Acaricidal Activity of Tonka Bean Extracts. Synthesis and Structure−Activity Relationships of Bioactive Derivatives

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The acaricidal effects of tonka bean, *Dipterix odorata*, extracts were investigated on *Dermatophagoides pteronyssinus*, the European house dust mite, and compared with benzyl benzoate as a standard acaricidal compound. A cyclohexane extract was the most effective, with an $EC_{50} = 0.075$ g/m^2 after a 24 h period, as compared with benzyl benzoate (0.025 g/m^2). Bioassay-guided fractionation of this extract led to the isolation of coumarin (**1**). Pharmacomodulation of this compound led us to test 20 analogues (**2**−**21**), which were either synthesized or purchased.

House dust mite allergy has been widely implicated in the etiology of nonseasonal asthma and atopic disorders, and this allergy is now considered to be a worldwide problem.[1] The control of insect pests by plants or their bioactive constituents is being widely investigated. However, only a few studies deal with acaricidal natural products.

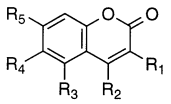
Tonka beans are the dried seeds of *Dipteryx odorata* (Aublet) Wild. (Fabaceae). Cultivated in Venezuela, this tree is used for its fruits to flavor tobacco or in perfumery. In the course of our systematic search for natural acaricidal compounds,[2,3] the aim of this study was to evaluate the activity of this species by a simple bioassay on the common pyroglyphid house dust mite (*Dermatophagoides pteronyssinus*). A cyclohexane extract of tonka beans exhibited a positive response to the bioassay, and this extract was thus selected for isolation of active compounds.

The cyclohexane extract was taken to dryness under reduced pressure to yield a brown gum. This extract showed a dose-dependent acaricidal effect. The crude extract was fractionated by column chromatography on silica gel. Bioassay-guided fractionation by flash chromatography afforded coumarin (**1**)[4] as the active component. The acaricidal properties of coumarin, a known compound of tonka beans, were hitherto unknown.[5,6] After 24 h, the EC_{50} for coumarin (0.032 g/m^2) was very similar to that of benzyl benzoate (0.025 g/m^2), a well-known and widely used acaricidal product.

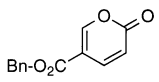
The coumarin skeleton and benzyl benzoate were then considered as lead structures in the present study. To improve the potency and acaricidal profile of these leads, different modifications were considered. The acaricidal activities of the synthetic coumarin derivatives are discussed below.

The derivatives can be classified into three classes: (1) compounds with a benzo α-pyrone ring (**2**−**8**); (2) compounds with a γ-pyrone ring (**9**−**16**); (3) compounds derivated from benzyl benzoate (**17**−**21**). These compounds were either obtained from commercial sources or prepared by synthesis.

Coumarin-3-carboxylic acid benzyl ester (**7**), benzyl coumalate (**8**), chromone-2-carboxylic acid benzyl ester (**11**), benzyl salicylate (**17**), 3-hydroxy benzoic acid benzyl ester (**18**), 4-hydroxybenzoic acid benzyl ester (**19**), and benzyl



1 : R_1 = H, R_2 = H, R_3 = H, R_4 = H, R_5 = H
2 : R_1 = H, R_2 = H, R_3 = H, R_4 = CH_3, R_5 = H
3 : R_1 = H, R_2 = OH, R_3 = H, R_4 = H, R_5 = H
4 : R_1 = H, R_2 = H, R_3 = H, R_4 = H, R_5 = OH
5 : R_1 = H, R_2 = H, R_3 = H, R_4 = CO_2-Bn, R_5 = H
6 : R_1 = H, R_2 = H, R_3 = CO_2-Bn, R_4 = H, R_5 = H
7 : R_1 = CO_2-Bn, R_2 = H, R_3 = H, R_4 = H, R_5 = H



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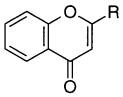
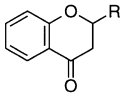
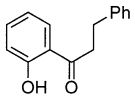


9 : R = H
10 : R = Ph
11 : R = CO_2-Bn



12 : R = H
13 : R = Ph



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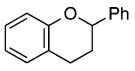

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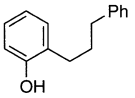

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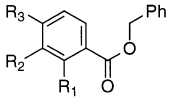

17 : R_1 = OH, R_2 = H, R_3 = H
18 : R_1 = H, R_2 =OH, R_3 = H
19 : R_1 = H, R_2 = H, R_3 = OH
20 : R_1 = H, R_2 =COH, R_3 = OH

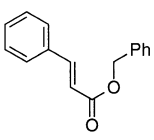

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Table 1. In Vitro Acaricidal Activities of Compounds **1**−**21** against *D. pteronyssinus* (EC_{50} values after 24 h)

tested compound	EC_{50} (g/m^2)
coumarin (**1**)	0.032
6-methylcoumarin (**2**)	0.040
4-hydroxycoumarin (**3**)	>1.50
7-hydroxycoumarin (**4**)	>1.50
coumarin-6-carboxylic acid benzyl ester (**5**)	0.495
coumarin-5-carboxylic acid benzyl ester (**6**)	0.495
coumarin-3-carboxylic acid benzyl ester (**7**)	0.280
benzyl coumalate (**8**)	0.495
chromone (**9**)	0.037
flavone (**10**)	0.500
chromone-2-carboxylic acid benzyl ester (**11**)	0.495
chromanone (**12**)	0.070
flavanone (**13**)	0.090
dihydrochalcone (**14**)	0.110
flavane (**15**)	0.150
chalcane (**16**)	0.310
benzyl salicylate (**17**)	0.025
3-hydroxybenzoic acid benzyl ester (**18**)	0.495
4-hydroxy benzoic acid benzyl ester (**19**)	0.495
3-formyl-4-hydroxy benzoic acid benzyl ester (**20**)	0.242
benzyltrans cinnamate (**21**)	0.180
benzyl benzoate (reference)	0.025

trans-cinnamate (**21**) were prepared by esterification of the commercial carboxylic acids by benzyl alcohol. 3-Formyl-4-hydroxybenzoic acid benzyl ester (**20**) was prepared in two steps from 4-hydroxybenzoic acid: (a) formylation according to Duff's process,[7] (b) esterification by benzyl alcohol. Cyclization of **20** into coumarin-6-carboxylic acid benzyl ester (**5**) was carried out by a Wittig reaction.[8] Coumarin-5-carboxylic acid benzyl ester (**6**) was prepared in the same manner from 3-hydroxybenzoic acid. Flavane (**15**) and chalcane (**16**) were obtained by reduction of flavanone by $NaBH_3CN$/TFA.[9] Dihydrochalcone (**14**) was prepared by catalytic hydrogenation of flavanone (**13**). The acaricidal activities of these coumarin and benzyl benzoate derivatives are shown in Table 1.

A methyl group at the C-6 position of **1** did not affect the acaricidal activity (compare **2** with **1**), whereas a hydroxy group at C-4 (**3**) or C-7 (**4**) led to a dramatic drop in activity. A benzyloxycarbonyl group at C-6 (**5**) or C-5 (**6**) had 10-fold lower activity, while the same group at C-3 (**7**) was 2-fold more active than these compounds. The combination of a coumarin skeleton substituted by a benzyloxycarbonyl group in these positions lowered the activity against the pyroglyphid house dust mite. Direct attachment of this substituent to the α-pyrone ring, as in benzyl coumalate (**8**), led to similar activity.

The acaricidal activity of compounds with a γ-pyrone ring was compared to compounds with an α-pyrone ring. In this group, chromone (**9**) showed the strongest activity, quite similar to coumarin (**1**). Reduction of the double bond at C-2/C-3 led to chromanone (**12**), which was slightly less active than chromone (**9**). In this group, it was interesting to note that flavanone (**13**), dihydrochalcone (**14**), flavane (**15**), and chalcane (**16**) preserved this activity, while flavone (**10**) was clearly less active.

As for coumarin (**1**), addition of a benzyloxycarbonyl group to position 2 of chromone (**9**) led to a negative effect on the bioactivity (compared to **9**−**11**). On the other hand, concerning the benzyl benzoate series, replacement of the benzoyl by a *trans*-cinnamoyl group led to a compound (**21**) that was 7-fold less active. Finally, substitution of benzyl benzoate at C-3 or C-4 with a polar hydroxy group led to greatly reduced activity (compare benzyl benzoate with **18** and **19**). However, activity was not reduced when the OH substituent was at C-2 (compare benzyl benzoate with **17**). These results could be explained by the occurrence in **17** of a hydrogen bond between the OH and the carbonyl of the ester group. This hypothesis is supported by the activity of compound **20**, in which a carbonyl substituent adjacent to the OH allowed a hydrogen bond.

In conclusion, coumarin (**1**) was isolated by bioassay-guided fractionation of tonka beans. In terms of acaricidal activity, this compound with an α-pyrone ring compared favorably with the known acaricidal benzyl benzoate. On the basis of these data, γ-pyrones may be worth considering as potential acaricidal compounds. Structural modifications on the α-pyrone and γ-pyrone rings showed that the benzyloxycarbonyl substitution is critical for acaricidal activity, and substitution by polar hydroxy groups causes reduction of activity.

Experimental Section

General Experimental Procedures. Melting points were determined with a Reichert apparatus and are uncorrected. ^{1}H NMR spectra were obtained with a Bruker AC-200 (at 200 MHz) NMR spectrometer. TLC was performed on precoated 0.25 mm thick Merck plates of silica gel 60 F_{254}. Column chromatography was carried out on Merck silica gel 60.

Plant Material. Tonka beans, *D. odorata*, were purchased from Herboristerie Cailleau, 5 Rue Robert d'Arbrissel, BP 69, 49210 Chemille, France, Lot No. 11013.

Extraction and Isolation of Coumarin (1). Powdered plant material (1 kg) was extracted by C_6H_{12} (cyclohexane) in a Soxhlet for 24 h. The C_6H_{12} extract was taken to dryness under reduced pressure to yield a brown gum. When tested for acaricidal activity, the C_6H_{12}-insoluble extract exhibited activity, with an IC_{50} value of 0.075 g/m^2. The C_6H_{12}-insoluble bioactive extract was subjected to column chromatography (CC) on silica gel, eluting with C_6H_{12}−*i*-PrOH, 95:5, to give coumarin (**1**), identical in all respects (NMR, MS) to a commercial product (Aldrich, France, C8, 557-7).

Materials. Compounds **2**, **3**, **4**, **9**, **10**, **12**, and **13** were commercially available. Compounds **15** and **16** were prepared by reduction of **13** using $NaBH_3CN$/TFA.[9] Compound **14** was prepared by catalytic hydrogenation (H_2/Pd) of **13**. Melting points and spectroscopic data for compounds **2**−**4**, **9**, **10**, and **12**−**16** agreed with those previously reported.[9]

General Procedures for the Synthesis of Benzyl Ester Derivatives 7, 8, 11, 17, 18, 19, and 21. A mixture of 200 mg of carboxylic acid, 3 mL of benzyl alcohol, and 25 mg of APTS was refluxed for 15 h. The reaction mixture was extracted with 200 mL of CH_2Cl_2, the CH_2Cl_2 layer was dried over Na_2SO_4 and filtered, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (CH_2Cl_2) over silica gel. Spectroscopic data agreed with those of commercial products (**17**, **19**, **21**) or with those previously reported in the literature (**7**,[10] **8**,[11] **18**[12]).

Chromone-2-carboxylic acid benzyl ester (11): orange solid; mp 104−105 °C (from CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 8.20 (1H, dd, J = 12, 3 Hz, H-5), δ 7.40 (9H, m, H-3, H-6, H-7, H-8, H−Bn), δ 5.40 (2H, s, CH_2−Bn); *anal.* C 72.93%, H 4.28%, calcd for $C_{17}H_{12}O_4$, C 72.85%, H 4.32%.

Procedures for the Synthesis of 20, 5, and 6. A mixture of 2.76 g (20 mmol) of 4-hydroxybenzoic acid and 2.8 g of hexamethylenetetramine (20 mmol) was heated at reflux in 50 mL of trifluoroacetic acid for 6 h. The products were concentrated and combined with ice water; the resultant mixture was stirred for 15 min and extracted with EtOAc. The solvent was washed several times with H_2O, dried over Na_2SO_4, then removed under reduced pressure. The crude residue (400 mg) was added to a stirred solution of APTS (60 mg) in benzyl alcohol (10 mL) and heated at reflux for 6 h. The resulting solution was extracted with CH_2Cl_2, dried over Na_2SO_4, and filtered, and solvent was removed under reduced pressure. The crude product was then purified with 20% cyclohexane in CH_2Cl_2 over silica gel to give **20** (510 mg, 10%). Reaction of **20** with carbethoxymethylenetriphenylphosphorane

(210 mg) in Et_2NPh (30 mL) under reflux was carried out for 90 min. The reaction mixture was diluted with 5% HCl solution and extracted with Et_2O. The residue in CH_2Cl_2 was chromatographed on silica gel. Elution with CH_2Cl_2 gave **5**. Compound **6** was identically synthesized from 3-hydroxybenzoic acid.

3-Formyl-4-hydroxybenzoic acid benzyl ester (20): white waxy solid; ^{1}H NMR ($CDCl_3$) δ 11.40 (1H, s, COH), δ 9.95 (1H, s, OH), δ 8.35 (1H, d, J = 3 Hz, H-2), δ 8.25 (1H, dd, J = 10, 3 Hz, H-6), δ 7.40 (5H, m, H−Bn), δ 7.00 (1H, d, J = 10 Hz, H-5), δ 5.40 (2H, s, CH_2−Bn); *anal.* C 70.22%, H 4.65%, calcd for $C_{15}H_{12}O_4$, C 70.30%, H 4.72%.

Coumarin-6-carboxylic acid benzyl ester (5): yellow solid; mp 135−136 °C (from CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 8.25 (2H, m, H-5, H-7), δ 7.70 (1H, d, J = 12 Hz, H-4), δ 7.40 (6H, m, H-8, H−Bn), δ 6.50 (1H, d, J = 12 Hz, H-3), δ 5.40 (2H, s, CH_2−Bn); *anal.* C 72.78%, H 4.40%, calcd for $C_{17}H_{12}O_4$, C 72.85%, H 4.32%.

Coumarin-5-carboxylic acid benzyl ester (6): yellow solid; mp 80 °C (from CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 8.90 (1H, d, J = 12 Hz, H-4), δ 8.00 (1H, dd, J = 9, 3 Hz, H-6), δ 7.70 (7H, m, H-7, H-8, H−Bn), δ 6.50 (1H, d, J = 12 Hz, H-3); δ 5.40 (2H, s, CH_2−Bn); *anal.* C 72.88%, H 4.26%, calcd for $C_{17}H_{12}O_4$, C 72.85%, H 4.32%.

House Dust Mites. *Dermatophagoides pteronyssinus* (Trouessart) strain, the European house dust mite, was provided by Stallergène Laboratories (Antony, France). The strain was maintained in a culture medium made of 90% *Saccharomyces cerevisiae* powder (Organotechnie, La Courneuve, France) enriched with 10% beard shavings at 25 °C and 75% relative humidity in darkness.

Biological Testing. Experiments were performed in 24-well tissue cultures (Costar No. 3524) using the Izri & Rousset method[13] for testing acaricidal compounds on a hard surface, with minor modifications. The surface of each well was 2 cm^2. Adequate dilutions (30 μL) of the compounds or crude extracts to be tested in absolute EtOH were applied on the bottom of the wells and allowed to dry for 24 h before the experiment. Concentrations of crude extracts and tested drugs were expressed in g/m^2. Control wells received only EtOH or crude extract solvent. To prevent the mites from escaping, the upper rims of the wells were covered with "Glue Pelton" (Rhône-Poulenc Jardin, Lyon, France). Treatments and blanks were executed in triplicate and repeated after one week. Benzyl benzoate, one of the most potent acaricidal compounds for human and domestic use (Sigma-Aldrich, Saint Quentin Fallavier, France), was used as the reference compound. One hundred mites (3.5 mg of culture medium) were added in each well, and the mortality was observed with a stereobinocular microscope after a 24 h incubation period at 25 °C, 75% relative humidity in darkness. Mites that did not move when prodded with a fine brush were considered as dead. The observed mortality in treated wells was corrected for the mortality observed in control wells according to Abbott's formula; concentrations were determined by means of linear regression analysis. For the determination of the activity of pure compounds, seven 2-fold dilutions in triplicate were tested, ranging from 0.01 to 0.640 g/m^2. Student's *t*-tests were used for comparison between treated and untreated groups of house dust mites. A P value $\leq$ 0.05 was considered to represent a significant difference.

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