Anthecotulide: Purification, Analytical Data, Absence from Chamomile Preparations, Stability and Reactivity, and Anti-infective Testing

Achim Meyer,[†] Stefan Zimmermann,[‡] Bernd Hempel,[§] and Peter Imming*,[†]

Institut für Pharmazeutische Chemie der Martin-Luther-Universität Halle, Wolfgang-Langenbeck-Strasse 4, D-06120 Halle, Germany, Institut für Medizinische Mikrobiologie und Krankenhaushygiene der Philipps-Universität Marburg, Pilgrimstein 2, D-35037 Marburg, Germany, and Robugen GmbH, Alleenstrasse 22, D-73730 Esslingen, Germany

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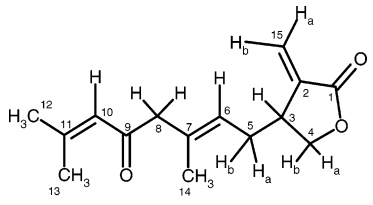
The sesquiterpene lactone anthecotulide (**1**) was extracted from *Anthemis cotula* in 0.7% w/w yield. A pure standard was prepared by preparative TLC and used for quantitative determination of **1** with a TLC scanner. The constitution of **1** was corroborated and the double-bond configuration determined. It was stable at room temparature in air and reacted very slowly with glutathione. It was not detectable in 34 chamomile (*Matricaria recutita*) preparations and did not show antibacterial activity against a range of clinically important strains at a concentration of <10 μg/mL.

Sesquiterpene lactones are plant constituents of very diverse physiological activity, being responsible for the pharmaceutical utility of some plants and plant extracts.[1] Unfortunately, certain sesquiterpenes and sesquiterpene lactones provoke allergic effects including contact allergy.[2] This causes severe problems, particularly in topical use. Anthecotulide (**1**) is a major constituent of *Anthemis cotula* L. (stinking mayweed, dog fennel; Asteraceae), indigenous to moderate zones of both the northern and southern hemisphere. Presently, *A. cotula* and the related species, *A. arvensis* L., have no agricultural nor medicinal uses and are weeds whose distribution has been greatly diminished by intensive agriculture in Germany, in contrast to the mayweeds of the genus *Matricaria* (*Matricaria recutita* L. and *M. inodora* L.).[3] Compound **1** was first isolated in 1969[4] and is considered to be one of the most potent contact allergens.[5] In view of the widespread use of chamomile (*M. recutita* L.) preparations, contamination by *A. cotula* and anthecotulide (**1**) is to be avoided.[6] We have reinvestigated anthecotulide (**1**) comprehensively in view of the scarcity of publications on this topic and inconsistencies in the literature. We also report an investigation of the presence of **1** in chamomile preparations. This complements a recent paper on the biosynthesis of **1**.[7]



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Anthecotulide (**1**) was isolated from *A. cotula* collected at the edge of a rape field near Marburg, Germany; for details of the isolation and purification procedure see the Experimental Section. Using dried aerial parts, a yield of 0.7% w/w was obtained (literature,[5] 0.3%). We found the content of **1** in the dried flower heads to be about 7-fold that of the leaves and stems. This probably explains a report that 7.3% (!) of **1** was "isolated from aerial parts of *Matricaria chamomilla* L."[8] Reinspection of the voucher specimen determined that *A. cotula* indeed had been mistaken for *Matricaria*.[9] In addition, it would appear the authors extracted flower heads of the latter species.

The published constitution and configuration of **1** mainly rest on a 100 MHz ^{1}H NMR spectrum.[4] We recorded one- and two-dimensional ^{1}H and ^{13}C NMR spectra and were able to prove all assignments (see formula **1**), corroborate the constitution, and determine the configuration of the stereogenic double bond (Δ^6) as *E* by a phase-sensitive NOESY experiment. This is much more conclusive than comparison of the chemical shifts of the olefinic carbon atoms with published ^{13}C NMR data.[10] The coupling constant between the H-8 geminal protons was zero, and the coupling pattern of both geminal H-5 protons was equal, with the expected ddd due to identical coupling constants being reduced to five lines. From this we have inferred that in chloroform solution the side chain adopts a straight, quasi-planar conformation. The signals of the methyl groups were recently assigned through deuterium labeling.[7]

A wide range of chamomile preparations (34 in all; see Table S2, Supporting Information) was analyzed for the presence of **1**. This included all preparations that were on sale in German public pharmacies, a number of herbal infusions from pharmacies and supermarkets, and some consumer products (e.g., shampoos) containing chamomile extracts. By quantitative TLC we excluded the presence of more than 100 ng of **1** in any of the preparations. This is well below the threshold amount that would provoke contact allergy.[5] Our study, of course, is a random sample at a certain point in time.

The stability of anthecotulide (**1**) was tested at various temperatures. Both in flower heads and as isolated substance, **1** was found to be completely stable at room temperature with and without exposure to direct light, for at least three months. Consequently, chamomile for pharmaceutical use has to be free of *A. cotula*, as **1** will persist for average shelf lives of teas and other preparations. Our finding also advocates the exclusive use of chamomile from controlled cultures.

The contact allergenic activity of many sesquiterpenes is closely linked to their ability to undergo Michael addi-

* To whom correspondence should be addressed. Tel: +49-(0)345-5525175. Fax: +49-(0)345-5527027. E-mail: imming@pharmazie.uni-halle.de.
[†] Martin-Luther-Universität Halle.
[‡] Philipps-Universität Marburg.
[§] Robugen GmbH.

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tion.[11] If they contain an α,β-unsaturated carbonyl moiety (aldehyde, ketone, or ester), they are reactive toward nucleophiles, e.g., thiol groups of proteins. Compound **1** contains two α,β-unsaturated carbonyl moieties. NMR proved that glutathione slowly reacted with both functionalities, much slower than the analogous conversion[11] of helenalin, a constituent of *Arnica montana*. In buffered solution (pH 7.4), one product was formed predominantly, as detected by TLC. It was too labile, however, for structure determination, as it decomposed on attempted isolation.

The antibacterial activity of a sesquiterpene lactone was reported recently.[12] Anthecotulide (**1**) was tested against a range of clinically important strains: *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, a methicillin-resistant *Staphylococcus aureus*, and a vancomycin-resistant *Enterococcus faecium*, with the latter two being clinical isolates. In no case was there activity at concentrations below 10 μg/mL. There is a slight possibility that this was due to the low solubility of **1** in water. Our findings add to a report on the antibacterial activity[13] of a methanolic extract of *A. cotula*. This extract, however, contained flavonoids only.

Experimental Section

General Experimental Procedures. Optical rotation was determined with a Perkin-Elmer polarimeter. UV spectra were obtained directly from silica gel with a CS-930 dual-wavelength scanner with data recorder DR-2 (Shimadzu Corp.), which was used for quantitative TLC as well. ^{1}H and ^{13}C NMR spectra were run on a JEOL Eclipse+ 500 in $CDCl_3$ solution at 500 and 125 MHz, respectively, with TMS as internal standard. Mass spectra were recorded with a Vacuum Generators VG 7070 H spectrometer at 70 eV. TLC, HPTLC, and RP-18 HPTLC and flash column chromatography (silica gel 60, 40−63 μm) were performed with products of Merck, Darmstadt, Germany. All solvents were analytical grade.

Plant Material. *Anthemis cotula* L. was collected in full bloom near Marburg, Germany, at the end of July 2001. Its taxonomic identification was confirmed by Prof. Dr. V. Melzheimer, Botanical Garden, University of Marburg. A voucher specimen was deposited in the herbarium of the corresponding author, acc. no. 3.

Isolation of Anthecotulide (1). Dried aerial parts of *A. cotula* L. (400 g) were macerated with 6 L of chloroform for 1−2 days. After filtration, the solvent was evaporated at reduced pressure and the residue re-macerated with another 6 L of chloroform for 12 h. The residue after filtration and evaporation was dissolved in 400 mL of *tert*-butylmethyl ether, washed with citric acid (5%), saturated potassium bicarbonate, and water, and dried over magnesium sulfate. After evaporation of the solvent, the residue was purified by flash chromatography, eluting with *n*-hexane−*tert*-butylmethyl ether (1:1) to separate nonpolar compounds. Anthecotulide (**1**) was eluted together with very little greenish impurity (yield, 0.7% w/w). For spectroscopical data, see below.

The anthecotulide (**1**) fraction from flash chromatography was purified by another flash chromatography eluting with toluene−*tert*-butylmethyl ether (3:1) followed by preparative thin-layer chromatography on silica gel 60 (200 × 200 × 2 mm) using the same eluent to give chromatographically and spectroscopically pure **1** as a pale yellow viscous oil: yield, 0.6% w/w of dried aerial parts.

Anthecotulide [*(E)*-(+)-4-(3,7-dimethyl-5-oxo-2,6-octadienyl)-dihydro-3-methylenetetrahydrofuran-2-one]: $[\alpha]_{25}^{578}$ +116° ($CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 231.5 (4.15), 203 (4,-16); IR ($CHCl_3$) ν_{max} 1780, 1693, 1670, 1625 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.13 (1H, d, J = 2.3 Hz, H-15a), 6.02 (1H, sept, J = 1.1 Hz, H-10), 5.64 (1H, d, J = 2.3 Hz, H-15b), 5.21 (1H, br qt, J = 7.1, 1.2 Hz, H-6), 4.37 (1H, dd, J = 8.2, 8.9 Hz, H-4a), 3.91 (1H, dd, J = 5.5, 8.9 Hz, H-4b), 3.15 (1H, dddd, J = 2.6, 5.3, 10.6, 13.1 Hz, H-3), 3.00 (2H, s, H-8), 2.42 (1H, ddd, J = 6.7, 6.7, 14.6 Hz, H-5a), 2.34 (1H, ddd, J = 7.6, 7.6, 14.6 Hz, H-5b), 2.09 (3H, d, J = 0.9 Hz, H-12), 1.88 (3H, d, J = 1.1 Hz, H-13), 1.62 (3H, d, J = 0.7 Hz, H-14); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 198.6 (C, C-9), 170.8 (C, C-1), 156.4 (C, C-11), 138.1 (C, C-2), 133.7 (C, C-7), 124.0 (CH, C-6), 123.0 (CH, C-10), 122.3 (CH_2, C-15), 70.6 (CH_2, C-4), 55.1 (CH_2, C-8), 38.7 (CH, C-3), 32.2 (CH_2, C-5), 27.7 (CH_3, C-13), 20.7 (CH_3, C-12), 16.9 (CH_3, C-14); EIMS *m/z* 248 $[M]^+$ (1%), 193 (1), 151 (2), 150 (1), 149 (1), 123 (2), 109 (2), 95 (1), 93 (1), 91 (2), 83 (100), 55 (20). All assignments except for individual methyl groups were proved by ^{1}H,^{1}H COSY, ^{1}H, ^{13}C COSY, phase-sensitive NOESY, and NOE difference spectra.

Quantitative Determination of Anthecotulide (1). Method: HPTLC on silica gel with toluene−*tert*-butylmethyl ether (3:1). Quantification: in reference to the pure sample of **1** we isolated. Detection: (a) visual fluorescence quenching; limit of quantification (LOQ) 200 ng; (b) colorimetrically, after derivatization (spraying) with a Zimmermann reagent ((I) 1-chloro-2,4-dinitrobenzene in toluene, (II) methanolic NaOH) gave a violet color that was stable for approximately 30 min; LOQ 500 ng; (c) UV reflection at 247 nm with a TLC scanner; LOQ 100 ng (see Table S1, Supporting Information).

Stability of Anthecotulide (1). Compound **1**, pure and in dried flower heads of *A. cotula*, was completely stable at −20, 4, and 21 °C for three months, with approximately 50% decomposition at 40 °C. Storage in closed containers, in direct light or in the open air, did not make any difference. Determination was by method (b) (see above).

Reaction of Anthecotulide (1) with Glutathione. TLC monitoring: 40 μmol of **1** was dissolved in a few drops of acetone and the solution added to 1 mL of phosphate buffer pH 7.4. Glutathione (20 μmol) was added, and the emulsion shaken at 700/min and 25 °C. After 5, 30, 90, 240, and 1380 min, 10 μL samples were analyzed by TLC on silica gel KG 60 F_{254} with *n*-butanol−water−acetic acid (5:4:1). Visual detection after spraying with ninhydrin reagent (0.2% ninhydrin in acetone) showed that after 30 min approximately 50% of **1** had reacted (R_f 0.72, ninhydrin negative). After 240 min, the remaining amount of **1** had not changed, and another 20 μmol of glutathione was added. After 23 h, the reaction had come to completion. Two ninhydrin-positive products had formed with R_f 0.18 (main product) and 0.50 (minor product).

^{1}H NMR monitoring: 40 μmol of **1** was dissolved in CD_3OD, mixed with an equimolar amount of glutathione in D_2O, and CD_3OD was added until the solution was clear. Spectra were recorded after 30, 60, 90, 120, 150, 180, 240, 300, and 360 min and during the following days. Even after 10 days, the reaction had not come to completion in this solvent. In **1**, the integrals of protons H-15 and H-10 slowly diminished in size, while in glutathione, the peak of the methylene group next to the thiol group at 3.30 ppm decreased (relative to internal standard, dioxane). New signals increased at 4.43 ppm (d), 3.92 ppm (d), 3.20 ppm (s), and 1.64 ppm (s). This is in accord with reaction at both the methylene lactone and α,β-unsaturated ketone moieties of **1**. The major reaction product decomposed on attempted isolation, so its structure could not be established fully.

Anti-infective Testing of Anthecotulide (1). Anthecotulide (**1**) was dissolved in dimethyl sulfoxide. Aliquots of the solution were added to Müller Hinton II broth pH 7.3 (±0.1) supplied by Becton-Dickinson, Heidelberg, Germany (cat. no. 4312322). The following strains were evaluated: *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, a methicillin-resistant *Staphylococcus aureus*, and a vancomycin-resistant *Enterococcus faecium*, the latter two being clinical isolates from the university hospital of Marburg, Germany.

Analysis of Chamomile Preparations for Anthecotulide (1). A total of 34 preparations containing chamomile were analyzed (see Table S2, Supporting Information). Herbal infusions were macerated for 1−2 days with chloroform and filtered, and the solvent was evaporated. Liquid preparations were analyzed as such. Body care products, lotions, and ointments, containing surfactants, were extracted by partitioning between chloroform and water. Emulsions were broken by

suction filtration through silica gel 60 (63−200 μm). In all cases, equal amounts were weighed, and the residue after evaporation of the chloroform was dissolved in 25 mL of *tert*-butylmethyl ether. Aliquots (10 and 20 μL) were analyzed by quantitative TLC (methods (b) and (c), above) on HPTLC 0.2 mm sheets (silica gel KG 60 F_{254}, toluene−*tert*-butylmethyl ether (3:1), and silica gel RP-18 WF_{254s}, diisopropyl ether). Anthecotulide (**1**) was not detected, so its amount in any sample did not exceed 100 ng. For the herbal infusions, this corresponds to a concentration of **1** of <0.0013%, and for the liquid preparations, <0.0001%.

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Supporting Information Available: Tables of the quantitative determination of **1** with a TLC scanner and the sources of 34 samples of chamomile-containing products analyzed for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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