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LC-MS for the identification of oxygen heterocyclic compounds in citrus essential oils

Paola Dugo [a,*], Luigi Mondello [b], Laura Dugo [b], Rosanna Stancanelli [b], Giovanni Dugo [b]

[a] *Dipartimento di Chimica Organica e Biologica, Facoltà di Scienze, Università di Messina, salita Sperone 31, 98166 Messina, Italy*

[b] *Dipartimento Farmaco-chimico, Facoltà di Farmacia, Università di Messina, viale Annunziata, 98168 Messina, Italy*

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Abstract

The oxygen heterocyclic compounds (coumarins, psoralens and polymethoxylated flavones) present in the non-volatile residue of the essential oils of Mandarin, Sweet Orange, Bitter Orange, Bergamot and Grapefruit were analysed with an HPLC/API/MS system equipped with an APcI probe in positive mode. The use of hyphenated techniques, such as LC/MS provides a great information about the content and nature of constituents of natural complex matrices, such as essential oils. In this work, MS spectra were recorded at different voltages, to obtain structural information in addition to molecular weight information. The different response of the compounds identified has been also evaluated. The method allowed the confirmation of the identification of the main components of the fraction, previously reported for the different oils. MS characteristics of coumarins, psoralens and polymethoxylated flavones with different substitution patterns were determined on the basis of the response obtained with the APcI interface. Interface parameters were optimised to obtain a contemporaneous response for all the three classes of components. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Citrus essential oils; Coumarins; Psoralens; Polymethoxylated flavones; HPLC/MS

1. Introduction

The coupling of mass spectrometry to chromatographic techniques represents a very powerful tool for the analysis of organic components in many areas of application. Until today, GC-MS has been the most popular and used technique, even though it permits only the analysis of compounds with sufficient volatility and thermal stability. In the past 20 years, the LC-MS technique has increased its popularity, mainly linked to the development of MS interface technology. In particular, the introduction of atmospheric pressure ionisation (API) techniques has dramatically influenced recent advances in the on-line coupling of LC and MS [1–4].

* Corresponding author. Fax: +39-090-6766532.
E-mail address: pdugo@pharma.unime.it (P. Dugo).

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Working with natural complex matrices, that may contain components with a possible biological activity, the HPLC-MS technique represents a rapid method to obtain on-line structural information and molecular weight, before isolation and biological assays. In fact, recently numerous studies on organic compounds extracted from plant material have been carried out by HPLC-MS and also by on-line coupling HPLC and NMR [5,6]. These detectors result more useful than other kind of LC detectors, such as UV, RI, fluorescence, either for the number of information that can be obtained, or for their universality. The main problem working with LC-MS of natural products is the choice of the ionisation technique. Unfortunately, there is not a single interface that allows the analysis of all the constituents of a natural matrix. Particle beam (PB) and thermospray (TSP) interfaces are the most commonly used for natural components analysis; both of them show many drawbacks, such as the difficulty to optimise ionisation conditions and the lack of sensitivity; moreover PB and TSP are suitable only for a restricted number of samples [7,8]. Meanwhile, electrospray (ESI) and atmospheric pressure chemical ionisation (APcI) techniques, which operate under atmospheric pressure, seem to be very promising. A combination of the use of these two interfaces seems to cover the analysis of a wide range of natural products [6]. In particular APcI has been labelled 'the chromatographer's LC-MS interface' [9] because of its high solvent flow rate capability, sensitivity, response linearity and fields of applicability.

This paper reports results obtained by HPLC-MS analysis by atmospheric pressure chemical ionisation (APcI) in positive mode for the characterisation of oxygen heterocyclic compounds of citrus essential oils. Literature does not report HPLC/API/MS data on oxygen heterocyclic compounds of citrus oils.

These components have an important role in the characterisation of citrus oils, since the qualitative and quantitative composition of the fraction is characteristic of each oil.

Moreover, many pharmacological and toxicological activities have been demonstrated for most of them, while other components have not extensively studied yet [10–16].

2. Experimental

This research was carried out on samples of genuine cold-pressed citrus oils (sweet orange, mandarin, bitter orange, grapefruit, bergamot).

All the analyses have been performed on a Shimadzu system equipped with two pumps LC 10-AD, a high pressure gradient, a UV detector SPD-10A, a controller SCL-10A and a degasser DGU-14A.

The system was coupled to a MS detector Shimadzu QP-8154 equipped with an APcI interface. UV and MS data were acquired and processed using QP-8000 software for Windows NT.

Essential oil samples were separated onto a C18 Pinnacle 250 × 4.6 mm column, with particle size of a 5 μm (Restek). Injected volume: 20 μl of a solution of oil in CH_3CN (1:10 v/v). Mobile phase: solvent A, tetrahydrofuran:acetonitrile:methanol: water (15/5/22/58); solvent B, acetonitrile. Flow: 1 ml/min. Sweet orange and mandarin were analysed under isocratic conditions, with 100% of solvent A; bitter orange, grapefruit and bergamot were analysed according to the following gradient program: 0–15 min: 100% A; 15–30 min: 50% A + 50% B; 30–40 min: 30% A + 70% B; UV detection at 315 nm. Standard solutions of tangeretin, 5-geranyloxy-7-methoxycoumarin, citropten, bergapten, bergamottin, aurapten, and meranzin were analysed in flow injection at a concentration of 10 ng/μl. Mobile phase: solvent A, water:acetonitrile (50/50). Flow: 1 ml/min.

The MS acquisition was performed under the following conditions: probe high voltage, 4 kV; APcI temperature, 400°C; nebulising gas (N_2) flow rate, 2.5 l/min; curved desolvation line (CDL) voltage, −25.5 V; CDL temperature, 230°C; deflector voltages, 25 and 60 V; acquisition mode, SCAN, 50–500 m/z.

3. Results

3.1. Choice of the interface and optimisation of the conditions

Electrospray (ESI) and atmospheric pressure chemical ionisation (APcI) differ in the way they

generate ions, but show many similarities: both operate at atmospheric pressure, giving molecular weigh information and additional structural information. Many classes of compounds can be analysed by both APcI and ESI; however, ESI is the technique of choice for polar and higher molecular weight compounds, while APcI is suitable for less polar compounds and of lower molecular weight than ESI. Preliminary experiments showed that polymethoxylated flavones, which are more polar than coumarins and psoralens, can be analysed both with positive ESI and APcI; coumarins and psoralens give a best response with $APcI^+$, while ions were not observed in ESI^+ mode. For this reason, further experiments were carried out using the APcI source in positive mode. APcI voltage, APcI temperature, CDL voltage, CDL temperature have been changed to optimise the ionisation. Analyses have been carried out at different deflector voltages to obtain different degrees of fragmentation, and, as a consequence, different structural information.

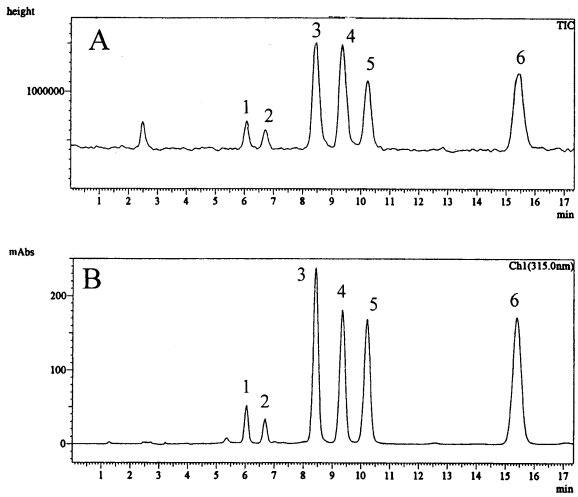

Fig. 1. (A) HPLC TIC chromatogram at 25 V and (B) HPLC UV chromatogram of the oxygen heterocyclic fraction of sweet orange oil. (1) Sinensetin; (2) Hexamethoxyflavone; (3) Nobiletin; (4) Heptamethoxyflavone; (5) Tetra-*O*-methylscutellarein; (6) Tangeretin.

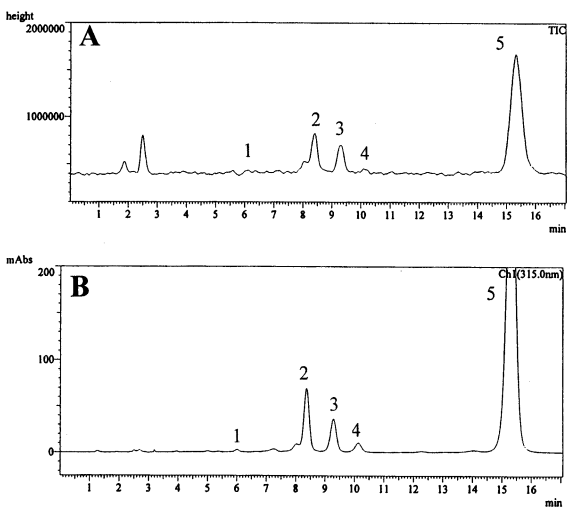

Fig. 2. (A) HPLC TIC chromatogram at 25 V and (B) HPLC UV chromatogram of the oxygen heterocyclic fraction of mandarin oil. (1) Sinensetin; (2) Nobiletin; (3) Heptamethoxyflavone; (4) Tetra-*O*-methylscutellarein; (5) Tangeretin.

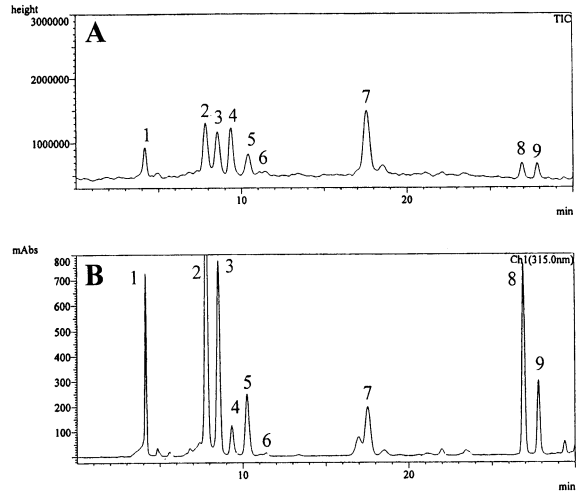

Fig. 3. (A) HPLC TIC chromatogram at 25 V and (B) HPLC UV chromatogram of the oxygen heterocyclic fraction of bitter orange oil. (1) Meranzin hydrate; (2) Meranzin; (3) Isomeranzin; (4) Nobiletin; (5) Heptamethoxyflavone + Bergapten; (6) Tetra-*O*-methylscutellarein; (7) Tangeretin; (8) Osthol; (9) Epoxybergamottin.

3.2. Identification of oxygen heterocyclic components

Figs. 1–5 show the HPLC-MS chromatogram at 25 V (low fragmentation voltage) of the five oils analysed, compared to the HPLC-UV

chromatogram at 315 nm, acquired contemporaneously. Under these conditions, the $[M+H]^+$ ion has been obtained for each component. Fig. 6 shows the basic structures of coumarin, psoralens and polymethoxylated flavones. Numbered position may be substituted. Table 1 reports the list of the oxygen heterocyclic compounds identified in citrus essential oils. Table 2 reports the list of the mayor ions observed at 25 V and 60 V for each compound. HPLC-API-MS analysis permitted the confirmation of the identification of the main components of the oxygen heterocyclic fraction, previously reported for the different oils [17]. As can be seen from date reported in Table 2, all the identified components show the $[M+H]^+$ ion in the MS spectrum acquired at low deflector voltage (25 V). Only the two components which have an epoxygeranyloxy group as a substituent show an adduct ion with acetonitrile $[M+H+41]^+$. Increasing the deflector voltage (60 V), coumarins and psoralens loss their side chains. 7,8-disubstituted coumarins give a tropilium ion at $m/z = 189$ [16]. Polymethoxylated flavones loss a methoxy-group; the ion corresponding to the loss of a methyl-group is also visible.

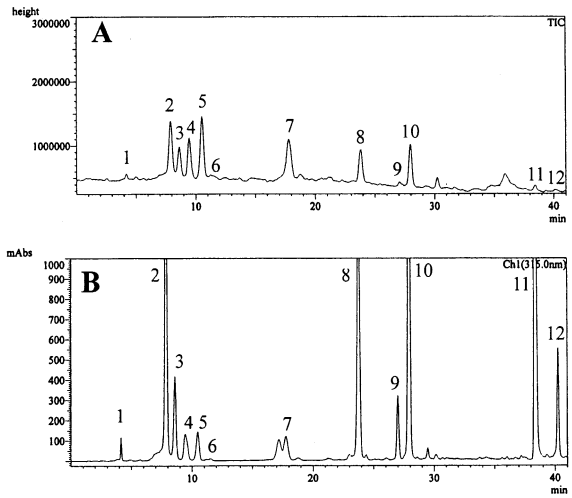

Fig. 4. (A) HPLC TIC chromatogram at 25 V and (B) HPLC UV chromatogram of the oxygen heterocyclic fraction of grapefruit oil. (1) Meranzin hydrate; (2) Meranzin; (3) Isomeranzin; (4) Nobiletin; (5) Heptamethoxyflavone + Bergapten; (6) Tetra-*O*-methylscutellarein; (7) Tangeretin; (8) Epoxyaurapten; (9) Osthol; (10) Epoxybergamottin; (11) Aurapten; (12) Bergamottin.

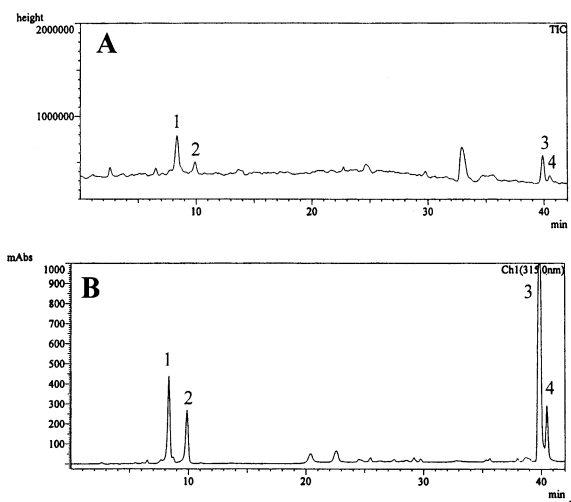

Fig. 5. (A) HPLC TIC chromatogram at 25 V and (B) HPLC UV chromatogram of the oxygen heterocyclic fraction of bergamot oil. (1) Citropten; (2) Bergapten; (3) Bergamottin; (4) 5-Geranyloxy-7-methoxycoumarin.

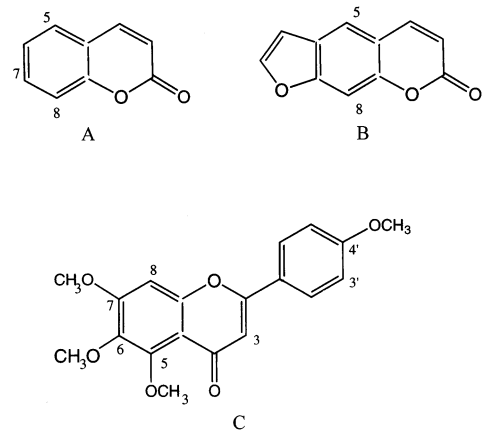

Fig. 6. Chemical structure of coumarins (A), psoralens (B) and polymethoxylated flavones (C).

3.3. APcI-MS characteristics of coumarins, psoralens and polymethoxylated flavones

As can be seen observing Figs. 1–5, the MS response varies depending on the class of the

component analysed and, in the same class, on the substitution pattern. In fact, comparing the HPLC-UV chromatogram to the HPLC-MS chromatogram of sweet orange or mandarin oils (Figs. 1 and 2), that contain only PMFs, the two chromatograms are very similar; making the same comparison in the case of bitter orange or grapefruit oils (Figs. 3 and 4), that contain coumarins, psoralens and PMFs, the two chromatograms show the same peaks, but the area % are very different.

Standard solutions of some of the identified components, chosen on the basis of the substitution pattern, have been injected into the LC-MS at different concentration, to evaluate the sensitivity. Table 3 reports the values of ions intensities for the standard components analysed. Table 3 also reports the average quantitative data obtained in our laboratory for the oxygen heterocyclic compounds of cold-pressed genuine samples of mandarin, sweet orange, bitter orange, grapefruit and bergamot oil.

We have observed that polymethoxylated flavones give a similar response independently from the position and the number of methoxy-groups. This can be easily deduced by observing Figs. 1 and 2. Coumarins and psoralens give a response that varies in accordance with the position and the nature of the substituent.

Bergapten (5-methoxypsoralen) gives a better response than the other psoralens analysed, substituted at position 5 with geranyloxy-group or its corresponding epoxide or vicinal diol groups. This can be easily observed looking Fig. 5, since the concentration of bergamottin in bergamot oil is about ten times higher than that of bergapten.

Citropten (5,7-dimethoxycoumarin) and 5-geranyloxy-7-methoxycoumarin differ for the substituent at position 5; also in this case, as for psoralens, the presence of the geranyloxy-group at

Table 1
Oxygen heterocyclic compounds identified in citrus essential oils*

	B.O.	S.O.	B	M	G
Coumarins					
Aurapten (7-geranyloxycoumarin)					X
Epoxyaurapten (7-(6′,7′-epoxygeranyloxy)coumarin)					X
Meranzin (7-methoxy-8(2′,3′-epoxy)isopentenylcoumarin)	X				X
Osthol (7-methoxy-8-isopentenylcoumarin)	X				X
Meranzin hydrate (7-methoxy-8(2′,3′-dihydroxy)isopentyloxycoumarin	X				X
Isomeranzin (7-methoxy-8-(2′-one-isopentyl)coumarin)	X				X
Citropten (5,7-dimethoxycoumarin)	X		X		X
5-Geranyloxy-7-methoxycoumarin			X		
Psoralens					
Bergapten (5-methoxypsoralen)	X		X		X
Epoxybergamottin (5-(6′,7′-epoxy-geranyloxy)psoralen)	X				X
Epoxybergamottin hydrate (5-(6′,7′-dihydroxy-geranyloxy)psoralen)	X				X
Bergamottin (5-geranyloxypsoralen)			X		X
Polymethoxylated flavones					
Tangeretin (4′,5,6,7,8-pentamethoxyflavone)	X	X		X	X
Nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone)	X	X		X	X
3,3′,4′,5,6,7,8-Heptamethoxyflavone	X	X		X	X
Sinensetin (3′,4′,5,6,7-pentamethoxyflavone)		X	X	X	
3,3′,4′,5,6,7-Hexamethoxyflavone		X			
Tetra-O-methylscutellarein (4′,5,6,7-tetramethoxyflavone)	X	X	X	X	

* B.O., Bitter Orange; S.O., Sweet Orange; M, Mandarin; G, Grapefruit; B, Bergamot; Isopentenyloxy, 3′-methylbut-2′-enyloxy; Geranyloxy, 3′,7′-dimethyloct-2′,6′-enyloxy.

Table 2
Major quasi-molecular and other ions observed in positive APcI-MS for compounds listed in Table 1

Compounds	Deflectors voltage 25 V		Deflectors voltage 60 V	
	Ion species	m/z	Ion species	m/z
Coumarins				
Meranzin	$[M+H]^+$	261	$[M+H\text{-}C_4H_7O]^+$	189
MW = 260			$[M+H]^+$	261
			$[M+H\text{-}H_2O]^+$	243
Meranzin hydrate	$[M+H\text{-}H_2O]^+$	261	$[M+H\text{-}H_2O\text{-}C_4H_7O]^+$	189
MW = 278			$[M+H\text{-}H_2O]^+$	261
Isomeranzin	$[M+H]^+$	261	$[M+H\text{-}C_4H_7O]^+$	189
MW = 260			$[M+H]^+$	261
			$[M+H\text{-}H_2O]^+$	243
Osthol	$[M+H]^+$	245	$[M+H\text{-}C_4H_7]^+$	189
MW = 244			$[M+H]^+$	245
Epoxyaurapten	$[M+H+CH_3CN]^+$	356	$[M+H\text{-}C_{10}H_{16}O]^+$	163
MW = 314	$[M+H]^+$	315	$[M+H\text{-}H_2O]^+$	297
	$[M+H\text{-}H_2O]^+$	297		
Aurapten	$[M+H]^+$	299	$[M+H\text{-}C_{10}H_{17}]^+$	163
MW = 298				
Citropten	$[M+H]^+$	207	$[M+H]^+$	207
MW = 206			$[M+H\text{-}OMe]^+$	193
5-Geranyloxy-7-methoxycoumarin	$[M+H]^+$	329	$[M+H\text{-}C_{10}H_{17}]^+$	193
MW = 328				
Psoralens				
Bergomottin	$[M+H]^+$	339	$[M+H\text{-}C_{10}H_{17}]^+$	203
MW = 338				
Epoxybergamottin	$[M+H]^+$	355	$[M+H\text{-}C_{10}H_{16}O]^+$	203
MW = 354	$[M+H+CH_3CN]^+$	396		
	$[M+H\text{-}H_2O]^+$	337		
Bergaptene	$[M+H]^+$	217	$[M+H\text{-}OMe]^+$	203
MW = 216			$[M+H]^+$	217
Polymethoxyflavones				
Sinensetin	$[M+H]^+$	373	$[M+H]^+$	373
MW = 372			$[M+H\text{-}OMe]^+$	343
			$[M+H\text{-}Me]^+$	358
Hexamethoxyflavone	$[M+H]^+$	403	$[M+H]^+$	403
MW = 402			$[M+H\text{-}OMe]^+$	373
			$[M+H\text{-}Me]^+$	388
Nobiletin	$[M+H]^+$	403	$[M+H\text{-}OMe]^+$	373
MW = 402			$[M+H]^+$	403
			$[M+H\text{-}Me]^+$	388
Heptamethoxyflavone	$[M+H]^+$	433	$[M+H\text{-}OMe]^+$	403
MW = 432			$[M+H]^+$	433
			$[M+H\text{-}Me]^+$	418
Tetra-*O*-methyl-scutellarein	$[M+H]^+$	343	$[M+H]^+$	343
MW = 342			$[M+H\text{-}OMe]^+$	313
			$[M+H\text{-}Me]^+$	328
			$[M+H\text{-}OMe\text{-}OMe]^+$	282
			$[M+H\text{-}OMe\text{-}Me]^+$	298
			$[M+H\text{-}Me]^+$	328
Tangeretin	$[M+H]^+$	373	$[M+H\text{-}OMe]^+$	343
MW = 372			$[M+H]^+$	373
			$[M+H\text{-}Me]^+$	358

Table 3
Average amount of oxygen heterocyclic compounds in citrus essential oils (ppm) [17] and intensity of standard components

	Intensity[a]	B.O.	S.O.	B	M	G
Coumarins						
Aurapten	$<1\times10^4$					11 240
Epoxyaurapten						9300
Meranzin	$>1\times10^4$	9260				5100
Osthol		1710				580
Meranzin hydrate		340				120
Isomeranzin		1880				810
Citropten	$>1\times10^6$			2200		
5-Geranyloxy-7-methoxycoumarin	$>1\times10^5$			1300		
Psoralens						
Bergapten	$>1\times10^4$	630		2100		110
Epoxybergamottin		2750				11 260
Epoxybergamottin hydrate		250				700
Bergamottin	$<1\times10^4$			18700		970
Polymethoxylated flavones						
Tangeretin	$>1\times10^6$	1100	480		2140	680
Nobiletin		640	520		740	460
3,3′,4′,5,6,7,8-Heptamethoxyflavone		100	840		370	370
Sinensetin			90		20	
3,3′,4′,5,6,7-Hexamethoxyflavone			130			
Tetra-*O*-methylscutellarein		140	310		50	

[a] Signal intensity is expressed as area counts.

position 5 decreases APcI-MS response. The response becomes significantly lower if the geranyloxy-group is in position 7, as for aurapten and epoxyaurapten. Thise behaviour can be observed looking respectively at Fig. 5 and Fig. 4, and at data of Table 3.

7,8-Disubstituted coumarins, with a methoxy-group in position 7 and a 3-methylbut-2-enyl group or its corresponding epoxide, vicinal diol or ketone group in position 8, give a better response than 7-geranyloxycoumarin, but lower than citropten.

The response of coumarins is higher than that of psoralens. This observation can be made comparing compounds with the same substitution pattern: citropten and bergapten, or 5-geranyloxy-7-methoxycoumarin and bergamottin, that differ only because at position 5 there is a methoxy-group (coumarins) or the oxygen of the furanic ring (psoralen).

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