Potential Anticancer Agents. Part 13.[1] Cytotoxic Constituents of *Acanthospermum glabratum* (Asteraceae)

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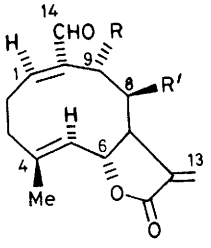
The Tanzanian plant *Acanthospermum glabratum* (DC.) Wild (Asteraceae) has yielded nine cytotoxic compounds, seven of which are new sesquiterpene lactones in the melampolide series. Structures of the new compounds were assigned based on spectra comparison with acantholide (3), a compound whose structure was determined by *X*-ray analysis. New compounds isolated in addition to acantholide (3), were acanthamolide (12), acanthospermolide (1), glabratolide (5), 9-hydroxyglabratolide (6), acanthoglabrolide (7), and dihydroacanthospermal A (10), and the known compounds acanthospermal A (8) and 3,6-dimethoxy-4',5,7-trihydroxyflavone (15).

In a continuing search for novel anticancer principles from higher plants, the Tanzanian plant *Acanthospermum glabratum* (DC.) Wild, a member of the subtribe Melampodiinae, tribe Heliantheae in the family Asteraceae, exhibited anticancer activity both *in vitro* and *in vivo*.[2] Separation of the constituents with coincidental *in vitro* bioassay of all appropriate fractions led to the isolation of nine constituents displaying cytotoxic activity. Some aspects of this work have been published [3-4] or described [6] previously.

RESULTS AND DISCUSSION

Initial work had demonstrated that antitumour activity was present in a 50% aqueous ethanol extract of the whole plant of *A. glabratum*, and after a re-collection had been obtained, this activity was found to be concentrated in the chloroform-soluble fraction after partition of a benzene extract of the plant. Chromatographic separation of the complex chloroform fraction on silica gel afforded the biologically active isolates described here.

Acanthospermolide (1).—Acanthospermolide crystallized from benzene–methanol, and from high-resolution mass spectrometry a molecular formula of $C_{20}H_{26}O_6$ was deduced. The carbonyl region of the i.r. spectrum was particularly complex and indicated the presence of an α,β-unsaturated lactone ($\nu_{max.}$ 1 776 cm^{-1}), a saturated ester ($\nu_{max.}$ 1 750 cm^{-1}), and an α,β-unsaturated aldehyde or ketone ($\nu_{max.}$ 1 690 cm^{-1}). No hydroxy-absorption was observed and acanthospermolide did not react with acetic anhydride in pyridine.



	R	R′	Others
(1)	OMe	$OCOCHMe_2$	
(2)	OMe	$OCOCHMe_2$	no 4,5-stereochemistry
(3)	$OCOCHMe_2$	OH	
(4)	$OCOCHMe_2$	OH	no 4,5-stereochemistry
(5)	H	$OCOCHMe_2$	
(6)	OH	$OCOCHMe_2$	
(7)	$OCOCHMe_2$	$OCOCHMe_2$	
(8)	$OCOCOHMe_2$	$OCOCHMe_2$	
(9)	$OCOCHMe_2$	$OCOCOHMe_2$	
(12)	$NHCOCHMe_2$	OH	
(16)	OCOMe	OCOMe	
(17)	$NHCOCHMe_2$	OCOMe	

The ^1H n.m.r. spectrum (Table 1) confirmed the presence of an α-methylene lactone with doublets (*J* 3.2 Hz) at δ 5.869 and 6.085 for H-13a and H-13b, respectively. An α-substituted α,β-unsaturated aldehyde was determined from an aldehyde signal at δ 9.40, and a complex, deshielded olefinic proton at δ 6.930 and the chemical shift of the aldehyde group suggested a *cis*-geometry for this functional moiety.[7] Three other signals were readily interpretable, a three-proton singlet at δ 3.128 for an aliphatic methoxy-group, a broad three-proton singlet at δ 1.938 for an olefinic methyl group, and a six-proton doublet at δ 1.149 attributed to two methyl groups on an isobutyrate residue. A loss of 71 Daltons from the molecular ion analysing for C_4H_7O ($OCCHMe_2$) substantiated this. In this way, all the oxygen atoms and seven of the eight degrees of unsaturation deduced from the molecular formula could be accounted for, and it was reasoned that the final unit of unsaturation was an alicyclic ring.

Biogenetic considerations led to an evaluation of the structure in terms of a germacradienolide in the melampolide series of compounds which typically possess a *cis*-1,10 double bond. The problem then centred on the placement of the remaining functional groups, methoxy and isobutoxy, and determination of the stereochemistry at all the appropriate positions.

The C-1 proton (δ 6.930) appeared as a broad doublet of doublets (*J* 11.0 and 6.4 Hz) indicating that it was adjacent to a methylene group and therefore that C-2 was unsubstituted. A pair of vicinally coupled doublets of doublets at 3.540 (*J* 8.8 and 2.4 Hz) and 6.533 (*J* 8.8 and 1.6 Hz) could be assigned to 8-H and 9-H, and since the aldehyde proton (δ 9.40) appeared as a doublet (*J* 2.4 Hz), the signal at δ 3.540 could be assigned to the 9-H. An examination of Dreiding models indicated that a W-spatial relationship was possible only when 9-H was β to the aldehyde group at C-10. This same spatial array also permits maximum π-orbital overlap of the

TABLE 1

^{1}H n.m.r. data of the melampolides of *Acanthospermum glabratum* [a]

Proton	Compound (1)	(3)	(3) [b]	(5)	(6)	(7)	(8)	(10)	(12) [c]	(16)	(17)
H-1	6.93 dd (6.4, 11.0)	6.74 dd (6.8, 10.4)	6.735 dd (6.8, 10.4)	6.62 bd dd	6.93 dd (6.4, 11.0)	6.766 dd	6.815 dd (8.6, 9.6)	5.670 dd (8.6, 10.8)	6.602 dd (6.9, 10.1)	6.68 dd (7.7, 10.6)	6.634 dd (6.8, 10.3)
H-5	5.009 m		4.871 d (10.4)	5.111 m	5.008 m	5.029 m	5.217 m	5.089 m	4.803 m	5.02 m	5.072 m
H-6	5.009 m		5.195 dd (10.1, 10.4)	5.111 m	5.008 m	5.029 m	5.217 m	5.089 m	4.803 m	5.02 m	5.072 m
H-8	6.533 dd (1.6, 8.8)		5.305 dd (1.75, 8.6)	6.32 ddd (1.6, 4.6, 8.3)	6.372 dd (1.6, 8.6)	6.694 dd (1.75, 8.6)	6.670 dd (1.4, 8.2)	6.142 dd (1.6, 8.2)	5.136 dd (2.2, 8.6)	6.65 dd (1.6, 9.0)	6.092 dd (2.1, 9.3)
H-9	3.54 dd (2.4, 8.8)		5.028 dd (1.8, 8.6)		3.918 dd (2.0, 8.6)	5.223 dd (1.8, 8.6)	5.264 dd	5.294 d (9.2)	4.407 dd (1.86, 8.6)	5.24 dd (1.75, 8.9)	4.666 dd (1.3, 9.2)
H-13a	5.869 d (3.2)	5.64 d (3.0)	5.643 d (3.0)	5.362 d (3.1)	5.639 d (3.1)	5.765 d (3.1)	5.710 d (3.0)	5.610 d (3.5)	5.888 d (3.2)	5.72 d (3.1)	5.585 d (3.1)
H-13b	6.085 d (3.2)	6.33 d (3.3)	6.333 d (3.5)	6.186 d (3.5)	6.237 d (3.6)	6.249 d (3.4)	6.297 d (3.4)	6.208 d (3.3)	6.221 d (3.6)	6.21 d (3.1)	6.291 d (3.6)
H-14	9.40 d (2.4)	9.41 d (1.8)	9.407 d (1.8)	9.43 d (1.4)	9.43 d (2.1)	9.518 d (1.8)	9.467 d (1.9)	4.225 AB (12)	9.351 d (1.9)	9.46 d (1.6)	9.417 d (1.3)
H-15	1.938 bs	1.97 bs	1.969 bs	1.933 bs	1.913 bs	2.007 bs	2.008 d (0.8)	1.979 bs	1.991 bs	1.97 bs	2.02 bs
COCHMe_2	1.149 d (7.0)	1.10 d (7.0) 1.15 d (7.0)	1.103 d (7.0) 1.149 d (7.0)	1.118 d (6.9) 1.137 d (6.9)	1.148 d (6.9) 1.175 d (6.9)	1.057 d (6.8) 1.087 d (6.8)	1.094 d (6.9)	1.142 d (6.8)	1.005 d (6.8) 1.089 d (6.8)		1.053 d (6.7) 1.183 d (6.7)
COCOHMe_2							1.288, 1.318 s	1.226, 1.340 s			
COMe										2.02 s	
OMe	3.128 s										

[a] Except where indicated, spectra were recorded at 60 MHz in $CDCl_3$ using $SiMe_4$ as an internal standard. Chemical shifts are given in p.p.m. relative from $SiMe_4$. Multiplicities are given as s = singlet, d = doublet, dd = doublet of doublets, AB = centre of AB system, m = multiplet. Figures in parentheses are coupling constants in Hz. [b] At 270 MHz in $CDCl_3$. [c] In [$^2H_4$]MeOH.

α,β-unsaturated aldehyde system. From the chemical shift of 9-H it was apparent that the methoxy-group was located at this position, and the isobutoxy-group at C-8.

The substantial coupling (*J* 8.8 Hz) of the C-9 proton with 8-H suggests a large dihedral angle (see below) between these protons and consequently an α-orientation for 8-H. Supporting evidence for this stereochemical assignment was deduced from the small (*J* 2.2 Hz) coupling of 8-H and 7-H, an arrangement possible only if 7-H and 8-H are *cis* and α.

The stereochemistry of the 7,11-bond in all well characterized sesquiterpene lactones is β, and since melampolides also obey Samek's rule [8] the magnitude of the coupling (*J* 3.2 Hz) of 7-H with the C-13 *exo*-methylene protons indicated a *trans*-fused lactone, placing the 6-H in a β-stereochemistry. At this point the structure of acanthospermolide could therefore be written as (2) in which the stereochemistry of the 4,5-double bond remained to be deduced.

The multiplicity of H-6 and H-5 and their coupling constant could not be determined due to overlap in the region of δ 5.01 and although the chemical shifts of these protons and of H-15 were similar to those melampolides of established stereochemistry,[9-14] this evidence was regarded as too tenuous to make a firm stereochemical assignment for the 4,5-double bond.

Several of the assignments made above were verified by irradiation studies. For example irradiation of the aldehyde doublet at δ 9.40 selectively reduced the multiplicity of the signal at δ 3.540 to a doublet and the reverse irradiation caused collapse of the aldehyde proton to a singlet. At the same time the doublet of doublets at δ 6.533 was reduced to a doublet, confirming that this was indeed the C-8 proton.

An n.O.e. study [15] confirmed the stereochemistry of the α,β-unsaturated aldehyde when irradiation of the aldehyde increased the intensity of H-1 by more than 20%. Molecular models indicated that *if* the 4,5-double bond has the *trans*-stereochemistry, one of two apparently stable conformers might have H-15 and H-9 proximate. Irradiation of this methyl signal did not increase the intensity of H-9 but did have an effect on the multiplet at δ 5.01 causing an intensity increase of about 12% in the region of this signal, assigned (because of its 'triplet' nature) to H-6. Once again models indicate that such a result is possible if the 4,5 double bond is *trans*. If this double bond is *cis*, H-6 is almost perpendicular or parallel (depending on conformational preferences) to the plane of the double bond and consequently the only n.O.e. affect observed should be between H-15 and H-5. Unfortunately the observed result could also be explained if H-15 had been misassigned and the 4,5 double bond was *cis*.

Another technique which was considered useful in deducing the C-4,5 stereochemistry was that of circular dichroism. Examination of the then (1976) available literature indicated that distinction between the 4,5-*cis* and 4,5-*trans* possibilities could not reliably be made by this method.

Consequently, although there was scant n.m.r. evidence for a 4,5-*trans*-stereochemistry, the dangers of relying on this for such an important stereochemical

assignment were considered too great, and single-crystal *X*-ray crystallography of acanthospermolide was undertaken. In spite of considerable effort these attempts failed to yield data which would could be analysed for structural elucidation purposes. The reasons for this are not clear at present.

Acantholide (3).—Obtained by column chromatography of the chloroform-soluble fraction, acantholide crystallized from methanol. In the mass spectrum was a molecular ion at *m/e* 348, analysing for $C_{19}H_{24}O_6$, and the oxygen atoms were accounted for by the hydroxy ($\nu_{max.}$ 3 500 cm^{-1}), α,β-unsaturated lactone (1 780, 1 760 cm^{-1}), saturated ester (1 725 cm^{-1}), and α,β-unsaturated carbonyl (1 690, 1 660 cm^{-1}) functionalities observed in the i.r. spectrum.

Several of the main features of the proton n.m.r. spectrum of acantholide were analogous to those present in acanthospermolide: an olefinic methyl group (δ 1.97), two three-proton doublets (*J* 7.0 Hz) at δ 1.15 and 1.10, two doublets (*J* 3.0 and 3.5 Hz) at δ 5.64 and 6.33 for the H-13a and H-13b protons respectively, a doublet (*J* 1.8 Hz) at δ 9.41 for an aldehyde proton, and the C-1 proton at δ 6.74. Consideration of these structural elements in concert with the molecular formula led to the conclusion that acantholide was very similar to acanthospermolide but contained a hydroxy-group in place of a methoxy-group.

Acantholide readily formed an acetate derivative (M^+ 362) in which both acetylation of the secondary hydroxy-group and ester exchange of the isobutyrate group had occurred. In support of this, the ^1H n.m.r. spectrum indicated the presence of two acetate groups (δ 2.02), but no signals in the region δ 1.1. One of the protons originally in the region δ 5.0 had been deshielded to δ 6.65, and from the coupling constants of 8.8 and 1.6 Hz it was clear that this was H-8. In this respect it is important to note, as Herz has indicated,[12] that when H-8 is α and lies in the deshielding region of the aldehyde carbonyl and the 11,13-*exo*-methylene, it is strongly deshielded. In acantholide, therefore, the isobutoxy-group should be located at C-9 and the hydroxy-group at C-8. From the multiplicity of the aldehyde proton, which indicated coupling with H-9, acantholide could be deduced to have the structure (4), where the 4,5 double-bond stereochemistry remained to be determined. Again attention was turned to deducing this stereochemical point by *X*-ray crystallography; this time it was successful.

Acantholide crystallized in the *P*2 monoclinic space group with *b* the unique axis, and the cell dimensions from preliminary Weissenberg and precession photographs were $a = 11.293$ Å, $b = 8.194$ Å, $c = 10.186$ Å, $\beta = 93.33°$. Analysis of 1 347 observed reflections using MULTAN and the *X*-ray System afforded the structure for acantholide shown in (3).[5] Acantholide therefore has the 1,10-*cis*-4,5-*trans*-stereochemistry of compounds in the melampolide series. By analogy the structure (1) could be assigned to acanthospermolide.

Glabratolide (5).—The compound was obtained as an amorphous gum and showed a molecular ion at *m/e* 332, sixteen mass units less than acantholide (3). The i.r. spectrum was quite similar to that of (3) showing α,β unsaturated lactone ($\nu_{max.}$ 1 770 cm^{-1}), saturated ester ($\nu_{max.}$ 1 740 cm^{-1}), and α,β-unsaturated aldehyde or ketone ($\nu_{max.}$ 1 690 cm^{-1}) moieties; but no hydroxy-absorption was observed.

The key to the structural relationship with acantholide was the ^1H n.m.r. spectrum, which confirmed the α-methylene lactone moiety with doublets (*J* 3.1 and 3.5 Hz) at δ 5.362 and 6.186 for H-13a and H-13b, respectively, a vinyl methyl group at δ 1.933, and the terminal methyls of an isobutyrate ester as doublets (*J* 6.9 Hz) at δ 1.137 and 1.118. The latter groups were substantiated by a loss of 71 (C_4H_7O) and 88 Daltons ($C_4H_8O_2$) from the molecular ion.

An α,β-unsaturated aldehyde having a *cis*-stereochemistry was deduced from a doublet (*J* 1.4 Hz) for the aldehyde proton at δ 9.43 and the β-proton as a multiplet at δ 6.62. A complex multiplet integrating for two protons in the region of δ 5.11 was attributed to the protons on C-5 and C-6. The only remaining recognizable signal to be assigned was a one-proton multiplet at δ 6.32. Analysis indicated an eight line system (although due to overlap with H-13b only five lines could be observed) having the coupling constants 8.3, 4.6, and 1.6 Hz. This signal is ascribed to H-8 and is strongly deshielded by the mechanisms discussed previously. From this evidence an isobutyrate ester could be placed at C-8 in a β-stereochemistry with C-9 unsubstituted. Glabratolide therefore has the structure (5).

9α-*Hydroxyglabratolide* (6).—The compound was obtained as an amorphous gum and was isomeric with acantholide (3), having a molecular ion at *m/e* 348 analysing for $C_{19}H_{24}O_6$. The isolate displayed i.r. absorptions at 3 400, 1 779, 1 730, 1 690, and 1 670 cm^{-1} for hydroxy, α,β-unsaturated lactone, saturated ester, and α,β-unsaturated aldehyde functionalities, respectively. The ^1H n.m.r. spectrum in $CDCl_3$ was very similar to that of glabratolide (5) except that the region around δ 6.2 was less complex, and a new doublet of doublets was observed at δ 3.918. Thus the isobutyrate methyl groups appeared as doublets (*J* 6.9 Hz) at δ 1.148 and 1.175, the vinyl methyl group as a broadened singlet at δ 1.91, the H-13a and H-13b doublets (*J* 3.1 and 3.6 Hz) at δ 5.639 and 6.237, respectively, the *cis*-aldehyde proton at δ 9.457 (*J* 2.1 Hz), and a broadened doublet of doublets (*J* 11.0, 6.4 Hz) at δ 6.93. The protons for H-5 and H-6 again appeared as a complex multiplet in the region of δ 5.00.

Two one-proton signals remained to be interpreted, a doublet of doublets (*J* 8.6, 1.6 Hz) at δ 6.372 and a doublet of doublets (*J* 8.6 and 2.0 Hz) at δ 3.918. The chemical shifts of these signals indicated that the former could be ascribed to H-8 and the latter to H-9. In agreement with this, acetylation caused a downfield shift of H-9 to δ 5.26 (compare with 'acantholide acetate'). Consequently this compound was assigned

the structure 9α-hydroxyglabratolide (6), the C-9 stereochemistry being assigned on the basis of the observed coupling (J 2.0 Hz) between H-14 and H-9β.

Acanthoglabrolide (7).—Acanthoglabrolide was obtained as an amorphous gum displaying a molecular ion at *m/e* 418, analysing for $C_{23}H_{30}O_7$. No hydroxy-absorption was observed in the region 3 000—3 600 cm^{-1} of the i.r. spectrum but absorptions characteristic of the α,β-unsaturated lactone ($\nu_{max.}$ 1 775 cm^{-1}), saturated ester ($\nu_{max.}$ 1 735 cm^{-1}), and α,β-unsaturated aldehyde ($\nu_{max.}$ 1 685 cm^{-1}) moieties were dominant features.

The mass spectrum showed two successive losses of 88 Daltons ($C_4H_8O_2$) giving rise to a base peak at *m/e* 242. Such losses suggest, by analogy with the previously described isolates, the presence of two isobutyrate ester moieties in acanthoglabrolide. This was indeed borne out by the ^1H n.m.r. spectrum, which showed two six-proton doublets of doublets at δ 1.057 and 1.087. Signals for the *exo*-methylene protons were observed as doublets (J 3.1 and 3.4 Hz) at δ 5.765 and 6.249, and for a vinyl methyl group at δ 2.007. An α,β-unsaturated aldehyde group having a *cis*-stereochemistry was deduced from the presence of a doublet (J 1.8 Hz) H-14 at δ 9.518 and a broadened doublet of doublets at δ 6.694 for the associated C-1 proton. The 5- and 6-protons were observed as a complex multiplet in the region δ 5.03.

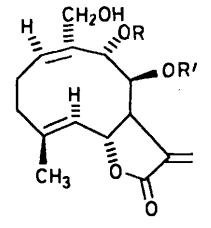
As in the previous compounds two doublets of doublets at δ 6.694 (J 8.6 Hz) and 5.223 (J 8.6, 1.85 Hz) could be attributed to H-8 and H-9, having the α- and β-stereochemistries respectively, as indicated in the complete structure (7) for acanthoglabrolide.

Acanthospermal A (8).—Acanthospermal A was obtained as a gum and from the mass spectrum, which showed a molecular ion at *m/e* 434 ($C_{23}H_{30}O_8$), and from other spectral data it was deduced that the compound differed from (7) in containing an additional oxygen atom. Since the i.r. spectrum showed hydroxy-absorption ($\nu_{max.}$ 3 500 cm^{-1}) not present in (7), but no oxymethine protons in the ^1H n.m.r. spectrum, the oxygen should be present in a hydroxy-group located at C-3 in one of the isobutyrate ester residues. Such an assignment was confirmed by the presence of a six-proton doublet (J 6.9 Hz) at δ 1.094 and two three-proton singlets at δ 1.288 and 1.318. The remaining ^1H n.m.r. spectral data were essentially identical to those of acanthoglabrolide and consequently two structures were considered possible for the isolate, namely (8) or the structural isomer (9).

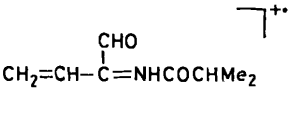
A distinction between those two was made possible by consideration of the predominating initial major loss in the mass spectrum which was of 103 Daltons, in agreement with an assignment of the hydroxy-isobutyrate residue to C-9 rather than C-8. Confirmation of this assignment was made possible by treatment of acanthospermal A with methoxide–methanol under mild conditions. The product was identical (t.l.c., n.m.r., m.s.) with acanthospermolide (1).

In previous work Herz and Kalyanaraman [12] had obtained the same compound from *Acanthospermum australe* (L.) Kuntze and observed the substantial losses of 103 and 104 Daltons in the mass spectrum and the facile displacement of the hydroxyisobutyrate residue at C-9. Our isolate from *A. glabratum* is therefore apparently identical with that of Herz and Kalyanaraman, although no direct comparison could be made.

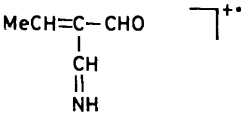
Dihydrocanthospermal A (10).—Closely related to acanthospermal A (8), but somewhat more polar in nature, was an amorphous gum showing a molecular ion at *m/e* 436 and analysing for $C_{23}H_{32}O_8$. The two additional hydrogen atoms compared with (8) were traced from the i.r. spectrum (no absorption in the region 1 650—1 700 cm^{-1}) and the ^1H n.m.r. spectrum (no aldehyde proton) to the presence of a C-10 hydroxymethyl group. In agreement with this, the n.m.r. spectrum displayed a two-proton AB pattern centred at δ 4.225 and two exchangeable protons at δ 3.42 and 3.63. Dramatic changes were observed in the region of δ 6, for now H-13b was the most downfield signal (doublet at δ 6.208) with H-8 as a doublet of doublets (J 8.2, 1.6 Hz) at δ 6.14 and H-1 as a broadened doublet of doublets (J 10.8, 8.9 Hz) at δ 5.67. The C-9 proton appeared as a doublet (J 8.2 Hz) at δ 5.29 indicating that coupling was only with H-8. On this basis the structures (10) or (11) could be proposed for dihydroacanthospermal A. A choice between the two was made from the mass spectrum which showed an important loss of 103 Daltons from the molecular ion, suggesting the hydroxyisobutyrate residue to be at C-8, and from manganese dioxide oxidation which afforded acanthospermal A (8) (identical by t.l.c. and mass-spectrometry). Dihydroacanthospermal A therefore has the structure (10).



	R	R'
(10)	$COCOHMe_2$	$COCHMe_2$
(11)	$COCHMe_2$	$COCOHMe_2$



(13) *m/e* 167



(14) *m/e* 97

Acanthamolide (12).—This unusual nitrogen-containing sesquiterpene lactone was obtained as colourless, rhomboid crystals from methanol–benzene, m.p. 249—251 °C. In the i.r. spectrum the α,β-unsaturated-γ-lactone ($\nu_{max.}$ 1 770 and 1 690 cm^{-1}) and α,β-unsaturated aldehyde or ketone moieties (1 670 cm^{-1}) were clearly visible, together with a medium-intensity band at 1 640 cm^{-1} characteristic of an amide moiety. The secondary nature of this group was supported by a sharp, quite intense absorption at 3 386 cm^{-1}. The ^1H n.m.r. spectrum confirmed the presence of the α-methylene with characteristic doublets (J 3.2 and 3.6 Hz) at δ 5.588

and 6.221, respectively and an α-substituted-α,β-unsaturated aldehyde, with the aldehyde proton as a doublet (J 1.9 Hz) at δ 9.351 and a complex β-proton at δ 6.602. Only two other signals were readily interpretable, a vinylic methyl group at δ 1.991 and two three-proton doublets (J 6.8 Hz) for an isobutyrate residue at δ 1.005 and 1.089.

A molecular ion was observed at m/e 347 which, by high-resolution mass spectrometry, was found to have the molecular formula $C_{19}H_{25}NO_5$. Treatment of acanthamolide with acetic anhydride afforded a monoacetate, indicating that the oxygen and nitrogen functional groups were an aldehyde, an α,β-unsaturated-γ-lactone, an isobutyryl amide, and a hydroxy-group.

The stereochemical assignments of acanthamolide were made by analogy with those of acantholide. A *cis*-geometry for the 1,10 double bond was deduced from the chemical shift of the aldehyde proton, and a *trans*-lactone from the magnitude of the coupling of 7-H in acanthamolide with the C-13 protons.

A broad doublet of doublets (J 10.1 and 6.9 Hz) for the C-1 proton appeared at δ 6.602 and was used as evidence that C-2 was unsubstituted. Two vicinally coupled doublets of doublets were observed in acanthamolide at δ 4.407 (J 8.6 and 1.86 Hz) and 5.136 (J 8.7 and 2.2 Hz), and from the J value of the aldehyde proton the former proton was assigned to be a β-proton at C-9. The large coupling constant of H-9 with H-8 (8.6 Hz) indicates the C-8 substituent to be β and the small (2 Hz) coupling of H-7 and H-8 substantiates this. Acetylation caused a downfield shift of the proton originally at δ 5.136 to 6.09, indicating the hydroxy-group to be at C-8 and the isobutyramide moiety at C-9.

Additional evidence for the location of the groups came from a rationalization of two important fragment ions at m/e 167 ($C_9H_{13}NO_2$) and m/e 97 (C_5H_7NO). The former ion appeared to be derived by allylic cleavage at the 2,3 and 8,9 bonds and to have the structure (13), while the latter would correspond to loss of the isobutyrate residue with concomitant proton transfer to afford an ion of m/e 97 having the structure (14). Thus acanthamolide was suggested to have the structure (12),[4] in which the 4,5-stereochemistry was assigned on the basis of similarity in chemical shift with compounds of established stereochemistry.

Stereochemical Considerations.—The 4,5-stereochemistry in acantholide was deduced[5] from single-crystal X-ray analysis to be unequivocally *trans*. However, this information could clearly not be obtained in this way for all the related compounds; time, expense, and the amorphous nature of certain of the compounds being just some of the reasons. Other techniques were therefore considered for the possibility of this stereochemistry; these included o.r.d., c.d., and ^{1}H n.m.r. spectroscopy.

With a 1,10-*cis*-stereochemistry, the undefined compounds could either be in the melampolide (1,10-*cis*-4,5-*trans*) or heliangolide (1,10-*cis*-4,5-*cis*) series,[16] and the initial comparison of these compounds was attempted on the basis of ^{1}H n.m.r. spectroscopy. At the time when these assignments were originally made[4,6] there were no appropriate data in the heliangolide series available and consequently comparison was made within the isolated group of compounds for signals which were thought to be significant. This first observation one can make when comparing Dreiding models of a *cis,cis*-compound and a *trans,trans*-compound is that the former is considerably more flexible than the latter,[17] and consequently one might expect (perhaps naïvely) that quite different chemical shifts and/or coupling constants would be observed since the preferred conformation should involve a different spatial array in each case. Thus our initial assignment of a 4,5-*trans*-stereochemistry to all of these sesquiterpene lactones was made based on the similarity of the chemical shifts of the C-8 and C-9 protons in appropriate compounds, and on the similarity of the various coupling constants involving H-15, H-9, H-8, and H-7 as discussed previously.

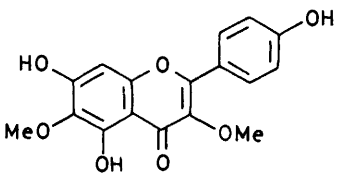
Very recently Fischer and his co-workers have reported on the melcanthins, a group of *cis,cis*-germacrenolides from *Melampodium leucanthum* Torr. and Gray (Asteraceae)[18] bearing similar functional groups to the compounds from *A. glabratum*. In particular, each compound was substituted at both C-8 and C-9 with an appropriate acyl function and moreover the stereochemistry at these centres was also deduced to be the same as that in our compounds.

Two significant observations can be made concerning the 4,5-stereochemistry as a result of these stereochemical assignments. Firstly, the 8α-H in these compounds appears in the region δ 5.89—5.95 and is therefore not as deshielded as in the *Acanthospermum* series of compounds. Secondly, the coupling constants of H-8 with H-9 in the *Melampodium* derivatives are in the region 3.5—5.0 Hz, substantially below the typical values of 8.5—9.0 Hz observed in all of the *Acanthospermum* derivatives. These data suggest that in the *cis,cis*-series this proton lies not in the plane of either the lactone ring or the C-15 carbonyl group, and that its preferred conformational angle is somewhat less than that observed for acantholide. Indeed Fischer and his co-workers deduced an approximate value of 130° for this dihedral angle.[18]

The reliability of such correlations from ^{1}H n.m.r. data remain to be established, but since the two stereochemical series of compounds may occur in one plant, stereochemical inferences of one compound by X-ray crystallography do not apparently have any biogenetic significance with respect to co-occurring derivatives. Therefore a method is needed which will demonstrate these stereochemical assignments more easily. It may be that the 8α-H is the key proton to be assigned and evaluated.

3,6-*Dimethoxy*-4′,5,7-*trihydroxyflavone* (15).—Principal i.r. absorptions at 3 470—3 100 and 1 651 cm^{-1} indicated strongly hydrogen-bonded hydroxy and α,β-unsaturated carbonyl groups. From the mass spectrum, which displayed a molecular ion at m/e 330 ($C_{17}H_{14}O_7$), and the

u.v. spectrum, with $\lambda_{max.}$ at 268 and 340 nm, the presence of a flavonoid having two methoxy and three phenolic functionalities was deduced.



(15)

The ^1H n.m.r. spectrum (determined in $[^2H_6]$DMSO for solubility reasons) indicated the presence of four aromatic AB related protons (J 8.7 Hz) on ring B and a singlet proton at δ 6.50. The band at 340 nm in the u.v. spectrum was shifted bathochromically by 55 nm in NaOMe–MeOH, indicating the C-4′-substituent to be a hydroxy-group.[19,20] A fragment ion at *m/e* 121 ($C_7H_5O_2$), derived by cleavage of ring C and containing ring B, O-1, and C-2, supports this interpretation.[21]

A six-proton, slightly broadened singlet at δ 3.81 confirmed the presence of two aromatic methoxy-groups, and signals exchanging with D_2O at δ 10.07 and 12.69 demonstrated the three phenolic groups alluded to previously. The location of these groups was determined principally by standard reactions in the u.v. spectrum.[19]

The $\lambda_{max.}$ at 340 nm suggested a flavone [19] and the mass spectrum displayed a moderate (23% base peak) fragment at *m/e* 287. This ion corresponds to loss of carbon monoxide and a methyl radical from ring C and is characteristic of a C-3 methoxy-group.[22,23] Placement of one hydroxy-group at C-7 could be made by the small (7 nm) bathochromic shift with no degeneration induced in the second band on the addition of acetate.[19,20,24]

Addition of $AlCl_3$–HCl caused a bathochromic shift of 25 nm, in agreement with the presence of a 6-methoxy-group [25] and this was substantiated by the mass spectrum which showed the facile loss of a methyl radical to yield a stable fragment ion (43% of base peak). The singlet aromatic proton could therefore be ascribed to H-8, and the complete molecular structure as 3,6-dimethoxy-4′,5,7-trihydroxyflavone (15).

This compound was previously isolated by Rosler *et al.*[26] from the flowers of *Centaurea jacea* L. (Asteraceae) and synthesized by Fukui and his co-workers.[27] Our sample was identical (u.v., i.r., m.p., mixed m.p., and t.l.c. on three systems) with an authentic sample provided by Professor L. Chopin.

Biological Activity of Isolates.—Each of the isolates was examined according to established protocols [2] in the Eagles carcinoma of the nasopharynx (KB) test system in the culture (Table 2). All the sesquiterpene lactones were cytotoxic as might reasonably be expected since each contains an α-methylene-butyrolactone moiety.[28] The flavone derivative (15) was inactive in this system,

TABLE 2

Anticancer activity of the isolates of *Acanthospermum glabratum*

Compound	NSC- No.	*In vitro* KB ED_{50} μg ml^{-1}	P-388 μg ml^{-1}	*In vivo* P-388 T/C, mg kg^{-1}
Acanthoglabrolide (7)	277280	3.1		inactive
Glabratolide (5)	277281	2.1		inactive
Acantholide (3)	277282	2.2		inactive *
Acanthospermolide (1)	255989	0.54		146, 12.5
Acanthospermal A (8)	277278	2.3		not tested
9α-Hydroxyglabratolide (6)	277283	2.0		144, 10
Dihydroacanthospermal A (10)	277279	2.6		136, 20
Acanthamolide (12)	280452	2.2		not tested
3,6-Dimethoxy-4,5,7-trihydroxyflavone (15)	271638	inactive	3.4	inactive

* Also inactive in the Lewis lung carcinoma, B16 melanoma, L-1210 lymphoid leukemia, and Colon 26 test systems.

although it did display marginal cytotoxicity in the P-388 lymphocytic leukemia system *in vitro*. Dihydroacanthospermal A (11) showed similar cytotoxicity to acanthospermal A (8) and consequently the *cis*-α,β-unsaturated aldehyde unit does not appear to enhance the cytotoxicity.

Seven of the isolates were obtained in quantity adequate for *in vivo* testing; three of these, acanthospermolide (1), 9α-hydroxyglabratolide (6), and dihydroacanthospermal A (10) were active in the P-388 lymphocytic leukemia system in mice, but insufficient data are available from which to draw conclusions concerning structure–activity relationships.

The flavone derivative (15) was previously shown by Wollenweber and Racker [29] to substantially reduce lactate production in Ascites cells, and in L-1210 cells alone and in the presence of sodium ion.

EXPERIMENTAL

Melting points were determined using a Kofler hot plate. The u.v. spectra were obtained with a Beckman model DB-G grating spectrophotometer. The i.r. spectra were determined with a Beckman model 18-A spectrophotometer with polystyrene calibration at 1 601 cm^{-1}. Absorption bands are recorded in wave numbers (cm^{-1}) and intensities as s (strong), m (medium), and w (weak). ^1H N.m.r. spectra were recorded in $CDCl_3$ or $[^2H_6]$DMSO solutions with a Varian model T-60A instrument, operating at 60 MHz with a Nicolet model TT-7 Fourier-transform attachment, or on a Bruker 270-MHz instrument. ^{13}C N.m.r. spectra were recorded at 25.034 MHz on a JEOL instrument. Tetramethylsilane was used as an internal standard and chemical shifts are reported in δ (p.p.m.) units. Low-resolution mass spectra were obtained with a Hitachi–Perkin-Elmer model RMU-6D single-focusing spectrometer operating at 70 eV. High-resolution mass spectra were obtained with a Varian 731 double-focusing spectrometer operating at 70 eV.

Plant Material.—The plant material was collected in Mufindi District, Tanzania, during November 1973. Whole

plants were collected, air-dried, and milled to a coarse powder.

The plant material was supplied through the auspices of the Drug Research and Development Branch, National Cancer Institute, by the Economic Botany Laboratory, Plant Genetics and Germplasm Institute, Agricultural Research Service, U.S.D.A., Beltsville, MD. A herbarium specimen documenting this collection (Spjut 3493) is deposited in the Herbarium of the National Arboretum, Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C.

Plant Extraction and Preliminary Fractionation.—The milled whole plant (5.5 kg) of *Acanthospermum glabratum* (DC). Wild was successively and thoroughly extracted at room temperature with light petroleum (b.p. 40—60 °C), benzene, and finally methanol. The residue (238 g) from the light petroleum extract was not cytotoxic in the KB test system and was not studied further.

After evaporation of solvent *in vacuo*, the residue (373 g) from the benzene extraction was partitioned between chloroform (1 l) and 2% aqueous hydrochloric acid (1.5 l). A solid (17 g) formed at the interface and was processed separately. The chloroform phase was evaporated to afford a residue (277 g) which displayed cytotoxic activity (ED_{50} 0.88 μg ml^{-1}) in the KB test system.

Separation of the Chloroform-soluble Fraction.—A sample of the chloroform-soluble fraction (60 g) was chromatographed on a column of silica gel PF-254 (2 kg) packed in benzene and eluted with benzene–ethyl acetate (99 : 1) and mixtures of successively increasing ethyl acetate concentration. Fractions of 100 ml were collected, analysed by t.l.c., combined on this basis, and then submitted for cytotoxic evaluation. Only those fractions which displayed activity according to established [2] protocol were further studied.

Acanthoglabrolide (7).—Column fractions 358—438 from the chromatography of the chloroform extract were combined and the residue (3.0 g) chromatographed on a column of silica gel PF-254 (100 g) eluting with chloroform and collecting 20-ml fractions. Column fractions 8—21 (540 mg) were further chromatographed on silica gel PF-254 (30 g) eluting with benzene to afford, from fraction 6, an amorphous gum (120 mg, 0.002%) identified as (7) from the following physical properties: $\nu_{max.}$ (film) 1 775, 1 735, 1 685, 1 640, 1 240, 1 180, 1 130, and 975 cm^{-1}; n.m.r., see Table 1; *m/e* 418 (M^+, 0.3%), 347 (0.2), 330 (0.5), 277 (0.5), 260 (1), 243 (3), 242 (8), 224 (1), 214 (2), 213 (3), 105 (4), 91 (9), 71 (33), and 43 (100).

Glabratolide (5).—Fraction 8 from the chromatography of fractions 8—21 described above was evaporated to yield an amorphous gum (115 mg, 0.002%) identified as (5) from the following spectral properties: $\nu_{max.}$ (film) 1 785, 1 770, 1 740, 1 730, 1 690, 1 670, 1 300, 1 240, 1 180, 1 140, 1 060, and 975 cm^{-1}; n.m.r., see Table 1; *m/e* 332 (M^+, 16%), 261 (12), 245 (24), 244 (28), 227 (5), 226 (6), 216 (9), 215 (10), 105 (16), 91 (21), 71 (73), and 43 (100).

Acantholide (3).—Column fractions 620—691 from the chromatography of the chloroform extract were combined and the residue (1.6 g) chromatographed on a column of silica gel PF-254 (100 g), eluting with chloroform and collecting 20-ml fractions. Fractions 48—53 were combined and re-chromatographed on Florisil, eluting with benzene, to afford acantholide (3), which crystallized from methanol as colourless needles (150 mg, 0.0027%), m.p. 208 °C; $\nu_{max.}$ (KBr) 3 500, 1 780, 1 760, 1 725, 1 690, 1 680, 1 660, 1 625, 1 240, 1 190, 1 140, 1 045, and 960 cm^{-1}; n.m.r., ^{1}H see Table 1; δ_C ($CDCl_3$) 16.9 (C-15), 18.9 (C-3′), 19.1 (C-3′), 26.3 (C-2), 34.1 (C-2′), 36.8 (C-3), 50.8 (C-7), 56.7 (OCH_3), 68.6 (C-8), 75.0 (C-6), 78.3 (C-9), 122.1 (C-13), 127.3 (C-5), 134.1 (C-11), 136.9 (C-4), 140.8 (C-10), 156.3 (C-1), 175.5 (C-1′), and 183.8 (C-14); C-12 was not observed; *m/e* 348 (M^+, 1.9%), 277 (M^+ — 71, 1.7), 260 (M_D — 88, 40), 242 (30), 214 (20), 213 (15), 177 (56), 91 (29), 84 (56), 71 (79), 69 (53), and 43 (100) (Found: M^+, 348.1571. Calc. for $C_{19}H_{24}O_6$: *M*, 348.1571).

Acetylation of acantholide. Acantholide (20 mg) was treated with acetic anhydride–pyridine (1 : 1, 1 ml) at room temperature overnight. Work-up in the usual way afforded an acetate derivative identified as (16); n.m.r., see Table 1; *m/e* 362 (M^+, 0.5%), 320 (M^+ — 42, 4), 302 (M^+ — 60, 2), 277 (2), 260 (8), 242 (25), 214 (10), 213 (15), 177 (24), 91 (13), and 43 (100).

3,6-Dimethoxy-4′,5,7-trihydroxyflavone (15).—Column fractions 701—950 (4.86 g) from the chromatography of the chloroform extract, eluted with benzene–ethyl acetate (9 : 1), were crystallized from methanol–chloroform to yield yellow needles (68 mg, 0.001%) of (15), m.p. 198—200 °C; $\nu_{max.}$ (KBr) 3 400m (OH), 1 651s (α,β-unsaturated CO), 1 610s, 1 585s, 1 565s, and 1 550s cm^{-1}; $\lambda_{max.}$ (MeOH) 268 and 340 nm; $\lambda_{max.}$ (NaOMe) 275, 329, and 395 nm; $\lambda_{max.}$ ($AlCl_3$) 238 (sh), 280, 300 (sh), 372, and 400 (sh) nm; $\lambda_{max.}$ ($AlCl_3$–HCl) 280, 300 (sh), 322, and 366 nm; $\lambda_{max.}$ (NaOAc) 275, 302, and 380 nm; $\lambda_{max.}$ (NaOAc–H_3BO_3) 277 and 350 nm; δ ([$^2H_6$]DMSO) 3.81 (6 H, s, 2 × OMe), 6.49 (1 H, s, C-8-H), 6.92 (2 H, m, C-3′-, -5′-H), 7.93 (2 H, m, C-2′-, -6′-H), 10.07 (2 H, br s, exchanged with D_2O, 2 × OH), and 12.69 (1 H, s, exchanged with D_2O, OH); *m/e* 330 (M^+, 100%, $C_{17}H_{14}O_7$), 329 (36), 315 (43), 297 (6, $C_{15}H_{11}O_6$), 287 (23), 269 (11, $C_{15}H_9O_5$), 244 (7, $C_{13}H_8O_5$), 165 (5), 136 (6), 121 (16, $C_7H_5O_2$), 71 (25), 69 (25), and 43 (42).

Acanthospermolide (1).—Column fractions 701—950, after removal of (15) by crystallization, were concentrated and part of the residue (3.46 g) was chromatographed on a column of silica gel PF-254 (75 g) eluting with benzene–methanol (99 : 1). Early fractions from the column afforded (1) as colourless needles (515 mg, 0.0094%) * from benzene–methanol, m.p. 154 °C; $\nu_{max.}$ (KBr) 1 776, 1 750, 1 690, 1 685, 1 630, 1 305, 1 240, 1 190, 1 155, and 970 cm^{-1}; n.m.r., see Table 1; *m/e* 362 (M^+, 1%), 333 (2), 292 (4), 291 (3), 275 (6), 274 (12), 256 (5), 246 (5), 245 (6), 242 (10), 214 (7), 213 (7), 197 (5), 196 (6), 183 (22), 178 (5), 177 (8), 134 (13), 113 (60), 112 (58), 91 (13), 84 (17), and 71 (100) (Found: M^+, 362 171 7. Calc. for $C_{20}H_{26}O_6$: *M*, 362.172 9).

Acanthospermal A (8).—Column fractions 951—962 from the chromatography of the chloroform extract were combined, and the residue (400 mg) subjected to preparative t.l.c on silica eluting with benzene–ethyl acetate (3 : 2) to yield an amorphous gum (83 mg, 0.001 5%) identified as (8); $\nu_{max.}$ (film) 3 500, 1 780, 1 740, 1 690, 1 625, 1 295, 1 235, 1 120, and 970 cm^{-1}; n.m.r., see Table 1; *m/e* 434 (M^+, 0.2%), 347 (0.4), 331 (0.9), 330 (0.8), 259 (0.6), 243 (2), 242 (5), 91 (27), 71 (54), 59 (82), and 43 (100).

9-Hydroxyglabratolide (6).—Column fractions 1 007—1 034 from the chromatography of the chloroform extract were combined and the residue (4.0 g) was chromatographed on a column of silica gel PF-254 (200 g), eluting initially

* This compound was detected in the column fractions 701—950 prior to *any* treatment with methanol, and is therefore not an artifact.

with benzene–ethyl acetate (97 : 3). Fractions 18—22 (245 mg), eluted with benzene–ethyl acetate (1 : 1), were further chromatographed on silica gel PF-254 (30 g), eluting with chloroform, to yield from column fractions 1—5 amorphous gum (89 mg, 0.001 6%), identified as (6); $\nu_{max.}$ (film) 3 400, 1 790, 1 750, 1 740, 1 690, 1 670, 1 635, 1 250, 1 195, 1 100, 1 075, and 980 cm^{-1}; n.m.r., see Table 1; *m/e* 348 (M^+, 16%), 331 (2), 278 (2), 277 (4), 261 (19), 260 (13), 243 (10.5), 242 (9), 232 (8), 231 (7), 214 (11), 213 (11), 177 (18), 163 (14), 119 (25), 105 (31), 91 (68), 71 (90), and 43 (100).

Acanthamolide (12).—Column fractions 1 075—1 080 from the chromatography of the chloroform extract were combined and the residue (5.12 g) chromatographed on a column of silica gel PF-254 (150 g) eluting with benzene–ethyl acetate. Fractions eluted with benzene–ethyl acetate (1 : 1) were combined and crystallized from benzene–methanol to afford colourless rhomboid crystals of (12) (60 mg, 0.001%), m.p. 249—251 °C; $\nu_{max.}$ (KBr) 3 520, 3 385, 1 770, 1 690, 1 670, 1 640, 1 390, 1 300, 1 245, 1 130, and 970 cm^{-1}; n.m.r., see Table 1; *m/e* 347 (M^+, 9%), 329 (8), 318 (10), 301 (2), 276 (4), 259 (4), 242 (5), 167 (85, $C_9H_{13}NO_2$), 139 (17), 98 (43), and 97 (100, C_5H_7NO) (Found, M^+, 347.173 4; Calc. for $C_{19}H_{25}NO_5$, *M* 347.173 3).

Acetylation of acanthamolide. Acanthamolide (15 mg) was treated with acetic anhydride–pyridine (1 : 1, 1 ml) at room temperature overnight. Work-up in the usual way afforded an acetate derivative as a gum, identified as (17); $\nu_{max.}$ (KBr) 3 360, 1 780, 1 740, 1 690, 1 670, 1 640, 1 620, 1 300, 1 230, 1 140, 1 030, and 975 cm^{-1}; n.m.r., see Table 1; *m/e*, 389 (M^+, 13%), 360 (3), 347 (2), 329 (15), 317 (2), 300 (6), 276 (6), 259 (6), 242 (10), 231 (4), 230 (4), 214 (4), 213 (7), 168 (11), 167 (53), 97 (43), 71 (30), and 43 (100).

Dihydrocanthospermal A (10).—Column fractions 1 083—1 092 from column chromatography of the chloroform extract were combined and the residue (1.9 g) chromatographed on Florisil (30 g), eluting with chloroform and collecting 100 ml fractions. Fraction 4 (400 mg) was further purified by preparative t.l.c. to yield an amorphous gum (162 mg, 0.003%) identified as (10); $\nu_{max.}$ (film) 3 400, 1 765, 1 730, 1 640, 1 240, 1 135, and 970 cm^{-1}; n.m.r., Table 1: *m/e* 436 (M^+, 4%), 333 (3), 314 (6), 262 (3), 244 (11), 243 (10), 227 (11), 226 (26), 213 (8), 199 (7), 105 (18), 91 (26), 71 (51), 59 (100), and 43 (95).

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