Antitumor Agents LII: The Effects of Molephantinin on Nucleic Acid and Protein Synthesis of Ehrlich Ascites Cells

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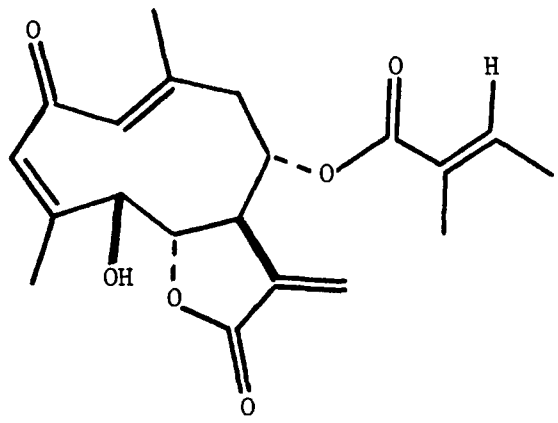
Received June 19, 1981, from the *Division of Medicinal Chemistry, School of Pharmacy, The University of North Carolina, Chapel Hill, NC 27514.* Accepted for publication October 7, 1981.

Abstract □ Molephantinin, a germacranolide, has previously been shown to possess antineoplastic activity in rodents. The principle effect of molephantinin on Ehrlich ascites carcinoma cells was to depress DNA and protein synthesis both *in vivo* and *in vitro*. DNA synthesis was inhibited at the following sites: DNA polymerase, purine synthesis specifically at inosinic acid dehydrogenase and to a lesser degree at dihydrofolate reductase, pyrimidine synthesis at orotidine monophosphate decarboxylase, thymidine kinase, histone phosphorylation, and oxidative phosphorylation processes. The protein synthesis inhibition pattern resembled more an initiation inhibitor as opposed to an elongation inhibitor.

Keyphrases □ Molephantinin—effects on nucleic acid and protein synthesis of Ehrlich ascites cells □ Antitumor agents—effects of molephantinin on nucleic acid and protein synthesis of Ehrlich ascites cells □ DNA—synthesis, effects of molephantinin, Ehrlich ascites cells □ RNA—synthesis, effects of molephantinin, Ehrlich ascites cells □ Protein synthesis—effects of molephantinin, Ehrlich ascites cells



I

Molephantinin (I), a germacranolide, was originally isolated from the winter collection of *Elephantopus mollis*, a member of the herbal Compositae family. Subsequently, molephantinin was shown to be active against Walker 256 carcinosarcoma growth in rats at 2.5 mg/kg/day resulting in a T/C% = 397 (1, 2). Further studies demonstrated that the agent was also active against P-388 lymphocytic leukemia and Ehrlich ascites cell growth (3). *In vitro* DNA polymerase activity and oxidative phosphorylation processes were reported to be inhibited by molephantinin at a relatively high concentration (3). At this time a more detailed examination of the effects of molephantinin on Ehrlich ascites tumor cell metabolism is reported.

EXPERIMENTAL

In vitro incorporation of [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine was determined using 10^6 Ehrlich ascites cells, 1 μCi of labeled precursor, minimum essential medium, and varying final concentrations of drug from 0.125–2.0 m*M* (4). The tubes were incubated at 37° for 60 min and inactivated by trichloroacetic acid. The insoluble acid, labeled DNA, was collected on glass filter discs[1], and RNA and protein were precipitated on nitrocellulose filters[2] by vacuum suction. Results are expressed as disintegrations per minute of incorporated precursor/hr/10^6 cells. For *in vitro* studies, cells were collected on Day 10 and the drug was incubated at 1.38 m*M*.

Ehrlich ascites cells (10^6) were injected intraperitoneally into CF_1 male mice (~22 g) on day 0. On Days 7, 8, and 9, molephantinin (12.5 mg/kg/day, ip in 0.05% polysorbate 80–water) was injected. Incorporation of thymidine into DNA was determined by the method of Chae *et al.* (5). One hour prior to the animal sacrifice on Day 10, 10 μCi, ip of [6-^{3}H]-thymidine (21.5 Ci/mmole) was injected. The DNA was isolated and the tritium content was determined in a toluene based scintillation fluid[3]. The DNA concentration was determined by the diphenylamine reaction using calf thymus DNA as a standard. Uridine incorporation into RNA was determined using 10 μCi of [5,6-^{3}H]uridine (22.4 Ci/mmole). RNA was extracted by the method of Wilson *et al.* (6). Using yeast RNA as a standard, the RNA content was assayed by the orcinol reaction. Leucine incorporation into protein was determined by the method of Sartorelli (7) using 10 μCi of [4,5-^{3}H]leucine (52.2 Ci/mmole). Extracted protein was determined by the Lowry procedure (8) using bovine albumin as a standard. *In vitro* and *in vivo* nuclear DNA polymerase activity was determined on isolated Ehrlich ascites cell nuclei (9). The incubation was that described by Sawada *et al.* (10) except that [methyl-^{3}H]deoxythymidine triphosphate (82.4 Ci/mmole) was used. The acid insoluble nucleic acid was collected on filters[1] and counted.

Nuclear RNA polymerase activities were determined on enzymes isolated from nuclei (9). Messenger, ribosomal, and transfer RNA polymerases were isolated using 0.3, 0.04, and 0.0 *M* concentrations of ammonium sulfate in magnesium chloride, respectively. The incubation medium was that of Anderson *et al.* (11) using [^{3}H]uridine triphosphate (23.2 Ci/mmole). The acid insoluble RNA was collected on nitrocellulose filters and counted.

Deoxythymidine as well as deoxythymidylate monophosphate and diphosphate kinase activities were measured spectrophotometrically at 340 nm at 20 min using reduced nadide (0.1 μmole) (12). [6-^{3}H]Thymidine (21.5 Ci/mmole) incorporation into the nucleotides was also measured using the reaction medium of Maley and Ochoa (12) and then plating the ether extracted reaction medium on polyethyleneiminecellulose plates. The plates were eluted with 0.5 *N* formic acid–0.6 *N* LiCl (1:1). After identifying R_f values consistent with the standards, thymidine, thymidylate monophosphate, thymidylate diphosphate, and thymidylate triphosphate, the areas on the plates were scraped and counted. Carbamyl phosphate synthetase activity was determined using the reaction medium of Kalman *et al.* (13) in the presence of ornithine and the enzyme ornithine transcarbamylase. Citrulline formed from ornithine was measured at 490 nm by the method of Archibald (14). Aspartate transcarbamylase activity was assayed using the incubation medium of Kalman *et al.* (13). The colorimetric determination of carbamyl aspartate was conducted by the procedure of Koritz and Cohen (15). Orotidine monophosphate decarboxylase activity was assayed by the method of Appel (16) using 0.1 μCi of [^{14}C]orotidine monophosphate (34.9 mCi/mmole). The [^{14}C]-carbon dioxide generated in 15 min was trapped in 1 *M* methanolic base[4] and counted. Thymidylate synthetase activity was determined using a

[1] Whatman GF/F.
[2] Millipore.
[3] Fisher Scintiverse.
[4] Hyamine Hydroxide, New England Nuclear.

0022-3549/82/0600-0687$01.00/0

Table I—The *In Vitro* Effects of Molephantinin at 1.38 m*M* Concentration on Ehrlich Ascites Cell Metabolism

Biochemical Parameter or Enzyme (n = 6)	% Control	
	Control x ± *SD*	Treated x ± *SD*
DNA polymerase	100 ± 14	55 ± 9[a]
Messenger RNA polymerase	100 ± 13	108 ± 11
Ribosomal RNA polymerase	100 ± 6	74 ± 7[a]
Transfer RNA polymerase	100 ± 10	128 ± 12[b]
Ribonucleotide reductase	100 ± 8	101 ± 15
[^{14}C]Formic acid incorporation into purines	100 ± 7	70 ± 9[a]
Phosphoribosyl pyrophosphate amido transferase	100 ± 11	77 ± 10[c]
Inosinic acid dehydrogenase	100 ± 6	43 ± 4[a]
Dihydrofolate reductase	100 ± 9	99 ± 12
Carbamyl phosphate synthetase	100 ± 10	104 ± 10
Aspartate transcarbamylase	100 ± 12	88 ± 9
Orotidine monophosphate decarboxylase	100 ± 9	20 ± 3[a]
Thymidylate synthetase	100 ± 13	92 ± 8
Thymidine kinase	100 ± 9	65 ± 6[a]
Thymidine monophosphate kinase	100 ± 8	18 ± 4[a]
Thymidine diphosphate kinase	100 ± 12	73 ± 7[b]
Oxidative Phosphorylation Processes Substrates:		
Succinate: State 4 respiration	100 ± 7	64 ± 9[a]
State 3 respiration	100 ± 4	75 ± 8[a]
α-Ketoglutarate: State 4 respiration	100 ± 5	69 ± 8[a]
State 3 respiration	100 ± 6	67 ± 9[a]

[a] $p \leq 0.001$. [b] $p \leq 0.005$. [c] $p \leq 0.010$.

Table II—The *In Vivo* Effects of Molephantinin at 12.5 mg/kg/day on Ehrlich Ascites Cell Metabolism

Biochemical Parameters or Enzymes (n = 6)	Control x ± *SD*	Treated x ± *SD*
[^{3}H]Thymidine incorporation into DNA	100 ± 8	19 ± 3[a]
[^{3}H]Uridine incorporation into RNA	100 ± 12	80 ± 12
[^{3}H]Leucine incorporation into protein	100 ± 13	56 ± 8[a]
DNA polymerase	100 ± 6	38 ± 4[a]
Messenger RNA polymerase	100 ± 12	90 ± 10
Ribosomal RNA polymerase	100 ± 8	70 ± 6[a]
Transfer RNA polymerase	100 ± 9	63 ± 4[a]
Ribonucleotide reductase	100 ± 8	90 ± 9
[^{14}C]Formic acid incorporation into purines	100 ± 12	19 ± 2[a]
Phosphoribosyl pyrophosphate amido transferase	100 ± 9	87 ± 5[b]
Inosinic acid dehydrogenase	100 ± 10	26 ± 7[a]
Dihydrofolate reductase	100 ± 12	67 ± 8[a]
Carbamyl phosphate synthetase	100 ± 10	96 ± 9
Aspartate transcarbamylase	100 ± 9	83 ± 8[a]
Orotidine monophosphate decarboxylase	100 ± 10	29 ± 3[a]
Thymidylate synthetase	100 ± 9	108 ± 10
Thymidine kinase	100 ± 9	63 ± 7[a]
Thymidine monophosphate kinase	100 ± 9	36 ± 6[a]
Thymidine diphosphate kinase	100 ± 7	84 ± 8[a]
[^{32}P]Phosphorylation of histones	100 ± 15	36 ± 8[a]
[^{32}P]Phosphorylation of nonhistones	100 ± 13	81 ± 11[b]
Cyclic adenosine monophosphate levels	100 ± 10	155 ± 14[a]
Number of tumor cells/per milliliter of ascites fluid	100 ± 6	24 ± 3[a]

[a] $p \leq 0.001$. [b] $p \leq 0.005$.

postmitochondrial supernate (9000×*g* for 10 min) and 5 μCi of [5-^{3}H]-deoxyuridine monophosphate (14 Ci/mmole) according to the method of Kampf *et al.* (17). [^{14}C]Formate incorporation into purines was determined by the method of Spassova *et al.* (18), using 0.5 μCi of [^{14}C]-formic acid (52.0 mCi/mmole). Purines were separated on silica gel TLC plates eluted with *n*-butanol–acetic acid–water (4:1:5). After identifying R_f values consistent with the standards, adenine and guanine, the plates were scraped and the radioactive content determined. Phosphoribosyl-1-pyrophosphate amidotransferase activity was determined on a supernatant fraction (600×*g* for 10 min) measuring the reduction of 0.6 μmole of nadide at 340 nm for 30 min (19). Inosinic acid dehydrogenase activity was determined spectrophotometrically at 340 nm for 30 min using a supernatant fraction (600×*g* for 10 min). The assay medium was that of Magasanik (20) which contained nadide. Dihydrofolate reductase activity was determined at 340 nm for 30 min as the oxidation of reduced nadide phosphate (21). Ribonucleotide reductase activity was determined by the method of Moore and Hurlbert (22) using [5-^{3}H]cytidine-5-diphosphate (25 Ci/mmole). Ribose and deoxyribose nucleotide were separated on polyethyleneiminecellulose plastic precoated plates eluted with 4% boric acid–4 *M* LiCl (4:3) and scraped at the R_f values consistent with the standard deoxycytidine diphosphate. *In vivo* phosphorylation of histones was determined by injecting 10 μCi of [γ^{32}P]adenosine triphosphate (30.0 Ci/mmole) into mice 1 hr prior to sacrifice. The nuclei were isolated (9), and the histone chromatin protein was extracted by the method of Raineri *et al.* (23). *In vitro* nonhistone protein phosphorylation, dependent on nuclear protein kinase, was determined using 2 n*M* of [γ-^{32}P]adenosine triphosphate (30.0 Ci/mmole) and isolated nuclei chromatin protein was collected on nitrocellulose filters (24). Cyclic 3′,5′-adenosine monophosphate levels were determined by the radioimmunoassay method of Gilman (25) using a commercial kit. An *in vitro* method was used to determine if the drug was an initiation or an elongation protein synthesis inhibitor by comparing with known standards, pyrocatechol violet and emetine, using 1 μCi of [^{3}H]leucine (52.2 Ci/mmole) incubated at 37° for 14 min. Aliquots of the reaction medium were spotted on filter paper[5], chemically extracted, and counted (4).

In vitro oxidative phosphorylation studies (26) were conducted on Ehrlich ascites cells using the substrates, α-ketoglutarate, or succinate. Basal oxygen consumption (State 4) was determined with an oxygen electrode connected to an oxygraph. The adenosine diphosphate was added to obtain State 3, or adenine diphosphate stimulated respiration. The number of microliters of oxygen consumed per hour per milligram of protein for States 3 and 4 was calculated. Protein was determined by the Lowry technique (8).

[5] Whatman No. 3.

Probable (p) significant differences were determined by the Student t test. Data are expressed in Tables I and II as percent of control with standard deviations; n is the number of animals per group.

RESULTS

The *in vitro* effects of molephantinin at 1.38 m*M* concentration are presented in Table I and the *in vivo* effects at 12.5 mg/kg/day are presented in Table II.

In vitro studies for the incorporation of labeled precursors into DNA, RNA, and protein revealed that all three were inhibited in the presence of molephantinin (Fig. 1). Thymidine incorporation into DNA was inhibited significantly with an $ID_{50} \cong 1.22$ m*M*. It may be noted that at the concentration that caused 50% inhibition of DNA synthesis, RNA synthesis was inhibited 30% and protein was inhibited 20% at 1.22 m*M*. The *in vivo* incorporation of thymidine incorporation into DNA was inhibited 81% after dosing for 3 days (Table II). The RNA synthesis was only inhibited 12%, whereas *in vivo* protein synthesis was inhibited 44% after 3 days dosing. The control value for thymidine incorporation was 107,533 dpm/mg of DNA, uridine incorporation was 51,192 dpm/mg of RNA, and leucine incorporation into protein was 19,181 dpm/mg of isolated protein.

Nuclear DNA polymerase activity for the control was 76,528 dpm/hr/mg of nucleoprotein which was suppressed *in vitro* 45% by drug presence (Table I) at 1.38 m*M*, and *in vivo* 62% (Table II) by 3-day administration of drug at 12.5 mg/kg/day. Messenger RNA polymerase activity for the control was 4867 dpm/hr/mg of protein, ribosomal RNA polymerase was 8751 dpm/hr/mg of protein, and transfer RNA polymerase activity was 10,792 dpm/mg of protein. Molephantinin caused little effect on messenger RNA polymerase activity either *in vitro* or after *in vivo* administration. However, ribosomal RNA polymerase activity was inhibited 26% *in vitro* in the presence of drug and 30% after *in vivo* administration of the drug. The transfer RNA polymerase activity was slightly elevated in both *in vitro* and *in vivo* studies.

Ribonucleotide reductase activity for the control was 153,791 dpm/mg of protein which was not affected by molephantinin administration. [^{14}C]Formate incorporation into purines for the control cells was 28,786 dpm/mg of protein which was suppressed 30% in the *in vitro* study and 81% after *in vivo* administration. Phosphoribosyl-pyrophosphate amido transferase activity for the control 10-day Ehrlich ascites cells was observed as an increase of 1.223 optical density unit/hr/mg of protein. *In vitro* presence of drug caused a 23% reduction while *in vivo* administration demonstrated only 13% reduction of enzyme activity. Inosinic acid dehydrogenase activity for the control was 0.358 optical density unit/hr/mg of protein. *In vitro* presence of drug caused 56% inhibition and *in*

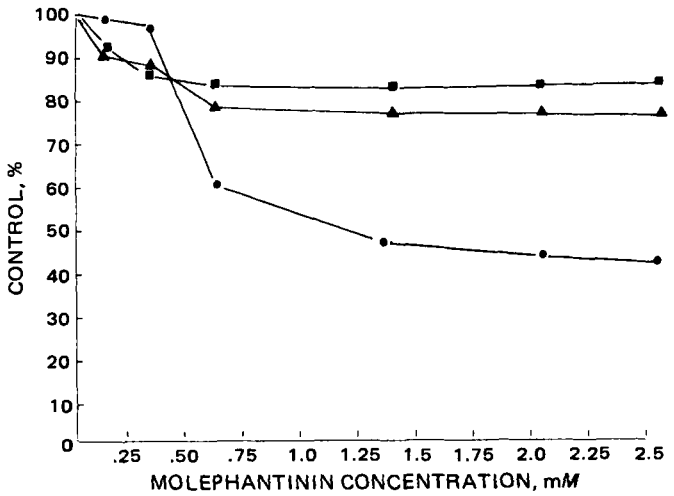


Figure 1—*The* in vitro *effects of molephantinin on nucleic acid and protein synthesis. Key:* ●, *[^{3}H]thymidine → DNA;* ▲, *[^{3}H]uridine → RNA;* ■, *[^{3}H]leucine → protein.*

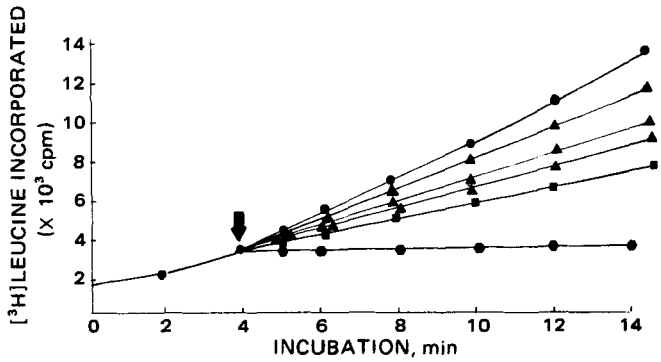


Figure 2—*Effect of pyrocatechol violet, emetine, and molephantinin on the protein synthesis of Ehrlich ascites homogenates using endogenous messenger RNA. Key:* ●, *control;* ▲, *molephantinin (10,100 and 1000 μM);* ■, *pyrocatechol violet (100 μM);* ●, *emetine (100 μM).*

vivo administration of drug resulted in 74% reduction of dehydrogenase activity. Dihydrofolate reductase activity for 10-day Ehrlich ascites cells was 0.514 optical density unit/hr/mg of protein which was unaffected by drug in the *in vitro* studies but was suppressed 33% by 3-day dosing.

Carbamyl phosphate synthetase activity for the control was 0.128 mg of carbamyl phosphate formed/hr/mg of protein. Aspartate carbamyl transferase activity for Day-10 Ehrlich ascites cells was 7.526 mg of carbamyl aspartate formed/hr/mg of protein. Neither of these enzymatic activities was suppressed significantly by molephantinin. Orotidine monophosphate decarboxylase activity for the control was 10,775 dpm of [^{14}C] carbon dioxide regenerated in 15 min/mg of protein. In the *in vitro* studies, there was an 80% reduction in the enzymatic activity, while in the *in vivo* studies there was a 71% reduction of the decarboxylase activity. Thymidylate synthetase activity for the control was 103,328 dpm/mg of protein which was not affected by molephantinin presence. Thymidine kinase activity for the control was 0.531 optical density unit/hr/mg of protein, which was reduced 35% in the *in vitro* studies and 37% in the *in vivo* studies. Thymidylate monophosphate kinase for the control was 0.305 optical density unit/hr/mg of protein which was suppressed 64% by the *in vitro* administration of molephantinin. Thymidylate diphosphate kinase activity in 10-day Ehrlich ascites cells was 0.238 optical density unit/hr/mg of protein which was reduced 16 and 27% in the *in vitro* and *in vivo* studies, respectively. The labeled nucleotide pool levels demonstrated a 54% reduction in the pool level of thymidylate diphosphate and a 58% reduction in thymidylate triphosphate level after 1-hr incubation with drug *in vitro*.

The basal respiration (State 4) of Day-10 Ehrlich ascites tumor cells with succinate as substrate was 5.273 μl of oxygen consumed/hr/mg of protein, while the adenosine diphosphate stimulate respiration (State 3) was 8.752 μl of oxygen consumed/hr/mg of protein. Molephantinin inhibited *in vitro* State 4 respiration by 36% and State 3 respiration by 25%. Using α-ketoglutarate as substrate resulted in a State 4 respiration of 3.569 μl of oxygen consumed/hr/mg of protein and in a State 3 respiration of 5.156 μl of oxygen consumed/hr/mg of protein. Molephantinin inhibited *in vitro* State 4 respiration 31% and State 3 respiration 33%.

Histone phosphorylation of chromatin protein for the control was at a rate of 3650 dpm/mg of isolated chromatin protein which was inhibited 64% by molephantinin administration for 3 days. Nonhistone chromatin phosphorylation for the control was 28,593 dpm/mg of chromatin protein isolation which was reduced 19% by molephantinin administration. Cyclic adenosine monophosphate levels for 10-day Ehrlich ascites cells was 3.65 pmole/10^6 cells. Drug administration for 3 days caused a 17% elevation of cyclic adenosine monophosphate levels of Ehrlich ascites cells. Molephantinin was shown to be a mild initiation inhibitor of protein synthesis (Fig. 2), which compared favorably to pyrocatechol violet, an initiation inhibitor, rather than to emetine, an elongation inhibitor.

DISCUSSION

Molephantinin administration at 12.5 mg/kg/day for 3 days significantly reduced DNA synthesis in Ehrlich ascites cells. The major sites of DNA synthesis inhibition appeared to occur at DNA polymerase, orotidine monophosphate decarboxylase, inosinic acid dehydrogenase, and thymidylate monophosphate kinase. These studies demonstrated that key enzymes in both the purine and pyrimidine pathways were markedly inhibited by molephantinin both *in vitro* and *in vivo*. The observed reduction in the level of either purine or pyrimidine nucleosides would account for the degree of reduction of labeled thymidine incorporation into DNA observed after administration of molephantinin. The germacronolides, eupaformosanin (27) and eupahyssopin (28), as well as the sesquiterpene lactones, helenalin and tenulin (29), have previously been shown to inhibit DNA polymerase activity, and thus, DNA synthesis in Ehrlich ascites cells. Eupaformosanin has been observed to reduce purine synthesis significantly (30). Supposedly, the moiety responsible for this inhibition is the O=CC=CH_2 system which exists both as an α-methylene-γ-lactone and as a C-8 ester side chain in molephantinin. The α-methylene-γ-lactone can undergo a rapid Michaelis-type addition with biological nucleophiles, *e.g.*, sulfhydryl, amino, and carboxyl group (29, 30), and has been shown to play a significant role in antineoplastic activity of sesquiterpene lactones.

Moderate reduction of other enzymes was observed after *in vivo* administration of molephantinin (*e.g.*, ribosomal RNA polymerase, dihydrofolate reductase, and thymidine kinase). *In vitro* administration of molephantinin resulted in a significant reduction in thymidylate diphosphate and triphosphate levels and an accumulation of thymidine. Inhibition of the phosphorylation of histone of the chromosomes was also observed. Inhibition of phosphorous 32 incorporation into histones has been demonstrated by eupaphyssopin, eupaformosanin, and helenalin (27–29). Supposedly, phosphorylation of histones helps to regulate cellular proliferation (31). Energy for this process is supplied by the mitochondrial oxidative phosphorylation process. As can be seen, molephantinin suppressed basal respiration and adenosine diphosphate stimulated respiration, thus reducing available energy sources. Preliminary studies have indicated molephantinin is an initiation inhibitor of protein synthesis in Ehrlich ascites cells. Formation of the initiation complex requires initiation factors which possess an exposed sulfhydryl group which may be alkylated by molephantinin's functional moieties. There appears to be a positive correlation between protein synthesis inhibitors and reduction of oxidative phosphorylation (32).

Molephantinin appears to be one of the more potent germacranolides and is more active than the sesquiterpene lactone, helenalin (27–29). Molephantinin has been shown to have a similar mode of action on the Ehrlich ascites cells as other germacranolides and sesquiterpene lactones.

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ACKNOWLEDGMENTS

Supported by American Cancer Society Grant CH-19 and National Cancer Institute Grant CA-26466.

The authors thank Larry Carpenter and Melba Gibson for their technical assistance on this project.

Herbal Remedies of the Maritime Indians: Sterols and Triterpenes of *Achillea millefolium* L. (Yarrow)

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Abstract □ As part of ongoing studies of the medicinal aspects of Maritime flora, particularly the herbal remedies of the Micmac and Malecite Indians, the sterols and triterpenes of *Achillea millefolium* L. (Compositae), a widely used herbal remedy known commonly as yarrow, were determined. Using modern techniques, including nuclear magnetic resonance spectroscopy and combined GC–mass spectrometry, β-sitosterol was identified as the major sterol and α-amyrin as the major triterpene of this plant. The sterols stigmasterol, campesterol, and cholesterol and the triterpenes β-amyrin, taraxasterol, and pseudotaraxasterol were also identified. Successful therapeutic application of yarrow may be partly due to the presence of one or more of these compounds since many sterols and triterpenes exhibit a wide range of pharmacological activities. This is the first reported occurrence of cholesterol, campesterol, and the four triterpenes in yarrow.

Keyphrases □ *Achillea millefolium* L.—herbal remedies, extraction of triterpenes and sterols □ Triterpenes—extraction from *Achillea millefolium* L., herbal remedies, sterols □ Sterols—extraction from *Achillea millefolium* L., herbal remedies, triterpenes

Since the Trojan war (~1200 BC), *Achillea millefolium* L. has been used extensively by many cultures on different continents as a herbal remedy for various afflictions (1). While conducting studies on the medicinal aspects of the Maritime flora, particularly the herbal remedies of the Micmac and Malecite Indians (2, 3), it was observed that although *A. millefolium* (yarrow) had been widely used and extensively studied (1), very little had been reported regarding its sterols and triterpenes.

Some early articles and various screening papers have reported sterols, triterpenes (3–5), and saponins (6, 7) present in yarrow, although at least one paper stated that the plant contained no steroids (8). Only two authors have previously attempted to determine the nature of the sterols and triterpenes present in this plant. One of those authors documented the presence of stigmasterol and a sitosterol (9), while another reported the presence of β-sitosterol, its acetate, a phytol, and a diol (10).

Therefore, the examination of yarrow for the presence and nature of these compounds is described.

EXPERIMENTAL

Collection and Extraction—The aerial parts of *A. millefolium* were collected during the flowering stage from an open field near Scots Bay, Kings Co., Nova Scotia in September, 1979[1]. The plant material was dried in a forced-air oven at 60° and ground in a Wiley mill to a coarse powder (0.5 cm). This material (2.09 kg) was placed in a stainless steel tank and

[1] The plant material utilized in this investigation was identified as *Achillea millefolium* (L.) (Compositae) by Dr. M. J. Harvey, Department of Biology, Dalhousie University. Herbarium samples (Number 79-03) representing material collected for this investigation are available for inspection at the College of Pharmacy, Dalhousie University.

0022-3549/82/0600-0690$01.00/0