New Semisynthetic Antimicrobial Labdane-Type Diterpenoids Derived from the Resin "Ladano" of *Cistus creticus*

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The antimicrobial activity of fifteen semisynthetic labdane-type diterpenes derived from the two major natural compounds **3** and **4** of the resin "ladano" of *Cistus creticus* is reported. The chloroethyl carbamidic esters **15** and **20** showed the strongest antimicrobial activity against Gram (+), Gram (–) bacteria and pathogenic fungi.

Introduction

Cistus creticus subsp. creticus L (Warburg, 1968) is a native shrub of the Mediterranean region. Its leaves are covered with glands secreting a brownish resin, which consists mainly of diterpenoids. Since antiquity the common greek name is "ladano". The chemical composition of the plant as well as of the resin has been the object of a few publications in our laboratory (Kalpoutzakis *et al.*, 1998; Demetzos *et al.*, 1989; Demetzos *et al.*, 1990; Demetzos *et al.*, 1994; Chinou *et al.*, 1994). On the other hand the antibacterial activity of labdane-type diterpenoids is well documented and diterpenes from Cistus creticus have been proved to possess interesting antibacterial properties (Kalpoutzakis *et al.*, 1998;. Chinou *et al.*, 1994).

As a part of our program concerning the discovery of new antimicrobial agents of natural origin, the antimicrobial activity of ten natural and fifteen semisynthetic compounds derived from the resin "ladano" of Cistus creticus is reported here. As starting material, for the synthetic compounds, 13(*E*)-labda-7,13-diene-15-ol (**1**), and 13(*E*)-labd-13-ene-8α,15-diol (**2**) have been used. These compounds were found as natural products in the leaves of *Cistus* creticus (Demetzos *et al.*, 1990) but also were obtained in quantitative yield by alkaline hydrolysis of the corresponding malonic esters **3** and **4** respectively. It is noteworthy that **3** and **4** are the major compounds of the resin "ladano" and constitute the 29% of the crude extract (Kalpoutzakis *et al.*, 1998).

Results and Discussion

A total of ten 13(*E*)-labda-7,13-diene-15–ol, and of eleven 13(*E*)-labd-13-ene-8α,15-diol, derivatives were isolated or prepared; the starting alcohol **1**, **2**, eight carboxylic esters, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, eleven carbamidic esters, **11**, **12**, **13**, **14**, **15**, **16**, **17**, **18**, **19**, **20** and **21**. Compounds **1**, **2**, **3**, **4**, **5** and **8** are natural diterpenes isolated from the resin "Ladano" and from the aerial parts of *Cistus creticus* subsp. *creticus*. All the others, are new semisynthetic compounds derived from **1** and **2**. All these components were identified by means of spectral data (CIMS, EIMS, 1D and 2D NMR). On the basis of these spectral data some previous ^{1}H and ^{13}C assignments for the natural compounds **1** (Demetzos *et al.*, 1990), **2** (Calabuic *et al.*, 1981; Forster *et al.*, 1985), **5** (Demetzos *et al.*, 1990) and **8** (Calabuic *et al.*, 1981) have been revised. The carboxylic esters were synthesized by treatment of the alcohols, with the corresponding anhydrides or chlorides. The carbamidic derivatives were synthesized by treatment of **1** and **2** with the corresponding isocyanate derivatives in the presence of pyridine (Antonini *et al.*, 1988). It is interesting to point out that prolonged reaction time and heating of **2**, yielded 15, 8 disubstituted compounds, such as **21**.

The antimicrobial activity of the tested compounds **1**–**21** (Table I) was evaluated against two Gram positive bacteria: *Staphylococcus aureus*, *Staphylococcus epidermidis*, four Gram negative bacteria: *Escherichia coli*, *Enterobacter cloacae*,

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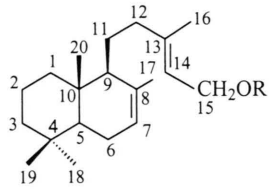


1 R = H

3 R = $COCH_2COOH$

5 R = $COCH_3$

6 R = COC_6H_5

7 R = $COC_6H_4NO_2$

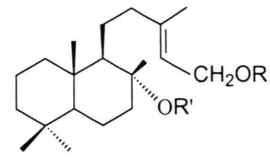
11 R = $CONHC_6H_5$

12 R = $CONHC_6H_4OCH_3$

13 R = $CONHCH_2(CH_2)_{10}CH_3$

14 R = $CONHCH_2(CH_2)_3CH_3$

15 R = $CONHCH_2CH_2Cl$



2 R = H, R' = H

4 R = $COCH_2COOH$, R' = H

8 R = $COCH_3$, R' = H

9 R = COC_6H_5, R' = H

10 R = $COC_6H_4NO_2$, R' = H

16 R = $CONHC_6H_5$, R' = H

17 R = $CONHC_6H_4OCH_3$, R' = H

18 R = $CONHCH_2(CH_2)_{10}CH_3$, R' = H

19 R = $CONHCH_2(CH_2)_3CH_3$, R' = H

20 R = $CONHCH_2CH_2Cl$, R' = H

21 R = R' = $CONHC_6H_5$

Klebsiella pneumoniae, *Pseudomonas aeruginosa* and three pathogenic fungi: *Candida albicans*, *Candida tropicalis* and *Torulopsis glabrata*. As far as structure-activity relationships are concerned, it is interesting to point out that i) while 13(*E*)-labda-7,13-diene-15-ol (**1**) was completely inactive, all the natural and semisynthetic derivatives (with one exception, the benzoic ester **6**) presented a wide spectrum of activities, against both Gram (+) and Gram (−) bacteria ii) the 13(*E*)-labd-13-ene-8α,15-diol (**2**) showed interesting activity against *S. aureus, K. pneumoniae and P. aeruginosa* and from its semisynthetic derivatives the p-nitrobenzoic ester **10**, and the carbamate esters **19** and **20** exhibited a good spectrum of activity. It is also noteworthy that while the 13(*E*)-labd-13-ene-8-α-ol-15-yl phenylcarbamate (**16**) is completely devoid of antibacterial activity, the corresponding diphenylcarbamate **21** exhibited a very interesting spectrum of activity iii) from all the semisynthetic compounds only the two chloroethyl carbamate derivatives **15** and **20** exhibited a good antimicrobial activity against the three pathogenic fungi iv) from the natural compounds 13(*E*)-labd-13-ene-8α,15-diol (**2**) showed a specific activity against the two tested *Candida* species v) the less active class of derivatives was the benzoic esters, **6** and **9**. The evaluation of the chloroethyl carbamate derivatives against clinical-isolated resistant bacterial strains and the synthesis of new analogs are currently in progress in our laboratory.

Experimental

General experimental procedures

Optical rotations were measured with a Perkin-Elmer 341 polarimeter. NMR spectra were recorded on a Bruker AC200 spectrometer and a Bruker DRX400. Chemical shifts are given in δ values with TMS as internal standard. The 2D experiments (COSY, HMQC and HMBC) were performed using standard Bruker microprograms. Mass spectra were recorded with a Nermag R 10–10C spectrometer using EIMS and CIMS (reagent gas, NH_3) technique. Column chromatography was conducted using silica gel [Merck, 0.04–0.06 mm].

Table I. Antimicrobial activity[a] of compounds **1–21**.

COMPOUND	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Escherichia coli*	*Klebsiella opneumoniae*	*Pseudomonas aeruginosa*	*Enterobacter cloacae*	*Candida albicans*	*Torulopsis glabrata*	*Candida tropicalis*
1	–	–	–	–	–	–	–	–	–
2	25	–	–	28	20	–	18	–	20
3	28	–	8	24	19	8	–	–	–
4	28	–	–	22	22	–	–	–	–
5	22	–	–	26	22	–	–	–	–
6	–	–	–	–	–	–	–	–	–
7	20	15	12	15	17	10	–	–	–
8	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–
10	20	15	10	12	14	12	–	–	–
11	20	12	10	15	12	10	–	–	–
12	15	14	11	10	10	12	–	–	–
13	10	14	15	10	10	15	–	–	–
14	10	–	–	–	–	10	–	–	–
15	14	14	16	18	18	16	10	11	12
16	–	–	–	–	–	–	–	–	–
17	–	–	–	–	–	–	–	–	–
18	–	–	–	–	–	–	–	–	–
19	11	11	12	12	12	14	–	–	–
20	18	12	12	16	18	18	10	12	12
21	18	17	12	14	18	17	–	–	–
Netilmicin	26	20	30	24	22	20	–	–	–
Ceftriaxon	18	20	25	24	22	22	–	–	–
Ceftazidin	30	22	30	18	32	22	–	–	–
Amoxicillin	14	18	20	14	20	16	–	–	–
5-Flucytocine	–	–	–	–	–	–	30	34	33
Amphotericin B	–	–	–	–	–	–	20	25	20
Intraconazole	–	–	–	–	–	–	25	32	28

[a] The results were reported as the diameter of the zone of inhibition around each disk (in mm) and the evaluation of inhibition corresponds at < 7 mm (–), 7–10 mm (+), 11–16 mm (++), > 16 mm (+++).

Analytical thin layer chromatography (TLC) was carried out on precoated (0.25 mm) Merck silica gel F-254 plates.

Plant material

The aerial parts as well as the resin "Ladano", of *Cistus creticus subsp. creticus*, were collected in July 1996 on the island of Crete (Greece). A voucher specimen has been deposited in the herbarium of the Laboratory of Pharmacognosy, University of Athens (KL057R).

Extraction and isolation

The compounds 13(*E*)-labda-7,13-diene-15-yl malonic acid (**3**) (15%) and 13(*E*)-labd-13-ene,8α-ol-15-yl malonic acid (**4**) (12%) were isolated from the resin "Ladano" (Kalpoutzakis *et al.*, 1998). The compounds 13(*E*)-labda-7,13-diene-15-ol (**1**), 13(*E*)-labd-13-ene-8α,15-diol (**2**), 13(*E*)-labda-7,13-diene-15-yl acetate (**5**) and 13(*E*)-labd-13-ene-8a-ol-15-yl acetate (**8**) were isolated from the aerial parts of *Cistus creticus* (Demetzos *et al.*, 1990; Chinou *et al.*, 1994). Compounds **1** and **2** were also obtained in quantitative yield by alkaline hydrolysis of **3** and **4** respectively (Kalpoutzakis *et al.*, 1998).

Antimicrobial activity

The antimicrobial activity of the tested compounds was determined by the standardized disk diffusion method of Bauer *et al.* (1966) as has been described in details previously (Kalpoutzakis *et al.*,

1998). Laboratory standard ATCC strains (*Staphylococcus aureus* # 25923, *Staphylococcus epidermidis* # 12228, *Escherichia coli* # 25922, *Enterobacter cloacae* # 13047, *Klebsiella pneumoniae* # 13883, *Pseudomonas aeruginosa* # 227853, *Candida albicans*, *Candida tropicalis* and *Torulopsis glabrata*) were used as testing bacteria. Netilmicin, ceftriaxon, ceftazidin, amoxicillin, 5-flucytocine, amphotericin B and intraconazole were used as standard antibiotics for comparison for the tested bacteria as well as for the tested fungi. The results were reported as the diameter of the zone of inhibition around each disk (in mm) and the evaluation of inhibition corresponds at < 7 mm (–), 7–10 mm (+), 11–16 mm (++), > 16 mm (+++).

Preparation of semisynthetic Derivatives; spectroscopic Data

General method (A) for the preparation of carboxylic ester by the treatment of the anhydride with the alcohols. Alcohol was dissolved in dry pyridine (2.0 ml), anhydride was added and the mixture was stirred under Ar atmosphere. The pyridine was removed under reduced pressure and the residue was dissolved in CH_2Cl_2. The solution was washed with saturated aqueous solution $NaHCO_3$ and the organic solvent was removed under reduced pressure. Carboxylic ester was isolated by column chromatography on silica gel.

General method (B) for the preparation of carboxylic ester by the treatment of the chloride with the alcohols. Alcohol was dissolved in dry pyridine (2.0 ml), chloride was added and the mixture was stirred under Ar atmosphere. The pyridine was removed under reduced pressure and the residue was chromatographed on silica gel.

General method (C) for the preparation of carbamate derivatives by the treatment of the corresponding isocyanate reagents with the alcohols. Alcohol was dissolved in dry pyridine (2.0 ml), isocyanate derivative was added and the mixture was stirred under Ar atmosphere. The pyridine was removed under reduced pressure and the residue was chromatographed on silica gel.

Spectral data (NMR, MS) of compounds **1–21** are available to interested readers on request from the authors.

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