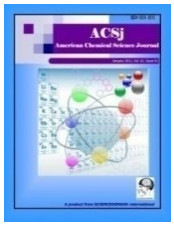

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New 5-substituted-1,3,4-thiadiazole-2(3*H*)-thione Derivatives: Synthesis and Their *In vitro* Antimicrobial Properties

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Authors' contributions

This work was carried out in collaboration between all authors. Author ŁP design the study, performed the synthesis of new derivatives of 1,3,4-thiadiazole, analyzed the spectral data and wrote the first draft of the manuscript excluding the in vitro antimicrobial section. Author AB performed the in vitro antimicrobial assays for synthesized compounds and wrote the antimicrobial section of this manuscript. Author AM analyzed the antimicrobial data. All authors read and approved the final manuscript.

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ABSTRACT

In this paper a new series of 1,3,4-thiadiazole derivatives have been designed, synthesized and evaluated as antimicrobial agents. New compounds were prepared by the reaction of 5-amino-1,3,4-thiadiazole-2(3*H*)-thione with appropriate aldehydes. The structures of the obtained compounds were confirmed by means of ^{1}H NMR and ^{13}C NMR. All synthesized compounds were tested for their *in vitro* antibacterial and antifungal activities using the broth microdilution method. Our results showed that the three of synthesized compounds had bactericidal or fungicidal effect against reference strains of Gram-positive bacteria, mainly opportunistic *S. epidermidis*, *M. luteus*, *Bacillus* spp., and yeast belonging to *Candida* spp.

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Keywords: 1,3,4-thiadiazole derivatives; antibacterial activity; antifungal activity; MIC; MBC/MFC.

1. INTRODUCTION

During the recent years there has been a large investigation on different classes of heterocyclic compounds as potential antimicrobial agents. The 1,3,4-thiadiazoles are a class of small molecules that have received much interest in the fields of agricultural chemistry, medicinal chemistry and biology due to their broad spectrum of activity [1]. The 1,3,4-thiadiazole derivatives have been reported to be analgesic [2], anti-inflammatory [2,3], anticonvulsant [4], antimicrobial [5-7], antitubercular [8-11], anti-leishmanial [12,13] and anticancer agents [14-18].

Some of the medicines with 1,3,4-thiadiazole scaffold such as Acetazolamide [19] or Methazolamide [20] are well-known for their therapeutic applications. Additionally the 1,3,4-thiadiazole scaffold is an interesting building block that has been used to synthesize a variety of useful bioactive compounds and it was reported that 1,3,4-thiadiazoles exhibit biological activity possibly due to the presence of the N=C=S moiety [21]. For this type of derivative, a different mechanism of action is assigned, depending on the type of modification of 1,3,4-thiadiazole ring.

In this study we design, synthesized and evaluated for *in vitro* antimicrobial activity new 1,3,4-thiadiazole compounds containing Schiff base side chains. This type of combination and rebuilding of these heterocyclic compounds is expected to have higher biological activity compared to standard medicines.

2. EXPERIMENTAL DETAILS

2.1 Chemistry

2.1.1 General`sq

All reagents were purchased from Sigma-Aldrich (Munich, Germany) and Merck Co. (Darmstadt, Germany) and used without further purification. Melting points were determined in Fisher-Johns blocks (Fisher Scientific, Schwerte, Germany) and presented without any corrections. The ^{1}H NMR spectra were recorded on a Bruker Avance 300 apparatus (Bruker BioSpin GmbH, Rheinstetten/Karlsruhe, Germany) in DMSO-d_6 with TMS as internal standard. The ^{13}C NMR spectra were recorded on a Bruker Avance 300 apparatus. Chemical shifts are given in ppm (δ-scale). The purity of obtained compounds was checked by TLC on aluminium oxide 60 F254 plates (Merck Co. Whitehouse Station, New Jersey, USA), in a $CHCl_3/C_2H_5OH$ (10:1, v/v) solvent system. The spots were detected by exposure to a UV lamp at 254 nm. Elemental analyses of the obtained compounds were performed for C, H, N on AMZ 851 CHX analyser (PG, Gdańsk, Poland). The maximum percentage differences between calculated and found values for each element were within the error and amounted to ±0.4%.

2.1.2 Preparation of 5-amino-1,3,4-thiadiazole-2(3*H*)-thione (1)

The compound was prepared according to the procedures described earlier [22]. A mixture of thiosemicarbazide (10 mmol), carbon disulphide (20 mmol), anhydrous sodium carbonate (1.0 g) and absolute ethanol (30.0 mL) was refluxed for 4 hrs. The solution was neutralized with potassium hydroxide. The precipitate was filtered and crystallized from ethanol.

CAS Registry Number: 2349-67-9. Yield: 78.0%, m.p. 230-232ºC. Physicochemical and spectroscopic data is consistent with the reference [22].

2.1.3 Preparation of 5-aminosubstituted-1,3,4-thiadiazole derivatives (2-9)

2.1.3.1 General method

To a suspension of 5-amino-1,3,4-thiadiazole-2(3*H*)-thione 1 (10 mmol) in ethanol (20 mL), an equimolar of various aldehydes (10 mmol) was added. The suspension was heated until clear solution was obtained. Then few drops of glacial acetic acid were added as a catalyst. The solution was refluxed for 4 hrs. After the completion of the reaction, the solution was cooled to room temperature. The obtained precipitate was filtered off and crystallized from ethanol.

5-[(2-methylpropylidene)amino]-1,3,4-thiadiazole-2(3*H*)-thione (2)

Yield: 78%; m.p.: 211-213ºC. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.34 (d, 6H, 2xCH_3), 2.22 (m, 1H, CH), 8.29 (s, 1H, =CH), 11.12 (s, 1H, NH); ^{13}C NMR (DMSO) δ (ppm) = 18.2 (2xCH_3), 30.3.

(CH), 159.6 (=CH), 163.9 ($C_{thiadiazole}$), 178.9 (C=S).

5-benzylideneamino-1,3,4-thiadiazole-2(3*H*)-thione (3)

CAS Registry Number: 137996-43-1. Yield: 47%; m.p.: 238-240ºC. ^{1}H NMR (DMSO-d_6) δ (ppm) = 7.31-7.77 (m, 5H, Ar-H), 8.77 (s, 1H, =CH), 11.12 (s, 1H, NH); ^{13}C NMR (DMSO) δ (ppm) = 129.0, 129.5, 133.1, 135.5 ($6C_{ar}$), 160.7 (=CH), 162.5 ($C_{thiadiazole}$), 173.6 (C=S).

5-[(2-hydroxybenzylidene)amino]-1,3,4-thiadiazole-2(3*H*)-thione (4)

CAS Registry Number: 94527-22-7. Yield: 61%; m.p.: 214-216ºC. ^{1}H NMR (DMSO-d_6) δ (ppm) = 7.00-7.07 (m, 2H, Ar-H), 7.52-7.59 (m, 1H, Ar-H), 7.73-7.91 (m, 1H, Ar-H), 8.91 (s, 1H, =CH), 10.30 (s, 1H, OH), 11.21 (s, 1H, NH); ^{13}C NMR (DMSO) δ (ppm) = 118.0, 120.5, 121.3, 130.6, 134.3, 162.1 ($6C_{ar}$), 162.5 ($C_{thiadiazole}$), 164.8 (=CH), 172.3 (C=S).

5-[(3-nitrobenzylidene)amino]-1,3,4-thiadiazole-2(3*H*)-thione (5)

CAS Registry Number: 135983-87-8. Yield: 28%; m.p.: 202-204ºC. ^{1}H NMR (DMSO-d_6) δ (ppm) = 7.25-7.78 (m, 4H, Ar-H), 8.97 (s, 1H, =CH), 10.20 (s, 1H, NH); ^{13}C NMR (DMSO) δ (ppm) = 124.8, 129.1, 129.8, 136.8., 137.5, 147.9 ($6C_{ar}$), 159.1 (=CH), 162.3 ($C_{thiadiazole}$), 172.8 (C=S).

5-[(4-methylbenzylidene)amino]-1,3,4-thiadiazole-2(3*H*)-thione (6)

CAS Registry Number: 256444-92-5. Yield: 37%; m.p.: 193-195ºC. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.11 (s, 3H, CH_3), 7.46-7.49 (dd, 2H, Ar-H, *J* = 7.5Hz), 7.85-7.88 (dd, 2H, Ar-H, *J* = 7.5 Hz), 8.62 (s, 1H, =CH), 10.01 (s, 1H, NH), ^{13}C NMR (DMSO) δ (ppm) = 21.1 (CH_3), 129.5, 130.1, 131.8. 143.6 ($6C_{ar}$), 160.7 (=CH), 162.8 ($C_{thiadiazole}$), 173.4 (C=S).

5-[(4-methoxybenzylidene)amino]-1,3,4-thiadiazole-2(3*H*)-thione (7)

CAS Registry Number: 256444-91-4. Yield: 24.7%; m.p.: 182-184ºC. ^{1}H NMR (DMSO-d_6) δ (ppm) = 3.92 (s, 3H, CH_3), 7.16-7.20 (dd, 2H, Ar-H, *J* = 10Hz), 8.00-8.04 (dd, 2H, Ar-H, *J* = 10Hz), 8.68 (s, 1H, =CH), 11.36 (s, 1H, NH); ^{13}C NMR (DMSO) δ (ppm) = 50.6 (CH_3), 114.5, 128.3, 132.3 ($5C_{ar}$), 160.7 (=CH), 162.3 ($C_{thiadiazole}$), 164.1 (C_{ar}), 174.1 (C=S).

5-[(4-bromobenzylidene)amino]-1,3,4-thiadiazole-2(3*H*)-thione (8)

CAS Registry Number: 256444-89-0. Yield: 61.5%; m.p.: 156-158ºC. ^{1}H NMR (DMSO-d_6) δ (ppm) = 7.66-8.01 (m, 4H, Ar-H), 8.81 (s, 1H, =CH), 10.05 (s, 1H, NH); ^{13}C NMR (DMSO) δ (ppm) = 128.8, 131.3, 132.3, 133.9 ($6C_{ar}$), 160.7 (=CH), 162.5 ($C_{thiadiazole}$), 171.4 (C=S).

5-[(4-fluorobenzylidene)amino]-1,3,4-thiadiazole-2(3*H*)-thione (9)

CAS Registry Number: 256444-84-5. Yield: 67.5%; m.p.: 219-221ºC. ^{1}H NMR (DMSO-d_6) δ (ppm) = 7.16-7.64 (m, 4H, Ar-H), 8.26 (s, 1H, =CH), 13.24 (s, 1H, NH); ^{13}C NMR (DMSO) δ (ppm) = 115.3, 131.7, 132.7, ($5C_{ar}$), 160.3 (=CH), 162.6 ($C_{thiadiazole}$), 165.3 (Car), 172.8 (C=S).

2.2 Microbiology

2.2.1 *In vitro* antimicrobial assay

The examined compounds (2-9): were screened *in vitro* for antibacterial and antifungal activities using the broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) [23] and Clinical and Laboratory Standards Institute guidelines [24] against a panel of reference strains of 20 microorganisms, including Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pyogenes* ATCC 19615, *Streptococcus pneumoniae* ATCC 49619, *Streptococcus mutans* ATCC 25175, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Micrococcus luteus* ATCC 10240), Gram-negative bacteria (*Escherichia coli* ATCC 3521, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 12453, *Bordetella bronchiseptica* ATCC 4617, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027) and fungi belonging to yeasts (*Candida albicans* ATCC 2091, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019). These microorganisms came from American Type Culture Collection (ATCC), routinely used for the evaluation of antimicrobials. All the used microbial cultures were first subcultured on nutrient agar or Sabouraud agar at 35ºC for 18-24 hrs or 30ºC for 24-48 hrs for bacteria and fungi, respectively.

The surface of Mueller-Hinton agar or Mueller-Hinton agar with 5% sheep blood (for bacteria) and RPMI 1640 with MOPS (for fungi) were inoculated with the suspensions of bacterial or fungal species. Microbial suspensions were prepared in sterile saline (0.85% NaCl) with an optical density of McFarland standard scale 0.5 - approximately 1.5 x 108 CFU (Colony Forming Units)/ml for bacteria and 0.5 McFarland standard scale – approximately 5 x 105 CFU/ml) for fungi.

Samples containing 5 mg, 1 mg and 0.5 mg of tested compounds 2-9 were dissolved in 1 ml dimethyl sulphoxide (DMSO). Next 50 µl of the tested compound was dropped into the wells (d = 6 mm) on the mentioned above agar media. The agar plates were preincubated at room temperature for 1h, next they were incubated at 37ºC for 24 hrs and 30ºC for 48hrs for bacteria and fungi, respectively. After the incubation period, the zones of growth inhibition were measured and average values were calculated. The wells containing DMSO without the tested compound was used as controls.

Furthermore, bacterial and fungal suspensions were put onto Petri dishes with solid media containing 1 mg/ml of tested compounds 2-9 followed incubation at 37ºC for 24 hrs and 30ºC for 48 hrs for bacteria and fungi, respectively. The inhibition of microbial growth was judged by comparison with a control culture prepared without any sample tested. Ciprofloxacin, vancomycin or fluconazole (Sigma) were used as a reference antibacterial or antifungal compounds, respectively.

Subsequently MIC (Minimal Inhibitory Concentration) of the compounds was examined by the microdilution broth method, using their two-fold dilutions in Mueller-Hinton broth or Mueller-Hinton broth with 5% sheep blood (for bacteria) and RPMI 1640 broth with MOPS (for fungi) prepared in 96-well polystyrene plates. Final concentrations of the compounds ranged from 1000 to 0.488 µg/ml. Microbial suspensions were prepared in sterile saline (0.85% NaCl) with an optical density of 0.5 McFarland standard. Next 2 µl of each bacterial or fungal suspension was added per each well containing 200 µl broth and various concentrations of the examined compounds. After incubation (37ºC, 24 hrs) the MIC was assessed spectrofotometrically as the lowest concentration of the samples showing complete bacterial or fungal growth inhibition. Appropriate DMSO, growth and sterile controls were carried out. The medium with no tested substances was used as control.

The MBC (Minimal Bactericidal Concentration) or MFC (Minimal Fungicidal Concentration) are defined as the lowest concentration of the compounds that is required to kill a particular bacterial or fungal species. MBC/MFC was determined by removing 20 µl of the culture using for MIC determinations from each well and spotting onto appropriate agar medium. The plates were incubated for 37ºC for 24 hrs and 30ºC for 48 hrs for bacteria and fungi, respectively. The lowest compounds concentrations with no visible growth observed was assessed as a bactericidal/fungicidal concentration. All the experiments were repeated three times and representative data are presented [25].

In this study, no bioactivity was defined as a MIC > 1000 µg/ml, mild bioactivity as a MIC in the range 501-1000 µg/ml, moderate bioactivity with MIC from 126 to 500 µg/ml, good bioactivity as a MIC in the range 26-125 µg/ml, strong bioactivity with MIC between 10 and 25 µg/ml and very strong bioactivity as a MIC < 10 µg/ml [26].

The MBC/MIC or MFC/MIC ratios were calculated in order to determine bactericidal/fungicidal (MBC/MIC ≤ 4, MFC/MIC ≤ 4) or bacteriostatic / fungistatic (MBC/MIC > 4, MFC/MIC > 4) effect of the tested compounds.

3. RESULTS AND DISCUSSION

3.1 Chemistry

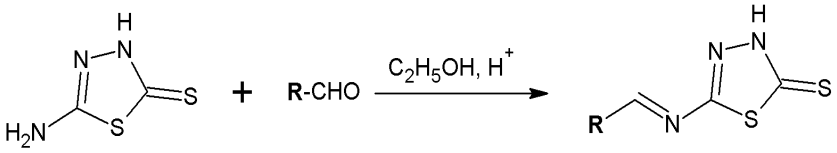
The synthetic pathway leading to the new 1,3,4-thiadiazole derivatives 2-9 was carried out according to the steps shown in Scheme 1. The new 5-substituted-1,3,4-thiadiazole derivatives were synthesized via condensation reaction of 5-amino-1,3,4-thiadiazole-2(3*H*)-thione 1 with appropriate aliphatic or aromatic aldehydes.

All the newly obtained compounds are air stable solids and soluble in DMSO at ambient temperature. The purity of the synthesized compounds was checked by elemental analyses and thin layer chromatography. The structures of prepared derivatives were determined on the basis of ^{1}H NMR and ^{13}C NMR spectra and all of the synthesized compounds have satisfactory analyses for their proposed structures. ^{1}H NMR spectral results for all compounds together with hydrogen assignments and ^{13}C NMR spectra results are presented in Experimental Section.

Table 1. The values of MIC and MBC/MFC [µg/ml] of tested compounds 2-9 against the reference strains of microorganisms.

	Species	MIC (MBC/MFC) [µg/ml] of the tested compounds								CIP/VA*/FLU**
		2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	
Gram-positive bacteria	*S. aureus ATCC 6538*	500(>1000)	-	125(250)	250(>1000)	-	125(1000)	1000(>1000)	125(250)	0.244
	S. aureus ATCC 43300	250(1000)	-	31.25(500)	125(500)	-	31.25(250)	500(>1000)	31.25(250)	0.244
	S. aureus ATCC 25923	250(1000)	-	62.5(500)	125(1000)	-	62.5(500)	250(>1000)	31.25(250)	0.488
	S. epidermidis ATCC 12228	500(>1000)	250(>1000)	31.25(62.5)	62.5(125)	-	31.25(31.25)	125(>1000)	15.62(31.25)	0.122
	M. luteus ATCC 10240	125(500)	125(500)	31.25(62.5)	62.5(62.5)	125(500)	31.25(62.5)	125(250)	31.25(62.5)	0.976
	B. subtilis ATCC 6633	62.5(62.5)	125(125)	15.62(15.62)	31.25(31.25)	31.25(31.25)	15.62(31.25)	15.62(15.62)	15.62(31.25)	0.031
	B. cereus ATCC 10876	-	500(500)	31.25(31.25)	250(1000)	-	31.25(62.5)	1000(>1000)	15.62(15.62)	0.061
	S. pneumonia ATCC 49619	-	-	250(250)	-	-	250(250)	-	125(250)	0.244*
	S. pyogenes ATCC 19615	500(>1000)	-	500(1000)	-	-	500(1000)	-	500(500)	0.244*
	S. mutans ATCC *25175*	-	-	500(>1000)	-	-	500(>1000)	-	500(500)	0.976*
Gram-negative bacteria	*E. coli* ATCC 3521	-	-	250(500)	-	-	-	-	500(500)	0.015
	E. coli ATCC 25922	-	-	1000(>1000)	-	-	1000(>1000)	-	500(500)	0.004
	K. pneumonia ATCC 13883	-	-	1000(>1000)	-	-	-	-	1000(>1000)	0.122
	S. typhimurium ATCC 14028	-	-	1000(>1000)	-	-	-	-	1000(1000)	0.061
	B. bronchiseptica ATCC 4617	-	-	125(500)	-	-	125(500)	-	125(500)	0.976
	P. mirabilis ATCC 12453	-	-	500(500)	-	-	500(1000)	-	500(1000)	0.031
	Ps. aeruginosa ATCC 9027	-	-	1000(>1000)	-	-	-	-	1000(>1000)	0.488
Fungi	*C. albicans ATCC 2091*	-	125(500)	31.25(62.5)	125(250)	-	62.5(62.5)	62.5(500)	31.25(125)	0.245**
	C. albicans ATCC 10231	-	31.25(62.5)	15.62(31.25)	31.25(125)	-	15.62(31.25)	31.25(125)	15.62(31.25)	0.976**
	C. parapsilosis ATCC 22019	-	125(1000)	31.25(125)	125(250)	-	31.25(62.5)	62.5(500)	31.25(125)	1.953**

The standard antibiotics used as positive controls: ciprofloxacin (CIP) for bacteria (except Streptococcus spp.), vancomycin (VA) for Streptococcus spp. and fluconazole (FLU**) for fungi*



(**1**) (**2** - **9**)

R = 2: i-Pr, **3:** C_6H_5, **4:** 2-OH-C_6H_4, **5:** 3-NO_2-C_6H_4, **6:** 4-CH_3-C_6H_4, **7:** 4-CH_3O-C_6H_4, **8:** 4-Br-C_6H_4, **9:** 4-F-C_6H_4

Scheme 1. Synthetic route to new 5-substituted-1,3,4-thiadiazole-2(3*H*)-thione derivatives (2-9)

In the ^{1}H NMR spectra of the compounds 2-9 the singlet peak due to the proton (=CH group) appeared in the region of δ 8.28-8.97 ppm, which confirmed the successful formation of the desired products. Whereas the singlet signal for NH 1,3,4-thiadiazole protons was observed in the region of δ 10.01-13.24 ppm. All other aliphatic and aromatic protons were observed at expected regions.

In the ^{13}C NMR spectra of 2-9 derivatives the carbon of =CH group had a signal at about 160 ppm. Similarly, the presence of the C=S group was also confirmed by a signal at about 170 ppm. All other aliphatic and aromatic protons were observed at expected regions.

3.2 Microbiology

Among the compounds examined, thiadiazoles 4, 7 and 9 demonstrated the widest spectrum of antibacterial activity with MICs ranging between 15.62 and 1000 µg/ml and MBCs ranging from 15.62 to ≥ 1000 µg/ml. These compounds indicated similar activity with bactericidal effect to some staphylococci (*S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228), micrococci, *Bacillus* spp. and streptococci (*S. pneumoniae* ATCC 49619, S. *pyogenes* ATCC 19615) or some Gram-negative bacteria (*E. coli* ATCC 3521, *P. mirabilis* ATCC 12453, *B. bronchiseptica* ATCC 4617). The compounds 4, 7 and 9 showed strong activity, especially to *B. subtilis* ATCC 6633 (MIC=15.62 µg/ml, MBC=15.62-31.25 µg/ml, MBC/MIC=1-2).

The remaining tested compounds 2, 3, 5, 6 and 8 indicated some bioactivity against to Gram-positive bacteria, especially to *S. epidermidis* ATCC 12228, *M. luteus* ATCC 10240 and *Bacillus* spp.

According to Table 1 above, on the basis of minimal inhibitory concentration values obtained by the broth microdilution method, it was shown that compounds 3, 4, 5, 7, 8 and 9 had also bioactivity against to tested yeasts belonging to *Candida* spp. with MIC = 15.62-250 µg/ml and MFC=15.62 - ≥ 1000 µg/ml. Among them, the compounds 4, 7 and 9 indicated high activity with fungicidal effect against these yeasts (MIC=15.62-62.5µg/ml, MFC=31.25-125 µg/ml). The most sensitive to these compounds was *C. albicans* ATCC 10231 with MIC=15.62 µg/ml, MFC=31.25 µg/ml and MFC/MIC=2.

4. CONCLUSION

In this paper we synthesized a new series of 5-substituted-1,3,4-thiadiazoles (2-9) by the condensation reaction of 5-amino-1,3,4-thiadiazole-2(3*H*)-thione with appropriate aldehydes. The structure of obtained compounds was confirmed by spectral methods. All synthesized derivatives were *in vitro* screened for their antimicrobial activity. Our results showed that the compounds 4, 7 and 9 indicated the highest activity of the investigated compounds, with bactericidal or fungicidal effect against reference strains of Gram-positive bacteria, mainly opportunistic *S. epidermidis*, *M. luteus*, *Bacillus* spp., and yeast belonging to *Candida* spp. The QSAR study for obtained compounds will be performed and the results will be presented in a future communication.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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