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J. Nat. Prod., **1992**, 55 (6), 780-785• DOI:
10.1021/np50084a012 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 55, No. 6, pp. 780-785, June 1992

ANTIBACTERIAL ACTIVITY OF CRINITOL AND ITS POTENTIATION

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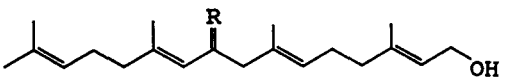
ABSTRACT.—An acyclic diterpene alcohol, crinitol [**1**], was identified in a marine brown alga, *Sargassum tortile*, as the principal antibacterial agent against Gram-positive bacteria, among which *Propionibacterium acnes* was most sensitive and, *Staphylococcus aureus* was least. To enhance its activity, crinitol was combined with several antioxidants, which presumably retard oxidative destruction of this molecule which possesses two easily oxidizable allylic alcohol groups. Two synthetic antioxidants, BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene), significantly enhanced the activity of crinitol, especially against *Streptococcus mutans*. Interestingly, crinitol also synergized BHT and BHA against this cariogenic bacterium.

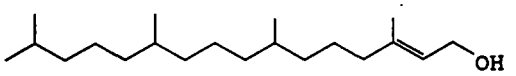
The marine brown alga *Sargassum tortile* C. Agardh (Sargassaceae), which is known as "yoremoku" in Japan, is commonly found on rocks from below the low-tide level to depths of several meters or more.

An acyclic diterpene alcohol, crinitol [**1**], was first isolated from one of the most studied brown algae, *Cystoseira crinita* (Cystoseiraceae) (1). Interestingly, the same compound was also identified in *Sarg. tortile* as an insect growth inhibitor against the pink bollworm *Pectinophora gossypiella* (2) by an artificial diet feeding assay (3).

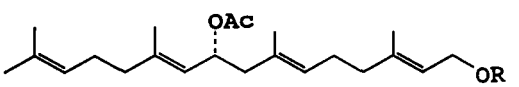
In our continuing search for new antimicrobial agents from botanical sources, crinitol was also found to exhibit moderate activity against *Bacillus subtilis*, a Gram-positive bacterium. In order to further evaluate the biological activity of crinitol against both bacteria and insects, more *Sarg. tortile* was collected from different geographic locations along coasts of the Pacific Ocean and the Sea of Japan during different seasons. The isolation of crinitol was monitored by an antibacterial assay against *Ba. subtilis*. As a result of this study, the quantity of crinitol in *Sarg. tortile* was also analyzed in terms of its geographical and season variation.



1 R=H, α=OH
2 R=H, H
4 R=O



3



5 R=H
6 R=Ac

We can control many troublesome microorganisms that contaminate food and cosmetics with antimicrobial agents that are currently available. Many of these agents are composed of plant secondary metabolites and their analogues such as hinokitiol, sorbic acid, salicyclic acid, *p*-hydroxybenzoic acid, and benzoic acid, and/or their various derivatives. Nevertheless, the need for new antimicrobial agents in cosmetic products still exists. The use of preservatives to control microorganisms that putrefy cosmetic products is a major problem which has yet to be solved. In the case of cosmetics, the control of specific microorganisms that cause skin, hair, and tooth infections is becoming even more important. With this in mind, twelve microorganisms were selected for our antimicrobial assay (4).

The application of phytochemicals to cosmetic products has produced a keen interest. A large number of phytochemicals have already been isolated as antimicrobial agents. Needless to say, they are all biodegradable and, more importantly, renewable. However, their biological activity is usually not potent enough to be considered for practical applications. The moderate antibacterial activity of crinitol is an example of this lack of potency. For this reason, an attempt to enhance crinitol's antibacterial activity by combining it with other substances was made. We combined crinitol with several antioxidants to, presumably, retard oxidative destruction of this molecule, which possesses two easily oxidizable allylic alcohol groups. This attempt was based on the fact that oxidation is, in general, one of the most important detoxification (or metabolic) pathways in living organisms.

EXPERIMENTAL

CHEMICALS.—Vitamins C and E, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO). DMF was purchased from EM Science (Gibbstown, NJ).

PLANT MATERIAL.—*Sarg. tortile* was collected at low tide from the beaches of Kata, Wakayama (the Pacific Ocean side of Japan), on September 18, 1982 (1.2 kg) and April 16, 1983 (2 kg), and of Maizuru, Kyoto (the Japan Sea side), on October 11, 1988 (2 kg) and April 11, 1989 (1.3 kg). The specimens were identified by Dr. H. Nakahara, Kyoto University, where the voucher specimens were deposited. The freshly collected samples were placed in brown bottles, covered with MeOH for up to 4 h, and stored prior to workup at ambient temperature. In addition, *Sargassum muticum* was also collected at low tide from beaches in the vicinity of Trinidad Head, California on November 15, 1990 by Prof. W.F. Wood of Humboldt State University, California. These plant samples were treated the same way as those described above.

EXTRACTION AND ISOLATION.—The above-mentioned *Sarg. tortile* was chopped finely and extracted with MeOH at ambient temperature. The aqueous MeOH solution was concentrated under reduced pressure, and the resulting extract was partitioned between H_2O and *n*-hexane to remove non-polar substances. The aqueous fraction was concentrated to about one third, saturated with NaCl, and then extracted with CH_2Cl_2 repeatedly. The CH_2Cl_2 extracts of the algae collected from different places and seasons yielded the following: extract A, 14.5 g (12.1 g/kg fresh wt), from Wakayama, September 1982; extract B, 18.6 g (9.3 g/kg fresh wt), from Kyoto, April 1983; extract C, 16.8 g (8.1 g/kg fresh wt), from Kyoto, October 1988; and extract D, 11.0 g (8.5 g/kg fresh wt), from Kyoto, April 1989. Interestingly, of these four CH_2Cl_2 extracts, A and B exhibited antibacterial activity against *Ba. subtilis*, whereas C and D did not show any activity. The antibacterial CH_2Cl_2 fractions, A and B, were then chromatographed on Si gel cc repeatedly, eluting with *n*-hexane containing increasing amounts of CH_2Cl_2 (50–100%) to yield 1.1 g and 2.1 g of the almost pure active principle from 1.2 g of A and 3.5 g of B, respectively. This antibacterial substance was identified as crinitol [**1**] by comparison of spectroscopic data (ir, eims, ^1H and ^{13}C nmr) with those of an authentic sample (2). During its structural elucidation, most of the ^{13}C-nmr assignments were established as follows: δ 15.92, 16.20, and 16.57 (Me-3, -7, or -11), 17.64 (Me-15), 25.61 (C-16), 25.96 (C-5), 26.46 (C-13), 39.23 and 39.54 (C-4 or -12), 48.12 (C-8), 59.22 (C-1), 65.65 (C-9), 124.02 (C-14), 124.57 (C-2), 127.22 (C-10), 128.12 (C-6), 131.57 (C-11), 132.26 (C-15), 138.19 (C-3), 138.94 (C-7).

In addition, two similar acyclic diterpene alcohols, geranylgeraniol [**2**] (8 mg) and phytol [**3**] (16 mg), were isolated from fraction A after repeated Si gel cc elution with *n*-hexane containing increasing concentrations of CH_2Cl_2 (0–50%). These common diterpene alcohols were also isolated from fraction B.

However, neither of them could be isolated from fractions C or D. These diterpene alcohols were identified by comparison of spectroscopic data with those of authentic samples (ir, eims). The compounds **4–6** utilized for additional antimicrobial assays for comparison purposes were obtained from previous studies (2). *Sarg. muticum* was also treated similarly.

ANTIMICROBIAL ASSAY.—*Test organisms.*—Twelve microorganisms used for the antimicrobial assay were purchased from American Type Culture Collection (Rockville, MD). They are *Bacillus subtilis* ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Propionibacterium acnes* ATCC 11827, *Staphylococcus aureus* ATCC 12598, *Streptococcus mutans* ATCC 25175, *Escherichia coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, *Saccharomyces cerevisiae* ATCC 7754, *Candida albicans* ATCC 18804, *Pityrosporum ovale* ATCC 14521, and *Penicillium chrysogenum* ATCC 10106.

To reactivate the freeze-dried microorganisms, *Ba. subtilis, Sacc. cerevisiae, C. albicans, Pit. ovale,* and *Pen. chrysogenum* were cultured with shaking at 30°. *Br. ammoniagenes* and *En. aerogenes* were cultured in stationary culture at 30°, and other microorganisms were cultured in stationary culture at 37°.

Media.—The culture media for the bacteria consisted of 0.8% nutrient broth (BBL), 0.5% yeast extract (Difco) and 0.1% glucose, except for the case of *Strep. mutans.* For the culture of *Strep. mutans,* 3.7% brain heart infusion broth (Difco) was utilized. The culture media for the fungi consisted of 2.5% malt extract broth (BBL), except for the case of *Pit. ovale.* For the culture of *Pit. ovale,* 1% bactopeptone (Difco), 0.5% yeast extract, 1% glucose, and 0.1% corn oil were used.

Method.—Because most of the compounds tested are hardly soluble in H_2O, the paper disk method is not an adequate assay, because an H_2O-insoluble compound cannot disperse into the media. Therefore, throughout this experiment, the bioassays were performed by a broth dilution method (5). The test compounds were first dissolved in DMF and diluted twofold; then 30 μl of each sample solution was added to 3 ml of the appropriate broth medium, to which 30 μl of 2-day-old test microorganisms (5-day-old *Pen. chrysogenum*) was inoculated. The lowest concentration of the test compounds in which no growth occurred was defined as the minimal inhibition concentration (MIC). The highest concentration used for the assay was 800 μg/ml because of the limited availability and solubility in the H_2O-based media of the samples. The concentration of DMF in the broth media was always 1%, which did not affect the growth of any of the test microorganisms employed.

For the antimicrobial assay, the microorganisms were cultured in stationary culture, except *Pen. chrysogenum,* which was cultured with shaking. After 2 days cultivation with the test substances (5 days for *Pen. chrysogenum*), the growth of microorganisms, except for *Pen. chrysogenum* and *Pit. ovale,* was examined by turbidity (OD at 660 nm). That of the two fungi was examined with the naked eye.

The combination data were obtained by the checkerboard method (6). The twofold dilutions of the crinitol were tested in combination with concentrations of twofold dilutions of one of the antioxidants. Each compound was tested against each bacterium at least twice.

RESULTS AND DISCUSSION

Sarg. tortile was collected from two different geographic locations in Japan, one at Kata, Wakayama, which is along the coast of the Pacific Ocean, and the other near Maizuru, Kyoto, which is along the coast of the Sea of Japan. The antibacterial acyclic diterpene alcohol, crinitol [**1**] was isolated as the only active principle based on bioassay-guided fractionation of the CH_2Cl_2 extracts of *Sarg. tortile* collected during both spring and fall at Kata. Crinitol was not only the antibacterial principle; it was also one of the major compounds (yielded about 1% of the fresh wt) from *Sarg. tortile* collected at Kata. However, it could not be isolated from those *Sarg. tortile* collected near Maizuru, regardless of the season. Although seasonal variation of crinitol in *Sarg. tortile* was not observed, the geographical variation was remarkable. This variation could be caused by genotypic and/or by phenotypic factors. These results seem to be interesting from a chemotaxonomic point of view, since species of *Sargassum* are often difficult to identify. It should, however, be noted that geographical and seasonal variation among marine natural products seems to be common. Morphologically, it is noticeable that *Sarg. tortile* grows better around Kata (the Pacific Ocean variety) than Maizuru (the Sea of Japan variety); the water temperature around the latter is cooler than the former. Thus, the algae collected from around Kata were larger than those of Maizuru.

The observed geographical variation of crinitol in *Sarg. tortile* may be caused by an

environmental difference, if crinitol is hypothesized to be a defense substance. The water temperature around Kata is warmer than around Maizuru, and bacteria at the former location may be more active than at the latter. If this hypothesis is true, then the algae growing around Kata may need more antibacterial agent to combat them. Clearly, further study is needed to confirm this.

In addition to crinitol, two other minor acyclic diterpene alcohols, geranylgeraniol [**2**] and phytol [**3**], were isolated from the active fraction, although neither of them exhibited any activity up to 800 μg/ml in our preliminary assay against four representative microorganisms (7). Nevertheless, more detailed antimicrobial activity of the purified crinitol together with these two acyclic diterpene alcohols was examined against the twelve selected microorganisms. The results are shown in Table 1. Crinitol exhibited weak to moderate activity against all the Gram-positive bacteria, *Ba. subtilis*, *Br. ammoniagenes*, *Strep. mutans*, *Staph. aureus*, and *Pr. acnes*, but not the Gram-negative bacteria, *En. aerogenes*, *Ps. aeruginosa* and *Esch. coli*, and fungi, *Sacc. cerevisiae*, *C. albicans*, *Pit. ovale*, and *Pen. crysogenum*. Among the Gram-positive bacteria, *Pr. acnes* was the most sensitive bacterium, with an MIC of 25 μg/ml. By contrast, *Staph. aureus* was the least sensitive, with an MIC of 400 μg/ml. Crinitol was found to be bactericidal against *Pr. acnes* (8). This was determined as follows: 30 μl taken from the medium that was incubated for 2 days with the MIC of crinitol was added into 3 ml of the crinitol-free fresh medium and incubated for 2 days. No recovery of *Pr. acnes* was observed.

TABLE 1. MICs (μg/ml) of Crinitol [**1**] Alone and in Combination with ½MICs of BHA and BHT.

Microorganism tested	Compound		
	1	**1** + BHA	**1** + BHT
Bacillus subtilis	50	50	12.5
Brevibacterium ammoniagenes	100	100	50
Staphylococcus aureus	400	400	400
Streptococcus mutans	50	12.5	0.78
Propionibacterium acnes	25	6.25	6.25

As expected, the two structurally related compounds, geranylgeraniol [**2**] and phytol [**3**], did not show any activity even against the same Gram-positive bacteria up to 800 μg/ml. In addition, several derivatives **4**–**6** of crinitol were assayed. The results are as follows. An additional hydroxy group at C-9 in **1** seemed to be essential for the antibacterial activity. In fact, the oxidized compound, 9-oxocrinitol [**4**] and mono- and diacetyl derivatives **5** and **6** did not exhibit any activity up to 800 μg/ml.

Although crinitol can be easily obtained in relatively large quantity from *Sarg. tortile* or other sources (1), its antibacterial activity may not be potent enough to be considered for practical use, even though it may play an important role in the defense of living plants. Hence, the enhancement of the antibacterial activity of crinitol by combining it with other substances was studied (9), although the rationale for selecting "other substances" is still in an embryonic stage.

The initial selection of "other substances" was based largely on the aforementioned structure-activity relationship study. Thus, an additional hydroxy group at C-9 in crinitol seems to be essential to its antibacterial activity. The C-9 hydroxy group is a so-called allylic alcohol, which is, chemically, easily oxidized. Because crinitol possesses two allylic alcohol groups in its molecule (at C-1 and C-9), we combined it with several antioxidants which have been used as food and cosmetic additives. Thus two natural antioxidants, vitamins C and E, and two synthetic antioxidants, BHA and BHT, were

chosen for the experiment. While the synthetic antioxidants themselves exhibited antimicrobial activity against all the test bacteria, natural antioxidants did not show any activity up to 800 μg/ml. Nevertheless, the antimicrobial activity of BHA and BHT is well documented (10).

In combination with crinitol, neither of the natural antioxidants (vitamins C and E) showed any enhancing activity against the bacteria tested. Neither synthetic antioxidant exhibited any synergistic effect against *Staph. aureus* at all.

Table 1 shows the MIC of crinitol in combination with the two synthetic antioxidants (BHA and BHT) at the concentration of each ½MIC. The activity of crinitol against *Strep. mutans* was significantly increased by both synthetic antioxidants. The activity of crinitol against this cariogenic bacterium was increased 64-fold by BHT; the MIC was lowered from 50 to 0.78 μg/ml. Similarly, BHA also synergized crinitol against *Strep. mutans*, but not as much as BHT; the MIC was reduced only from 50 to 12.5 μg/ml. This result indicates that BHT was the most effective enhancer of crinitol against *Strep. mutans*. In the case against three other bacteria, *Ba. subtilis*, *Br. ammoniagenes*, and *Pr. acnes*, the enhancing activity of BHA and BHT to crinitol was only 2–4-fold.

Interestingly, crinitol also enhanced the activity of BHT and BHA against *Strep. mutans* 16- and 8-fold, respectively (Table 2). The MICs were reduced from 25 to 1.57 and from 100 to 12.5 μg/ml, respectively. Crinitol also enhanced their activity against *Ba. subtilis* and *Br. ammoniagenes*, but the effect was only additive. Against *Pr. acnes*, crinitol lowered the MICs of BHT and BHA from 25 to 6.25 and from 50 to 6.25 μg/ml, respectively. These MICs were, however, gradually increased when the assay was of longer duration, indicating that the combination was not bactericidal against these bacteria. The enhancing activities of BHA and BHT seem to depend on the chemicals that they are combined with. The rational basis of these combination effects is currently under investigation.

TABLE 2. MICs (μg/ml) of BHA and BHT in Combination with ½MIC of Crinitol [**1**].

Microorganism tested	Compound			
	BHA	BHA + **1**	BHT	BHT + **1**
Bacillus subtilis	100	50	25	6.25
Brevibacterium ammoniagenes	200	100	25	12.5
Staphylococcus aureus	200	200	>800	—
Streptococcus mutans	100	12.5	25	1.57
Propionibacterium acnes	50	6.25	50	6.25

Combining two or more substances to enhance the total biological activity may not only be a most efficient way to use natural products, but also the bacteria may take longer to develop their resistance to two or more toxins whose mode of action is diverse. Yet another benefit of utilizing BHA and BHT is their antioxidant activity.

Based on these findings, we also collected several other species of *Sargassum* algae along the coast of the Western United States. For example, *Sarg. muticum* was collected near Trinidad, California. Although its *n*-hexane extract exhibited weak antibacterial activity against *Ba. subtilis*, its CH_2Cl_2 extract (from which crinitol was isolated from *Sarg. sargassum*) showed no activity. More specifically, crinitol was not identified in *Sarg. muticum* at all.

ACKNOWLEDGMENTS

This work is a result of research sponsored in part by NOAA, National Sea Grant College Program,

Department of Commerce, under grant number NA85AA-D-SG140, project number R/MP-32, through the California Sea Grant College Program, and in part by the California State Resources Agency. The U.S. Government is authorized to reproduce and distribute this material for government purposes. The authors are indebted to Dr. H. Nakahara and Prof. W.F. Wood for plant collection and identification, and to Prof. Y. Hayashi and Mr. H Naoki for nmr measurements.

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Received 15 October 1991