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Identification of 15-Hydroperoxyabietic Acid as a Contact Allergen in Portuguese Colophony

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Abstract—15-Hydroperoxyabietic acid (15-HPA) has been isolated from Portuguese colophony of the gum rosin type and identified as its methyl ester. The structure of the compound was elucidated using UV, IR, NMR and mass spectrometry. 15-HPA methyl ester was found to be an elicitor when tested in colophony-sensitized guinea-pigs. The sensitizing capacity was verified in the same species and 15-HPA methyl ester was considered to be a strong allergen. The eliciting potential was also verified in patients with known allergy to colophony. The Portuguese gum rosin investigated contained approximately 1% of 15-HPA. Based on its allergenicity and the amounts isolated, we conclude that 15-HPA is a main contact allergen in Portuguese gum rosin.

Colophony and its derivatives are among the most common causes of allergic contact dermatitis. It is a widespread material, the global production approaching 1 million tons per year. Every year about 60 new patents on colophony derivatives are published. The main areas of use are paper size, adhesives, emulgators and soldering fluxes (Hausen et al 1982a; Adams 1983).

An increasing incidence of allergic reactions to colophony has been reported in West Germany (Hausen et al 1982b) and in Sweden (pers. comm. I. Jeansson, Dept. of Derm., Kalmar; H. Möller, Dept of Derm., Malmö; J. E. Wahlberg, Dept. of Occ. Derm., Stockholm). Allergic contact dermatitis to colophony has been connected particularly with the use of plasters (Bonnevie 1939; Hausen et al 1982b). Since colophony is present in so many different products, the exact origin for a patients's contact allergy may be difficult to determine (Karlberg & Lidén 1985).

Colophony is complex chemically. It contains about 90% resin acids and 10% neutral matter. Three main types of rosin are produced (gum rosin, wood rosin, and tall oil rosin). Gum rosin and tall oil rosin are the dominant products on the market. Gum rosin is obtained from living pine trees by oleoresin tapping and steam distillation to remove the turpentine constituents. Tall oil rosin is derived from crude tall oil obtained as a by-product in paper pulp production. The chemical compositions as well as the allergenic activities of the rosins differ from each other (Karlberg et al 1986). The allergenic components have never been isolated and identified, but abietic acid is often claimed to be the compound responsible (Hausen et al 1982b; Foussereau et al 1982).

As part of our research on allergic contact dermatitis, we describe the isolation and identification of a main contact allergen in Portuguese colophony of the gum rosin type.

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Materials and Methods

Materials

Portuguese colophony of the gum rosin type produced by SOCER, Lisbon, Portugal was of commercial quality. White petrolatum (Vaselinum album) produced by Witco Chemical Corp., New York, USA was of pharmaceutical quality. Silica gel 60, for column chromatography, dichloromethane of HPLC grade, ethyl acetate and toluene of analytical grade were obtained from Merck, Darmstad, FRG. Diethyl ether of analytical grade was obtained from May & Baker Ltd., Dagenham, UK.

Column chromatography

A solution of 50 g of gum rosin in toluene (200 mL) was applied to a column of 1400 g of silica gel. The column was eluted with toluene 4000 mL and then with toluene–ethyl acetate mixtures of increasing polarity to a total amount of 16 L. Fractions of 1 L each were collected, evaporated and analysed by TLC. Fractions with similar TLC-properties were pooled, giving six different fractions.

Flash chromatography, using dichloromethane or ethyl acetate as eluents, was performed as described by Still et al (1978). The system was pressurized with N_2. Each fraction was analysed by TLC.

Thin layer chromatography (TLC)

TLC was performed using silica gel G fluorescent plates, 0·2 mm thick (HPTLC Fertigplatten, Kieselgel 60, F 254, Merck, Darmstadt). The spots were applied with Camac Nanomat II and the plates were developed in ethyl acetate–toluene 1 : 3. The spots were detected in UV light at 254 nm.

High performance liquid chromatography (HPLC)

For HPLC, the equipment used was: Waters pump model 590, LKB 2151 variable wavelength monitor, LKB Super Rac 220 V fraction collector, Nucleosil 50-7 preparative column from Macherey-Nagel Düren, West Germany, size 250 × 20 mm (l. × i.d.) and 7 μm particle size. Precolumn

size 30 × 20 mm (l. × i.d.) was packed with Nucleosil 100-15 from the above company. The eluent consisted of ethanol–dichloromethane (0·5 : 99·5 v/v) which was pumped at a rate of 20 mL min^{-1}. Injections were made with samples of 100 mg dissolved in 1 mL dichloromethane. The wavelength detector was set at 254 nm and fractions were collected every 30 s.

Spectroscopy

Infrared spectra were obtained using a Perkin Elmer 580 model. An ether solution of the sample was evaporated in a stream of N_2 on the surface of a KBr disc. The UV-spectra were recorded on a Perkin Elmer Lambda 5 instrument using methanol solutions. The NMR-spectra were recorded on a Jeol FX 90 Q instrument using $CDCl_3$ solutions with TMS as internal standard. For mass spectrometry a Shimadzu QP-1000 instrument with a direct insertion probe was used. The parameters used in chemical ionization mode were: ionization energy 200 eV, ion source temperature 200–250 °C, scan range 110–450 *m/z*, scan cycle time 3·0 s and isobutane as reagent gas. Since the instrument has no direct reading facilities of the ion source pressure, the conditions for chemical ionization were tuned by adjusting the ratio between 43 and 57 *m/z* to approx. 1 : 2.

Reduction of the isolated compound with $LiAlH_4$

About 50 mg of $LiAlH_4$ was added to a solution of 2 mg of the isolated compound in 10 mL of dry ether. The mixture was stirred for 1 h, whereupon 1 M H_2SO_4 was added to bring the mixture into solution. The ether layer was separated, dried (Na_2SO_4), filtered and concentrated.

Esterification of the fractions

Two grams of a fraction from the chromatography column was dissolved in ether. A solution of diazomethane was added until the reaction mixture remained yellow. The solution was left at room temperature (20 °C) overnight and was then concentrated.

Sensitization experiments in guinea-pigs

Albino guinea-pigs of Dunkin Hartley strain from H. B. Sahlin, Malmö, Sweden were tested according to the modified Freund's complete adjuvant test method (FCAT) (Klecak 1985) for details see Boman et al (1987).

Induction. The animals were induced with three intradermal injections of 5% solutions of the test materials in Freund's complete adjuvant (FCA). Gum rosin was used for induction when the allergenicity of different fractions was studied. The isolated compound was used both for induction and challenge when investigating its sensitizing capacity.

Challenge. Challenge testing was performed using the closed patch test method with Finn Chambers (Epitest, Helsinki, Finland). The patches were removed after 24 h and assessment of the reactions was made at 48 h and at 72 h after start of exposure. The vehicle for the test preparations was petrolatum. The test concentrations were chosen from experience (Karlberg et al 1980, 1985, 1986) and are given in Tables 1, 2, 4, and 5. Gum rosin was used as a positive and petrolatum as a negative control. Results and statistical analyses are presented as outlined by Wahlberg & Boman (1985).

Patch testing in man

Patch testing was carried out according to the internationally accepted method for diagnosis of contact allergy (Fregert 1981). Ten patients with previously verified sensitivity to colophony of the gum rosin type and eleven healthy control subjects were patch tested with Portuguese gum rosin and the isolated compound. In these tests, Finn Chamber and Scanpor surgical tape (Norgesplaster, Kristiansand, Norway) were used. The vehicle for the test preparations was petrolatum. The patches were removed after 48 h and readings were made 72 h after application. This study was approved by the local ethical committee.

Results and Discussion

Colophony is believed to contain several allergens. Both the acid and neutral parts of gum rosin have been found to be allergenic (Karlberg et al 1986). Abietic acid (I) is the

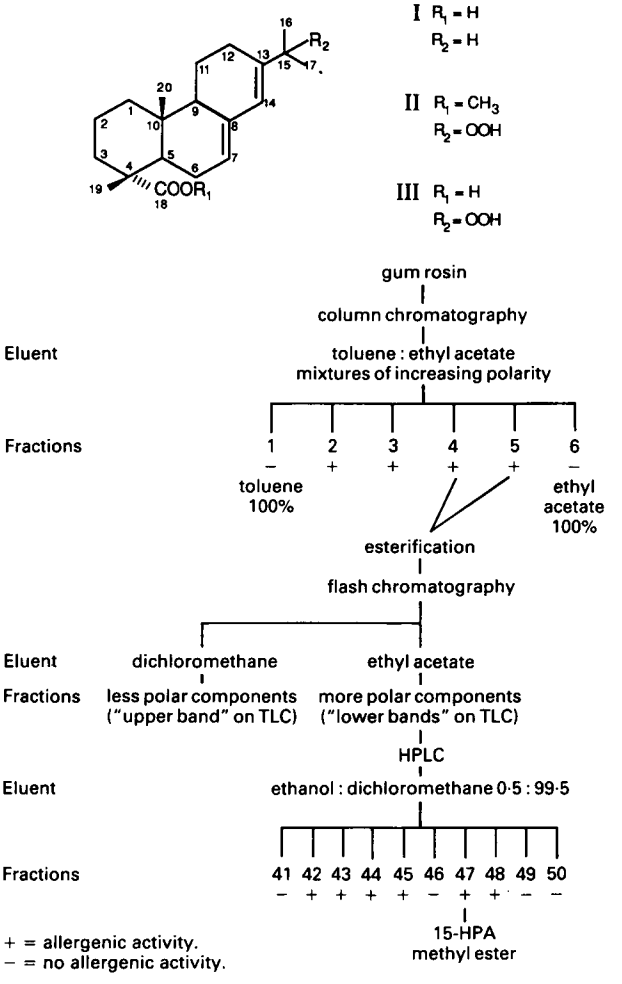


FIG. 1. Flow chart of the fractionation of Portuguese gum rosin.

major component of colophony (40–50%) and has been claimed to be the dominant allergen (Adams 1983; Foussereau et al 1982). However, we have recently demonstrated in guinea-pigs that purified abietic acid itself is not a contact allergen suggesting that allergenic compounds could be formed from abietic acid, probably by air oxidation (Karlberg et al 1985).

We avoided the use of acid-base treatment of the natural material since an allergen in colophony might be a labile compound. Chromatographic procedures were therefore

Table 1. Eliciting activity of the fractions from silica gel column chromatography of gum rosin. (Guinea-pigs: modified FCAT method.) *Induction*: gum rosin 5% w/w. *Challenge*: The animals were tested with the different fractions, gum rosin and petrolatum. The number of animals with positive test reactions 48 h and 72 h after start of exposure is given.

		Challenge material (10% w/w in petrolatum)							
Fractions		1	2	3	4	5*	6	Gum rosin pos. contr.	Petrolatum vehicle control
Yield (g) from 50 g of gum rosin		0·8	1·5	10·6	22·7	12·8	3·5		
Exposed (n = 9)	48 h	1	4	4	6	9	1	8	0
	72 h	1	5	5	7	9	3	7	0
Controls (n = 5)	48 h	0	1	0	1	1	0	1	0
	72 h	0	0	0	1	1	0	1	0

* 7/9 animals reacted after esterification with diazomethane.

Table 2. Eliciting activity of fractions from the HPLC separation (guinea-pigs; modified FCAT method). *Induction*: gum rosin 5% w/w. *Statistical method*: χ^2 analysis.

	Challenge material											
Fractions	41	42	43	44	45	46	47	48	49	50	Gum rosin	Petro-latum
	Concn in petrolatum (% w/w)											
	1	1	1	1	1	1	5*	1	1	1	10	
Exposed												
n	13	12	13	13	13	12	13	12	13	12	21	21
48 h	0	6	2	10	6	1	8	4	4	1	17	1
72 h	0	2	4	8	8	1	7	3	3	1	17	0
Controls												
n	12	12	12	12	12	12	12	12	12	12	20	20
48 h	0	0	0	1	0	0	0	0	1	1	1	2
72 h	0	0	0	0	0	0	0	0	0	0	0	1
P												
48 h	NS	<0·01	NS	<0·001	<0·01	NS	<0·001	<0·05	NS	NS	<0·001	NS
72 h	NS	NS	<0·05	<0·001	<0·001	NS	<0·01	NS	NS	NS	<0·001	NS

* Fraction 47 was also found to have eliciting activity at 1% concentration in a subsequent test.
NS = Not significant.

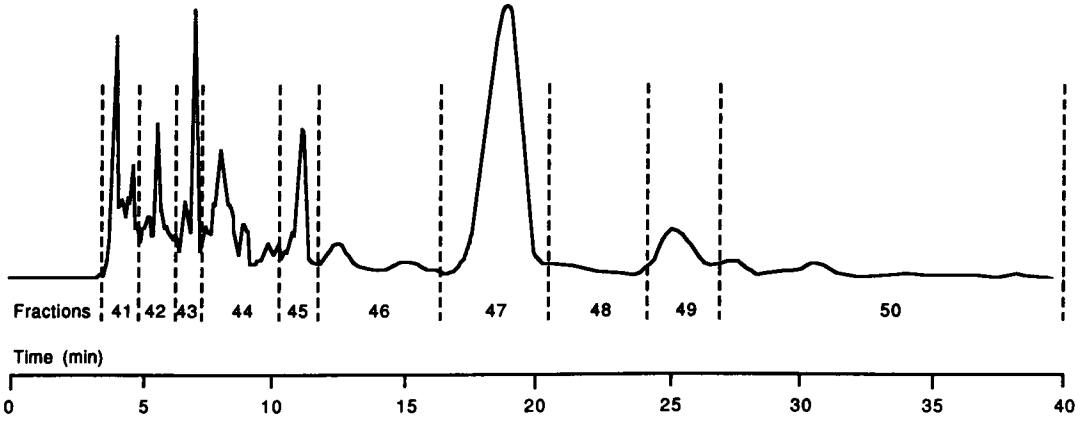


FIG. 2. HPLC-separation of different compounds from Portuguese gum rosin.

employed for the isolation, instead of the commonly used partitioning separation (Holmbom 1978).

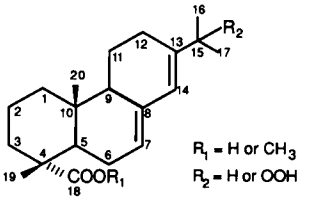
To define the capacity of a chemical to cause contact allergy, there are various experimental methods using guinea-pigs. The methods have different advantages depending on the aim of the study (Andersen & Maibach 1985). We found Freund's Complete Adjuvant Test (FCAT) method to be the most suitable for our study (Boman et al 1987).

A large silica gel column was used for a first crude separation (Fig. 1). The amounts of material in each of the six fractions are shown in Table 1. Each fraction was tested for eliciting activity using guinea-pigs sensitized to gum rosin (Table 1). Most animals (6/9 and 9/9, respectively) reacted to fractions 4 and 5, so these two fractions were chosen for further fractionation.

Esterification of the material using diazomethane improved the chromatographic properties considerably. To determine if the esterification had any influence on the eliciting activity, the animals were retested with the esterified and the non-esterified fraction 5. This gave a result very similar to that shown in Table 1. Thus, the esterification did not change the eliciting potential.

Thin layer chromatography of the methylated material from fractions 4 and 5 showed a major band with the same R_F-value as abietic acid methyl ester (upper band) and several lower bands (more polar components). The major components were separated by flash chromatography (Fig. 1). The material corresponding to the upper band in TLC was eluted with dichloromethane, while the more polar compounds were eluted with ethyl acetate. The material eluted with dichloromethane had no significant eliciting potential (data not shown).

Table 3. NMR spectral data of abietic acid, its methyl ester and the isolated compound of Portuguese gum rosin.



Position	Abietic acid methyl ester*	Abietic acid**	Isolated compound	
	^1H	^{13}C	^1H	^{13}C
1		38·3		38·3
2		18·1		18·1
3		37·2		37·1
4		46·3		46·5
5		44·9		45·0
6		25·6		25·6
7	5·36	120·5	5·46	122·3
8		135·5		135·0
9		51·0		50·6
10		34·5		34·5
11		22·5		22·5
12		27·5		25·8
13		145·1		144·6
14	5·77	122·5	6·06	122·9
15		34·8		68·1
16	1·00 d	20·9	1·32	28·5
17		21·4	1·34	28·7
18		185·4		185·4
19	1·25	16·7	1·26	17·0
20	0·83	14·0	0·82	14·0
R_1	3·62	—	3·63	51·9

* The sample was purified immediately before the spectrum was recorded as described by Karlberg et al (1985).
** Data according to Smith (1978).

Table 4. Sensitizing capacity of 15-hydroperoxy abietic acid methyl ester (15-HPA-Me-ester) (guinea-pigs; modified FCAT method). *Induction*: 15-HPA-Me-ester 5% w/w. *Statistical method*: χ^2 analysis.

		Challenge material				
		15-HPA-Me* ester	15-HPA-Me* ester	15-HPA-Me** ester	Gum rosin	
		Concn in petrolatum (% w/w)				
		5	1	5	10	Petrolatum
Exposed (n = 11)	48 h	11	8	10	6	2
	72 h	7	8	11	7	3
Controls (n = 15)	48 h	0	0	0	0	0
	72 h	1	0	0	1	1
P	48 h	<0·001	<0·001	<0·001	<0·01	NS
	72 h	<0·01	<0·001	<0·001	<0·01	NS

NS = Not significant.
* The test material was prepared just before challenge.
** The test material was prepared 3 months before challenge and kept in a refrigerator.

The material eluted with ethyl acetate was separated into ten fractions using preparative straight phase HPLC (Figs 1, 2). Each fraction was then tested for eliciting activity. The challenge test concentrations in petrolatum were chosen to correspond to the approximate relations in natural colophony (1 and 5% w/w). Several fractions were found to have eliciting activity (Table 2). Most of the chromatographed material appeared in fraction 47 which elicited allergic contact dermatitis at the 5% challenge concentration. A concentration of 1% also gave allergic reactions in a subsequent test.

TLC, HPLC and the NMR-spectra of fraction 47 indicated the presence of only one compound. Around 10 mg of this could be isolated from 1 g of colophony but was obtained as an oil that did not crystallize. Its structure was determined primarily from spectral data. The UV-spectrum (MeOH) showed absorption maxima at 232 (sh), 240 and 249 (sh) nm; cf. abietic acid methyl ester with maxima at 233 (sh), 240 and 249 (sh) nm. The infrared spectrum showed prominent peaks at 3450 cm^{-1} (OH), 1725 cm^{-1} (C=O), 1245 cm^{-1} (C–O) and 730 cm^{-1}. The band at 730 cm^{-1} might be due to a C–OOH group although this band in most cases is reported to appear at 920–830 cm^{-1} (Bellamy 1975; Socrates 1980); cf. abietic acid methyl ester with strong peaks at 1725 cm^{-1} and 1245 cm^{-1}.

The mass spectrum (CI) showed an ion at m/z = 349, consistent with an (M + 1) ion corresponding to abietic acid methyl ester plus two oxygen atoms (M + 32). This might either correspond to two OH-groups or one -OOH group added to the abietic acid structure. To differentiate between these two possibilities, a sample was treated with $LiAlH_4$, and the crude product was analysed by mass spectrometry (CI). The spectrum showed an ion at m/z = 305 consistent with a (M + 1) ion for a compound formed

Table 5. Eliciting activity of abietic acid and its methyl ester (guinea-pigs; modified FCAT method). *Induction*: gum rosin 5%. *Statistical method*: χ^2 analysis.

		Challenge material (10% w/w in petrolatum)			
		Abietic acid	Abietic acid methyl ester	Gum rosin	Petrolatum
Exposed (n = 15)	48 h	1	0	11	0
	72 h	2	3	12	0
Controls (n = 15)	48 h	0	0	0	0
	72 h	1	1	1	2
P	48 h	NS	NS	<0·001	NS
	72 h	NS	NS	<0·001	NS

NS = Not significant.

by reduction of an ester and a hydroperoxy group to $-CH_2OH$ and -OH, respectively. This indicates that the isolated compound is a hydroperoxy derivative of abietic acid methyl ester.

^1H- and ^{13}C-NMR spectral data of abietic acid, its methyl ester and the isolated compound (Table 3) show great similarities but also some specific differences. In the proton spectrum of abietic acid methyl ester, the protons in the two methyl groups C-16 and C-17 give rise to a doublet centred at δ 1·00 ppm; whereas in the isolated compound, they appear as two signals at δ 1·32 and 1·34 ppm. Also the vinylic protons at positions C-7 and C-14 appear at higher shifts in the spectrum of the isolated compound compared with those in abietic acid methyl ester. The shifts in the two ^{13}C-NMR spectra are similar except for the carbons of the isopropyl group. The C-15 appear at δ 68·1 ppm in the isolated compound compared with δ 34·8 ppm in abietic acid (Table 3). Also, the two methyl groups C-16 and C-17 appear at higher shifts. This indicates that the hydroperoxy group is located at C-15.

Taken together, the chemical and spectral data are consistent with the structure 15-hydroperoxyabietic acid methyl ester (15-HPA-Me-ester; II) a compound not previously described. It is apparently formed by air oxidation of the abietic acid in colophony. It is known that abietic acid is readily oxidized by atmospheric oxygen (Sandermann 1960). Schuller & Lawrence (1961) suggested the formation of a hydroperoxy-transannular peroxide from neoabietic acid. Auto-oxidation of abietic acid has been studied by Enoki & Kitao (1975) who did not find a hydroperoxy compound.

Table 6. Test reactions in 10 dermatitis patients sensitive to colophony of the gum rosin type when patch tested with 15-hydroperoxyabietic acid methyl ester (15-HPA-Me-ester), Portuguese gum rosin and petrolatum.

	15-HPA-Me* ester	15-HPA-Me* ester	15-HPA-Me** ester	Gum rosin	
	Concn in petrolatum (% w/w)				
	5	1	5	10	Petrolatum
Patient reactions:					
Positive	8	8	7	10	0
Negative	2	2	3	0	10

* The test material was prepared just before challenge.
** The test material was prepared 3 months before test and kept in a refrigerator.

According to MS analyses 15-HPA-Me-ester is stable enough to be kept in a refrigerator for at least six months without apparent chemical deterioration. Its sensitizing capacity was verified in guinea-pigs (Table 4) induced with 15-HPA-Me-ester and then challenged with 15-HPA-Me-ester in two concentrations. The result shows 15-HPA-Me-ester to be a strong allergen. A concentration of 1% gave allergic reactions in 8 out of 11 induced animals. 15-HPA (III) was isolated and tested only as its methyl ester and not in its native acid form. We have earlier shown that pure abietic acid is not allergenic (Karlberg et al 1985) and esterification does not add any allergenic activity (Table 5). Furthermore, the esterification did not effect the allergenic potential of the material from the first column separation (above and Table 1). The allergenicity of the isolated compound is thus not related to the esterification, but rather to an inherent property of 15-HPA. The positive reactions to gum rosin in the test (Table 4) indicate that 15-HPA is an ingredient of the resin and not formed in the isolation procedure.

Patch testing with 15-HPA-Me-ester (5 and 1% w/w) in dermatitis patients with known allergy to colophony of the gum rosin type gave allergic reactions in most of the patients (8 out of 10) (Table 6). Two patients did not react, indicating allergy to other compounds in gum rosin. Patch testing a control group of 11 persons gave no allergic reactions. Based on its allergenicity and the amount isolated, we consider 15-HPA to be a main contact allergen in Portuguese gum rosin.

Hydroperoxides have previously been suggested to be contact allergens, but to our knowledge, there are no reports which conclusively show the allergencity of a hydroperoxide of a known structure. Hydroperoxides of Δ-3-carene are considered to be the main allergens of turpentine (Hellerström et al 1955; Pirilä et al 1966), although turpentine, like colophony, contains several allergens (Grimm & Gries 1967, 1968). None of the suggested hydroperoxides have, to our knowledge, been isolated and identified (Pirilä 1970).

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