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Efficient Isolation of an Anti-Cancer Sesquiterpene Lactone from *Calomeria amaranthoides* by Steam Distillation

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Abstract

An efficient method of isolating an anti-cancer sesquiterpene lactone, eremophila-1(10)-11(13)-dien-12,8β-olide, was developed from fresh *Calomeria amaranthoides* plant material on the basis of its non-polar volatile property. Steam distillation of fresh plant material gave a high recovery of sesquiterpene-rich oil, 0.56%, compared with the estimated 0.66% calculated from solvent extraction of dried plant material. The sesquiterpene-rich oil containing 58% of the sesquiterpene lactone was fractionated by the short-column vacuum chromatography method, using minimal stationary-phase and solvent, to give the sesquiterpene lactone 95% purity, yield 41%.

1. Introduction

Calomeria amaranthoides Vent.*,* is a shrub of the Asteraceae (Compositae) family and belongs to a monotypic genus endemic to NSW and Victoria, Australia [1]. It is a biennial plant that can grow to more than three metres high, with plume-like flowers and wrinkly leaves. The plant has a strong aromatic scent and its common name is incense plant. The chemical constituents composition of aerial parts of *C. amaranthoides* were examined initially by Zdero *et al.* [2], however, none the constituents reported by them were identified in the *C. amaranthoides* extracts described in a study by van Haaften *et al.* [3] where two main constituents isolated from the aerial parts of *C. amaranthoides* were identified as eremophila-1(10)-11(13)-dien-12,8β-olide (**1**) [4] and eremophila-1(10), 11(13)-dien-12-oic acid (**2**) [5] (**Figure 1**). **1** and **2** had been isolated and reported previously from plants of the Asteraceae family [4,5]. The study by van Haaften *et al.* [3] showed a potent cytotoxic effect of the non-polar extract from the leaves of *C. amaranthoides* towards ovarian cancer cells. Much of the *in vitro* cytotoxicity towards ovarian cancer cells appeared to be due to **1**. This was supported by studies *in vivo* where, on treatment with **1**, anti-cancer effects were observed with nude mice inoculated with OVCAR3 cells intraperitoneally [3].

An ester substituted eremophilane marine natural product, cryptospaerolide, showed significant cytotoxicity towards HCT-116 human colon carcinoma cell line [6]. This finding indicates that the plant derived **1** eremophilane skeleton has potential to serve as a scaffold for synthesis of effective anti-cancer agents. Efficient methods were sought to produce larger quantities of extract of **1**, required for further biological studies. The odour of the plant, the extract and **1** were very similar, so a method of isolating the volatile biologically active material by steam distillation was developed.

2. Results and Discussion

The yield of sesquiterpenes, **1** and **2**, from fresh plant material was estimated to be approximately 0.66% based on the content of the CH_2Cl_2 extract of freshly dried leaves. Hydrodistillation conditions were initially used
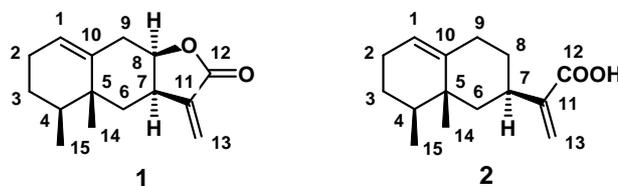

Figure 1. Structures of eremophila-1(10)-11(13)-dien-12,8β-olide (1) and eremophila-1(10),11(13)-dien-12-oic acid (2).

with a relatively large amount of plant material torn into large pieces and using minimal added water (Experiment 1, **Table 1**) and this resulted in relatively low recovery of sesquiterpene-rich oil steam distillate. Using less plant material and a larger volume of added water (Experiment 2, **Table 1**) gave a higher recovery of sesquiterpene-rich oil steam distillate, however, the yield was only about half of the estimated maximum. Separation of the sesquiterpene-rich oil from the steam distillate required solvent extraction and salting-out due to the small proportion and the stable emulsion of sesquiterpene-rich oil steam distillate. Extraction with CH_2Cl_2 alone proved to be ineffective in Experiments 1 & 2 as EtOAc was required to complete the extraction. Efficient extraction was achieved with EtOAc alone in later experiments. For Experiments 3 and 4 plant material was blended to a fine suspension and after the first distillation the process of adding water and distilling was repeated twice. In Experiment 3, the yield of sesquiterpene-rich oil from the steam distillate was comparable to the estimated maximum, however, the amount of sesquiterpene-rich oil produced from the third distillation was comparable to the first and second indicating that a higher yield may have been possible. In Experiment 4 the amount of plant ma-

terial was halved and this provided conditions which gave the highest yield. Most of the sesquiterpene-rich oil was obtained in the first distillation, and the amount obtained in the second distillation was approximately half that of the first and approximately halved again in the third distillation indicating that close to maximum yield is likely to have been achieved with Experiment 4. Even with the addition of NaCl to salt-out the sesquiterpene-rich oil from the steam distillate, extraction with EtOAc to remove the cloudy emulsion was slow and a clear aqueous phase was not achieved until the third EtOAc extraction. This was an unexpected property as the two sesquiterpenes have a low polarity and are very lipophilic. The apparent affinity for aqueous media may prove a useful property in terms of increased bio-availability in the evaluation of **1** for potential application to treat cancer [3].

Short-column vacuum chromatography was selected to fractionate and refine the sesquiterpene-rich oil, as the method required a relatively small quantity of TLC grade silica gel and solvent and it was carried as a single stage large-scale separation process. A single application of this process gave good yields of highly refined **1** and **2** (**Table 2**). The yield and high recovery of **1** and **2** showed

Table 1. Yields of sesquiterpene-rich oil and sesquiterpene lactone (1) obtained by steam distillation processes.

Expt	steam distillation	fresh plant material grams	vol added hot water (mL)	vol steam distillate (mL)	extraction solvent	yield grams	oil yield %	AV **1** *%	STDev **1** %
1	single	500	1000	600	CH_2Cl_2	0.1803		69.5	1.2
					EtOAc	0.0287		72.8	2.0
					Total	0.209	0.042		
2	single	345	2000	1800	CH_2Cl_2	0.5802		68.3	2.0
					EtOAc	0.2933		70.5	2.2
					Total	0.8735	0.253		
3	triple	370	2000	1500	EtOAc	0.8589		70.2	2.4
			2000	1600	EtOAc	0.5529		66.5	1.2
			1600	1600	EtOAc	0.5115		61.9	1.7
					Total	1.9233	0.520		
4	triple	190	3000	2000	EtOAc	0.6147		66.8	1.6
			1600	1600	EtOAc	0.2778		57.6	2.3
			1500	1500	EtOAc	0.162		48.9	2.8
					Total	1.0545	0.555		

AV: Average; STDev: Standard deviation, *Proportion of **1** in relation to **1** plus **2** content.

Table 2. Short-column vacuum chromatography fractionation of steam distillate from experiment 3.

Fract	Hexane (mL)	CH$_2$Cl$_2$ (mL)	EtOAc (mL)	Fract (mg)	yield* %	1 %	2 %
1	25	25		89	8	26	
2	12.5	12.5		457	41	94.7 ± 1.8	
3		25		134	12	78.8 ± 1.8	20
4		25		157	14	54.7 ± 0.8	44
5		25		61	5.4		92
6		22.5	2.5	40	3.6		84
7		22.5	2.5	70	6.2		91
8		20	5	31	2.7		95
9		20	5	22	1.9		95
10		12.5	12.5	13	1.2		64
TOTAL				1074	95.8		

*based on the 1120.3 mg portion of sesquiterpene-rich oil fractionated.

that both of these sesquiterpenes were stable under the steam distillation conditions. There are no references for the steam distillation of sesquiterpene lactones in the literature as in many cases thermal degradation occurs, commonly by rearrangement, dehydration and elimination processes [7]. The volatile nature of both **1** and **2** may explain their loss over time from stored dried plant material [3].

3. Conclusions

Steam distillation by the hydro-distillation method is a convenient and efficient method to isolate from fresh plant material a highly refined product free from waxes and other non-volatile material. The pale yellow liquid product contained approximately 58% **1** with most of the remaining material being **2**. Through the application of short-column vacuum chromatography both **1** and **2** are rapidly and efficiently refined to high levels of purity.

This method is of great importance for obtaining the larger quantities of **1** required for further studies on the treatment of ovarian and other cancers.

4. Experimental Section

4.1. Plant Material

A voucher specimen of *Calomeria amaranthoides*, collected near Old Bell's Line of Road, Mount Tomah NSW 2758, Australia, is held in the John Ray Herbarium, University of Sydney, Collection number: Silvester 11011801.

Leaves and stems of the upper aerial parts of *C. amaranthoides,* gathered in the Blue Mountains (Mount Tomah, NSW, Australia), were kept fresh at 4°C for no more than 4 days before steam distillation.

4.2. 1H-NMR Analyses

^{1}H-NMR spectroscopy was performed using a Varian 400 MHz instrument (Palo Alto, CA, USA). The solvent used was CDCl$_3$ and the spectra were measured in parts per million (ppm) referenced to tetramethylsilane (TMS = 0 ppm).

Short-Column Vacuum Chromatography (SCVC) [8] was performed using a column packed with TLC-grade silica gel H60 (Merck, Darmstadt, Germany) and applying a step-wise gradient of solvents with increasing polarity.

1 and **2** content of the steam distillate and chromatographic fractions were determined by integration of proton signals for H-1, H-13 and H-13' for both sesquiterpenes. The content based on the integration for each proton type was calculated as the average and standard deviation.

4.3. Steam Distillation Method and Apparatus

Plant material was finely chopped in deionised water us-

ing a 550 W Breville Blender. Steam distillation was carried out by the hydrodistillation method at atmospheric pressure. Heat was provided using the lower mantle setting of a 5 L heating mantle. Apparatus consisted of a B34 5 L round bottom flask, stillhead, a double-coiled single-jacketed water cooled condenser and a receiver head. The 5 L RB flask and stillhead were wrapped with a single layer of aluminium foil throughout the distillation.

4.4. Steam Distillation of Leaves and Stems (Optimised Procedure, Experiment 3)

Fresh leaves and stems (370 g) and hot water (2000 mL) were blended to a fine suspension, placed in a 5 litre flask which was heated and the water distilled conden-

sate (1500 mL) collected. The apparatus was allowed to cool and hot water was added to the 5 L flask which was again heated and further water distilled condensate (1600 mL) collected. The process described in the last sentence was repeated giving 3 lots of condensate in total. Each lot of condensate was mixed with NaCl (approx. 12.5 g per 100 mL of condensate) until the NaCl had all dissolved, then, thrice extracted with EtOAc (3 × 50 mL). The pooled EtOAc for each lot was collected, dried with anhydrous Na_2SO_4, filtered and concentrated to a very pale yellow slightly viscous liquid (0.86 g, 0.55 g, 0.51 g, total 1.9 g). ^{1}H-NMR analysis (**Figure 2**) indicated that the EtOAc extracted pale yellow viscous liquid was mainly a mixture of **1** (62% to 70%) and **2** (28% to 30%) (**Table 1**).
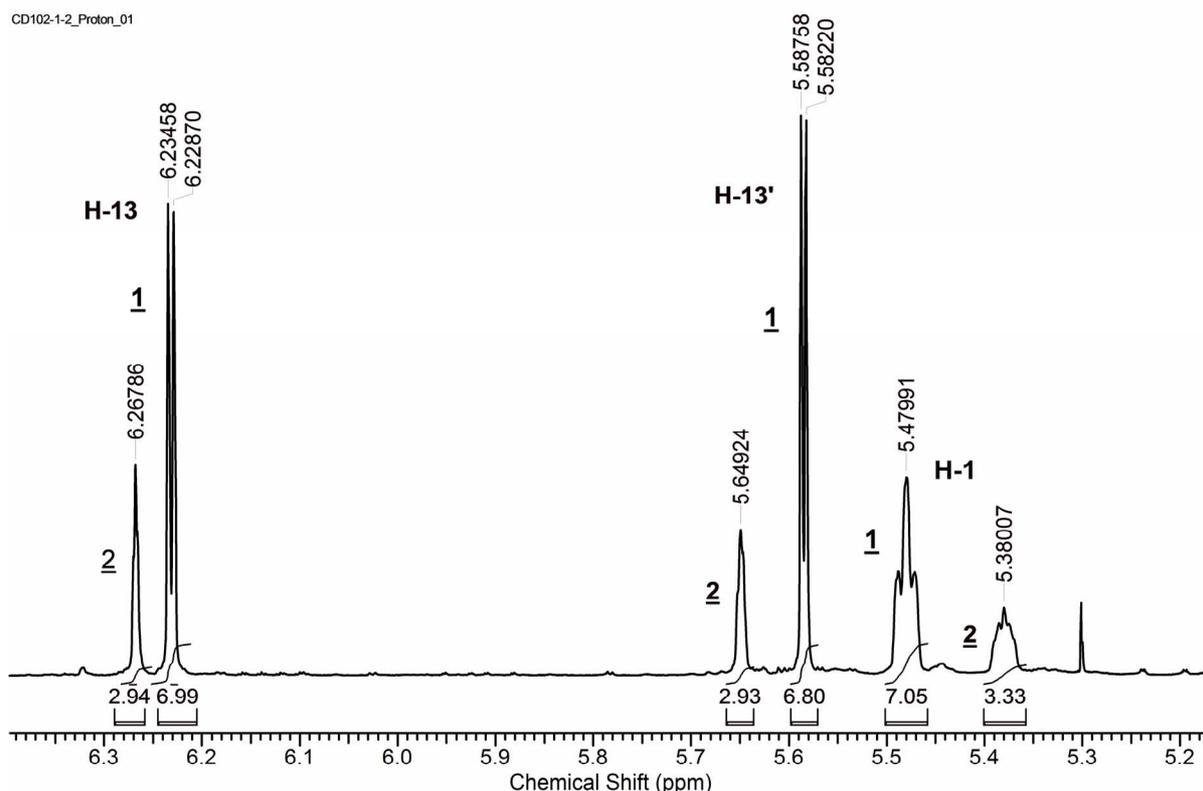

Figure 2. Partial ^{1}H-NMR spectrum of sesquiterpene-rich oil steam distillate showing an example integration of the H-1, H-13 and H-13' signal to determine the 1 and 2 content.

A portion of the EtOAc extracted steam distillates were combined (1.12 g, ^{1}H-NMR analysis **1** 69.4% ± 1.4%) and fractionated by normal-phase short column vacuum chromatography, silica gel H, diameter 50 mm, height 25 mm, eluted with a stepwise solvent gradient of hexane:dichloromethane 1:1 (50 mL and 25 mL); dichloromethane (3 × 25 mL); dichloromethane:ethyl acetate 9:1 (2 × 25 mL); dichloromethane:ethyl acetate 4:1 (2 × 25 mL); dichloromethane:ethyl acetate 1:1 (2 × 25

mL); ethyl acetate (2 × 25 mL). For each fraction the solvent was evaporated under reduced pressure and the residue was weighed.

4.5. Normal-Phase Short-Column Vacuum Chromatography

Fraction (weight): F1 (88.8 mg); F2 (457 mg); F3 (134 mg); F4 (157 mg); F5 (60.6 mg); F6 (39.8 mg); F7 (70

mg); F8-F10 (49.6 mg). Recovery, as a proportion of the total weight oil, 95.8%. ^{1}H-NMR analysis showed that fractions 2 consisted mainly of **1**, 94.7%. Fractions 7 to 9 (122 mg) consisted mainly of **2**, 91-95% (**Table 2**). The sesquiterpene-rich oil from experiment 3 was found to consist of approximately 85% of a mixture of compounds **1** and **2** from ^{1}H NMR analyses of Fractions 1 to 10.

4.6. Cold Extraction of Leaves and Stems with Dichloromethane

Fresh plant material (208 g) was dried over a few days in subdued light to give dried plant material (46.7 g, 22.5 %). A portion of this dried plant material (1.63 g) was extracted with CH_2Cl_2 (100 mL) to give yellow-brown oil (62.6 mg, 3.9%). ^{1}H-NMR analysis indicated that approximately 75% of the mixture was **1** and **2** and the remainder was a complex mixture of unidentified material.

Yields and Proportions of **1** and **2** steam distilled from fresh plant material under a range of conditions are summarised in **Table 1**.

5. Acknowledgements

6. References

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