Identification of the cat attractants isodihydronepetalactone and isoiridomyrmecin from *Acalypha indica*.

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§ In honour of the late Professor Emilio Ghisalberti.

Abstract

Acalypha indica is a herb that grows throughout the tropical regions of the world. As well as being exploited for medicinal use, the roots of this plant are known to elicit a drug-like effect on cats. Recent research into feral cat control on Christmas Island has investigated whether a preparation of the roots of *A. indica* might be effective in traps to attract feral cats. However, the volatile nature of the attractants made it unviable for use in traps for more than a few days. In this study we investigated the volatile components emitted by the plant roots and identified two iridoid compounds, (4*R*,4a*R*,7*S*,7a*R*)-isodihydronepetalactone and (4*R*,4a*S*,7*S*,7a*R*)-isoiridomyrmecin, which are known to affect behavioural activity in cats. Synthesis of standards confirmed the stereochemistry of both compounds emitted by the plant. Potential application for these compounds in feral cat control is discussed.

Introduction

Christmas Island is a small island off the north-west coast of Australia that is well known for its unique flora and fauna. Recently there has been concern over the high level of introduced species that have had a detrimental effect on many of the native species that inhabit the island, causing some of the native species to be declared rare or endangered. Some of the more damaging introduced species include cats (*Felis catus*), yellow crazy ants (*Anoplolepsis gracilipes*) and black rats (*Rattus rattus*).[1-3] As a result a number of control measures have recently been implemented. One particular project has been attempting to reduce the large number of feral cats on the island[4] and during this project, the research team became aware of a plant that several local residents suggested had a peculiar effect on cats when the roots of the plant were exposed. When the plant was removed from the soil, both feral and domestic cats were noticed to chew the roots and roll in them in what appeared to be a drug induced stupor (Fig. 1). This behaviour has been observed on multiple occasions and verified by a qualified veterinarian (Dr Don Nickels). Furthermore, if the plant was withheld, the cats became aggressive in an attempt to regain the material.

On further investigation the plant was identified as *Acalypha indica* L. (Euphorbiaceae) which is commonly known as Indian acalypha, Indian nettle or three-seeded mercury. *A. indica* is a common herb growing up to 75 cm tall with ovate leaves, and occurs throughout tropical Africa, India, Sri Lanka, Yemen and Pakistan.[5] It was probably introduced to Christmas Island where it has become prevalent and grows as a weed. *A. indica* has a long history of medicinal use and has been reported to possess hepatoprotective, anti-inflammatory, antitussive, antifungal and antibacterial activity.[5] Although there are no scientific reports to our knowledge of this drug-like effect on cats, there is much anecdotal evidence in the public domain.

Detailed phytochemical analysis of *A. indica* is lacking, especially of the roots, but some screening studies of the aerial parts have indicated that alkaloids, sterols, saponins, flavonoids and tannins are present.[6] GC-MS analysis has revealed the presence of flavonoids, amino acids and nitrile containing compounds,[7] while HPLC has indicated a number of phenolic acids such as gallic, ferulic and caffeic acids along with flavonoid glycosides such as rutin and naringenin.[8]

To our knowledge, there have been no studies investigating this plant for the specific compounds that cause behavioural effects on cats. There are plants such as catnip (*Nepeta cataria*) which have similar effects on cats due to the presence of nepetalactone.[9] However, investigation of catnip by the Christmas Island research team found that this plant had no effect on the Christmas Island cats. In this study, we undertake a chemical investigation to identify the compounds in *A. indica* that are likely to be responsible for this drug-like effect on cats.

Results and Discussion

Initially, a number of plants were brought to the mainland, under Australian quarantine (AQIS) permits, to provide root material for chemical analysis. Following fumigation and the regulatory quarantine period, roots were extracted with dichloromethane and ethanol and analysed by GC-MS. A large range of compounds were detected including aromatic aldehydes, phenols and fatty acids, although no plausible compounds for causing the 'cat effect' could be identified. It was noted however, that roots lost their impact on cats after a few days exposed to the atmosphere. To circumvent this problem, chemical extractions of fresh root material was conducted at Christmas Island and placed in sealed vials and sent back to the mainland for chemical analysis. In addition, volatile absorption traps packed with Tenax® were used to trap volatile compounds emitted from fresh root material and were also sent back for analysis.

GC-MS analysis of the solvent extractions of the fresh root material revealed similar compounds as in the analysis of previous extractions. However, GC-MS analysis of the absorption traps from the headspace collection of *A. indica* exposed roots, either extracted by thermal desorption or by solvent elution, resulted in detection and partial identification of two candidate compounds that have previously been reported to have activity on cats (Fig. 2). These two candidates were confirmed as only minor compounds in solvent extracts of root tissue. The mass spectrum of the first compound (RI=1421) was in good agreement with the library spectrum of dihydronepetalactone (NIST database, 2011), as was the molecular formula of $C_{10}H_{16}O_2$, as indicated by high resolution mass spectrometry (HRMS). The second compound (RI=1454) also had the formula $C_{10}H_{16}O_2$ and gave a good match to iridomyrmecin (NIST database, 2011) with a comparable retention index to that reported in the literature.[10] Both compounds belong to the class of iridoid compounds and have been

previously implicated to elicit drug-like activity on cats. To ultimately confirm our identification, a synthesis of these compounds was conducted.

To prepare dihydronepetalactone, it was noted that 'catnip' (*Nepeta cataria*) was a good source of the *cis*-fused (4a*R*,7*S*,7a*R*)-nepetalactone (**1**) and *trans*-fused (4a*S*,7*S*,7a*S*)-nepetalactone (**2**),[11] and hydrogenation of each of these would provide access to four of the sixteen possible stereoisomers. Steam distillation of the plant is reported to give an oil containing 98% of these two isomers in about a 6:1 ratio.[12] In our hands, hydrodistillation of fresh aerial material afforded an oil consisting mostly of the two isomers (~92%) but in this case they were in a 2:5 ratio of *cis*:*trans*-fused nepetalactones (Fig. S1). The two compounds were separated by semi-preparative HPLC to give the pure diastereoisomers. Hydrogenation of the individual isomers afforded two main compounds for each nepetalactone, with one compound in each case dominating as expected.[13] GC-MS analysis revealed that the *A. indica* derived dihydronepetalactone matched the minor product from hydrogenation of the *cis*-fused nepetalactone. Separation of the two *cis*-fused dihydronepetalactones **3** and **4** by semi-preparative HPLC yielded each isomer in pure form. Analysis of the matching compound by NMR spectroscopy revealed it to be the (4*R*,4a*R*,7*S*,7a*R*)-isodihydronepetalactone (**4**).[13] Comparison with the *A. indica*-derived dihydronepetalactone on two different GC columns (DB-wax and BPX-5) and an enantioslective GC column (Cydex B) confirmed the natural compound as (4*R*,4a*R*,7*S*,7a*R*)-isodihydronepetalactone (**4**).

For iridomyrmecin there are also sixteen stereoisomers, several of which are known to be derived biologically.[10] A synthesis of (4*S*,4a*S*,7*S*,7a*R*)-iridomyrmecin (**5**) was initially undertaken[14] and subsequent analysis by GC-MS suggested that the mass spectra matched that for the *A. indica* derived compound. However, the retention index was different by 12 units (0.3 min). Fortunately, it was noted that a minor by-product of the (4*S*,4a*S*,7*S*,7a*R*)-iridomyrmecin synthesis returned its 4-epimer, (4*R*,4a*S*,7*S*,7a*R*)-isoirodomyrmecin (**6**).[14] On

comparison, it was found that (4*R*,4a*S*,7*S*,7a*R*)-isoiridomyrmecin (**6**) matched both the mass spectrum and retention time by GC-MS. By co-injection on the three GC columns we confirmed that (4*R*,4a*S*,7*S*,7a*R*)-isoirodomyrmecin (**6**) was indistinguishable from the *A. indica* natural compound.

Although we did not have the enantiomers readily available to confirm separation of these iridoids on our enantioselective GC column, a previous study using a similar *β*-cyclodextrin phased column (Beta-Dex 225, Sigma-Aldrich) has shown that the enantiomers of iridomyrmecin and isoiridomyrmecin are all easily separated using a similar method to ours.[10] In our analysis we observed a one minute separation of (4*S*,4a*S*,7*S*,7a*R*)-iridomyrmecin (**5**) and (4*R*,4a*S*,7*S*,7a*R*)-isoiridomyrmecin (**6**), in agreement with the reported separation of these compounds by Stökl and coworkers.[10] In addition, most naturally occurring dihydronepetalactones have been reported with the 7*R* configuration of the methyl substituent with only a few exceptions.[15,16] Hence, our results suggest these compounds are present as single enantiomers derived from *A. indica*.

The two compounds (4*R*,4a*R*,7*S*,7a*R*)-isodihydronepetalactone (**4**) and (4*R*,4a*S*,7*S*,7a*R*)-isoiridomyrmecin (**6**) have previously been reported to have cat attractant activity and both have been identified from plant and animal sources.[15,17] Most notably is that many species of insects, especially *Iridomyrmex* ants, use these compounds as defensive compounds, hence the name iridoids.[18] It has previously been suggested that these compounds are produced by plants to deter insects from feeding.[19] To our knowledge, this is the first report of iridoids from the *Acalypha* genus.[20] However, these iridoids along with several others, have previously been identified and implicated in the cat attracting activity of *Actinidia polygama* also.[21]

At least fourteen compounds have been reported with cat attractant activity ranging from structurally similar iridoids known as 7-methylcyclopentapyranones,[22] to which our identified *A. indica* compounds belong. In addition, 7-methyl-2-pyridines such as actinidine (**7**) as well as furanones such as actinidiolide (**8**) are also cat attracting compounds.[22] Further work will be aimed at targeting some of these compounds for appropriate physicochemical properties to investigate their efficacy in traps and to increase the attractiveness of baits for aiding feral cat control measures on Christmas Island. Furthermore, the results from this study may also be widely applicable to the Australian mainland, which also has a significant problem with feral cats. Finally, there is also the potential to use these compounds as attractants to monitor other felid species around the world in conservation projects.

Experimental

General Experimental

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AV600 spectrometer. Deuterochloroform ($CDCl_3$) was used as the solvent with residual $CHCl_3$ (δ_H 7.26) or $CDCl_3$ (δ_C 77.0) being employed as internal standards. GC-MS were recorded on either an Agilent 6890 GC connected to an Agilent 5973 mass selective detector (Agilent technologies, MA, USA) or a Shimadzu GCMS-QP2010 (Kyoto, Japan). GC columns used included a BPX-5 column (5% phenyl polysilphenylene-siloxane, 30 m × 0.25 mm × 0.25 μm film thickness, SGE Australia), a DB-wax column (polyethylene glycol, 30 m × 0.25 mm × 0.25 μm film thickness, J&W Scientific, USA), and a Cydex B column (permethylated β-cyclodextrin in OV1701, 30 m × 0.25 mm × 0.22 μm film thickness, SGE Australia). In all cases, helium was used as the carrier gas with a constant flow rate of 1.0 mL/min. A scan range of *m/z* 45 – 400 and a solvent delay of 5 min were used with splitless injections of 1.0 μL for 1.0 min. The ion source was set to 230 °C, and the transfer line temperature to 250 °C. The oven temperature program was 40 °C, held for 1 min then ramped at 7 °C /min to 250 °C, and held for 10 min. For chiral separations using the Cydex B column, similar conditions as above were used except the ramp rate was 3 °C/min. Retention indices (RI) were calculated on the BPX-5 column using a linear gradient method with comparison to an *n*-hydrocarbon mix (Sigma-Aldrich, p/n 46827-U). Thermal desorption was conducted using a short path thermal desorption injector (TD-2, Scientific Instrument Services, Inc., USA) connected to the Agilent 6890 GC inlet. The BPX-5 column was used with similar conditions as above and a ramp rate of 5 °C/min. High resolution (HR)-EIMS were recorded on a Waters GCT Premier time-of-flight (TOF) MS connected to an Agilent 7890 GC, equipped with a BPX-5 column as above and using He as a carrier gas. Semi-preparative high pressure

liquid chromatography (HPLC) was performed on an Agilent 1200 system with a photodiode array detector (DAD) and fraction collector. Separation was achieved using a 250 x 10 mm i.d. 5 µm, Apollo C18 reversed-phase column (Grace-Davison, USA) operating at 4 mL/min with 500 µL injections. HPLC grade solvents were used and all experiments were carried out under an inert atmosphere with solvents dried prior to use. Thin-layer chromatography (TLC) was effected on Merck silica gel 60 F254 aluminium-backed plates. Percentage yields for chemical reactions as described are quoted only for those compounds that were purified by HPLC and the purity assessed by TLC and NMR.

Extraction of *Acalypha indica* root material

Voucher specimens of *A. indica* from Christmas Island have previously been identified and deposited at the Western Australian herbarium (e.g. Swarberick, #13119, 1996). For extractions, ground air-dried root material from *A. indica* (2 g) was extracted with either dichloromethane or ethanol (ca. 10 mL) while stirred at RT overnight. The solutions were filtered and concentrated with a stream of nitrogen to ca. 1 mL and subjected to GC-MS analysis. For extractions on Christmas Island, freshly picked and chopped *A. indica* root material (ca. 200 mg) was extracted with dichloromethane or ethanol (ca. 1 mL) overnight before filtering into a GC vial which was sent back to the mainland for GC-MS analysis.

Vacuum absorption and GC-MS of compounds emitted by *Acalypha indica* roots

Thermal desorption traps were packed with Tenax™ (200 mg, Tenax TA 60/80, Supelco, Bellefonte, PA, USA) and activated at 200 ºC for 1 hr under a stream of nitrogen. The activated traps were sealed and sent to Christmas Island for use. The traps were connected to a flask with a gas-purge fitting containing freshly picked and chopped *A.indica* root material. The headspace was drawn through the Tenax trap for 1 hr at an airflow rate of 1.5 L/min using a portable air sampling pump (224-PCXR8, SKC Inc.). The traps were sealed and

brought back for analysis. For thermal desorption, the absorbent trap was desorbed for 5 min at 200 ºC using helium (2 mL/min) into the splitless injection port of the GC. A small portion of the column (BPX-5) was cooled to -20 to -40 ºC by dipping the column into an ethanol/dry ice bath for the desorption period to trap the eluting compounds. The ethanol/dry ice bath was removed and the column was equilibrated to 40 ºC before starting the general temperature program as above. In addition to thermal desorption, two traps were eluted with ethyl acetate (ca. 2 mL) to extract the absorbed compounds. The collected eluate was concentrated to ca. 100 µL under a stream of nitrogen. This sample was used for RI calculations and comparison with authentic samples using different columns by GC-MS.

Synthesis of dihydronepetalactones

A mixture of *cis* and *trans*-fused nepetalactones were isolated from the aerial parts (ca. 250 g) of *Nepeta cataria* ('Cat Nip', Swan Valley Nursery, Australia) by hydrodistillation overnight using a modified Clevenger apparatus. The aqueous distillate was extracted in dichloromethane, dried ($MgSO_4$) and evaporated under reduced pressure. Analysis by GC-MS and NMR revealed a ratio of 2:5 for the *cis:trans*-fused nepetalactones respectively (Fig. S1). The two isomers were separated by semi-preparative HPLC using a mobile phase of 40% acetonitrile/water. The fractions containing each isomer were pooled separately and the acetonitrile removed by rotary evaporation. The resulting aqueous layer was extracted with dichloromethane and the organic layer dried ($MgSO_4$) and concentrated to give the (4a*R*,7*S*,7a*R*)-nepetalactone (**1**, 30.1 mg) and (4a*S*,7*S*,7a*S*)-nepetalactone (**2**, 86.4 mg) as colourless liquids. The (4a*R*,7*S*,7a*R*)-nepetalactone (**1**, 25 mg, 0.15 mmol) was reduced overnight in methanol (5 mL) under an atmosphere of hydrogen using a catalytic amount of 10% Pd/C. Upon completion by TLC, the Pd/C was filtered off and the mixture purified by semi-preparative HPLC using 35% acetonitrile/water as the mobile phase. The fractions containing each isomer were treated as above to return (4*S*,4a*R*,7*S*,7a*R*)-dihydronepetalactone

(**3**, 11.1 mg, 44%) and (4*R*,4a*R*,7*S*,7a*R*)-isodihydronepetalactone (**4**, 2.6 mg, 10%) as colourless oils.

(4*S*,4a*R*,7*S*,7a*R*)-dihydronepetalactone (3)

^{1}H NMR (600 MHz, $CDCl_3$) δ: 4.08 (t, *J* = 10.7 Hz, 1H), 4.05-4.00 (m, 1H), 2.56-2.48 (m, 1H), 2.42 (dd, *J* = 10.6 Hz, 9.8 Hz, 1H), 2.28-2.20 (m, 1H), 2.05-1.95 (m, 1H), 1.95-1.85 (m, 1H), 1.77-1.70 (m, 1H), 1.49-1.39 (m, 1H), 1.20-1.14 (m, 1H), 1.19 (d, *J* = 6.5 Hz, 3H), 0.9 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) 174.52, 70.06, 50.68, 41.67, 40.59, 35.18, 31.12, 26.49, 19.44, 13.23.

RI = 1468, HRMS (EI): for $C_{10}H_{16}O_2$ require *m/z* [M^+] 168.1150, observed 168.1158

(4*R*,4a*R*,7*S*,7a*R*)-isodihydronepetalactone (4)

^{1}H NMR (600 MHz, $CDCl_3$) δ: 4.15 (dd, , *J* = 10.9 Hz, 3.4 Hz, 1H), 3.88 (t, *J* = 10.5 Hz, 1H), 2.37-2.31 (m, 1H), 2.31-2.25 (m, 1H), 2.12-2.00 (m, 2H), 1.90-1.83 (m, 1H), 1.30-1.23 (m, 2H), 1.21 (d, *J* = 6.5 Hz, 3H), 1.20-1.13 (m, 1H), 1.00 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) 175.20, 73.05, 49.20, 44.85, 38.94, 35.36, 34.56, 32.02, 20.45, 15.93.

RI = 1421, HRMS (EI): for $C_{10}H_{16}O_2$ require *m/z* [M^+] 168.1150, observed 168.1144

Synthesis of (4*S*,4a*S*,7*S*,7a*R*)-iridomyrmecin and (4*R*,4a*S*,7*S*,7a*R*)-isoiridomyrmecin

(4*S*,4a*S*,7*S*,7a*R*)-Iridomyrmecin and (4*R*,4a*S*,7*S*,7a*R*)-isoiridomyrmecin were prepared from (*S*)-citronellene as previously reported[14] and the spectral data were consistent with literature reports.[10] Separation of the two compounds was achieved by semi-preparative HPLC using 40% acetonitrile/water as the mobile phase. The fractions containing each compound were pooled and evaporated to dryness under reduced pressure.

4*S*,4a*S*,7*S*,7a*R*-iridomyrmecin ((+)-iridomyrmecin, 5)

^{1}H NMR (600 MHz, $CDCl_3$) δ: 4.26 (dd, *J* = 11.8 Hz, 3.3 Hz, 1H), 4.18 (d, *J* = 11.7 Hz, 1H), 2.72 (dt, *J* = 6.7 Hz, 1H), 2.60 (m, 1H), 1.88-1.74 (m, 4H), 1.15 (d, *J* = 6.8, 3H), 1.06-1.02 (m, 4H). ^{13}C NMR (150 MHz, $CDCl_3$) 176.39, 68.06, 45.64, 41.32, 38.04, 37.44, 34.31, 29.95, 18.51, 12.87.

RI = 1442, HRMS (EI): for $C_{10}H_{16}O_2$ require *m/z* [M^+] 168.1150, observed 168.1149

4*R*,4a*S*,7*S*,7a*R*-isoiridomyrmecin ((-)-isoiridomyrmecin, 6)

^{1}H NMR (600 MHz, $CDCl_3$) δ: 4.35 (dd, *J* = 11.2 Hz, 6.1 Hz, 1H), 3.95 (t, *J* = 11.2 Hz, 1H), 2.33-2.28 (m, 1H), 2.15-2.10 (m, 1H), 2.08-2.00 (m, 2H), 1.92-1.86 (m, 1H), 1.70-1.62 (m, 1H), 1.35-1.25 (m, 2H), 1.19 (d, *J* = 6.6, 3H), 1.05 (d, *J* = 6.7, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) 176.43, 69.43, 45.26, 43.15, 39.07, 38.27, 35.68, 33.10, 19.14, 13.93.

RI = 1454, HRMS (EI): for $C_{10}H_{16}O_2$ require *m/z* [M^+] 168.1150, observed 168.1152

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Figure Legends

Figure 1. A cat on Christmas Island in an apparent drug-induced stupor after chewing the roots of *Acalypha indica*.

Figure 2. Thermal desorption GC-MS chromatogram of the volatile components of *A. indica* roots trapped by an absorbent trap. The two peaks of interest, dihydronepetalactone and iridomyrmecin are indicated by **D** and **I** respectively. Peaks marked with * indicate siloxane contaminants from the thermal desorption. For tentative library matches of compounds to other peaks based on their mass spectra see supplementary material.

Figure 3. Chemical structures of (4a*R*,7*S*,7a*R)*-nepetalactone (**1**), (4a*S*,7*S*,7a*S)*-nepetalactone (**2**), (4*S*,4a*R*,7*S*,7a*R*)-dihydronepetalactone (**3**), (4*R*,4a*R*,7*S*,7a*R*)-isodihydronepetalactone (**4**), (4*S*,4a*S*,7*S*,7a*R*)-iridomyrmecin (**5**) and (4*R*,4a*S*,7*S*,7a*R*)-isoiridomyrmecin (**6**).

Figure 4. Chemical structures of actinidine (**7**) and actinidiolide (**8**).

Figures

Fig. 1

Fig. 2

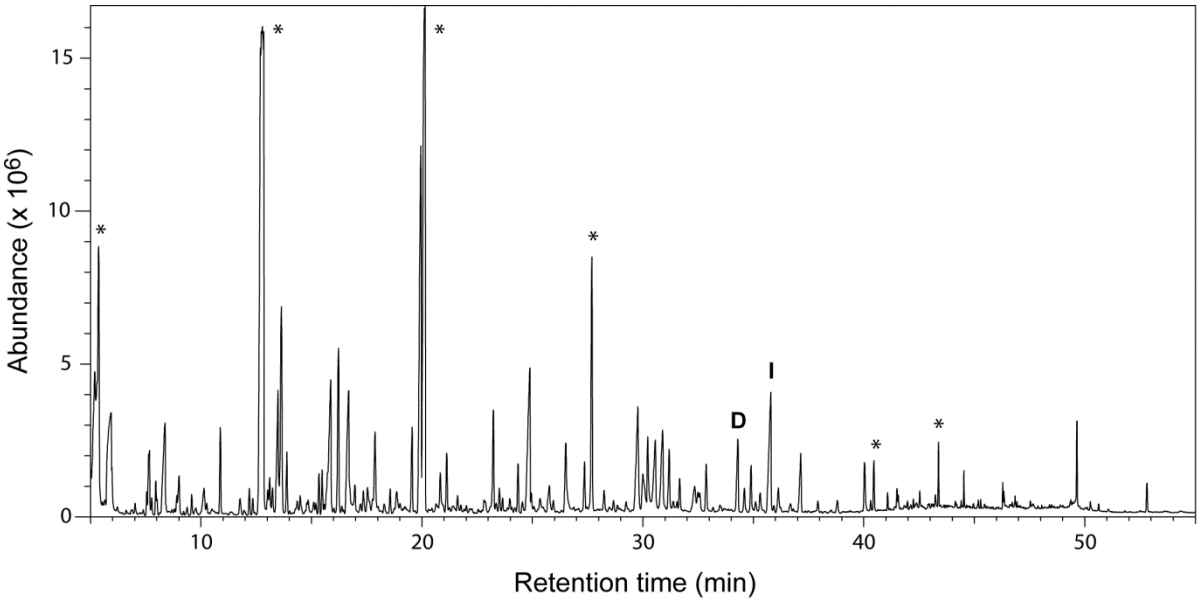
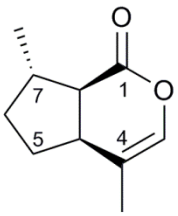


Fig. 3



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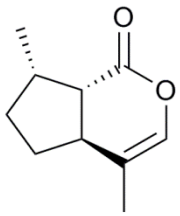

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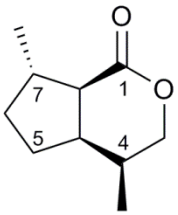

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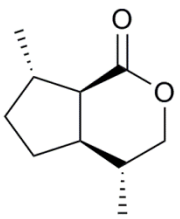

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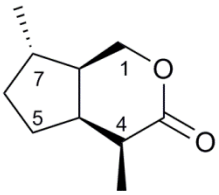

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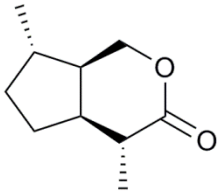

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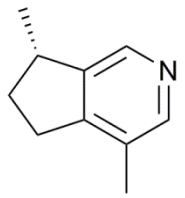
Fig. 4



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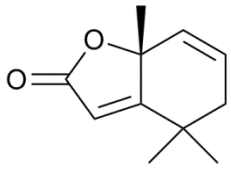

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