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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*. ©2000 Elsevier Science Ltd. All rights reserved.

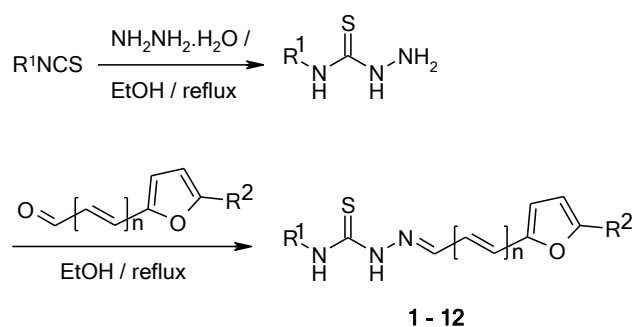
The number of reported cases of *Clostridium difficile* associated diarrhoea (CDAD) has increased dramatically over the last decade. In 2005, more than 55, 620 cases of CDAD in patients over the age of sixty-five were reported in England, many of which were associated with the hypertoxin-producing strain 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}, antiamoebic⁸ and antibacterial[JMC1967 530] activity. In some of these

the 5-nitrofuryl moiety has proved to be essential either connected directly as the thiosemicarbazone derivative or attached via an ethylene spacer. 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 (Scheme1) 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 procedures⁸. The

appropriate isothiocyanates were added to an excess of hydrazine in boiling ethanol to give the corresponding substituted thiosemicarbazides as crystalline solids that 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 two hours with 1.3 molar equivalents 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.



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

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

Compound	R ¹	n	R ²	cLogP ^a	Ref.
1	Me	0	NO ₂	2.1	6, 7, 9
2	Et	0	NO ₂	2.4	6, 7, 9
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, 9
7	Et	1	H	2.1	
8	Et	1	NO ₂	2.8	6, 7, 9
9	i-Pr	1	H	2.5	
10	i-Pr	1	NO ₂	3.2	10
11	t-Bu	1	H	2.5	
12	t-Bu	1	NO ₂	3.3	11

^aCAChe WorkSystem Pro Version 6.1.12.33 (Fujitsu Ltd)

The test compounds were solubilised in DMSO and diluted with nutrient broth (Oxoid Ltd) prior to screening for 24h at 37°C against an initial panel of organisms in using 2.5 x 10⁷ 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 sub-set 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 Chlagent 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-nitrofuranyl 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**.

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 [JMC1967]. For example, the N-4 diethyl analogue of compound **2** has good activity against *E. coli*, *M. pyogenes*, *S. pyogenes*, *B. subtilis* and moderate activity against *T. mentagrophytes* and *C. 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 thirty-three 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 bacterium *Acinetobacterium* or *C. albicans* yeast strains.

The mechanism of action of the lead compound **8** is unknown at this time. On the basis that the LogP 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 be similar. Thus the observed SAR may indeed be because of interactions at particular targets rather than differences in cellular penetration. In addition the acute *in vivo* toxicity of compounds **2** and **8** indicated nontoxic effects on the basis of clinical biochemistry,

haematology and the histological studies of organs.⁶

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.

Table 2. Minimum inhibitory concentration ($\mu\text{g mL}^{-1}$) of compounds **1-12** against an initial panel of organisms.

Organisms	Compounds												Vanc. ^a
	1	2	3	4	5	6	7	8	9	10	11	12	
<i>C. difficile</i> NCTC11204	>256	64-128	>256	128-256	>256	64-128	>256	4-8	>256	>256	>256	>256	0.5
<i>C. difficile</i> 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
<i>C. difficile</i> Z162	>256	128-256	>256	128-256	>256	32-64	>256	0.125-	>256	>256	>256	128-256	
<i>C. difficile</i> 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	
<i>S. epidermidis</i> NCTC 11047	16-32	32-64	64-128	128-256	>256	32-64	>256	4-8	>256	64-128	>256	>256	2
<i>S. epidermidis</i> 9	128-256	64-128	>256	>256	>256	64-128	>256	1-2	>256	>256	>256	>256	
<i>S. epidermidis</i> 4	32-64	32-64	>256	>256	128-256	32-64	>256	4-8	>256	64-128	>256	>256	
<i>S. aureus</i> ATCC 6538	64-128	32-64	64-128	64-128	>256	32-64	>256	4-8	>256	>256	>256	>256	1.5
<i>S. aureus</i> H1	64-128	16-32	128-256	>256	>256	16-32	>256	16-32	>256	256	>256	64-128	
<i>S. aureus</i> 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	
<i>P. acnes</i> NCTC 737	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
<i>P. acnes</i> B5	64-128	32-64	>256	32-64	32-64	1-2	>256	8-16	>256	32-64	64-128	32-64	
<i>P. acnes</i> 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	
<i>P. acnes</i> 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	

^a Vancomycin

Table 3. Minimum inhibitory concentration ($\mu\text{g mL}^{-1}$) of compound **8** against an expanded panel of organisms.

Organism	MIC	Organism	MIC	Organism	MIC
<i>C. difficile</i> Z16	8-16	<i>C. difficile</i> Z179	2-4	MRSA A3	4-8
<i>C. difficile</i> Z21	16-32	<i>C. difficile</i> Z251	1-2	MRSA B1	4-8
<i>C. difficile</i> Z66	4-8	<i>C. difficile</i> Z387	16-32	MRSA B3	4-8
<i>C. difficile</i> Z150	4-8	<i>C. difficile</i> Z1568	1-2	MRSA D1	4-8
<i>C. difficile</i> Z158	2-4	<i>C. difficile</i> Z1577	0.5-1	MRSA C1	2-4
<i>C. difficile</i> Z159	8-16	<i>C. difficile</i> Z1578	1-2	EMRSA 15	4-8
<i>C. difficile</i> Z160	8-16	<i>C. difficile</i> Z1579	2-4	<i>P. acnes</i> B3	8-16
<i>C. difficile</i> Z161	0.5-1	<i>C. difficile</i> Z1580	1-2	<i>P. acnes</i> C1	8-16
<i>C. difficile</i> Z163	2-4	<i>C. difficile</i> Z1591	1-2	<i>P. acnes</i> D1	4-8
<i>C. difficile</i> Z164	1-2	<i>S. epidermidis</i> NCTC 9865	2-4	<i>P. acnes</i> D3	4-8
<i>C. difficile</i> Z166	2-4	<i>S. epidermidis</i> RP62A	4-8	<i>C. albicans</i> 1013781	128-256
<i>C. difficile</i> Z167	2-4	<i>S. epidermidis</i> 3	2-4	<i>C. albicans</i> 1012155	128-256
<i>C. difficile</i> Z168	2-4	<i>S. epidermidis</i> 7A	2-4	<i>C. albicans</i> 1009116	128-256
<i>C. difficile</i> Z169	2-4	<i>S. epidermidis</i> 8A	2-4	<i>C. albicans</i> 1011377	128-256
<i>C. difficile</i> Z170	2-4	<i>S. epidermidis</i> 10	4-8	<i>C. albicans</i> 1015391	128-256
<i>C. difficile</i> Z171	2-4	<i>S. epidermidis</i> 11	1-2	<i>Acinetobacterium</i> spp. 1	128-256
<i>C. difficile</i> Z172	4-8	<i>S. aureus</i> T1	4-8	<i>Acinetobacterium</i> spp. 2	128-256
<i>C. difficile</i> Z173	8-16	<i>S. aureus</i> V1	2-4	<i>Acinetobacterium</i> spp. 2	128-256
<i>C. difficile</i> Z175	0.5-1	<i>S. aureus</i> H1	64-128	<i>Acinetobacterium</i> spp. 4	128-256
<i>C. difficile</i> Z176	2-4	<i>S. aureus</i> MSSA2	4-8	<i>Acinetobacterium</i> spp. 5	128-256
<i>C. difficile</i> Z177	2-4	<i>S. aureus</i> MSSA3	4-8		

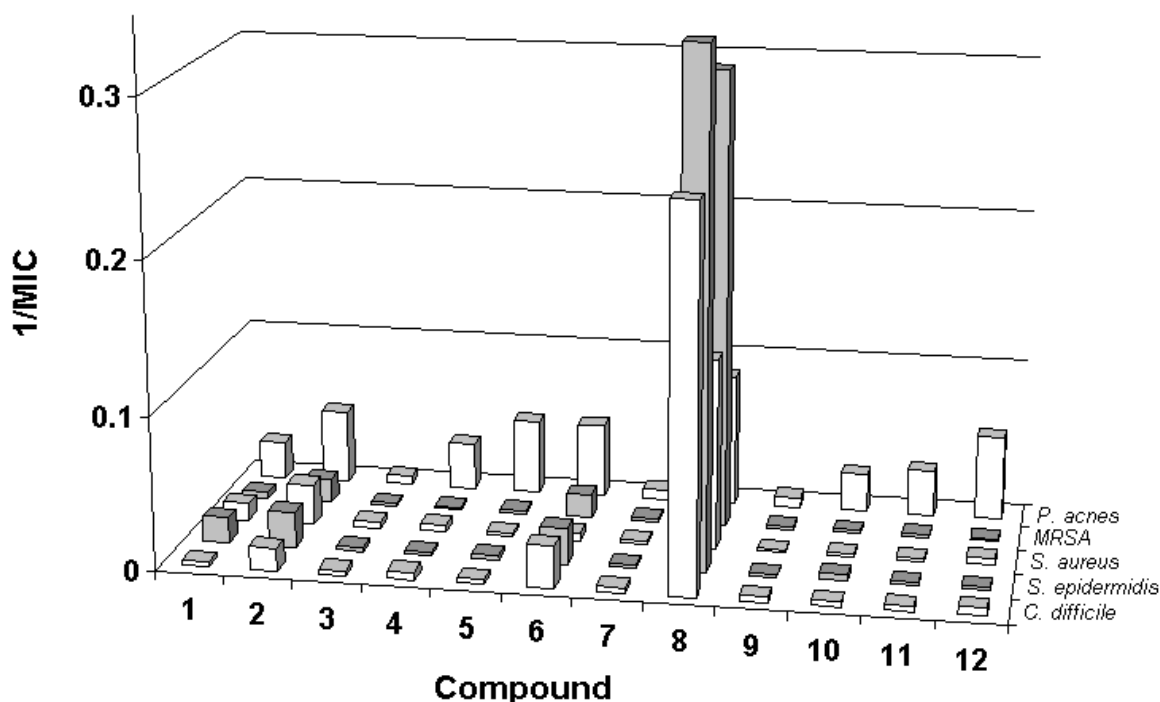


Figure 1. Bar chart of averaged 1/MIC values ($\text{mL } \mu\text{g}^{-1}$) for compounds 1-12 for the various classes of organisms tested.

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- 4-(i-Propyl)-1-[3-(5-nitrofuryl)propenilidene]thiosemi carbazide (**10**)
Dark red powder. 86% Yield. Melting Point 219.4-222.0 °C (corrected). ¹H NMR (D₆-DMSO; δDMSO = 2.50ppm) 1.18 (6H, d, J=6.9 Hz, Me₂), 4.47 (1H, m, CHMe₂), 7.04 (3H, overlapping m, Furan-H3 & N=CH-CH=CH), 7.75 (1H, d, J=3.8 Hz, Furan-H4), 7.86 (1H, dd, J=6.95 1.90 Hz, N=CH-CH), 8.15 (1H, d, J=8.9Hz, Me₂CHNH), 11.62 (s, 1H, NH-N=C) ppm. IR (KBr) 3358, 3320, 3123, 2971, 1531, 1464, 1347, 1231, 1190 cm⁻¹. High resolution MS (+electrospray): (M+H)⁺ calc 283.0865, found 283.0845.
- 4-(t-Butyl)-1-[3-(5-nitrofuryl)propenilidene]thiosemi carbazide (**12**)
Orange powder. 93% Yield. Melting Point decomp. 209.5 °C (Corrected). ¹H NMR (D₆-DMSO; δDMSO = 2.50ppm), 1.51 (9H, s, Me₃), 7.02 (2H, overlapping m, N=CH-CH=CH), 7.08 (d, J=3.8 Hz, 1H, Furan-H3), 7.57 (1H, s, NHCMe₃), 7.76 (1H, d, J=4.4 Hz, Furan-H4), 7.87 (dd, J=5.06 3.16 Hz, 1H, N=CH-CH), 11.56 (s, 1H, NH-N=C) ppm. IR (KBr) 3312, 3142, 2973, 1529, 1509, 1466, 1417, 1390, 1355, 1260, 1241 cm⁻¹. High resolution MS (+electrospray): (M+H)⁺ calc 297.1021, found 297.1013.