Antibacterial Activity of Totarol and Its Potentiation

Isao Kubo, Hisae Muroi, and Masaki Himejima

J. Nat. Prod., **1992**, 55 (10), 1436-1440• DOI:
10.1021/np50088a008 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 55, No. 10, pp. 1436-1440 October 1992

ANTIBACTERIAL ACTIVITY OF TOTAROL AND ITS POTENTIATION

ISAO KUBO,* HISAE MUROI, and MASAKI HIMEJIMA

Division of Entomology and Parasitology, College of Natural Resources, University of California, Berkeley, California 94720

ABSTRACT.—Antimicrobial activity of six diterpenoids isolated from the bark of *Podocarpus nagi* (Podocarpaceae) has been tested against twelve selected microorganisms. Totarol [**1**], the most abundant compound among the six, exhibited potent bactericidal activity only against Gram-positive bacteria, among which *Propionibacterium acnes* was the most senstitive bacterium. Totarol also showed strong activity against four other Gram-positive bacteria tested: *Streptococcus mutans, Bacillus subtilis, Brevibacterium ammoniagenes,* and *Staphylococcus aureus* (both penicillin-resistant and penicillin-susceptible strains).

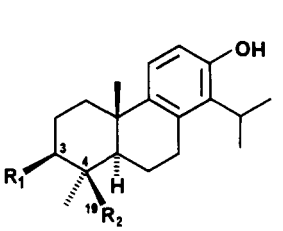
The bactericidal activity of totarol was enhanced when it was tested in combination with several other natural products. Noticeably, the activity of totarol against *Sta. aureus* was increased eightfold when tested in combination with ½MIC of anacardic acid [**9**]. The synergistic activity of anacardic acid caused the minimum bactericidal concentration (MBC) of totarol to be lowered from 1.56 to 0.2 μg/ml.

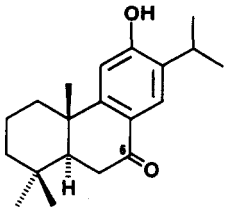
The MeOH extract of the bark of *Podocarpus nagi* (Thunberg) Pilger (Podocarpaceae) exhibited a rather broad antimicrobial spectrum in our preliminary screening against four representative microorganisms (1). The MeOH extract, after removal of solvent, was suspended in H_2O and then partitioned between H_2O and, sequentially, *n*-hexane, Et_2O, and EtOAc. Subsequent bioassay indicated that both the Et_2O and EtOAc portions were active. Bioassay-guided fractionation of the EtOAc portion has led to the isolation of several antifungal principles active against *Saccharomyces cerevisiae, Candida albicans,* and *Pityrosporum ovale*. These active compounds have been identified, based on spectroscopic studies, as norditerpene and bisnorditerpene dilactones, which are characteristic compounds in *Podocarpus* species with various biological activities (2–7). This finding has already been reported in part (8). However, none of these norditerpene dilactones exhibited any activity against bacteria when tested up to 800 μg/ml; thus they could not account for the antibacterial activity of earlier fractions.

Isolation, guided by antibacterial activity using *Bacillus subtilis*, from the Et_2O fraction has been carried out. This paper reports in detail the antibacterial and bactericidal activity of the principal active compound, totarol [**1**], emphasizing the activity against a penicillin-resistant *Staphylococcus aureus*, and the combination study with several other antibacterial natural products.

RESULTS AND DISCUSSION

Guided by bioassay using *Bac. subtilis*, two antibacterial principles have been isolated from the Et_2O portion after repeated various chromatographies. The active compounds have been identified as totarol [**1**] and its congener, totaradiol [**2**], based on spectroscopic studies and particularly the nmr data (9). These two diterpenoids have been previously described together with three closely related analogues 19-hydroxytotarol [**3**], totaral [**4**], and 4β-carboxy-19-nortotarol [**5**] from the same source (9). Although compounds **3–5** did not exhibit any activity against *Bac. subtilis* when tested up to 400 μg/ml, the antimicrobial activity of the five purified diterpenoids **1–5** was tested against twelve selected microorganisms. The minimum inhibitory concentrations (MICs) and MBCs of the two active diterpenoids, totarol [**1**] and totaradiol [**2**], are listed in Table 1. None of them showed any activity against Gram-negative bacteria (*Pseudomonas aeruginosa, Enterobacter aerogenes,* and *Escherichia coli*) or fungi (*Sac. cerevisiae, C. albicans, Pit. ovale,* and *Penicillium chrysogenum*) when tested up to 400 μg/ml. How-





1 R_1=H, R_2=Me
2 R_1=OH, R_2=Me
3 R_1=H, R_2=CH_2OH
4 R_1=H, R_2=CHO
5 R_1=H, R_2=COOH

6

ever, totarol [**1**] exhibited potent antibacterial activity against all the Gram-positive bacteria tested, among which *Propionibacterium acnes* was the most sensitive, with MIC and MBC of 0.39 and 0.78 μg/ml, respectively. In contrast, *Sta. aureus* (a penicillin-susceptible strain) was the bacterium least sensitive to totarol, but the MIC and MBC were still as low as 1.56 μg/ml. Furthermore, the other three Gram-positive bacteria, *Streptococcus mutans*, *Brevibacterium ammoniagenes*, and *Bac. subtilis*, were also sensitive to totarol, with MICs of 0.78, 0.78, and 1.56 μg/ml and MBCs of 0.78, 6.25, and 1.56 μg/ml, respectively.

Although a large number of phytochemicals have been found to show antibacterial activity against Gram-positive bacteria, only a few exhibit activity against *Sta. aureus*. Hence, this bacterium, especially the penicillin- and methicillin-resistant strains, remains difficult to control. As shown in Table 2, totarol is one of the rare phytochemicals that exhibits potent antibacterial activity against *Sta. aureus*, both the penicillin-susceptible and penicillin-resistant strains tested.

Totarol's congener, totaradiol [**2**], also exhibited a similar antimicrobial spectrum but one much less potent than totarol. Thus, its MICs against the Gram-positive bacteria were in the range of 25–200 μg/ml. In addition, three other structurally similar diterpenoids **3**–**5** isolated from the same source did not show any antimicrobial activity when tested up to 400 μg/ml. As a result, structure-activity relationships can be proposed as the five diterpenoids tested are different oxidation peers. By using the simplest, totarol, as a standard, the following points can be summarized: (a) an additional hydroxy group at the 3-position on **1** transforms it to **2** and a decrease in the activity results; (b) different levels of oxidation at the 4β-Me group of **1** convert it to 4β-

TABLE 1. Antibacterial and Bactericidal Activity of Totarol [**1**] and Totaradiol [**2**].

Bacteria tested	Compound		
	1		**2**[a]
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)
Bacillus subtilis	1.56	1.56	25
Brevibacterium ammoniagenes	0.78	6.25	100
Staphylococcus aureus[b]	1.56	1.56	50
Streptococcus mutans	0.78	0.78	200
Propionibacterium acnes	0.39	0.78	25

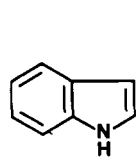
[a]MBCs were not tested.
[b]*Sta. aureus* is a penicillin-susceptible strain.

TABLE 2. MICs of Totarol [1] Against Gram-positive Bacteria, Compared with Those of Penicillin G.

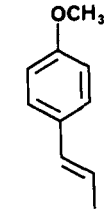
Bacteria tested	Compound	
	1	Penicillin G
Bacillus subtilis	1.56	50
Brevibacterium ammoniagenes	0.78	0.39
Staphylococcus aureus ATCC 12598	1.56	0.049
Staphylococcus aureus ATCC 29247	0.78	>800
Streptococcus mutans	0.78	0.049
Propionibacterium acnes	0.39	0.012

CH_2OH (**3**), 4β-CHO (**4**), and 4β-COOH (**5**), and a dramatic decrease in the activity occurs. Thus, when the methyl group of **1** is oxidized, the biological activity is completely lost when tested up to 400 μg/ml. In addition, a similar diterpenoid, sugiol [**6**], oxidized at C-6, was also isolated from the same part of the plant collected from different geographic locations (9). However, it did not show any antimicrobial activity up to 400 μg/ml. Interestingly, the diterpenoids **1** and **2** showed antimicrobial activity specifically against Gram-positive bacteria, while the norditerpene dilactones exhibited antifungal activity against *Sac. cerevisiae, C. albicans,* and *Pit. ovale*. Thus, it seems that two different classes of diterpenoids combine to yield the original antimicrobial activity of the crude MeOH extract.

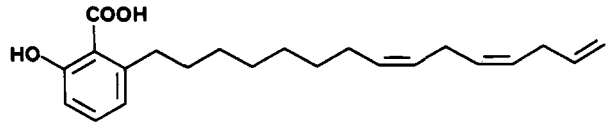
In view of increasing importance of controlling specific bacteria such as *Sta. aureus, Str. mutans,* and *Pr. acnes* that cause skin and tooth problems, the antibacterial activity of totarol may be potent enough to be considered for practical use. However, it may be advisable to use it in combination with other compounds (10). Combining more than two compounds might be superior to the use of a single antibacterial compound in order to make the development of resistance mechanisms in microorganisms less likely, as well as enhancing and broadening the total biological activity. Because of these concerns, we attempted to enhance the antibacterial activity of totarol through combination of two or more natural substances. The initial selection of other substances was based largely on our previous study, because a rational basis for the selection of other substances is still in an embryonic stage. We have recently reported that indole [7] sig-



7



8



9

nificantly enhanced the antibacterial activity against *Str. mutans* of several sesquiterpene hydrocarbons, δ-cadinene and β-caryophyllene, identified in green tea flavor (11) and that anethole [**8**] isolated from the seeds of *Pimpinella anisum* (Umbelliferae) exhibited a significant synergistic effect on the antifungal activity of polygodial against *C. albicans* and *Sac. cerevisiae* (12). In addition, we have recently reported that several phenolic compounds isolated from the shell oil of the cashew *Anacardium occidentale* (Anacardiaceae), such as anacardic acids and cardols (13), exhibit potent antibacterial activity against Gram-positive bacteria. Among them, the principal active compound in the cashew nut shell oil, anacardic acid, 6-[8(*Z*),11(*Z*),14-pentadecatrienyl]salicylic acid [**9**], was also tested in combination with totarol. Therefore, indole [**7**], anethole [**8**] and anacardic acid [**9**] were selected for testing in combination with totarol to determine their synergistic effect on the antibacterial activity of totarol.

Table 3 shows the MIC and MBC of totarol in combination with ½MIC of indole, anethole, or anacardic acid against *Sta. aureus* (a penicillin-susceptible strain) and *Str. mutans*. Most noticeably, the activity of totarol against *Sta. aureus* was increased 8-fold by anacardic acid [**9**]. The MIC and MBC were lowered from 1.56 to 0.2 μg/ml. The other combinations were not significant. Their MICs and MBCs were enhanced at most by twofold. The basis of these combination effects is currently under investigation.

TABLE 3. MICs of Totarol [**1**] in Combination with ½MICs of Indole [**7**], Anethole [**8**], and Anacardic Acid [**9**] Against *Staphylococcus aureus* and *Streptococcus mutans*.

Compound	MIC (μg/ml)	
	Sta. aureus[a]	*Str. mutans*
Indole	1.56→0.78	0.78→0.39
Anethole	1.56→1.56	0.78→0.39
Anacardic Acid	1.56→0.2	0.78→0.39

[a]*Sta. aureus* is a penicillin-susceptible strain.

EXPERIMENTAL

CHEMICALS.—Isolation and identification from the Et_2O fraction of the bark of *Pod. nagi* of the six diterpenoids **1**–**6** used for the assay have been previously described (9). Anethole [**8**] was previously isolated from the seeds of *Pim. anisum* (12). Anacardic acid, 6-[8(*Z*),11(*Z*),14-pentadecatrienyl]salicylic acid [**9**], was from our previous work (13). Indole **7** and penicillin G were purchased from Sigma Chemical Co. (St. Louis, MO). DMF was obtained from EM Science (Gibbstown, NJ).

MICROORGANISMS AND MEDIA.—The twelve microorganisms used for the assay were purchased from American Type Culture Collection (Rockville, MD). They are *Bac. subtilis* ATCC 9372, *Bre. ammoniagenes* ATCC 6872, *Sta. aureus* ATCC 12598 (a penicillin-susceptible strain) and *Sta. aureus* ATCC 29247 (a penicillin-resistant strain), *Pr. acnes* ATCC 11827, *Str. mutans* ATCC 25175, *Esch. coli* ATCC 13048, *Ps. aeruginosa* ATCC 10145, *Ent. aerogenes* ATCC 13048, *Sac. cerevisiae* ATCC 7754, *C. albicans* ATCC 18804, *Pit. ovale* ATCC 14521, and *Pen. chrysogenum* ATCC 10106. Their appropriate media were previously reported (11–13).

ANTIMICROBIAL ASSAY.—The bioassay was performed by a broth dilution method as previously described (14). The highest concentration tested was 400 μg/ml unless otherwise specified because of limited availability of some of the samples.

The MIC was measured by twofold serial broth dilution. It should be noted that the concentration of DMF in the medium was always 1% which did not affect the growth of any of the microorganisms tested. The MIC was the lowest concentration of sample in which no growth was visible.

The MBC was the lowest concentration of totarol that decreased the initial inoculum concentration by >99.9%. After determining the MIC, 10-fold dilutions from each test tube showing no turbidity were

plated onto the totarol-free Brain Heart Infusion (BHI) (DIFCO) agar medium. After 2 days of incubation, MBC break points were determined by using rejection values as previously described (15). MIC and MBC were determined in duplicate.

The synergism data against two bacteria, *Sta. aureus* and *Str. mutans*, were obtained by a broth checkerboard method (15). The twofold dilutions of totarol were tested in combination with concentrations of twofold dilutions of **7**, **8**, and **9**. Each bacterium was tested in duplicate.

ACKNOWLEDGMENTS

The authors are grateful to Mr. B.P. Ying for technical assistance, Prof. M. Kozuka and Dr. T. Nakatsu for collection of the plant, and Prof. K. Goto for identification of the plant. H.M. thanks Takasago Institute for Interdisciplinary Science for financial support.

LITERATURE CITED

1. M. Taniguchi, A. Chapya, I. Kubo, and K. Nakanishi, *Chem. Pharm. Bull.*, **26**, 2910 (1978).
2. B.P. Ying, I. Kubo, Chairul, T. Matsumoto, and Y. Hayashi, *Phytochemistry*, **29**, 3953 (1990).
3. I. Kubo and B.P. Ying, *Phytochemistry*, **30**, 1967 (1991).
4. I. Kubo, M. Sutisna, and K.S. Tan, *Phytochemistry*, **30**, 455 (1991).
5. K.S. Tan and I. Kubo, *Experientia*, **46**, 478 (1990).
6. I. Kubo, T. Matsumoto, and J.A. Klocke, *J. Chem. Ecol.*, **10**, 547 (1984).
7. I. Kubo, T. Matsumoto, F.J. Hanke, M. Taniguchi, and Y. Hayashi, *Experientia*, **41**, 1462 (1985).
8. I. Kubo, M. Himejima, and B.P. Ying, *Phytochemistry*, **30**, 1467 (1991).
9. B.P. Ying and I. Kubo, *Phytochemistry*, **30**, 1955 (1991).
10. I. Kubo and M. Taniguchi, *J. Nat. Prod.*, **51**, 22 (1988).
11. I. Kubo, H. Muroi, and M. Himejima, *J. Agric. Food Chem.*, **40**, 245 (1992).
12. I. Kubo and M. Himejima, *J. Agric. Food Chem.*, **39**, 2290 (1991).
13. M. Himejima and I. Kubo, *J. Agric. Food Chem.*, **39**, 418 (1991).
14. M. Taniguchi and Y. Satomura, *Agric. Biol. Chem.*, **36**, 2169 (1972).
15. C.W. Norden, H. Wentzel, and E. Keleti, *J. Infect. Dis.*, **25**, 629 (1979).

Received 2 March 1992