(ester and lactone CO) cm^{-1}. PMR ($CDCl_3$) δ 0.99 (d, J = 6 Hz, 9H, CH_3-4 and two CH_3-23), 1.40 (s, CH_3-10), 3.86 (s, $COOCH_3$), and 6.40 (d, J = 13 Hz, H-15).

Anal.—Calc. for $C_{26}H_{36}O_{11}$: *m/z* 524.2255 (M^+). Found: *m/z* 524.2259.

Compound XXXV could also be prepared by hydrogenation of II (504 mg) in 90% ethanol (100 ml) with platinum oxide (50 mg) in water at room temperature and atmospheric pressure. The reaction mixture was filtered by use of a silica gel column. The filtered solution was evaporated to yield colorless XXXV (498 mg).

Synthesis of XXXVI—To a solution of XXXV (66 mg) in methyl alcohol (10 ml) was added a solution of sodium borohydride (2 mg) in water (5 ml). The mixture was stirred at room temperature for 23 hr and then subjected to preparative TLC (chloroform-acetone 1:1) to give 2-hydroxy-2-deoxytetrahydrobrusatol lactol (XXXVI) as colorless crystals (31.6 mg).

XXXVI: mp 121–123°; IR (potassium bromide) 3440 (OH), 1715 (ester CO), 1040 (cyclic *sec*-OH); PMR ($CDCl_3$) δ 0.91 (d, J = 6 Hz, 9H, CH_3-4 and two CH_3-23), 1.50 (s, CH_3-10), 3.81 (s, $COOCH_3$), 4.13 (m, H-12), 4.20 (m, H-11), 4.60 (m, H-2 and H-3) and 5.52 (m, H-15 and H-16).

Anal.—Calc. for $C_{26}H_{40}O_{11}$: *m/z* 528.2571 (M^+). Found: *m/z* 528.2573.

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Antitumor Agents XLVI: *In Vitro* Effects of Esters of Brusatol, Bisbrusatol, and Related Compounds on Nucleic Acid and Protein Synthesis of P-388 Lymphocytic Leukemia Cells

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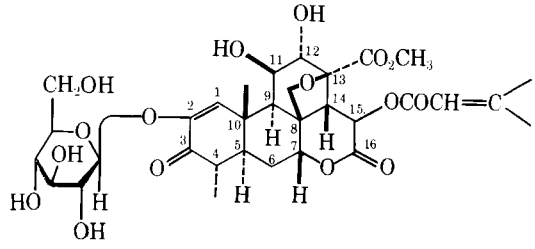
Abstract □ A series of esters of brusatol, bisbrusatol, and bruceantin were shown to have potent antileukemic activity. Antineoplastic activity was correlated with the ability of the compounds to suppress DNA and protein synthesis in P-388 lymphocytic leukemia cells. Compounds with high T/C% values successfully inhibited DNA polymerase activity and purine synthesis. The ability to inhibit protein synthesis during the elongation process also correlated positively with high antileukemic activity in this series of quassinoids. Dihydrofolate reductase activity and basal and adenosine diphosphate stimulated respiration of P-388 cells were also inhibited.

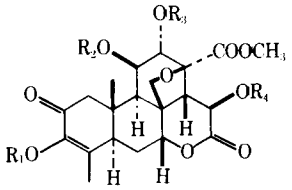
Keyphrases □ Brusatol—esters, *in vitro* effects on nucleic acid and protein synthesis, P-388 lymphocytic leukemia cells □ Bisbrusatol—esters, *in vitro* effects on nucleic acid and protein synthesis, P-388 lymphocytic leukemia cells □ Antitumor agents—*in vitro* effects of brusatol, bisbrusatol, and related compounds on nucleic acid and protein synthesis, P-388 lymphocytic leukemia cells

Bruceantin was first isolated from *Brucea antidysenterica* (1, 2) and is currently in Phase II clinical trials as an antineoplastic agent (3, 4). Brusatol, a derivative of bruceantin, was prepared by Lee *et al.* (5, 6) and shown to be active against P-388 lymphocytic leukemia cell growth (7). This laboratory demonstrated that bruceantin and brusatol reduced nucleic acid and protein synthesis (7, 8), purine synthesis (7), and oxidative phosphorylation processes (8) of P-388 cells. Based on the observation that a number of potent antileukemic agents have an ester in their structure, a series of brusatol related esters as well

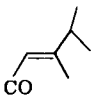


I: Bruceoside-A

0022-3549/82/0300-0345$01.00/0



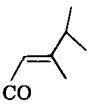
II: Brusatol: $R_1 = R_2 = R_3 = H$, $R_4 =$ 

III: Bruceantin: $R_1 = R_2 = R_3 = H$, $R_4 =$ 

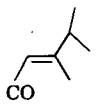
IV: Brusatol triacetate: $R_1 = R_2 = R_3 = COCH_3$, $R_4 =$ 

V: Bruceolide: $R_1 = R_2 = R_3 = R_4 = H$

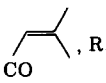
VI: 3-(3,4-Dimethyl-2-pentenoyl)bruceolide:

$R_1 =$  , $R_2 = R_3 = R_4 = H$

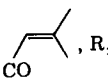
VII: 3-(3,4-Dimethyl-2-pentenoyl)bruceantin:

$R_1 = R_4 =$  , $R_2 = R_3 = H$

VIII: 12-Acetyl-3,15-disenecioyl bruceolide:

$R_1 = R_4 =$  , $R_2 = H$, $R_3 = COCH_3$

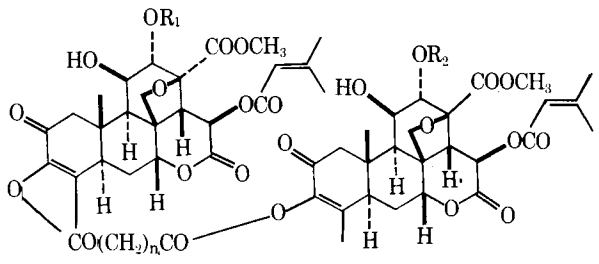
IX: 3,15-Disenecioyl bruceolide:

$R_1 = R_4 =$  , $R_2 = R_3 = H$

as bisbrusatolyl esters and related moieties were synthesized. These compounds were tested for antineoplastic activity and were active against P-388 lymphocytic leukemia growth (10). These ester derivatives of brusatol were examined for their *in vitro* effects on nucleic acid and protein synthesis of P-388 cells and the results are now reported.

EXPERIMENTAL

The compound bruceoside-A (I) was originally isolated from *Brucea javanica* (5). Brusatol (II) was obtained by treating bruceoside-A with 3*N* H_2SO_4–methanol (1:1) to hydrolyze the glycosidic linkage (5, 6). Bruceantin (III) was obtained from bruceoside-A by synthetic methods (11). The chemical synthesis, purification, and physical characterization



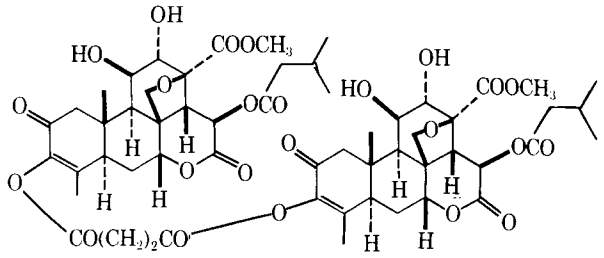
X: Bis-12,12′-diacetyl brusatolyl succinate: $R_1 = R_2 = COCH_3$, n = 2

XI: Bisbrusatolyl adipate: $R_1 = R_2 = H$, n = 4

XII: Bisbrusatolyl glutarate: $R_1 = R_2 = H$, n = 3

XIII: Bisbrusatolyl succinate: $R_1 = R_2 = H$, n = 2

XIV: Bisbrusatolyl malonate: $R_1 = R_2 = H$, n = 1



XV: Bisdihydrobrusatolyl succinate

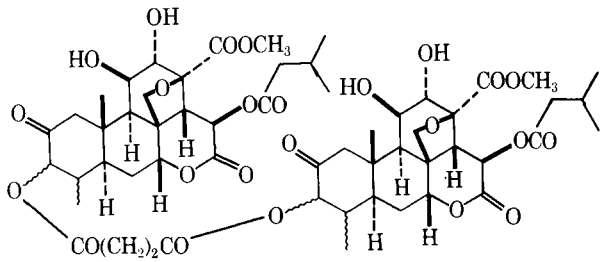
of compounds IV–XIX, *i.e.*, brusatol esters, bisbrusatolyl esters, and related derivatives, were reported recently by this laboratory (10, 11).

Antileukemic Screen—The UNC P-388 lymphocytic leukemia tumor line was maintained in DBA/2 male mice (~20 g) by inoculation with 10^6 P-388 cells on day 0, intraperitoneally. The tumor was transferred on day 8 to BDF_1 male mice (~18 g) for the testing of new agents. Test drugs were suspended by homogenization in 0.05% polysorbate 80 and administered 0.6 mg/kg intraperitoneally on days 1–14, utilizing 5-fluorouracil as a standard (12).

***In Vitro* Incorporation Studies**—BDF_1 ($C_{57}B1/6 \times DBA/j$) male mice were inoculated with 10^6 tumor cells as previously described. On day 9, the P-388 ascites cells were collected from the peritoneal cavity. *In vitro* incorporation studies (8) were carried out using 10^6 P-388 cells in minimum essential medium in 10% fetal calf serum and either 1 μCi of [6-^{3}H] thymidine (21.8 Ci/mmole), [6-^{3}H]uridine (22.4 Ci/mmole) or [4,5-^{3}H(N)]-L-leucine (56.5 Ci/mmole) in a total volume of 1 ml and incubated for 90 min at 37°. The final concentration of I–XIX was 10 μ*M*. Thymidine incorporation into DNA was terminated with perchloric acid containing pyrophosphate which was filtered on glass fiber paper by vacuum suction. The control value was 137,346 dpm/10^6 cells/90 min. RNA and protein incorporation assays were terminated with trichloroacetic acid and the macromolecule was collected on nitrocellulose membrane filter paper. The acid insoluble precipitates on the filter papers were placed in scintillation vials and counted in a toluene–oxtoxynol scintillation fluid. The control value for uridine incorporation was 36,442 dpm/10^6 cells/90 min and for leucine incorporation into protein the value was 8477 dpm/10^6 cells/90 min. Nuclear DNA polymerase activity was determined on isolated P-388 lymphocytic leukemia cell nuclei. The incubation medium was that used by Sawada *et al.* (13) except that [^{14}C]thymidine triphosphate (78.1 Ci/mmole) was used; the insoluble nucleic acids were collected on glass fiber paper discs[1], resulting in a control value of 24,568 dpm/mg nucleic protein. [^{14}C]Formic acid incorporation into purines was measured using 0.5 μCi of [^{14}C]formic acid (4.95 mCi/mmole) (14). The reaction medium was spotted on silica gel TLC plates and eluted with *n*-butanol–acetic acid–water (4:1:5). Using adenosine and guanosine as standards, the plates were scraped and the radioactivity determined. The control value was 10,621 dpm/mg of protein. Dihydrofolate reductase was determined by a spectrophotometric method (15) based on the rate of disappearance of 0.1 μmole of reduced NADP. The control resulted in an absorbance difference of 0.760 o.d. unit/hr/mg of protein using a 600×*g* (10 min) supernate.

Utilizing a test system (7) that differentiates between initiation and elongation inhibitors of protein synthesis, P-388 tumor cells were homogenized and incubated in tromethamine buffer (pH 7.6) containing KCl, $MgCl_2$, adenosine triphosphate, guanosine triphosphate, phosphoenolpyruvate, pyruvate kinase, dithiothreitol, the 19 basic amino acids, and [^{3}H]leucine (56.5 Ci/mmol) for 0–14 min at 37°. Test drugs were added at 10 μ*M* concentration to the incubation medium 90 sec after initiating the experiment. Samples were taken from the assay medium at 1 min intervals and spotted on filter paper[2]. The reaction was terminated with trichloroacetic acid and the protein extracted and counted. The test drugs were compared to the standards pyrocatechol violet, an initiator inhibitor, and emetine, an elongation inhibitor of protein synthesis at 75 μmoles. Oxidative phosphorylation studies were carried out on 9 day P-388 cells (9). Oxygen consumption was measured with an oxygen electrode[3] connected to an oxygraph. The reaction vessel contained 55 μmoles of sucrose, 22 μmoles of monobasic potassium phosphate, 22 μmoles of potassium chloride, 90 μmoles of succinate or 60 μmoles of α-ketoglutarate as substrate, and 22 μmoles of adenosine triphosphate. Test drugs were present in a final concentration of 10 μ*M*. After the basal metabolic level (state 4) was obtained, 0.257 μmole of

[1] GF/F.
[2] Whatman No. 3.
[3] Clark.

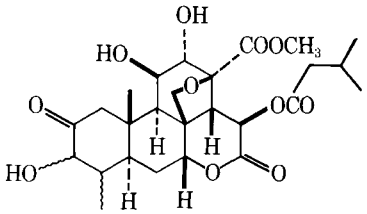


XVI: Bistetrahydrobrusatolyl succinate

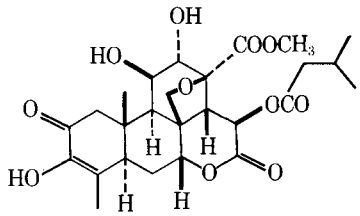
adenosine diphosphate was added to the vessel to obtain the adenosine diphosphate-stimulated respiration rate (state 3). The control values for states 4 and 3 respiration were 11.44 and 20.76 μl of oxygen consumed/min/mg of protein, respectively, using succinate as substrate. When α-ketoglutarate was used as substrate, states 4 and 3 respiration were 10.01 and 19.17 μl of oxygen consumed/min/mg of protein.

RESULTS

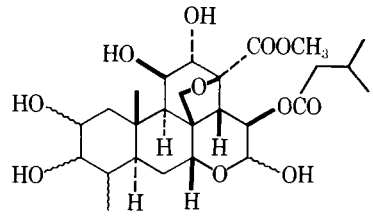
Using a dose of 0.6 mg/kg/day, compounds II, III, V–VII, IX, XI–XV, and XVIII demonstrated significant inhibitory activity against P-388 lymphocytic leukemia cell growth in BDF_1 mice. The bisbrusatolyl esters (XI–XIV) were in general more potent than brusatol (II), especially the succinate (XIII) and the malonate (XIV) with T/C = 217 and 272%, respectively. Reduction of the C-15 ester double bond did not decrease the activity (compare XV to XIII and XVIII to III). However, saturation of the diosphenol double bond gave either less active or inactive compounds



XVII: Tetrahydrobrusatol



XVIII: Dihydrobrusatol



XIX: 2-Hydroxy-2-deoxotetrahydrobrusatol lactol

(XVI, XVII, and XIX). Esterification of II at C-3 afforded VII and IX which were more active than II. Acetylation of hydroxyl groups at C-11 and C-12 gave compounds (IV, VIII, and X) which were either less active or inactive, indicating the importance of these hydroxyl groups for enhanced antileukemic activity.

Examination of the *in vitro* incorporation studies in P-388 lymphocytic leukemia cells demonstrated that compounds II, III, VII, IX, XI–XIV, and XVIII suppressed thymidine incorporation into DNA 41–59%. These same compounds inhibited uridine incorporation into RNA 16–59%. L-Leucine incorporation into protein was suppressed more than 70% by XIV and VIII and more than 50% by II and IX. Compounds III, V, VII, XI, XII, XV, and XVIII caused at least 40% inhibition of protein synthesis of P-388 cells.

Detailed examination of a number of enzymatic activities which are known to be suppressed by quassinoids subsequently followed. Nucleic DNA polymerase activity in P-388 was inhibited at least 50% by compounds XI, XII, and XIV and at least 40% by compounds II, III, VII, and IX. Purine synthesis was inhibited more than 50% by compounds III, VII, IX, and XIII–XV and more than 40% by II, X, XI, and XII. Dihydrofolate reductase activity was suppressed at least 40% by compounds I, III, IV, XI, XIII, XIV, and XV. The elongation process of protein synthesis compared with the emetine standard was inhibited greater than 60% by II, VII, XI, XIII, XIV, IX, and XII at 10 μ*M*. The quassinoids inhibited ongoing polypeptide synthesis. Oxidative phosphorylation processes were not inhibited as significantly as other metabolic events. However, state 4 (basal) respiration with succinate as the substrate was inhibited greater than 15% by compounds II, III, V–VII, IX, XI–XIII, XV, and XVIII. With α-ketoglutarate as the substrate, compounds II, III, VI, VII, IX–XV, XVII, and XVIII caused 15% state 4 inhibition. Adenosine diphosphate-stimulated respiration was inhibited 15% using succinate as substrate by compounds II, III, V, IX, XI, XII, XIV, and XVIII and by compounds II, III, VII, IX, XI–XV, XVII, and XVIII using α-ketoglutarate as substrate.

DISCUSSION

The quassinoids demonstrated that protein and nucleic acid metabolism were inhibited in a manner which correlated positively with their antileukemic activity when tested against P-388 lymphoid leukemia growth. Compounds with the higher T/C % values had a greater ability

Table 1—*In Vitro* Effects of Quassinoids on Nucleic Acid and Protein Synthesis of P-388 Lymphocytic Leukemia Cells

Drug, 10 μ*M* (*n* = 6)	% Control			*In Vivo* 0.6 mg/kg/day, T/C %
	Thymidine into DNA	Uridine into RNA	L-Leucine into Protein	
I Bruceoside A	65 ± 4[a]	72 ± 5[a]	54 ± 5[a]	118
II Brusatol	51 ± 5[a]	44 ± 4[a]	44 ± 3[a]	149
III Bruceantin	41 ± 3[a]	53 ± 4[a]	57 ± 3[a]	162
IV Brusatol triacetate	98 ± 7	94 ± 6	89 ± 6	102
V Bruceolide	74 ± 6	100 ± 7	60 ±4[a]	139
VI 3-(3,4-Dimethyl-2-pentonyl) bruceolide	91 ± 4[b]	106 ± 5	64 ± 4[a]	131
VII 3-(3,4-Dimethyl-2-pentonyl) bruceantin	53 ± 6[a]	69 ± 3[a]	59 ± 5[a]	194
VIII 12-Acetyl-3,15-disenecioyl bruceolide	112 ± 5	91 ± 4[b]	78 ± 6[a]	103
IX 3,15-Disenecioyl bruceolide	56 ± 4[a]	53 ± 5[a]	38 ± 3[a]	185
X Bis-12,12′-diacetyl brusatolyl succinate	98 ± 5	87 ± 6[c]	75 ± 4[a]	118
XI Bisbrusatolyl adipate	59 ± 3[a]	61 ± 5[c]	60 ± 5[a]	176
XII Bisbrusatolyl glutarate	55 ± 6[a]	75 ± 5[a]	57 ± 4[a]	176
XIII Bisbrusatolyl succinate	45 ± 3[a]	41 ± 4[a]	28 ± 4[a]	217
XIV Bisbrusatolyl malonate	55 ± 5[a]	52 ± 3[a]	17 ± 3[a]	272
XV Bisdihydrobrusatolyl succinate	62 ± 3[a]	60 ± 5[a]	57 ± 4[a]	193
XVI Bistetrahydrobrusatolyl succinate	111 ± 5	83 ± 5[a]	66 ± 5[a]	113
XVII Tetrahydrobrusatol	89 ± 6[b]	108 ± 6	76 ± 3[a]	120
XVIII Dihydrobrusatol	54 ± 5[a]	61 ± 5[a]	58 ± 4[a]	150
XIX 2-Hydroxy-2-deoxotetrahydrobrusatol lactol	88 ± 4[c]	93 ± 4	60 ± 4[a]	107
XX 0.05% Polysorbate 80 water	100 ± 5	100 ± 4	100 ± 6	100

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

Table II—*In Vitro* Effects of Quassinoids on Specific Processes of Nucleic Acid and Protein Synthesis and Respiration

Drugs, 10 μM ($n = 6$)	% Control							
	DNA Polymerase Activity	[^{14}C]Formate into Purines	Dihydrofolate Reductase Activity	Elongation Process of Protein Synthesis	Oxidative Phosphorylation			
					Succinate		α-Ketoglutarate	
					State 4	State 3	State 4	State 3
I	89 ± 6[a]	82 ± 6[b]	50 ± 4[b]	54 ± 6[b]	86 ± 4[b]	99 ± 7	85 ± 4[b]	93 ± 4
II	57 ± 5[b]	58 ± 4[b]	64 ± 5[b]	28 ± 3[b]	80 ± 3[b]	78 ± 5[b]	69 ± 3[b]	74 ± 4[b]
III	56 ± 7[b]	49 ± 4[b]	47 ± 4[b]	77 ± 4[b]	81 ± 5[b]	81 ± 4[b]	82 ± 4[b]	75 ± 3[b]
IV	79 ± 8[b]	93 ± 3	55 ± 8[b]	60 ± 5[b]	87 ± 3[b]	95 ± 3	86 ± 2[c]	114 ± 5
V	74 ± 7[b]	86 ± 5[c]	65 ± 4[b]	64 ± 6[b]	80 ± 4[b]	81 ± 4[b]	87 ± 5[c]	89 ± 4[c]
VI	77 ± 6[b]	81 ± 4[b]	81 ± 7[c]	57 ± 5[b]	82 ± 5[b]	90 ± 5[a]	82 ± 4[b]	89 ± 5[c]
VII	56 ± 5[b]	17 ± 2[b]	87 ± 6[a]	33 ± 4[b]	72 ± 3[b]	89 ± 3[b]	64 ± 6[b]	67 ± 3[b]
VIII	91 ± 4	91 ± 4	69 ± 7[b]	78 ± 4[b]	98 ± 5[b]	89 ± 5[c]	95 ± 6	92 ± 5
IX	58 ± 3[b]	46 ± 5[b]	58 ± 6[b]	53 ± 6[b]	66 ± 3[b]	72 ± 4[b]	67 ± 4[b]	72 ± 5[b]
X	91 ± 7	81 ± 5[b]	72 ± 5[b]	67 ± 5[b]	89 ± 3[c]	92 ± 6	83 ± 5[b]	101 ± 4
XI	47 ± 6[b]	55 ± 5[b]	65 ± 5[b]	38 ± 3[b]	83 ± 2[b]	73 ± 5[b]	68 ± 4[b]	73 ± 4[b]
XII	47 ± 5[b]	55 ± 6[b]	65 ± 4[b]	46 ± 4[b]	83 ± 3[b]	77 ± 5[b]	70 ± 4[b]	68 ± 5[b]
XIII	70 ± 6[b]	43 ± 5[b]	53 ± 3[b]	35 ± 4[b]	73 ± 4[b]	89 ± 6[c]	57 ± 4[b]	64 ± 6[b]
XIV	43 ± 4[b]	45 ± 4[b]	50 ± 2[b]	20 ± 3[b]	85 ± 5[b]	76 ± 5[b]	70 ± 5[b]	76 ± 5[b]
XV	78 ± 5[b]	43 ± 3[b]	59 ± 4[b]	31 ± 5[b]	75 ± 6[b]	90 ± 4[c]	52 ± 5[b]	61 ± 4[b]
XVI	72 ± 4[b]	100 ± 7	64 ± 5[b]	70 ± 4[b]	91 ± 4	90 ± 5[c]	94 ± 4	96 ± 6
XVII	91 ± 5	108 ± 4	62 ± 4[b]	89 ± 4[c]	87 ± 3[b]	85 ± 3[b]	83 ± 6[b]	70 ± 3[b]
XVIII	81 ± 4[b]	69 ± 3	—	64 ± 5[b]	61 ± 5[b]	62 ± 5[b]	70 ± 4[a]	74 ± 4[b]
XIX	85 ± 3[b]	96 ± 5	—	80 ± 6[b]	90 ± 6	96 ± 4	102 ± 3	86 ± 5[c]
XX	100 ± 5	100 ± 6	100 ± 7	100 ± 4	100 ± 5	100 ± 4	100 ± 5	100 ± 4

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

to suppress protein synthesis (compounds XIII, XIV, XV, XI, and VII). The elongation step of translocation appeared to be affected preferentially over initiation events of protein synthesis. This observation is consistent with previous data for compound II on rabbit reticulocyte protein synthesis and compound III on yeast. The inhibition of DNA and RNA was also positively correlated with antineoplastic activity although DNA synthesis was suppressed slightly less than the inhibition of protein synthesis. Nuclear DNA polymerase activity was inhibited ~50% by compounds with T/C % >150 although there were exceptions. Purine synthesis inhibition correlated more positively with inhibition of nucleic acid synthesis and antineoplastic activity than inhibition of DNA polymerase activity by these quassinoids. Dihydrofolate reductase activity was affected in a similar manner although the regulatory enzyme of purine synthesis, phosphoribosyl pyrophosphate aminotransferase, was identified as being inhibited when these drugs are administered *in vivo*. However, only marginal inhibitory effects were observed after *in vitro* (6) incubation of the quassinoids with this regulatory enzyme. Surprisingly, 3-(3,4-dimethyl-2-pentonyl) bruceantin (VII) inhibited purine synthesis 83%, apparently at some site other than dihydrofolate reductase. The inhibition of purine synthesis by VII probably contributes significantly to the T/C % = 194. Previously, data indicated that quassinoids affected anaerobic and aerobic respiration of P-388 cells and that state 4 and state 3 respiration were inhibited ~50% by quassinoids at 0.015 mM. However, at 0.010 mM concentrations, the effect on oxidative phosphorylation was not as dramatic. Basal and coupled oxidative phosphorylation was inhibited ~15% by those compounds possessing a T/C % ≥125 in the antineoplastic screen. Reduction of oxidative phosphorylation processes would reduce the available energy for macromolecular synthesis in rapidly dividing cells, thus possibly indirectly affecting nucleic acid and protein synthetic pathways. Hence, it may be concluded that the optimum structural requirements for potent antileukemic activity and metabolic inhibition of P-388 cells are (*a*) bis-esters of brusatol with an ester carbon chain length of one or two and (*b*) a C-3, C-15 disenecioate or di-3,4-dimethyl-2-pentenoate side chain of bruceolide. Both types of structure require an enone system in ring A and a free hydroxyl group at positions C-11 and C-12.

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