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COMBINATION EFFECTS OF ANTIFUNGAL NAGILACTONES AGAINST *CANDIDA ALBICANS* AND TWO OTHER FUNGI WITH PHENYLPROPANOIDS

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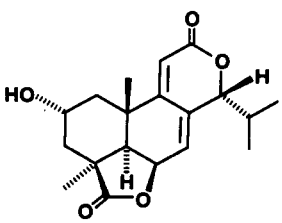
ABSTRACT.—Antifungal activity of three nagilactones isolated from the root bark of *Podocarpus nagi* (Podocarpaceae), alone and in combination with a variety of phenylpropanoids, was investigated against three fungi, *Candida albicans, Saccharomyces cerevisiae,* and *Pityrosporum ovale.* Nagilactone E [**2**], the most abundant norditerpene dilactone, showed moderate to weak activity against these fungi. This activity was dramatically enhanced by several naturally occurring phenylpropanoids such as anethole [**5**] and isosafrole. For example, anethole enhanced the activity of nagilactone E as much as 128-fold for *C. albicans*, decreasing the MIC of this nagilactone from 800 to 6.25 μg/ml.

In the past two decades more than 60 nor- and bisnor-diterpene dilactones have been isolated from various parts of fifteen species of the gymnosperm genus *Podocarpus* (Podocarpaceae) (1,2), among which *Podocarpus nagi* (Thunberg) Pilger has been studied most extensively. These dilactone compounds have been shown to exhibit diverse biological activities (3–10), to which we have recently added antifungal activity (11). A new antifungal norditerpene dilactone, 2α-hydroxynagilactone F [**1**], isolated from the root bark of *Pod. nagi* has been reported. Among the twelve selected microorganisms tested (12), *Saccharomyces cerevisiae* was susceptible to this new norditerpene dilactone only when tested at 800 μg/ml. However, no studies have been made for the antimicrobial activity of nagilactone E [**2**], the major component of a *P. nagi* root bark extract. In our preliminary assay, nagilactone E [**2**] exhibited antifungal activity against not only *Sa. cerevisiae,* but also *Candida albicans* and *Pityrosporum ovale.* The range of the activity of nagilactone E was moderate.

In view of the importance of controlling filamentous fungi such as *C. albicans*, an attempt to enhance the activity of these nagilactones by combining them with other substances was made. In addition, for comparative purposes, nagilactone C [**3**] was also tested, although it did not exhibit any activity against these fungi up to 800 μg/ml. The approach to the enhancement of total biological activity by combining two or more substances seems to be a most promising strategy for efficient utilization of renewable natural substances. However, a rationale for selecting other substances for an appropriate combination is still in an embryonic stage. The aim of this report is to compare the minimum inhibitory concentrations (MICs) of the nagilactones **1–3** against *C. albicans, Sa. cerevisiae,* and *Pit. ovale*, alone and in combination with other substances.



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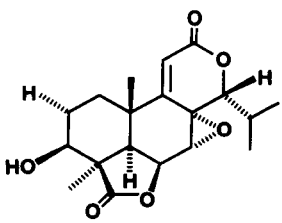

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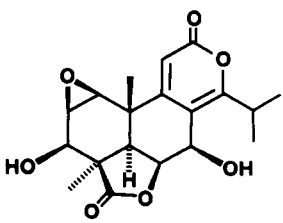

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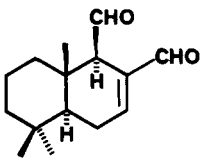
RESULT AND DISCUSSION

The antimicrobial activity against twelve selected microorganisms of three nagilactones **1**–**3** was tested. In addition to the aforementioned antifungal activity of 2α-hydroxynagilactone F [**1**] against *Sa. cerevisiae* with MIC of 800 μg/ml (11), nagilactone E [**2**] also showed moderate activity against *C. albicans, Sa. cerevisiae,* and *Pit. ovale*, with MICs of 800, 100, and 50μg/ml, respectively. However, nagilactone C [**3**] did not exhibit any activity up to 800 μg/ml. None of them exhibited any activity against five Gram-positive bacteria, three Gram-negative bacteria, and *Penicillium chrysogenum* up to 800 μg/ml. Nevertheless, the antifungal activity of these nagilactones was not potent enough to be considered for practical use. Therefore, they were examined in combination with polygodial [**4**] and anethole [**5**] in order to enhance the activity. The initial selection of these two substances was based largely on our previous study (13–15). Because of limited availability of the samples of two nagilactones **1** and **3**, most of the detailed study was carried out with the principal active compound, nagilactone E [**2**], isolated in rather large quantity.



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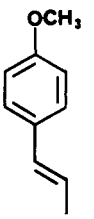

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We have recently reported that a drimane sesquiterpene dialdehyde, polygodial [**4**], isolated from various plants (16), increased the antifungal activity against *Sa. cerevisiae* and *Candida utilis* of several antibiotics such as actinomycin D and rifampicin (17). This sesquiterpenoid also enhanced the antifungal activity of maesanin, a benzoquinone isolated from the berries of *Maesa lanceolata* (Myrsinaceae), specifically against *C. utilis* (18). The rationale for these synergistic effects is based on the seemingly increased permeability of the plasma membrane to the aforementioned antimicrobial agents when they are combined with polygodial (19). It should be noted that this enhancing activity did not occur vice versa; polygodial enhances the antifungal activity of the aforementioned antimicrobial agents but none of them was synergistic to polygodial. Nevertheless, the nagilactones **1**–**3** were first combined with polygodial to examine whether or not they had the same enhancing activity against *C. albicans, Sa. cerevisiae,* and *Pit. ovale*. Polygodial did not synergize the antifungal activity of the nagilactones tested. Unexpectedly, polygodial displayed somewhat antagonistic activity. Thus, as shown in Figure 1, 0.098 μg/ml (1/16 of the MIC) of polygodial antagonized the antifungal activity of even 400 μg/ml (4 times of the MIC) of nagilactone E when this combination was tested against *Sa. cerevisiae*. Similar results were obtained against *C. albicans*. However, this combination showed no antagonistic activity against *Pit. ovale*.

Nagilactone E [**2**] was also combined with amphotericin B, known to be one of the most potent antibiotics against fungi. This combination was based on the fact that the mode of action of amphotericin B is also known to damage the plasma membrane by interacting with sterols (20) in fungal cell membranes (21). In contrast to polygodial, 0.78 μg/ml (=½MIC) of amphotericin B enhanced the activity of nagilactone E

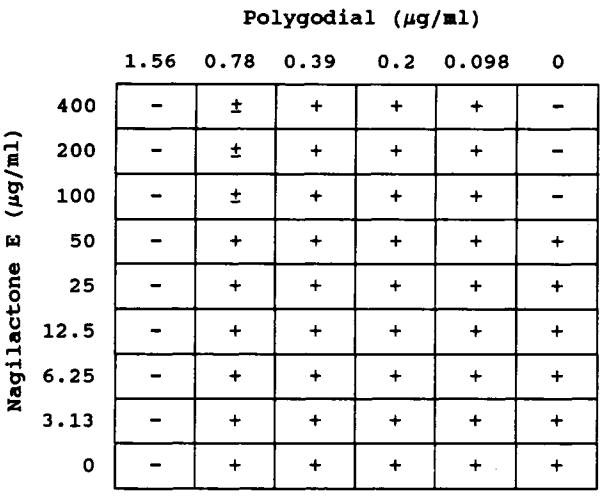

FIGURE 1. Checkerboard determination of nagilactone E in combination with polygodial against *Saccharomyces cerevisiae*. (+) = Visible growth, (±) = partial growth, and (−) = no growth.

against *Sa. cerevisiae* 4-fold; the MIC was reduced from 100 to 25 μg/ml. Similarly, the MIC against *C. albicans* was lowered from 800 to 100 μg/ml when nagilactone E was combined with ½MIC of amphotericin B. In addition, noticeably, this enhancing activity was observed vice versa as well. Thus, in the case against *Sa. cerevisiae*, nagilactone E also synergized amphotericin B fourfold; the MIC of amphotericin B was lowered from 1.56 to 0.39 μg/ml, when combined with 50 μg/ml (= ½MIC) of nagilactone E.

In addition, we have recently described a common naturally occurring phenylpropanoid, anethole [**5**], isolated from aniseed, *Pimpinella anisum* L. (Umbelliferae), that exhibited significant enhancement of polygodial activity against *C. albicans* and *Sa. cerevisiae* (13). Therefore, the nagilactones **1–3** were tested in combination with anethole against the same fungi. The results of testing are listed in Table 1. The activity of the nagilactones against these three fungi was significantly increased by ½MIC of anethole. In particular, the activity of 2α-hydroxynagilactone F [**1**] against *Sa. cerevisiae* was enhanced 128-fold. Thus, the MIC was decreased from 800 to 6.25 μg/ml. Similarly, the activity of nagilactone E [**2**] was also increased 128-fold, 16-fold, and 32-fold by anethole when tested against *C. albicans*, *Sa. cerevisiae*, and *Pit. ovale*, respectively. The MICs were lowered from 800 to 6.25 μg/ml, from 100 to 6.25 μg/ml, and from 50 to 1.56 μg/ml, respectively. Moreover, in combination with anethole,

TABLE 1. Antifungal Activity of the Three Nagilactones **1–3** Alone and in Combination with ½MIC of Anethole Against *Candida albicans*, *Saccharomyces cerevisiae* and *Pityrosporum ovale*.

Compound	MIC (μg/ml) Nagilactone alone→plus anethole		
	C. albicans	*Sa. cerevisiae*	*Pit. ovale*
1	—[a]	800→6.25	—
2	800→6.25	100→6.25	50→1.56
3	—	>1600→400	>1600→50

[a]—, Not tested.

nagilactone C [**3**] exhibited activity against both *Sa. cerevisiae* and *Pit. ovale* at 400 and 50 μg/ml, although it did not show any activity against these fungi even at 1600 μg/ml when it was tested alone.

Based on this finding, several other common naturally occurring phenylpropanoids such as safrole, isosafrole, eugenol, isoeugenol, methyleugenol, methylisoeugenol, and allylanisole together with several related substances such as allylbenzene, *trans*-β-methylstyrene, α-asarone, and 4-methoxycinnamic acid were also tested to examine if they had the same synergistic activity. In addition, because of the structural similarity, paraben (propyl 4-hydroxybenzoate) and BHA (butylated hydroxyanisole), which have been used in cosmetic products as preservatives and/or antioxidants, were assayed for comparative purposes. Again, because of limited availability, the assay was performed with only nagilactone E [**2**]. Table 2 displays the activity against *C. albicans*, *Sa. cerevisiae*, and *Pit. ovale* when the phenylpropanoids were tested alone. Most of them exhibited moderate activity against these three fungi. Table 3 shows the MICs of nagilactone E in combination with the phenylpropanoids at a concentration of ½MIC for each. Besides anethole, isosafrole also significantly enhanced the activity of nagilactone E for *C. albicans* and *Sa. cerevisiae*. Thus, the activity of nagilactone E was increased 256-fold against *C. albicans*; the MIC was lowered from 800 to 3.13 μg/ml, and the MIC of nagilactone E against *Sa. cerevisiae* was also reduced from 100 to 6.25 μg/ml. For *Pit. ovale*, methylisoeugenol and α-asarone enhanced the activity of nagilactone E, the activity of these compounds increasing 32-fold. Overall, the phenylpropanoids tested against *C. albicans* and *Pit. ovale* conferred significant enhancing activity to nagilactone E. In contrast, the phenylpropanoids tested against *Sa. cerevisiae*, excepting anethole and isosafrole, showed slight effects in combination with nagilactone E. Moreover, in these combinations for *Pit. ovale*, the enhancing effects occurred vice versa. Table 4 shows the MICs of phenylpropanoids in combination with nagilactone E at concentration of ½MIC. Thus, nagilactone E increased the antifungal activity of the phenylpropanoids tested against *Pit. ovale* from 8-fold to 32-fold. The enhancing activity of

TABLE 2. Antifungal Activity of Phenylpropanoids Against *Candida albicans*, *Saccharomyces cerevisiae*, and *Pityrosporum ovale*.

Compound	MIC (μg/ml)		
	C. albicans	*Sa. cerevisiae*	*Pit. ovale*
Anethole	200	200	200
Safrole	200	200	200
Isosafrole	100	200	100
Eugenol	800	800	200
Isoeugenol	400	400	200
Methyleugenol	800	800	200
Methylisoeugenol	400	400	200
Allylanisole	200	200	200
Allylbenzene	400	400	>800
trans-β-Methylstyrene	400	400	>800
α-Asarone	800	800	200
4-Methoxycinnamic acid	200	100	>800
Paraben	—[a]	200	100
BHA	—	200	200

[a] —, Not tested.

TABLE 3. Antifungal Activity of Nagilactone E [2] in Combination with ½MIC of Each of Various Phenylpropanoids Against *Candida albicans*, *Saccharomyces cerevisiae*, and *Pityrosporum ovale*.

Compound	MIC (μg/ml)		
	C. albicans	*Sa. cerevisiae*	*Pit. ovale*
None	800	100	50
Anethole	6.25	6.25	1.56
Safrole	12.5	50	6.25
Isosafrole	3.13	6.25	6.25
Eugenol	50	50	6.25
Isoeugenol	25	50	3.13
Methyleugenol	—[a]	25	6.25
Methylisoeugenol	—	25	1.56
Allylanisole	—	25	6.25
Allylbenzene	—	50	—
trans-β-Methylstyrene	—	50	—
α-Asarone	—	25	1.56
4-Methoxycinnamic acid	—	100	—
Paraben	—	100	6.25
BHA	—	100	3.13

[a]—, Not tested.

the nagilactones seems to depend on the species of fungi being tested and the phenylpropanoids being combined. A rational approach to these combination effects is currently in development.

Combining two or more substances to enhance total biological activity may not only be the most efficient way to utilize renewable natural products; it is also possible that the microorganisms may take longer to develop their resistance to two or more toxins in which the mode of action is diverse. This study may provide a new approach to a more efficient utilization of renewable natural products for these purposes.

TABLE 4. Antifungal Activity of Phenylpropanoids in Combination with ½MIC of Nagilactone E [2] Against *Saccharomyces cerevisiae* and *Pityrosporum ovale*.

Compound	MIC (μg/ml)	
	Sa. cerevisiae	*Pit. ovale*
Anethole	25	6.25
Safrole	100	25
Isosafrole	50	6.25
Eugenol	400	25
Isoeugenol	200	6.25
Methyleugenol	400	25
Methylisoeugenol	100	6.25
Allylanisole	50	12.5
α-Asarone	200	6.25
Paraben	200	12.5
BHA	200	12.5

EXPERIMENTAL

CHEMICALS.—All the nagilactones **1–3** used for the assay were previously isolated from the root of *Pod. nagi* (11,22). Polygodial [**4**] was isolated from the sprouts and seeds of *Polygonum hydropiper* (Polygonaceae) (16). Anethole [**5**], amphotericin B, and BHA were purchased from Sigma Chemical Co. (St. Louis, MO). Safrole, isosafrole, eugenol, methyleugenol, isoeugenol, methylisoeugenol, *trans*-β-methylstyrene, allylanisole, allylbenzene, α-asarone, 4-methoxycinnamic acid, and paraben were obtained from Aldrich Chemical Co. (Milwaukee, WI). *N,N*-Dimethylformamide (DMF) was purchased from EM Science (Gibbstown, NJ).

MICROORGANISMS AND MEDIA.—All microorganisms for the antimicrobial assay were purchased from American Type Culture Collection (Rockville, MD). They are *Bacillus subtilis* ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Staphylococcus aureus* ATCC 12598, *Streptococcus mutans* ATCC 25175, *Propionibacterium acnes* ATCC 11827, *Escherichia coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, *Sa. cerevisiae* ATCC 7754, *C. albicans* ATCC 18804, *Pit. ovale* ATCC 14521, and *Pe. chrysogenum* ATCC 10106. Their appropriate media and cultivation conditions have been previously described (12).

ANTIMICROBIAL ASSAY.—The bioassay was performed by a broth dilution method as previously described (12). The test compound was dissolved in DMF, and 30 μl of the sample solution was added to 3 ml of the appropriate medium, to which 30 μl of 2-day-old culture of test microorganisms (5-day-old of *Pe. chrysogenum*) was inoculated. After 2 days cultivation (3 days for *Pit. ovale* and 5 days for *Pe. chrysogenum*), the growth of the microorganisms was examined. The MIC was defined as the lowest concentration of the test compound in which no growth was visible. The highest concentration tested was 800 μg/ml, unless otherwise specified, because of limited availability and solubility in the H_2O-based media of some of the samples.

The combination data of the nagilactones against *C. albicans*, *Sa. cerevisiae* and *Pit. ovale* with other substances were evaluated by the broth dilution checkerboard method (23), with incubations at 30° for 48 h (72 h for *Pit. ovale*). The twofold dilutions of the nagilactones were tested in combination with concentrations of twofold dilutions of the other compound. It should be noted that the concentration of DMF in each medium was always 1%, which did not affect the growth of any microorganisms employed. Each fungus was tested at least twice with the checkerboard method.

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