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Irreversible inhibitors of the proline racemase (PRAC) unveil innovative mechanism of action as antibacterial against *Clostridioides difficile*

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Abstract

Proline racemases (PRAC), catalyzing the *L*-proline and *D*-proline interconversion, are essential factors in eucaryotic pathogenes such as *Trypanosoma cruzi*, *Trypanosoma vivax* and *Clostridioides difficile*. If the discovery of irreversible inhibitors of *Trypanosoma cruzi* PRAC (*TcPRAC*) led to innovative therapy of the Chagas disease, no inhibitors of *CdPRAC* have been discovered to date. However, *Clostridioides difficile*, due to an increased incidence in recent years, is considered as a major cause of health threat.

In this work, we have taken into account the similarity between *TcPRAC* and *CdPRAC* enzymes to design new inhibitors of *CdPRAC*.

Starting from (*E*) 4-oxopent-2-enoic acid *TcPRAC* irreversible inhibitors, we synthesized 4-aryl substituted analogues and evaluated their *CdPRAC* enzymatic inhibition against eleven strains of *Clostridioides difficile*. This study resulted in promising candidates and allowed for identification of (*E*)-4-(3-bromothiophen-2-yl)-4-oxobut-2-enoic acid **20** that was chosen for complementary *in vivo* studies and did not reveal *in vivo* toxicity.

Keywords: Structure-activity relationship, Proline racemase, Enzyme inhibitors, *Clostridioides difficile*, Antibacterial.

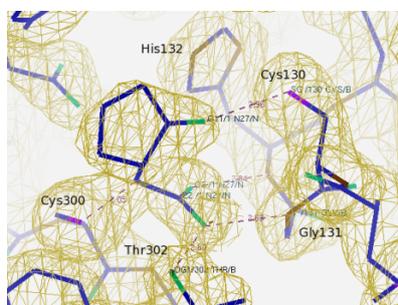
1 INTRODUCTION

Racemases, including proline racemases (PRAC), enzymes which catalyze the interconversion of *L*-proline and *D*-proline are frequently found as essential factors in pathogenes.¹ The first eukaryotic proline racemase was discovered and isolated by Minoprio's team from the human parasitic pathogen *Trypanosoma cruzi*.^{1,2} The group used the *Trypanosoma cruzi* proline racemase (*TcPRAC*) to design PRAC signatures and search for homologs in other microorganisms. They discovered novels PRACs from pathogenic microorganisms and characterized them; one had already been isolated in 1957 from *Clostridioides stricklandii* (*CsPRAC*), and more interestingly, two new functional PRACs, were respectively identified and isolated from *Trypanosoma vivax* (*TvPRAC*) and *Clostridioides difficile* (*CdPRAC*).^{3,4} *CdPRAC* closely resembled to *TcPRAC* and is also a strong B-lymphocyte mitogen. *TvPRAC* is an PLP-independent enzyme which kinetic properties were fully characterized.^{4,5} Importantly, genes

presenting similarities in human and other bacterial genomes lack the essential catalytic residues or the whole domain.⁵ They are not functional or encode other enzymes (e.g. hydroxyproline isomerase), which conforts *CdPRAC* as an interesting and specific target for drug development against *Clostridioides difficile*.⁶

Clostridioides difficile is considered as the major cause of healthcare-associated diarrhea in hospitalized adults. This anaerobic spore-forming bacterium is responsible for 15-25% of antibiotic-associated diarrhea and for virtually all cases of pseudomembranous colitis in humans.⁷ It has also recently emerged as an important enteropathogen both in the community and in animals. The epidemiology of *Clostridioides difficile* infections (CDI) has been characterized by an increase of its incidence over the last 15 years and the emergence of hypervirulent clones (e.g. 027) is responsible for large outbreaks of severe cases worldwide. The medical treatment of CDI currently relies on three oral drugs: metronidazole, vancomycin and fidaxomicin.⁸ However, recent guidelines from the Infectious Diseases Society of America recommend excluding metronidazole as the first line therapy of mild CDI due to the high rate of clinical failures and recurrences following this treatment.⁹

We previously reported the discovery of irreversible inhibitors of the *Trypanosoma cruzi* PRAC (*TcPRAC*) using a combination of computational and a classical drug design approaches. This led, in particular to compound **NG-P27**, which could be co-crystallized with the *TcPRAC* (Fig 1).^{10,11} Using the structural data, more efficient inhibitors, bearing an aryl substituent in C-4 (Fig1), were subsequently designed. The activity of the inhibitors on both promastigote and epimastigote forms of the parasite was established *in vitro*. Recently, the efficacy of the association of one of these aromatic inhibitors in prodrug form with benznidazole was described in mice.¹²



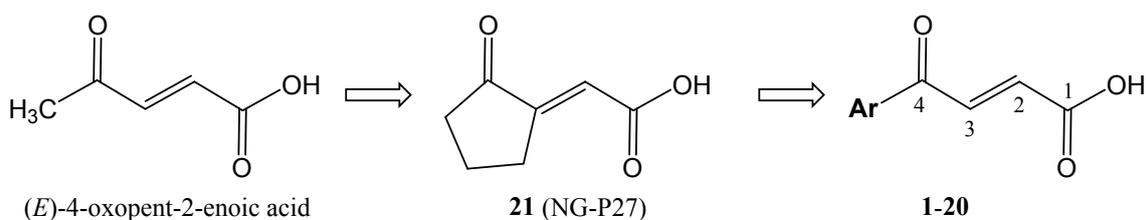


FIGURE 1: Co-crystallization of **21** (NG-P27) with TcPRAC and general structure of inhibitors **1-20**

2 EXPERIMENTAL PART

2.1 Chemistry

General Experimental Procedures

All reagents of high quality were purchased from commercial suppliers, and used without further purification or were purified/dried according to Armarego W. L. F. and Chai C. L. L. (Purification of Laboratory Chemicals, 6th edition, Elsevier). ¹H and ¹³C NMR were recorded at 300 and 75 MHz respectively, (using TMS as an internal standard). δ Values are given in parts per million (ppm), coupling constants (*J* values) are given in Hertz (Hz), and multiplicity of signals are reported as follows: s (singlet), d (doublet), t (triplet), q (quadruplet), quint (quintet), sext (sextet), m (multiplet), bs (broad singlet), dt (doublet of triplet), td (triplet of doublet). Thin layer chromatography was performed using precoated silica gel plate (0.2 mm thickness). LogD of compounds (Table 1) were determined using ChemAxon software.

Final compounds **1**, **2**, **3**, **4**, **6**, **8**, **13** and **16** were obtained according to Bianchi, A. *et al.*¹³

General method A (Compounds **9**, **10**, **11**, **19**, **20**)

To a suspension of AlCl₃ (2.14 g, 16.0 mmol) in 1,2-dichloroethane (10 mL) was added slowly at 0° C, a solution of an aromatic substrate (4.44 mmol) and maleic anhydride (0.47 g, 4.84 mmol) in 1,2-dichloroethane (10 mL). The mixture was allowed to react at room temperature for 15 min. then at 60° C for 2 hrs. The reaction mixture was acidified with aqueous HCl 6N and extracted with ethyl acetate (3 x 20 mL). The organic mixture was dried over MgSO₄, chromatographed over silica gel (CH₂Cl₂/ Et₂O/ 1% AcOH) to yield the desired compound (32% to 81%).

General method B1 (Compounds **14**, **15**, **17**)

To a suspension of glyoxylic acid monohydrate (1.2 g, 13 mmol) in a mixture of acetic acid (15 mL) and concentrated aqueous HCl solution (2.5 mL) was added slowly an arylmethylketone (13 mmol). The reactional mixture was then heated to reflux for 18 hrs. Water was added and the mixture was extracted with diethyl ether (3 x 30 mL). After drying over MgSO₄ and evaporation of diethyl ether, the resulting oil slowly crystallized. It was filtered and crystallized from ethyl acetate to yield the pure compound (25% to 27%).

General method B2 (Compounds 5, 7, 12, 18)

A mixture of an arylmethylketone (15 mmol) and glyoxylic acid monohydrate (0.92 g, 10 mmol) in 1,4-dioxane (15 mL) and concentrated H₂SO₄ (2 mL) was refluxed for 1.5 hrs. Water (50 mL) was added and the mixture was extracted with 3 x 30 mL of ethyl acetate. The solvent was dried over Na₂SO₄ and evaporated under reduced pressure to yield a solid that was chromatographed over silica gel (CH₂Cl₂/ Et₂O/ 1% AcOH) to yield the desired compound (47% to 63%).

(E)-4-oxo-4-phenylbut-2-enoic acid 1

¹H NMR (300MHz, CDCl₃) δ (ppm): 8.01 (m, 3H), 7.65 (m, 1H), 7.53 (m, 2H), 6.90 (d, *J* = 15.5 Hz, 1H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 189.2, 170.0, 138.4, 136.4, 134.1, 131.3, 129.0, 128.9. HRMS (ESI) calcd. for C₁₀H₇O₃ [M-H]⁻: 175.04007; found: 175.0399

(E)-4-(furan-2-yl)-4-oxobut-2-enoic acid 2

¹H NMR (300MHz, CDCl₃) δ (ppm): 7.84 (d, *J* = 15.6 Hz, 1H), 7.71 (m, 1H), 7.40 (m, 1H), 6.99 (d, *J* = 15.6 Hz, 1H), 6.64 (m, 1H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 176.2, 169.3, 152.8, 148.0, 137.4, 130.9, 119.8, 113.1. HRMS (ESI) calcd. for C₈H₅O₄ [M-H]⁻: 165.01933; found: 165.0191.

(E)-4-oxo-4-(thiophen-2-yl)but-2-enoic acid 3

¹H NMR (300MHz, Acetone-D₆) δ (ppm): 8.17 (dd, *J* = 3.8 Hz, *J* = 1.1 Hz, 1H), 8.05 (dd, *J* = 4.9 Hz, *J* = 1.1 Hz, 1H), 7.86 (d, *J* = 15.5 Hz, 1H), 7.32 (dd, *J* = 4.9 Hz, *J* = 3.8 Hz, 1H), 6.83 (m, 1H). ¹³C NMR (75MHz, Acetone-D₆) δ (ppm): 181.9, 166.4, 145.3, 137.1, 137.0, 135.2, 132.6, 129.8. HRMS (ESI) calcd. for C₈H₅O₃S [M-H]⁻: 180.9965; found: 180.9963.

(E)-4-(4-nitrophenyl)-4-oxobut-2-enoic acid 4

¹H NMR (300MHz, Acetone-D₆) δ (ppm): 8.42 (m, 2H), 8.33 (m, 2H), 7.95 (d, *J* = 15.6 Hz, 1H), 6.83 (d, *J* = 15.6 Hz, 1H). ¹³C NMR (75MHz, Acetone-D₆) δ (ppm): 189.2, 165.9, 151.3, 141.8, 136.7, 133.9, 130.6, 124.5. HRMS (ESI) calcd. for C₁₀H₆NO₅ [M-H]⁻: 220.02515; found: 220.0250

(E)-4-(4-cyanophenyl)-4-oxobut-2-enoic acid 5

Compound **5** was obtained from general method **B2** as a pale solid (0.84 g, 42%).

¹H NMR (300MHz, Acetone-D₆) δ (ppm): 8.25 (d, *J* = 8.7 Hz, 2H), 8.02 (d, *J* = 8.7 Hz, 2H), 7.93 (d, *J* = 15.6 Hz, 1H), 6.83 (d, *J* = 15.6 Hz, 1H). ¹³C NMR (75MHz, Acetone-D₆) δ (ppm): 188.7, 165.4, 139.7, 136.0, 132.8, 129.3, 117.7, 116.5. HRMS (ESI) calcd. for C₁₁H₆NO₃ [M-H]⁻: 200.03532; found: 200.0352.

(E)-4-(4-hydroxyphenyl)-4-oxobut-2-enoic acid 6

¹H NMR (300MHz, Acetone-D₆) δ (ppm): 8.02 (m, 2H), 7.95 (d, *J* = 15.5 Hz, 1H), 7.00 (m, 2H), 6.74 (d, *J* = 15.5 Hz, 1H). ¹³C NMR (75MHz, Acetone-D₆) δ (ppm): 187.8, 166.7, 163.6, 137.7, 132.4, 132.0, 129.8, 116.5. HRMS (ESI) calcd. for C₁₀H₇O₄ [M-H]⁻: 191.03498; found: 191.0348.

(E)-4-(3-methoxyphenyl)-4-oxobut-2-enoic acid 7

Compound **7** was obtained from general method **B2** as a pale solid (1.27 g, 62%).

¹H NMR (300MHz, CDCl₃) δ (ppm): 7.99 (d, *J* = 15.8 Hz, 1H), 7.60 (m, 2H), 7.54 (m, 1H), 7.46 (t, *J* = 7.9 Hz, 1H), 7.21 (m, 1H), 6.92 (d, *J* = 15.8 Hz, 1H), 3.91 (s, 3H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 189.0, 169.6, 160.1, 138.3, 137.7, 131.3, 129.9, 121.7, 120.9, 112.7, 55.6. HRMS (ESI) calcd. for C₁₁H₉O₄ [M-H]: 205.05063; found: 205.0505.

(E)-4-(4-acetamidophenyl)-4-oxobut-2-enoic acid 8

¹H NMR (300MHz, DMSO-D₆) δ (ppm): 10.37 (s, 1H), 8.02 (d, *J* = 8.8 Hz, 2H), 7.88 (d, *J* = 15.5 Hz, 1H), 7.76 (d, *J* = 8.8 Hz, 2H), 6.66 (d, *J* = 15.5 Hz, 1H), 2.10 (s, 3H). ¹³C NMR (75MHz, DMSO-D₆) δ (ppm): 187.6, 169.1, 166.4, 144.5, 136.2, 132.4, 130.7, 130.3, 118.4, 24.2. HRMS (ESI) calcd. for C₁₂H₁₀NO₄ [M-H]: 232.0615; found: 232.0615.

(E)-4-(4-(2-benzamidoethyl)phenyl)-4-oxobut-2-enoic acid 9

Compound **9** was obtained from *N*-phenethylbenzamide¹⁴ using general method **A** as a white solid (1.00 g, 70%).

¹H NMR (300MHz, Acetone-D₆) δ (ppm): 8.02 (m, 2H), 7.94 (d, *J* = 15.5 Hz, 1H), 7.85 (m, 2H), 7.47 (m, 5H), 6.77 (d, *J* = 15.5 Hz, 1H), 3.70 (m, 2H), 3.05 (t, *J* = 7.2 Hz, 2H). ¹³C NMR (75MHz, Acetone-D₆) δ (ppm): 189.6, 167.5, 166.6, 147.5, 137.6, 136.0, 135.9, 132.9, 132.0, 130.4, 129.9, 129.2, 128.0, 41.6, 36.5. HRMS (ESI) calcd. for C₁₉H₁₆NO₄ [M-H]: 322.10848; found: 322.1083.

(E)-4-oxo-4-(4-(2-(phenylsulfonamido)ethyl)phenyl)but-2-enoic acid 10

Compound **10** was obtained from *N*-phenethylbenzenesulfonamide¹⁵ using general method **A** as a pale solid (0.85 g, 53%).

¹H NMR (300MHz, CDCl₃) δ (ppm): 9.59 (s, 1H), 7.86 (m, 5H), 7.49 (m, 4H), 7.25 (m, 1H), 6.83 (d, *J* = 15.5 Hz, 1H), 5.32 (m, 1H), 3.25 (m, 2H), 2.86 (t, *J* = 6.9 Hz, 2H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 188.7, 169.9, 144.8, 139.7, 138.1, 134.9, 132.8, 131.4, 129.4, 129.3, 129.2, 129.1, 126.9, 126.3, 43.7, 36.0. HRMS (ESI) calcd. for C₁₈H₁₆NO₅S [M-H]: 358.07547; found: 358.0756.

(E)-4-(4-(2-(4-methoxybenzamido)ethyl)phenyl)-4-oxobut-2-enoic acid 11

A solution of 4-methoxybenzoic acid (3.0 g, 19.7 mmol) was solved and refluxed in thionyl chloride (15 mL) for 1.5 hrs. Excessive thionyl chloride was evaporated under reduced pressure then phenethylamine (1.55 g, 12.8 mmol) was slowly added at 0° C under stirring. An aqueous saturated solution of NaHCO₃ (20 mL) was then added dropwise. The solid residue was collected by filtration, washed with water and crystallized from cyclohexane/ethyl acetate (80/20) to yield 4-methoxy-*N*-phenethylbenzamide¹⁶ as a pale solid (0.82 g, 25 %).

Compound **11** was obtained from 4-methoxy-*N*-phenethylbenzamide using general method **A** as a white solid (0.35 g, 32%).

¹H NMR (300MHz, Acetone-D₆) δ (ppm): 8.02 (d, *J* = 8.3 Hz, 2H), 7.94 (d, *J* = 15.5 Hz, 1H), 7.84 (m, 3H), 7.49 (d, *J* = 8.3 Hz, 2H), 6.96 (m, 2H), 6.77 (d, *J* = 15.5 Hz, 1H), 3.84 (s, 3H), 3.68 (m, 2H), 3.04 (t, *J* = 7.2 Hz, 2H). ¹³C NMR (75MHz, Acetone-D₆) δ (ppm): 189.6, 166.6, 162.9, 147.6, 137.5, 135.8, 132.9, 130.4, 129.9, 129.7, 128.1, 114.3, 55.7, 41.5, 41.4, 36.6. HRMS (ESI) calcd. for C₂₀H₁₈NO₅ [M-H]⁻: 352.11905; found: 352.1191.

(E)-4-(3-fluorophenyl)-4-oxobut-2-enoic acid 12

Compound **12** was obtained from general method **B2** as a pale solid (0.65 g, 47%).

¹H NMR (300MHz, Acetone-D₆) δ (ppm): 7.96 (m, 1H), 7.94 (d, *J* = 15.5 Hz, 1H), 7.81 (m, 1H), 7.68 (m, 1H), 7.51 (m, 1H), 6.82 (d, *J* = 15.5 Hz, 1H). ¹³C NMR (75MHz, Acetone-D₆) δ (ppm): 188.2, 165.5, 162.9 (*J*_{C-F} = 246.2 Hz), 138.9 (*J*_{C-F} = 6.4 Hz), 136.1, 132.8, 131.1 (*J*_{C-F} = 7.8 Hz), 124.9 (*J*_{C-F} = 2.9 Hz), 120.6 (*J*_{C-F} = 21.5 Hz), 115.0 (*J*_{C-F} = 22.6 Hz). HRMS (ESI) calcd. for C₁₀H₆O₃F [M-H]⁻: 193.03065; found: 193.0307.

(E)-4-(4-fluorophenyl)-4-oxobut-2-enoic acid 13

¹H NMR (300MHz, CDCl₃) δ (ppm): 8.06 (m, 2H), 7.98 (d, *J* = 15.5 Hz, 1H), 7.22 (m, 2H), 6.90 (d, *J* = 15.5 Hz, 1H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 187.5, 170.5, 166.3 (d, *J* = 257.0 Hz), 138.1, 132.8 (d, *J* = 2.9 Hz), 131.8, 131.6 (d, *J* = 5.0 Hz), 116.3 (d, *J* = 22.1 Hz). HRMS (ESI) calcd. for C₁₀H₆O₃F [M-H]⁻: 193.03065; found: 193.0307.

(E)-4-(2,5-difluorophenyl)-4-oxobut-2-enoic acid 14

Compound **14** was obtained from general method **B1** as a white solid (0.28 g, 25%).

¹H NMR (300MHz, CDCl₃) δ (ppm): 7.84 (dd, *J* = 15.5 Hz, *J* = 3.5 Hz, 1H), 7.55 (m, 1H), 7.29 (m, 1H), 7.18 (m, 1H), 6.87 (dd, *J* = 15.5 Hz, *J* = 1.4 Hz, 1H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 186.3 (d, *J* = 4.5 Hz), 170.3, 158.8 (d, *J* = 245.6 Hz), 157.9 (d, *J* = 249.1 Hz), 140.4 (d, *J* = 8.0 Hz), 131.6, 126.3 (dd, *J* = 14.9 Hz, *J* = 6.7 Hz), 122.3 (dd, *J* = 24.5 Hz, *J* = 9.3 Hz), 118.4 (dd, *J* = 26.3 Hz, *J* = 8.0 Hz), 117.0 (dd, *J* = 25.1 Hz, *J* = 2.7 Hz). HRMS (ESI) calcd. for C₁₀H₅O₃F₂ [M-H]⁻: 211.02123; found: 211.0211.

(E)-4-oxo-4-(3-(trifluoromethyl)phenyl)but-2-enoic acid 15

Compound **15** was obtained from general method **B1** as a white solid (0.35 g, 27%).

¹H NMR (300MHz, CDCl₃) δ (ppm): 8.26 (s, 1H), 8.20 (d, *J* = 7.9 Hz, 1H), 8.00 (d, *J* = 15.5 Hz, 1H), 7.91 (d, *J* = 7.8 Hz, 1H), 7.70 (t, *J* = 7.8 Hz, 1H), 6.95 (d, *J* = 15.5 Hz, 1H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 188.0, 170.4, 137.4, 136.9, 132.5, 132.0, 131.4 (q, *J* = 33.2 Hz), 130.4 (q, *J* = 3.5 Hz), 129.7, 125.6 (q, *J* = 3.7 Hz), 123.6 (q, *J* = 272.6 Hz). HRMS (ESI) calcd. for C₁₁H₆O₃F₃ [M-H]⁻: 243.02745; found: 243.0273.

(E)-4-(4-chlorophenyl)-4-oxobut-2-enoic acid 16

¹H NMR (300MHz, CDCl₃) δ (ppm): 7.98 (m, 3H), 7.54 (m, 1H), 7.52 (m, 1H), 6.93 (d, *J* = 15.5 Hz, 1H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 187.9, 169.8, 140.8, 137.8, 134.7, 131.7, 130.3, 129.4. HRMS (ESI) calcd. for C₁₀H₆O₃Cl [M-H]⁻: 209.0011; found: 209.0009.

(E)-4-(3-chlorophenyl)-4-oxobut-2-enoic acid 17

Compound **17** was obtained from general method **B1** as a white solid (0.75 g, 27%).

¹H NMR (300MHz, CDCl₃) δ (ppm): 7.98 (m, 1H), 7.93 (d, *J* = 15.5 Hz, 1H), 7.89 (m, 1H), 7.62 (m, 1H), 7.48 (t, *J* = 7.9 Hz, 1H), 6.92 (d, *J* = 15.5 Hz, 1H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 188.0, 169.4, 137.9, 137.6, 135.4, 134.0, 132.0, 130.3, 128.9, 127.0. HRMS (ESI) calcd. for C₁₀H₆O₃Cl [M-H]⁻: 209.0011; found: 209.0010.

(E)-4-(3-bromophenyl)-4-oxobut-2-enoic acid 18

Compound **18** was obtained from general method **B2** as a pale solid (1.58 g, 63%).

¹H NMR (300MHz, CDCl₃) δ (ppm): 8.16 (m, 1H), 7.95 (m, 2H), 7.80 (m, 1H), 7.44 (m, 1H), 6.93 (d, *J* = 15.5 Hz, 1H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 187.8, 169.7, 138.1, 137.7, 136.9, 132.0, 131.8, 130.5, 127.4, 123.4. HRMS (ESI) calcd. for C₁₀H₆O₃Br [M-H]⁻: 252.95058; found: 252.9505.

(E)-4-(3-chlorothiophen-2-yl)-4-oxobut-2-enoic acid 19

Compound **19** was obtained from general method **A** as a white solid (1.10 g, 61%).

¹H NMR (300MHz, CDCl₃) δ (ppm): 8.15 (d, *J* = 15.4 Hz, 1H), 7.68 (d, *J* = 5.2 Hz, 1H), 7.10 (d, *J* = 5.2 Hz, 1H), 6.90 (d, *J* = 15.4 Hz, 1H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 181.0, 170.2, 139.3, 136.5, 133.7, 131.2, 130.6. HRMS (ESI) calcd. for C₈H₄O₃ClS [M-H]⁻: 214.9575; found: 214.9575.

(E)-4-(3-bromothiophen-2-yl)-4-oxobut-2-enoic acid 20

Compound **20** was obtained from general method **A** as a white solid (1.30 g, 81%).

¹H NMR (300MHz, CDCl₃) δ (ppm): 8.15 (dd, *J* = 15.3 Hz, *J* = 0.7 Hz, 1H), 7.65 (d, *J* = 5.2 Hz, 1H), 7.19 (d, *J* = 5.2 Hz, 1H), 6.90 (d, *J* = 15.3 Hz, 1H). ¹³C NMR (75MHz, CDCl₃) δ (ppm): 181.0, 169.4, 139.2, 137.6, 134.0, 133.8, 130.9, 116.3. HRMS (ESI) calcd. for C₉H₇O₃BrNaS [M+Na]⁺: 296.9192; found: 296.9193.

Compound	Chemical formula	Molecular weight	LogD at pH 5	LogD at pH 7	LogD at pH 9	Number v (μmol/50μg)
1*	C ₁₀ H ₈ O ₃	176.17	1.71	-1.68	-1.81	n=0.284
2*	C ₈ H ₆ O ₄	166.13	0.77	-2.75	-2.76	n=0.301
3*	C ₈ H ₆ O ₃ S	182.19	1.63	-1.86	-1.90	n=0.274
4*	C ₁₀ H ₇ NO ₅	221.17	1.65	-1.86	-1.88	n=0.226
5	C ₁₁ H ₇ NO ₃	201.18	1.57	-1.95	-1.96	n=0.248

6*	C ₁₀ H ₈ O ₄	192.17	1.41	-2.18	-3.32	n=0.260
7	C ₁₁ H ₁₀ O ₄	206.20	1.56	-1.90	-1.97	n=0.242
8*	C ₁₂ H ₁₁ NO ₄	233.22	0.95	-2.49	-2.57	n=0.214
9	C ₁₉ H ₁₇ NO ₄	323.35	2.80	-0.6	-0.73	n=0.154
10	C ₁₈ H ₁₇ NO ₅ S	359.40	2.56	-0.86	-0.99	n=0.139
11	C ₂₀ H ₁₉ NO ₅	353.37	2.64	-0.86	-0.99	n=0.141
12	C ₁₀ H ₇ FO ₃	194.16	1.86	-1.63	-1.67	n=0.257
13*	C ₁₀ H ₇ FO ₃	194.16	1.86	-1.63	-1.67	n=0.257
14	C ₁₀ H ₆ F ₂ O ₃	212.15	2.50	-1.50	-1.53	n=0.235
15	C ₁₁ H ₇ F ₃ O ₃	244.17	2.59	-1.04	-1.05	n=0.211
16*	C ₁₀ H ₇ ClO ₃	210.61	2.32	-1.16	-1.21	n=0.237
17	C ₁₀ H ₇ ClO ₃	210.61	2.32	-1.16	-1.21	n=0.237
18	C ₁₀ H ₇ BrO ₃	255.07	2.48	-1.04	-1.05	n=0.196
19	C ₈ H ₅ ClO ₃ S	216.64	2.23	-1.29	-1.30	n=0.231
20	C ₈ H ₅ BrO ₃ S	261.09	2.39	-1.14	-1.13	n=0.191
21	C ₇ H ₈ O ₃	140.17	1.71	-1.68	-1.81	n=0.284

*Reported in ref 13

TABLE 1: Calculated LogD of compounds **1-21**.

2.2 Bacteriology

Strains Eleven clinical strains (10 toxigenic, 1 non toxigenic) of *C. difficile* were selected to perform antimicrobial screening assays of the 21 selected TcPRAC inhibitors. These strains belonged to PCR ribotype 002, 10 (non toxigenic), 014/020/077, 015, 018, 027, 056, 078, 106, 126, and FR056. For MIC determination, 14 strains of *C. difficile* (from PCR ribotypes 002 (n=1), 10 (n=1), 014/20/77 (n=1), 015 (n=1), 027 (n=1), 056 (n=1), 078 (n=3) 106 (n=1), 126 (n=3) and FR 056 (n=1). were used.

PCR ribotyping Strains were characterized by capillary gel electrophoresis PCR ribotyping as previously described by Bidet *et al.* [P. Bidet, F. Barbut, V. Lalande, B. Burghoffer, J.C. Petit. Development of a new PCR-ribotyping method for *Clostridioides difficile* based on ribosomal RNA gene sequencing. *FEMS Microbiol. Lett.*,1999;175:261-266]. After DNA amplification, 1 µL of a 1/200 dilution of each PCR product was mixed with 10.5 µL formamide and 0.5 µL GeneScan LIZ600 (Applied Biosystems®, Foster City, USA) as an internal marker. After 30 s of denaturation at 90 °C, capillary electrophoresis was performed on an 8-capillary 3500 Genetic Analyzer (Applied Biosystems®, Foster City, USA). The GeneMapper software (Thermo Fisher Scientific,

Villebon-sur-Yvette, France) was used to analyze the banding patterns. PCR ribotypes (RTs) were identified using the webribo software (<https://webribo.ages.at/>).

2.2.1 Screening of compound activity by the disk diffusion technique on agar

Antimicrobial susceptibility to the 21 selected TcPRAC inhibitors was determined by the disk diffusion method as described elsewhere [F. Barbut, B. Gariazzo, L. Bonne, V. Lalande, B. Burghoffer, R. Luiuz, et al. *Clinical features of Clostridioides difficile-associated infections and molecular characterization of strains: results of a retrospective study, 2000-2004. Infect. Control Hosp. Epidemiol.* 2007;28:131-139]. Briefly, colonies of *C. difficile* were suspended in sterile saline buffer (no. 1 McFarland standard) and were swabbed on prereduced Brucella agar. A 12 mm-diameter disk was placed on the agar and 50 µl of each TcPRAC inhibitors (1 mg/ml) were deposited on the disk. The diameter of inhibition was read after 48h-incubation in anaerobic jars. Other antimicrobials including vancomycin (30 µg), metronidazole (4 µg [Sanofi Diagnostics Pasteur, Marnes la Coquette, France] and 16 µg [Rosco, Taastrup, Denmark]), erythromycin (15 IU), clindamycin (2 IU), moxifloxacin (5 µg), chloramphenicol (30 µg), imipenem (10 µg) and tetracycline (30 IU) were also tested. The results were interpreted according to the French Society of Microbiology's "Comité de l'Antibiogramme" (2013, available at: https://resapath.anses.fr/resapath_uploadfiles/files/Documents/2013_CASFM.pdf). Strains were considered susceptible if the inhibition diameters for vancomycin (VA), metronidazole (MZ), erythromycin (ERY), clindamycin (CM), moxifloxacin (MXF), chloramphenicol (C), imipenem (IMI) and tetracycline (TE) were greater than or equal to 17 mm, 21 mm, 22 mm, 15 mm, 23 mm, 23 mm, 24 mm and 23 mm respectively.

2.2.2 Minimum inhibitory concentration (MIC) determination

MICs were determined by the agar dilution method described by the National Committee for Clinical Laboratory Standards (CLSI, Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria, Approved standard Wayne, Pa: National Committee for Clinical Laboratory Standards M11-A7, 2007) with a Steers replicator. Serial twofold dilutions of NG-P68 were incorporated into *Brucella* agar (Oxoid, Dardilly, France), with antibiotic concentrations ranging from 0.125 to 256 µg/ml. Inocula were prepared from brain heart infusion broth (Diagnostics Pasteur, Marnes-la-Coquette, France) in which the organisms were grown at 37°C for 24 h. Cultures were adjusted to an optical density on the McFarland scale of 0.5, and 10 µl (10⁵ CFU/spot) was applied with a Steers replicator to prereduced Brucella agar. Assays were performed at least in duplicate for each strain. The plates were observed after 48 h of incubation

in anaerobic jars (HP11; Oxoid, Dardilly, France) at 37°C. The MIC was defined as the lowest concentration of each antibiotic that inhibited visible growth.

The quality control strains used in susceptibility testing included *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridioides perfringens* ATCC 13124, and *Clostridioides difficile* ATCC 9689.

2.3 Biology

Preparation of recombinant CdPRAC: Recombinant *Clostridioides difficile* proline racemase (EC 5.1.1.4) was produced in *E. coli* BL21 (DE3) (Invitrogen) and purified by immobilized metal affinity chromatography on nickel columns, as previously described for *Trypanosoma cruzi* proline racemase (1).

Racemization of L-Proline and inhibition assays: Proline racemization conditions for CdPRAC were determined as previously described for TcPRAC (3). Briefly, L- to D- proline conversion by CdPRAC and TcPRAC took place in 1.0 mL reaction containing 40 mM of L-Proline in 0.2 M sodium acetate pH 6.0 and 5 µg/mL of CdPRAC. The concentrations of D-proline were determined by optical rotation of the solution at 365 nm with a 10 cm optical path cell, at 37°C during one hour using a polarimeter (Jasco, P-2000, Bouguenais, France). Inhibition assays were performed in the same conditions as above with 5 µM of potential CdPRAC/TcPRAC inhibitors during 500 seconds (exponential phase of L-proline racemization).

In vivo toxicity of CdPRAC inhibitor prodrugs in Zebrafish embryos: AB wild-type zebrafish (*Danio rerio*) embryos were raised at 28°C in embryo water composed of mineral water supplemented with 280 µg/mL methylene blue (M-4159, Sigma-Aldrich) and 30 µg/mL 1-phenyl-2-thiourea (P-7629, Sigma-Aldrich), and handled according to the institutional approved guidelines. 72 hours post-fertilization (hpf) embryos were distributed in 24-well plates (10 embryos/well) in 500 µL of embryo water containing freshly-diluted drugs and incubated at 28°C during 72 hours in the dark. The quantification of live embryos and possible malformations was achieved using a stereomicroscope.

In vivo activity of the CdPRAC inhibitor NG-P68 in the golden Syrian hamster model: Male golden Syrian hamsters (*Mesocricetus auratus*) of 6 weeks of age (average weight 60-80 grams) were purchased from Janvier Laboratories (RjHan:AURA) and handled under specific pathogen-free conditions. The animals were housed with ad libitum access to water and food. Five days before infection, the animals received a subcutaneous injection of clindamycin (30 mg/kg) (5). The infection was performed by oral gavage with approximately 1×10^4 spores of *C. difficile* (strain K027) in 300 µL of PBS. Mock-infected animals received the PBS only. Infected and mock-infected animals were housed in separated cages in a BSL-3 environment. Starting on day 1

post-infection, the animals were treated by oral gavage with 100 mg/kg of the compound **20** diluted in PBS + 6.7% DMSO in a final volume of 300 μ L/animal. Treatment was administered once a day, during four days. Control animals received the PBS+DMSO only. All hamsters were followed-up twice a day.

Ethical statement: Animals were housed in the Institut Pasteur animal facilities accredited by the French Ministry of Agriculture for performing experiments on live rodents. Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (EC Directive 2010/63, French Law 2013-118, February 6th, 2013). All experiments were approved by the Ethics Committee #89 and registered under the reference #2013-0047.

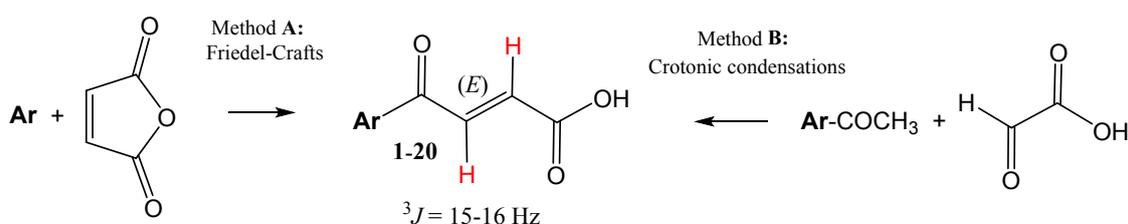
3 RESULTS AND DISCUSSION

The structural similarity between *TcPRAC* and *CdPRAC* enzymes led us to evaluate the activity of selected aromatic *TcPRAC* irreversible inhibitors against *Clostridioides difficile*. We report in this work the evaluation of their biochemical and biological effects as antibacterial agent, both *in vitro* and *in vivo*.

3.1 Synthesis of selected *TcPRAC* irreversible inhibitors (Scheme 1)

Following the hypothesis that *TcPRAC* inhibitors could have an antibacterial activity against *Clostridioides difficile*, we selected twenty 4-aryl substituted derivatives of the (*E*) 4-oxopent-2-enoic acid (**1-20**) efficiently inhibiting *TcPRAC*, as well as NG-P27 (**21**) for testing. These compounds were prepared according to two strategies reported in the literature¹³ and following the fundamental rules of drug design:¹⁷

- A Friedel and Crafts reaction (A) involving an aromatic or heteroaromatic compound and the maleic anhydride,
- A crotonic condensation (B) between an arylmethylketone and the glyoxylic acid monohydrate.



SCHEME 1: Synthesis of the PRAC inhibitors

The (*E*) configuration of the double bond was established by ¹H NMR from the values of the ³*J* coupling constants. Eight compounds have been previously reported.¹³ Relevant data (chemical formula, molecular weight, LogD at pH 5, 7 and 9, number *v* of μmol in 50μg) are reported in the experimental section (Table 1).

3.2 *In vitro* Antibacterial Activity of 21 TcPRAC irreversible inhibitors against *Clostridioides difficile* species

The compound inhibitory potential against *Clostridioides difficile* was tested *in vitro* against eleven strains of different PCR-ribotypes. Susceptibility of these strains to different antimicrobial drugs is reported in **Table 2**.

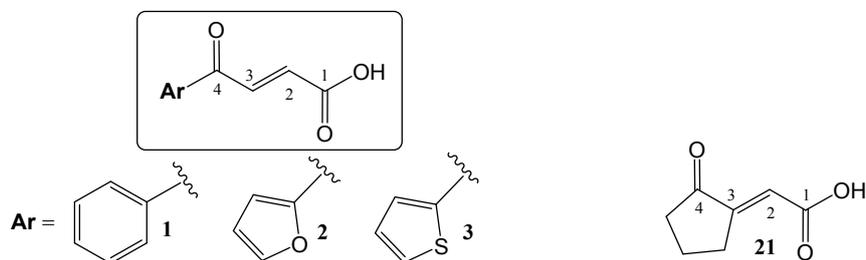
Strain Number	PCR-Ribotype	ERY	CIP	CM	MOXI	MTZ 5μg	MTZ 16μg	VA	TE	IMP	C
CD17-027	002	25	6	8	25	36	60	30	50	28	30
CD17-029	106	30	6	8	24	34	50	35	50	30	35
CD17-159	027	6	6	6	6	22	43	36	50	22	36
CD17-033	015	25	6	13	21	33	46	30	42	24	29
CD17-118	010	6	8	6	20	6	28	30	50	24	27
CD17-035	FR056	30	6	6	24	34	51	34	46	24	32
CD17-037	014/020/07	23	6	6	6	29	44	30	46	30	25
CD17-038	018	6	6	6	6	35	50	33	46	14	28
CD17-049	056	27	9	13	24	36	52	30	54	26	34
CD17-142	126	6	6	6	21	38	55	32	21	25	30
CD16-220	078	31	6	13	28	34	66	32	20	26	32

ERY: Erythromycin, **CIP:** Ciprofloxacin, **CM:** Clindamycin, **MOXI:** Moxifloxacin, **MTZ:** metronidazole, **VA:** Vancomycin, **TE:** Tetracyclin, **IMP:** imipenem, **C:** chloramphenicol. Strains were considered susceptible if the inhibition diameters for vancomycin, metronidazole, erythromycin, clindamycin, moxifloxacin, chloramphenicol, imipenem and tetracycline were greater than or equal to 17 mm, 21 mm, 22 mm, 15 mm, 23 mm, 23 mm, 24 mm and 23 mm respectively.

Table 2: Antimicrobial susceptibility of the studied strains

Depending on the nature of the substituent in 4-position the 21 compounds were divided in three families. The data corresponding to the inhibition diameter values (δ) induced by

50 μ g of each compound are reported in the **Tables 3-5**. In order to compare and select inhibitors, the total of diameter values, $\Sigma\delta$, and the diameter cumulative increment, $\Sigma\Delta\delta$, were also calculated. Absence of activity of all compounds against *E. coli* and *E. faecalis*, lacking a PRAC enzyme, was also confirmed with δ values = 12mm (contact).

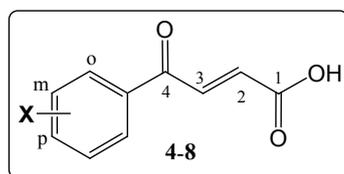


<i>Clostridioides difficile</i>		Compounds (inhibition diameter at 50 μ L)			
strain	PCR-ribo	1	2	3	21
CD17-027	2	23	19	21	12
CD17-029	106	23	17	22	12
CD17-159	27	24	14	23	12
CD17-033	15	22	19	22	12
CD17-118	10	21	16	22	12
CD17-035	FR056	22	17	23	12
CD17-037	014/020/077	23	16	20	12
CD17-038	18	22	12	22	12
CD17-049	56	22	19	21	12
CD17-142	126	12	12	12	12
CD16-220	78	13	12	12	12
	$\Sigma\delta$	227	173	220	133
		-12x11=	-12x11=	-12x11=	-12x11=
	$\Sigma\Delta\delta$	95	41	88	0

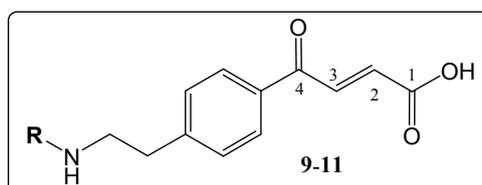
TABLE 3: Activities of compounds 1-3, 21

As reported in Table 3, non-aromatic compound **NG-P27 (21)** was completely inactive but introduction of an aromatic substituent in position 4 induced an antibacterial activity (compounds 1-3). In particular, phenyl and thiophenyl groups were quite equivalent with $\Sigma\Delta\delta = 88-95$, whereas the presence of a more hydrophilic furanyl substituent decreased the activity ($\Sigma\Delta\delta = 41$). No activity was observed against strains with 126 and 78 PCR-ribotypes.

Further investigating the positive impact of the aromatic ring in 4-position, eight substituted derivatives were prepared (**Table 4**). Polar substituents such as NO₂, CN, and OH in *para* position (compounds **4-6**) gave overall a low antibacterial activity as compared to compound **1** ($\Sigma\Delta\delta = 40, 3, 14$, v.s. 95, respectively). Interestingly, methylation of the OH (compound **7**) induced a strong activity increasing ($\Sigma\delta = 112$ versus 14). Addition of a long lipophilic chain in *para* position -compounds **9-11**- possibly able to bind in the canal entrance was poorly efficient ($\Sigma\Delta\delta = 35-37$). As observed with compounds **1-3**, these eight derivatives were also inactive against strains with PCR-ribotypes 126 and 78. Noticeably, compound **8** possessing an acetamido group in *para* position presented an interesting profile with ($\Sigma\Delta\delta = 100$) with detectable activity against the latter strains.



4: X= *p*-NO₂, 5: X= *p*-CN, 6: X= *p*-OH,
7: X= *m*-OCH₃, 8: X= *p*-NHCOCH₃

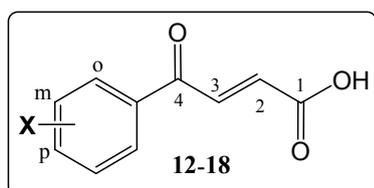


9: R= C₆H₅CO-, 10: R= C₆H₅SO₂-, 11: R= *p*-MeO-C₆H₄CO-

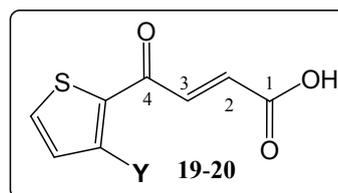
<i>Clostridioides difficile</i>		Compounds (inhibition diameter at 50 μ L)							
strain	PCR-ribo	4	5	6	7	8	9	10	11
CD17-027	2	17	12	13	23	22	16	16	16
CD17-029	106	12	12	12	25	22	17	12	17
CD17-159	27	19	12	16	26	23	18	18	15
CD17-033	15	17	12	12	24	20	16	16	17
CD17-118	10	17	12	14	24	22	16	15	17
CD17-035	FR056	16	12	12	24	22	15	16	16
CD17-037	014/020/077	18	12	14	25	24	16	19	15
CD17-038	18	18	12	12	24	20	12	14	15
CD17-049	56	14	15	17	25	23	17	17	17
CD17-142	126	12	12	12	12	17	12	12	12
CD16-220	78	12	12	12	12	17	12	12	12
	$\Sigma\delta$	172	135	146	247	238	167	167	169
	$\Sigma\Delta\delta$	40	3	14	112	100	35	35	37

TABLE 4: Activities of compounds **4-11**

Finally, introduction of a halogen substituent (F, Cl and Br) on the aromatic ring was successful (**Table 5**). This pharmacomodulation increased the overall lipophilicity in a limited volume. Thus, mono- or di-fluorination (compounds **12-14**) slightly improved the activity ($\Sigma\delta = 240-250$) except for strains with ribotypes 126 and 78 as already observed. However, introduction of CF_3 in *meta* position (compound **15**) induced one of the best antibacterial effect with $\Sigma\delta = 272$ but also an interesting activity against strains with ribosomes 126 and 78. Except on these latter strains, the nature (F, Cl, Br) and the position (*o*, *m*, *p*) of the halogen atom did not induce important differences between compounds **12-14** and **16-18** ($\Sigma\delta = 240-250$). In this case, a chlorine or a bromine atom is needed (compounds **16-18**). Finally, the best profiles were obtained with the chloro- and -bromothiophen compounds **19** and **20**.



12: X= *m*-F, 13: X= *p*-F, 14: X= *o,p*-F₂, 15: X= *m*-CF₃,
16: X= *p*-Cl, 17: X= *m*-Cl, 18: X= *m*-Br



19: Y= -Cl, 20: Y= -Br

<i>Clostridioides difficile</i>		Compounds (inhibition diameter at 50 μ L)								
strain	PCR-ribo	12	13	14	15	16	17	18	19	20
CD17-027	2	23	24	24	27	24	24	23	24	26
CD17-029	106	24	25	25	13	25	25	24	25	27
CD17-159	27	25	25	24	26	25	25	25	/	26
CD17-033	15	23	26	23	26	24	24	23	25	25
CD17-118	10	22	24	23	26	23	24	22	/	25
CD17-035	FR056	23	25	23	25	23	24	23	24	25
CD17-037	014/020/077	23	26	24	27	25	25	24	27	26
CD17-038	18	22	24	23	24	22	23	22	23	24
CD17-049	56	23	26	25	27	25	24	24	29	25
CD17-142	126	14	14	15	21	19	17	19	22	19
CD16-220	78	17	16	15	21	20	20	19	22	20
	$\Sigma\delta$	239	255	244	263	255	255	248	221*	268
	$\Sigma\Delta\delta$	107	123	112	131	123	123	116	113*	136

TABLE 5: Activities of compounds **12-20**

In conclusion, this *in vitro* study highlighted six compounds, namely compounds **15-20**, as promising candidates against *Clostridioides difficile* species. These derivatives possess a (*E*) 4-oxobut-2-enoic acid core substituted in position 4 by a halogeno phenyl or thiophenyl ring. In addition, the *para*-acetamido derivative **8** represented also an interesting candidate. Indeed, they presented the best inhibition diameters and were active on the PCR-ribotypes 078 and 126. It should be noticed that these compounds presented a Hammett σ and Hantzsch π positive constant¹⁸ giving similar Log D values as illustrated in Table 6.

Compound	Log D (pH 5)	Log D (pH 7)	Log D (pH 9)
1	1,71	-1.68	-1.81
8	0.95	-2.49	-2.57
15	2.59	-1.04	-1.05
16	2.32	-1.16	-1.21
17	2.32	-1.16	-1.21
18	2.48	-1.04	-1.05
19	2.23	-1.29	-1.30
20	2;39	-1.14	-1.13

TABLE 6: Log D at pH 5, 7 and 9

3.3 Inhibiting Activity of compounds **8, 15-20** on CdPRAC

In order to identify a hit for further development, evaluation of the *cdPRAC* inhibition in connection with the antibacterial activity was assessed on the seven selected compounds (**8, 15-20**) (Figures 2 A, B). The highest inhibition activity (38%) was observed in the presence of compound **20**. For comparison inhibition obtained with the *Trypanosoma cruzi* proline racemase [6] with derivatives **8, 16, 17, 19** and **20** are reported on Figures 2 C, D. Of note, the level of *TcPRAC* inhibition was higher than for *CdPRAC* (for example **20** inhibited 76% of *TcPRAC* activity *versus* 38% for *CdPRAC*) and that the activity sequence differed (Figure 2). Derivative **8**, the most hydrophilic compound, was the best inhibitor for *TcPRAC* but the worse for *CdPRAC*. If the catalytic site sequence in both

enzymes is conserved, this result indicated that variations of other amino-acids may induce recognition modifications.

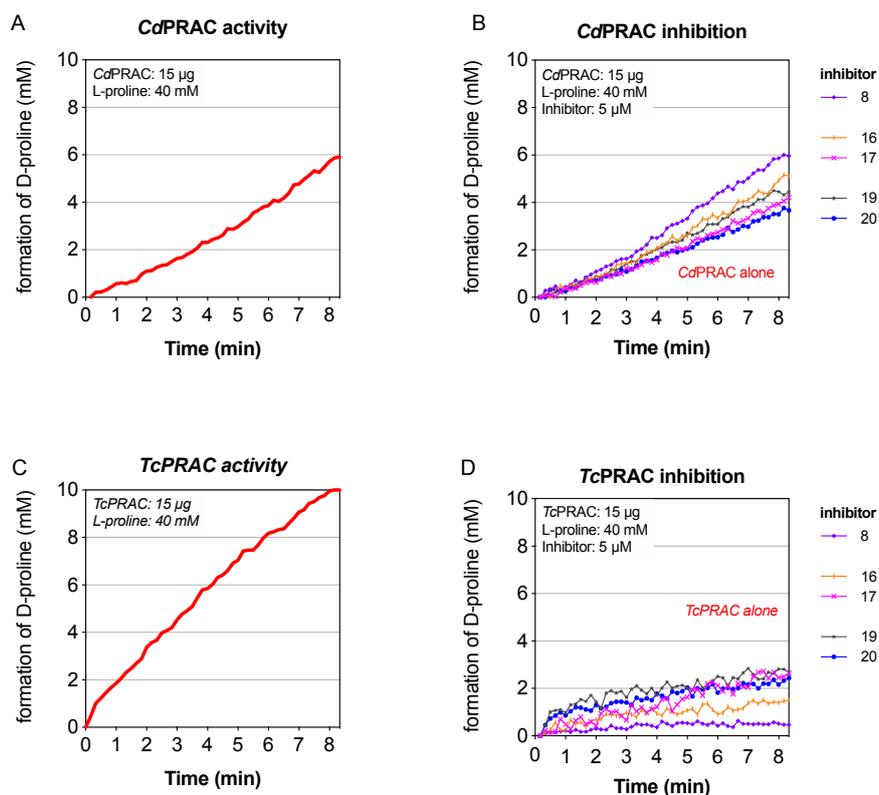


FIGURE 2 Inhibition of proline racemases by specific inhibitors.

(A) Kinetic curves of 40 mM L-proline racemization catalyzed by 15 μg of CdPRAC. (B) Kinetic curves of 40 mM L-proline racemization catalyzed by 15 μg of CdPRAC in the presence of 5 μM of potential CdPRAC inhibitors. (C) Kinetic curves of 40 mM L-proline racemization catalyzed by 15 μg of TcPRAC. (D) Kinetic curves of 40 mM L-proline racemization catalyzed by 15 μg of TcPRAC in the presence of 5 μM of potential TcPRAC inhibitors. Curves of **15** and **18** idem as **16**. The red crosshatched areas indicate the activity of the enzyme alone.

Finally, it appeared that lipophilic inhibitors were more efficient to inhibit CdPRAC. This data could be synergistic with a better penetration of the compounds into the bacteria. Therefore, compound **20** was chosen for complementary studies and *in vivo* test.

3.4 *In vivo* study of compound **20**

Before the *in vivo* evaluation of compound **20** on infected hamsters, minimum inhibitory concentrations (MICs) were determined and the data are reported on Figure 3. MICs

ranged from 16 mg/l to 64 mg/l (Figure 3). Consistently with previous results, strains belonging to PCR ribotype 126 or 78, except for CD17-019 to a lower extent, displayed the highest MIC among all strains.



FIGURE 3: Minimum inhibitory concentration (MIC) values of **20**

Furthermore, the *in vivo* toxicity test using zebrafish embryos¹⁹ was performed. No mortality was observed for **20** up to 100 μ M (Figure 4).

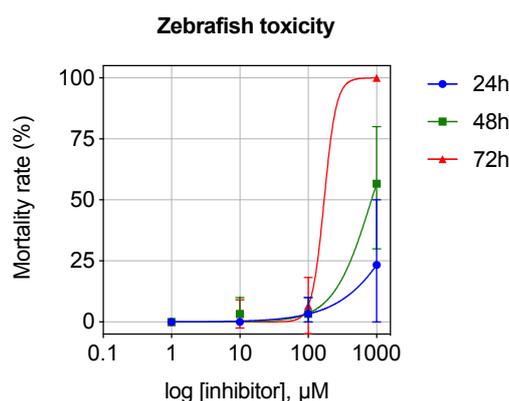


FIGURE 4: CdPRAC inhibitor **20** does not induce mortality in zebrafish embryos.

Dose-response curves for zebrafish embryos mortality after 24-, 48- and 72-hours exposition to 20. The graphs show data obtained from three independent experiments.

Interestingly compound RU 38086, analog of **20** was tested for its anti-secretory, anti-ulcer and cytoprotective properties¹³ and clinically evaluated²⁰ indicating the good tolerance of this family.

Finally, as an *in vivo* proof of concept, 1×10^4 *C. difficile* spores were orally inoculated to clindamycin-pretreated golden hamsters.²¹ After 24 hours, the infected animals received the CdPRAC inhibitor **20** (also called NG-P68) orally 100 mg/kg, once a day during four

days. This treatment allowed a discrete augmentation in the survival rate (Log-rank (Mantel-Cox) test, $p=0.0114$ (Figure 5).

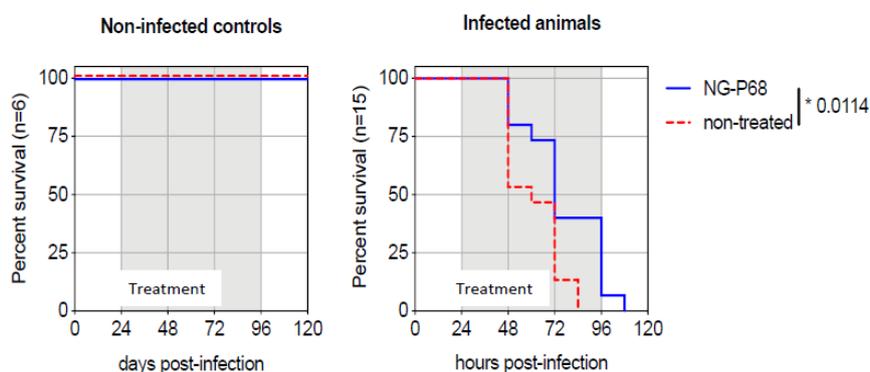


FIGURE 5: Treatment with **20** in *Clostridioides difficile* infected hamsters.

Cumulative Kaplan–Meier survival curves of hamsters treated with the compound **20** (NG-P68) or not. Treatment in non-infected control hamsters (left panel) presented no effect. Treatment in infected hamsters (right panel) produced an increase in the survival rate. Log-rank (Mantel-Cox) test to compare the treated group with the infected, non-treated group. The graphs show data obtained from two independent experiments.

4 Conclusion

In this study we demonstrated that PRAC enzyme constituted an interesting and specific target for the development of new antibacterial agents against *Clostridioides difficile* infections. The use of local or gastro-resistant galenic forms as well as the administration frequency are certainly to explore. Finally, prevention of *Clostridioides difficile* infections by use of this new specific class of antibacterial agents could represent a promising indication that should be explored. Therefore, its co-administration in association with typical antibiotics used for primary infection could inhibit the development of *Clostridioides difficile*.

Data availability statement

Data available on request from the authors: the data that support the findings of this study are available from the corresponding author upon reasonable request.

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