Antileishmanial Activity of Natural Diterpenes from *Cistus* sp. and Semisynthetic Derivatives Thereof

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Eleven *cis*-clerodane diterpenes, seven labdane type diterpenes and one triterpene isolated from *Cistus monspeliensis* and the resin "Ladano" of *Cistus creticus* subsp. *creticus* were evaluated against *Leishmania donovani* promastigotes, the causative agent for visceral leishmaniasis. In addition, eleven semisynthetic manoyl oxide, seventeen labdane type derivatives and a triterpene were also evaluated for their antileishmanial activity. 18-Acetoxy-*cis*-clerod-3-en-15-ol, 15,18-diacetoxy-*cis*-clerod-3-ene and 13-(*E*)-8*a*-hydroxylabd-13-en-15-ol 2-chloroethylcarbamate exhibited the most potent and selective leishmanicidal activity with IC_{50} values of 3.3 μg/ml, 3.4 μg/ml and 3.5 μg/ml, respectively.

Key words *Cistus* sp.; *Leishmania donovani*; *cis*-clerodane; labdane; manoyl oxide; diterpene; antileishmanial activity

Leishmaniasis is a public health problem in many parts of the world with approximately 90% of the cutaneous form of the disease occurring in the Mediterranean region, South America and parts of Africa and Asia.[1)] Recent reports indicate an increase in the incidence of leishmaniasis in Middle Eastern countries.[2)] Diagnosis and treatment of cutaneous leishmaniasis pose considerable problems. An added complication is the reported development of resistance to pentavalent antimonial agents in countries like Sudan and India, where the visceral form is endemic.[3)]

The high toxicity of and resistance to currently available drugs used for these parasitic diseases is an impetus for the development of new therapeutic approaches. Plants have been used traditionally for the treatment of protozoan diseases, and phytotherapy has received considerable attention recently in the search for alternative compounds with antiparasitic activity.

We have previously shown that several plant derived extracts of the Mediterranean basin possess activity against *Leishmania donovani*, the causative agent for visceral leishmaniasis.[4)] Some of the active plant extracts were from different species of the genus *Cistus*. *Cistus* species are widespread in the Mediterranean basin, especially in Greece which is represented by six taxa.[5)]

Cistus creticus L. subsp. *creticus* is a shrub belonging to the family Cistaceae found in all kinds of soil, and its leaves are covered with glands secreting a brownish resin (Ladano), consisting mainly of diterpenoids. The chemical composition of the plant as well as the resin has been studied extensively in our laboratory in the past.[6,7)] Another interesting species is *Cistus monspeliensis* L., which is a compact, aromatic bush up to 1 m tall, widespread in Greece and one of the most common *Cistus* species in the Mediterranean basin. This plant also has been studied by our lab, and several clerodane-type diterpenes were isolated.[8)]

Taking into consideration the fact that *Cistus* species are widespread in the Mediterranean basin, we investigated the antileishmanial activity of their secondary metabolites. In particular, the present study assesses the antileishmanial potential of clerodane, labdane and manoyl oxide type diterpenes from these species. In this paper we report the antileishmanial activity of nineteen compounds isolated from *Cistus monspeliensis* and *Cistus creticus* subsp. *creticus* and twenty nine semisynthetic derivatives of these natural compounds.

MATERIALS AND METHODS

Plant Material The aerial parts of *C. monspeliensis* were collected in June 2002 from East Crete, Greece, and the resin of *C. creticus* subsp. *creticus* was collected in July 2002 from central Crete. All plant materials were identified by Dr. E. Kalpoutzakis. Voucher specimens of *C. monspeliensis* and *C. creticus* subsp. *creticus* were deposited in the herbarium of Laboratory of Pharmacognosy and Natural Products Chemistry, Department of Pharmacy, University of Athens, Greece, under the numbers KL 060 and KL057R, respectively.

Extraction and Isolation The air-dried plant material of *C. monspeliensis* was extracted at room temperature with CH_2Cl_2, and then with MeOH. The CH_2Cl_2 extract was chromatographed over a silica gel column with cyclohexane–CH_2Cl_2 (80 : 20 → 0 : 100) and CH_2Cl_2–MeOH (100 : 0 → 50 : 50) to afford twelve fractions. Fractions three and four were combined and rechromatographed over silica gel using cyclohexane–EtOAc (98 : 2 → 50 : 50) to give compounds **3**, **9**, **8**, **12**, **11**, **2**, and **4**. Fraction six, was further purified by silica gel column chromatography using cyclohexane–EtOAc (95 : 5 → 50 : 50) to afford compounds **10**, **1**, **7**, and the mixture of the two isomers **5** and **6**.

The resin of *C. creticus* subsp. *creticus* was extracted with CH_2Cl_2/MeOH (50 : 50). The extract was fractionated over a silica gel column with CH_2Cl_2–MeOH (100 : 0 → 50 : 50) and afforded fourteen fractions. Fraction two was further fractionated with CH_2Cl_2 to afford compound **14**. Fraction three and fraction five were fractionated in a similar way to afford compounds **15** and compound **16** respectively. Fraction nine was further fractionated with a CH_2Cl_2–MeOH gradient sys-

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tem, leading to the isolation of compound **17**. Further chromatographic separation of fraction eleven, using a gradient system CH_2Cl_2–MeOH, afforded compounds **18**, **19** and **20**.

Preparation of Semisynthetic Derivatives The semisynthetic labdane type derivatives **21**—**37** and the semisynthetic derivatives of manoyl oxide **38**—**48** were prepared as previously described.[6,9] Compound **13** was obtained by alkaline hydrolysis of compound **12**.

Antileishmanial Assay Antileishmanial activity was tested *in vitro* on a culture of *Leishmania donovani* promastigotes (1S2D strain provided by Professor R. Balana-Fouce, University of Leon, Spain). In a 96-well microplate assay, 5 μl of solutions of the tested compounds were added to 195 μl of the leishmania promastigotes cultures (2×10^6 cell/ml). The plates were incubated at 26 °C for 72 h, and growth of leishmania promastigotes was determined by the Alamar blue assay.[10] Pentamidine and amphotericin B were used as the standard antileishmanial agents.

Pure compounds were also tested for cytotoxicity in VERO cells (monkey kidney fibroblast, obtained from ATCC) up to a highest concentration of 47.6 μg/ml. The assay was performed in 96-well tissue culture-treated microplates, as described earlier.[11] Briefly, cells (approximately 25000 cells/well) were seeded to the wells of the plate and incubated for 24 h. Diluted samples were added and plates were again incubated for 48 h. The number of viable cells was determined according to a modified version of neutral red assay procedure.[12] IC_{50} values were determined from logarithmic graphs of growth inhibition versus concentration. Doxorubicin was used as a positive control, while DMSO was used as the vehicle control.

RESULTS AND DISCUSSION

Recently, we demonstrated that crude extracts of *Cistus creticus* subsp. *creticus* and *Cistus monspeliensis* possess significant antileishmanial activity.[4] In a continuation of our research for discovering new antileishmanial agents or new lead compounds, we have focused on the isolation of active compounds from these plants that have shown to possess activity.

Sequential fractionation of the CH_2Cl_2 extract of the aerial parts of *C. monspeliensis* led to the isolation of eleven clerodane type diterpenes: 15,18-dihydroxy-*cis*-clerod-3-ene or cistadiol (**1**), 18-acetoxy-*cis*-clerod-3-en-15-ol (**2**), 15,18-diacetoxy-*cis*-clerod-3-ene (**3**), 15-acetoxy-*cis*-clerod-3-en-18-ol (**4**), 18-hydroxy-*cis*-clerod-3-en-15-oic acid (**5**), 15-hydroxy-*cis*-clerod-3-en-18-oic acid (**6**), 18-acetoxy-*cis*-clerod-3-en-15-oic acid (**7**), 15-acetoxy-*cis*-clerod-3-en-18-oic acid (**8**), 15-acetoxy-*cis*-clerod-3-en-18-al (**9**), 15-hydroxy-*cis*-clerod-3-en-18-al (**10**), *cis*-clerod-3-en-15-oic acid or epipopulifolic acid (**11**) and one triterpene 3-*O*-acetyl oleanolic acid (**12**). Although it is known that the genus *Cistus* contains some triterpenes, to our knowledge this is the first report of the presence of 3-*O*-acetyl oleanolic acid in *Cistus* sp. The chemical structures of the above compounds were established by extensive one- and two-dimensional NMR experiments in comparison with authentic samples.[8]

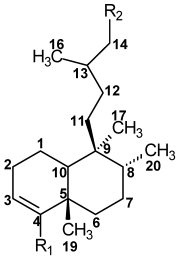
The phytochemical study of the resin extract of *Cistus creticus* subsb. *creticus*, led to the isolation of seven labdane type diterpenes: *ent*-13-*epi*-manoyl oxide (**14**), *ent*-3β-acetoxy-13-*epi*-manoyl oxide (**15**), *ent*-3β-hydroxy-13-*epi*-manoyl oxide (**16**), 13(*E*)-labda-7,13-diene-15-ol malonate (**17**), 13(*E*)-8*a*-hydroxy-labd-13-en-15-ol malonate (**18**), 13(*E*)-labda-7,13-diene-15-ol (**19**), 13(*E*)-labd-13-en-8*a*,15-diol (**20**). All the isolated compounds were identified from their NMR spectra in comparison with authentic samples.[6,7]

The biological evaluation of the antileishmanial activity of the above isolated compounds was performed by testing them *in vitro* against promastigote cultures of *L. donovani* promastigotes at three concentrations: 1.6, 8, and 40 μg/ml and then calculating IC_{50} and IC_{90} values (the concentration that caused 50% and 90% cell death, respectively). Compounds **1**—**4**, **7**, **9**, **10**, **13**, **15**, **19**, **20** were active with IC_{50} and IC_{90} values below 20 μg/ml and 40 μg/ml, respectively

Table 1. Antileishmanial Activity of Natural and Semisynthetic Diterpene Derivatives

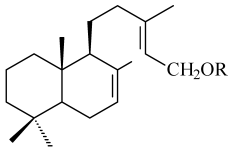
Compound	IC_{50} (μg/ml)	IC_{90} (μg/ml)
1	18	40
2	3.3	6.8
3	3.4	7.3
4	15	37
5, 6	30	>40
7	18	40
8	NA	NA
9	5	>40
10	13	40
11	NA	NA
12	36	>40
13	5.3	34
14	27	>40
15	17	35
16	24	>40
17	NA	NA
18	NA	NA
19	18	35
20	17	34
21	17	34
22	15	33
23	20	>40
24	18	35
25	37	>40
26	NA	NA
27	18	36
28	11	32
29	18	34
30	8	29
31	NA	NA
32	6	27
33	13	32
34	NA	NA
35	7	28
36	3.5	7.5
37	11	32
38	23	>40
39	NA	NA
40	4.3	20
41	5	29
42	7.7	29
43	NA	NA
44	15	33
45	18	34
46	17	34
47	17	34
48	19	35
Pentamidine	1.6	8.0
Amphotericin B	0.17	0.34

NA: Not active up to the highest concentration tested (40 μg/ml).



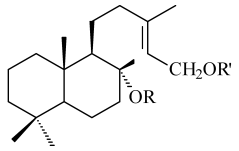
1 $R_1 = CH_2OH$ $R_2 = CH_2OH$
2 $R_1 = CH_2OCOCH_3$ $R_2 = CH_2OH$
3 $R_1 = CH_2OCOCH_3$ $R_2 = CH_2OCOCH_3$
4 $R_1 = CH_2OH$ $R_2 = CH_2OCOCH_3$
5 $R_1 = CH_2OH$ $R_2 = CO_2H$
6 $R_1 = CO_2H$ $R_2 = CH_2OH$
7 $R_1 = CH_2OCOCH_3$ $R_2 = CO_2H$
8 $R_1 = CO_2H$ $R_2 = CH_2OCOCH_3$
9 $R_1 = CH{=}O$ $R_2 = CH_2OCOCH_3$,
10 $R_1 = CH{=}O$ $R_2 = CH_2OH$,
11 $R_1 = CH_3$ $R_2 = CO_2H$

Fig. 1. *cis*-Clerodane Diterpenes from *C. monspeliensis*



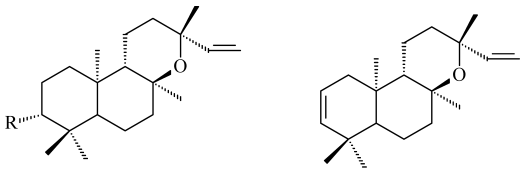
17 R = $COCH_2COOH$
19 R = H
22 R = COC_6H_5
23 R = $COC_6H_4NO_2$
24 R = $CONHC_6H_5$
25 R = $CONHC_6H_4OCH_3$
26 R = $CONHCH_2(CH_2)_{10}CH_3$
27 R = $CONHCH_2(CH_2)_3CH_3$
28 R = $CONHCH_2CH_2Cl$

Fig. 2. Naturally Occurring and Semisynthetic 13(*E*)-Labda-7,13-dien-15-ol Derivatives



18 R = H R' = $COCH_2COOH$
20 R = H R' = H
21 R = H R' = $COCH_3$
29 R = H R'= COC_6H_5
30 R = H R' = $COC_6H_4NO_2$
31 R = H R' = $CONHC_6H_5$
32 R = R' = $CONHC_6H_5$
33 R = H R' = $CONHC_6H_4OCH_3$
34 R = H R' = $CONHCH_2(CH_2)_{10}CH_3$
35 R = H R' = $CONHCH_2(CH_2)_3CH_3$
36 R = H R' = $CONHCH_2CH_2Cl$
37 R = H R' = $COCH{=}CHC_6H_4OCOCH_3$

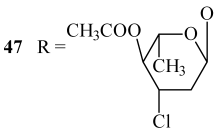
Fig. 3. Naturally Occurring and Semisynthetic 13(*E*)-Labd-13-ene-8α,15-diol Derivatives



14 R = H
15 R = $OCOCH_3$
16 R = OH
38 R = $OCOC_6H_5$
39 R = $OCOC_6H_4NO_2$
40 R = $OCONHC_6H_5$
41 R = $OCONHC_6H_4OCH_3$
42 R = $OCONHCH_2CH_2Cl$
43 R = $OCONHCH_2(CH_2)_{10}CH_3$
44 R = $OCONH(CH_2)_3CH_3$
45 R = OSO_2CH_3

46

47 R =



48 R =

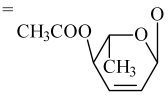


Fig. 4. Natural and Semisynthetic Manoyl Oxide Derivatives

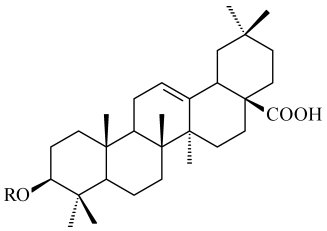
(Table 1). Among these natural compounds, the most potent ones were the *cis*-clerodane diterpenes **2**, **3** and **9** (Fig. 1), with IC_{50} values of 3.3, 3.4 and 5.0 μg/ml. The substitution in position 4 of these clerodanes seems to affect their leishmanicidal activity. Compounds with an acid or methyl group in that position are inactive (see **8**, **11**). However, the compounds with an aldehyde (see **9**, **10**) or hydroxymethylene group (see **1**, **4**, **5**) at the same position are active. The compounds become even more active with an acetoxymethylene group, and, furthermore, the most potent compounds have the combination of the above substitution and a hydroxymethylene group (see **2**) or an acetoxymethylene (see **3**) in position 14. In contrast, the presence of a carboxylic acid group in position 14 decreases the activity of the clerodanes (compare **7** to **2**, **3**, and **5** to **4**).

These promising results led us to proceed in the biological evaluation of several semisynthetic derivatives of the above diterpenes recently prepared by our research team.[6,9] Compounds **17** and **18** are the major constituents of *Cistus creticus* subsp. *creticus* and could easily be transformed to compounds **19** and **20**, respectively, by an alkaline hydrolysis. These compounds (**19**, **20**) were used as starting materials for further modification. From compound **19**, compounds **22**—**28** (Fig. 2) and from compound **20**, compounds **21** and **29**—**37** were synthesized, respectively (Fig. 3). In addition the manoyl oxide derivatives **38**—**48** (Fig. 4), were prepared using compound **16** as the starting material.

In the case of 13(*E*)-labd-13-ene-8α,15-diol derivatives, the natural compound **18** is inactive (similar to derivatives **31** and **34**), but its hydrolyzed derivative **20** has moderate activity with IC_{50} value <20 μg/ml. Compounds **21**, **29**, **33** and **37** also have moderate activity ($10\ \mu g/ml < IC_{50} < 20\ \mu g/ml$), while compounds **30**, **32**, **35** and **36** are more potent ($IC_{50} < 10\ \mu g/ml$). Especially 13-(*E*)-8*a*-hydroxylabd-13-en-15-ol 2-chloroethylcarbamate (**36**) with the chlorinated carbamate side chain, was one of the most active derivatives with IC_{50} and IC_{90} values of 3.5 and 7.5 μg/ml, respectively (Table 1).

Most of the semisynthetic derivatives of 13(*E*)-labda-7,13-dien-15-ol, like **22**, **23**, **24**, **27**, exhibited similar activity to compound **19** ($15\ \mu g/ml < IC_{50} < 20\ \mu g/ml$), and only **28** with the chlorinated carbamate side chain had slightly increased activity ($IC_{50} < 11\ \mu g/ml$). Compounds **17** and **26** were inactive or almost inactive like the case of compound **25** (IC_{50} 37 μg/ml).

The derivatives of manoyl oxide **38**—**48**, seem to be more



12 R = $COCH_3$
13 R = H

Fig. 5. Natural and Semisynthetic Triterpenes

active than the parent compound **16**. Compound **16** has moderate activity but its natural acetoxy derivative **15** has activity with $IC_{50}<20\,\mu g/ml$. Compounds **44**—**48**, have a moderate activity ($10\,\mu g/ml<IC_{50}<20\,\mu g/ml$), while compounds **40**—**42** are highly active ($IC_{50}<10\,\mu g/ml$). Compound **40** was very active with an IC_{50} of $4.3\,\mu g/ml$.

The natural triterpene **12** (Fig. 5) was almost inactive (IC_{50} $36\,\mu g/ml$), but its hydrolyzed derivative **13** was highly active with IC_{50} $5.3\,\mu g/ml$, which is in accordance with previously published data.[13)]

All natural and semisynthetic derivatives were also evaluated for their cytotoxicity against VERO cells (monkey kidney fibroblasts). None of them were cytotoxic to the mammalian cells up to the highest concentration of $47.6\,\mu g/ml$.

The structural diversity of the natural labdane type diterpenes and semisynthetic derivatives evaluated in our studies allows us to make some comments about structure–activity relationships. In both cases of 13(*E*)-labda-7,13-dien-15-ol and 13(*E*)-labd-13-ene-8α,15-diol derivatives the presence of a carboxylic acid side chain leads to inactive compounds (compare **17** to **19** and **18** to **20**), and it seems that the basic skeleton rings of the above two groups of compounds with no substituents are more effective than the manoyl oxide skeleton (compare compounds **19** and **20** to **16**). In addition, in all three cases the presence of a long side carbamide chain makes the derivatives (compounds **26**, **34**, **43**) inactive, while a shorter carbamate chain had no negative effects (compounds **27**, **35**, **44**). On the other hand, the presence of the ethylclorinated carbamate chain in all three cases increased the activity, and in some cases afforded some of the most potent semisynthetic derivatives (compounds **28**, **36**, **42**). This is in accordance with previous results obtained for these compounds against a wide range of microbes.[6,9)] In the case of the manoyl oxide derivatives, it seems that the substitution of the side chain by a sugar (compound **47**, **48**) or the formation of the double bond (compound **46**) has no significant impact on leishmanicidal activity of the manoyl oxide skeleton.

In summary, two species from the genus *Cistus* contain several non-polar bioactive compounds that are mainly diterpenes, that possess significant antileshmanial activity. The IC_{50} concentratrions of none of the compounds were as low as those of pentamidine or amphotericin B, however, **2**, **3** and **36** were more active than pentamidine at their I_{90} concentrations. In addition, the biological evaluation of a large number of semisynthetic compounds led to the identification of some potent derivatives and to some structure–activity relationship findings that could be useful leads in the design of new, highly potent antileishmanial agents.

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