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Isolation and Antibacterial Activity of Acylphloroglucinols from *Myrtus communis*

A. ROTSTEIN, A. LIFSHITZ, AND Y. KASHMAN

Departments of Biochemistry and Chemistry, Tel-Aviv University, Tel-Aviv, Israel

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Isolation procedures of two new acylphloroglucinols, myrtucommulone-A and myrtucommulone-B, from *Myrtus communis* leaves are given. Myrtucommulone-A was highly antibacterial against gram-positive bacteria but was not active against gram negatives. The chemical relation to other acylphloroglucinols and the antibacterial activity of the compounds isolated are discussed.

The occurrence of antibacterial and fungitoxic compounds in higher plants has been demonstrated in many instances (5, 6, 9, 16). These compounds may have significant physiological roles such as protection against plant pathogens (2, 18).

As part of the work done in our laboratory to isolate antibacterial compounds with possible pharmaceutical value (10, 15), various sources were investigated. Promising results were obtained from the total chloroform and ethanol extracts of *Myrtus communis* leaves. The occurrence of antibacterial activity in this plant was previously reported by Russian workers (1, 3), but no chemical structure or isolation procedure of the substances responsible was given.

We report here the isolation procedure and biological activity of two compounds isolated from *M. communis* leaves and named myrtucommulone-A and myrtucommulone-B (11) (Fig. 1).

M. communis is a shrubby plant belonging to the family *Myrtaceae* subfamily *Myrtoideae* and is widely distributed in the Mediterranian area.

MATERIALS AND METHODS

Isolation procedures. The criterion of a biological assay was used as a guide in the isolation procedure. Every stage of the isolation was followed by an assay for antibacterial activity, and the results determined the direction and development of the work. Once a small quantity of the pure active compound was obtained, its chromatographic behavior and some of its chemical characteristics were established. These later served as criteria for seeking less complicated and higher-yielding isolation procedures.

Myrtucommulone-A: procedure (i). A total of 500 g of dried, powdered leaves of *M. communis* was extracted with 5 liters of chloroform. The extract was evaporated under reduced pressure and the residual green, oily material was dissolved in 0.5 liter of warm ether. The ether was extracted with 3 volumes of 100 ml of 4% NaOH. After filtration of the precipitating compounds, the basic water phase was reextracted with a small volume of ether, which was discarded. The basic water solution was then acidified with HCl (1:1) and extracted with 0.5 liter of ether, which was dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The remaining amorphous mass was dissolved in hot MeOH, from which yellow-brown crystals of myrtucommulone-A precipitated. Myrtucommulone-A thus obtained was further purified by repeating part of the isolation procedure, namely by dissolving it in 4% NaOH, extracting impurities with ether, and then acidifying the basic water phase, extracting it with ether, and crystallizing it from MeOH, $CHCl_3$, or MeOH. Myrtucommulone-A was found to have the chemical formula $C_{35}H_{52}O_{10}$, a melting point at 186 C and a molecular weight of 668.

Procedure (ii). To verify that the basic and acidic treatments used in procedure (i) did not cause any change in the natural product, procedure (ii) was used. Two grams of the green, oily material obtained after evaporating the chloroform extract was separated on a silica column (100 g of Kieselgel, 0.06 to 0.2 mm; Merk & Co.). Elution with benzene caused a yellow band, which was discarded, to descend quickly. The elution was continued with chloroform. Two green chlorophyl bands appeared, and under the lower one a yellow band separated. This yellow band was shown by thin-layer chromatography (TLC) to contain myrtucommulone-A. The band was collected and evaporated to dryness, and the residue was submitted to crystallization from MeOH to give myrtucommulone-A. Myrtucommulone-A obtained in the two procedures was proved to be identical.

Myrtucommulone-B. TLC on basic silica plates (according to Stahl and Schorn [20]) showed the presence of an additional compound accompanying myrucommulone-A ($R_f \simeq 0.8$ developed in EtOAc red with blue salt B).

After myrtucommulone-A was filtered off, the MeOH solution was evaporated to dryness, dissolved in a small volume of EtOAc, and filtered through a small column (3 by 3 cm) of basic silica (150 g Kieselgel 60 [Merk & Co.], 0.06 to 0.2 mm suspended

in 200 ml of 0.3 M CH_3COONa and dried at 110 C overnight), and eluted with EtOAc. The solution obtained was further separated on a larger basic silica column (30 by 1.5 cm) eluting with EtOAc. The first concentrated, fast-moving band was collected and served as material for preparatory separation on basic TLC plates developed in EtOAc. The proper band was scraped off and extracted with chloroform, which was then evaporated, leaving amorphous myrtucommulone-B. Amorphous myrtucommulone-B was found to have the chemical formula $C_{34}H_{50}O_6$ and a molecular weight of 442.

Antibacterial bioassay: Paper disk inhibition zone. Paper disks of 6-mm diameter made from Whatman no. 3 filter paper and containing the compound tested were placed on seeded nutrient agar plates. The inhibition zones were recorded after 24 h of incubation.

For following the isolation procedure, four test microorganisms were used: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Saccharomyces cerevisiae*. The microorganisms were grown on slants for 2 days. The slants were washed with 10 ml of saline, and the dispersion obtained was diluted 10^4-fold. A 0.1-ml amount of this dilution was used for seeding each plate. A wider spectrum of microorganisms was used to test the pure compound (Table 1).

Growth curve. Microorganisms were grown in nutrient broth, and the turbidity changes were followed by using a Klett instrument (filter, 540 nm). Myrtucommulone-A was added dissolved in EtoH, and proper controls were run.

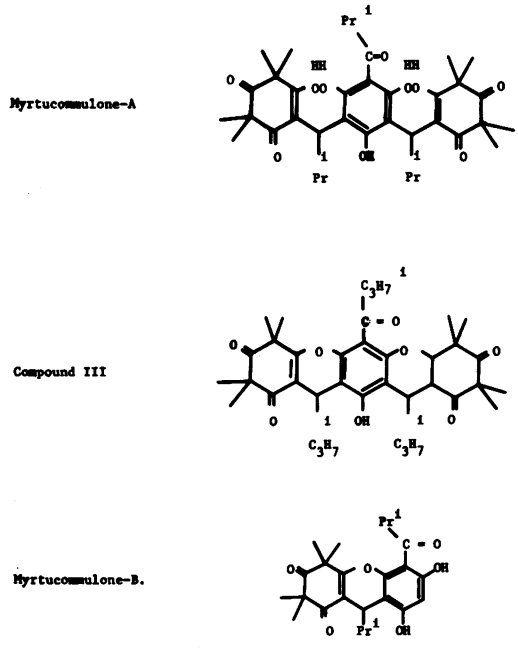

FIG. 1. *Structures of myrtucommulone-A, compound III, and myrtucommulone-B.*

RESULTS AND DISCUSSION

Table 1 records the sensitivity of 20 test microorganisms to myrtucommulone-A. The gram-positive bacteria were profoundly effected by myrtucommulone-A. The effect was of the same magnitude as that of penicillin or streptomycin. None of the gram-negative bacteria were affected.

Figure 2 shows the effect of various concentrations of mytrucommulone-A on the growth of *B. subtilis*. Concentrations as low as 0.5 μg/ml showed clear inhibition. Similar results were obtained with *S. aureus*. With both test microorganisms, 10 μg/ml was more than enough for total inhibition.

Addition of 20 μg of myrtucommulone-A per ml to a growing culture of *B. subtilis* stopped growth (Fig. 3).

The effect of blood on the antibacterial activity of myrtucommulone-A was tested. Nutrient agar containing 50% whole human blood to which various concentrations of myrtucommulone-A were added was prepared. The microorganisms were added to the solid medium suspended in a drop of saline, and after incubation the plates were inspected for growth. Inhibition of growth in the control plates was caused at

TABLE 1. *Effect of myrtucommulone-A on various organisms*

Organism	Inhibition zone (mm)		
	Myrtucommulone-A (80 μg/disk)	Penicillin (10 U/disk)	Streptomycin (10 μg/disk)
Escherichia coli B		22	16
E. coli CW 3747		11	18
E. coli K-12		8	18
Klebsiella pneumoniae			12
Proteus mirabilis		22	13
Proteus morganii		14	10
Shigella dysenteriae		18	14
S. flexneri		9	12
Salmonella typhimurium		26	18
Pseudomonas fluorescens		8	8
Vibrio cholerae		20	26
Serratia			9
Staphylococcus aureus	35	46	22
S. albus	13	40	18
Bacillus subtilis W23	28	32	24
B. subtilis 16	25	32	19
B. pumilus	25	32	16
Streptococcus faecalis	24	50	30
Corynebacterium diphtheriae	30	40	20
C. xerosis	28	42	

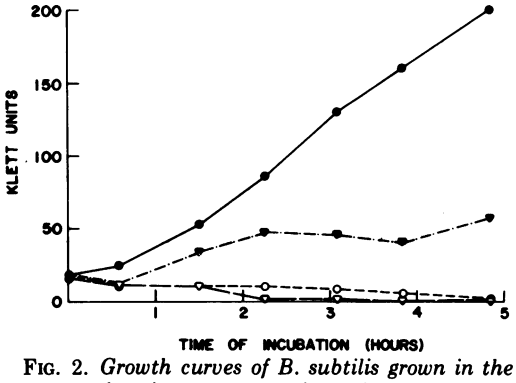

FIG. 2. *Growth curves of B. subtilis grown in the presence of various concentrations of myrtucommulone-A. Symbols:* ▽, *10 μg/ml;* ○, *0.5 μg/ml;* ▼, *0.1 μg/ml;* ●, *control.*

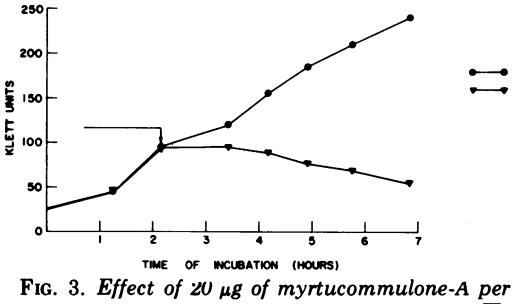

FIG. 3. *Effect of 20 μg of myrtucommulone-A per ml on a growing culture of B. subtilis. Symbols:* ▼, *20 μg/ml;* ●, *control. The arrow shows the point of myrtucommulone-A addition.*

concentrations as low as 1 μg/ml, but in the blood-containing plates, the antibacterial activity was totally lost and growth occurred even at concentrations as high as 1,000 μg of myrtucommulone-A per ml.

In vivo infections in mice were caused by intraperitoneal injection of *S. aureus* (susceptible in vitro). Myrtucommulone-A was applied externally as a paste. All results were negative.

Myrtucommulone-A and -B are chemically related to acylphloroglucinols of ferns (17) and also to the uliginosins (16) and Kosins (13) isolated from higher plants. Some of these compounds are known to have antibacterial properties (22, 23), e.g., aspidind, desaspidin, paraaspidin, filixic acid, flavaspidic acid, uliginosin-I, and others (Fig. 4). Many other phenolic and polyphenolic compounds like hummulon and lupulone (19), flavonoids (4), and many phytoalexines like orchinol, phaseollin, chlorogenic acid, caffeic acid, and others are known to have antibacterial and fungitoxic properties.

The role of phenolic compounds in plant pathology is the subject of many publications (2, 7, 12, 18, 21). As a general rule, phenolic compounds are more active against gram-positive than gram-negative bacteria (22), and the presence of organic matter such as milk or serum effectively reduces their antibacterial activity (8). This may explain the ineffectiveness of myrtucommulone-A and uliginosin-I in

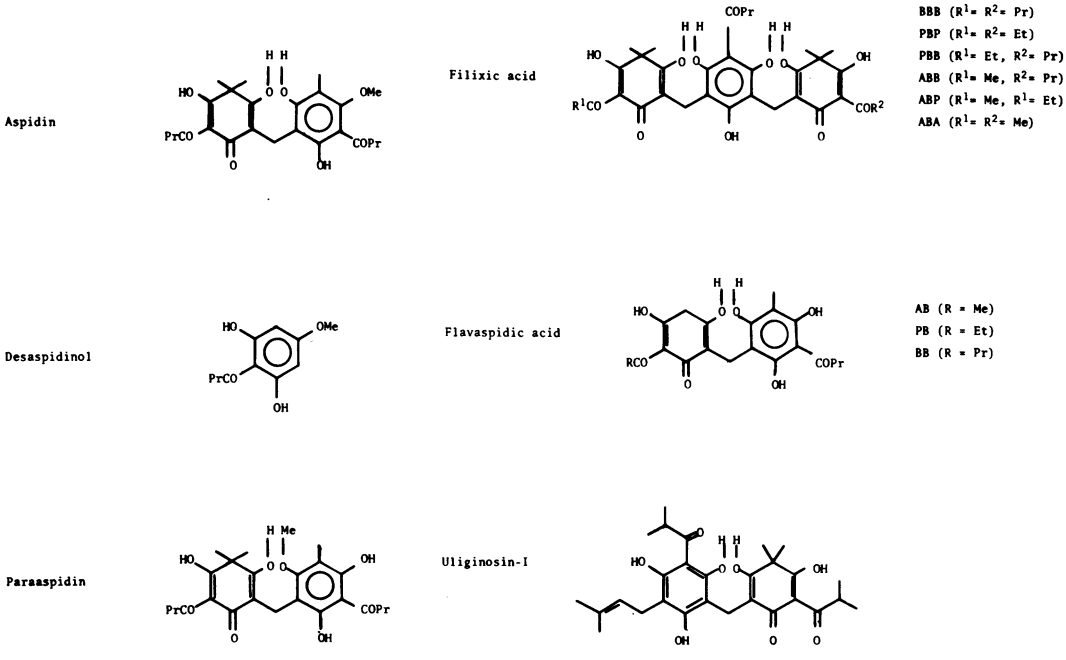

FIG. 4. *Structures of antibacterial acylphloroglucinols.*

combatting in vivo infections, as well as the negative results obtained with blood-containing medium.

To better understand the importance of the phenolic nature of myrtucommulone-A for its antibacterial activity, compound III was prepared (Fig. 1). This was done by boiling myrtucommulone-A in benzene in the presence of p-TsOH or in alcohol in the presence of diluted HCl. The formation of compound III from myrtucommulone-A can be explained by the acid-catalyzed hemiketal formation between the phenolic hydroxyl and the neighboring ketone followed by elimination of water. Compound III, from which four phenolic groups were removed, had no antibacterial activity, and myrtucommulone-B had very little. In the face of the known importance of the surface-active character of the phenolic compounds for antibacterial activity, the loss of activity caused by the above transformation is clear.

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