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Triketones active against antibiotic-resistant bacteria: Synthesis, structure–activity relationships, and mode of action

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Abstract—A series of acylated phloroglucinols and triketones was synthesized and tested for activity against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE) and multi-drug-resistant *Mycobacterium tuberculosis* (MDR-TB). A tetra-methylated triketone with a C_{12} side chain was the most active compound (MIC of around 1.0 μg/ml against MRSA) and was shown to stimulate oxygen consumption by resting cell suspensions, suggesting that the primary target was the cytoplasmic membrane.

1. Introduction

Soon after the introduction of antibiotics in the 1940s, resistant microorganisms appeared.[1] In recent years, the number of bacteria with multiple resistance to drugs now poses a serious threat to human health, with methicillin-resistant *Staphylococcus aureus* (MRSA) a particular problem.[2] Natural products are a vital source of structurally diverse compounds[3,4] and we have been searching for antibacterial compounds from New Zealand's unique native flora.[5]

Natural products called β-triketones (Fig. 1) are responsible for the antimicrobial activity, including activity against MRSA, of essential oils from the New Zealand shrub manuka, *Leptospermum scoparium* J. R. et G. Forst.[6–9] β-Triketones are rare natural products, found mainly in trees and shrubs of the Myrtaceae family, particularly in the genera *Eucalyptus* and *Leptospermum*.[10,11] We have reported the syntheses of the triketones **4**, **6**, **8**, and **10** of manuka oil plus another natural triketone **12** (Fig. 1).[12] We now report the activities of these natural triketones, plus a series of synthetic analogues[13] and the phloroglucinols from which they were derived, against MRSA, vancomycin-resistant *Enterococcus faecalis* (VRE) and multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB). Evidence of the mode of action of the most potent MRSA-inhibitory triketone is presented.

2. Results and discussion

Sixteen triketones (Fig. 1) were prepared by the Friedel–Crafts reaction to give C-acylated phloroglucinols (yields 16–61%), followed by alkylation (yields 4–92%), as described previously for syntheses of isoleptospermone **6** and papuanone **12**.[12]

2.1. Syntheses

We found that longer alkyl chain acids gave increasing amounts of O-acylated reaction products rather than

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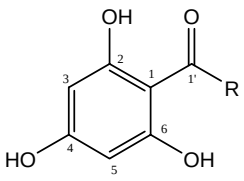
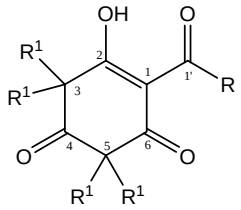
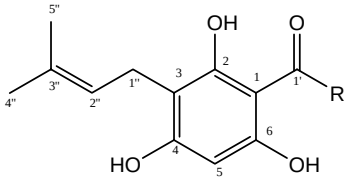
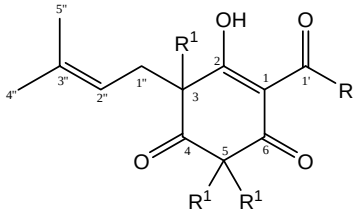




	Phloroglucinols		Triketones	
	R		R	R^1
1	methyl	**2**	methyl	methyl
3	1-methylethyl	**4**	1-methylethyl	methyl(flavesone)
5	1-methylpropyl	**6**	1-methylpropyl	methyl (isoleptospermone)
7	2-methylpropyl	**8**	2-methylpropyl	methyl (leptospermone)
9	2-phenylethyl	**10**	2-phenylethyl	methyl (grandiflorone)
11	pentyl	**12**	pentyl	methyl (papuanone)
13	nonyl	**14**	nonyl	methyl
15	undecyl	**16**	undecyl	methyl
17	pentadecyl	**18**	pentadecyl	methyl
19	cyclohexyl	**20**	cyclohexyl	methyl
21	2-cyclohexylethyl	**22**	2-cyclohexylethyl	methyl
23	phenylmethyl	**24**	phenylmethyl	methyl
		25	undecyl	ethyl
		26	undecyl	3-methylbut-2-enyl
		27	1-methylethyl	3-methylbut-2-enyl





28	undecyl	**29**	undecyl	methyl

Figure 1. Structures of phloroglucinols and triketones (natural product common name).

the required C-acylated products. For example, dodecanoic acid with phloroglucinol, $AlCl_3$, and $POCl_3$ gave the C-acylated phloroglucinol **15** in 40% yield, after silica column chromatography to remove mono- and bis-O-acylated products. The reaction of the hexadecanoic (palmitic) acid under these conditions gave a 1:1 mixture of the mono-O- and mono-C-acylated products, which co-eluted from a silica column. Crystallisation from dichloromethane gave the mono C-acyl compound **17** cleanly, as a white solid. Acylated phloroglucinols **23** and **28** (Fig. 1) are previously unreported, and their ^1H and ^{13}C NMR data are given in Tables 1 and 2.

There have been reports of C-acylated phloroglucinols **13** and **15** as natural products from the Myristicaceae family,[14,15] of **17** from marine algae of the order Dictyotales[16] and of phloroglucinols **19** and **21** as synthetic products.[17,18] However, the NMR data reported were incomplete, so their NMR data are also included in Tables 1 and 2. The ^1H NMR spectra of the mono-substituted phloroglucinols all showed broad, two proton signals at about 11.9 ppm (Table 2). The signals of 2-OH and 6-OH are averaged by fast exchange between two rotamers with an intramolecular hydrogen bond to C=O at 1′ (Fig. 2). The 4-OH signal is at about 9.3 ppm due to weaker hydrogen bonding to the solvent C=O of acetone. The disubstituted phloroglucinol **28** shows one strong intramolecularly hydrogen-bonded signal at 14.5 ppm, with the other two OH signals at 9.11 and 9.55 ppm (Table 2). We interpret this as being due to the predominance of one rotamer and suggest that this is rotamer A (Fig. 2) with the prenyl group ortho to the hydrogen-bonded OH, as found for analogous acyl phloroglucinols.[19]

Table 1. ^{13}C NMR data for selected phloroglucinols

Position	**13**[a]	**15**[a]	**17**[a]	**19**[a]	**21**[a]	**23**[a]	**28**[b]
1	105.8	105.7	105.8	105.1	105.9	105.8	105.8
2	165.8	165.7	165.8	166.1	165.9	166.1	163.2
3	96.5	96.3	96.5	96.6	96.6	96.5	108.5
4	166.1	166.1	166.1	165.7	166.2	166.2	165.8
5	96.5	96.3	96.5	96.6	96.6	96.5	95.6
6	165.8	165.7	165.8	166.1	165.9	166.1	161.1
1′	207.2	207.1	207.2	210.4	207.7	204.3	207.4
2′	45.1	43.1	45.1	50.7	42.8	50.9	45.2
3′	26.2	26.2	26.2	27.5	33.9	137.7	24.0
4′	31.0[c]	31.0[c]	30.7[c]	27.5	39.2	131.3	31.0[c]
5′	30.9[c]	30.9[c]	30.9[c]	31.0	34.8	129.5	31.0[c]
6′	30.9[c]	30.9[c]	31.0[c]	27.5	27.8	127.7	31.0[c]
7′	30.7[c]	30.9[c]	31.0[c]	27.5	28.1	129.5	30.0[c]
8′	33.3	30.8[c]	31.1[c]	—	27.8	131.3	30.0[c]
9′	24.0	30.7[c]	31.1[c]	—	34.8	—	30.0[c]
10′	15.0	33.3	31.1[c]	—	—	—	30.0[c]
11′	—	24.0	31.1[c]	—	—	—	22.6
12′	—	15.0	31.1[c]	—	—	—	15.0
13′	—	—	31.1[c]	—	—	—	—
14′	—	—	33.3	—	—	—	—
15′	—	—	24.0	—	—	—	—
16′	—	—	15.0	—	—	—	—
1″	—	—	—	—	—	—	33.3
2″	—	—	—	—	—	—	124.9
3″	—	—	—	—	—	—	131.4
4″	—	—	—	—	—	—	26.4
5″	—	—	—	—	—	—	18.5

[a] In deuteroacetone.
[b] In deuterochloroform.
[c] Signals interchangeable within columns.

Table 2. ^{1}H NMR data for selected phloroglucinols at 300 MHz at 25 °C

H	**13**[a]	**15**[a]	**17**[a]	**19**[a]	**21**[a]	**23**[a]	**28**[b]
2-OH	11.86, br s	11.93, br s	11.85, br s	11.92, br s	11.87, br s	11.83, br s	14.15, s
4-OH	11.86, br s	11.93, br s	11.85, br s	11.92, br s	11.87, br s	11.83, br s	9.11, s
6-OH	9.33, br s	9.48, br s	9.33, br s	9.38, br s	9.32, br s	9.39, br s	9.53 s
3 + 5	6.02, s	6.02, s	6.01, s	6.02, s	6.02, s	6.04, s	6.15, s
2′	3.16, t (7 Hz)	3.16, t (7 Hz)	3.16, t (7 Hz)	3.79, tt (5, 13 Hz)	3.17, t (7 Hz)	4.51, s	3.17, t (7 Hz)
3′	1.76, m	1.73, m	1.76, m	2.00, br d (13 Hz), 1.47 m	1.52 m	—	1.77 m
4′	1.42, m	1.37, m	1.38, m	1.87 dt (13, 3 Hz), 1.40 m	1.24 m	7.37 m	1.39 m
5′	1.42, m	1.37, m	1.38, m	1.78 dm (14 Hz), 1.30 m	1.03, 1.82 m	7.31 m	1.39 m
6′	1.42, m	1.37, m	1.38, m	1.87 dt (13, 3 Hz), 1.40 m	1.27, 1.74 m	7.31 m	1.39 m
7′	1.42, m	1.37, m	1.38, m	2.00, br d (13 Hz), 1.47 m	1.76 m	7.31 m	1.39 m
8′	1.42, m	1.37, m	1.38, m	—	1.27, 1.74 m	7.37 m	1.39 m
9′	1.42, m	1.37, m	1.38, m	—	1.03, 1.82 m	—	1.39, m
10′	0.97, t (7 Hz)	1.37, m	1.38, m	—	—	—	1.39, m
11′	—	1.37, m	1.38, m	—	—	—	1.39, m
12′	—	0.97 t (7 Hz)	1.38, m	—	—	—	0.98, t (7 Hz)
13′-15′	—	—	1.38, m	—	—	—	—
16′	—	—	0.95, t (7 Hz)	—	—	—	—
1″	—	—	—	—	—	—	3.33, d (7 Hz)
2″	—	—	—	—	—		5.32, t (7 Hz)
4″	—	—	—	—	—	—	1.83, s
5″	—	—	—	—	—	—	1.72, s

[a] In deuteroacetone.
[b] In deuterochloroform.

Using these acylated phloroglucinols, alkylations were carried out to form triketones. The reaction of phloroglucinols with methyl iodide in methanolic sodium methoxide at room temperature gave the new compounds **14**, **16**, **18**, **20**, **22**, and **24** (Fig. 1) in good yield. Ethyl groups could be added in place of the methyl groups using ethyl iodide, to give the new tetra-ethyl product, **25**. Reports in the literature of a method of adding just one geranyl group, to a trihydroxy-acetophenone system,[14] led us to try adding just one prenyl (i.e., 3-methylbut-2-enyl)

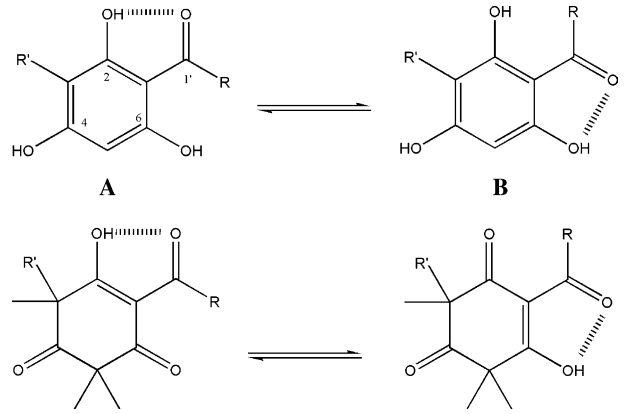


Figure 2. Phloroglucinol and triketone rotamers.

group to the dodecanoic acid-derived phloroglucinol **15**. The reaction of phloroglucinol with one equivalent of both potassium carbonate and prenyl bromide in refluxing acetone gave some mono-prenylated material, which was separated by column chromatography. This proved to be a 1:1 mixture of O- and C-prenylated phloroglucinols. However, crystallisation led to a sample of pure new mono-C prenylated product **28**. Methylation of this compound as before led to the new mono-prenyl trimethyl compound **29**. Complete prenylation to form the new tetraprenyl compound **26** could be effected by carrying out the reaction with excess potassium carbonate and prenyl bromide. Similarly, the isobutyric acid-derived phloroglucinol **3** gave the tetraprenyl triketone **27,** known as a natural product from hops.[20]

NMR data are reported in Tables 3 and 4. The ^{1}H NMR spectra of all but one triketone showed sharp one-proton singlets around 18 ppm, due to the enol tautomer with a very strong intramolecular hydrogen bond between 2-OH and C=O at 1′.[12] However, the asymmetrically alkylated compound **29** showed two sets of NMR signals due to a slow exchange between two tautomeric rotational isomers (Fig. 2). These endocyclic enol tautomers have been proven to be present in the low polarity $CDCl_3$,[12] but Kavana and Moran have shown the presence of exocyclic enol tautomers of a simpler triketone in aqueous solution at pH 7.0.[21] It would be useful to know the tautomeric configuration of the most active (see below) triketone **16** under these biologically relevant conditions, but this would be experimentally difficult to determine due to the low aqueous solubility of **16**.

3. Bioactivity and mode of action

The triketones from manuka oil are much more active against Gram-positive bacteria than Gram-negative bacteria[6–8] The natural mix of triketones is reported as having minimum inhibitory concentrations (MICs) against MRSA of 65–125 μg/ml.[7] We now report on the activities of the individual pure triketones for the first time, including the newly discovered activity against VRE and MDR-TB. The results summarised in Table 5, for the intermediate phloroglucinols and triketones, comprise MIC data and results of disc-diffusion assays against other microorganisms. Table 5 also includes cytotoxicity against P388 mouse leukaemia cells and calculated log*P*, octanol/water partition coefficient (C log *P*) values.

Of the naturally occurring triketones, leptospermone **8** (Fig. 1), the main manuka oil triketone, had antibacterial activity against MRSA similar to that of isoleptospermone **6** and was more active than flavesone **4**. Two other natural triketones, grandiflorone **10,**a minor component of manuka oil, and papuanone **12,** found in a related Myrtaceous plant,[12] were the most active of the natural triketones (Table 5). Of the new synthetic triketones, compound **16** was the most active, with an MIC against MRSA down to 0.5 μg/ml. Side-chain lengths less than or greater than C_{12}have less activity. Variation of the alkyl substituents on the ring of the synthetic triketone C_{12} analogues led to loss of activity as the substituents increased in bulk: from tetra methyl **16** to trimethyl prenyl **29** to tetra ethyl **25** to tetra prenyl **26** (Table 5).

The activity of the triketones against MRSA was mirrored in the other MIC-type antibacterial assays, against VRE and MDR-TB (Table 5). We used the cheaper disc-diffusion assays as an initial prescreen to the MIC-type assays. However, inhibition zones in disc-diffusion assays against the Gram-positive *B. subtilis* did not correlate with MIC values (Table 5). For example, the lipophilic tetra methyl C_{12} analogue **16** (MIC 0.5 μg/ml, C log *P* 5.86) showed only a small zone of inhibition (3 mm), whereas the less potent (MIC 8 μg/ml) and more lipophilic (C log *P* 3.75) **22** showed the largest inhibition zone recorded (12 mm). This is because size of the zone of inhibition found in disc-diffusion assays is indicative of both diffusion effects and antimicrobial potency.[22] Bearing this in mind, the disc-diffusion data can be used to reach some conclusions on the spectrum of antimicrobial activity. None of the triketones showed activity against the Gram-negative bacterium *Pseudomonas aeruginosa* (data not shown). There was a rough positive trend between zone sizes against *B. subtilis* and activity against the dermatophyte *Trichophyton mentagrophytes* (Table 5). This may also be the case for the yeast *Candida albicans*, since compound **22** with the largest zone against *B. subtilis* was the only one to show*C. albicans* inhibition (2 mm zone). Therefore, the spectrum of antimicrobial activity of the individual synthetic triketones is similar to that of the mix of natural triketones in manuka oil.[5–7]

The antimicrobial activity of some of the phloroglucinols was also examined. The C_{12} phloroglucinol **15** was the most active with an MIC about four times less potent than that of the corresponding triketone **16**. In disc-diffusion assays, most of the phloroglucinols showed some inhibition of both *B. subtilis* and *T. mentagrophytes* (Table 5). Compounds **21** and **23** also showed activity (2 and 1 mm inhibition zones, respectively) against *Escherichia coli* and **21** was also active against *C. albicans* (data not shown). The antifungal activities of a series of acylated phloroglucinols, including **13** and **15**, have been reported.[23] These authors found that MICs against *T. mentagrophytes* were lowest

Table 3. ^{13}C NMR data for new triketones[a]

Position	**14**	**16**	**18**	**20**	**22**	**24**	**25**	**26**	**27**	**29**[b]	**29**[c]
1	109.0	109.1	109.0	108.2	109.9	109.1	111.0	113.3	112.5	110.8	110.1
2	199.1	199.0	199.1	199.8	199.0	198.3	198.3	197.0	197.8	197.7	199.1
3	52.1	52.1	52.1	52.4	52.1	51.9	61.2	60.9	61.1	56.1	51.9
4	210.0	210.0	210.2	210.0	210.0	209.7	207.8	207.5	208.3	210.2	209.8
5	56.8	56.9	56.8	57.0	56.8	56.7	65.7	65.6	65.7	57.2	61.0
6	196.8	196.7	196.8	196.9	196.8	196.7	195.7	194.8	194.5	196.5	196.2
1′	204.8	204.7	204.8	207.3	205.2	201.9	204.3	204.4	207.5	204.7	204.6
2′	39.2	39.2	39.2	45.1	37.5	45.0	39.3	39.4	35.5	39.4	39.3
3′	25.1	25.2	25.1	29.3	32.5	134.1	25.1	25.1	19.2	25.3	25.0
4′	29.4[d]	29.4[d]	29.7[d]	25.7	36.8	129.8	29.3[d]	29.6[d]	19.2	29.6[d]	29.6[d]
5′	29.4[d]	29.4[d]	29.7[d]	25.8	33.1	128.5	29.4[d]	29.6[d]	—	29.6[d]	29.6[d]
6′	29.3[d]	31.9[d]	29.6[d]	25.7	26.2	127.2	29.5[d]	29.5[d]	—	29.5[d]	29.5[d]
7′	29.2[d]	29.6[d]	29.6[d]	29.3	26.5	128.5	29.6[d]	29.4[d]	—	29.4[d]	29.4[d]
8′	31.9	29.5[d]	29.5[d]	—	26.2	129.8	29.6[d]	29.4[d]	—	29.4[d]	29.4[d]
9′	22.6	29.5[d]	29.5[d]	—	33.1	—	29.7[d]	29.3[d]	—	29.3[d]	29.3[d]
10′	14.1	32.0[d]	29.5[d]	—	—	—	31.9	31.9	—	31.9	31.9
11′	—	22.7	29.3[d]	—	—	—	22.7	22.7	—	22.6	22.6
12′	—	14.1	29.3[d]	—	—	—	14.1	14.1	—	14.1	14.1
13′	—	—	29.3[d]	—	—	—	—	—	—	—	—
14′	—	—	31.9	—	—	—	—	—	—	—	—
15′	—	—	22.7	—	—	—	—	—	—	—	—
16′	—	—	14.1	—	—	—	—	—	—	—	—
3-*R*											
1″	24.3	24.3	24.3	24.3	24.3	24.3	28.2	36.8	37.0	38.9, 20.3	38.0, 20.8
2″	—	—	—	—	—	—	9.4	118.9	118.9	117.5	118.0
3″	—	—	—	—	—	—	—	136.2	136.3	137.1	135.8
4″	—	—	—	—	—	—	—	25.9	25.9	25.8	25.9
5″	—	—	—	—	—	—	—	17.9	17.9	17.7	17.8
5-*R*											
1‴	23.8	23.9	23.9	23.9	23.8	23.6	28.2	33.8	33.7	22.2, 26.1	22.3, 26.2
2‴	—	—	—	—	—	—	9.2	118.3	118.3	—	—
3‴	—	—	—	—	—	—	—	134.6	134.6	—	—
4‴	—	—	—	—	—	—	—	25.8	25.8	—	—
5‴	—	—	—	—	—	—	—	17.8	17.8	—	—

[a] In $CDCl_3$.
[b] Major tautomer.
[c] Minor tautomer.
[d] Signals interchangeable within columns.

for the C_9 phloroglucinol (6.25 μg/ml), slightly higher for the C_{10} phloroglucinol **13** (12.5 μg/ml), and much higher for the C_{12} phloroglucinol **15** (>200 μg/ml).[23]

Our initial structure–activity relationship (SAR) investigations examined the literature of related compounds. Bioactivities of phloroglucinols (including chalcones) and structures resembling triketones reported previously recognised the β-di-carbonyl system and hydrogen-bonding parameters as being essential for activity.[24–27] Structure–activity relationships have been examined for the antibacterial hop bitter acids with several key-points noted: antibacterial activity was coupled to hydrophobic activity of the compounds; and conversion of the six-membered ring into a five-membered ring resulted in decreased activity.[28] Several studies on natural products containing a syncarpic acid residue, the core part of the triketone skeleton, omit details of the biological activity or quantitative assay data.[29–31] It was these studies, combined with this observation that the naturally occurring triketones with more carbons in the acyl side chain (R, Fig. 1) were more active (Table 5), which led us to synthesize compounds with longer acyl side chains.

The relationship we found between chain length and activity against MRSA for the *n*-alkyl compounds parallels the results reported by Kubo et al. for the activity of simple *n*-alkan-1-ols and *n*-alkyl derivatives of salicylic acid.[32,33] Their reported activity reached a maximum at C_{12}: MIC 12.5 μg/ml for dodecan-1-ol and 3.13 μg/ml for 6-*n*-decyl-2-hydroxy benzoic acid, compared with that of 0.5–1.0 μg/ml for our C_{12} triketone **16**. In a more recent publication, Kubo et al. examined the antibacterial activity of dodecyl gallate and found that the mode of action was effected through inhibition of the membrane respiratory chain, observed as inhibition of O_2 consumption by whole cells and oxidation of NADH in membrane preparations.[34] It was thought that a surfactant effect is responsible for the bacterial affinities to hydrophobic portions of the molecules and therefore influences their antimicrobial spectra and potency.[34] Similarly, Kopecky noted that *n*-alkyl quaternary ammonium salts are also antimicrobial, with maximum activity for C_{12} to C_{16}

Table 4. ^{1}H NMR data for new triketones[a]

H	14	16	18	20	22	24	25	26	27	29[b]
2-OH	18.34, s	18.37, s	18.33, s	18.54, s	18.34, s	17.95, s	18.41, s	18.34, s	18.38, s	18.34, s*
2′	2.96 t (8 Hz)	2.98, t (8 Hz)	2.94, br t (8 Hz)	3.50, br t (8 Hz)	2.98, br t (8 Hz)	4.25 , s	2.98 t (8 Hz)	2.88, m	3.64, sept (7 Hz)	2.95, m
3′	1.63, m	1.65, m	1.60, m	1.82, br m, 1.47, m	1.52, m	—	1.63, m	NR	1.15 d (7 Hz)	1.65, m
4′	1.25, m	1.26, m	1.22, m	1.79, br m, 1.38, m	1.28, m	7.21, m	1.25, m	1.25, m	1.15, d (7 Hz)	1.26, m
5′	1.25, m	1.26, m	1.22, m	1.72, m, 1.24, m	0.91, 1.69, m	7.21, m	1.25, m	1.25, m	—	1.26, m
6′	1.25, m	1.26, m	1.22, m	1.79, br m, 1.38, m	1.16, 1.59, m	7.21, m	1.25, m	1.25, m	—	1.26, m
7′	1.25, m	1.26, m	1.22, m	1.82, br m, 1.47, m	1.62, m	7.21, m	1.25, m	1.25, m	—	1.26, m
8′	1.25, m	1.26, m	1.22, m	—	1.16, 1.59, m	7.21, m	1.25, m	1.25, m	—	1.26, m
9′	1.25, m	1.26, m	1.22, m	—	0.91, 1.69, m	—	1.25, m	1.25, m	—	1.26, m
10′	0.86 t (7 Hz)	1.26, m	1.22, m	—	—	—	1.25, m	1.25, m	—	1.26, m
12′	—	0.88, t (7 Hz)	1.22 m	—	—	—	0.86, t (8 Hz)	0.86, t (7 Hz)	—	0.86, t (7 Hz)
16′	—	—	0.84, t (7 Hz)	—	—	—	—	—	—	—
3-Me_2	1.41, s	1.46, s	1.41, s	1.43, s	1.44, s	1.36, s	—	—	—	1.44, 1.40, 1.39
5-Me_2	1.35, s	1.37, s	1.33, s	1.35, s	1.35, s	1.20, s	—	—	—	1.30, 1.28, 1.26
1″ + 1‴	—	—	—	—	—	—	1.79, m	2.51, m	2.51, m	2.50, m
2″ + 2‴	—	—	—	—	—	—	0.55, m	4.89, m	4.89, m	4.73, m
4″ + 4‴	—	—	—	—	—	—	—	1.61, 1.58	1.63, 1.59	1.56*, 1.59*
5″ + 5‴	—	—	—	—	—	—	—	1.56, 1.52	1.56, 1.53	1.47*, 1.52*

[a] In $CDCl_3$, NR =not resolved.
[b] Tautomers not resolved, though some (*) signals broadened.

compounds, suggesting that this is due to interaction with cytoplasmic membranes of the bacterial cells, thus interfering with respiration and ATP synthesis.[35] Alkyl chain length of similar compounds has also been implicated in other types of biological activity, including antiviral and antifungal activities.[36,37] However, variation of the alkyl substituents on the ring also changes the antimicrobial activity. Tetraethyl triketone **25** is less active than tetramethyl **16** and tetraprenyl **26** is much less active (Table 5). This could be due to steric crowding of the acidic enol group, which may be important for the biological activity.[38] On the other hand, the tetra prenyl analogue **27** was more antimicrobial than flavesone **4**. Plotting liphophilicity of the triketones, expressed as C log *P* values,[37]against their antimicrobial activity (Fig. 3) helped us to rationalize these apparently contradictory substituent effects. The optimum C log *P* is around 6, corresponding to tetramethyl triketone **16**. The tetraethyl **25** analogue of **16** had a higher C log *P* value (Table 5), and tetraprenyl analogue **26** is expected to have an even higher value (compare **4** and **27**, Table 5). Flavesone **4** has a C log *P* a long way from the optimum, whereas tetraprenyl analogue **27** has a C log *P* closer to optimum.

The studies noted above and our results suggest that the triketones may act by disrupting the cytoplasmic membrane due to their hydrophobic nature.[39] Because vitally important energy generating systems are located in the cytoplasmic membrane (i.e., electron transport chain, F_1F_0 ATP synthase), this could lead to toxicity against mammalian cells, and lack of selective antimicrobial action. Activity against the mouse leukaemia P388 cell line did show some parallels to the antimicrobial activity against MRSA (Table 5), though no clear relationship was observed. For example, the tetra ethyl C_{12} compound **25** had a P388 IC_{50} to MRSA MIC ratio of about 1:20, that is, it was apparently more cytotoxic than antimicrobial. On the other hand, the tetra methyl C_{10} and C_{12} compounds **14** and **16** had ratios of about 2:1, more favourable for selective antimicrobial activity.

Activity against a range of bacteria, fungi and mammalian cells showed that the triketones were relatively nonspecific in their target, consistent with the target being the cytoplasmic membrane. We examined their mode of action by looking at the effects of the most potent antibacterial compound **16** on energy transduction of *S. aureus*. In general, the addition of protonophores (compounds that interact with the cytoplasmic membrane), such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), to resting bacterial cell suspensions (oxidizing added substrate) causes a transient decline in the membrane potential. The potential is reestablished via an increase in the rate of proton pumping through the membrane-bound respiratory chain components, and thus a stimulation of oxygen consumption is observed.[40,41] However, O_2 consumption is stimulated only over a narrow concentration range and may be inhibited at high concentrations of protonophore.[42] The effect on oxygen consumption by resting cell suspensions was examined and the results are presented in

Table 5. Summary of phloroglucinol and triketone activity

Phloroglucinol	MRSA[b]	*B.sub*[c]	*T.ment*[d]	P388[e]	Triketone	MRSA[b]	VRE[f]	MDR-TB[f]	*B. sub*[c]	*T. ment*[d]	P388[e]	C log *P*[g]
1	NT	1	0	>25	**2**	1000	NT	NT	1	0	>25	0.56
3	NT	6	5	>25	**4**[a]	500–1000	>500	125	0	0	>25	1.41
5	NT	5	4	>25	**6**[a]	250	>500	125	2	1	>25	1.94
7	NT	8	8	>25	**8**[a]	250	>500	125	2	3	>25	2.02
9	NT	2	0	>25	**10**[a]	125	125	31	6	5	10.5	2.66
11	NT	6	3	10.5	**12**[a]	125	63	31	3	3	5.5	2.68
13	8–15	1	1	16.9	**14**	1–2	4	4	7	5	3.0	4.80
15[a]	4-8	0	3	>25	**16**	0.5–1	4	2	3	2	1.5	5.86
17[a]	NT	0	0	8.8	**18**	2–8	31	31	0	0	1.0	7.97
19	15–31	2	0	>25	**20**	15–63	250	125	0	0	20.9	2.60
21	4–8	4	6	>25	**22**	4–8	31	15	12	5	1.8	3.75
23	NT	3	4	>25	**24**	500	125	63	3	2	9.9	2.41
28	4–15	0	0	7.5	**25**	4–8	31	31	0	0	0.4	7.47
					26	>500	250	125	0	0	>25	>7.5 (NA)
					27	15–31	500	125	5	0	4.0	7.29
					29	1–2	4	31	0	0	1.7	7.36

NT = not tested; NA = not able to be calculated.
[a] Naturally occuring compounds.
[b] Methicillin-resistant *Staphylococcus aureus* MIC in μg/ml, vancomycin MIC 1.6–3.1 μg/ml.
[c] *Bacillus subtilis* inhibition zone (mm) at 60 μg/disc.
[d] *Trichophyton mentagrophytes* inhibition zone (mm) at 60 μg/disc.
[e] IC_{50}μg/ml.
[f] Vancomycin-resistant *Enterococcus faecalis* MIC μg/ml.

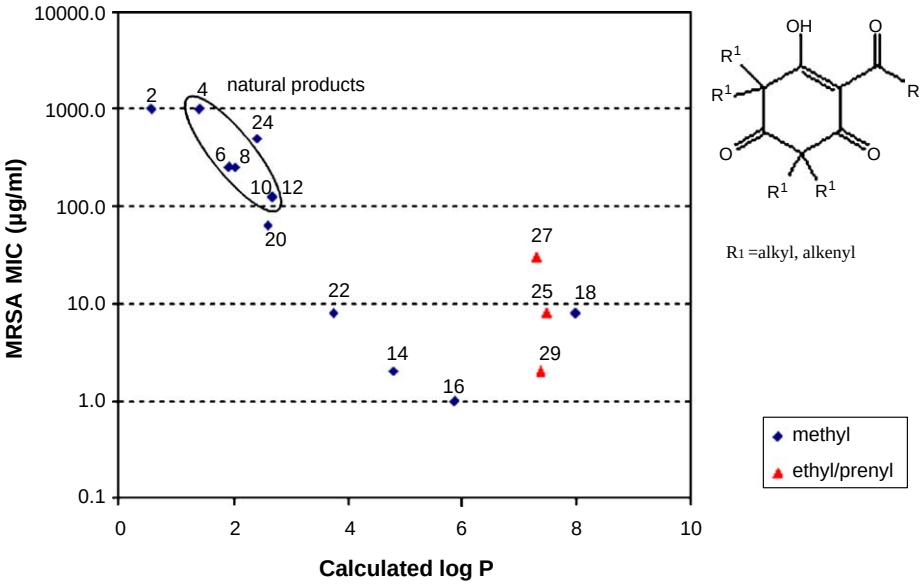


Figure 3. Comparison of MRSA activity versus calculated log *P* of triketones.

Figure 4a. Addition of triketone **16** (up to 30 μg/ml) stimulated oxygen consumption in resting cell suspensions that were oxidizing glucose. Identical results were confirmed when succinate was used as the oxidizable substrate (data not shown). Nisin, a bacterial peptide antibiotic, has also been shown to stimulate oxygen consumption by resting cells of *S. aureus*.[40] The effect of triketone **16** on the membrane potential (i.e., energized cell membrane) of *S. aureus* cells was examined using tritiated tetraphenylphosphonium ions (TPP) as a probe. It was observed that triketone **16** at the concentration tested did not dissipate the membrane potential over the time course of the assay compared to nigericin and valinomycin (Fig. 4b), proven inhibitors of the membrane potential Gram-positive bacteria. When triketone **16** was incubated with cell suspensions, intracellular ATP decreased with time, suggesting that this compound was able to inhibit ATP production (Fig. 4c). No effect on intracellular K^+ concentration was noted (Fig. 4d).

4. Conclusions

We have sought to identify novel structures with significant activity against antibiotic-resistant Gram-positive bacteria that could be used as therapeutic agents. The series of naturally occurring triketones led us to new synthetic analogues with much more potent activities

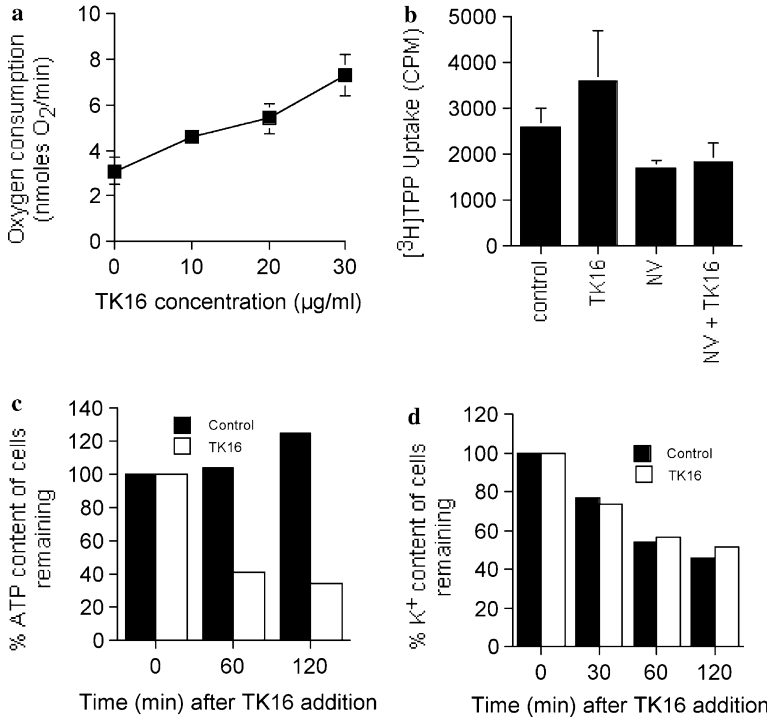


Figure 4. Mode of action of triketone **16** with *Staphylococcus aureus* cells. (a) The effect of triketone **16** concentration (0–30 μg/ml) on oxygen consumption of glucose oxidizing cells. (b) The effect of triketone **16** (10 μg/ml) on the membrane potential of *S. aureus*. NV = nigericin and valinomycin added in combination at 15 mM each). (c) The effect of triketone **16** (10 μg/ml) on intracellular ATP levels. (d) The effect of triketone**16** (10 μg/ml) on intracellular potassium levels.

against MRSA, VRE and MDR-TB (Table 5). Compound **16** with an *n*-alkyl C_{12} side chain was the most active, apparently due to its optimum lipophilicity. The antibacterial activity of **16** (MIC 0.5–1.0 mg/ml) is of the same order of magnitude as the commercial antibiotic vancomycin, which is widely used clinically for treating MRSA.[43]

Triketone **16** was shown to stimulate oxygen consumption by resting cell suspensions, suggesting that it does indeed interact with the cytoplasmic membrane. This is also consistent with the compounds' ability to inhibit a wide range of Gram-positive bacteria but not Gram-negative bacteria in which the outer membrane may serve as a barrier to triketone **16**. Furthermore, ATP generation was also affected by triketone **16**, a process that is carried out by the membrane-bound F_1F_0 ATP synthase during oxidation of succinate and requires an intact (undisturbed) cytoplasmic membrane. Neither the membrane potential nor intracellular potassium levels were affected by triketone **16**, suggesting that this compound did not promote critical ion loss from the cell, even though it was able to disrupt the cytoplasmic membrane by some unknown mechanism.

5. Experimental

5.1. General experimental procedures

All solvents were distilled before use and were removed by rotary evaporation at temperatures up to 35 °C. Merck silica gel 60, 200–400 mesh, 40–63 μm, was used for silica gel flash chromatography. TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F254, visualised with a UV lamp. Mass, UV, and IR spectra were recorded on Kratos MS-80, Shimadzu UV 240 and Perkin-Elmer 1600 FTIR instruments, respectively. NMR spectra, at 25 °C, were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Varian INOVA 300 spectrometer. Chemical shifts are given in ppm on the δ scale referenced to the solvent peaks $CHCl_3$ at 7.25 and $CDCl_3$ at 77.0 ppm or $(CH_3)_2CO$ at 2.15 and $(CD_3)_2CO$ at 30.5 ppm.

5.2. Acylations

Compounds were synthesized by the method described previously[12] and the previously undescribed compounds are characterized below:

5.2.1. 1-(2,4,6-Trihydroxyphenyl)-1-decanone (13). White crystals (23% yield); mp 127 °C. TLC: R_f 0.24 (10:1 DCM/EtOAc); UV (MeOH) λ_{max} (log ε): 230 (3.94), 282 (4.05), 320 (3.35) nm. IR (dry film): ν_{max}: 3300, 1650, 1580, 1470, 1072 cm^{-1}. EIMS (70 eV) *m/z* (rel. int.): 280 $[M]^+$ (32%), 181 (78) 153 (100). Anal. Calcd for $C_{16}H_{24}O_4$: C, 68.57; H, 8.57. Found: C, 68.31; H, 8.64.

5.2.2. 1-(2,4,6-Trihydroxyphenyl)-1-dodecanone (15). White crystals (25% yield); mp 134 °C. TLC: R_f 0.50 (1:1 hexane/EtOAc); UV (hexane) λ_{max} (log ε): 286 (4.13), 226 (4.09) nm. IR (dry film) ν_{max}: 3250, 2900, 2840, 1650, 1600, 1556, 1523, 1460, 1380, 1290, 1205, 1060, 770 cm^{-1}. EIMS (70 eV) *m/z* (rel. int.): 308 $[M]^+$ (12), 290 (10), 181 (25), 168 (49), 153 (100). Anal. Calcd

for $C_{18}H_{28}O_4$: C, 70.09; H, 9.15. Found: C, 69.83; H, 9.45.

5.2.3. 1-(2,4,6-Trihydroxyphenyl)-1-hexadecanone (17). White crystals (16% yield); mp 129 °C. TLC: R_f 0.27 (10:1 DCM/EtOAc); UV (MeOH) λ_{max} ($\log\varepsilon$): 230 (3.96), 288 (4.05), 320 (3.34) nm. IR (dry film) ν_{max}: 3300, 1660, 1560, 1480, 1200 cm^{-1}. HR-EIMS (70 eV) *m/z* (rel. int.): 364.26236 $[M]^+$ (4%, $C_{22}H_{36}O_4$ calcd 364.26202), 346 (9), 236 (11), 181 (23), 168 (42), 153 (100).

5.2.4. 1-(2,4,6-Trihydroxyphenyl)-cyclohexylmethanone (19). Oily solid (27% yield); TLC: R_f 0.30 (10:1 DCM/EtOAc); UV (MeOH) λ_{max} ($\log\varepsilon$): 230 (4.04), 288 (4.15), 320 (3.47) nm. IR (dry film) ν_{max}: 3290, 2929, 2853, 1702, 1631, 1605, 1451, 1214, 1174, 828 cm^{-1}. HR-EIMS (70 eV) *m/z* (rel. int.): 236.10465 $[M]^+$ (44%, $C_{13}H_{16}O_4$ calcd 236.10378), and 153 (100).

5.2.5. 1-(2,4,6-Trihydroxyphenyl)-3-cyclohexyl-1-propanone (21). White crystals (51% yield); mp 138 °C. TLC: R_f 0.32 (10:1 DCM/EtOAc); UV (MeOH) λ_{max} ($\log\varepsilon$): 230 (4.14), 288 (4.26), 320 (3.52) nm; IR (dry film) ν_{max}: 3308, 2923, 2851, 1704, 1632, 1605, 1453, 1234, 827 cm^{-1}. EIMS (70 eV) *m/z* (rel. int.): 264 $[M]^+$ (10%), 246 (22), 168 (86), 153 (100). Anal. Calcd for $C_{15}H_{20}O_4$: C, 68.18; H, 7.58. Found: C, 68.38; H, 7.68.

5.2.6. 1-(2,4,6-Trihydroxyphenyl)-2-phenylethanone (23). White crystals (20% yield); mp 162 °C. TLC: R_f 0.31 (1:1 hexane:EtOAc); UV (MeOH) λ_{max} ($\log\varepsilon$): 287 (3.96), 226 (3.84) nm; IR (dry film) ν_{max}: 3310, 2958, 1635, 1600, 1522, 1456, 1394, 1347, 1295, 1228, 1167, 1074, 975, 824, 730, 668 cm^{-1}. EIMS (70 eV) *m/z* (rel. int.): 244 $[M]^+$ (12), 154 (15), 153 (100). Anal. Calcd for $C_{14}H_{12}O_4$: C, 68.18; H, 7.58. Found: C, 68.38; H, 7.68.

5.3. Alkylations

Compounds were synthesized by the method described previously[12] and the previously undescribed compounds are characterized below.

5.3.1. 5-Hydroxy-4-(1-oxodecyl)-2,2,6,6-tetramethyl-4-cyclohexene-1,3-dione (14). Colourless oil (73% yield); TLC: R_f 0.28 (1:1 hexane/DCM); UV (MeOH) λ_{max} ($\log\varepsilon$): 278 (4.0), 238 (3.8) nm. IR (dry film) ν_{max}: 2927, 2855, 1723, 1672, 1666, 1581, 1564, 1552, 1049 cm^{-1}. HR-EIMS (70 eV) *m/z* (rel. int.): 336.23116 $[M]^+$ (30%, $C_{20}H_{32}O_4$ calcd 336.23006), 321 (21), 266 (62), 237 (89), 224 (63), 154 (53) 96 (100).

5.3.2. 5-Hydroxy-4-(1-oxododecyl)-2,2,6,6-tetramethyl-4-cyclohexene-1,3-dione (16). Pale yellow oil (92% yield); TLC: R_f 0.45 (9:1 hexane:EtOAc); UV (hexane) λ_{max} ($\log\varepsilon$): 277 (4.23), 236 (3.86) nm. IR (dry film) ν_{max}: 2924, 1721, 1670, 1559, 1465, 1381, 1048; EIMS (70 eV) *m/z* (rel. int.) 364 $[M]^+$ (95), 349 (23), 346 (20), 294 (70), 237 (93), 224 (96), 209 (40), 196 (35), 167 (40), 154 (58), 113 (31), 96 (100), 81 (44), 70 (60), 69 (62), 55 (71). Anal. Calcd $C_{22}H_{36}O_4$: C, 72.48; H, 9.95. Found: C, 72.52; H, 9.81.

5.3.3. 5-Hydroxy-4-(1-oxohexadecyl)-2,2,6,6-tetramethyl-4-cyclohexene-1,3-dione (18). White crystals (86% yield); mp 39 °C. TLC: R_f 0.31 (1:1 hexane/DCM); UV (MeOH) λ_{max} ($\log\varepsilon$): 278 (4.0), 239 (3.8) nm. IR (dry film) ν_{max}: 2927, 2855, 1723, 1672, 1666, 1581, 1564, 1552, 1049 cm^{-1}; HR-EIMS (70 eV) *m/z* (rel. int.) 420.32577 $[M]^+$ (23%, $C_{26}H_{44}O_4$ calcd 420.32396), 350 (32), 237 (99), 224 (79), 154 (52), 96 (100).

5.3.4. 5-Hydroxy-4-(1-oxocyclohexyl)-2,2,6,6-tetramethyl-4-cyclohexene-1,3- dione (20). White crystals (54% yield); mp 48 °C. TLC: R_f 0.23 (1:1 hexane/DCM); UV (MeOH) λ_{max} ($\log\varepsilon$): 280 (4.1), 240 (3.8) nm. IR (dry film) ν_{max}: 2932, 2854, 1723, 1672, 1666, 1581, 1563, 1549, 1049 cm^{-1}. HR-EIMS (70 eV) *m/z* (rel. int.): 292.16837 $[M]^+$ (94%, $C_{17}H_{24}O_4$ calcd 292.16746), 277 (47), 222 (56), 204 (47), 83 (100).

5.3.5. 5-Hydroxy-4-(1-oxo-ethylcyclohexyl)-2,2,6,6-tetramethyl-4-cyclohexene-1, 3-dione (22). Yellow crystalline solid (58% yield); mp 45 °C; TLC: R_f 0.27 (1:1 hexane/DCM); UV (MeOH) λ_{max} ($\log\varepsilon$): 279 (4.2), 238 (4.0) nm. IR (dry film) ν_{max}: 2924, 2852, 1723, 1672, 1666, 1581, 1563, 1549, 1049 cm^{-1}. EIMS (70 eV) *m/z*: 320 $[M]^+$ (17%), 237 (100). Anal. Calcd for $C_{19}H_{28}O_4$: C, 71.25; H, 8.75. Found 71.54; H, 8.74.

5.3.6. 5-Hydroxy-4-(1-oxophenylacetyl)-2,2,6,6-tetramethyl-4-cyclohexene-1,3- dione (24). Pale yellow oil (90% yield); TLC: R_f 0.22 (9:1 hexane:EtOAc); UV (MeOH) λ_{max} ($\log\varepsilon$) 279 (3.9), 230 (4.0) nm. IR (film) ν_{max}: 2983, 2939, 1722, 1671, 1560, 1494, 1471, 1382, 1304, 1183, 1048, 964, 874, 848, 750, 701, 668 cm^{-1}. HR-EIMS 70 eV *m/z* (rel. int.) 300.13608 $[M]^+$ (8%, $C_{18}H_{20}O_4$ calcd for 300.13616), 229 (29), 209 (100), 191 (29), 139 (41), 96 (40), 91 (92), 70 (30), 69 (33).

5.3.7. 5-Hydroxy-4-(1-oxododecyl)-2,2,6,6-tetraethyl-4-cyclohexene-1,3-dione (25). Sodium metal (0.3 g) was slowly added to MeOH (5 ml) to form a solution. To this was added the phloroglucinol **15** (200 mg), followed by CH_3CH_2I (5 ml). The solution was refluxed under N_2 for 3 h. Addition of HCl (1 M) until just acidic, followed by extraction into EtOAc, gave the crude product. Column chromatography over silica gel eluting with hexane containing increasing amounts of DCM gave the tetraethyltriketone **25** as a colourless oil (109 mg, 40%); TLC: R_f 0.51 (1:1 hexane/DCM); UV (MeOH) λ_{max} ($\log\varepsilon$) 320 sh (3.3), 281 (4.1) 238 (3.9) nm. IR (dry film) ν_{max}: 2926, 2854, 1711, 1668, 1666, 1581, 1564, 1552, 1068 cm^{-1}. HR-EIMS (70 eV) *m/z* (rel. int.): 420.32390 $[M]^+$ (2%, $C_{26}H_{44}O_4$ calcd 420.32396), 391 (42), 265 (12), 237 (54), 149 (26), 111 (40), 57 (100).

5.3.8. 5-Hydroxy-4-(1-oxododecyl)-2,2,6,6-tetrakis(3-methyl-2-butenyl)-4-cyclohexene-1,3-dione (26) and 1-(2,4,6-trihydroxyphenyl)-3-(3-methyl-2-butenyl)-1-dodecanone (28). To a stirred suspension of K_2CO_3 (240 mg) and phloroglucinol **15** (0.5 g) in dry acetone (5 ml) was added prenyl bromide (270 μl), and the mixture was refluxed for 5 h. The solvents were removed in vacuo to give an orange solid. Column chromatography over Si gel eluting with hexane containing increasing

amounts of DCM and then EtOAc first gave **26** as a colourless oil (40 mg, 4%); TLC: R_f 0.66 (1:1 hexane/DCM); UV (MeOH) λ_{max} ($\log\varepsilon$): 283 (3.9), 235 (3.8), 201 (4.2) nm. IR (dry film) ν_{max}: 2925, 2855, 1712, 1672, 1666, 1581, 1564, 1549, 1038 cm^{-1}. HR-EIMS (70 eV) *m/z* (rel. int.): 580.44942 $[M]^+$ (1.3%, $C_{38}H_{60}O_4$ calcd 580.44986), 511 (21), 443 (47), 387 (100); Followed by a mixture of C and O mono-prenyl compounds (227 mg, 37%). Recrystallisation from DCM gave **28** as a white crystalline solid (82 mg, 13%); mp 114 °C TLC: R_f 0.54 (10:1 DCM/ EtOAc); UV (MeOH) λ_{max} ($\log\varepsilon$) 325 (3.2), 290 (4.1), 226 (3.9), 202 (4.1) nm. IR (dry film) ν_{max}: 3450, 1622, 1460, 1250, 1080 cm^{-1}. HR-EIMS (70 eV) *m/z* (rel. int.) 376.26253 $[M]^+$ (12%, $C_{23}H_{36}O_4$ calcd 376.26136), 359 (100), 321 (15), 221 (30) 165 (35).

5.3.9. 5-Hydroxy-4-(2-methyl-1-oxopropyl)-2,2,6,6-tetrakis(3-methyl-2-butenyl)-4-cyclohexene-1,3-dione (27). Phloroglucinol **3** (100 mg) was reacted with K_2CO_3 (0.5 g) and prenyl bromide (0.2 ml) in acetone. Column chromatography over Si gel eluting with hexane containing increasing amounts of DCM gave **27** as a colourless oil (67 mg, 28%); TLC: R_f 0.58 (1:1 hexane/DCM); UV (MeOH) λ_{max} (log ε): 284 (4.1), 234 (4.0), and 202 (4.3) nm. IR (dry film) ν_{max}: 2968, 2926, 1713, 1672, 1666, 1581, 1564, 1549, 1062 cm^{-1}. HR-EIMS (70 eV) *m/z* (rel. int.): 468.32438 $[M]^+$ (1%, $C_{30}H_{44}O_4$ calcd 468.32528), 399 (24), 331 (34), 275 (100).

5.3.10. 5-Hydroxy-4-(1-oxododecyl)-2,2,6-trimethyl-6-(3-methyl-2-butenyl)-4- cyclohexene-1,3-dione (29). Sodium metal (0.15 g) was slowly added to MeOH (3 ml) to form a solution. To this was added the phloroglucinol **28** (50 mg), followed by CH_3I (3 ml). The solution was refluxed under N_2 for 3 h. Addition of HCl (1 M) until just acidic, followed by extraction into EtOAc, gave the crude product. Column chromatography over Si gel eluting with hexane containing increasing amounts of DCM gave **29** as a colourless oil (45 mg, 82%); TLC: R_f 0.42 (1:1 hexane/DCM); UV (MeOH) λ_{max} ($\log\varepsilon$) 279 (3.9), 238 (3.7) nm. IR (dry film) ν_{max}: 2926, 2854, 1721, 1672, 1666, 1580, 1564, 1549, 1034 cm^{-1}. HR-EIMS (70 eV) *m/z* (rel int.): 418.30909 $[M]^+$ (10%, $C_{26}H_{42}O_4$ calcd 418.31096), 375 (14), 350 (35), 322 (16), 293 (17), 69 (100).

5.4. Biological assays

MRSA assays: were performed against methicillin-resistant *Staphylococcus aureus* (SRL 62.537) with methicillin MIC of greater than or equal to 128 μg/ml and an oxacillin MIC in the range of 64 to greater than 128 μg/ml. Minimum inhibitory concentrations (MICs) were determined by preparing doubling dilutions of each test sample in Mueller–Hinton broth containing 0.5% Tween 80. Each dilution was then inoculated with a standard inoculum of an overnight culture of bacteria adjusted so that each test received 3×10^5–5×10^5 CFU (colony-forming units)/ml. All tests were performed in duplicate or triplicate with control growth controls (liquid medium plus inoculum) and sterility controls (liquid medium plus test sample only). A positive control was performed using vancomycin (MIC 1.25 μg/ml). The MIC was defined as the lowest drug concentration that prevented visible growth.

Anti-mycobacterial assays: were performed against multi-drug resistant *Mycobacterium tuberculosis* (MDR-TB). A clinical isolate of MDR-TB (IMCJ945), obtained from International Medical Center of Japan Hospital, was subcultured on 1% Ogawa agar medium (Kyokuto Seiyaku Co. Ltd. Tokyo, Japan). The isolate IMCJ945 was resistant to isoniazide, rifampicin, ethombutol, streptomycin, kanamycin, ethionamide and *p*-aminosalicylic acid. Bacterial suspensions were incubated at 37 °C in 7H9GC broth (4.7 g of Middlebrook 7H9 broth base, BBLTM Middlebrook OADC Enrichment; 8.5 g NaCl, 50.0 g bovine albumin, 20.0 g dextrose, 0.03 g catalase and 0.6 ml oleic acid in 1.0 l H_2O containing 2.0 ml glycerol) so that their turbidities matched that of a McFarland no. 1 turbidity standard. One hundred microlitres of 7H9 GC broth was added to all outer-perimeter wells of sterile 96-well plates to minimize evaporation of the medium in the test wells during incubation. Next, 100 μl of $2\times$ drug solutions was added to the first wells. By using a multichannel pipette, 100 μl was transferred from first well to next well, and the contents of the wells were mixed well. Identical serial 1:2 dilutions were continued through final wells, and 100 μl of excess medium was discarded from the final wells. Final drug concentration ranges were 2–125 μg/ml. Ten microliters of *M. tuberculosis* inoculum was added to all the wells (yielding a final volume of 210 μl per well). The wells filled with 7H9 GC broth served as drug-free (inoculum-only) controls. The plates were sealed with Parafilm and were incubated with CO_2 at 35 °C for 7 days. The MIC was defined as the lowest drug concentration that prevented visible growth. A positive control was performed using rifampicin (MIC 0.02 μg/ml for TB, >12.5 μg/ml for MDR-TB).

VRE assays: A vancomycin-resistant strain of *Enterococcus faecalis* ATCC 700802 was obtained from the American Type Culture Collection. One hundred microlitres of the compounds in solution was serially diluted into Mueller–Hinton broth by using a 96-well microtitre plate. After the constituents were diluted, a 10 μl aliquot of the test isolate (5×10^5 CFU/ml) was added to each well of the microtiter plate. Inocula of the test organisms were prepared from colonies grown on Mueller–Hinton plates, incubated for 20–24 h. Colonies were suspended in 0.9% saline to obtain a suspension equivalent to the turbidity of a 0.5 McFarland standard and diluted further 1:20 in 0.9% saline within 15 min. This provided a final inoculum density of approximately 5×10^5 CFU/ml in the wells of the microdilution panels. The inoculated plates were incubated at 35 °C in ambient air for 18–24 h. The MIC was defined as the lowest drug concentration that prevented visible growth.

Antimicrobial disc-diffusion assays: were performed against *Bacillus subtilis* (ATCC Strain number 19659), positive control 30 μg/disc chloramphenicol (12 mm zone), *Escherichia coli* (25922), positive control 10 μg/disc gentamicin (10 mm zone), *Candida albicans*

(14053), positive control 100 units/disc nystatin (12 mm zone), *Trichophyton mentagrophytes* (28185), positive control 100 units/disc nystatin (6 mm zone), *Pseudomonas aeruginosa* (27853), positive control 10 μg/disc gentamicin (10 mm zone) and *Cladosporium resinae* positive control 100 units/disc nystatin (10 mm zone). Solutions of compounds for assay were dried onto 6 mm filter paper disks, which were then placed onto seeded agar petri dishes and incubated. Activity showed as a zone of inhibition around the disc, with its width recorded from the edge of the disc in millimeters.

For the P388 assay, a twofold dilution series of the sample was incubated for 72 h with murine leukaemia cells (ATCC CCL 46 P388D1). The concentration of the sample required to inhibit cell growth to 50% of the growth of a solvent control was determined using the absorbance obtained upon staining with MTT tetrazolium. As a positive control for this assay, mitomycin C at a concentration of 0.06 μg/ml inhibited the growth of P388 cells by 43–75%.

5.4.1. Effect of triketone 16 on oxygen consumption. *Staphylococcus aureus* ATCC25923 cells in the exponential phase of growth (tryptic soy broth, TSB) were harvested by centrifugation, washed twice with 100 mM sodium phosphate buffer (pH 7.0) and then resuspended to a final OD_{600} of 0.2–1.0. A Clark-type oxygen electrode (Digital Model 10, Rank Brothers Ltd, England) was employed to measure the oxygen consumption rates. When oxygen consumption had reached steady state, measurement of respiration began by the addition of either glucose or succinate (final concentration 20 mM). After 5 min, steady-state respiration, triketone **16** was added to the chamber at a final concentration of 10 μg/ml. Identical experiments were performed over a range of triketone **16** concentrations. The same process was performed with cells grown to OD_{600} 0.2 and OD_{600} 0.5, testing the cells with 10, 20 and 30 μg/ml triketone **16**.

5.4.2. Effect of triketone 16 on the membrane potential ($\Delta\psi$). The $\Delta\psi$ was determined on mid-log phase cultures (grown at pH 7.0) that were harvested by centrifugation (8000*g*, 20 min, 4 °C) and washed twice in 100 mM sodium phosphate buffer (pH 7.0). Cells were energized with 20 mM glucose for 15 min, followed by the addition of $[^3H]TPP^+$ (1 μM final concentration). After incubation for 10 min at 37 °C, the cultures were centrifuged through 0.35 ml silicon oil (BDH Laboratory Supplies, Poole, England) in 1.5 ml microcentrifuge tubes (13,000 × g, 5 min, 22 °C) and 20 μl samples of the supernatant were removed. The tubes and contents were frozen (−20 °C), and cell pellets were removed with dog nail-clippers. Supernatant and cell pellets were dissolved in scintillation fluid and counted. The silicon oil mix was a 40 % mixture of phthalic acid bis(2-ethyl-hexyl ester) and 60 % silicone oil (40 % part mixture of DC200/200 silicone oil and 60 % DC 550). Silicon oils were left overnight at room temperature to equilibrate. Non-specific TPP^+ binding was estimated from cells that had been treated with valinomycin and nigericin (15 μM each) for 25 min.

5.4.3. Effect of triketone 16 on intracellular ATP levels and K^+. ATP was extracted from glucose-energized cell suspensions (100 mM potassium phosphate buffer, pH 7.0, and 5 mM $MgCl_2$), with and without the addition of triketone 16, by perchloric acid and $KOH/NaHCO_3$ treatment and frozen at −70 °C. Prior to analysis, samples were thawed and the potassium perchlorate was removed by centrifugation (13,000*g*, 5 min, 22 °C). ATP was determined by the luciferin–luciferase method.[44] The samples (50 μl) were then diluted in 400 μl of 40 mM Tris–acetate buffer (pH 7.75) containing 2 mM EDTA and 50 mM $MgCl_2$. The luciferase reaction was initiated by adding 50 μl of a purified luciferin–luciferase mix to 450 μl of the diluted extract, according to the supplier's recommendations (Sigma). Light output was immediately measured with a luminometer (Model LB953, Eg and G Berthold) using ATP as a standard.

Internal K^+ measurements were performed on glucose-energized cells that were separated from the external medium by silicon oil centrifugation (see above). For all samples, the cell pellets and supernatant samples were digested at room temperature for 24 h in 3 N HNO_3. Potassium concentrations were determined by flame photometry. Corrections were made for extracellular contamination of the cell pellet by K.

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