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Synthesis, Crystal Structure and Antifungal/Antibacterial Activity of Some Novel Highly Functionalized Benzoylaminocarbothioyl Pyrrolidines

H. Ali DÖNDAŞ[1*], **Yahya NURAL**[1], **Nizami DURAN**[2], **Colin KILNER**[3]
[1]*Department of Chemistry, Faculty of Pharmacy, Mersin University, Yenişehir, 33343, Mersin-TURKEY*
e-mail: yakdas25@mersin.edu.tr
[2]*Department of Microbiology, Faculty of Medicine, Mustafa Kemal University, 31100, Hatay-TURKEY*
[3]*School of Chemistry, Leeds University, Leeds LS2 9JT, UNITED KINGDOM*

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A series of novel highly functionalized benzoylaminocarbothioyl pyrrolidines were prepared from benzoylisothiocyanate and substituted pyrrolidines in excellent yield. The crystal structure of the novel 1-benzoylaminocarbothioyl-5-(naphthyl)-pyrrolidine-2,3,4-tricarboxylicacid trimethyl ester **(3a)** was determined by X-ray crystal structure analysis. The synthesized compounds were characterized and screened for their *in vitro* antibacterial and antifungal activities and toxicity. The prepared compounds were tested against the standard strains: *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213) and *Staphylococcus epidermidis* (ATCC 12228) and the yeasts *Candida albicans* (ATCC 90028), *Candida krusei* (ATCC 6258), *Candida parapsilosis* (ATCC 22019), *Candida tropicalis* (ATCC 22019) and *Candida glabrata* (ATCC 32554).

Key Words: Pyrrolidine derivatives, aminocarbothioyl derivatives, bioactivity, antibacterial activity, antifungal activity, toxicity

Introduction

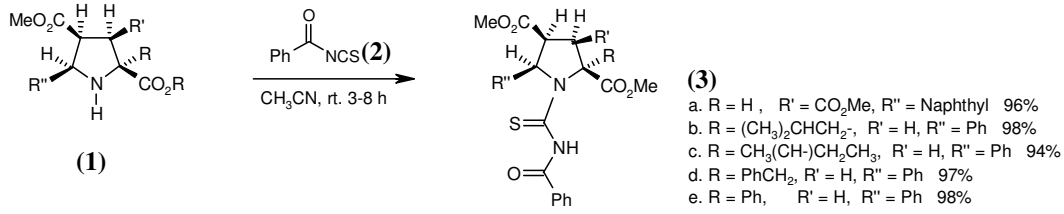
It has been reported that thiourea moiety containing aminocarbothioyl derivatives and their metal complexes show a wide range of biological activities such as antifungal,[1−6] antitumour[7] and antifouling[8] properties, and they have been screened for some other pharmaceutical actions.[1−8] The potential technical and analytical applications of aminocarbothioyl derivatives are well known[9−18] and some of these compounds have been found to be useful ligands for the liquid-liquid extraction and separation of several metal ions.[13,20−28] Some favourable physiochemical properties of their metal complexes[9−12] and potential determination of traces of the transition metals[9−12,21−28] were also reported.

*Corresponding author

A range of thermal and metal catalysed imine-azomethine ylides-1,3-dipolar cycloaddition cascades reactions furnishing azomethine ylides and their cycloadduct pyrrolidine compounds derived from amine and α-aminoacid ester were reported and reviewed by Grigg et al. in good to excellent yield.[29−32] They applied this cascade chemistry to provide a series of chiral and achiral peptidomimetics,[31] nikkomycin analogues,[33] spirobenzodiazepines related to MK-329,[34,35] pyrrolidine β-lactam analogues[36] and fused, bridged ring containing pyrrolidine compounds.[33−38] We reported a strategy to synthesise potential bioactive carbothioyl pyrrolidines from the corresponding pyrrolidine cycloadduct in excellent yield.[39]

In view of these favourable properties of these types of compound, we prepared some novel bioactive arylaminocarbothioyl pyrrolidines from corresponding arylisothiocyanate and substituted pyrrolidines, derived from α-aminoacid ester, in excellent yield as indicated in Scheme 1. The structures and stereochemistry of the novel compounds prepared were determined by ^{1}H-NMR, ^{13}C-NMR, ^{2}D-COSY, DEPT, FT-IR, MS, microanalyses and an X-ray crystal structure analysis of compound **3a**.

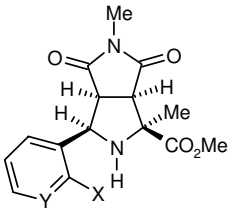
Results and Discussion: The highly functionalized benzoylaminocarbothioyl pyrrolidines **(3a-c)** were obtained in excellent yield from substituted pyrolidine cycloadducts **(1a-c)**[32,40] and benzoylisothiocyanate **(2)** in acetonitrile at ambient temperature for 6-10 h as a 3:1 and 2:1 rotamer (Scheme 1). In addition to these, 2 new highly functionalized bicyclic benzoylaminocarbothioyl pyrrolidines **(5a,b)** were also prepared from corresponding pyrrolidine ring compounds **(4a,b)**[41,42] and benzoylisothiocyanate **(2)** under similar conditions (CH_3CN, rt) in 90% and 92% yield, respectively. The structure and rotamer ratio of **3a-c** and **5a,b** were determined on the basis of ^{1}H-NMR, ^{13}C-NMR, DEPT and ^{2}D-COSY studies (see Experimental). The stereochemistry of substitution on pyrrolidine ring was assigned from nOe data, an X-ray crystal structure analysis of compound **(3a)** and related previous work.[30,31,38−42]



Scheme 1

The structures of the new compounds **(3a-c** and **5a,b)** are consistent with those of other thiourea derivatives as reported in our previous work.[39] Thus, in the ^{1}H-NMR spectra of the novel compounds **(3a-c)**, the NH proton appears at 8.60, 7.85 and 7.90 ppm as a broad singlet, respectively. In the ^{13}C-NMR spectra, the carbon atom of the thiocarbonyl group for compounds **3a-c** appears at 179.7, 186.6 and 189.9 ppm, respectively. In the IR spectra, all the ligands **(3a-c)** show NH stretching vibrations in the 3250-3360 cm^{-1} range. The bands at 1740-1750 and 1235-1245 cm^{-1} are assigned to the corresponding ester υ(C=O) and υ(C-O) vibration bands, respectively. All the compounds show a (C=O) amide vibration band in the 1680-1640 cm^{-1} range owing to conjugation with the aromatic ring. The thionyl (C-S) vibration bands appear at 1210-1205 cm^{-1} (see Experimental). In the case of bicyclic benzoylaminocarbothioyl pyrrolidines **(5a, b)**, the NH protons appear at 8.90 and 7.85 ppm as broad singlets, respectively. The carbon atom of the thiocarbonyl group appears at 187.9 and 187.85 ppm. Both **5a** and **5b** showed NH stretching vibrations at 3360 and 3355 cm^{-1}, respectively. The bands at 1743 and 1745 cm^{-1} are assigned to the corresponding ester

(C=O) vibration, and the amide (C=O) vibration at 1686 and 1688 cm^{-1}, respectively (see Experimental) in the IR spectrum. Compounds **3d,e** were prepared as reported previously[39] and their bioactivities were tested in this study.



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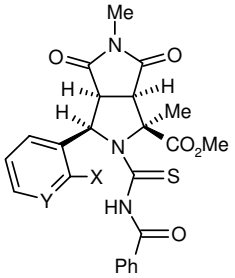


(5)

a. X = Br , Y = N, 92%
b. X = I, Y = CH 90%

Crystal structure of 3a. The crystal structure of **3a,** $C_{28}H_{26}N_2O_7S$ (Figure 1), was solved in space group $Pna2_1$ with Z'=16, that is with 4 molecules in the asymmetric unit. All 4 molecules have the same numbering scheme and are distinguished with the suffixes A, B, C and D. Some hydrogen atoms could be located in the Fourier difference map but, in the final stages of the refinement, hydrogen atoms bonded to carbon were placed in calculated positions and refined using a riding model. C-H distances: CH_3, 0.98Å; CH, 1.00 Å; aromatic, 0.95 Å. H11 attached to N11 was located in the Fourier map and freely refined. It was positioned in a planar configuration. Although anomalous dispersion effects were sufficient to allow the Flack parameter to refine to –0.01(4), inspection of the 4 molecules in the asymmetric unit shows that 2 enantiomers are present, with each enantiomer being represented by 2 examples that differ slightly in conformation. Thus the crystal structure is racemic, with molecules A and C representing one enantiomer and molecules B and D representing the other. This is illustrated in the view of the 4 molecules of the asymmetric unit (Figure 1), where all the molecules are oriented so that N1 occludes C2, and S11 is positioned to the right of N1. Selected bond atomic distances and angles of **3a** (molecule A) are shown in Table 2. Thus the bond length of N(11a)-C(11a) = 1.384(3) Å and N(11a)-C(12a) = 1.386(3) Å are different and shortened in comparison with the typical N-C single bond values. However, the bond S(11a)-C(11a) = 1.669(3) Å and O(12a)-C(12a) = 1.210(3) are significantly longer than C=S and C=O double bonds. These bond lengths suggest the existence of a partial electron delocalisation in the N-C(S)-NH-C(O) structural moieties, which is also observed in some other reported thiourea derivatives.[6,43]

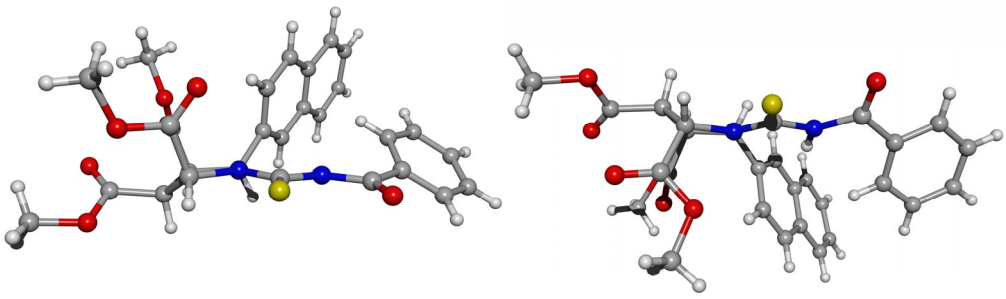
Antifungal and Antibacterial Activity: The synthesized cyclic and bicyclic benzoylaminocarbothioyl pyrrolidines **3a-e** and **5b** were screened for their *in vitro* antibacterial and antifungal activities as well as for toxicity (Tables 3 and 4). The prepared compounds were tested against the standard strains: *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213) and *Staphylococcus epidermidis* (ATCC 12228) and the yeasts *Candida albicans* (ATCC 90028), *Candida krusei* (ATCC 6258), *Candida parapsilosis* (ATCC 22019), *Candida tropicalis* (ATCC 22019) and *Candida glabrata* (ATCC 32554). All the tested compounds showed moderate antimicrobial activity; however, the antifungal efficacy is better than antibacterial activity. The tested compounds inhibited the growth of Gram-positive bacteria (*Staphylococcus epidermidis* and *Staphylococcus aureus*) at MIC values between 100 and 200 μg/mL (Table 3). The tested compounds showed an antifungal activity with a range of MICs between 25 and 100 μg/mL (Table 4). Microbiological results showed that the synthesized compounds possessed a broad spectrum of antifungal activity

Table 1. Summary of crystallographic data and structural parameters of compound **3a.**

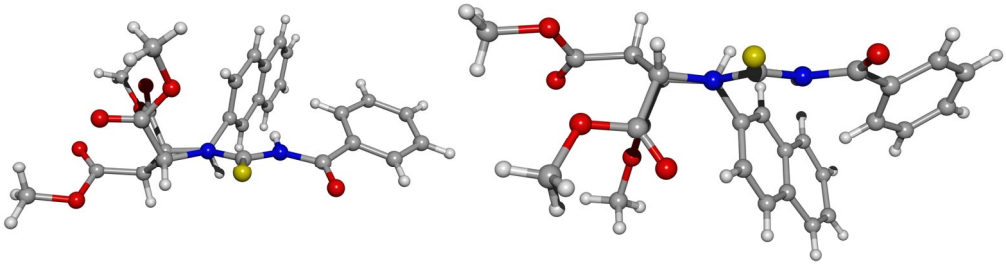
Formula	$C_{28}H_{26}N_2O_7S$	D_c(Mg.M^{-3})	1.356
M	534.57	*F*(000)	4480
Crystal Size (mm)	0.32 x 0.16 x 0.13	μ (mm^{-1})	0.174
Crystal color and shape	Pale yellow prism	Diffractometer	Nonius-Kappa CCD
Crystal system	Orthorhombic	Data collection range	$2.23 \leq \theta \leq 26°$
Space group	$Pna2_1$	No. of unique reflections	83770
a (Å)	18.27360(10)	No of observations	19308 [$I > 2\sigma(I)$]
b (Å)	21.74840(10)	Temperature (K)	150(2)
c (Å)	26.3497(2)	R_1	0.0496
α(°)	90°	*Rw*	0.1300
β(°)	90°	GOF	1.033
γ(°)	90°	$\Delta\rho$ max., min (e.Å^{-3})	1.217 -0.337
Z	16		

Table 2. Selected interatomic distances (Å) and torsion angles (°) with s.u.s. in parentheses.

Atomic distances			
S(11a)-C(11a)	1.669(3)	O(12a)-C(12a)	1.210(3)
N(11a)-C(11a)	1.384(3)	N(11a)-C(12a)	1.386(3)
Torsion angles			
C(11a)-N(11a)-C(12a)-O(12a)	6.5(4)		
C(12a)-N(11a)-C(11a)-S(11a)	1.8(4)		



3a, molecule A. **3a,** molecule B.



3a, molecule C. **3a,** molecule D.

Figure 1. Crystal structures of 4 molecules in the asymmetric unit with the suffixes A, B, C and D for compound **3a.**

against the tested micro-organisms. Compound **3a** was the most active compound against the screened the yeast-like fungi strains at a minimum inhibitory concentration (MIC) value of 25 μg/mL. On the other hand, compounds **3b-e** had significant antifungal activity. Moreover, the synthesized compounds also possessed antibacterial activity against the Gram-negative and Gram-positive bacteria showing MIC values between 100 and 400 μg/mL (Table 3).

Table 4 reveals that the synthesized compounds **3a-e** and **5b** provided antifungal activity against *C. albicans, C. krusei, C. parapsilosis, C. tropicalis* and *C. glabrata*, possessing MIC values between 50 and 100 μg/mL, except for derivative **3a**, which was active at a MIC value of 25 μg/mL against *C. krusei*. These results indicated that the addition of phenyl groups as substituents enhances the antifungal activity. Thus compound **3a,** which showed better activity against *C. krusei,* is a very promising antifungal structure to be modified. The same possibility is valid for compounds **3b** and **3d,** concerning their activities against *C. parapsilosis*. Moreover, the compounds tested here showed more antifungal activity than antibacterial activity. The antifungal activity against the yeast-like fungi may be ascribed to the difference between the cell structures of bacteria and fungi.[44,45]

Table 3. The MIC values (μg/mL) of the tested compounds against the bacteria.

Compounds	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. epidermidis*	*E. faecalis*	*E. cloacae*
3a	100	100	100	200	>200	100
3b	100	100	100	100	100	100
3c	100	100	>100	200	>100	>100
5b	>200	>400	200	100	>200	>100

Table 4. The MIC values (μg/mL) of the tested compounds for yeast-like fungi.

Compounds	*C. albicans*	*C. krusei*	*C. parapsilosis*	*C. tropicalis*	*C. glabrata*
3a	50	25	100	50	50
3b	75	75	50	50	50
3c	50	50	100	50	100
5b	100	100	50	100	50
3d	100	50	50	50	50
3e	100	100	100	50	50

In summary, the technically simple imine-azomethine ylides-1,3-dipolar cycloaddition cascades chemistry[29] has been applied to prepare some novel highly functionalized pyrrolidine cycloadducts[32,39−42], which can easily be converted to potential bioactive cyclic and bicyclic benzoylaminocarbothioyl pyrrolidines. The compounds were obtained under mild conditions in good to excellent yield and provide a major increase in functionalized molecules. The results of the present investigation may encourage us to develop and/or improve similar other related compounds, and test them for a wide range of biological activities. The antifungal effect of the compounds studied, especially compounds **3a-e** could be considered for testing in plant pathogenic fungi.

Some more chiral versions of these processes and their metal complexes, which can be used as chiral catalysts, are under investigation. The further bioactivities of the final compounds will be examined in conjunction with a pharmaceutical company.

Experimental

General Technical Data: Nuclear magnetic resonance spectra and decoupling experiments were determined at 300 MHz on a Q.E. 300 instrument and at 500 MHz on a Bruker AM500 spectrometer as specified. Chemical shifts are given in parts per million (δ) downfield from tetramethylsilane as internal standard. Spectra were determined in deuterochloroform, except where otherwise stated. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad and brs = broad singlet. IR spectra were recorded in the 4000-400 cm^{-1} range on a Shimadzu 435 spectrophotometer, using KBr pellets**.** Flash column chromatography was performed using silica gel 60 (230-400 mesh). Microanalyses were obtained using a Carlo-Erba Model 1106 instrument. Mass spectra were recorded at 70 eV on a VG Autospec mass spectrometer. The X-ray crystal structural data were collected on a Stadi 4-circle diffractometer by Colin Kilner. All solvents were purified according to procedures given in "Purification of Laboratory Chemicals", D.D. Perrin, W.L.F. Armarego, D.R. Perrin, Permagon Press, 1980. Benzoylisothiocyanate was prepared according to the literature.[16,17] 1-Benzoylaminocarbothioyl-2-benzyl-5-phenyl-pyrrolidine-2,4-dicarboxylic acid dimethyl ester **(3d)** and 1-benzoylaminocarbothioyl-5-phenyl-pyrrolidine-2,4-dicarboxylic acid dimethyl ester **(3e)** were prepared as reported previously.[39]

General Procedure for the synthesis of Benzoylaminocarbothioyl pyrrolidines: A solution of pyrrolidine cycloadduct (1.22 mmol) in freshly distilled dry degassed acetonitrile (20 mL) was added to a stirred solution of benzoylisothiocyanate (1.22 mmol) in 10 mL of acetonitrile over 15 min, followed by stirring at ambient temperature under nitrogen atmosphere for the appropriate time. The solvent was evaporated under reduced pressure (bath temperature not higher than 30 °C) and the residue was crystallised from an appropriate solvent.

1-Benzoylaminocarbothioyl-5-(naphthyl)-pyrrolidine-2,3,4-tricarboxylic acid trimethyl ester (3a): After a reaction time of 6 h and work up the product as a 1:1 mixture of rotamers crystallised from petroleum ether-hexane as pale yellow prisms in 96% yield. The rotameric mixture disappeared when heated to 333 K in $CDCl_3$. m.p. 156-158 °C (Found: C, 62.65, H, 5.1, N, 5.45, $C_{28}H_{26}N_2O_7S$ requires: C, 62.90, H, 4.90, N, 5.25). m/z (%) (ES): 535.1 (M+1, 100), 114.8 (20) and 100.0 (10). νgax (KBr): 3353, 2950, 1740, 1675, 1489, 1437, 1230, 1208 and 1055 cm^{-1}. δ_H(500 MHz) ($CDCl_3$) (333 K): 8.76 (br s, 1H, N-H), 7.85-8.02 (m, 12H, Ar-H), 5.89 (d, 1H, J=8.1 Hz, H-5), 5.79 (br d, 1H, J=7.9 Hz, H-2), 3.89 (s, 3H, OMe), 3.89 (m, 1H, H-4), 3.80 (m, 1H, H-3), 3.73 (s, 3H, OMe), 3.03 (s, 3H, OMe). δ_C($CDCl_3$): 179.7 (C=S), 170.1 (C=O), 168.0 (2C=O), 164.4 (C=O), 133.0 (2C), 130.5 (2C), 128.4, 128.1 (2C), 127.8, 127,6 (2C), 127.5, 127.3 (2C) 127.0, 126.3, 124.7, 77.3, 67.8, 65.8, 53.0, 52.4, 51.5, 46.7.

1-Benzoylaminocarbothioyl-5-phenyl-2-isobutyl-pyrrolidine-2,4-dicarboxylic acid dimethyl ester (3b): After a reaction time of 6 h and work up the product as a 3:1 mixture of rotamers crystallised from petroleum ether-hexane as a pale yellow amorphous solid in 98% yield, m.p. 72-74 °C (Found: C, 64.95, H, 6.5, N, 6.15, $C_{26}H_{30}N_2O_5S$ requires: C, 64.75, H, 6.25, N, 5.8). m/z (%) (ES): 483.1 (M+1, 100), 318.1 (12) and 105.7 (8). νgax (KBr): 3369, 2949, 1743, 1675, 1508, 1401, 1238, 1207 and 1025 cm^{-1}. δ_H(500 MHz) ($CDCl_3$): 8.10 (m, 1H, Ar-H), 7.8 (br s, 1H, N-H), 7.2-7.7 (m, 9H, Ar-H), 5.8 (d, 1H, J=9.5 Hz, H-5, minor rotamer), 5.7 (d, 1H, J=9.5 Hz, H-5, major rotamer), 3.90 (s, 3H, OMe major rotamer), 3.8 (m, 1H, H-4), 3.72 (s, 3H, OMe minor rotamer), 3.32 (s, 3H, OMe minor rotamer), 3.22 (s, 3H, OMe major rotamer) 3.05 (m, 1H, H-3b), 2.75 and 2.70 (2xm, 1H, CH$\underline{CH_2}\underline{a}$, minor and major

rotamers), 2.45 (m, 1H, H-3a), 2.20 and 2.15 (2xm, 1H, CH$\underline{CH_2}\underline{b}$, minor and major rotamers), 2.05 (m, 1H, $\underline{CH}(CH_3)_2$), 0.95-1.15 (m, 6H, CH$\underline{CH_3}$, 2 rotamers). δ_C($CDCl_3$): 186.6 and 178.5 (C=S, rotamers), 174.0 and 172.7 (C=O, rotamers), 172.05 and 170.0 (C=O, rotamers), 169.75 and 164.64 (C=O, rotamers).

1-Benzoylaminocarbothioyl-2-*sec*-butyl-5-phenylpyrrolidine-2,4-dicarboxylic acid dimethyl ester (3c): After a reaction time of 6 h and work up the product as a 3:1 mixture of rotamers crystallised from petroleum ether-hexane as a pale yellow amorphous solid in 94% yield, m.p. 97-99 °C (Found: C, 64.75, H, 6.5, N, 5.8, $C_{26}H_{30}N_2O_5S$ requires: C, 64.75, H, 6.25, N, 5.8). m/z (%) (ES): 483.2 (M+1, 100), 318.1 (4) and 115.8 (5). νg*ax* (KBr): 3375, 2951 1741, 1684, 1508, 1406, 1235, 1204 and 1025 cm^{-1}. δ_H(500 MHz) ($CDCl_3$): 8.12 (m, 1H, Ar-H), 7.9 (br s, 1H, N-H), 7.05-7.8 (m, 9H, Ar-H), 6.05 (br, 1H, H-5, minor rotamer), 5.75 (br, 1H, H-5, major rotamer), 3.91 (m, 1H, H-4), 3.84 (s, 3H, OMe major rotamer), 3.71 (s, 3H, OMe minor rotamer), 3.27 (s, 3H, OMe minor rotamer), 3.16 (s, 3H, OMe major rotamer) 3.0 (m, 1H, H-3b), 2.50 (m, 1H, H-3a), 2.0 (m, 1H, CH), 0.85-1.22 (m, 8H, CH($\underline{CH_3}$)$\underline{CH_2}\underline{CH_3}$). δ_C($CDCl_3$): 186.9 and 178.9 (C=S, rotamers), 174.05 and 172.8 (C=O, rotamers), 172.3 and 170.1 (C=O, rotamers), 169.9 and 164.6 (C=O, rotamers).

2-Benzoylaminocarbonothioyl-3-(2-bromopyridin-3-yl)-1,5-dimethyl-4,6-dioxooctahydropyrrolo[3,4-*c*]pyrrole-1-carboxylic acid methyl ester (5a): After a reaction time of 8 h and work up the product as a 2:1 mixture of rotamers crystallised from petroleum ether-hexane as a yellow amorphous solid in 90% yield, m.p. 187-189 °C (decomp) (Found: C, 51.6, H, 4.25, N, 10.2, $C_{23}H_{22}N_4O_5BrS$ requires: C, 50.7, H, 4.45, N, 10.3). m/z (%) (ES): 545.2 (Br= 79) (M+1, 100), 547.1 (Br=81) (M+1, 100), 465.2 (15), 342.1 (22) and 100 (80). νg*ax* (KBr): 3360, 2949, 1745, 1686, 1521, 1205 and 1091 cm^{-1}. δ_H(500 MHz) ($CDCl_3$): 8.0-8.3 (m, 2H, Ar-H), 7.9 (br s, 1H, N-H), 7.1-7.6 (m, 6H, Ar-H), 6.3 (d, 1H, J=11.1 Hz, H-5), 4.1 (m, 1H, H-4), 3.85 (s, 3H, OMe minor rotamer), 3.81 (s, 3H, OMe major rotamer), 3.7 (d, 1H, J=9.3 Hz, H-3 major rotamer), 3.58 (d, 1H, J=9.3 Hz, H-3 minor rotamer), 2.78 (s, 3H, N-Me, major rotamer), 2.60 (s, 3H, N-Me, minor rotamer) 2.18 (s, 3H, Me major rotamer), 2.09 (s, 3H, Me minor rotamer δ_C($CDCl_3$): 187.92 and 179.75 (C=S, rotamers), 174.09 and 173.56 (C=O, rotamers), 172.59 and 172.52 (C=O, rotamers), 170.67 and 170.32 (C=O, rotamers), 164.18 (C=O, rotamer).

2-Benzoylaminocarbonothioyl-3-(2-iodophenyl)-1,5-dimethyl-4,6-dioxooctahydropyrrolo [3,4-*c*] pyrrole-1-carboxylic acid methyl ester (5b): After a reaction time of 8 h and work up the product as a 2:1 mixture of rotamers crystallised from petroleum ether-hexane as a pale yellow prisms in 90% yield, m.p. 200-202 °C (decomp.) m/z (%) (ES): 592.2 (M+1, 100), 532.3 (10), 369.2 (15), 172.0 (12) and 100.9 (40). νg*ax* (KBr): 3359, 2948, 1743, 1688, 1526, 1244 and 1089 cm^{-1}. δ_H(500 MHz) ($CDCl_3$): 8.0-8.13 (m, 2H, Ar-H), 7.85 (br s, 1H, N-H), 6.95-7.6 (m, 7H, Ar-H), 6.25 (br m, 1H, H-5), 4.15 (m, 1H, H-4), 3.83 (s, 3H, OMe), 3.63 (d, 1H, J=9.5 Hz, H-3 major rotamer), 3.58 (d, 1H, J=9.3 Hz, H-3 minor rotamer), 2.74 (s, 3H, N-Me, major rotamer), 2.53 (s, 3H, N-Me, minor rotamer) 2.18 (s, 3H, Me major rotamer), 2.08 (s, 3H, Me minor rotamer). δ_C($CDCl_3$): 187.86 and 179.84 (C=S, rotamers), 174.269 (2C=O, rotamers), 173.56 and 172.91 (C=O, rotamers), 172.58 and 172.29 (C=O, rotamers), 164.10 (2C=O, rotamers). NOE data: Irradiation of 5-H resulted in enhancement of 4-H (11), irradiation of 4-H resulted in enhancement of 5-H (6.5) and 3-H (7.3) and irradiation of 3-H caused enhancement of 4-H (6.5).

Preparation of the cell culture.[46] To evaluate the cytotoxicity of the compounds for human cells, HEp-2 cell line (HEp-2 cell line no: ATCC CCL23) was selected. In preparation of the cell cultures, EMEM

(Eagle's Minimum Essential Medium) was used as the medium with fetal bovine serum (Seromed) at a rate of 10% as the growth factor. Incubation of the cells was performed in an atmosphere of 5% carbon dioxide at 37 °C.

Cytotoxicity assay. To determine the effects of chemical compounds on HEp-2 cells, they were infected with chemical compounds, and control cells (uninfected with chemical compounds HEp-2 cells) were observed. The non-toxic concentration was determined to be up to 1024 μg/mL. We used these concentrations in all of the experiments to test the effects of chemical compounds on all bacterial growth.

In order to test the effects of the compounds on HEp-2 cells, 5×10^4 cells were seeded into each well of 12-well plates, cultured for 6 h at 28 °C, and cells were allowed to grow for an additional 48 h in the presence of increasing amounts of chemicals, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 and 2048 μg/mL. The cytotoxicity of the compounds was determined by a conventional haemocytometer using the trypan blue-exclusion method.[47−49] The highest noncytocidal (on HEp-2 cells) concentration of the chemical compounds were determined to be 1024 μg/mL. Therefore, up to 1024 μg/mL concentrations of chemicals were used for the determination of antimicrobial activities.

Antimicrobial activity. The stock solutions of the chemical compounds were dissolved in dimethylsulfoxide and then diluted in Mueller-Hinton broth (Difco, USA) to give an initial concentration of 8 mg/mL. Further dilutions of the compounds and standard drugs in the test medium were prepared at the required quantities of 800, 400, 200, 100, 75, 50, 25, 12.5 and 6.25 μg/mL concentrations. In order to ensure that the solvents had no effect on microbial growth, a control test was also performed containing inoculated broth supplemented with dimethylsulfoxide at the same dilutions used in our experiments and was found to be inactive in culture medium.

Minimal inhibitory concentrations for each compound were investigated against standard bacterial strains: *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213), *Enterobacter cloacae* (ATCC 13047) and *Staphylococcus epidermidis* (ATCC 12228), and yeast-like fungi: *Candida albicans* (ATCC 90028), *Candida krusei* (ATCC 6258), *Candida parapsilosis* (ATCC 22019), *Candida tropicalis* (ATCC 22019) and *Candida glabrata* (ATCC 32554) obtained from the Refik Saydam Hifzisihha Institute, Ankara, Turkey. The observed data on the antimicrobial activity of the compounds are given in Tables 3 and 4.

Antibacterial assay. The cultures were obtained in Mueller-Hinton broth for all the bacteria after 24 h of incubation at 37 ± 1 °C. Testing was carried out in Mueller-Hinton broth at pH 7.4 and the 2-fold serial dilution technique was applied. The micro-organisms were grown overnight in Mueller-Hinton broth at 37 ± 1 °C and the final inoculum size was 10^5 CFU/mL for the antibacterial assay. A set of tubes containing only inoculated broth was kept as controls. After inoculation for 24 h at 37 ± 1°C, the last tube with no growth of micro-organism was recorded to represent MIC expressed in μg/mL.

Antimycotic assay. The yeasts were maintained in Sabouraud dextrose broth (Difco, USA) after incubation for 24 h at 25 ± 1 °C. Testing was carried out in Sabouraud dextrose broth at pH 7.4, and the 2-fold serial dilution technique was applied. The final inoculum size was 10^4 CFU/mL for the antifungal assay. A set of tubes containing only inoculated broth was used as controls. After incubation for 48 h at 25 ± 1 °C for the antifungal assay, the last tube with no growth of yeast was recorded to represent the MIC expressed in μg/mL. Each experiment for antimycotic activity and the antibacterial assay was replicated twice in order to define the MIC values.

Determination of Minimal Inhibitory Concentration (MIC). MIC values and bacterial killing

were determined as described.[47] Briefly, bacterial suspensions corresponding to a McFarland value of 1.0 were diluted 1:1000 in 2-fold concentrated Mueller-Hinton broth. The MIC was defined as the lowest concentration of the analogue inhibiting visible growth after incubation at 37 °C for 24 h. Microbial killing was determined by cultivating 10 μL of a suitable dilution, made from micro dilution wells, on Mueller-Hinton agar plates and by counting colony forming units after overnight incubation at 37 °C. For colony counting only plates having 20-500 bacterial colonies per plate were used.

Safety tests including cytotoxicity assays are required for all products to be used in contact with humans. Cytotoxicity tests using culture cells have been accepted as the first step in identifying active compounds and for biosafety testing. This cytotoxicity test demonstrates the cytotoxic effect of the compounds submitted for testing. Samples are placed in contact with a monolayer of an appropriate layer of cells (such as HEp-2 cells) and incubated. The cells are then scored for cytopathic effect.[48−49]

Supplementary crystallographic data: Full crystallographic data, which include hydrogen coordinates, thermal parameters and complete bond lengths and angles for the structure reported in this paper, have been deposited at the Cambridge Crystallographic Data Centre [CCDC- 615063] and are available on request [e-mail: deposit@ccdc.cam.ac.uk].

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