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A Simple and Rapid Method for the Differentiation of C-13 Manoyl Oxide Epimers in Biologically Important Samples Using GC–MS Analysis Supported with NMR Spectroscopy and Computational Chemistry Results†

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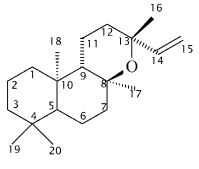
Abstract—There is confusion in the literature related to the characterization of biologically important C13 manoyl oxide epimers by using their mass spectrometric data. Furthermore a method for calculating the epimeric purity has not been established. In this work mixtures of manoyl oxide C13 epimers and pure *ent*-13-*epi*-manoyl oxide have been isolated from plant extracts. The GC–MS analysis allows the characterization of each stereoisomer in a sample of natural origin: *the ratio of intensities of peaks m/z* 275 : 257 is lower in ent-13-*epi*-manoyl oxide than in manoyl oxide. NMR spectroscopy is used to give experimental evidence and simple calculations were performed to support the MS data. On the basis of these results the characterization of the two stereoisomers and the calculation of their ratio in plant extracts and essential oils can be done in a routine basis. The biological activity evaluation of mixtures with different epimeric composition of manoyl oxide showed that the ratio of the two epimers is important for their antibacterial activity. *Ent*-13-*epi*-manoyl oxide seems to be more active than its epimeric congener against Gram-positive bacteria.

Introduction

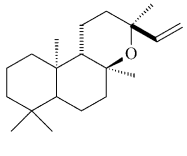
Manoyl oxide (8,13-epoxy-labd-14-ene) belongs to labdane diterpenes. Labdanes is a family of natural products isolated from several plant families[1] with a wide range of biological activities.[2] Forskolin is the most medicinally important labdane representing a high functionalized manoyl oxide isolated from the plant extract of *Coleus forskohlii* Briq. (Labiatae).[3]

Manoyl oxide and most of the labdane diterpenes with unsaturated side chain were present as mixtures of C-13 epimers. Recent publications are referred to manoyl oxide isolation from plant extracts and essential oils and its characterization using GC–MS without any clarification for the presence of epimeric mixtures. In some cases, the MS of 13-*epi*-manoyl oxide was confused with that of manoyl oxide.[4]

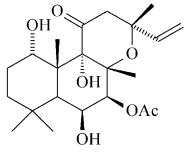
Derivatives of 13-*epi*-manoyl oxide have been reported to be biologically active.[5] Recent studies published from our research group on the genus *Cistus* i.e, *C. creticus* subsp. *creticus* and subsp. *eriocephalus*, and *C. monspeliensis*, showed that the percentage content of ent-manoyl oxide epimers **1** and **2** in plant extracts (fruits, leaves, essential oils) varies, depending on the part of the plant and the polarity of the solvent used. The biological evaluation of the extracts suggest a possible correlation between biological activity and C-13 manoyl oxide epimeric composition.[6a,b] This can be verified only through the biological activity evaluation of mixtures with different epimeric composition.



ent-13-epi manoyl oxide, **1**



ent-manoyl oxide, **2**



Forskolin

Since C-13 manoyl oxide epimers are often isolated as mixtures or found as mixtures in complex isolations

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from natural sources, that is, plant extracts or essential oils, a rapid and straightforward method of epimer characterization and calculation of the epimeric composition is needed to be established.

NMR Spectroscopy using chiral shift reagents has been suggested as a method to differentiate manool from 13-*epi*-manool and for the characterization of the relevant epimeric composition.[7] This method is important but requires chiral shift reagents, the isolation of the manoyl oxide mixture of epimers and sufficient quantity of sample to run a ^{1}H NMR spectrum.

In this paper we present a simple method for the differentiation between manoyl oxide and its 13-*epi* isomer in biologically important plant extracts or essential oils using GC–MS analysis. NMR spectroscopy and computational chemistry were used to support the GC–MS analysis results. Using the same methodology the epimeric purity can be estimated. Moreover our purpose is to contribute in the relationship between the stereochemistry and the biological activity of the isomers.

Results and Discussion

Natural product material

Mixtures of *ent*-manoyl oxide C-13 epimers **1** and **2** have been isolated from the methanolic extracts of fruits, of the resin Ladano and of the leaves from *C. creticus* subsp. *Creticus*.[6a,8] From the hexane extract of *C. monspeliensis* leaves a pure C-13 manoyl oxide epimer was isolated.[6b]

GC–MS analysis—NMR spectroscopy

We tried on several temperature programs during the GC and GC–MS analysis[9a] before finding the best one for separation of the two C-13 manoyl oxide epimers **1** and **2**.[6a] Retention Indices (RI) were calculated according to Van den Dool and Kratz and were 2010 and 1989 on HP-5 column and 2351 and 2331 on CP-wax column, for **1** and **2** epimers respectively.[6a,c] The characterization of the GC peaks and the unambiguous assignment of the corresponding MS spectra to a certain epimer was accomplished after the inspection of the ^{1}H NMR spectra[9b] of the isolated pure epimer and the epimers mixture.

The ^{1}H NMR spectrum of the pure epimer was assigned to 13-*epi*-isomer (Fig. 1): the olefinic region of the spectrum shows a vinylic ABX pattern, because the two methylene vinylic protons are diastereotopic. The olefinic protons H-14, H-15 *cis* and H-15 *trans* appeared as doublets of doublets. H-14 resonates at 6.0 ppm, H-15 *cis* and H-15 *trans* at 4.90 and 4.95 ppm; the coupling constants between H-14 and H-15 *cis*, H-15 *trans* are $J_{cis} = 11.1$ Hz, $J_{trans} = 18$ Hz. Then the ^{1}H NMR spectrum of C-13 manoyl oxide epimers mixture could be

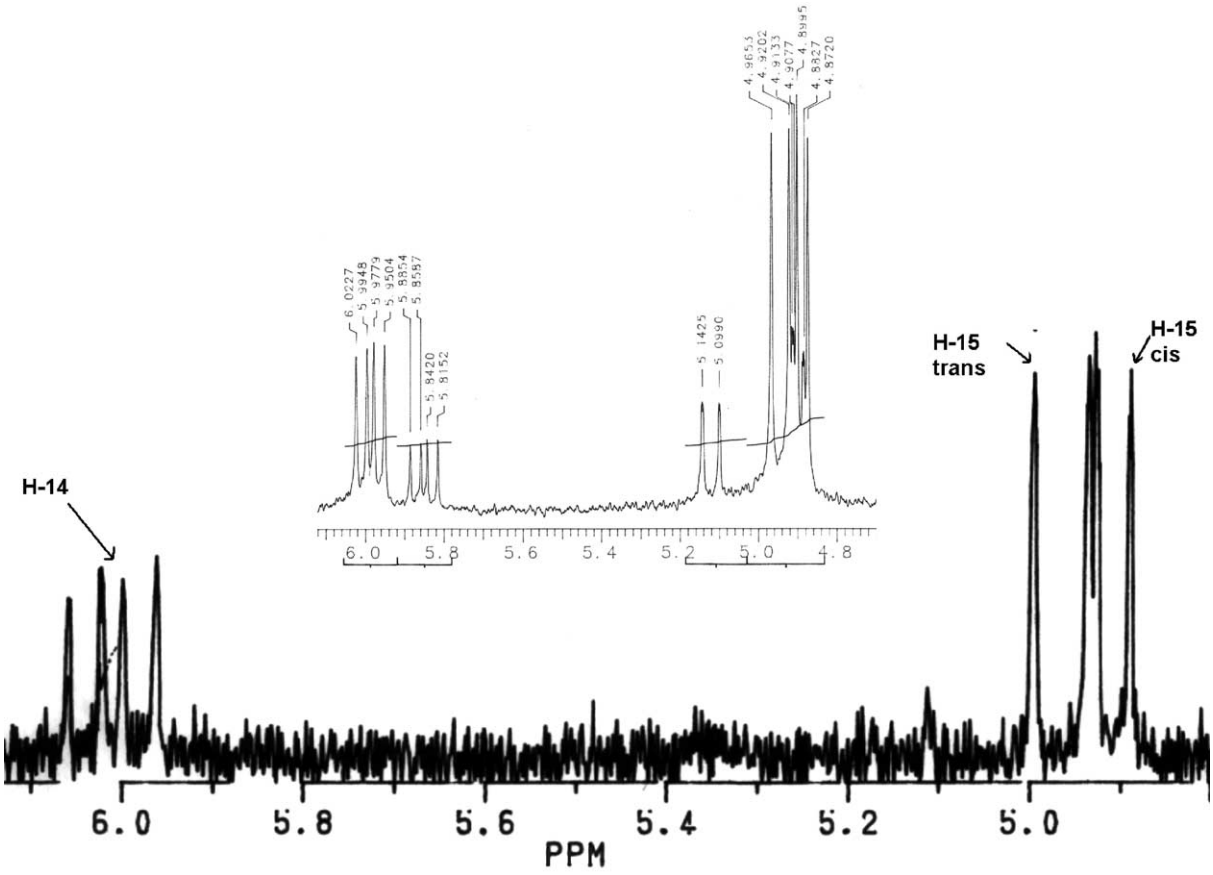


Figure 1. Olefinic region of pure *ent*-13-*epi*-manoyl oxide **1** (down) and of mixture of manoyl oxide epimers **1** and **2** (up).

easily assigned (Fig. 1). The major isomer corresponds to 13-*epi* isomer. The minor epimer has similar spectral characteristics with forskolin[10] since the two molecules have similar relative stereochemistry, that is H-14 resonates at 5.85 ppm, H-15 *cis* and H-15 *trans* resonate at 4.88 and 5.12 ppm; the coupling constants between H-14 and H-15 *cis*, H-15 *trans* are $J_{cis} = 10.6$ Hz, $J_{trans} = 17.4$ Hz. According to the NMR analysis of forskolin the coupling constant between H-15 *cis* and H-15 *trans* is 1.4 Hz; our ^{1}H NMR spectrum was not enough resolved to allow the observation of this small coupling constant.

Using this methodology the GC peaks and MS spectra were assigned to each isomer. The most characteristic spectral feature was the different relative intensity of the peaks with *m*/*z* 275 and *m*/*z* 257 between the two epimers. For 13-*epi* manoyl oxide the 275:257 peak intensities ratio was lower than that for manoyl oxide (Fig. 2).

The occurrence of pure *ent*-13-*epi*-manoyl oxide in plants is rare.[6b] When this labdane was noted in the literature no clear spectral data were given.[4b,11] Thus, it can be existed as an uncharacterized mixture with its epimer. The method developed can be used for the rapid characterization and percentage estimation of manoyl oxide epimers and related compounds in complex samples of biological origin.

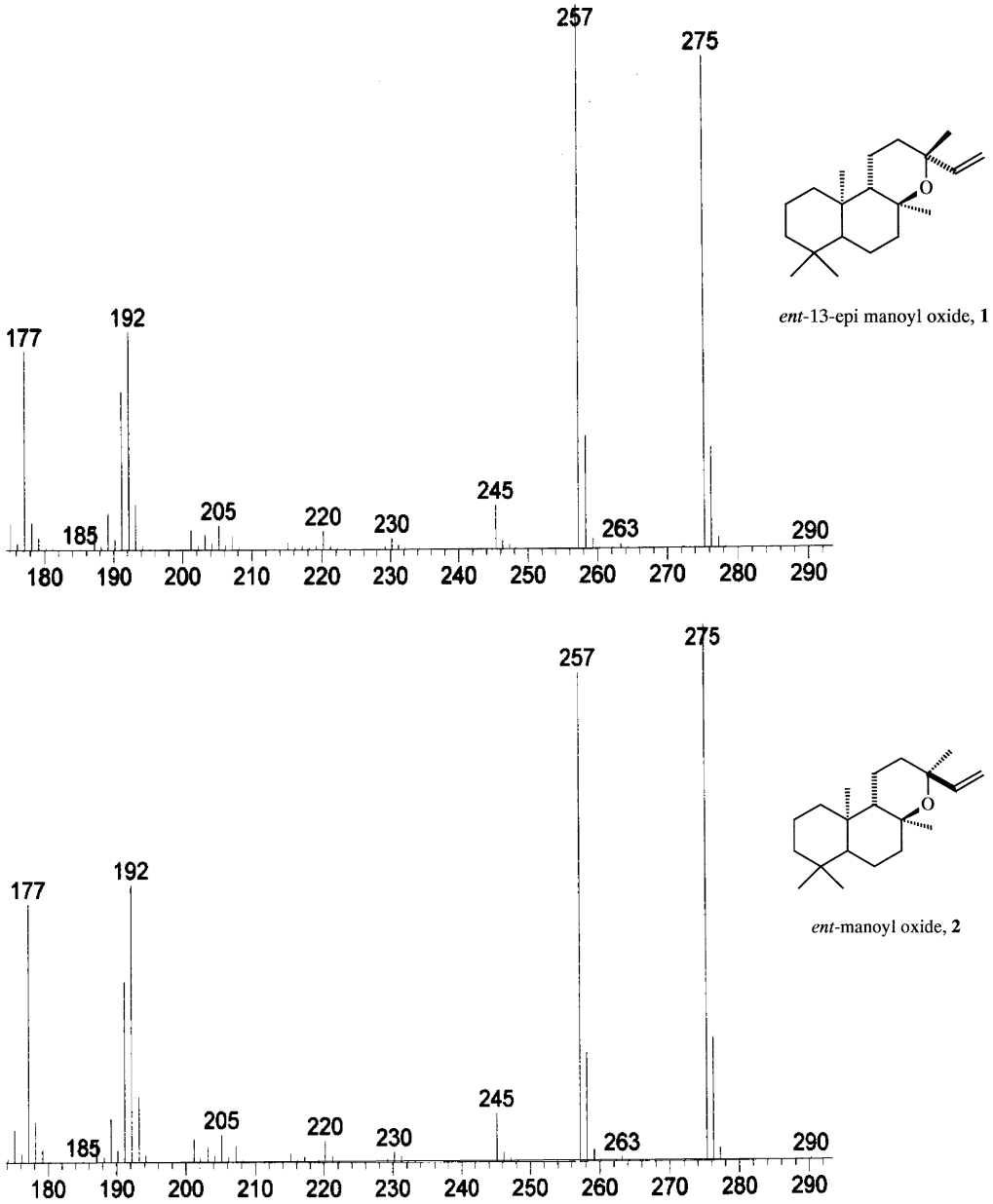
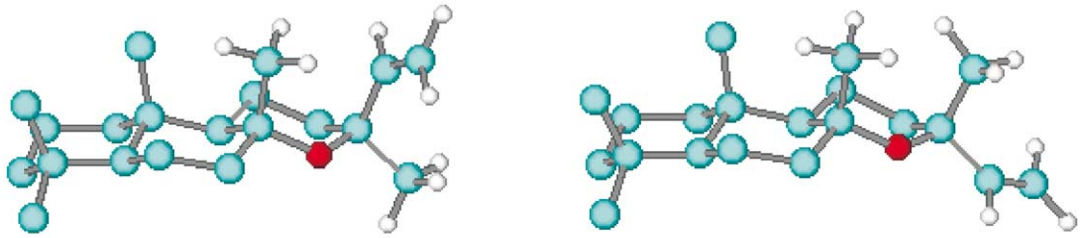


Figure 2. Part of the mass spectrum of *ent*-13-*epi*-manoyl oxide **1** (up) and *ent*-manoyl oxide **2** (down) showing the lower ratio of *m*/*z* 275:257 peak intensities for 13-*epi* manoyl oxide **1** relative to manoyl oxide **2**.



1 **2**

Figure 3. Lowest energy conformations of diastereomeric *ent*-13-*epi*-manoyl oxide **1** (left) and *ent*-manoyl oxide **2** (right). In manoyl oxide **2** energy consuming 1,3-diaxial interactions between methyl groups are present.

Table 1. Antimicrobial activity of mixtures of manoyl oxides **1** and **2** and pure *ent*-13-epi isomer **1** isolated from plant extracts and essential oils

Plant extract	Composition%			MIC_{50} [a] (mg/mL)				
	Pure **1**	**1**	**2**	*S. aureus* (ATCC 6538)	*S. epidermidis* (ATCC 12228)	*S. hominis* (ATCC 27844)	*K. pneumoniae* (ATCC 13883)	*E. coli* (ATCC 25922)
C. creticus subsp. *creticus*								
Fruits	—	70	30	750	750	750	>2	>2
Resin	—	45	55	>2	>2	>2	>3	>3
Leaves	—	75	25	600	600	600	>2	>2
C. monspeliensis								
Fruits	—	—	—	—	—	—	—	—
Resin	N.S	N.S	N.S	—	—	—	—	—
Leaves	100	—	—	250	250	250	>2	>2
Streptomycin[b]				0.0021	0.0021	0.0021	0.0006	0.0006

[a]MIC_{50} was defined as the lowest concentration that inhibited visible growth. All data represent mean values for at least two separate experiments.
[b]Sulfate salt; N.S: not studied.

Computational chemistry

To account for the lower ratio of *m*/*z* 275:257 peak intensities for 13-*epi* manoyl oxide **1** relative to manoyl oxide **2**, some simple calculations were undertaken. Since the two molecular ions lead to the same carbocation fragments *m*/*z* 275: M-15 and 257: M-(15+18) the energy required for the carbocation formation from **1** must be higher, that is the energy content of **1** must be lower than **2**. Indeed, MM+ molecular mechanics calculations[12] predict that 13-epi manoyl oxide **1** is more stable than manoyl oxide **2** by 3.2 kcal mol^{-1}. The higher energy content of manoyl oxide **2** can be explained in terms of energy consuming 1,3-dimethyl diaxial interactions because of their 2.1 Å interproton distance. This interaction is not present in 13-epi manoyl oxide **1** where axial methyl and vinyl groups are 2.6 Å apart (Fig. 3).

Biological activity evaluation

Manoyl oxide mixtures, containing different percentage of epimers 1 and 2 (Table 1), and a sample of pure *ent*-13-*epi*-manoyl oxide 1 were tested for their antimicrobial activity against Gram positive and Gram negative bacteria: *Staphyloccoccus aureus* (ATCC 6538), *S. epidermides* (ATCC 12228) and *S. hominis* (ATCC 27844), *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 25922).

The percentage content of isomers into the mixture seems to be important for the activity. Pure 13-*epi* isomer was very active against *Staphylococci* with a MIC_{50} = 250 mg mL^{-1}. The mixture of isomers with a molar ratio **1**:**2** being 75:25 (leaves of *C. creticus* subsp. *Creticus*) exhibited better activity than the mixture with a molar ratio 70:30 (fruits), and the mixture with a molar ratio 45:55 (resin) was inactive. Thus, by increasing the percentage content of **1** the activity against *Staphylococci* becomes better and it can be surmised that **1** is more active than its epimer **2**. This fact is in accordance with reports obtained from locals in the island of Crete, Greece stating the traditional use of leaves from *C. creticus* subsp. *creticus* and from *C. monspeliensis* as antimicrobial remedies.

Pure 13-*epi* isomer and all the manoyl oxide mixtures were inactive against Gram-negative bacteria.

Conclusion

A simple and rapid method for the differentiation of manoyl oxide epimers based on GC–MS spectra was established: the intensities ratio of peaks at *m*/*z* 275 and 257 is lower in *ent*-13-*epi*-manoyl oxide than in manoyl oxide. The method allows also the calculation of epimer content in natural isolations of biological importance, without the need to isolate the pure compounds not

even the mixture of compounds. An experimental support based on NMR spectroscopy and a qualitative theoretical background was presented. An application of the method was given through the antimicrobial evaluation of manoyl oxide isomers. It was found that the biological activity of mixtures of *ent*-13-epi manoyl oxide and manoyl oxide against Gram-positive bacteria improves by increasing the percentage content of 13-epi isomer.

The results are useful for other labdanes like manool and possibly for medicinally important derivatives of manoyl oxide like forskolin.

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References and Notes

1. Demetzos, C.; Dimas, K. Labdane-Type Diterpenes; Chemistry and Biological Activity, In *Studies in Natural Product Chemistry*, Atta-Ur-Rahman, Ed.; Elsevier, 2001; Vol. 25, Part F, pp 235–292.
2. (a) Singh, M.; Pal, M.; Sharma, R. P. *Planta Med.* **1999**, *65*, 2. (b) Demetzos, C.; Dimas, K.; Hatziantoniou, S.; Anastasaki, T.; Angelopoulou, D. *Planta Med.* **2001**, *67*, 614. (c) Dimas, K.; Demetzos, C.; Vaos, V.; Ioannidis, P.; Trangas, T. *Leukemia Res.* **2001**, *25*, 21.
3. (a) Gabetta, B.; Zini, G.; Danielli, B. *Phytochemistry* **1989**, *28*, 859. (b) De Souza, N. J.; Dohadwaal, A. N. *J. Med. Res. Reviews* **1983**, *3*, 201. (c) Garcia-Granados, A.; Martinez, A.; Jimenez, M. B.; Onorato, E. M.; Rivas, F.; Arias, M. J. *J. Chem. Research (S)* **1990**, 94.
4. (a) See for example: Adams, R. P. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*; Allured Publ. Corp.: Carol Stream, IL, 1995. (b) Torres, M. E.; Velasco-Negueruela, A.; Perez-Alonso, J. M.; Pinilla, M. G. *J. Ess. Oil Res.* **1997**, *9*, 27.
5. Garcia-Granados, A.; Martinez, A.; Onorato, M. E.; Parra, A.; Recondo, M. B.; Rivas, F.; Arrebola, M. L.; Socorro, O. *Phytochemistry* **1994**, *35*, 645.
6. (a) Anastasaki, T.; Demetzos, C.; Perdetzoglou, D.; Gazouli, M.; Loukis, A.; Harvala, C. *Planta Med.* **1999**, 735. (b) Angelopoulou, D.; Demetzos, C.; Dimas, K.; Perdetzoglou, D.; Loukis, A. *Planta Med.* **2001**, 66. (c) Van den Dool, H.; Kratz, P. D. *J. Chromatogr.* **1963**, *11*, 463.
7. Conner, A. H.; Rowe, J. W. *Phytochemistry* **1976**, *15*, 1949.
8. Demetzos, C.; Stahl, M.; Anastasaki, T.; Gazouli, M.; Tzouvelekis, L.; Rallis, M. *Planta Med.* **1999**, *65*, 76.
9. (a) A Hewllet Packard 8500 was used for the GC–MS analysis. (b) NMR spectra were run in Bruker and Varian 400 MHz machines.
10. Kogler, H.; Fehlhaber, H. W. *Magnetic Resonance in Chemistry* **1991**, *29*, 993.
11. Pietsch, M.; Konig, A. W. *Phytochem. Anal.* **2000**, *11*, 99.
12. Molecular mechanics were performed using the MM+ force field provided by the Hyperchem. This force field is an extension of MM2 force field. Molecular mechanics calculations give the most consistent results with experimental data for cycloalkane derivatives.