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Bioorganic & Medicinal Chemistry Letters 13 (2003) 3993–3996

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Rational Design of Antimicrobial Agents: Antifungal Activity of Alk(en)yl Dihydroxybenzoates and Dihydroxyphenyl Alkanoates

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Received 18 June 2003; revised 15 August 2003; accepted 27 August 2003

Abstract—A homologous series (C_3–C_{14}) of each alkyl 3,4- and 3,5-dihydroxybenzoates, and 3,4- and 3,5-dihydroxyphenyl alkanoates exhibit similar antifungal activity against *Saccharomyces cerevisiae*. Their nonyl derivatives exhibit the most potent antifungal activity against this yeast with the minimum fungicidal concentration (MFC) in the range between 12.5 and 50 μg/mL. In addition, various 3,4-dihydroxybenzoates, possessing different side chains, namely unsaturated, branched and alicyclic were synthesized and their activity was compared.

In our previous reports on structure–antifungal activity relationship (SAR) studies with a series of aliphatic alcohols using *Saccharomyces cerevisiae* ATCC 7754 as a model, their maximum antifungal activity was described to be dependent on the hydrophobic alkyl (tail) chain length from the hydrophilic hydroxyl group (head).[1] Biophysical processes appears to be a major contributor to the antimicrobial activity of amphipathic alkanols. Antifungal agents that primarily act as surfactants may target the extracytoplasmic region and thus do not need to enter the cell, they avoid most cellular pump-based resistance mechanisms. Structurally simple alkanols were a superior example to understand the role of the hydrophobic alkyl portion. However, alkanols may not be an ideal model to proceed with further SAR study since their derivatization is limited. This restriction limits the synthesis of many diverse related molecules for comparison that is essential for SAR study. On the basis of the data obtained, the hydrophilic hydroxyl group can be replaced by any hydrophilic groups as long as the 'head and tail' structure is balanced. This prompted us to search for novel antifungal agents through synthetic optimization.

In order to have more diverse structural analogues, various phenolic acids were selected as alternative hydrophilic head portions. The antifungal activity of the selected phenolic acids themselves was tested against *S. cerevisiae* first. All possessed little or no activity against this yeast. For example, 3,4,5-trihydroxybenzoic acid (gallic acid) (**1**) did not exhibit any fungicidal activity up to 3200 μg/mL. However, this phenolic acid was first selected as the hydrophilic (head) part because gallic acid is a common natural product isolated from many edible plants and more importantly, its three esters—propyl, octyl, and dodecyl (lauryl)— are currently permitted for use as antioxidant additives in food.[2] Furthermore, synthesis of a series of the esters and their related analogues is essential for SAR study and this can be easily carried out. It should be noted that various additional biological activities, which are needed to protect foods can be introduced by selecting appropriate head portions.

Among the alkyl 3,4,5-trihydroxybenzoates tested, nonyl 3,4,5-trihydroxybenzoate (C_9) (**2**) was found to be the most effective against *S. cerevisiae* with the minimum fungicidal concentration (MFC) of 12.5 μg/mL (42 μM), followed by octyl 3,4,5-trihydroxybenzoate (**3**) with an MFC of 25 μg/mL (89 μM).[3] Interestingly, decyl 3,4,5-trihydroxybenzoate (C_{10}) (**4**) did not show any activity.[3] It is worth noting that undecyl (C_{11}) (**5**) and dodecyl (C_{12}) (**6**) 3,4,5-trihydroxybenzoates did not exhibit any antifungal activity against *S. cerevisiae*, but still showed antibacterial activity against Gram-positive bacteria.[4] The maximum activity of the carbon chain lengths in alkyl 3,4,5-trihydroxybenzoates differed between the microorganisms tested, similar to those

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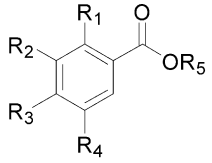
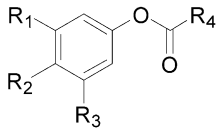
doi:10.1016/j.bmcl.2003.08.057

found for alkanols.[5] The antifungal activity of alkyl 3,4,5-trihydroxybenzoates is due primarily to their ability to act as nonionic surface-active agents (surfactants) and the alkyl chain length is largely related to the activity.[3,6,7]

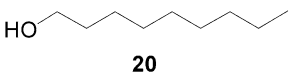
Subsequently, nonyl 2-hydroxybenzoate (**7**), nonyl 3-hydroxybenzoate (**8**), nonyl 4-hydroxybenzoate (**9**), nonyl 2,3-dihydroxybenzoate (**10**), nonyl 2,4-dihydroxybenzoate (**11**), nonyl 2,5-dihydroxybenzoate (**12**), nonyl 3,5-dihydroxybenzoate (**13**), nonyl 3,4-dihydroxybenzoate (protocatechuate) (**14**), nonyl 3-hydroxy-4-methoxybenzoate (**15**), nonyl 4-hydroxy-3-methoxybenzoate (**16**), 3,4-dihydroxyphenyl decanoate (**17**), and 3,5-dihydroxyphenyl decanoate (**18**) were synthesized (Fig. 1). The synthesis of nonyl 3,4-dihydroxybenzoate, 3,4-dihydroxyphenyl decanoate, and 3,5-dihydroxyphenyl



1: $R_1 = H, R_2 = R_3 = R_4 = OH, R_5 = H$
2: $R_1 = H, R_2 = R_3 = R_4 = OH, R_5 = CH_2(CH_2)_7CH_3$
3: $R_1 = H, R_2 = R_3 = R_4 = OH, R_5 = CH_2(CH_2)_6CH_3$
4: $R_1 = H, R_2 = R_3 = R_4 = OH, R_5 = CH_2(CH_2)_8CH_3$
5: $R_1 = H, R_2 = R_3 = R_4 = OH, R_5 = CH_2(CH_2)_9CH_3$
6: $R_1 = H, R_2 = R_3 = R_4 = OH, R_5 = CH_2(CH_2)_{10}CH_3$
7: $R_1 = OH, R_2 = R_3 = R_4 = H, R_5 = CH_2(CH_2)_7CH_3$
8: $R_1 = R_3 = R_4 = H, R_2 = OH, R_5 = CH_2(CH_2)_7CH_3$
9: $R_1 = R_2 = R_4 = H, R_3 = OH, R_5 = CH_2(CH_2)_7CH_3$
10: $R_1 = R_2 = OH, R_3 = R_4 = H, R_5 = CH_2(CH_2)_7CH_3$
11: $R_1 = R_3 = OH, R_2 = R_4 = H, R_5 = CH_2(CH_2)_7CH_3$
12: $R_1 = R_4 = OH, R_2 = R_3 = H, R_5 = CH_2(CH_2)_7CH_3$
13: $R_1 = R_3 = H, R_2 = R_4 = OH, R_5 = CH_2(CH_2)_7CH_3$
14: $R_1 = R_4 = H, R_2 = R_3 = OH, R_5 = CH_2(CH_2)_7CH_3$
15: $R_1 = R_4 = H, R_2 = OH, R_3 = OCH_3, R_5 = CH_2(CH_2)_7CH_3$
16: $R_1 = R_4 = H, R_2 = OCH_3, R_3 = OH, R_5 = CH_2(CH_2)_7CH_3$
19: $R_1 = R_4 = H, R_2 = R_3 = OH, R_5 = H$
21: $R_1 = R_4 = H, R_2 = R_3 = OH, R_5 = CH_2(CH_2)_6CH_3$



17: $R_1 = R_2 = OH, R_3 = H, R_4 = CH_2(CH_2)_7CH_3$
18: $R_1 = R_3 = OH, R_2 = H, R_4 = CH_2(CH_2)_7CH_3$
32: $R_1 = R_2 = OH, R_3 = H, R_4 = CH_2(CH_2)_8CH_3$



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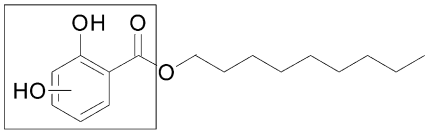

a

Figure 1. Structures of alkyl hydroxybenzoates.

decanoate were carried out by one step esterification utilizing *N*,*N*′-dicyclohexylcarbodiimide (DCC) as an activating reagent.[3] However, this method was not appropriate for syntheses of **7**–**13**, **15**, and **16** because of low yield and hard for eliminating remaining alcohol. The corresponding benzyloxybenzoic acids were obtained by the methods previously reported.[8–14] The final product alkyl hydroxybenzoates, were obtained in high yield by two step procedures, Mitsunobu reaction as a key step followed by hydrogenation for the removal of protecting group.[15]

The synthesized nonyl hydroxybenzoates (**7**–**16**) were tested for their antifungal activity against *S. cerevisiae*.[16,17] Among them, nonyl 3,5-dihydroxybenzoate and nonyl 3,4-dihydroxybenzoate were the only active compounds each with MFC of 12.5 μg/mL, respectively.[18] In contrast, neither monohydroxybenzoates (**7**–**9**) nor salicylic acid derivatives (**10**–**12**) showed any antifungal activity. It appears therefore that two hydroxyl groups are essential to elicit the activity as the hydrophilic 'head' portion. This can be supported by the observation that neither nonyl 3-hydroxy-4-methoxybenzoate nor nonyl 4-hydroxy-3-methoxybenzoate showed any fungicidal activity. In the case of nonyl 2,3-, 2,4-, and 2,5-dihydroxybenzoate, the salicylate moiety of '***a***' is in a sterically crowded environment and forms an intramolecular hydrogen bond. This may suggests that the moiety of ***a*** is not able to bind the membrane surface and hence cannot act as surfactants. A small change in chemical structures, especially in the head portion, was noted to affect biological activity to a large extent.

The 'hydrolyzable' ester group was selected in order to avoid undesired side effects; particularly endocrine disrupting activity of environmentally persistent estrogen mimics[19] such as alkylphenolic compounds.[20] The ester group is not directly related to the activity since 3,4- and 3,5-dihydroxyphenyl decanoate exhibited the comparable fungicidal activity against *S. cerevisiae* with that of nonyl 3,4-dihydroxybenzoate. The possibility that *S. cerevisiae* cells do secrete nonspecific extracellular esterases that hydrolyzes nonyl 3,4-dihydroxybenzoate to 3,4-dihydroxybenzoic acid (**19**) and 1-nonanol (**20**), the antifungal activity observed could be expected from the hydrolysates. However, the MIC and MFC of 1-nonanol against *S. cerevisiae* were 100 and 200 μg/mL, respectively. 3,4-Dihydroxybenzoic acid (**19**) did not exhibit any antifungal activity against *S. cerevisiae* up to 3200 μg/mL.

The same series of alkyl 3,4-dihydroxybenzoates was synthesized and tested for their antifungal activity against the same *S. cerevisiae* strain for comparison. The results are listed in Table 1. The potency of alkyl 3,4-dihydroxybenzoates is noted to be nearly comparable with those of the corresponding alkyl 3,4,5-trihydroxybenzoates,[3] indicating that the additional hydroxyl group is not essential. The range of the antifungal activity of alkyl 3,4-dihydroxybenzoates tested against *S. cerevisiae* is between 12.5 and 400 μg/mL. The maximum potency of both the growth inhibitory

Table 1. Antifungal activity of alkyl 3,4-dihydroxybenzoate against *S. cerevisiae* ATCC 7754

Compd tested	μg/mL	log *P*
	MIC (*MFC*)	
C_3	>400 (>*400*)	1.90
C_4	200 (*400*)	2.32
C_5	100 (*100*)	2.73
C_6	25 (*25*)	3.15
C_7	12.5 (*12.5*)	3.57
C_8	6.25 (*12.5*)	3.99
C_9	6.25 (*12.5*)	4.40
C_{10}	>400 (>*400*)	4.82
C_{11}	>400 (>*400*)	5.24
Miconazole	6.25 (*50*)	—

Numbers in *italic* type in parentheses are *MFC*.
Log *P* values were calculated by the method described.[21]

Table 2. Antifungal activity of bulky alkyl 3,4-dihydroxybenzoates against *S. cerevisiae* ATCC 7754

Comp tested	μg/mL	log *P*
	MIC (*MFC*)	
trans-2-Nonene-1-yl (**22**)	6.25 (*6.25*)	4.22
cis-2-Nonene-1-yl (**23**)	6.25 (*6.25*)	4.22
2-Nonanyl (**24**)	25 (*25*)	4.30
3-Nonanyl (**25**)	12.5 (*12.5*)	4.37
Cyclohexylmethyl (**26**)	50 (*50*)	3.05
Geranyl (**27**)	12.5 (*12.5*)	3.84
Neryl (**28**)	12.5 (*25*)	3.84
Menthyl (**29**)	12.5 (*12.5*)	4.10
Bornyl (**30**)	50 (*50*)	3.78
Decahydro-2-naphtyl (**31**)	25 (*25*)	3.62

Numbers in *italic* type in parentheses are *MFC*.

(MIC) and the fungicidal effect (MFC) was found in octyl (C_8) (**21**) and nonyl (C_9) (**14**) 3,4-dihydroxybenzoates each at 6.25 and 12.5 μg/mL, respectively. The differences between the MIC and MFC values are not more than 2-fold, suggesting that their activity is fungicidal.

As long as the head and tail structure is balanced, the hydrophobic portion seems to be flexible. In the surfactant concept, the solubility of molecules in the water based medium should be an important concern for synthetic optimization. The introduction of branching or unsaturation into the hydrophobic group is known to increase the solubility of the surfactant in water.[22] If the hydrophobic portion of the molecule enters into the membrane lipid bilayer and creates disorder in the fluid bilayer, increasing the volume of the hydrophobic portion through synthetic modification may enhance the activity. This phenomenon can be expressed by log *P* values. Since decyl 3,4,5-trihydroxybenzoate (**4**) did not exhibit any antifungal activity against *S. cerevisiae*, the target molecule's log *P* value should be as close to 4.5 but not largely exceed it. The hydrophobic portion is not a major contributor but obviously associated with the activity to a large extent. The more bulky alkyl groups may form large size pores and as a result, may increase the activity. Hence, various alcohols were selected and the log *P* values of their 3,4-dihydroxybenzoates were calculated. The values calculated are listed in Table 1 and most are around between 3.5 and 4.5. The 3,4-dihydroxybenzoates in the range of this log *P* values possess the optimum balance between hydrophilicity and lipophilicity to act as surfactants.

Similarly bulkier alkyl 3,4-dihydroxybenzoates were synthesized in the same manner.[3] The synthesized alkyl 3,4-dihydroxybenzoates; *trans*-2-nonene-1-yl (**22**), *cis*-2-nonene-1-yl (**23**), 2-nonanyl (**24**), 3-nonanyl (**25**), cyclohexylmethyl (**26**), geranyl (**27**), neryl (**28**), menthyl (**29**), bornyl (**30**), and decahydro-2-naphtyl (**31**), were assayed against the same *S. cerevisiae* strain for comparison (Fig. 2). The results are listed in Table 2. As expected, all the alkyl 3,4-dihydroxybenzoates tested, regardless of their alkyl shape exhibited the activity. The MFC to MIC ratios are no greater than 2-fold, indicating that

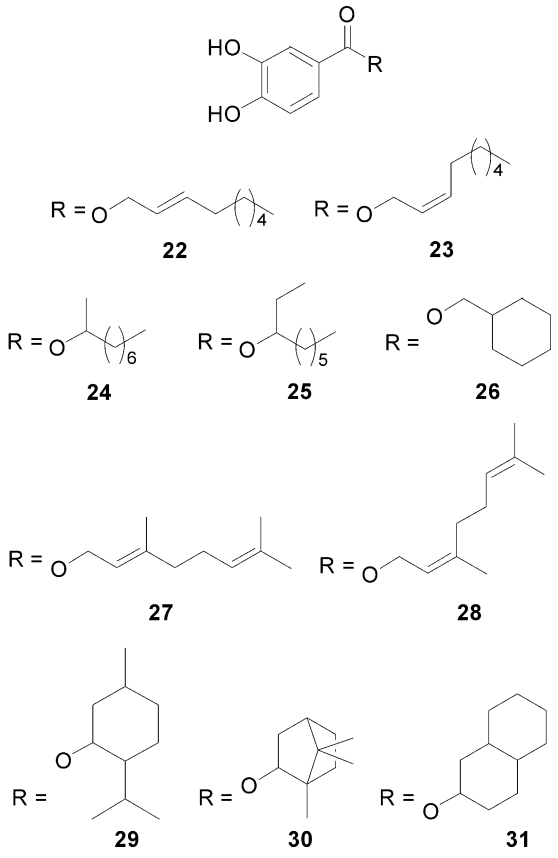

Figure 2. Structures of bulky alkyl 3,4-dihydroxybenzoates.

Table 3. Antifungal activity of 1,2,4-benzenetriol alkanoates against *S. cerevisiae* ATCC 7754

Comps tested	μg/mL	log *P*
	MIC (*MFC*)	
C_7	200 (*200*)	3.16
C_8	50 (*100*)	3.58
C_9	25 (*25*)	4.00
C_{10}	12.5 (*25*)	4.42
C_{11}	25 (*25*)	4.83
C_{12}	>400 (>*400*)	5.25
C_{13}	>400 (>*400*)	5.67

Numbers in *italic* type in parentheses are *MFC*.

their activity is fungicidal. Both *trans*- and *cis*-2-nonene-1-yl-3,4-dihydroxybenzoates were found to be the most effective against *S. cerevisiae* each with an MFC of 6.25 μg/mL (23 μM). As far as alkyl 3,4-dihydroxybenzoates are compared, the compounds possessing similar log *P* values exhibit similar MFC values, and 6.25 μg/mL of both *trans*- and *cis*-2-nonene-1-yl-3,4-dihydroxybenzoates was the maximum activity obtained through synthetic optimization. Overall, the molecular shape does not appear to be a major contributor to the activity.

Subsequently, the same series of 3,4-dihydroxyphenyl alkanoates was synthesized and tested for their antifungal activity against *S. cerevisiae* for comparison. The results are similar to those found for alkyl 3,4-dihydroxybenzoates as listed in Table 3. 3,4-Dihydroxyphenyl nonanoate (Cg), 3-4-dihydroxyphenyl deconoate (C_{10}) (**17**) and undecanoate (C_{11}) (**32**) were found to be the most effective against *S. cerevisiae* each with an MFC of 25 μg/mL. Furthermore, the same series of 3,5-dihydroxybenzoates and 3,5-dihydroxyphenyl alkanoates were synthesized and examined for their antifungal activity. The results are similar to those found for alkyl 3,4-dihydroxybenzoates (data not listed). The activity was found to correlate with the hydrophobic alkyl chain length. The compounds possessing similar log *P* values exhibit similar activity.

Acknowledgements

The authors are grateful to Dr. K. Fujita for his continuing advises, and Dr. K. Tsujimoto, Dr. K. Shimizu and Dr. N. Masuoka for obtaining the spectroscopic data.

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15. Nonyl 3,5-dihydroxybenzoate was synthesized as follows. A mixture of 3,5-dibenzyloxybenzoic acid (200 mg, 0.60 mmol),[11] 1-nonanol (95 mg, 0.66 mmol), and PPh_3 (190 mg, 0.72 mmol) in THF (4 mL) was cooled to 0 °C and treated with diisopropyl azodicarboxylate (146 mg, 0.72 mmol). After being stirred for 2 h at room temp, the solvent was removed in vacuo. The residue was subjected to silica gel chromatography eluted with 1–8% AcOEt–hexane to give an ester as white solid, which was used in the next step without further purification. The ester was hydrogenated over 20% $Pd(OH)_2$ on carbon (10 mg) in 1% AcOH–AcOEt (4 mL) for 12 h. Filtration through Celite and concentration followed by silica gel chromatography (15–30% AcOEt–hexane) gave 155 mg (92%) of the title compound as a white solid. The structure was established by spectroscopic methods (IR, NMR, and MS).
16. The procedures used for antifungal assay were the same as previously described.[3] The highest concentration of some esters remain uncertainty because of their solubility in limited the water-based medium.
17. The test strains, *S. cerevisiae* ATCC 7754 used for this study was purchased from American Type Culture Collection (Manassas, VA, USA).[3]
18. Nonyl 3,4-dihydroxybenzoate was also found to inhibit the growth of *Candida albicans* ATCC 18804 and *Zygosaccharomyces* ATCC 60483 each with MFC of 50 μg/mL.
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