Biological evaluation of certain substituted hydantoins and benzalhydantoins against microbes

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Abstract. Twenty-three synthetic (thio)hydantoins and benzalhydantoins were evaluated for antimicrobial activity against \textit{Candida albicans}, \textit{Malassezia furfur}, \textit{Escherichia coli} and \textit{Staphylococcus aureus}, by the paper disc diffusion method. 3-n-butyl-4'-nitrobenzalhydantoin showed very high activity against \textit{E. coli} and high selectivity with respect to the other microorganisms, while 3-n-butyl-2'-bromo-4',5'-dimethoxybenzal hydantoin demonstrated very high selectivity in its activity against \textit{M. furfur} and \textit{S. aureus}. These compounds show the most promise as drug lead compounds.

1. Introduction
Infectious diseases are still a major cause of deaths, especially in developing countries, as new infectious diseases arise and an increasing number of multi-drug resistant strains of microbial pathogens emerge. The latter problem applies particularly through Gram positive bacteria, such as \textit{S. aureus}. Despite remarkable advances over the past decades, the search for new drugs to combat and control this threat is ongoing\cite{1-9}

Oxazolidinones have emerged as a promising new class of antimicrobial agents. The first of these to be authorised for use in Australia, linezolid \textsuperscript{1}, was approved in 2003 for use in humans. Oxazolidinones operate through a unique mechanism that result in the absence of cross-resistance with other antibiotics. The mechanism involves early inhibition of protein synthesis by preventing the formation of the 70S initiation complex through binding to the 20S ribosomal RNA of the 50S bacterial ribosomal subunit.\cite{9-17}

\textbf{Linezolid 1}

\textbf{2 Linezolid mimic.}
Our group has prepared a wide range of new, substituted benzalhydantoins (5-arylideneimidazolidine-2,4-diones) as part of a synthetic study toward tandem approaches to the molecules. [18] It occurred to us that these can be viewed as spatial mimics of linezolid 1. Like the oxazolidinones, 5-benzalhydantoins and 5-benzylhydantoins have attracted medicinal chemistry interest in their own rights because of their phenethylamine skeleton.[19] It was decided to evaluate a representative selection of hydantoin (imidazolidine-2,4-dione) precursors and final substituted benzalhydantoin compounds, in in vitro assays for antimicrobial activity.

2. Experimental

2.1. Procedure for Assessment of Antimicrobial Activity [20]

The culture medium for the fungi, *C. albicans* and *M. furfur*, was nutrient broth on agar medium 25 g/L at pH 7.0. The test organisms were inoculated in 10 mL assay broth in 50 mL Falcon™ tubes at 37 ºC and incubated overnight. An aliquot (100 μL) of test organisms was then added to each of the assay plates and the broth spread on the agar. Paper discs (6 mm diameter size) impregnated with solutions of Amphotericin B, hydantoins 6-8, thiohydantoins 3-5, benzalhydantoins 9-22, 24-25, and benzylhydantoin alcohol 26 (20 μL of 1.0 mg/mL), were applied to the test plates and the plates were incubated for 24-36 h at 37 ºC for *E. coli* and *S. aureus*. After the first 24 h the plates were examined and the diameter of any inhibition zone recorded. This process was repeated on all plates. A clear zone (inhibitory zone) surrounding the test disc indicated the presence of bioactivity in the compound (Table 1). Meanwhile, the bacterial strains *E. coli* and *S. aureus* were maintained in malt extract agar medium 13 g/L at pH 7.0. A method similar to the bacterial test protocol was adopted, except that incubation was carried out at each step at 30 ºC. The results are presented in table1.

3. Results and Discussions

In addition to the compounds mentioned above, it was thought worthwhile to study an example of an intermediate in the synthetic pathway, an hydroxybenzylhydantoin, as well as to study the effect of replacing the C(2)-carbonyl group oxygen with sulfur in both classes of compound. Thus, selected hydantoins 3-7, benzalhydantoins 8-26, were tested.

3.1. Antimicrobial Test Methodology

Two common strains of fungi, *Candida albicans* and *Malassezia furfur*, and the benchmark Gram negative and Gram positive bacteria, *Escherichia coli* and *Staphylococcus aureus*, respectively, were chosen as test organisms for compounds 3-26, and the paper disc diffusion test method [20] was employed for their screening. Amphotericin B [21, 22] a well known commercial antifungal drug, was chosen as a reference substance for assessing the antimicrobial performance of these compounds and they were both tested against methanol as the control solvent.

The inhibition associated with each of the sample substances against each of the four test organisms was recorded in Table 1. Inhibition was recorded numerically in Table 1 as the diameter (width) of the inhibitory zone (clear zone). In addition, inhibition was accorded a ranking of “**”, “***”, or “****”, corresponding to clear zones measured in the ranges 7–11 mm, 12–15 mm, and 16–20 mm, respectively.

3.2. General Observations

Eight compounds, namely hydantoin 8 and benzalhydantoins 12, 17-21, 24, and 4, had antimicrobial activity against all four test organisms (table 1). These compounds had in common the presence of halides...
(chlorine or bromine) at the 2-position and/or 3,4-dimethoxy substituents in the benzenoid ring of the arylmethylidene group.

Interestingly, the highest and broadest activity across all four microorganisms was demonstrated by 3,4-dimethoxybenzenemethylidene examples 17-21. The introduction of an ortho-bromo substituent into these derivatives 21-25, and in one example, replacement of the 3,4-dimethoxy substituents with a 3,4-methylenedioxy equivalent 5, gave almost as much activity in many cases but with total loss of activity against some other species. This group included the most active substance, the n-butyl derivative 24, and the least active compound, with N,N'-diallylation of the hydantoin ring.

Incorporation of such methoxy substituents therefore had a positive effect on activity but 2-halogenation and even replacement of the dimethoxy motif with the methylenedioxy motif has a significant impact on selectivity. In contrast, removal of the N(1)-H proton was detrimental and this suggested the positive involvement of an hydrogen bonding interaction associated with N(1).

3.3. Antifungal Activity

When the antifungal properties of the sample library members were assessed against C. albicans and M. furfur, the results in Table 1 revealed that the compounds generally had poor or no activity against C. albicans to the extent that all were of lower activity than the control, Amphotericin B. The most active compounds, benzalhydantoin 16, 17, and 20, all exhibited inhibitory zones of 11 mm, which corresponded to the upper limits of the "*" (= weak activity) category, and there was no obvious correlation between the position and/or type of substituents and the activity.
Table 1. Inhibitory zones (clear zone, diameter in mm) of hydantoin derivatives against four test organisms [20] (Red coloured entries indicate results for compounds with at least one (***) level of microbial activity.)

| Compound Number | C. Albicans | M. furfur | E. coli | S. aureus |
|-----------------|-------------|-----------|---------|-----------|
| 6               | 9 (*)       | 10 (*)    | 10 (*)  | 0         |
| 7               | 0           | 0         | 12 (**)| 0         |
| 8               | 7 (*)       | 10 (*)    | 12 (**)| 12 (***) |
| 3[23]           | 0           | 0         | 10 (*)  | 0         |
| 4               | 7 (*)       | 10 (*)    | 8 (*)   | 17 (***) |
| 9               | 9 (*)       | 10 (*)    | 10 (*)  | 0         |
| 10              | 0           | 0         | 10 (*)  | 14 (**)  |
| 11              | 7 (*)       | 16 (***)| 12 (**)| 14 (**)  |
| 12              | 0           | 16 (***)| 14 (**)| 14 (**)  |
| 13              | 0           | 12 (**)| 13 (**)| 12 (**)  |
| 14              | 0           | 7 (*)  | 13 (**)| 12 (**)  |
| 15              | 11(*)       | 0       | 11(*)   | 13(*)    |
| 16              | 7 (*)       | 0       | 16 (***)| 8 (*)    |
| 17              | 7 (*)       | 14 (**)| 16 (***)| 10 (*)   |
| 18              | 11(*)       | 13 (**)| 12 (**)| 11 (*)   |
| 19              | 7 (*)       | 10 (*0| 12 (**)| 11 (*)   |
| 20              | 9(*)        | 14 (**)| 16 (***)| 14 (**)  |
| 21              | 11(*)       | 10 (*) | 0       | 12 (**)  |
| 22              | 0           | 8 (*)  | 12 (**)| 15 (**)  |
| 24              | 7 (*)       | 17 (***)| 11 (*) | 17 (***)|
| 25              | 0           | 10 (*) | 0       | 0        |
| 26[26]          | 7 (*)       | 10 (*) | 0       | 15 (**)  |
| 5[24,25]        | 0           | 0      | 12 (**)| 12 (***)|
| 20              | 0           | 0      | 12 (**)| 11 (*)   |

**Amphotericin B** 12 (**), 0, 0, 0

**Control (MeOH)** 0, 0, 0, 0

Disc size: 6 mm (width), volume: 20 μL /disc (c = 1 mg/mL). Clear zone (inhibitory zone): * = 7–11 mm (weak activity); ** = 12–15 mm (high activity); *** = 16–20 mm (very high activity). Compound numbers are coded red in order to highlight the most active compounds.

In contrast, *M. furfur* was susceptible to eighteen of the twenty-three test substances, and of these eight showed "***" (= high activity) to "****" (= very high activity). The most active compounds, namely benzal hydantoin 11, 12, and 24, had chlorine or bromine substituents in the benzenoid ring, and in one case a 3-allyl group and the other two cases a 3-butyl substituent in the hydantoin ring, with inhibitory zones within the range 16-17 mm (very high activity; ***).

There was therefore considerable variation in the bioactivity of the test compounds against fungi, but some examples of high activity were observed. As a result, it is suggested that compounds 11, 12, and 24 might serve as useful lead compounds for the development of drugs that are selective antifungal agents, particularly against *M. furfur.*
3.4. Antibacterial Activity
Further examination of the results in Table 1 indicated that bioactivity of the test compounds against bacteria appeared more common and higher than against fungi. Antibacterial activity against Gram-negative *E. coli* was only slightly more widespread (twenty bioactive out of twenty-three test compounds) than against Gram-positive *S. aureus* (eighteen bioactive), but the number that were observed to have "high" to "very high" activity were about the same. Almost all compounds showed activity against Gram-negative *E. coli*, except compounds 21, 25 and 5. Interestingly, compound 25 with allyl substituents in both N1 and N3 hydantoin ring positions was again inactive against Gram-positive *S. aureus* but compounds 21 and 5 showed high activity against Gram-positive *S. aureus*, with inhibitory zones within the range 12 - 15 mm (high activity; **).

The most active compounds against *E. coli* were compounds 16, 17, and 20 while those most active against *S. aureus* were compounds 24 and 4. Compound 16, was unusual in bearing a nitro group in the benzenoid ring, while all the other most active antibacterial compounds, namely 17, 20, 24 and 4, bore two methoxy substituents. In addition, it was noted that of the most active antibacterials, compounds 16, 20 and 24, bore 3-n-butyl substituents.

Further reflection on the results in Table 1 revealed that compound 24 had quite modest levels of activity against *C. albicans* and *E. coli*, but was the only compound of those tested that showed the maximum level of bioactivity, "very high activity", against two or more microorganisms. Compound 24 therefore stands out as a major lead compound for further development in terms of drug development, especially as it demonstrates high selectivity in its biological activity, but also since it is active against a fungus and a Gram positive bacterium.

In a complementary sense, compound 16 appeared to be the only compound tested that showed very high activity against the Gram-negative bacterium, *E. coli*, as well as very high selectivity towards this microorganism (compounds 17 and 20 were more widespread inactivity).

In speculating on the most important features of compounds 16 and 24, and indeed the other most bioactive compounds, it appeared that the 3-n-butyl substitution was important. If this meant that lipophilicity alone was important, then compounds 11, 15, 19, 23, and 25, with their N-allyl substituents, should have shown promising bioactivity. Compound 23 was not available for testing, but compounds 11, 15 and 19 did show parallel activity to the N-n-butyl analogues. Notably, compound 25, was observed to have the least antimicrobial activity of all the benzal hydantoins and be one of the least active compounds overall of those tested. This reinforced the concept that a free N(1)-H group was needed for H-bonding to illicit high activity. Alternatively, the close proximity of the bromine and N(1)-allyl substituents in compound 25 might have created steric hindrance and/or a change in conformation of the molecule that interfered with biological activity. Whatever the cause, the poor bioactivity of compound 25 together with the promising activity of compounds 16 and 24 reveal the need for further structure-activity relationship studies on these compounds in the future.

4. Summary
A total of twenty three hydantoin and benzalhydantoin derivatives were investigated for antimicrobial activity against fungal (*C. albicans, M. furfur*) and bacterial (*E. coli* and *S. aureus*) microorganisms. A preliminary scan of the data showed that compounds had poor or no activity against *C. albicans*. All had lower activity than the control, Amphoterisin B, and within experimental error, all but two had lower activity against *C. albicans* than against the other three organisms. The two exceptions, benzalhydantoins 15 and 16, were nitro derivatives, and of the set of nitro derivatives, 13-17, these bore the most lipophilic groups at N(3). Curiously, those with N(3)-H and N(3)-phenyl substituents had similar activity in the bacterial assays but opposite selectivity towards *C. albicans* and *M. furfur*. 
Eight compounds, hydantoins 8 and 4 and benzalhydantoins 11, 17-20 and 24, exhibited relatively strong activity against all four test organisms, although amongst these, compounds 17-20 showed the most consistent activity across all four organisms and compounds 24 and 4 showed the highest degree of selectivity. Compounds 17-20 all contained 3,4-dimethoxy substituents, which are commonly found in natural products and associated with biological activity. These substituents might have imparted a good degree of solubility and lipophilicity to the molecules. However, compounds 18-21 also had this motif and yet their bioactivity was very varied. Similarly, compound 5 had an equivalent 3,4-methylenedioxy substituent, and yet its bioactivity was also inconsistent against the four organisms.

It was concluded that compounds 21-24 and 5 might have had the conjugation of the 4’-methoxy substituent with the C(4)=O carbonyl group on the hydantoin segment of the more active molecules disrupted in the latter molecules. In particular, the large size of the bromine atom in compounds 21-25 might have caused a twist in the 5-methylene bond on the hydantoin rings due to steric hindrance. For compound 5, the combined effect of the C(2)=S thiono group, which would be less polarized that the equivalent C(2)=O carbonyl bond in the other examples, and the subsequent strengthening of the N(3)-C(4) with the opportunity for conjugation with the N(3)-phenyl substituent, might provide a different explanation for the supposed reduced conjugation between the 4’-methoxy group and the C(4)=O group.

Another general observation was that the relatively small, hydantoin derivatives 6, 7 and 3 were active against the Gram negative bacterium, E. coli but ineffective against the Gram positive bacterium S. aureus. This might be explained by the more fragile cell wall of E. coli, which is supported by the observation that this was not the case for hydantoin analogues 6 and 4, wherein the molecules were more lipophilic and able to penetrate the relatively thick wall of S. aureus.

Compounds 16 and 24 showed the most promise of all the candidates for further investigation because of their very high activity and specificity towards particular microorganisms.

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