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Martin M. Pedersen, Jude C. Chukwujekwu, Carmen A. Lategan, Johannes van Staden, Peter J. Smith, Dan Staerk

*Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark*

*Research Centre for Plant Growth and Development, School of Biological and Conservation Science, University of KwaZulu-Natal Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa*

*Department of Medicine, Division of Pharmacology, University of Cape Town, Observatory 7925, Capetown, South Africa*

*Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg, Denmark*

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*Antiplasmodial activity*

**ABSTRACT**

Combined use of bioassay-guided fractionation based on *in vitro* antimalarial assay and dereplication based on HPLC–PDA–MS–SPE–NMR led to isolation of 65,7R,8S)-14-acetoxy-8-[2-hydroxymethacrylate]-15-heliana-1(10),4,11(13)-trien-15-al-6,12-olid and (5R,6S,7R,8S,10S)-14-acetoxy-8-[2-hydroxymethylacrylate]-elema-1,3,11(13)-trien-15-al-6,12-olid, along with vernodalol, vernodalin, and 11,13-dihydroxyvernodalin from extract of *Distephanus angulifolius*. All compounds were identified by spectroscopic methods, including 1D and 2D homo- and heteronuclear NMR experiments. The isolated compounds showed IC50 values in the range 1.6–3.8 μM and 2.1–4.9 μM against chloroquine sensitive D10 and chloroquine resistant W2 *Plasmodium falciparum* strains, respectively.

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1. Introduction

Drug discovery based on results from *in vitro* antimalarial bioassays, i.e., bioassay-guided isolation, has revealed many active constituents from complex mixtures of biological origin, e.g., plant extracts, marine organisms, and microorganisms. However, the method suffers from several problems, including limited compatibility with high-throughput technologies typically used in pharmaceutical drug discovery programmes (Butler, 2004), results from *in vitro* assays do not necessarily reflect *in vivo* activity (Bourdy et al., 2008), need for time-consuming preparative-scale isolation for *in vitro* bioassays, and the risk of spending time on isolation of known compounds with known activity. Urgent need for new antimalarial drug candidates calls for alleviation of some of these drawbacks, and the latter can be partly solved by applying chemical screening technologies that identifies the extract-constituents before or simultaneous with the bioassay-guided fractionation. Although LC–MS is a fast and sensitive technique that is frequently used for chemical screening, mass spectrometry lacks the possibility of rigorous structure elucidation that is provided by NMR spectroscopy. In a series of recent papers, it has been demonstrated that hyphenated HPLC–SPE–NMR is a fast, sensitive and efficient technique for analytical-scale identification of constituents in complex mixtures (Clarkson et al., 2006a; Schmidt et al., 2008; Sprogøe et al., 2007; Sørensen et al., 2007; Tatsis et al., 2007), including full structure elucidation of very complex structures (Clarkson et al., 2006b) and assignment of absolute stereochemistry by combining the technique with circular dichroism (Sprogøe et al., 2008). The HPLC–SPE–NMR technique has been extended to include direct on-line identification of radical scavenging activity (Pukalskas et al., 2005), but the simultaneous identification of chemical structure and biological activity is not possible in most cases. In this work, the two complementary strategies, i.e., chemical screening based on HPLC–PDA–MS–SPE–NMR and biological screening based on results from *in vitro* antimalarial assays, were performed simultaneous on an extract of *Distephanus angulifolius* (DC.) H. Rob. & B. Kahn [syn. *Vernonia angulifolia* DC.] (Robinson and Kahn, 1986) to avoid preparative-scale isolation of uninteresting constituents, both in terms of chemical structure and biological activity.

*Distephanus* and *Vernonia* are closely related genera within the *Asteraceae* (Robinson, 2006), but whereas phytochemical information on *Distephanus* is scarce (Jakupovic et al., 1987), *Vernonia* is known to be a rich source of sesquiterpene lactones. *Vernonia amygdalina* is probably one of the most intensively investigated *Vernonia* species due to reports of its use as an antiparasitic remedy by wild chimpanzees (Huffman and Seifu, 1989; Jisaka et al., 1992;...
Koshimizu et al., 1994; Ohigashi et al., 1994) but other activities, including antitumor (Jisaka et al., 1993), antibacterial (Erasto et al., 2006; Rabe et al., 2002; Reid et al., 2001), antioxidant (Erasto et al., 2007), anti-inflammatory (Cioffi et al., 2004), insect antifeedant (Ganjian et al., 1983), and cytotoxicity (Koul et al., 2003), have been reported from various Vernonia species.

Disstephanus angulifolius is a scrambling shrub or climber which in South Africa is found from the Eastern Cape to Mozambique. It is commonly known as “Trailing Vernonia” due to its growth habit, and in Zulu, it is called impoqompoqwane. According to Pooley (1998), D. angulifolius is used traditionally to treat stomach ailments, and an earlier investigation reported the presence of four germacrane-type sesquiterpene lactones (Bohlmann et al., 2007), anti-inflammatory (Cioffi et al., 2004), insect antifeedant (Ganjian et al., 1983), and cytotoxicity (Koul et al., 2003), have been reported from various Vernonia species.

2. Results and discussion

HPLC–PDA–MS–SPE–NMR was used for initial analysis of a defatted dichloromethane–methanol extract (extract A) of D. angulifolius. A total of 17 peaks were analyzed (Fig. 1), showing the presence of chlorogenic acid analogs (peak 1–3), vernodinal (peak 4), vernoldinal (peak 5), 11,13-dihydrovernodinal (peak 6), 1 (peak 7), 2 (peak 9) and seven unidentified steroid saponins (peak 8, 10–17) (Phenomenex Luna C18(2) column, 150 × 4.6 mm i.d., 3 µm, flow rate 0.8 mL/min, acetonitrile gradient in water (0.1% formic acid) as shown).

Koshimizu et al., 1994; Ohigashi et al., 1994) but other activities, including antitumor (Jisaka et al., 1993), antibacterial (Erasto et al., 2006; Rabe et al., 2002; Reid et al., 2001), antioxidant (Erasto et al., 2007), anti-inflammatory (Cioffi et al., 2004), insect antifeedant (Ganjian et al., 1983), and cytotoxicity (Koul et al., 2003), have been reported from various Vernonia species.

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contrary to their own analysis of coupling constants and a molecular modeling study, which both suggest a chair conformation. In addition, there is no further evidence for the boat-conformation, e.g., information about a strong ROE between H-6 and H-9b, or other ROE’s supporting the boat conformation. In this study, both coupling constants, observed NOE’s and molecular modeling studies suggests that the cyclohexane part of 2 adopts a pseudo-chair conformation. The absolute configuration of 2 is 5R,6R,7R,8S,10R based on comparison of the optical rotation (\(\frac{\alpha}{c} = +26.3\)) with data from related structures possessing the same chiral skeleton with achiral substituents (Roselli et al., 2003; Karamenderes et al., 2007; Cardona et al., 1997). Compound 2 is a new compound for which the name vernangulide B is suggested.

Compounds 3–5 were identified as vernodolol, vernodalin and 11,13β-dihydrovernodalin, respectively, based on comparison of spectroscopic data (optical rotation, \(^1\)H and \(^13\)C NMR; see Supplementary Tables 3–5) with data from literature (Erasto et al., 2007; Abegaz et al., 1994; Reid et al., 2001; Al Magboul et al., 1997) as well as by full assignment of 1D and 2D NMR spectroscopic data.

Compounds 6 and 7 were isolated as a mixture and identified as the C6,C12-ring-opened forms of vernodalin and 11,13β-dihydrovernodalin. Lactones exist in equilibrium with their ring-opened analogs, and consequently 6 and 7 should not be considered as independent compounds. However, because 6 and 7 could be isolated as the ring-opened form without their lactonized analogs, \(^1\)H NMR data are given for future reference (Supplementary Tables 6 and 7).

Antiplasmodial activity of 1–4 against chloroquine sensitive D10 and chloroquine resistant W2 P. falciparum strains are shown.
All tested compounds have IC_{50} values in the low μM range against both D10 and W2 strains. Compounds 1 and 2 show slightly better effect than 3, with 2 having the lowest resistance index. Compounds 2 and 3 show a selectivity index 2.8 and 3.6 times higher, respectively, than 1.

3. Conclusion

In this work, fast dereplication of extract constituents avoided time-consuming preparative-scale isolation of steroid saponins, which due to their amphiphilic and/or detergent properties are likely to give false-positive response in in vitro antiplasmodial assays due to membrane alterations (Ziegler et al., 2002, 2004; Sairafianpour et al., 2003). In addition, results from bioassay-guided fractionation targeted preparative-scale isolation towards the sesquiterpene lactones. It is well-established that sesquiterpene lactones possess a general cytotoxicity mediated by their α,β-unsaturated carbonyls (Scotti et al., 2007; Zhang et al., 2005). These structural elements react with cystein sulphydryl groups by a Michael type addition, thus making covalent adducts with cystein residues of the proteins. However, 2 and 3 showed both low resistance index and high selectivity index, which make them interesting as chemotherapeutic lead structures against chloroquine resistant P. falciparum. Future improvements in antimalarial chemotherapy based on sesquiterpene lactones could originate from medicinal chemistry studies aiming at synthesis of analogs with increased selectivity and lowered resistance index, and further understanding of drug-delivery mechanisms, including parasite transporter-based strategies (Biagini et al., 2005), opens new horizons for targeting the increased mortality due to drug-resistant malaria.

4. Experimental

4.1. General

Optical rotations were recorded using a Perkin–Elmer 241 polarimeter. NMR spectra of isolated compounds were recorded at 25 °C using a Bruker Avance 600 MHz equipped with a 5 mm 1H{13C} probe or a Bruker Avance 400 MHz spectrometer equipped with a 5 mm 13C{1H} probe (1H resonance frequency 600.13 and 400.13 MHz, respectively). Spectra were calibrated using TMS as internal standard. High-resolution mass determinations were carried out using a Micromass LCT mass spectrometer equipped with an electro-spray ion source operated in positive-ion mode. The spectra were calibrated relative to Leu-Enkephalin (556.2771 g/mol) added as an internal standard. Preparative-scale separations were performed using an Agilent 1100 LC system (two preparative pumps, an autosampler, a sample collector, and a multiple-wave-length UV detector; controlled with ChemStation rev. B.01.01 software; HPLC system I), a HPLC system consisting of a Waters 590 pump, a Rheodyne 7125 injector, and a Lambda-Max Model 481 LC UV detector (HPLC system II) or a Shimadzu HPLC system (SCL-10A system controller, SIL-10AD autoinjector, LC-10AT pump, CTO-10AC column oven, FRC-10A fraction collector, and SPD-M10A PDA detector; controlled with Shimadzu Class-VP ver. 6.10 software; HPLC system III). HPLC–PDA–MS–SPE–NMR experiments were performed as described in details previously (Sprogøe et al., 2008).

4.2. Plant material

Aerial parts of D. angulifolius (DC.) H. Rob. & B. Kahn [syn. V. angulifolia DC.] (Asteraceae) were collected at an altitude of 665 m in the Botanical Garden of University of KwaZulu-Natal, Pietermaritzburg, on January 5th 2006. The identity of the plant was confirmed by Professor T.J. Edwards and a voucher specimen (Accession Number: Chukwujekwu #1 NU) has been deposited in the Herbarium at the University of KwaZulu-Natal, Pietermaritzburg.
with a flow rate of 0.8 ml/min using a mixture of water–acetonitrile 5:148.5:6.54 (4:142.3:145.2).

Leaves (350 g) were sonicated in 2 l, b.p. 80–100°C, overnight in vacuo, and freeze dried to give 67 g of raw extract. The extract was defatted by redissolving in 1 l of methanol–water (9:1) and extracting with petroleum ether (20 × 0.5 l, b.p. 80–100°C), which yielded 56 g of defatted extract. Leaves (350 g) were sonicated for 1 h with 1 l acetone and then left for extraction overnight. Extraction was subsequently repeated three times with 0.5 l acetone, and the combined extracts were evaporated under reduced pressure at 30°C to give 19 g of extract.

Separation of defatted extract was performed at 40°C on a 150 × 4.6 mm i.d. Phenomenex C18(2) Luna column (3 μm, 100 Å) with a flow rate of 0.8 ml/min using a mixture of water–acetonitrile 95.5:0.1% formic acid (eluent A) and acetonitrile–water 95.5:0.1% formic acid (eluent B). Five repeated separations (30 μl injection volume, 100 mg/ml, gradient profile: 0 min, 18% B; 13 min, 18% B; 28 min, 32% B; 43 min 32% B; 53 min, 100% B; 60 min, 100% B; 62 min, 18% B; 72 min 18% B) were performed using UV threshold-based trapping (220 and 254 nm) of analytes on GP phase SPE cartridges (general-purpose poly(divinylbenzene)-based resin from Spark Holland, 10 × 2 mm i.d.; water post-column dilution, 2 ml/min).

4.5. Preparative-scale isolation

Initial separation of defatted extract was performed with HPLC system I on a 250 × 21.2 mm i.d. Phenomenex C18(2) Luna column (5 μm, 100 Å) with a flow rate of 20 ml/min. Mixtures of water–acetonitrile 95.5:0.1% TFA (eluent A) and acetonitrile–water 95.5:0.1% TFA (eluent B) were used for separation of 3.1 g (900 μl pr. injection, 250 mg/ml solution) using the following gradient profile: 0 min, 25% B; 5 min, 25% B; 20 min, 32% B; 33 min, 60% B.

Table 2
Antiplasmodial and cytotoxic activity of 1-4 against P. falciparum D10 and W2 strains and Chinese hamster ovarian cell line.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 (µM)</th>
<th>Resistance indexb</th>
<th>Selectivity indexc</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10 (n = 2)a</td>
<td>W2 (n = 2)</td>
<td>CHO (n = 3)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.90 ± 0.10</td>
<td>3.24 ± 0.27</td>
<td>3.52 ± 1.63</td>
</tr>
<tr>
<td>2</td>
<td>1.55 ± 0.02</td>
<td>2.10 ± 0.17</td>
<td>8.28 ± 0.39</td>
</tr>
<tr>
<td>3</td>
<td>3.82 ± 0.08</td>
<td>4.04 ± 0.18</td>
<td>26.38 ± 1.85</td>
</tr>
<tr>
<td>4</td>
<td>1.75 ± 0.35</td>
<td>2.69 ± 0.26</td>
<td>4.47 ± 0.64</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.04 ± 0.004</td>
<td>0.16 ± 0.055</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Emetine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a n = number of replicates.

b Resistance index = IC50 W2/IC50 D10.

c Selectivity index = IC50 CHO/IC50 D10.
32% B; 38 min, 100% B; 43 min, 100% B; 45 min, 25% B; 55 min, 25% B. Manual collection based on absorption levels at 220 and 254 nm afforded seven fractions (A–G) and $^1$H NMR spectra of the fractions showed that only fraction A (34 mg) and fraction C (112 mg) contained sesquiterpene lactones. HPLC system II and the above-mentioned HPLC column were used to rechromatograph fraction A and C. Thus, isocratic elution of fraction A (water–acetonitrile–formic acid 80:20:0.1) yielded 3.2 mg of (3) whereas isocratic elution of fraction C (water–acetonitrile–formic acid 65:35:0.1) yielded fractions C1–C5. Fraction C4 (2 mg, norartocarpetin and luteolin, 2:1 mixture) was not further purified, but fraction C2 (13 mg) and C5 (52 mg) were rechromatographed on HPLC system III using the same HPLC column and eluents as for the HPLC–PDA–MS–SPE–NMR analysis. Isocratic separation of 7.7 mg of fraction C2 (water–acetonitrile–formic acid 82:18:0.1) yielded 2.7 mg of 2:1 mixture of 6 and 7, whereas isocratic separation of 13.6 mg of fraction C5 (water–acetonitrile–formic acid 80:20:0.1) yielded 1.3 mg of 4 and 3.0 mg of 5.

4.6. Bioassay-guided isolation

Column chromatography (Merck 9385, 200 g, 2.5 × 73 cm) of the acetone extract, using hexane–acetonitrile step gradients (10:0, 9:1, 4:1, 7:3, 3:2, 1:1, 2:3, 3:7, 1:4, and 0:10; 0.5 l each), yielded 355 fractions which were pooled into 16 fractions based on similarity of TLC profiles using UV light at 254 and 365 nm for visualization. All fractions were assessed for antimalarial activity against chloroquine sensitive D10 P. falciparum strain at concentrations of 5, 2.5, and 1.25 μg/ml. Fractions 11 (1.197 g) and 12 (5.6 g) were further purified by preparative TLC using a hexane–EtOAc (3:1) solvent system, and this yielded 14.5 mg of 1, 1 mg of 2, 13 mg of 3, and 59 mg of a mixture of 4 and 5.

4.7. Antiplasmodial assay

Continuous in vitro cultures of asexual erythrocyte stages of chloroquine sensitive D10 strain and chloroquine resistant W2 strain of P. falciparum were maintained using a modified method of Trager and Jensen (1976), and quantitative assessment of antimalarial activity in vitro was determined via the parasite lactate dehydrogenase assay (Makler et al., 1993) as described in details elsewhere (Clarkson et al., 2003; Pillay et al., 2007).

4.8. Cytotoxicity assay

Quantitative assessment of cytotoxicity was assessed with an in vitro assay (Mossman, 1983; Rubinstein et al., 1990) using the Chinese Hamster Ovarian cell line as described previously (Clarkson et al., 2003; Pillay et al., 2007).

4.9. Compound 1


4.10. Compound 2

Vernangulide B [5(S),7R,8S,10S)-14-acetoxy-8-[2-hydroxymethylacylactyl]-15-heligna-1(10),4,11(13)-trien-15-α-d-6,12-olid] (2): $^1$H NMR and $^{13}$C NMR data see Table 1; HREIMS m/z 427.1442 [M+Na]$^+$ (calc. for $[^{12}C_2H_3O_2Na]^+$, 427.1363).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.02.005.

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