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Design, synthesis and evaluation of clinafloxacin triazole hybrids as a new type of antibacterial and antifungal agents

Yan Wang, Guri L. V. Damu [1], Jing-Song Lv, Rong-Xia Geng, Da-Cheng Yang, Cheng-He Zhou *

Laboratory of Bioorganic & Medicinal Chemistry, School of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, PR China

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ABSTRACT

A series of clinafloxacin triazole hybrids as a new type of antibacterial and antifungal agents were synthesized for the first time and screened for their antimicrobial efficacy against four Gram-positive bacteria, four Gram-negative bacteria and two fungi by two fold serial dilution technique. The bioactive assay indicated that most of the target compounds displayed broad antimicrobial spectrum and good antibacterial and antifungal activities with low MIC values ranging from 0.25 to 2 μg/mL against all the tested strains which exhibited comparable or even better efficiency in comparison with the reference drugs Chloramphenicol, Clinafloxacin and Fluconazole, respectively. Notably, some synthesized clinafloxacin triazoles showed stronger efficacy against methicillin-resistant *Staphylococcus aureus* than their parent Clinafloxacin.

Clinafloxacin is a newly synthesized fluoroquinolone antimicrobial agent that has been demonstrated to be more active than currently available fluoroquinolones with expanded activity against most Gram-positive, Gram-negative and anaerobic bacteria.[1] It acts by inhibiting the essential bacterial enzyme DNA gyrase, thereby preventing the synthesis of bacterial DNA and RNA.[2] The extensive in vitro and in vivo investigations found that Clinafloxacin has broad antibacterial spectrum, good bioavailability and tissue penetration, prolonged serum half-life, improved safety and tolerability as well as favorable pharmacokinetics.[3] More importantly, Clinafloxacin is active against bacterial strains that are highly resistant to other quinolone agents including vancomycin-resistant *enterococci*, ciprofloxacin-resistant *staphylococci* as well as Methicillin-resistant *Staphylococcus aureus* (MRSA).[4] Nevertheless, Clinafloxacin is not permitted to go on the market because of the exposed severe side effects such as phototoxicity, DNA cleavages, lipid peroxidation, and photohemolysis in vivo.[5] Moreover, Clinafloxacin has bad water-solubility and is unstable in water solution. As a result, the structural modification of Clinafloxacin for its new derivatives with lower toxicity, better stability, stronger dissolvability and bioactivity is the most effective strategy to develop new antibacterial agents.

Many researches showed that the property of C-7 substituent on the standard structure of 4-quinolone-3-carboxylic acid in quinolones could greatly influence the inhibition of DNA gyrase and cell permeability, and ultimately impact the bioactivity, spectrum, solubility and pharmacokinetics.[6] So far much effort has been done toward the structural modification at C-7 position in quinolone by incorporating various types of substituents including some five- and six-membered heterocycles especially N-containing heterocycles like pyridine,[7] thiodiazole,[8] and piperidine[9] etc. into the C-7 position of quinolone which have been investigated very well for improving their antibacterial potential. A large number of structurally novel quinolone derivatives were synthesized and exhibited good antibacterial potency.[10] Particularly, in recent years, the researches and developments of quinolone hybrids as a new class of antimicrobial agents have been attracting an expanded interest.[11] The combination of antibacterial quinolones with other pharmacophores or drug fragments might produce medical hybrids with difunctional targets, drug synergism or new action mechanisms to overcome drug resistance, which is an innovative method to develop new types of antimicrobial drugs. A large number of works have been directed toward the synthesis of quinolone hybrids by incorporating various types of bioactive moieties including aminoglycosides,[12] macrolides[13] and oxazolidinones[14] etc. into the C-7 position to prepare drug conjugates, which have shown great potential as novel antimicrobial agents. All these mentions above overwhelmingly compel us with great interest to focus on further structural modification at the C-7 position of Clinafloxacin in order to find more active and safe clinafloxacin derivatives. In spite that a few heterocyclic rings such as thiophene, furan, pyridine and so on have been incorporated into Clinafloxacin in recent years,[7] however, to our best knowledge, so far the hybrid of Clinafloxacin with 1,2,4-triazole ring has not been observed. In view of this, here we combined Clinafloxacin and 1,2,4-triazole for the first time to generate a series of

* Corresponding author. Tel./fax: +86 23 68254967.
E-mail address: zhouch@swu.edu.cn (C.-H. Zhou).
[1] Postdoctoral fellow from Indian Institute of Chemical Technology (IICT), India

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clinafloxacin triazole hybrids which were expected as new type of antimicrobial agents with potentially good bioactivity and broad antimicrobial spectrum.

Fluconazole (Fig. 1) is one of the most important triazoles recommended by World Health Organization (WHO) as the first-line antifungal drug. It has become the first choice in the treatment of Candida infections and established an exceptional therapeutic record due to its potent activity, excellent safety profile, and favorable pharmacokinetic characteristics.[15] However, the prolonged clinical use of Fluconazole has resulted in the increasing fluconazole-resistant *Candida albicans* isolates. In addition, Fluconazole can not effectively inhibit the growth of invasive aspergillosis spp.[16] Therefore the structural modification of Fluconazole has attracted much attention in order to broaden its antifungal spectrum and enhance its antifungal efficacy. Recently, the structural modifications have mainly focused on the optimization of the side chain of Fluconazole by replacing with diverse functional groups for the enhancement of the binding ability between the drugs and the active sites of lanosterol 14α-demethylase of fungus.[17]

Prompted by above observations and on the basis of our previous work on triazoles,[18] herein we would like to combine the antifungal Fluconazole with antibacterial Clinafloxacin to prepare a class of new clinafloxacin-based triazole hybrids **5a–g** and evaluate their antibacterial and antifungal efficacy in vitro. 1,2,4-Ttriazole ring possesses the unusual five-membered tri-nitrogen aromatic heterocyclic structure, could exert interactions with DNA, enzymes and receptors and so on via hydrogen bonds, coordination, ion-dipole, cation-π, π–π stacking, hydrophobic effect and/or van der Waals force etc. This means that the introduction of 1,2,4-triazole ring into clinafloxacin skeleton to generate clinafloxacin triazole hybrids might exert diverse interactions with various targets which are helpful to overcome the side effects or resistance problems, and may beneficially not only improve their physicochemical property and binding affinity, thereby effectively increase their biological activities and broaden active spectrum in comparison with their precursor Clinafloxacin and Fluconazole, but also improve their water solubility by the hydrogen bonds formed by the nitrogen atoms in triazole ring. This new type of compounds might not only inhibit the growth of fungi but also treat bacterial infection effectively. Moreover, a lot of researches have provided evidences that the substituents on the benzene ring in drug molecules have great influence on their biological activity.[18a,19] Reasonably, various substituted phenyl groups were introduced to the target compounds.

The synthetic route of target clinafloxacin triazole hybrids was outlined in Scheme 1. The desired clinafloxacin triazoles were prepared via multistep reactions from commercially available substituted benzene, triazole and Clinafloxacin. The intermediates **2a**–**g** could be efficiently prepared in satisfactory yields (85–95%) by the acetylation of substituted benzenes **1a–g** by chloroacetyl chloride, and were N-alkylated with 1,2,4-triazole, respectively, in acetonitrile in the presence of potassium carbonate to afford the corresponding triazo1yl ethanones **3a**–**g** in good yields (78–85%). The further epoxidation of compounds **3a**–**g** in toluene by trimethyl sulfoxonium iodide (TMSI) and 20% sodium hydroxide

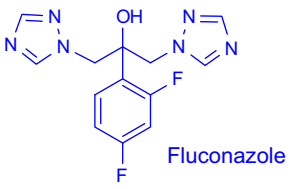


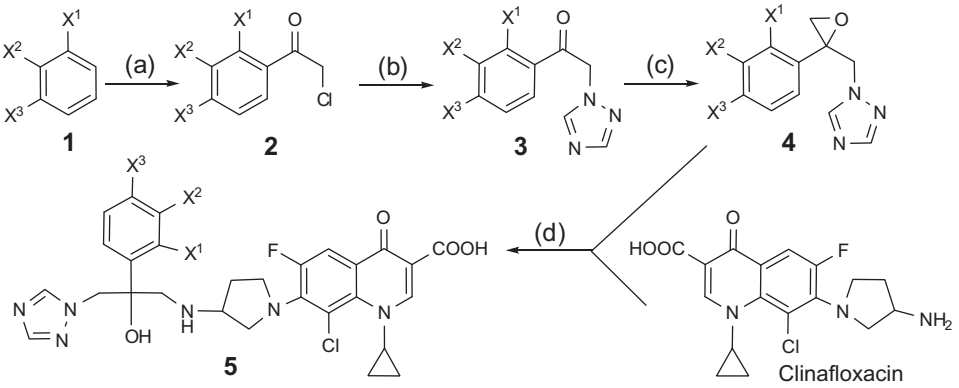
Figure 1. Structure of fluconazole.

at 60 °C produced the corresponding triazolyl oxiranes **4a**–**g** in 29–49% yields.[20] The target Clinafloxacin triazole hybrids **5a**–**g** were conveniently obtained by the reaction of Clinafloxacin with compounds **4a**–**g** in ethanol using sodium bicarbonate as base at 70 °C and all of them were prepared as racemates. All new compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR, MS and HRMS spectra.[21]

The in vitro antimicrobial screening for all prepared compounds was evaluated against four Gram-positive (*S. aureus* ATCC 25923, methicillin-resistant *S. aureus* N 315 (MRSA), *Bacillus subtilis* ATCC 6633 and *Micrococcus luteus* ATCC 4698), four Gram-negative bacteria (*Escherichia coli* JM109, *Pseudomonas aeruginosa*, *Shigella dysenteriae* and *Bacillus proteus*) as well as two fungal strains (*C. albican* ATCC 76615 and *Candida mycoderma*) using twofold broth dilution method in 96-well micro-test plates recommended by National Committee for Clinical Laboratory Standards (NCCLS).[22] Clinically antimicrobial Clinafloxacin, Chloramphenicol and Fluconazole were used as the positive control. The obtained results, depicted in Table 1, revealed that the prepared compounds **4–5** could effectively, to some extent, inhibit the growth of all tested strains in vitro. Triazolyl ethanones **3a**–**g** showed poor or even no activity against most of the tested bacterial strains except for Gram-negative bacteria *E. coli* and *P. aeruginosa* which were sensitive toward compounds **3a** and **3d–g** with MIC values ranging from 32 to 256 μg/mL.

In comparison with triazo1yl ethanones **3a**–**g**, triazolyl oxiranes **4a**–**g** displayed stronger antimicrobial efficacy and broader antimicrobial spectrum. Almost all these triazolyl oxiranes **4a**–**g** showed effective activities against all the tested bacteria and fungi with MIC values of 1–256 μg/mL except that compound **4a** had no obvious activity toward MRSA. Notably, bacteria *B. subtilis* and *M. luteus* and fungus *C. mycoderma* were quite sensitive to compounds **4a**–**g** with MIC values of 1–32 μg/mL. Especially, compound **4a** with methyl group at the 4-position of benzene ring gave quite low inhibitory concentration (MIC = 1 μg/mL) toward *M. luteus*, which was eight times more active than clinical drug Chloramphenicol, and only slightly weaker than Clinafloxacin (0.5 μg/mL). The anti-*C. mycoderma* activity (MIC = 1 μg/mL) also was comparable to reference drug Fluconazole (MIC = 0.5 μg/mL). These antimicrobial results indicated that the transformation of carbonyl group into an oxirane moiety should be beneficial for the antibacterial and antifungal potency.

For the tested clinafloxacin triazoles **5a–g**, except for 4-chlorophenyl compound **5b**, all other compounds displayed good inhibitory efficacy toward both Gram-positive and Gram-negative bacteria as well as fungi with MIC values in the range of 0.25–32 μg/mL, which were much more active than their precursor triazolyl oxiranes **4a**–**g**. This phenomenon indicated that the combination of clinafloxacin with triaozle ring should have significant influence on antimicrobial activities. Among them, compounds **5a**, **5c**, **5e** and **5g** with 4-toyl, 4-fluorophenyl, 3,4-difluorophenyl and 2,4-difluorophenyl groups respectively, exhibited excellent activities against pathogenic bacterial strains with the MIC values in the range of 0.25–2 μg/mL, which were much more potent than reference drug Chloramphenicol (MIC = 8–32 μg/mL) and equipotent to Clinafloxacin (MIC = 0.5–1 μg/mL). These compounds also showed significant antifungal activity against *C. albicans* (MIC = 0.5–1 μg/mL) and *C. mycoderma* (MIC = 0.5 μg/mL), which were comparable to reference drug Fluconazole. Especially compound **5g** could remarkably inhibit the growth of all the tested strains with MIC values ranging from 0.25 to 1 μg/mL, which was more active than other clinafloxacin triazoles. The result indicated that the 2,4-difluorphenyl moiety should play an important role in the antibacterial and antifungal profiles. Notably, compound **5g** exhibited the best bioactivity against *B. proteus* with MIC value of 0.25 μg/mL, which was 64-fold more potent than the reference



2-5: **a**, X^1 = H, X^2 = H, X^3 = CH_3, **b**, X^1 = H, X^2 = H, X^3 = Cl, **c**, X^1 = H, X^2 = H, X^3 = F, **d**, X^1 = H, X^2 = Cl, X^3 = Cl, **e**, X^1 = H, X^2 = F, X^3 = F **f**, X^1 = Cl, X^2 = H, X^3 = Cl, **g**, X^1 = F, X^2 = H, X^3 = F

Scheme 1. Reagents and conditions: (a) $ClCOCH_2Cl$, $AlCl_3$, CH_2Cl_2, rt, 2–3 h; (b) 1,2,4-triazole, K_2CO_3, CH_3CN, rt–80 °C, 1–2 h; (c) TMSI, 20% NaOH, PhMe, 60 °C, 4–8 h; Yield: **4a** (41%), **4b** (42%), **4c** (46%), **4d** (29%), **4e** (30%), **4f** (35%), **4g** (40%); (d) $NaHCO_3$, EtOH, 70 °C, 8–18 h; HCOOH, rt; Yield: **5a** (35%), **5b** (32%), **5c** (37%), **5d** (34%), **5e** (38%), **5f** (33%), **5g** (36%).

Table 1
In vitro antimicrobial activities for compounds **3–5** expressed as MIC (μg/mL)

Compds	Gram-positive bacteria				Gram-negative bacteria				Fungi	
	MRSA	*S. aureus*	*B. subtilis*	*M. luteus*	*E. coli*	*S. dysenteriae*	*P. aeruginosa*	*B. proteus*	*C. albicans*	*C. mycoderma*
3a	>512	>512	256	>512	32	>512	32	>512	>512	>512
3b	512	512	>512	>512	>512	512	512	512	512	>512
3c	>512	>512	>512	>512	>512	>512	512	>512	>512	>512
3d	>512	256	512	512	32	512	128	256	>512	512
3e	>512	>512	512	512	64	512	256	>512	>512	512
3f	>512	>512	512	>512	128	512	256	512	512	>512
3g	>512	512	512	512	32	512	256	512	512	>512
4a	512	256	8	1	256	256	256	256	256	1
4b	256	64	16	32	32	32	32	16	32	32
4c	128	256	8	8	128	64	128	128	64	8
4d	256	128	16	16	128	64	128	32	64	16
4e	256	256	32	8	128	64	64	64	128	8
4f	256	256	32	32	256	64	128	64	32	16
4g	128	256	16	32	32	32	16	32	64	8
5a	0.25	2	0.5	2	1	0.5	0.5	0.5	0.5	0.5
5b	>512	>512	>512	>512	32	>512	>512	512	>512	>512
5c	2	2	1	0.5	1	0.5	0.5	0.5	1	0.5
5d	0.25	8	32	0.5	4	0.5	0.5	0.25	2	0.5
5e	0.5	0.5	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5
5f	4	4	32	0.5	4	0.5	2	1	4	0.5
5g	0.25	0.5	0.5	0.5	1	0.5	0.5	0.25	0.5	0.5
Clinafloxacin	1	0.5	0.5	0.5	0.5	1	0.5	0.5	>512	>512
Chloramphenicol	16	16	32	8	32	32	32	32	>512	>512
Fluconazole	>512	>512	>512	>512	>512	>512	>512	>512	0.5	0.5

MRSA = Methicillin-resistant *Staphylococcus aureus*, *S. aureus* = *Staphylococcus aureus*, *B. subtilis* = *Bacillus subtilis*, *M. luteus* = *Micrococcus luteus*, *E. coli* = *Escherichia coli*, *S. dysenteriae* = *Shigella dysenteriae*, *P. aeruginosa* = *Pseudomonas aeruginosa*, *B. proteus* = *Bacillus proteus*, *C. albicans* = *Candida albicans*, *C. mycoderma* = *Candida mycoderma*.

drug Chloramphenicol and twofold more active than Clinafloxacin. These results demonstrated that the existence of fluorine atom on the benzyl moieties in this series of clinafloxacin triazole hybrids should be of special importance in microbial inhibition probably due to its easy and efficient formation of non-covalent forces with biosystem which could be helpful for the biological transportation and distribution in organism.[23]

In addition, bacterium *S. dysenteriae* also was more sensitive to all these target compounds **5a** and **5c–g** (MIC = 0.5 μg/mL) than Chloramphenicol (MIC = 32 μg/mL) and Clinafloxacin (MIC = 1 μg/mL) except for 4-chlorophenyl compound **5b** with no remarkable antimicrobial activity. Similarly, compounds **5a**, **5c–e** and **5g** displayed excellent activity against bacteria *P. aeruginosa* and *B. proteus* as well as fungi *C. albicans* and *C. mycoderma* with low MIC values ranging from 0.5 to 4 μg/mL, but no inhibitory activity was observed for compound **5b**. This demonstrated that the 4-chlorophenyl group in the clinafloxacin triazole hybrids seemed to be unfavorable for their antimicrobial efficacy.

It was known that MRSA was the most virulent organism that caused a huge array of problems to hospitalized and community-acquired patients, and showed severe multi-drug resistance to numerous currently available agents including the standard drugs.[18d] Excitedly, the target compounds **5a** and **5c–g** exhibited remarkable biological activity against MRSA with quite low MIC values of 0.25–4 μg/mL which were more active than Chloramphenicol (MIC = 16 μg/mL), especially 4-fluorophenyl compound **5d** and 2,4-difluorophenyl derivative **5g** gave the strong anti-MRSA activity with MIC value of 0.25 μg/mL which was twofold lower

than that of Clinafloxacin. These implied that this new type of clinafloxacin triazoles should be potential as anti-MRSA agents.

In conclusion, a series of new clinafloxacin triazole hybrids were successfully synthesized from commercially available substituted benzene, triazole and Clinafloxacin. Their structures were confirmed by ^{1}H NMR, ^{13}C NMR, IR, MS and HRMS spectra. The in vitro antibacterial and antifungal evaluation showed that most of the synthesized clinafloxacin triazole compounds could effectively inhibit the growth of all tested bacteria and fungi including methicillin-resistant *S. aureus*, and some clinafloxacin triazoles displayed better antimicrobial activities in comparison with their positive control. Particularly, clinafloxacin triazole **5g** with a 2,4-difluorophenyl group gave the most potent antibacterial and antifungal efficacy (MIC = 0.25–1 μg/mL) among these tested compounds including the standard drugs. Importantly, 4-fluorophenyl compound **5d** and 2,4-difluorophenyl triazole **5g** showed the strongest activity against drug-resistant MRSA (MIC = 0.25 μg/mL), which were superior to their precursor Clinafloxacin (MIC = 1 μg/mL). These results suggested that this type of clinafloxacin triazole hybrids should have great potential as new type of antibacterial agents to treat the drug-resistant bacteria infection. Moreover, these results also confirmed that the hybrid of antibacterial Clinafloxacin with antifungal triazole moiety could not only remarkably enhance the antimicrobial activity, but also broaden the antimicrobial spectrum. Further researches, including the in vivo bioactive evaluation along with toxicity investigation, the effect factors on antimicrobial activities such as other heterocyclic azole rings (benzotriazole, imidazole, benzimidazole and their derivatives) and other substituents on skeleton quinolone ring as well as their corresponding metal complexes and salts (hydrochloride, nitrate, acetate and lactate) are now in progress. All these will be discussed in the future paper.

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21. *Experimental:* Melting points were determined on X-6 melting point apparatus and were uncorrected. IR spectra were determined on a Bio-Rad FTS-185 spectrophotometer in the range of 400–4000 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV 300 spectrometer using TMS as an internal standard. The mass spectra were confirmed on FINIGAN TRACE GC/MS and HRMS. Some synthetic data were given for some representative compounds.
Synthesis of [1-((2-p-tolyloxiran-2-yl)methyl)-1H-1,2,4-triazole] (***4a***). To a solution of **3a** (1.00 g, 5 mmol) in toluene (55 mL) was added trimethylsulfoxonium iodide (2.20 g, 10 mmol) followed by the addition of 20% sodium hydroxide solution (1.89 mL). The reaction mixture was then heated at 60 °C for 6 h. After the reaction was completed (monitored by TLC, ethyl acetate/light petroleum (1/1, V/V)), it was diluted with ethyl acetate (3 × 20 mL). The organic layer was washed with water (2 × 30 mL), brine (20 mL), dried over anhydrous sodium sulfate. The filtrate was concentrated under reduced pressure and the residue was purified by flash silica gel column eluting with ethyl acetate/light petroleum (1/1, V/V) to give 4a as light yellow solid (0.44 g). Yield: 41%, mp: 52–53 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.06 (s, 1H, Tri 3-H), 7.91 (s, 1H, Tri 5-H), 7.22 (d, J = 6 Hz, 2H, Ph 2, 6-H), 7.15 (d, J = 6 Hz, 2H, Ph 3, 5-H), 4.61–4.81 (dd, J^1 = 45 Hz, J^2 = 15 Hz, Tri-CH_2), 2.83 (s, 2H, OCH_2), 2.33 (s, 3H, Ph-CH_3) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 151.8, 144.4, 138.3, 132.9, 129.5, 125.7, 58.6, 53.6, 53.4, 21.1 ppm; MS (*m*/*z*): 238 $[M+Na]^+$; HRMS (TOF) calcd for $C_{12}H_{13}N_3O$: [M]+, 215.2511; found, 215.2519. Synthesis of [8-Chloro-1-cyclopropyl-7-(3-(2-(2,4-difluorophenyl) -2-hydroxy -3-(1H–1,2,4-triazol-1-yl)propylamino)pyrrolidin-1-yl) -6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid] (5 g). To a solution of 4 g (0.323 g, 1.5 mmol) in ethanol (20 mL) was added clinafloxacin (0.547 g, 1.5 mmol) followed by the addition of sodium bicarbonate (0.126 g, 1.5 mmol). The reaction mixture was stirred at 70 °C for 18 h. After the reaction was completed (monitored by TLC, methanol/dichloromethane (1/100, V/V)), the reaction was cooled to room temperature and treated with formic acid to adjust the pH value to 5.5–6.5. After the ethanol was removed under reduced pressure, the mixture was purified by flash silica gel column eluting with methanol/dichloromethane (1/100, V/V) to give the pure target compound 5 g as white solid (0.322 g). Yield: 36%, mp: 210–211 °C; IR (KBr) : 3419, 3044, 2964, 2924, 2849, 2822, 2725, 1729, 1629, 1614, 1500, 1446, 1382, 1349, 1259, 1136, 965 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 14.37 (s, 1H, COOH), 8.90 (s, 1H, quinolone 2-H), 8.19 (s, 1H, Tri 3-H), 8.02 (d, 1H, J = 12 Hz, quinolone 5-H), 7.83 (s, 1H, Tri 5-H), 7.57–7.62 (m, 1H, Ph 6-H), 6.80–6.87 (m, 2H, Ph 3, 5-H), 4.51–4.64 (m, 3H, Tri-CH2, cycl-CH), 4.31 (s, 1H, OH), 3.27 (s, 4H, pyrr 2 × N-CH2), 2.73–2.96 (dd, 2H, J^1 = 78 Hz, J2 = 57 Hz, NH–CH_2), 2.54 (bs, 4H, pyrr CH_2CH, NH), 0.93–1.26 (m, 4H, cycl-2 × CH_2) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.6, 165.9, 164.4, 157.5, 154.4, 151.8, 150.9, 144.1, 144.8, 137.6, 129.1, 123.4, 111.8, 111.5, 108.5, 104.7, 104.3, 104.0, 72.1, 62.9, 56.0, 54.8, 51.0, 41.1, 11.6 ppm; MS (*m*/*z*): 625 $[M+Na]^+$, 603 $[M+H]^+$; HRMS (TOF) calcd for $C_{28}H_{26}ClF_3N_6O_4$: [M+H]+, 602.9920; found, 603.1722.
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