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Thiosemicarbazones active against *Clostridium difficile*

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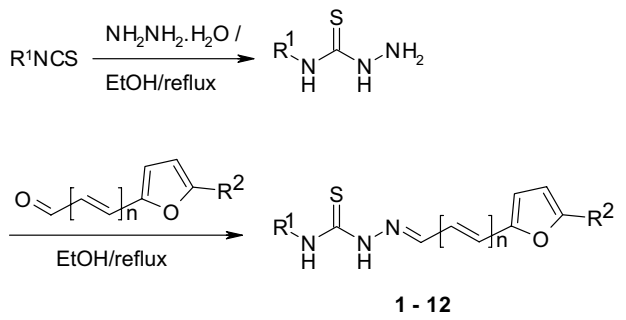
Abstract—A set of closely related furylidene thiosemicarbazones was prepared and screened against various clinically important Gram-positive bacteria. One compound containing an ethylene spacer and a 5-nitrofuryl group was found to have promising activity against *Clostridium difficile*.

The number of reported cases of *Clostridium difficile* associated diarrhoea (CDAD) has increased dramatically over the last decade. In 2005, more than 55,620 cases of CDAD in patients over the age of 65 were reported in England, many of which were associated with the hypertoxin-producing strain ribotype 027. Not surprisingly, *C. difficile* is now the leading hospital associated infection in the UK.[1] Furthermore, many strains of *C. difficile* are now emerging with increased resistance and multiple resistance to many common antibiotics including several fluoroquinolones, erythromycin, clarithromycin, fusidic acid and clindamycin.[2,3] Such strains have been associated with outbreaks of CDAD in the clinical setting. In light of this, there is a clear need to find novel antimicrobial agents with activity against both spores and vegetative cells of *C. difficile.*

Arylidene thiosemicarbazones have been explored in recent years for trypanocidal,[4–7] antiamoebic[8] and antibacterial[9,10] activity. In some of these the 5-nitrofuryl group has proved to be essential either connected directly as the thiosemicarbazone derivative or attached via an ethylene spacer. Biological activity associated with the 5-nitrofuryl moiety is understood to be a complex sequence of events starting with reduction of the nitro group.[11,12] In addition, the corresponding copper and palladium complexes of these ligands have shown similar or enhanced activity against the same targets.[7,8]

We now present the biological activities of a set of substituted furyl-containing thiosemicarbazones (Scheme 1) against a panel of Gram-positive bacteria including *Staphylococcus aureus* (methicillin-sensitive and -resistant strains), *Staphylococcus epidermidis*, *Propionibacterium acnes* and *C. difficile*. This was undertaken with a view to establishing the influence of the following structural features on the antimicrobial activity: the size of the alkyl group at the N-4 position; the presence of a nitro group at the 5-position on the furan; the presence of an ethylene spacer group in the furylidene portion.

The thiosemicarbazones were prepared in two steps using variations on literature procedures.[8] The appropriate isothiocyanates were added to an excess of hydrazine in boiling ethanol to give the corresponding substituted thiosemicarbazides as crystalline solids that



Scheme 1. Preparation of the target thiosemicarbazones (compounds **1**–**12**).

Keywords: Thiosemicarbazone; *Clostridium difficile*; Antimicrobial; Nitrofuran.
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precipitated out of the reaction mixture upon cooling. These were sufficiently clean to be used in the next step without purification. The thiosemicarbazides were suspended in ethanol and heated at reflux for 2 h with 1.3 mequiv of the appropriate furyl aldehyde to give the target compounds **1**–**12** (Scheme 1 and Table 1). Again, these compounds precipitated cleanly out of the reaction mixture. By this simple procedure the nitrofuranyl compounds (**1**–**4**, **6**, **8**, **10**, **12**) were obtained in 59–93% yield and the furanyl compounds (**5**, **7**, **9**, **11**) in 27–57% yield. The compounds were characterised by melting point, infra-red spectroscopy, mass spectrometry (+ve electrospray accurate mass) and proton NMR spectroscopy and had properties consistent with the proposed structures.

The test compounds were solubilised in DMSO and diluted with nutrient broth (Oxoid, UK) prior to screening for 24 h at 37 °C against an initial panel of organisms in using 2.5×10^7 CFU/mL. Anaerobic microorganisms were incubated in an anaerobic cabinet. Controls consisted of incubations of the specified organisms as above but omitting the test compounds. Compound **8** was tested further against an expanded panel of organisms. The resulting minimum inhibitory concentrations are presented in Tables 2 and 3. Vancomycin susceptibility was investigated for a subset of organisms and the results are presented in Table 2. A suspension of each organism (at a concentration of McFarland 0.5) was used to inoculate the entire surface of appropriate agar plates. Vancomycin E-test strips (AB BIODISK, Solna, Sweden) were placed in the middle of the agar plates and incubated under the appropriate conditions: Staphylococci, Nutrient agar (Oxoid, UK) plates, 37 °C, aerobic, 24 h; *C. difficile*, Wilkins Chlagren agar WCA (Oxoid, UK) plates, 37 °C, anaerobic, 48 h; *P. acnes*, nutrient agar plates, 37 °C, anaerobic, 48 h.

Compounds **1**–**12** were screened against an initial panel of Gram-positive organisms in nutrient broth that included representative strains of *C. difficile*, *S. epidermidis*, *S. aureus* (including MRSA strains) and *P. acnes* (Table 2). Compound **8** was significantly more active against each of the different types of organism. This can be seen in Figure 1 where the reciprocal of the averaged MIC values for the different types of organism for each of the compounds has been presented as a bar chart. Excluding *P. acnes*, the structure–activity profile was quite sharp. Apart from compound **8** only compounds **1**, **2** and **6** had noticeable antimicrobial activity. Each of these four compounds has the 5-nitrofuran moiety. For *P. acnes,* again, compound **8** was the most active but this time moderate antimicrobial activity was spread across the test compounds with the exception of **3**, **7** and **9**.

Table 1. Structures of the compounds synthesised and screened in this study

Compound	R^1	*n*	R^2	*c*Log *P*[a]	Reference
1	Me	0	NO_2	2.1	6,7,13
2	Et	0	NO_2	2.4	6,7,13
3	*i*-Pr	0	NO_2	2.8	7,8
4	*t*-Bu	0	NO_2	2.9	
5	Me	1	H	1.7	
6	Me	1	NO_2	2.5	6,7,13
7	Et	1	H	2.1	
8	Et	1	NO_2	2.8	6,7,13
9	*i*-Pr	1	H	2.5	
10	*i*-Pr	1	NO_2	3.2	14
11	*t*-Bu	1	H	2.5	
12	*t*-Bu	1	NO_2	3.3	15

[a] CAChe WorkSystem Pro Version 6.1.12.33 (Fujitsu Ltd).

Table 2. Minimum inhibitory concentration (μg mL^{-1}) of compounds **1**–**12** against an initial panel of organisms

Organisms	Compound												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Vancomycin**
C. difficile NCTC11204	>256	64–128	>256	128–256	>256	64–128	>256	4–8	>256	>256	>256	>256	0.5
C. difficile O27	64–128	64–128	64–128	>256	64–128	32–64	64–128	8–16	64–128	64–128	64–128	64–128	0.75
C. difficile Z162	>256	128–256	>256	128–256	>256	32–64	>256	0.125–0.25	>256	>256	>256	128–256	
C. difficile Z229	64–128	8–16	128–256	16–32	128–256	16–32	64–128	4–8	128–256	64–128	64–128	64–128	
S. epidermidis NCTC 11047	16–32	32–64	64–128	128–256	>256	32–64	>256	4–8	>256	64–128	>256	>256	2
S. epidermidis 9	128–256	64–128	>256	>256	>256	64–128	>256	1–2	>256	>256	>256	>256	
S .epidermidis 4	32–64	32–64	>256	>256	128–256	32–64	>256	4–8	>256	64–128	>256	>256	
S. aureus ATCC6538	64–128	32–64	64–128	64–128	>256	32–64	>256	4–8	>256	>256	>256	>256	1.5
S. aureus H1	64–128	16–32	128–256	>256	>256	16–32	>256	16–32	>256	256	>256	64–128	
S. aureus K1	128–256	64–128	>256	64–128	>256	>256	>256	4–8	>256	>256	>256	32–64	
MRSA A1	64–128	64–128	>256	>256	>256	64–128	>256	2–4	>256	>256	>256	>256	
MRSA C3	>256	64–128	>256	>256	>256	64–128	>256	4–8	>256	>256	>256	>256	
MRSA E1	64–128	64–128	>256	>256	>256	64–128	>256	4–8	>256	>256	>256	>256	
P. acnes NCTC737	16–32	16–32	64–128	64–128	32–64	16–32	16–32	4–8	32–64	32–64	64–128	16–32	0.5
P. acnes B5	64–128	32–64	>256	32–64	32–64	1–2	>256	8–16	>256	32–64	64–128	32–64	
P. acnes C3	16–32	2–4	32–64	16–32	16–32	1–2	32–64	2–4	1–2	32–64	4–8	8–16	
P. acnes C5	64–128	32–64	64–128	16–32	1–2	64–128	32–64	32–64	64–128	64–128	1–2	16–32	

Table 3. Minimum inhibitory concentration (μg mL^{-1}) of compound **8** against an expanded panel of organisms

Organism	MIC
C. difficile Z16	8–16
C. difficile Z21	16-32
C. difficile Z 66	4–8
C. difficile Z150	4–8
C. difficile Z158	2–4
C. difficile Z159	8–16
C. difficile Z160	8–16
C. difficile Z161	0.5–1
C. difficile Z163	2–4
C. difficile Z164	1–2
C. difficile Z166	2–4
C. difficile Z167	2–4
C. difficile Z168	2–4
C. difficile Z169	2–4
C. difficile Z170	2–4
C. difficile Z171	2–4
C. difficile Z172	4–8
C. difficile Z173	8–16
C. difficile Z175	0.5–1
C. difficile Z176	2–4
C. difficile Z177	2–4
C. difficile Z179	2–4
C. difficile Z251	1–2
C. difficile Z387	16–32
C. difficile Z1568	1–2
C. difficile Z1577	0.5–1
C. difficile Z1578	1–2
C. difficile Z1579	2–4
C. difficile Z1580	1–2
C. difficile Z1591	1–2
S. epidermidis NCTC 9865	
S. epidermidis RP62A	4–8
S. epidermidis 3	2–4
S. epidermidis 7A	2–4
S. epidermidis 8A	2–4
S. epidermidis 10	4–8
S. epidermidis 11	1–2
S. aureus T1	4–8
S. aureus V1	2–4
S. aureus H1	64–128
S. aureus MSSA2	4–8
S. aureus MSSA3	4–8
MRSA A3	4–8
MRSA B1	4–8
MRSA B3	4–8
MRSA D1	4–8
MRSA C1	2–4
EMRSA 15	4–8
P. acnes B3	8–16
P. acnes C1	8–16
P. acnes D1	4–8
P. acnes D3	4–8
C. albicans 1013781	128–256
C. albicans 1012155	128–256
C. albicans 1009116	128–256
C. albicans 1011377	128–256
C. albicans 1015391	128-256
Acinetobacter spp. 1	128–256
Acinetobacter spp. 2	128–256
Acinetobacter spp. 3	128–256
Acinetobacter spp. 4	128–256
Acinetobacter spp. 5	128–256

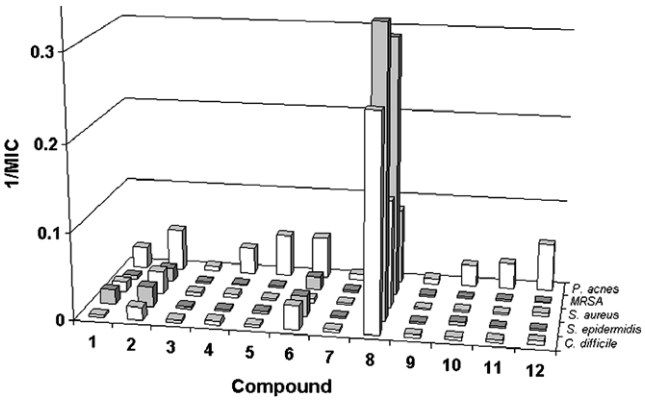

Figure 1. Bar chart of averaged 1/MIC values (mL μg^{-1}) for compounds **1**–**12** for the various classes of organisms tested.

Thiosemicarbazones of 5-nitro-2-furaldehyde also containing N-4 dialkyl/cycloalkyl substituents are known to have antifungal properties and activity against mainly Gram-positive bacteria.[9] For example, the N-4 diethyl analogue of compound **2** has good activity against *Escherichia coli*, *Micrococcus pyrogenes*, *Streptococcus pyogenes*, *Baccillus subtilis* and moderate activity against *Trichophyton mentagrophytes* and *Candida albicans*. Although these are different organisms from those presented in this article it appears that the addition of a second alkyl substituent at the N-4 position of the thiosemicarbazones without the ethylene spacer ($n = 0$) improves activity against Gram-positive bacteria.

Compound **8** was tested against an expanded panel of organisms (Table 3). It had good activity against the organisms mentioned above. For *C. difficile* in particular the reference strain NCTC 11204 was inhibited at 4–8 μg/mL and a set of 33 clinical strains exhibited minimum inhibitory concentrations in the range 0.125–0.25 to 16–32 μg/mL. In addition, no activity was observed against the Gram-negative microorganism *Acinetobacter* spp. or *C. albicans* yeast strains. The mechanism of action of the lead compound **8** is unknown at this time. On the basis that the Log *P* values for the inactive compounds **3**, **4**, **6**, **9**, **10** and **11** (Table 1) are very similar to that of the active compound **8**, it may be expected that the cellular penetrations of these compounds are similar. Thus the observed SAR may indeed reflect interactions at particular targets rather than differences in cellular penetration. In addition previous authors[6] have investigated the acute in vivo toxicity of compounds **2** and **8**. These indicated nontoxic effects on the basis of clinical biochemistry, haematology and the histological studies of organs.

In summary, amongst a set of closely related thiosemicarbazones, compound **8** was found to have promising antimicrobial activity against clinically important Gram-positive skin organisms. Its key structural features included an ethyl group at N-4, an ethylene spacer in the furylidene portion and a nitro group at the 5-position of the furan ring.

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14. *4-(i-Propyl)-1-[3-(5-nitrofuryl)propenilidene]thiosemi carbazide* (**10**): Dark red powder. 86% Yield. Mp 219.4–222.0 °C (corrected). ^{1}H NMR (d_6-DMSO; δ DMSO = 2.50 ppm) 1.18 (6H, d, J = 6.9 Hz, Me_2), 4.47 (1H, m, C*H*Me_2), 7.04 (3H, overlapping m, Furan-*H*3 and N=C*H*—CH=C*H*), 7.75 (1H, d, J = 3.8 Hz, Furan-*H*4), 7.86 (1H, dd, J = 6.95, 1.90 Hz, N=CH—C*H*), 8.15 (1H, d, J = 8.9 Hz, Me_2CHN*H*), 11.62 (s, 1H, N*H*—N=C) ppm. IR (KBr) 3358, 3320, 3123, 2971, 1531, 1464, 1347, 1231, 1190 cm^{-1}. High-resolution MS (+electrospray): $(M+H)^+$ calcd 283.0865, found 283.0845.
15. *4-(tert-Butyl)-1-[3-(5-nitrofuryl)propenilidene]thiosemi carbazide* (**12**): Orange powder. 93% Yield. Mp dec 209.5 °C (corrected). ^{1}H NMR (d_6-DMSO; δ DMSO = 2.50 ppm), 1.51 (9H, s, Me_3), 7.02 (2H, overlapping m, N=C*H*—CH=C*H*), 7.08 (d, J = 3.8 Hz, 1H, Furan-*H*3), 7.57 (1H, s, N*H*CMe_3), 7.76 (1H, d, J = 4.4 Hz, Furan-*H*4), 7.87 (dd, J = 5.06 3.16 Hz, 1H, N=CH—C*H*), 11.56 (s, 1H, N*H*—N=C) ppm. IR (KBr) 3312, 3142, 2973, 1529, 1509, 1466, 1417, 1390, 1355, 1260, 1241 cm^{-1}. High-resolution MS (+electrospray): $(M+H)^+$ calcd 297.1021, found 297.1013.