron carriers in an anaerobic environment.^{67,68} The active form of the drug interacts with DNA by a mechanism not fully elucidated, causing DNA damage and non specific macromolecular alterations leading to cell death.^{69,70} Similarly, many *N*-oxides have been described as bio-reducible drugs, *i.e.* they are inactive *per se*, but become cytotoxic after a reduction step which in many cases requires hypoxic conditions.⁷¹ According to these facts, it would be reasonable that nitrones **1** and **2** would not exert their action as such but after activation through a bio-reduction step. To investigate this mechanistic hypothesis, we calculated the electron affinity (EA) of the compounds (Table 4), as an indicator of their redox potential, and related it to their antimicrobial activity as logCIM₉₀ (μM).

Fig. 2 shows the relationship between the calculated EA and logCIM₉₀ (with CIM values expressed as μM) for *C. difficile*, chosen as a representative example due to its clinical relevance. Gram negative bacilli were extremely susceptible and the MIC₉₀ was in many cases less than the minimum concentration tested (Table 2, values $\leq 0.06 \mu\text{g mL}^{-1}$). Even so, results presented in Fig. 2 were consistent for the 6 groups of bacteria classified according to Table 2.

Fig. 2 shows that compounds with the highest EA are the most active within each group. The behavior is not linear since, as expected, antibacterial activity does not depend exclusively on a single parameter such as electronic affinity. Imidazoquinoxaline derivatives **1i-l** always show higher electron affinities and are more active than the homologous pyrimidoquinoxalines **1a-d**. Additionally, pyrimidoquinoxaline **1b** and diazepinoquinoxaline *N*-oxides **1m** have comparable electron affinity values and display similar activities.

The relationship between the electron affinity and antibacterial activity supports the hypothesis that the mechanism of action of the *N*-oxides would include a reduction step to transform the compound into its active form. Compounds **1c,k** and **2c**, where both the *N*-oxide and the nitro functional groups may undergo reduction, are more complex to analyze, although it is worth highlighting that these compounds were the most active within each series.

3.5 Cytotoxicity

Cell viability data at $100 \mu\text{g mL}^{-1}$ (the maximum concentrations tested) are shown in Table 5. In all cases CC₅₀ were higher than $100 \mu\text{g mL}^{-1}$. Considering that values of CC₅₀ $> 50 \mu\text{g mL}^{-1}$ correspond to non-cytotoxic compounds,⁷² the results of these

Table 6 Predicted ADME related properties computed by Qikprop and selected experimental logP values^a

Comp	CNS	MW	SASA	Vol	DHB	AHB	logP	logS	PCaco	logBB	PMDCK	nM	HOA	%HOA	PSA	VR5	VR3
1a	1	277.325	523.783	902.771	0	2.500	-0.149	-4.671	4160.303	0.147	2309.675	3	3	100.000	32.038	0	0
1b	1	311.770	545.805	944.637	0	2.500	0.836	-5.400	4205.739	0.319	5764.794	3	3	100.000	31.973	0	0
1c	-1	322.323	562.589	977.280	0	3.500	0.657	-4.720	466.578	-0.859	217.018	3	3	94.215	78.769	0	0
1d	1	307.351	558.782	975.816	0	3.250	-0.128	-4.779	4179.554	0.080	2321.229	4	3	100.000	40.297	0	0
1e	1	335.405	598.020	1071.044	0	3.250	—	-5.522	4378.159	0.095	2440.677	4	3	100.000	39.808	0	0
1f	1	278.313	516.286	889.391	0	3.500	0.311	-4.005	3398.734	0.068	1856.269	3	3	100.000	41.993	0	0
1g	1	283.347	500.778	868.766	0	2.500	-0.010	-4.636	4678.076	0.401	5214.276	4	3	100.000	31.116	0	0
1h	0	291.352	591.760	1000.602	0	2.500	—	-5.796	3341.608	-0.053	1822.568	3	3	100.000	33.186	0	1
1i	1	263.298	502.438	854.774	0	2.500	—	-4.267	4124.038	0.147	2287.921	2	3	100.000	33.198	0	0
1j	1	297.743	525.850	898.135	0	2.500	1.292	-5.073	4093.646	0.311	5601.908	1	3	100.000	33.222	0	0
1k	-1	308.296	543.140	931.304	0	3.500	—	-4.352	458.238	-0.859	212.828	2	3	92.378	80.021	0	0
1l	1	293.324	540.213	930.486	0	3.250	1.247	-4.427	4111.646	0.074	2280.490	3	3	100.000	41.477	0	0
m	1	325.797	563.828	986.954	0	2.500	1.247	-4.427	4111.646	0.074	2280.490	3	3	100.000	41.477	0	0

^a MW: molecular weight (recommended range 130–725 Da); SASA: total solvent-accessible surface area (recommended range 300.0–1000.0); Vol: total solvent-accessible volume (recommended range 500.0–2000.0); DHB: estimated number of hydrogen bond donors (recommended range 0.0–6.0); AHB: estimated number of hydrogen bond acceptors (recommended range 2.0–20.0); logP: experimental log of the octanol/water partition coefficient determined in PBS buffer (data taken from ref. 47) (recommended range -2.0 – 6.5); logS: predicted aqueous solubility (recommended range -6.5 – 0.5); PCaco: predicted apparent Caco-2 cell permeability (<25 poor, >500 great); logBB: predicted log of the brain/blood partition coefficient (recommended range -3.0 – 1.2); PMDCK: predicted apparent MDCK cell permeability (<25 poor, >500 great); nM: number of likely metabolic reactions (recommended range 1 – 8); HOA: qualitative human oral absorption - 1, 2, or 3 for low, medium, or high; % HOA: predicted human oral absorption (>80% is high <25% is poor); PSA: polar (N and O) van der Waals surface area (recommended range 7.0–200); VR5: number of violations for Lipinski's rule of five (MW < 500, logP < 5, DHB $\leq$ 5, acceptHB $\leq$ 10); VR3: number of violations for Jørgensen's rule of three: logS $>$ -5.7; PCaco $>$ 22 nm s⁻¹; nM < 7. The Qikprop ranges/recommended values were determined with 95% of known drugs.



tests are excellent and show the low cytotoxicity of the compounds in the eukaryotic cell model RAW 264.7.

The Selectivity Index (SI), defined as $SI = CC_{50}/MIC$, allows to relate the MIC value with the cytotoxicity. The greater the SI, the higher is the cytotoxic concentration (represented by the CC_{50}) with respect to the active concentration (symbolized by the MIC), and the greater the probability that the compounds will not be toxic to host cells *in vivo*. According to literature reports⁷³ values of $SI \geq 10$ are considered suitable. SI values calculated as $SI > 100 \mu\text{g mL}^{-1}/\text{CIM}_{90}$ for the six bacteria groups presented in Table 2, show very promising results (Table 5). Even if the most favorable structural variations in terms of antibacterial activity seem to be associated with higher cytotoxicity of the compounds (Table 5, second column), the increase in antibacterial activity is so important that the Selectivity Indexes still show very favourable results. Interestingly, the most active compounds of the series **1k**, shows $SI > 800$ for all groups of bacteria. Results presented in Table 5 demonstrate the low toxicity of the compounds in the eukaryotic cell model, in comparison to their antianaerobic activity.

3.6 ADME properties

The predominant role of pharmacokinetics in drug discovery has been recognized for years. Ideally, a new drug should be target-specific, orally-absorbed, cause minimal or no adverse effects and be distributed and excreted in a way that allows its administration once a day.^{74,75} Many drug candidates fail in clinical trials due to their pharmacokinetic properties, causing enormous loss of time and funds. Therefore *in silico* ADME (absorption, distribution, metabolism, and excretion) predictions are nowadays an important tool to identify problematic compounds at an early stage and to rationalize the overall development progression. The drug-like properties of the *N*-oxides **1** were calculated as a first approximation to investigate the pharmacokinetic features (ADME) of the new compounds. Selected molecular descriptors are presented in Table 6, which also includes the experimental logP values of some derivatives.⁵⁰

Nitrones **1** comply with Lipinski's rule of five,⁷⁶ Jorgensen's rule of three (except for **1h** whose logS is too low),⁷⁷ indicating that the new compounds are drug-like and, according to their predicted bioavailability, could be orally administered. Other individual parameters like logS, logP, PCaco and the predicted HOA are also very encouraging concerning the gut-blood barrier penetration, making these nitrones good candidates for oral absorption. This is a fundamental feature in a potential drug, since the oral route facilitates its administration and contributes to patient's compliance.

4 Conclusions

The activity of 13 amidinoquinoxaline *N*-oxides **1** and **3** related quaternary salts with selected structural variations against Gram-positive and Gram-negative anaerobic bacteria was evaluated, as well as their cytotoxicity and ADME (calculated) properties. Six and seven membered derivatives were synthesized by a method previously developed by our group, which was

not efficient for imidazoquinoxaline *N*-oxides. Such compounds were prepared in high yields by base-promoted ring closure of the quinoxaline ring.

The majority of the *N*-oxides under study showed high to excellent antianaerobic activity together with low cytotoxicity and suitable selectivity indexes, with an activity spectrum similar to that of metronidazole. Among them, six out of 13 compounds (**1c,f,i-l**) exhibited MIC_{90} values significantly lower than metronidazole, its analogues and other reference drugs. It is also remarkable that these compounds are very active against *C. difficile*, a multidrug-resistant anaerobe which can cause severe intestinal disease. Regarding their structure–activity relationship, the dominating factor was the ring size of the amidine ring: derivatives containing imidazoline rings were the most active within each series. Substitution with strong electron withdrawing groups either in the aryl moiety adjacent to the *N*-oxide function or in the benzofused ring significantly improved the activity. A combination of both relevant structural features led to the most active compound **1k**. The *N*-oxides also showed favourable drug likeness profiles.

It is known that metronidazole, a first choice antianaerobic agent, is a prodrug which needs a bioreductive step to yield the active species. A preliminary study on the mechanism suggested that the *N*-oxides **1** would also undergo reductive activation in the biological medium in order to exert their activity. In fact, compounds with the highest electron affinities were the most active within each group. Taken together with the ease of preparation of the compounds (3–4 steps with high overall yields), all these features make amidinoquinoxalines *N*-oxides attractive candidates for further studies on their therapeutic potential.

Author contributions

Synthesis: NG, NBK, JAB, MBG, LROMIC determinations: LFC, NG Cytotoxicity: DGM, MLP, NG. Experimental planning: NG, LFC, DGM, LRO. DFT calculations: PS, NG. Paper writing: NG, LRO.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the Universidad de Buenos Aires [Grant 20020170100189BA] and by CONICET [Grant 11220200101694CO].

References

- 1 H. Jousimies-Somer, P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler and S. M. Finegold, *Wadsworth-KTL Anaerobic Bacteriology Manual*, Star, Corea, 6th edn, 2002.
- 2 I. Brook, *Antimicrobial Resistance of Anaerobic Bacteria*, in *Antimicrobial Drug Resistance*, ed. D.L. Mayers, J. D. Sobel,



- M. Ouellette, K. S. Kaye and D. Marchaim, Springer International Publishing, 2017, p. 1007.
- 3 S. Löfmark, C. Edlund and C. E. Nor, *Clin. Infect. Dis.*, 2010, **50**, 16.
 - 4 (a) M. A. Pence, *Clin. Microbiol. Newsl.*, 2019, **41**, 1; (b) L. Fernández-Canigia, M. Litterio, M. C. Legaria, L. Castello, S. C. Predari, A. Di Martino, A. Rossetti, R. Rollet, G. Carloni, H. Bianchini, D. Cejas, M. Radice and G. Gutkind, Anaerobe Surveillance Team, *Antimicrob. Agents Chemother.*, 2012, **56**(3), 1309, DOI: [10.1128/AAC.05622-11](https://doi.org/10.1128/AAC.05622-11).
 - 5 H. R. Ingham, S. Eaton, C. W. Venables and P. C. Adams, *Lancet*, 1978, **1**, 214.
 - 6 J. S. Brazier, S. L. Stubbs and B. I. Duerden, *J. Antimicrob. Chemother.*, 1999, **44**, 580.
 - 7 V. O. Rotimi, M. Khoursheed, J. S. Brazier, W. Y. Jamal and F. B. Khodakhast, *Clin. Microbiol. Infect.*, 1999, **5**, 166.
 - 8 S. P. Sadarangani, S. A. Cunningham, P. R. Jeraldo, J. W. Wilson, R. Khare and R. Patel, *Antimicrob. Agents Chemother.*, 2015, **59**, 4157, DOI: [10.1128/AAC.00677-15](https://doi.org/10.1128/AAC.00677-15).
 - 9 A. A. Elsaghier, J. S. Brazier and E. A. James, *J. Antimicrob. Chemother.*, 2003, **51**(6), 1436, DOI: [10.1093/jac/dkg265](https://doi.org/10.1093/jac/dkg265).
 - 10 C. Alauzet, A. Lozniewski and H. Marchandin, *Anaerobe*, 2019, **55**, 40.
 - 11 S. Sethi, R. Shukla, K. Bala, V. Gautam, A. Angrup and P. Ray, *J. Global Antimicrob. Resist.*, 2019, **16**, 210.
 - 12 (a) A. N. Schuetz, Anaerobic Bacteria: Antimicrobial Susceptibility Testing and Resistance Patterns, in *Antimicrobial Resistance in the 21st Century, Emerging Infectious Diseases of the 21st Century*, ed. I. W. Fong, D. Shlaes and K. Drlica, Springer International Publishing AG, 2018, DOI: [10.1007/978-3-319-78538-7_6](https://doi.org/10.1007/978-3-319-78538-7_6); (b) A. C. M. Veloo, H. B. Tokman, H. Jean-Pierre, Y. Dumont, S. Jeverica, R. Lienhard, A. Novak, A. Rodloff, V. Rotimi, I. Wybo and E. Nagy, ESGAI study group, *Anaerobe*, 2020, **61**, 102111, DOI: [10.1016/j.anaerobe.2019.102111](https://doi.org/10.1016/j.anaerobe.2019.102111); (c) B. Fox, M. A. Berger, M. Roncallo, L. Pinoche, M. E. Ibáñez, S. González-Fraga and L. Fernández-Canigia, *Anaerobe*, 2018, **54**, 267, DOI: [10.1016/j.anaerobe.2018.04.003](https://doi.org/10.1016/j.anaerobe.2018.04.003); (d) S. C. Predari, A. N. de Paulis, E. Bertona, D. Guevara Núñez, J. P. Suárez and L. Castello, *Rev. Argent. Microbiol.*, 2017, **49**(2), 146, DOI: [10.1016/j.ram.2016.12.008](https://doi.org/10.1016/j.ram.2016.12.008).
 - 13 K. M. Land and P. J. Johnson, *Drug Resistance Updates*, 1999, **2**, 289.
 - 14 L. Pumbwe, A. Chang, R. L. Smith and H. M. Wexler, *Microb. Drug Resist.*, 2007, **13**, 96, DOI: [10.1089/mdr.2007.719](https://doi.org/10.1089/mdr.2007.719).
 - 15 R. Ghotaslou, M. Yekani and M. Y. Memar, *Microbiol. Res.*, 2018, **210**, 1.
 - 16 R. Ghotaslou, H. B. Baghi, N. Alizadeh, M. Yekani, S. Arbabi and M. Y. Memar, *Infect., Genet. Evol.*, 2018, **64**, 156.
 - 17 H. K. Leiros, S. Kozielski-Stuhrmann, U. Kapp, L. Terradot, G. A. Leonard and S. M. McSweeney, *J. Biol. Chem.*, 2004, **279**, 55840.
 - 18 G. Reysset, *Anaerobe*, 1996, **2**, 59.
 - 19 M. M. Theron, M. N. Janse Van Rensburg and L. J. Chalkley, *J. Antimicrob. Chemother.*, 2004, **54**, 240.
 - 20 S. Lofmark, H. Fang, M. Hedberg and C. Edlund, *Antimicrob. Agents Chemother.*, 2005, **49**, 1253.
 - 21 E. Otte, H. L. Nielsen, H. Hasman and D. Fuglsang-Damgaard, *Anaerobe*, 2017, **43**, 91.
 - 22 M. M. Theron, M. N. Janse van Rensburg and J. Chalkley, *J. Antimicrob. Chemother.*, 2004, **54**, 240.
 - 23 (a) F. C. Lessa, Y. Mu, W. M. Bamberg, Z. G. Beldavs, G. K. Dumyati, J. R. Dunn, M. M. Farley, S. M. Holzbauer, J. I. Meek, E. Phipps, L. E. Wilson, L. G. Winston, J. A. Cohen, B. M. Limbago, S. K. Fridkin, D. N. Gerding and L. C. McDonald, *N. Engl. J. Med.*, 2015, **372**(9), 825, DOI: [10.1056/NEJMoa1408913](https://doi.org/10.1056/NEJMoa1408913); (b) A. Cheknis, S. Johnson, L. Chesnel, L. Petrella, S. Sambol, S. E. Dale, J. Nary, P. Sears, D. M. Citron, E. J. C. Goldstein and D. N. Gerding, *Anaerobe*, 2018, **53**, 38, DOI: [10.1016/j.anaerobe.2018.05.009](https://doi.org/10.1016/j.anaerobe.2018.05.009).
 - 24 https://www.cdc.gov/hai/organisms/cdiff/cdiff_infect.html.
 - 25 K. Z. Vardakas, K. A. Polyzos, K. Patouni, P. I. Rafailidis, G. Samonis and M. E. Falagas, *Int. J. Antimicrob. Agents*, 2012, **40**, 1.
 - 26 Z. Peng, D. Jin, H. B. Kim, W. Stratton, B. Wu, Y.-W. Tang and X. Sun, *J. Clin. Microbiol.*, 2017, **55**(7), 1998, DOI: [10.1128/JCM.02250-16](https://doi.org/10.1128/JCM.02250-16).
 - 27 A. M. Jarrad, T. Karoli, M. A. T. Blaskovich, D. Lyras and M. A. Cooper, *J. Med. Chem.*, 2015, **58**(13), 5164, DOI: [10.1021/jm5016846](https://doi.org/10.1021/jm5016846).
 - 28 A. Malath Al-Qtaitat, A. Haythem, A. Saadeh, A. G. Al-Bakri, K. Hargobinder, G. Kapil, R. Sehgal and M. S. Mubarak, *Monatsh. Chem.*, 2015, **146**, 705, DOI: [10.1007/s00706-014-1352-0](https://doi.org/10.1007/s00706-014-1352-0).
 - 29 Y. Miyamoto, J. Kalisiak, K. Korthals, T. Lauwaet, D. Young Cheung, R. Lozano, E. R. Cobo, P. Upcroft, J. A. Upcroft, D. E. Berg, F. D. Gillin, V. V. Fokin, K. B. Sharpless and L. Eckmann, *Proc. Natl. Acad. Sci. U.S.A.*, 2013, **110**(43), 17564.
 - 30 H. A. Saadeh, I. M. Mosleh, A. G. Al-Bakri and M. S. Mubarak, *Monatsh. Chem.*, 2010, **141**, 471, DOI: [10.1007/s00706-010-0281-9](https://doi.org/10.1007/s00706-010-0281-9).
 - 31 M. M. Bkhaitan, M. Alarjah, A. Z. Mirza, A. N. Abdalla, H. M. El-Said and H. S. Faidah, *Chem. Biol. Drug Des.*, 2018, **92**, 1954, DOI: [10.1111/cbdd.13366](https://doi.org/10.1111/cbdd.13366).
 - 32 A. A. Oliveira, A. P. A. Oliveira, L. L. Franco, M. O. Ferencs, J. F. G. Ferreira, S. M. P. S. Bachi, N. L. Speziali, L. M. Farias, P. P. Magalhães and H. Beraldo Alexandre, *BioMetals*, 2018, **31**, 571, DOI: [10.1111/cbdd.13366](https://doi.org/10.1111/cbdd.13366).
 - 33 B. R. Prajapati and A. K. Seth, *Pharma Sci. Monit.*, 2018, **9**(1), 318.
 - 34 T. E. Ballard, X. Wang, I. Olekhovich, T. Koerner, C. Seymour, P. S. Hoffman and T. L. Macdonald, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 3537, DOI: [10.1016/j.bmcl.2010.04.126](https://doi.org/10.1016/j.bmcl.2010.04.126).
 - 35 T. E. Ballard, X. Wang, I. Olekhovich, T. Koerner, C. Seymour, J. Salamoun, M. Warthan, P. S. Hoffman and T. L. Macdonald, *ChemMedChem*, 2011, **6**, 362, DOI: [10.1002/cmdc.201000475](https://doi.org/10.1002/cmdc.201000475).



- 36 G. A. Pankuch and P. C. Appelbaum, *Antimicrob. Agents Chemother.*, 2006, **50**, 1112, DOI: [10.1128/AAC.50.3.1112-1117.2006](https://doi.org/10.1128/AAC.50.3.1112-1117.2006).
- 37 C. Costello, T. Karpanen, P. A. Lambert, P. Mistry, K. J. Parker, D. L. Rathbone, J. Ren, L. Wheeldon and T. Worthington, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 1708, DOI: [10.1016/j.bmcl.2008.01.041](https://doi.org/10.1016/j.bmcl.2008.01.041).
- 38 *Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline, including tuberculosis*, World Health Organization, Geneva, 2017, WHO/EMP/IAU/2017.11.
- 39 G. E. Adams, E. M. Fielden, M. A. Naylor and I. J. Stratford, UK Patent Application No: GB2257360, 1993.
- 40 M. A. Naylor, M. A. Stephens, J. Nolan, B. Sutton, J. H. Tocher, E. M. Fielden, G. E. Adams and I. J. Stratford, *Anti Cancer Drug Des.*, 1993, **8**, 439.
- 41 (a) M. A. Naylor, *Oncol. Res.*, 1994, **6**, 483; (b) M. A. Naylor, G. E. Adams, A. Haigh, S. Cole, T. Jenner, N. Robertson, D. Siemann, M. A. Stephens and I. J. Stratford, *Anti Cancer Drugs*, 1995, **6**, 259; (c) H. M. Barham and I. J. Stratford, *Biochem. Pharmacol.*, 1996, **51**, 829; (d) B. M. Sutton, N. J. Reeves, M. A. Naylor, E. M. Fielden, S. Cole, G. E. Adams and I. J. Stratford, *Int. J. Radiat. Oncol., Biol., Phys.*, 1994, **29**, 339.
- 42 P. C. Parthasarathy, B. S. Joshi, M. R. Chaphekar, D. H. Gawad, L. Anandan, M. A. Likhate, M. Hendi, S. Mudaliar, S. Iyer, D. K. Ray and V. B. Srivastava, *Indian J. Chem., Sect. B: Org. Chem. Incl. Med. Chem.*, 1983, **22**, 1250.
- 43 G. J. Ellames and A. A. Jaxa-Chamiec, *US Pat.*, 4696928, 1987.
- 44 G. J. Ellames, K. R. Lawson, A. A. Jaxa-Chamiec and R. M. Upton, *European Pat.*, EP0256545, 1988.
- 45 G. J. Ellames, R. M. Upton, A. A. Jaxa-Chamiec and P. L. Myers, *US Pat.*, 4761414, 1988.
- 46 J. Segreti, L. J. Goodman and G. M. Trenholme, *Diagn. Microbiol. Infect. Dis.*, 1993, **17**, 177.
- 47 N. Gruber, L. Orelli, C. Minnelli, L. Mangano, E. Laudadio, G. Mobbili and P. Stipa, *Antioxidants*, 2021, **10**, 1185, DOI: [10.3390/antiox10081185](https://doi.org/10.3390/antiox10081185).
- 48 N. Gruber, L. L. Piehl, E. Rubin de Celis, J. E. Díaz, M. B. García, P. Stipa and L. R. Orelli, *RSC Adv.*, 2015, **5**, 2724.
- 49 N. Gruber, L. R. Orelli, R. Cipolletti and P. Stipa, *Org. Biomol. Chem.*, 2017, **15**, 7685.
- 50 M. B. García, L. R. Orelli, M. L. Magri and I. A. Perillo, *Synthesis*, 2002, 2687.
- 51 N. A. Isley, R. T. H. Linstadt, S. M. Kelly, F. Gallou and B. H. Lipshutz, *Org. Lett.*, 2015, **17**(19), 4734.
- 52 Clinical and Laboratory Standards Institute (CLSI), *Methods for antimicrobial susceptibility testing of anaerobic bacteria*, Approved standard M11-A7, CLSI, Wayne, PA, 7th edn, 2007.
- 53 Clinical and Laboratory Standards Institute (CLSI), *Performance standards for antimicrobial susceptibility testing: 24th informational supplement*, CLSI, Wayne, PA, 2012, CLSI M100-S22.
- 54 *Manual of clinical microbiology*, ed. J. H. Jorgensen, M. Pfaller and K. C. Carroll, ASM press, Washington, DC, 11th edn, 2015, vol. 1.
- 55 J. O'Brien, I. Wilson, T. Orton and F. Pognan, *Eur. J. Biochem.*, 2000, **267**, 5421, DOI: [10.1046/j.1432-1327.2000.01606.x](https://doi.org/10.1046/j.1432-1327.2000.01606.x).
- 56 (a) R. G. Parr and W. Yang, *Density Functional Theory of Atoms and Molecules*, Oxford University, New York, NY, 1998; (b) W. Koch and M. C. Holthausen, *A Chemist's Guide to Density Functional Theory*, Wiley-VCH, Weinheim, Germany, 2000.
- 57 M. J. Frisch, *et al.*, *Gaussian 09 (Revision D.01)*, Gaussian Inc., Wallingford, CT, 2009.
- 58 J. C. Rienstra-Kiracofe, G. S. Tschumper and H. F. Schaefer, *Chem. Rev.*, 2002, **102**, 231.
- 59 H. van de Waterbeemd, D. A. Smith, K. Beaumont and D. K. Walker, *J. Med. Chem.*, 2001, **44**, 1313.
- 60 (a) C. W. Muth, J. C. Ellers and O. F. Folmer, *J. Am. Chem. Soc.*, 1957, **79**, 6500; (b) R. P. Barnes, J. H. Graham and M. A. Salim Qureshi, *J. Org. Chem.*, 1963, **28**, 2890; (c) G. Tennant, *J. Chem. Soc.*, 1963, 2428; (d) R. Fusco and S. Rossi, *Gazz. Chim. Ital.*, 1964, **94**, 3.
- 61 I. Perillo, B. Fernandez and S. Lamdan, *J. Chem. Soc., Perkin Trans. 1*, 1977, 2068.
- 62 O. Aspevall, A. Lundberg, L. G. Burman, T. Åkerlund and B. Svenungsson, *Antimicrob. Agents Chemother.*, 2006, **50**, 1890.
- 63 J. Freeman, J. Vernon, K. Morris, S. Nicholson, S. Todhunter, C. Longshaw and M. H. Wilcox, *Clin. Microbiol. Infect.*, 2015, **21**, 248.
- 64 J. Arca-Suárez, F. Galán-Sánchez, F. Cano-Cano, G. García-Santos and M. A. Rodríguez-Iglesias, *Anaerobe*, 2018, **54**, 146.
- 65 M. Sebahia, B. W. Wren, P. Mullany, N. F. Fairweather, N. Minton, R. Stabler, *et al.*, *Nat. Genet.*, 2006, **38**, 779, DOI: [10.1038/ng1830](https://doi.org/10.1038/ng1830).
- 66 K. Nepali, H.-Y. Lee and J.-P. Liou, *J. Med. Chem.*, 2019, **62**(6), 2851.
- 67 K. M. Land and P. J. Johnson, *Drug Resistance Updates*, 1999, **2**, 289.
- 68 H. K. Leiros, S. Kozielski-Stuhrmann, U. Kapp, L. Terradot, G. A. Leonard and S. M. McSweeney, *J. Biol. Chem.*, 2004, **279**, 55840.
- 69 J. Muller, P. Schildknecht and N. Muller, *J. Antimicrob. Chemother.*, 2013, **68**, 1781, DOI: [10.1093/jac/dkt106](https://doi.org/10.1093/jac/dkt106).
- 70 C. G. Diniz, S. G. Santos, A. C. Pestana, L. M. Farias and M. A. Carvalho, *Anaerobe*, 2000, **6**, 149.
- 71 H. Cerecetto and M. González, *Mini-Rev. Med. Chem.*, 2001, **1**, 219.
- 72 A. B. Patel, K. H. Chikhalia and P. Kumari, *Eur. J. Med. Chem.*, 2014, **79**, 57.
- 73 (a) R. Yendapally, J. G. Hurdle, E. I. Carson, R. B. Lee and R. E. Lee, *J. Med. Chem.*, 2008, **51**, 1487; (b) T. Yempala, J. P. Sridevi, P. Yogeewari, D. Sriram and S. Kantavari, *Eur. J. Med. Chem.*, 2014, **71**, 160.
- 74 K. M. Merz and J. J. Baldwin, *J. Med. Chem.*, 2000, **43**, 3867.
- 75 U. Norinder and C. A. S. Bergström, *ChemMedChem*, 2006, **1**, 920.
- 76 C. A. Lipinski, F. Lombardo, B. W. Dominy and P. J. Feeney, *Adv. Drug Delivery Rev.*, 1997, **23**, 3.
- 77 W. L. Jorgensen, *Acc. Chem. Res.*, 2009, **42**, 724.

