CHAPTER 3

Antiplasmodial Activity of *Vernonia staehelinoides*

3.1 *Vernonia staehelinoides* Harv.

Vernonia belongs to the botanical family Asteraceae and there are over 1000 different species of which approximately 50 species occur in Southern Africa.[1] *Vernonia* species have been used traditionally to treat rheumatism, dysentery, diabetes, jaundice as well as malaria.[2] Many *Vernonia* species have been studied chemically. Highly oxygenated germacranolides such as glaucolides and hirsutinolides seem to be characteristic for many members of this genus, though many other compounds have also been isolated.[3]

Vernonia staehelinoides, commonly known as "blouteebossie", is a multi-stemmed perennial shrublet. Stems and leaves are covered with numerous short hairs, giving it a greyish appearance. Leaves are very narrow and long flexuous stems give rise to numerous flower heads. It is indigenous to South Africa and grows on rocky ridges in summit grasslands predominantly in Gauteng as well as in parts of Mpumalanga and the Limpopo Province.

Figure 3.1 *Vernonia staehelinoides* growing at Klapperkop, Pretoria. Photo by Jean Meyer (SANBI)

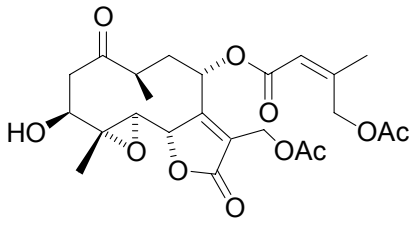
---

[1] http://www.plantzafrica.com/planttuv/vernonhirsut.htm

[2] B.E. van Wyk, B. van Oudtshoorn and N. Gericke, 'Medicinal Plants', Briza Publications, Pretoria, 2000, 268.

[3] F. Bohlmann, C. Zdero, R.M. King and H. Robinson, *Phytochemistry*, 1982, **21**, 695.

V. staehelinoides is reported to be used medicinally but no details have been specified.[4] Previous chemical investigations of this plant revealed that the roots contain squalene, stigmasterol and sitosterol while the aerial parts afforded caryophyllene, α-humulene, germacrene D and a novel glaucolide, 3β-hydroxy-stilpnotomentolide-8-*O*-(5-acetoxysenecioate) **(49)**.[5]



(49)

3.2 *In vitro* Antiplasmodial Activity of *V. staehelinoides* Extracts

A dichloromethane, a 1:1 dichloromethane/methanol and an aqueous extract were prepared from the leaves of *V. staehelinoides* (Table 3.1).

Table 3.1 Yield of extracts obtained from *V. staehelinoides* leaves

Extract code	Extract description	% Yield
P01009A	Dichloromethane	3.3
P01009B	Dichloromethane/Methanol (1:1)	3.8
P01009C	Water	1.3

The concentrated *V. staehelinoides* extracts were tested *in vitro* against a chloroquine-sensitive (D10) strain in duplicate (Table 3.2). Those extracts showing a D10 IC_{50} of less than 10 μg/ml were tested against the chloroquine-resistant (K1) strain of *P. falciparum*.

The antiplasmodial component in the leaves of *Vernonia staehelinoides* was concentrated in the organic extracts. P01009A and P01009B, were found to be significantly active against both strains of the parasite having 50% inhibitory concentrations (IC_{50}) values of less than 10μg/ml. The dichloromethane extract was to some extent more active than the dichloromethane/methanol (1:1) extract

[4] J.M. Watt and M.G. Breyer-Brandwyk, 'The Medicinal and Poisonous Plants of Southern Africa', 2nd Edition. Livingstone, London, 1962.
[5] F. Bohlmann, M. Wallmeyer and J. Jakupovic, *Phytochemistry*, 1982, **21**, 1445.

against both the D10 and K1 strains of *P. falciparum*. The aqueous extract, P01009C, was found to be relatively inactive.

Table 3.2 *In vitro* antiplasmodial activity of *V. staehelinoides* extracts

Extract	D10 (Experiment 1) IC_{50} (μg/ml)	D10 (Experiment 2) IC_{50} (μg/ml)	K1 IC_{50} (μg/ml)
P01009A	2.0	4.0	2.8
P01009B	3.0	9.0	4.5
P01009C	>10	>10	-

3.3 Bioassay-guided Fractionation of the *V. staehelinoides* Extracts

Bioassay-guided fractionation based on *in vitro* antiplasmodial activity against the D10 *P. falciparum* strain was used to identify the compounds from the organic extracts of *V. staehelinoides* which are responsible for the observed activity. Details of the purification techniques are given in Chapter 5 (Experimental).

3.3.1 Bioassay-guided Fractionation of P01009A

Figure 3.2 outlines the stepwise fractionation of the dichloromethane extract, P01009A, guided by observed antiplasmodial activity against the D10 *P. falciparum* strain. Enhanced antiplasmodial activity was observed for fractions 1C, 1D and 1E. Based on their similar TLC profiles and the yield of the three fractions, fraction 1D was selected for further purification. This process led to the identification (by TLC analysis) of two semi-pure compounds in fractions 2C and 2D with significant antiplasmodial activity (0.65 and 0.8 μg/ml, respectively). The two compounds gave distinctive colours (orange and brown) with vanillin spray reagent and were therefore easily discernible. Further purification of the two compounds for structure determination was impractical due to the low yields of fractions 2C and 2D.

3.3.2 Bioassay-guided Fractionation of P01009B

Figure 3.3 summarizes the stepwise fractionation of the 1:1 dichloromethane/ methanol extract, P01009B, which was guided by the observed antiplasmodial activity against the D10 *P. falciparum* strain. Increased antiplasmodial activity was

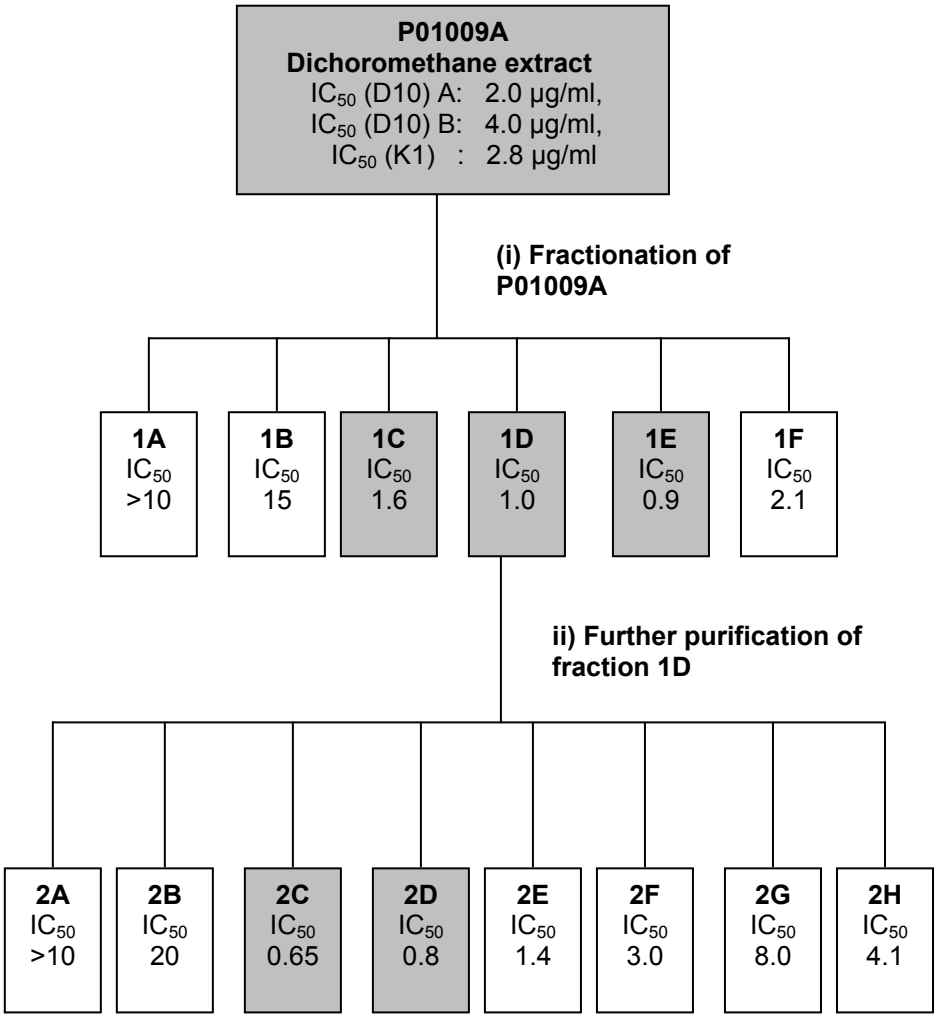


Figure 3.2 Fractionation of the dichloromethane extract of *V. staehelinoides.* IC_{50} values are in µg/ml.

observed in fractions 3D and 3E. Based on the similar TLC profiles and the yields of the two fractions, fraction 3E was selected for further purification. This resulted in a single fraction, 4C, with improved antiplasmodial activity. TLC analysis of fraction 4C revealed that it contained two major compounds: the same compounds identified in fractions 2C and 2D of the dichloromethane extract. Due to the low yield of fraction 4C, further purification was not feasible.

3.4 Targeted Purification of Active Compounds from *V. staehelinoides*

Once it was established that essentially the same two compounds were responsible for the antiplasmodial activity in both the dichloromethane (P01009A) and the 1:1 dichloromethane/methanol (P01009B) extracts of *V. staehelinoides*,

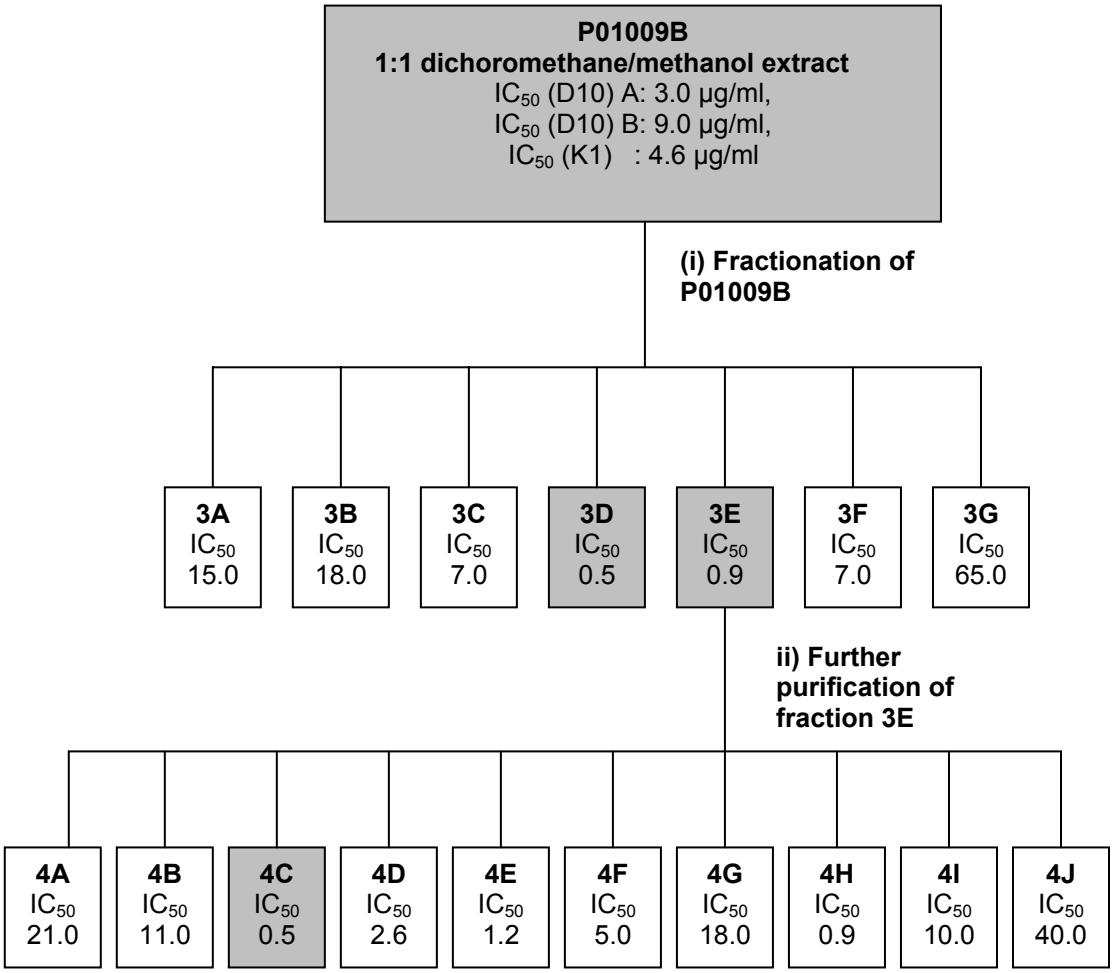


Figure 3.3 Fractionation of the dichloromethane/methanol (1:1) extract of *V. staehelinoides.* IC_{50} values are in µg/ml.

targeted isolation of the active compounds from P01009A was conducted. Liquid-liquid partitioning of the crude extract served to concentrate the active components in a less complex matrix. TLC analysis of the three fractions generated from liquid/liquid partitioning of the crude extract revealed that the target compounds were concentrated in the dichloromethane-soluble fraction, (5A). This fraction was subjected to a series of successive flash silica gel purifications to yield compounds **(50)** and **(51)** (Figure 3.4). This proved to be an effective and time economical method for the isolation of the active compounds.

The yield of **(50)** was 0.39% by weight of the dichloromethane extract and 0.01% by weight of the dried ground plant material. For **(51)** the yields were marginally higher; 0.56% and 0.02% respectively. The relatively low yields of the active compounds indicate that the antiplasmodial component was probably a minor

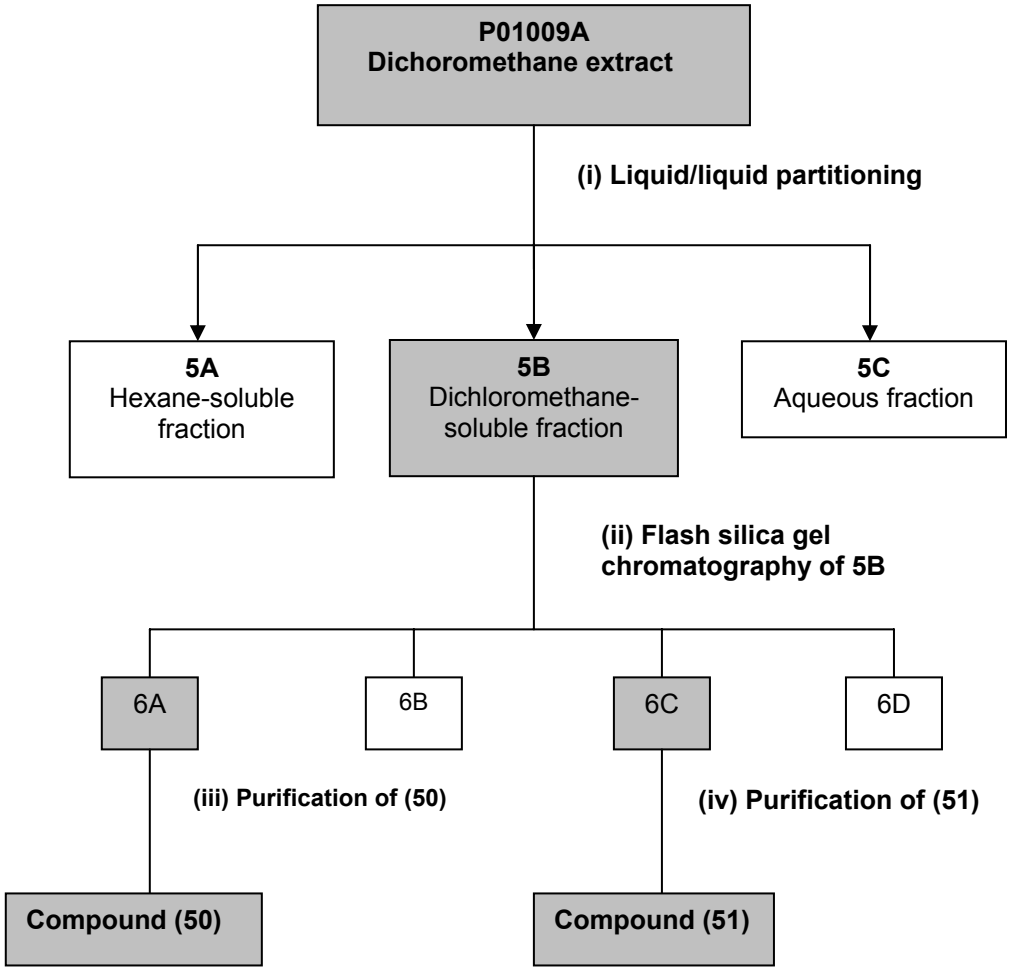


Figure 3.4 Targeted purification of compounds **(50)** and **(51)** from P01009A

constituent of the leaves of the plant. The low yields could also be attributed to the fact that compounds **(50)** and **(51)** are relatively unstable and showed evidence of decomposition during silica gel purification and on standing. The yields could possibly be improved to some extent by optimising the extraction and separation procedures. Despite the low yields, the quantities of **(50)** and **(51)** isolated were sufficient for structure elucidation and *in vitro* assaying.

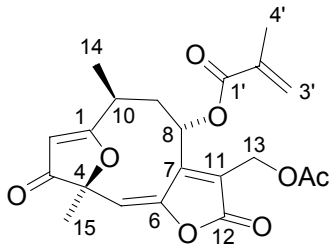
3.5 Identification and Characterization of Compounds (50) and (51) from *V. staehelinoides*

Compounds **(50)** and **(51)** were identified and characterized by mass spectrometry (HR EI-MS) and NMR experiments. The structural assignments of **(50)** and **(51)** are based on detailed studies of the high-field ^{1}H and ^{13}C NMR spectral data

(chemical shifts and coupling constants) and the following two-dimensional (2D) NMR techniques.[6,7]

The proton-proton connectivity patterns were established by 2D (^{1}H,^{1}H) correlation spectroscopy (COSY) experiments. The multiplicities of the different resonances in the ^{13}C spectra were deduced from the proton-decoupled CH, CH_2 and CH_3 subspectra obtained using the DEPT (distortionless enhancement by polarisation transfer) pulse sequence. The ^{13}C resonances were partly assigned by correlation of the proton-bearing carbon atoms with specific proton resonances in 2D (^{13}C,^{1}H) heteronuclear single quantum correlation (HSQC) experiments. The assignment of the quaternary carbon atoms and the deduction of the long-range (more than one bond) connectivity pattern was facilitated by 2D (^{13}C,^{1}H) heteronuclear multiple bond correlation (HMBC) experiments. Correlations observed in nuclear Overhauser effect spectroscopy (NOESY) experiments provided information on the relative stereochemistry of the compounds.

3.5.1 Structural Elucidation of 13-Acetoxy-1,4*β*-epoxy-8*α*-(2-methylpropenoyl)-3-oxo-1,5,7(11)-germacratrien-12,6-olide (50)



(50)

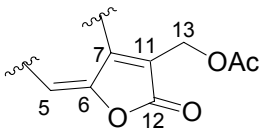
Compound **(50)** was isolated as a colourless gum and had $[\alpha]_D$ –77.4 (*c* 0.31, $CHCl_3$). The HR EI-MS did not show the molecular ion peak but showed a $[M–C_3H_5COOH]^+$ fragment at *m/z* 316.0868. The molecular formula of **(50)** was deduced to be $C_{21}H_{22}O_8$, corresponding to a molecular weight of 402 Dalton. The ^{1}H, ^{13}C, HSQC, HMBC, COSY and NOESY data are summarized in Table 3.3.

---

[6] W. R. Croasmun and R.M.K. Carlson, "Two Dimensional NMR Spectroscopy: Applications for Chemists and Biochemists", VCH Publishers, New York, 1987.

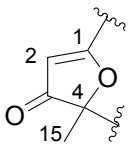
[7] J.M. Saunders and B.K. Hunter, "Modern NMR Spectroscopy. A Guide for Chemists", Oxford University Press, Oxford, 1987.

The ^{1}H NMR spectrum of (**50**) showed a pair of broad doublets (J 13.2 Hz) at δ_H 4.91 and 5.09, which were assigned to the C(13) (δ_C 55.65T) protons. The chemical shift of this AB system suggested that the C(13) protons were allylic and this was confirmed by their HMBC correlations to the olefinic carbons, C(7) (δ_C 151.12S) and C(11) (δ_C 132.66S). H(13) also showed an HMBC correlation to an ester carbonyl carbon atom at δ_C 170.15S, which in turn was correlated to a methyl signal in the acetate region (δ_H 2.03) indicating that there was an *O*-acetate group at C(13). The HMBC correlation of H(13) with another ester carbonyl, C(12) (δ_C 166.14S), as well as the correlation between C(7) and the C(5) (δ_C 117.89D) olefinic proton at δ_H 5.89 which was in turn correlated to the olefinic carbon atom C(6) (δ_C 146.19S), established the presence of an enol 2(5H)-furanone moiety **(50 i)** in the compound.



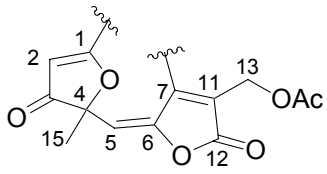
50 i

The methyl resonance at δ_H 1.58, assigned to H(15), correlated with the signal at δ_C 86.90S, assigned to an oxygen-substituted quaternary carbon, in the HMBC spectrum and placed the methyl group at C(4). H(15) also showed an HMBC correlation with a ketone carbonyl carbon at δ_C 202.27S, which was subsequently identified as C(3). The correlation of an olefinic proton signal at δ_H 5.40, H(2), with C(3) and an oxygen-bearing olefinic carbon, C(1), at δ_C 194.93S, revealed the presence of a dihydrofuran-4-one moiety **(50 ii)** formed by an oxygen bridge between C(1) and C(4).



50 ii

The two furanone moieties **(50 i)** and **(50 ii)** were subsequently linked by the long-range (HMBC) correlations between the C(5) and H(15) signals to generate the fragment **(50 iii)**.



50 iii

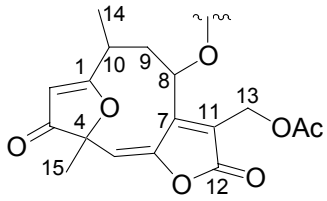
The signal for C(1) showed HMBC correlations to a proton resonating at δ_H 3.01, identified as H(10), as well as to a methyl signal resonating at δ_H 1.26, H(14). The methyl group, H(14), was subsequently located at C(10) (δ_C 31.31D). HMBC correlations also linked C(1) with two proton signals at δ_H 2.77 and 1.70, which were assigned to the C(9) (δ_C 40.23T) methylene protons. The (^{1}H,^{1}H) COSY spectrum showed cross peaks between H(14) and H(10), as well as between H(10) and H(9), supporting the observed multiplicities of H(14) as a doublet ($J_{10,14}$ 7.0 Hz) and H(10) as a multiplet (ddq) signal ($J_{10,14}$ 7.0 Hz, $J_{10,9a}$ 5.1 Hz $J_{10,9b}$ 11.0 Hz).

The chemical shift value for H(8), a broad doublet resonating at δ_H 6.45, suggested that this proton is dehielded by the neighbouring double bond π-system. In addition the C(8) signal appeared at δ_C 65.70D, and represents an oxygen-bearing carbon atom. The C(8) proton displayed a coupling (J 9.0 Hz) only with H(9a) (δ_H 2.77 ddd, $J_{9a,10}$ 5.1, $J_{8,9a}$ 9.0, $J_{9a,9b}$ 14.9 Hz). The dihedral angle between H(8) and H(9b) obtained from a molecular model was close to 90°, and based on the Karplus equation[8] explains the absence of vicinal coupling with H(9b). This absence of coupling was confirmed by the COSY correlation of H(8) only with H(9a) and by the observed multiplicities of H(9a) and H(9b) (δ_H 1.70 dd, $J_{9b,10}$ 11.0, $J_{9a.9b}$ 14.9 Hz). Geminal coupling between H-9a and H-9b (J 14.9 Hz) was also observed.

The HMBC data linking C(7) and C(8) with H(9a), together with the above corroborations and published data,[9,10] led to the deduction that a sesquiterpene

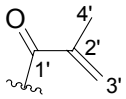
[8] R.J. Abraham, " Introduction to NMR spectroscopy.", John Wiley & Sons, Essex, 1988.
[9] S. Borkosky, A. Bardón, César, A.N. Catalán, J.G. Díaz and W. Herz, *Phytochemistry*, 1997, **44**, 465.
[10] F. Bohlmann, G. Brindöpke and R.C. Rastogi, *Phytochemistry*, 1978, **17**, 475.

lactone skeleton such as **(50 iv)**, commonly isolated from *Vernonia* spp. and named as a hirsutinolide type sesquiterpene, was present.



50 iv

The unaccounted ^{13}C and ^{1}H resonances were assigned to a 2-methylpropenoyl moiety **(50 v)** located at C(8). This was demonstrated by the HMBC correlation between the pair of broad signals at δ_H 5.63 and 6.22, identified as H(3′), and the signals for a carbonyl carbon atom (δ_C 166.90S, C(1′)), an olefinic quaternary carbon (δ_C 135.67S, C(2′)) and a methyl carbon atom at δ_C 18.12Q, identified as C(4′). These data were supported by the observed multiplicities and COSY correlations between the C(3′) and C(4′) protons. H(3′a) (δ_H 5.63) was observed as a doublet of quartets and showed a geminal coupling (J 1.6 Hz) to H(3′b) (δ_H 6.22) typical of a terminal methylene group, and allylic coupling (J 1.6 Hz) to H(4′). Allylic coupling (J 1.0 Hz) was also observed between H(3′b) and H(4′).



50 v

Inspection of a molecular model and the observed proton-proton couplings indicated that the ester group at C(8) was probably α-orientated. The stereochemistry at C(10) and C(4) followed from a molecular model and the observed NOESY correlations between H(2) and H(14) and that between H(5) and H(15). Thus compound **(50)** was identified as 13-acetoxy-1,4*β*-epoxy-8*α*-(2-methylpropenoyl)-3-oxo-1,5,7(11)-germacratrien-12,6-olide.

A search of the chemical literature revealed that **(50)** is a known compound, previously isolated from *Vernonia poskeana* and referred to as 8*α*-(2-methylacryl-

oyloxy)-3-oxo-1-desoxy-1,2-dehydrohirsutinolide-13-*O*-acetate.[11] The ^{1}H NMR data compared favourably with published data.[11]

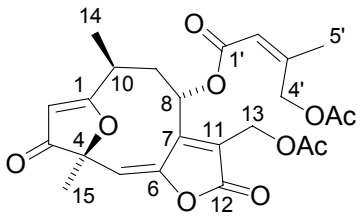
Table 3.3 ^{1}H and ^{13}C NMR data (in $CDCl_3$) for 13-acetoxy-1,4*β*-epoxy-8*α*-(2-methylpropenoyl)-3-oxo-1,5,7(11)-germacratrien-12,6-olide **(50)**

Atom	^{1}H δ_H (J in Hz)	^{13}C δ_C	HMBC ^{13}C↔^{1}H	COSY ^{1}H↔^{1}H	NOESY ^{1}H↔^{1}H
1		194.93 S	H(14),H(9a),H(10), H(2)		
2	5.40 (s)	98.28 D			H(14)
3		202.27 S	H(15), H(2)		
4		86.90 S	H(15), H(2), H(5)		
5	5.89 (s)	117.89 D	H(15)		H(15)
6		146.19 S	H(15), H(5)		
7		151.12 S	H(9a), H(13), H(5)		
8	6.45 (br d) (J 9.0)	65.70 D	H(9a)	H(9a)	H(9a),H(9b). H(13a),H(13b)
9	H(9a) 2.77 (ddd) (J 5.1, 9.0, 14.9) H(9b) 1.70 (dd) (J 11.0, 14.9)	40.23 T	H(14)	H(10), H(8), H(9b) H(10), H(9a)	H(14),H(9b), H(10), H(8) H(14); H(9a); H(8)
10	3.01 (ddq) (J 5.1, 11.0, 7.0)	31.31 D	H(14), H(9a)	H(9a), H(9b), H(14)	H(14), H(9a)
11		132.66 S	H(13)		
12		166.14 S	H(13)		
13	H(13a) 4.91 (d) (J 13.2) H(13b) 5.09 (d) (J 13.2)	55.65 T	13-OCOC**H**$_3$	H(13b) H(13a)	H(13b), H(8) H(13a), H(8)
14	1.26 (d) (J 7.0)	15.52 Q	H(9a), H(10)	H(10)	H(9a), H(9b), H(10), H(2)
15	1.58 (s)	20.68 Q	H(5)		H(5)
1′		166.90 S	H(4′); H(3′)		
2′		135.67 S	H(4′); H(3′)		
3′	H(3′a) 5.63 (dq) (J 1.6,1.6)	126.98 T	H(4')	H(3′b), H(4′)	H(3′b)

[11] F. Bohlmann, N. Ates (Gören) and J. Jakupovic, *Phytochemistry*, 1983, **22**, 1159.

	H(3′b) 6.22 (br dq) (J 1.6; 1.0)			H(3′a); H(4′)	H(3′a)
4′	1.93 (dd) (J 1.6,1.0)	18.12 Q	H(3′)	H(3′)	H(3′a)
13-*O*-**C**OCH$_3$		170.15 S	13-OCOC**H**$_3$); H(13)		
13-*O*-CO**CH**$_3$	2.03 (s)	20.68 Q			

3.5.2 Structural Elucidation of 13-Acetoxy-8*α*-(4-acetoxy-3-methyl-2*Z*-buten-oyl)-1,4*β*-epoxy-3-oxo-1,5,7(11)-germacratrien-12,6-olide (51)



(51)

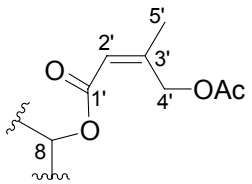
Compound **(51)** {[α]$_D$ -209.8 (*c* 0.51, $CHCl_3$)} was isolated as a colourless gum. The EI-MS spectrum of **(51),** as in the case of **(50)**, did not show a molecular ion peak but rather showed a fragment at *m/z* 316.0885, corresponding to the loss of a $C_7H_{10}O_4$ fragment *i.e.* 4-acetoxy-3-methyl-2*Z*-butenoic acid, from the molecular ion peak. The molecular formula of **(51)** was established as $C_{24}H_{26}O_{10}$ and corresponded to a molecular weight of 474 Dalton. The ^{1}H, ^{13}C, HSQC, HMBC, COSY and NOESY data of this compound are summarized in Table 3.4.

The ^{1}H NMR spectrum of compound **(51)** closely resembled that of compound **(50)**. The most obvious differences were the additional methyl signal in the acetate region (δ_H 2.05) and the additional olefinic proton at δ_H 5.75 in the ^{1}H spectrum of **(51)**. A thorough investigation of the ^{1}H, ^{13}C, HSQC, HMBC, COSY and NOESY experiments performed on this compound showed that the main skeleton was identical to that of **(50)** and that only the ester group at C(8) was different.

The olefinic proton H(2′) (δ_H 5.75) appeared as a broad multiplet and showed HMBC correlations to a broad methyl signal (δ_H 1.91) assigned as H(5′) and to a

pair of broad doublets (J 15.5 Hz) resonating at δ_H 5.12 and δ_H 5.22. Closer inspection of the pair of doublets, identified as H(4′), revealed that it was not an ideal AB system, and each signal was a multiplet due to allylic coupling (J ~1Hz) with H(5′) and H(2′). This was supported by cross peaks observed in the COSY spectrum.

An HMBC correlation between H(4′) with a carbonyl carbon atom at δ_C 170.54, which in turn correlated to the methyl signal at δ_H 2.05, revealed that there was an acetate at C(4′). This led to the conclusion that there was a 4-acetoxy-3-methyl-2-butenoyl moiety attached to C(8) as shown in **(51 i)**.



51 i

The *Z* configuration of the 2′ double bond followed from the strong NOE observed between H(2′) and H(5′). This was confirmed by comparison of the H(4′) chemical shift (δ_H 5.17) with published data for methyl *Z*- and *E*-4-acetoxy-3-methyl-2-butenoates: the *Z*-CH_2 signals were shown to occur at δ_H 5.15 whereas the *E*-CH_2 signals were observed at δ_H 4.56.[12]

Compound **(51)** was previously isolated from *V. poskeana*[11]and *V. erinacea*.[12] A comparison of the data for (**51**) with the published ^{1}H NMR data[11] revealed that the authors had transposed the H-13 and H-4' resonances. In this account an unequivocal assignment of these resonances based on 2D NMR data, which the original reporters did not have access to, is reported. All other data compared favourably, indicating that **(51)** was identical to 8α-(5'-acetoxysenecioyloxy)-3-oxo-1-desoxy-1,2-dehydrohirsutinolide-13-*O*-acetate.

[12] L.E. Tully, M.S. Carson and T.B.H. McMurray, *Tetrahedron Lett.*, 1987, **28**, 5925.

Table 3.4 ^{1}H and ^{13}C NMR data (in $CDCl_3$) for 13-acetoxy-8*α*-(4-acetoxy-3-methyl-2*Z*-butenoyl)-1,4*β*-epoxy-3-oxo-1,5,7(11)-germacratrien-12,6-olide **(51)**

Atom	^{1}H δ_H (J in Hz)	^{13}C δ_C	HMBC $^{13}C \leftrightarrow {}^{1}H$	COSY $^{1}H \leftrightarrow {}^{1}H$	NOESY $^{1}H \leftrightarrow {}^{1}H$
1		194.92 S	H(14), H(9a)		
2	5.43 (s)	99.93 D			H(14)
3		202.16 S	H(15)		
4		86.93 S	H(15), H(5)		
5	5.88 (s)	117.35 D	H(15)		H(15)
6		146.19 S	H(15), H(5)		
7		151.19 S	H(9a), H(13), H(5)		
8	6.23 (br d) (J 8.0)	65.61 D	H(9a)	H(9a)	H(9a),H(9b), H(13)
9	H(9a) 2.75 (ddd) (J 5.5, 8.0, 14.7) H(9b) 1.70 (dd) (J 9.0, 14.7)	40.09 T	H(14), H(10)	H(10), H(8), H(9b) H(10), H(9a)	H(14),H(9b), H(10), H(8) H(14), H(9a), H(8)
10	3.01 (ddq) (J 5.5; 9.0; 7.0)	31.92 D	H(14), H(9a)	H(9a), H(9b), H(14)	H(14), H(9a)
11		131.79 S	H(13a)		
12		166.23 S	H(13)		
13	H(13a) 4.93 (d) (J 13.1) H(13b) 5.02 (d) (J 13.1)	55.79 T	13-OCOC**H**$_3$	H(13b) H-13a	H(13b), H(8) H(13a), H(8)
14	1.25 (d) (J 7.0)	15.38 Q	H(9a), H(10)	H(10)	H(9a),H(9b), H(10), H(2)
15	1.59 (s)	20.64 Q	H(5)		H(5)
1′		164.04 S			
2′	5.75 (br m*)	116.49 D	H(5′), H(4′)	H(4′), H(5′)	H(5′)
3′		156.77 S	H(5′), H(4′)		
4′	H(4′a) 5.12 (br dm*) (J 15.5) H(4′b) 5.22 (br dm*) (J 15.5)	63.60 T	H(5′), H(2′)	H(4′b), H(2′); H(5′) H(4′a), H(2′); H(5′)	H(5′)
5′	1.91 (br m*)	21.48 Q	H(4′)	H(2′), H(4′)	H(2′)

13-*O*-**C**OCH_3		170.18 S	13-OCOC**H**$_3$, H(13)		
13-*O*-CO**CH**$_3$	2.03 (s)	20.69 Q			
4′-O **C**OCH_3		170.54 S	4′-OCOC**H**$_3$, H(4′)		
4′-*O*-CO**CH**$_3$	2.05 (s)	20.69 Q			

* allylic coupling of ~1Hz

3.5.3 Characterization and Biosynthesis of Compounds (50) and (51)

Although **(50)** and **(51)** have been isolated previously from other South African *Vernonia* species,[11,12] this is the first report on the isolation of these compounds from *V. staehelinoides*. Compounds **(50)** and **(51)** fall into a class of sesquiterpene lactones which are typically isolated from *Vernonia* species and named as hirsutinolides.[9,10]

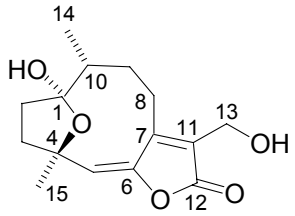
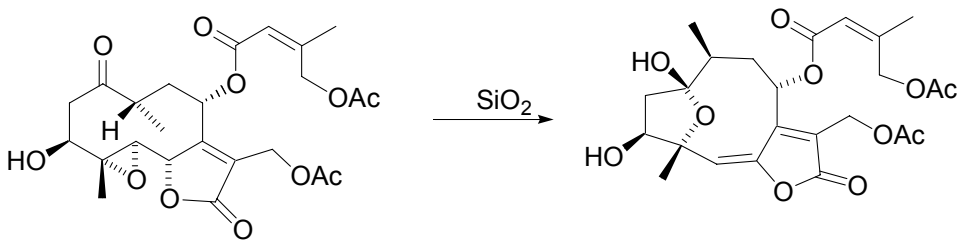


Figure 3.5 Basic hirsutinolide skeleton

Thus **(50)** and **(51)** are ketohirsutinolides in which the C(8) hydroxyl group is utilized for ester formation with two different acids. As plant metabolites often exist as part of a family of related compounds it is likely that other less active analogues and precursors were also present in the crude extract. In order to isolate sufficient material for structural characterization the focus in this investigation remained on **(50)** and **(51)** as these compounds showed the greatest antiplasmodial activity.

It has been suggested that hirsutinolides are not natural products but are rather artifacts formed from glaucolides on exposure to slightly acidic silica gel during chromatography.[9,12] Thus the glaucolide **(49)** could be chemically transformed into the hirsutinolide **(52)** by treatment with slightly acidic silica gel as depicted in Figure 3.6.[5]



(49) **(52)**

Figure 3.6 Silica gel induced transformation of glaucolide **(49)** to hirsutinolide **(52)**

Contrary to this suggestion, **(50)** and **(51)** were easily detected through their characteristic colours and R_f values by TLC analysis of the dichloromethane-soluble fraction (5B) following liquid/liquid partitioning of the crude dichloro-methane extract. This detection prior to any silica gel chromatography suggests that **(50)** and **(51)** were originally present in the dichloromethane extract of *V. staehelinoides*.

The demonstrated transformation of glaucolide **(49)** into hirsultinolide **(52)** provides some insight into the biosynthesis of compounds **(50)** and **(51)**. Glaucolide **(49)** was previously isolated from the roots of *V. staehelinoides* and hirsutinolide **(52)** can be considered a precursor of compound **(51)**. Based on this and literature precedent a biosynthetic pathway to the formation of compounds **(50)** and **(51)** is postulated in Figure 3.7. In recognising compounds **(50)** and **(51)** as 6,7-germacranolide derivatives it is safe to assume that they are formed from the most elementary germacrene sesquiterpene lactone, costunolide **(25)**. Epoxidation of the 1,10 and 4,5 double bonds of **(25)** and hydroxylation at the C(7) allylic position would yield the diepoxide **(53)**. Subsequent rearrangement and esterifications of **(53)** would result in the formation of **(54)**. Selective hydrolysis of the 1,10 epoxide would afford **(55)**, which could dehydrate to give **(56)**. Hydrolysis at the C(3) position of **(56)** would yield the glaucolide **(57)**. This glaucolide could then, as demonstrated, be transformed into hirsutinolide **(58)**. An ensuing dehydration at C(1) and oxidation of the C(3) hydroxyl would yield compound **(50)** or compound **(51).**

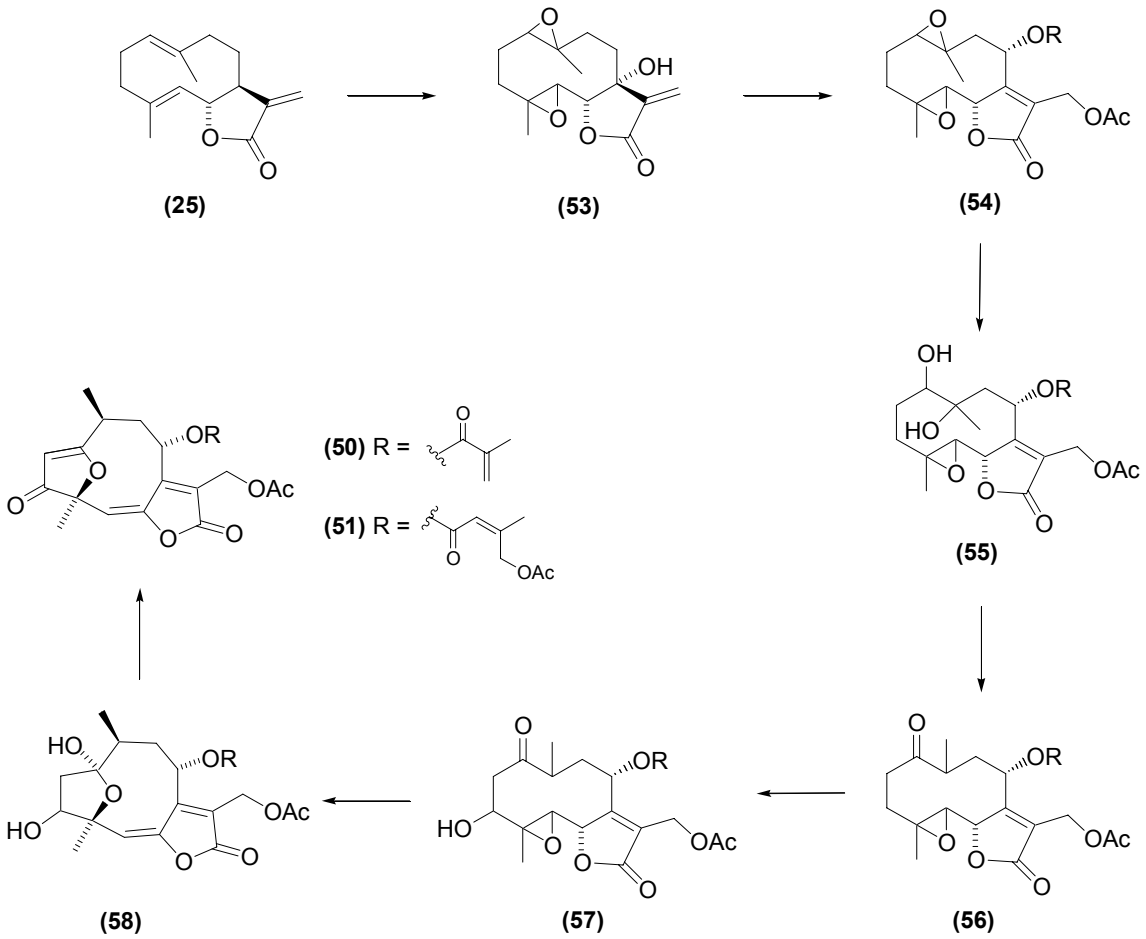


Figure 3.7 Postulated biosynthesis of compounds **(50)** and **(51)**.

3.6 *In vitro* Antiplasmodial Activity and Cytotoxicity of Compounds (50) and (51)

Compounds **(50)** and **(51)** were tested for *in vitro* antiplasmodial activity against the D10 and K1 *P. falciparum* strains. The sensitivity of the two strains to chloroquine **(2)** was also evaluated. The corresponding IC_{50} values are listed in Table 3.5.

Table 3.5 *In vitro* antiplasmodial activity of chloroquine and **(50)** and **(51)**

Tested sample	**D10 IC_{50} (μg/ml)**	**K1 IC_{50} (μg/ml)**	**RI***
Chloroquine	11.83×10^{-3}	181.76×10^{-3}	15.36
Compound **(50)**	0.26	1.80	6.92
Compound **(51)**	0.24	2.60	10.83

* RI = K_1 IC_{50} / D_{10} IC_{50}

There are no previous reports of **(50)** and **(51)** having been investigated for any biological activity. In this account the compounds are demonstrated to have *in vitro* antiplasmodial activity against two strains of *P. falciparum*. The difference in their C(8) ester groups does not appear to significantly influence the observed antiplasmodial activities of **(50)** and **(51)** as they were equipotent against the chloroquine sensitive D10 strain. There was a marginal difference in their observed activity against the chloroquine resistant K1 strain.

Both compounds, like chloroquine, showed greater activity against the D10 strain than against the K1 strain as indicated by their resistance index values being greater than 1. Compounds **(50)** and **(51)** are, however, in all respects significantly less active than chloroquine against both strains. They were found to be more than 20 times less active than chloroquine against the D10 strain. The difference in activity between chloroquine and **(50)** against the K_1 strain was 10 fold and for **(51)** this difference was approximately 14 fold.

The *in vitro* cytotoxicity of the compounds was determined against Chinese Hamster Ovarian (CHO) cells using the MTT assay. The IC_{50} values of the positive control (Emetine), chloroquine and compounds **(50)** and **(51)** are summarised in Table 3.6.

Table 3.6 *In vitro* cytotoxicity results

Tested sample	**CHO IC_{50} (μg/ml)**
Emetine	0.03
Chloroquine	18.53
Compound **(50)**	2.89
Compound **(51)**	0.97

Although both compounds had significantly higher IC_{50} values than Emetine, they can still be considered as cytotoxic to CHO cells. This data suggests that **(50)** and **(51)** were cytotoxic to mammalian cells at similar concentrations, indicating that the observed antiplasmodial activity could be due to general toxicity. Further

investigation would be required to confirm this but the fact that **(50)** and **(51)** have similar IC_{50}'s against the D10 strain but show a notable difference in their activity against the K1 strain, suggests that there may be more than general cytotoxicity coming into play.

The apparent non-selectivity of **(50)** and **(51)** is emphasised by the selectivity index values for the two compounds, summarised in Table 3.7. The selectivity index (SI) is a ratio of cytotoxicity to antiplasmodial activity and gives a general indication of the specific activity.

Table 3.7 Selectivity index values for chloroquine and compounds **(50)** and **(51)**

Tested sample	**SI***
Chloroquine	1566
Compound **(50)**	11.12
Compound **(51)**	4.04

* SI = cytotoxicity CHO IC_{50} / antiplasmodial D_{10} IC_{50}

The SI value for **(50)** indicates that it is approximately 11 times more potent to the parasites than to mammalian cells. For **(51)** this ratio is of the order of 4, indicating less selectivity.

In considering a recent publication[13] outlining criteria for antiparasitic drug discovery, a compound can be considered a hit if it is:

- Active *in vitro* against whole protozoa with an IC_{50} of ≤ 1 µg/ml
- Selective (at least tenfold more active against the parasite than against a mammalian cell line)

Based on these criteria and taking into account the activity of the compounds against the D10 *P. falciparum* strain, **(50)** can be considered a hit. Compound **(51)** was not sufficiently selective to kill the parasites without damaging mammalian cells.

[13] R. Pink, A. Hudson, M. Mouriès and M. Bendig, *Nature Rev. Drug Discov.*, 2005, **4**, 727.

3.7 Conclusion and Research Prospects

Although *Vernonia staehelinoides* is used traditionally to treat malaria and compounds showing significant *in vitro* antiplasmodial activity have been isolated from extracts of the plant; the active components, **(50)** and **(51)**, cannot be considered as viable antimalarial drugs – as their activity does not compare to that of chloroquine and **(51)** is non-selective (SI 4) while **(50)** showed only limited selectivity (SI 11). Ideally, for an effective therapeutic window, selectivity index values should be greater than 100 - the SI value for chloroquine is in excess of 1500.

The hirsutinolides could, however, prove to be attractive scaffolds for chemical modification with the view of exploring structure-activity relationships. Medicinal chemistry approaches could be used to modify the natural product core structure so as to optimise the activity of these compounds and reduce their cytotoxicity, generating lead compounds for antimalarial drug development. The hirsutinolide skeleton could be modified through hydrogenation of the double bonds and hydrolysis of the ester groups. Alternatively potential pharmacaphores or substructures which may be responsible for the observed antiplasmodial activity, can be used as scaffolds for new derivatives. The 2(5H)-furanone and dihydrofuran-4-one (Figure 3.8) are two such privileged substructures identified for derivatisation and antimalarial drug lead development. This research prospect has been taken up by the University of Cape Town, Department of Chemistry.

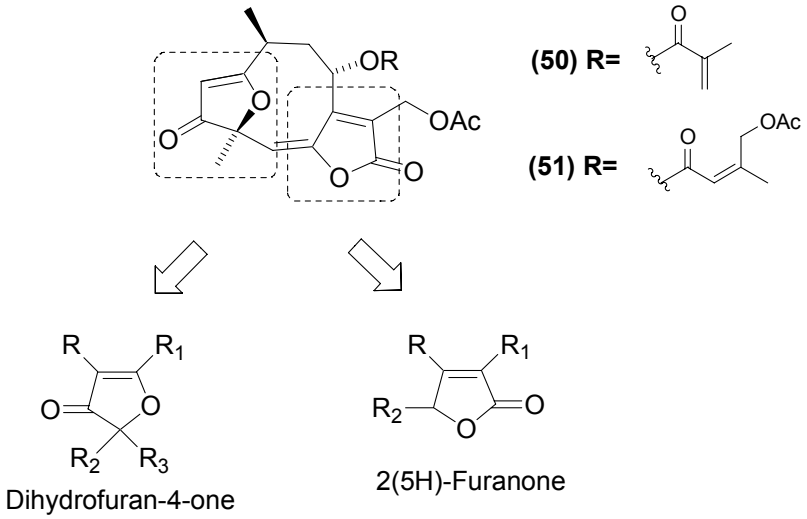


Figure 3.8 Potential pharmacophores in compounds **(50)** and **(51)**