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15-HYDROPEROXYDEHYDROABIETIC ACID—A CONTACT ALLERGEN IN COLOPHONY FROM *PINUS* SPECIES

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Key Word Index—*Pinus*; Pinaceae; colophony; rosin; gum rosin; tall oil rosin; contact allergen; synthesis; dehydroabietic acid; hydroperoxide.

Abstract—A new hydroperoxide, 15-hydroperoxydehydroabietic acid (15-HPDA), with contact allergenic properties has been detected in rosin obtained from *Pinus* species. Detection was facilitated using a synthetic preparation of 15-HPDA for reference purposes. The synthesis and the detection (HPLC and GC) of 15-HPDA in rosin are described. The allergenic activity of 15-HPDA was studied in an experimental sensitization test on guinea-pigs.

INTRODUCTION

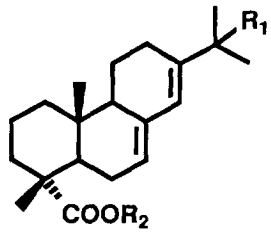
In our research on naturally occurring allergenic substances we have especially studied the allergenic activity of compounds found in colophony (gum rosin), the distillation residue of oleoresin from *Pinus palustris* Mill. and other *Pinus* species [1, 2]. Colophony is among the 10 most common causes of delayed hypersensitivity [3–5]. Gum rosin and tall oil rosin, a by-product in the pulp industry, are used almost interchangeably in technical products and world production is about one million tons a year. Colophony is a complex mixture of diterpene resin acids (85–90%), diterpene alcohols, aldehydes and hydrocarbons. The major resin acids are abietic acid (**1**) and dehydroabietic acid (**3**) [6]. In particular, abietic acid is easily oxidized by atmospheric oxygen. In nature the oxidation gives an antimicrobial effect to the exudate when this comes in contact with air owing to wounding of the tree. The tree is then protected from microbes until healed.

In previous studies we have isolated oxidation products of abietic acid and dehydroabietic acid and identified them as contact allergens [7–10]. 15-Hydroperoxyabietic acid (15-HPA) (**2**) in gum rosin and tall oil rosin has been identified as a major contact allergen among the oxidation products [7]. However, the corresponding hydroperoxide of dehydroabietic acid (15-HPDA) (**4**) has not been previously identified. Since didehydroabietic acid peroxide (DeA-OO-DeA) (**5**) is another of the identified allergenic compounds [10], it is reasonable to assume that 15-HPDA could be an intermediate in the formation of this peroxide. Owing to the structural similarity between 15-HPDA and the earlier identified 15-HPA it is probable that 15-HPDA could be a contact allergen with a potency similar to that of 15-HPA. We, therefore, decided to develop a synthetic route to 15-HPDA and use this compound as a reference material to facilitate the detection of 15-HPDA in colophony. In the present paper we describe the synthesis of 15-HPDA and its subsequent identification in methyl esterified Portuguese colophony and Swedish tall oil rosin. The sensitizing potential of 15-HPDA was also studied in guinea-pigs. The hydroperoxide of dehydroabietic acid was synthesized, detected in rosin and studied in guinea-pigs as its methyl ester. We use the abbreviation 15-HPDA for the methyl ester also.

RESULTS AND DISCUSSION

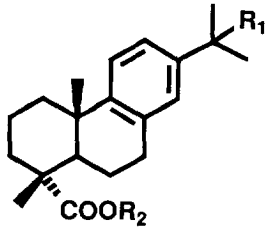
For the synthesis of the hydroperoxide **4a** the synthetic route depicted in Scheme 1 was followed. Dehydroabietic acid methyl ester (**3a**) was used as starting material. This compound was dehydrogenated to **6** using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). The 15-hydroxydehydroabietate (**7**) was obtained in 80% yield by oxymercuration followed by reduction using alkaline sodium borohydride. While this paper was in preparation **7** was reported [11] prepared essentially according to the same procedure. In our synthesis the hydroxy compound **7** was transferred to the corresponding hydroperoxide **4a** using excess aqueous hydrogen peroxide and acid catalysis. The new compounds were identified with IR, NMR and mass spectrometry.

Portuguese colophony (gum rosin) and Swedish tall oil rosin were analysed by means of HPLC. The synthesized 15-HPDA was used as reference substance. Tall oil rosin contained a peak eluting at the same retention time as 15-HPDA (Fig. 1). This peak was isolated for subsequent



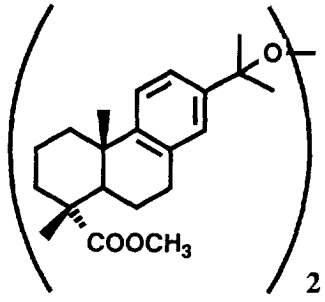
1 R1=R2=H,
1a R1=H, R2=CH3

2 R1=OOH, R2=H,
2a R1=OOH, R2=CH3



3 R1=R2=H,
3a R1=H, R2=CH3

4 R1=OOH, R2=H,
4a R1=OOH, R2=CH3



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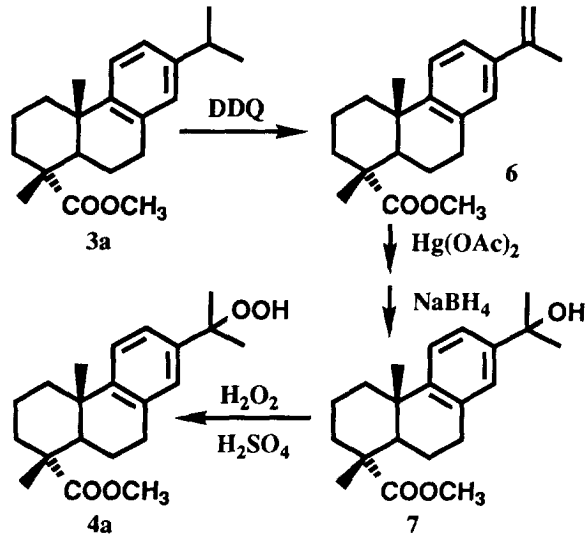

analysis with mass spectrometry. Owing to the thermal instability of 15-HPDA, the sample was introduced to the mass spectrometer with a direct insertion probe (DIP). The mass spectrum obtained from the compound in the collected HPLC fraction showed an identical fragmentation pattern to, and the same molecular ion as that of the synthesized reference compound. This confirms that 15-HPDA is present in Swedish tall oil rosin. 15-HPDA was not detected in the Portuguese gum rosin. Theoretically, 15-HPDA should be present, since the didehydroabietic acid peroxide has been identified in gum rosin [10]. This peroxide is probably formed from 15-HPDA.

It is not likely that 15-HPDA is an artefact from the distillation process. The high temperature will rather decrease the level of hydroperoxides present. 15-HPDA has not been reported in pine exudate, but several dehydroabietic acid derivatives with a 15-OH group have

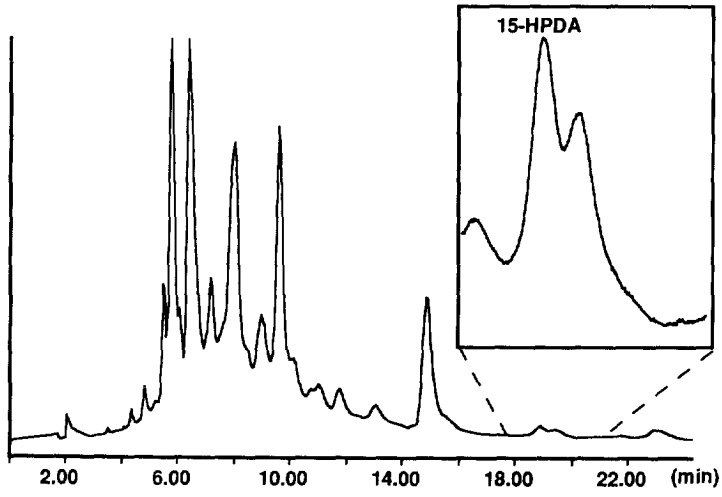

Fig. 1. HPLC-chromatogram of tall oil rosin. Peak eluting at 18.9 was identified as 15-HPDA with MS (first eluting peak in the magnified section). For HPLC conditions, see Experimental.

been found [12]. These alcohols detected may have been formed from the corresponding hydroperoxides. The isolation process involves the use of aqueous NaOH to separate acids from neutral matter. Strong bases will probably destroy any hydroperoxides present. Moreover, GC analyses have often been used for the analysis of exudates [13, 14]. According to our experience it is not possible to detect hydroperoxides of diterpenes using the GC-technique, since the hydroperoxides will decompose at the high temperature of the gas chromatograph. Thus, it is likely that 15-HPDA is a true plant product, although present in small amounts.

15-HPDA was shown to be a sensitizer of the same magnitude as 15-HPA [7]. The animals reacted significantly to 1 and 5% 15-HPDA in petrolatum (pet.). However, a non-significant response was observed for gum rosin (Table 1).

15-HPDA is present in free acid form in natural rosin but was tested here as the methyl ester. The change in structure owing to methyl esterification has little effect on the allergenic activity of rosin allergens [7, 8, 15].

The low reactivity to gum rosin among the 15-HPDA-sensitized animals reflects the low concentration of 15-HPDA in rosin, where the minute amounts may be too low to induce sensitivity. This would also explain the absence of reactions to 15-HPDA when tested in rosin-allergic individuals [16]. However, it cannot be excluded that 15-HPDA may be present in larger amounts in other types/batches of rosin since the composition varies considerably with origin, production and handling of the material [17–20] and thus 15-HPDA may contribute to the allergenic potential of rosin.

EXPERIMENTAL

Dehydroabietic acid was prepared from Portuguese colophony according to procedures described in the literature [21]. Diazogen® and DDQ were obtained from Janssen Chimica, Beerse, Belgium. Portuguese colophony of the gum rosin type was obtained from Socer-Comercio et Industria de Resinas, SA, Pombal, Portugal. Tall oil rosin was obtained from Bergvik Kemi AB, Söderhamn, Sweden. A Merckoquant 10011 Peroxide Test, Merck, was used for hydroperoxide screening.

IR: Perkin–Elmer 298 instrument. ^{1}H and ^{13}C NMR: Jeol EX 270 instrument using $CDCl_3$ solns with TMS as int. standard. HPLC analyses of rosin were performed on a cyanopropyl modified silica column (250 × 4 mm, 5 μm, LiChrosorb, Merck, Germany) with UV detection (254 nm). The mobile phase was hexane–EtOH (499:1) with a flow rate of 2.0 ml min^{-1}. An HPLC fraction of tall oil rosin was collected at the retention time corresponding to that of the reference substance methyl 15-hydroperoxydehydroabietate (15-HPDA). The fraction was analysed with DIP-MS. A spectrum of the compound showed a fragmentation pattern and molecular ion, $[M]^+$ 346, identical to those of the synthesized reference compound identified as 15-HPDA.

MS: the methylated compounds except for 15-HPDA were analysed with GC–MS using a DB-5 capillary column and on-column injection. During injection the column temperature was held at 85° for 1 min. It was then increased linearly at a rate of 10° min^{-1} up to 295°. The temp. of GC–MS interface was held at 310°. Owing to its thermal instability 15-HPDA could not be analysed with GC–MS, but was introduced with a direct insertion probe (DIP) to the ion source at a temperature of 50°. The temperature was increased linearly at a rate of 30° min^{-1} up to 350°. The scan range was *m/z* 50–500. For GC–MS the scan cycle time was 0.6 sec; for DIP-MS 2.5 sec. The ion source was held at a temp. of 150° and the electron energy was set to 70 eV.

Methyl dehydroabietate (**3a**). Dehydroabietic acid was esterified quantitatively with diazomethane generated from Diazogen®.

Methyl 8,11,13,15-*abietatetraen*-18-*oate* (**6**). This compound was prepared using a synthetic route described in ref. [22]. A mixture of methyl dehydroabietate (**3a**) (1.05 g, 3.34 mmol) and DDQ (0.95 g, 4.19 mmol) in 1,4-dioxane (20 ml) was reflexed for 5 hr. The mixture was passed through an alumina column eluted with toluene (100 ml). Compound **6** was obtained in 70% yield as an oil after purification with CC on silica gel eluted with hexane–Et_2O (9:1). IR ν cm^{-1}: 1715, 1625 (ester).

Table. 1. Sensitizing capacity of methyl 15-hydroperoxydehydroabietate (15-HPDA) according to Freund's complete adjuvant test (FCAT)

		Exposed group (n = 15)*		Control group (n = 15)
Challenge material	Conc. (%)	Positive reactions	P(exp/co)	Positive reactions
15-HPDA	5	14	< 0.001	0
15-HPDA	1	13	< 0.001	0
Gum rosin	10	2	NS†	0
Vehicle control (pet.)		1	NS	0

*Induction: 15-HPDA 5% (w/w) (0.14 mmol g^{-1}) intradermally.
†NS, Not significant.

^{1}H NMR: δ 7.05–6.93 (3H, *m*, Ar-H), 5.17 (1H, *br s*, H-16), 4.88 (1H, *br s*, H-16), 3.61 (3H, *s*, H-21), 2.08 (3H, *br s*, H-17), 1.22 (3H, *s*, H-19), 1.19 (3H, *s*, H-20), ^{13}C NMR: δ179.0 (C-18), 142.0 (C-15), 111.5 (C-16), 149.1, 134.8, 126.7, 125.9, 123.9, 122.8 (aromatic carbons). GC-EI-MS 70 eV, *m/z* (rel. int.): 312 $[M]^+$ (18), 297 $[M - Me]^+$ (8), 237 $[M - 75]^+$ (100). These spectral data are consistent with the structure of **6**.

Methyl 15-hydroxydehydroabietate (**7**). For this synthesis, a procedure described in ref. [23] was used. $Hg(OAc)_2$ (0.98 g, 3.1 mmol) was dissolved in 3 ml H_2O and then 0.95 g (3.0 mmol) of **6** in 3 ml THF was added. The reaction mixture was stirred at room temp. under N_2 for 3 hr. Then 3 ml of 3.0 M NaOH was added, followed by 3 ml of a soln of 0.5 M $NaBH_4$ in 3.0 M NaOH. The mixture was stirred for 3 hr. The mercury was allowed to settle and NaCl was added to saturate the H_2O layer. The upper layer of THF was evapd and passed through an alumina column eluted with 30% EtOAc in hexane. This yielded 2.4 g (80%) of an oil with the following spectral data: IR ν cm^{-1}: 3450 (OH), 1730 (C=O). ^{1}H NMR: δ7.26–7.15 (3H, *m*, Ar-H), 3.66 (3H, *s*, H-21), 1.56 (6H, *s*, H-16, H-17), 1.28 (3H, *s*, H-19), 1.21 (3H, *s*, H-20),. ^{13}C NMR: δ179.1 (C-18), 148.0, 146.1, 134.8, 124.9, 124.2, 122.0 (aromatic carbons), 72.3 (C-15). GC-EI-MS 70 eV, *m/z* (rel. int.): 330 $[M]^+$ (18), 315 $[M - Me]^+$ (87), 312 $[M - H_2O]^+$ (17), 297 $[M - H_2O - Me]^+$ (11), 255 $[M - 75]^+$ (100), 237 $[M - 93]^+$ (75). The spectral data are consistent with the structure of **7**.

Methyl 15-hydroperoxydehydroabietate (15-HPDA) (**4a**). The synthesis of **4a** from **7** was based on a procedure described in ref. [24]. The solution of methyl 15-hydroxydehydroabietate (**7**) (0.4 g, 1.2 mmol) in THF (15 ml) was stirred with H_2O_2 (10 ml, 35%, 97.2 mmol) and conc H_2SO_4 (3 drops). The mixture was stirred at room temp. under N_2 for 3 hr. The soln was satd with NaCl and extracted with EtOAc. After chromatography the methyl 15-hydroperoxydehydroabietate (15-HPDA) (**4a**) was obtained in 26% yield. The compound gave a positive peroxide test result. IR ν cm^{-1}: 3505 (OOH), 1730 (C=O). ^{1}H NMR: δ7.28–6.98 (3H, *m*, Ar-H), 3.69 (3H, *s*, H-21), 1.59 (6H, *s*, H-16, H-17), 1.29 (3H, *s*, H-19), 1.22 (3H, *s*, H-20). ^{13}C NMR: δ179.1 (C-18), 148.8, 141.3, 135.2, 126.0, 124.6, 122.9 (aromatic carbons), 83.8 (C-15). DIP-EI-MS 70 eV, *m/z* (rel. int.): 346 $[M]^+$ (2), 313 $[M - OOH]^+$ (68), 297 $[M - 49]^+$ (8), 255 $[M - 91]^+$ (38), 237 $[M - 109]^+$ (100). The signals at *m/z* 315 and 330, respectively, probably originate from 15-hydroxydehydroabietate (**7**). This compound is most likely formed by degradation of 15-HPDA when the probe is heated. The spectral data are consistent with structure **4a**.

Sensitizing capacity of 15-HPDA according to Freund's complete adjuvant test. The experiment was performed as described by Klecak but slightly modified according to earlier experience [25, 26]. Of 30 guinea-pigs 15 (group I) were intradermally injected with 5% 15-HPDA (corresponding to 0.14 mmol g^{-1}) in FCA–H_2O-emulsion. The other 15 animals (group II) were the control group and were injected with neat FCA–H_2O-emulsion. The concentration of 15-HPDA was chosen to correspond to the molar concentration of 15-HPA according to previous experience [7]. The animals were challenge-tested with 5 and 1% 15-HPDA in petrolatum (corresponding to 0.14 and 0.03 mmol g^{-1}). The test concentrations of 15-HPDA were shown in a pretest on FCA-treated animals to be non-irritating. The animals were also tested with Portuguese gum rosin 10% in petrolatum.

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