## CONCLUSIONS

The first 1 mm or so of frozen and reconstituted cattleskin[4] acts as a homogeneous barrier to penetration of the skin by chemicals. The diffusion coefficient of levamisole in this barrier is close to its expected value in hair follicles or sweat ducts. The results of the present study suggest that polar molecules such as levamisole will penetrate cattle skin much more rapidly than human skin.

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[4] It should be noted that freezing the skin could very well alter its cellular geography to such an extent that the results of this study may not reflect properties of intact fresh skin. If this is true, then the value of the present results to *in vivo* behavior will only be realized when *in vivo* experiments are completed. However, the results clearly indicate that frozen and reconstituted cattle skin has very different barrier properties to similarly treated human skin and that the stratum corneum of the former skin does not appear to be the rate-determining barrier to penetration.

## ACKNOWLEDGMENTS


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Grateful acknowledgment is made to Professor P. Bhathal, University of Melbourne, for assistance with the human skin measurements and to Mr. Ian Ray, Victorian College of Pharmacy and Mr. John Standing, Alfred Hospital, Melbourne for assistance in harvesting animal skins.


# Antitumor Agents XLVIII: Structure–Activity Relationships of Quassinoids as *In Vitro* Protein Synthesis Inhibitors of P-388 Lymphocytic Leukemia Tumor Cell Metabolism


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Received April 6, 1981, from the *Division of Medicinal Chemistry, School of Pharmacy, and the *Department of Biochemistry, School of Medicine, University of North Carolina, Chapel Hill, NC 27514.* Accepted for publication August 4, 1981.



**Abstract** □ A series of brusatol, bisbrusatol, and bruceantin esters were examined for their ability to inhibit protein synthesis in P-388 lymphocytic leukemia cells. Compounds which produced high T/C % values (170–272) resulted in $ID_{50}$ of 5.4–15.5 $\mu M$ for inhibition of whole cell protein synthesis, $ID_{50}$ of 1.3–13 $\mu M$ for inhibition of endogenous protein synthesis in cell homogenates, and $ID_{50}$ of 1.9–6 $\mu M$ for inhibition of polyuridine directed polyphenylalanine synthesis using "runoff" ribosomes and a "pH 5" enzyme preparation. The polyuridine directed polyphenylalanine synthesis requires neither initiation nor termination factors, suggesting that quassinoids are exclusively elongation inhibitors. Bruceantin, brusatol, and bisbrusatolyl malonate allowed a runoff of the polyribosomes to 80S free ribosomes. However, formation of the ternary complex and 80S initiation complex were not inhibited by the quassinoids. Thus, these agents do not affect the individual steps leading to the formation of a stable 80S initiation complex in P-388 cells. Brusatol, bruceantin, and bisbrusatolyl malonate inhibited the formation of the first peptide bond between puromycin and [$^3$H]methionyl-transfer RNA bound to the initiation complex, indicating peptidyl transferase activity is inhibited by the quassinoids in P-388 cells. These studies also suggest that the free 80S ribosome is the site of binding by the quassinoid. Ribosomes actively conducting protein synthesis will continue protein synthesis and terminate before the quassinoids bind. This proves quassinoids are elongation inhibitors of tumor cells. A strong correlation was observed between potent antileukemic activity and the ability to inhibit protein synthesis in P-388 lymphocytic leukemia cells.



**Keyphrases** □ Protein synthesis—inhibition by quassinoids, P-388 lymphocytic leukemia cells □ Quassinoids—inhibition of protein synthesis, P-388 lymphocytic leukemia cells □ Structure–activity relationships—quassinoids, inhibition of protein synthesis, P-388 lymphocytic leukemia cells □ Antitumor agents—quassinoids, inhibition of protein synthesis, P-388 lymphocytic leukemia cells


Bruceantin, a quassinoid now in phase II clinical trials, was first isolated from *Brucea antidysenterica* (1, 2). Subsequently, bruceoside A was isolated from *Brucea javanica* (3) and brusatol was derived chemically from bruceoside A. It was demonstrated (4) that bruceantin inhibited protein synthesis in HeLa cells by 90% at 2 $\mu M$, whereas DNA and RNA synthesis were inhibited 60 and 15%, respectively. Protein synthesis was inhibited 79% in rabbit reticulocytes by bruceantin at 0.1 $\mu M$ (4). Liao (4) postulated that bruceantin was an initiation inhibitor of




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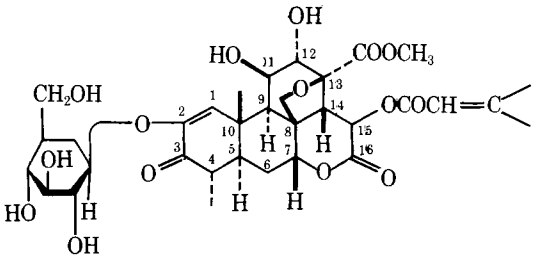
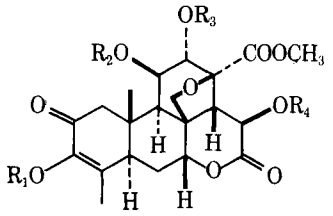
protein synthesis. However, it was shown (5), using the yeast organism *Saccharomyces cereviseae*, that bruceantin blocks the peptidyl transferase site of the ribosome and inhibits the peptide chain elongation reaction. Interestingly, the drug binds to the free ribosome as opposed to the ribosome actively engaged in protein synthesis (5).

Brusatol has been observed to inhibit DNA, RNA, and protein synthesis of P-388 lymphocytic leukemia cells at 0.015 m*M* concentration resulting in 84, 62, and 86% inhibition, respectively. At 0.005 m*M* the inhibitions are 38, 44, and 60%, respectively (6). Brusatol was shown to be an elongation inhibitor by using the rabbit reticulocyte pro-

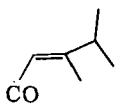


I[bruceoside A]



II[brusatol]: $R_1 = R_2 = R_3 = H$, $R_4$ =

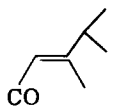
III[bruceantin]: $R_1 = R_2 = R_3 = H$, $R_4$ =



IV[brusatol triacetate]: $R_1 = R_2 = R_3 = COCH_3$, $R_4$ =

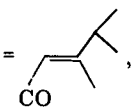
V[bruceolide]: $R_1 = R_2 = R_3 = R_4 = H$

VI[3-(3,4-dimethyl-2-pentenoyl)bruceolide]: $R_1$ =



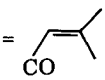
, $R_2 = R_3 = R_4 = H$

VII[3-(3,4-dimethyl-2-pentenoyl)bruceantin]: $R_1 = R_4$ =



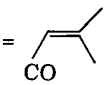
, $R_2 = R_3 = H$

VIII[12-acetyl-3,15-disenecioyl bruceolide]: $R_1 = R_4$ =

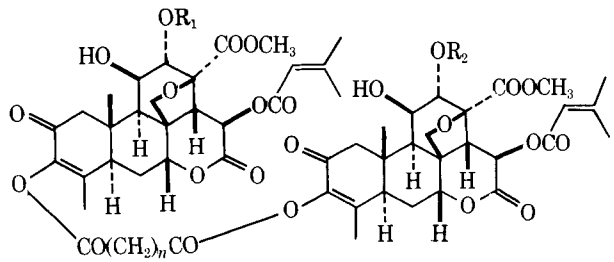


, $R_2$ = H, $R_3 = COCH_3$

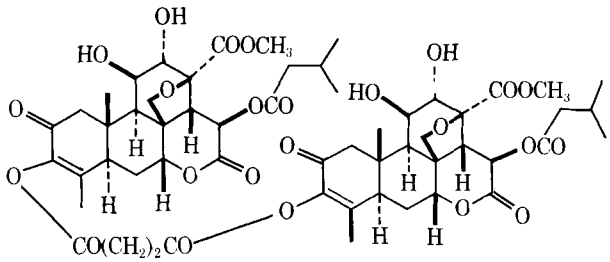
IX[3,15-disenecioyl bruceolide]: $R_1 = R_4$ =



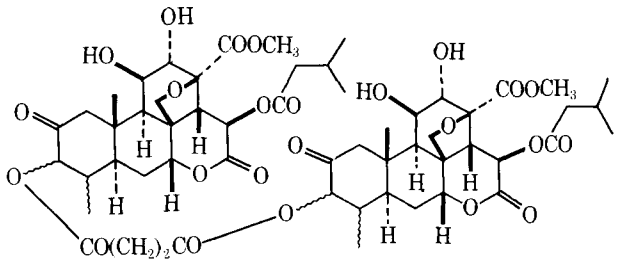
, $R_2 = R_3 = H$



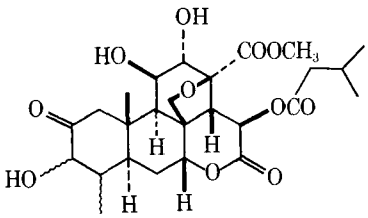
X[bis-12,12′-diacetyl brusatolyl succinate]: $R_1 = R_2 = COCH_3$, n = 2
XI[bisbrusatolyl adipate]: $R_1 = R_2 = H$, n = 4
XII [bisbrusatolyl glutarate]: $R_1 = R_2 = H$, n = 3
XIII[bisbrusatolyl succinate]: $R_1 = R_2 = H$, n = 2
XIV[bisbrusatolyl malonate]: $R_1 = R_2 = H$, n = 1



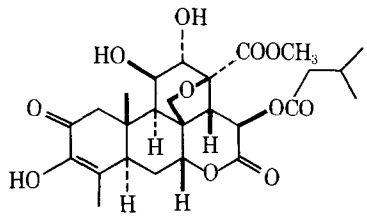
XV[bis-dihydrobrusatolyl succinate]



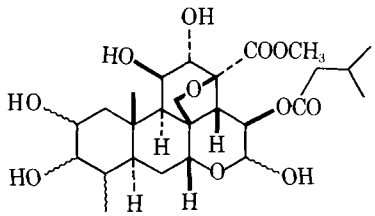
XVI[bis-tetrahydrobrusatolyl succinate]



XVII [tetrahydrobrusatol]



XVIII [dihydrobrusatol]



XIX[2-hydroxy-2-deoxotetrahydrobrusatol lactol]

tein synthesis system. Brusatol probably inhibits peptidyl transferase activity (7). Bruceantin and brusatol are active against P-388 lymphocytic leukemia growth *in vivo* between 100 μg/kg to 1 mg/kg depending on the host strain of mice used (8). A series of brusatol esters and bisbrusatolyl esters were synthesized and characterized previously (9).

The present study reports the structure–activity relationships between the antileukemic activity and the ability to suppress protein synthesis elongation processes.

## RESULTS AND DISCUSSION

Potent antileukemic activity against P-388-UNC lymphocytic leukemia was observed with compounds VII, IX, XIII, XIV, and XV giving T/C % ≧ 180 at 0.6 mg/kg/day in $BDF_1$ male mice. The results are comparable with the standard, fluorouracil, at 25 mg/kg/day. Moderate activity (T/C % ≧ 140–176) was observed with compounds II, III, XI, XII, and VIII. The bisbrusatolyl esters (XI–XIV) were more potent than brusatol (II) or bruceoside A (I), respectively, the succinate (XIII) and malonate (XIV) esters with T/C % of 217 and 272, respectively. Reduction of the $C_{15}$ ester double bond did not decrease the antileukemic activity significantly (compare XV to XIII and XVIII to III). However, saturation of the diosphenol double bond resulted in either less activity or an inactive compound (XVI, XVII, and XIX). Esterification of II at the C-3 position gave VII and IX which were more active than II. Compounds formed by acetylation of the hydroxyl groups at C-11 and C-12 possessed little antileukemic activity (IV, VIII, and X).

Whole cell protein synthesis of P-388 cells was inhibited maximally (92%) by compound XIV (Table I) followed by compounds II, IX, and XIII (78–69%) and compounds I, III, V, VII, XII, XV, and XVIII (60–53%) at 15 μ*M* final concentration. The quassinoids followed a dose response from 5–15 μ*M*. Examination of the lysate protein synthesis assay demonstrated that compounds II, IX, XIII, XIV, and XV caused >80% inhibition, whereas compounds VII, XI, and XII resulted in >70% inhibition of protein synthesis (Table II).

Compounds II and III have been previously demonstrated to be elongation inhibitors in normal rabbit reticulocytes and compound XIV in the P-388 tumor line. For this reason a series of experiments were conducted to establish the inhibition mechanism by compounds II and III in the P-388 leukemia cells. A comparison of Fig. 2 with Fig. 1 shows that compounds II, III, and XIV all allow accumulation of the 80S ribosome peak similar to pyrocatechol violet rather than emetine. The result suggests the quassinoids allow completion of the already initiated polypeptide chain synthesis and release of the free 80S ribosome from the polyribosome before totally inhibiting protein synthesis. The quassinoids had little or no effect on the formation of either the ternary complex[1] or the 80S initiation complex[2] (Table III). The quassinoids II, III, and XIV behaved in these assays more like emetine than pyrocatechol violet indicating that they did not inhibit initiation events of polypeptide chain synthesis.

In an additional study, the formation of the 80S initiation complex[2] and peptide bond formation was examined by treating P-388 cell lysates with the elongation inhibitor chlortetracycline, which specifically inhibited binding of the aminoacyl-transfer RNA to the ribosome A site but did not inhibit the peptidyl transferase reaction. When [$^3$H]methionyl-transfer RNA was added to the system, most of the radioactivity was found associated with the 80S initiation complex. Addition of poly-adenosine-uridine-guanosine to the chlortetracycline treated lysate allowed formation of an 80S initiation complex (Fig. 3). The complex then reacted with puromycin followed by the puromycin-induced release of [$^3$H]methionine from the 80S complex (Fig. 4). The quassinoids II, III, and XIV did not appear to inhibit the formation of the 80S initiation complex, but did inhibit puromycin release of methionine indicating that the quassinoids inhibit P-388 peptide bond formation. Examination of the formation of aminoacyl-transfer RNA with phenylalanine, leucine, or methionine indicated that these agents had no effect on the activation of amino acid for incorporation into polypeptides. These studies suggest that the quassinoids are elongation inhibitors of protein synthesis, probably inhibiting peptidyl transferase activity. The quassinoids do not inhibit the synthesis of ongoing polypeptide synthesis but rather appear to bind to free P-388 80S ribosomes. The 80S peak which accumulates in the presence of the quassinoids is actually an 80S initiation complex

[1] eIF·Guanosine triphosphate·[$^3$H]methionyl-transfer RNA.

[2] 80S·Adenosine-uridine-guanosine·eIF·guanosine triphosphate·[$^3$H]methioinyl-transfer RNA.

**Table I—Effect of Quassinoids on Protein Synthesis of P-388 Lymphocytic Leukemia**

| Drugs | Control Protein Synthesis, % Concentration, $\mu M$ 5 | 10 | 15 | T/C | $ID_{50}$ |
|---|---|---|---|---|---|
| I. Bruceoside A | 70 | 48 | 42 | 121 | 9.7 |
| II. Brusatol | 61 | 44 | 22 | 149 | 7.5 |
| III. Bruceantin | 74 | 57 | 46 | 146 | 13.1 |
| IV. Brusatol triacetate | 94 | 89 | 79 | 102 | 110.0 |
| V. Bruceolide | 72 | 60 | 40 | 139 | 12.1 |
| VI. 3-(3,4-Dimethyl-2-pentenoyl)-bruceolide | 68 | 64 | 57 | 131 | 24.8 |
| VII. 3-(3,4-Dimethyl-2-pentenoyl)-bruceantin | 66 | 59 | 48 | 194 | 13.9 |
| VIII. 12-Acetyl-3,15-disenecioyl bruceolide | 91 | 78 | 69 | 103 | 37.2 |
| IX. 3,15-Disenecioyl bruceolide | 52 | 38 | 31 | 185 | 6.0 |
| X. Bis-12,12′-diacetyl brusatolyl succinate | 83 | 75 | 67 | 118 | 37.0 |
| XI. Bisbrusatolyl adipate | 70 | 60 | 51 | 176 | 15.3 |
| XII. Bisbrusatolyl glutarate | 63 | 57 | 43 | 176 | 11.9 |
| XIII. Bisbrusatolyl succinate | 58 | 40 | 30 | 217 | 6.8 |
| XIV. Bisbrusatolyl malonate | 54 | 17 | 8 | 272 | 5.4 |
| XV. Bisdihydrobrusatolyl succinate | 60 | 57 | 47 | 193 | 13.8 |
| XVI. Bistetrahydrobrusatolyl succinate | 75 | 66 | 57 | 113 | 21.6 |
| XVII. Tetrahydrobrusatol | 85 | 76 | 56 | 120 | 17.8 |
| XVIII. Dihydrobrusatol | 72 | 58 | 43 | 150 | 12.3 |
| XIX. 2-Hydroxy-2-deoxotetrahydrobrusatol lactol | 77 | 70 | 65 | 107 | 48.1 |
| XX. 0.05% Polysorbate·water | 100 | 100 | 100 | 100 | |

rather than true runoff ribosomes. The acceleration of 80S initiation complex has been reported previously with bruceantin in yeast, brusatol in rabbit reticulocytes, and bisbrusatolyl malonate in P-388 cells. Table IV demonstrates that the quassinoids inhibit polyuridine directed synthesis of polyphenylalanine, a study carried out on purified runoff ribosomes isolated from 10-day P-388 cells. Polyuridine directed polyphenylalanine synthesis does not require the normal initiation and termination reactions; consequently, this inhibition is of the elongation type exclusively. Table IV shows that compound XIV caused 92% inhibition at 15 $\mu M$. Compounds VII and XIII result in greater than 80% inhibition of polyphenylalanine synthesis, whereas compounds II, IX, XI, and XII caused greater than 75% inhibition at 15 $\mu M$.

The structural rquirements for inhibition of protein synthesis of P-388 leukemia cells are bis-esters of brusatol with an alkyl side chain of the ester of 1 or 2 carbons, or a C-3 or C-15 disenecioate or di-3,4-dimethyl-2-pentenoate side chain of bruceolide. Those compounds which demonstrated potent activity possessed an enone system in ring A and free hydroxy groups at C-11 and C-12. The same structural requirements are needed for antileukemic activity and *in vitro* inhibition of protein synthesis of P-388 cells. Analysis of the inhibition of polyuridine directed polyphenylalanine synthesis in Table IV and T/C % values obtained in the antileukemic screen indicated that there was a negative correlation coefficient of 0.84, *i.e.*, the higher the T/C % value obtained for the esters, the greater the degree of inhibition of protein synthesis in P-388 lymphocytic leukemic cells.

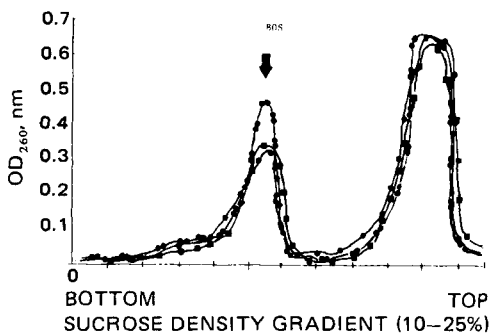



**Figure 1**—*Effects of pyrocatechol violet and emetine on the P-388 ribosome profile. Key:* ●—●, *control;* —● ●—, *pyrocatechol violet; and* ■—■, *emetine.*

**Table II—Effects of Quassinoids on P-388 Lymphocytic Leukemia Cell Lysate Using Endogenous mRNA**

| Drugs | Control, % 1 $\mu M$ | 10 $\mu M$ | 100 $\mu M$ | $ID_{50}$ |
|---|---|---|---|---|
| I. Bruceoside A | 76.8 | 53.8 | 47.3 | 38.3 |
| II. Brusatol | 76.8 | 28.4 | 10.9 | 6.0 |
| III. Bruceantin | 85.8 | 76.5 | 61.9 | 6.0 |
| IV. Brusatol triacetate | 87.4 | 60.2 | 53.6 | 125.0 |
| V. Bruceolide | 76.4 | 64.4 | 55.4 | 268.0 |
| VI. 3-(3,4-Dimethyl-2-pentenoyl)bruceolide | 70.6 | 57.3 | 53.9 | 695.0 |
| VII. 3-(3,4-Dimethyl-2-pentenoyl)bruceantin | 54.2 | 33.1 | 26.0 | 1.7 |
| VIII. 12-Acetyl-3,15-disenecioyl bruceolide | 91.9 | 77.6 | 67.0 | 125.0 |
| IX. 3,15-Disenecioyl bruceolide | 69.6 | 52.8 | 16.8 | 13.0 |
| X. Bis-12,12′-diacetyl brusatolyl succinate | 85.7 | 67.1 | 62.4 | 1400.0 |
| XI. Bisbrusatolyl adipate | 50.5 | 37.6 | 21.5 | 1.3 |
| XII. Bisbrusatolyl glutarate | 69.4 | 46.1 | 21.4 | 6.8 |
| XIII. Bisbrusatolyl succinate | 62.0 | 34.9 | 18.1 | 2.7 |
| XIV. Bisbrusatolyl malonate | 57.8 | 19.9 | 8.7 | 1.7 |
| XV. Bisdihydrobrusatolyl succinate | 57.9 | 31.3 | 13.9 | 2.0 |
| XVI. Bistetrahydrobrusatolyl succinate | 84.7 | 69.9 | 54.9 | 122.0 |
| XVII. Tetrahydrobrusatol | 89.9 | 83.9 | 78.3 | 1930.0 |
| XVIII. Dihydrobrusatol | 69.4 | 63.8 | 43.0 | 50.0 |
| XIX. 2-Hydroxy-2-deoxotetrahydrobrusatol lactol | 86.3 | 79.8 | 67.1 | 1120.0 |
| XX. 0.05% Polysorbate·water | 100.0 | 100.0 | 100.0 | |

## EXPERIMENTAL

**Source of Compounds**—Bruceoside A (1) originally was isolated from *Brucea javanica* (1, 2). Brusatol (II) was obtained by treating bruceoside A with 3 $N$ $H_2SO_4$–methanol (1:1) to hydrolyze the glycosidic linkage (3). Bruceantin (III) was obtained from bruceoside A by the synthetic method (20). The chemical synthesis, purification, and physical characteristics of compounds IV–XIX, *i.e.*, brusatol esters, bisbrusatolyl esters, and related derivatives, were reported elsewhere (9).

**P-388 Lymphocytic Leukemia Antitumor Screen**—The P-388 lymphocytic leukemia cell line was maintained in DBA/2 male mice (~20 g). For the antineoplastic screen, $10^6$ cells were injected intraperitoneally into $BDF_1$ male mice (~20 g) on day 0. Test compounds were homogenized in 0.05% polysorbate 80–water and administered on days 1–14. The average number of days survived for each group was determined and T/C % values were calculated (10). Fluorouracil was used as a positive standard.

Studies of the effects of quassinoid esters on protein synthesis were conducted on P-388 cells harvested on day 10. P-388 lymphocytic leukemia lysates were prepared by the method of Kruh *et al.* (11). The following were isolated from P-388 lysates by literature techniques: runoff ribosomes (12), "pH 5" enzyme (11), and uncharged transfer RNA (13). The P-388 lymphocytic leukemia cell initiation factors for protein synthesis were prepared as decribed previously (14). [$^3$H]Methionyl-transfer RNA was prepared from P-388 cell transfer RNA by the method of Takeishi *et al.* (15). The effects of the brusatol esters on endogenous protein synthesis (4) of P-388 lysates were carried out in a reaction

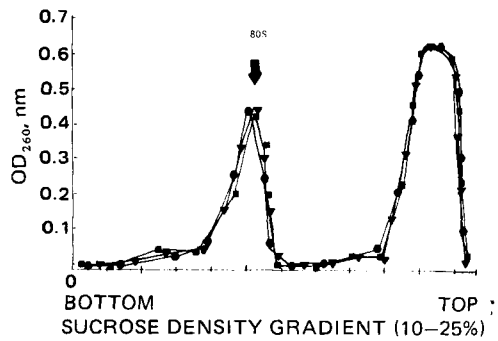



**Figure 2**—*Effects of brusatol, bruceantin, and bisbrusatolyl malonate on the P-388 ribosomal profile. Key:* ■, *brusatol;* ●, *bruceantin; and* ▼, *bisbrusatolyl malonate.*

**Table III—Effects of Bisbrusatolyl Malonate on Ternary and 80S Complex Formation**

| | Concentration, $\mu M$ | Complex Formation, pmole | Control, % |
|---|---|---|---|
| **Ternary Complex Formation** | | | |
| Control | | 2.10 | 100 |
| + Emetine | 100 | 2.02 | 96 |
| + Pyrocatechol violet | 100 | 0.21 | 10 |
| III. + Bruceantin | 25 | 2.01 | 96 |
| II. + Brusatol | 25 | 1.97 | 94 |
| XIV. + Bisbrusatolyl malonate | 25 | 1.81 | 86 |
| **80S Initiation Complex Formation** | | | |
| Control | | 1.82 | 100 |
| + Emetine | 100 | 1.49 | 82 |
| + Pyrocatechol violet | 100 | 0.36 | 20 |
| III. + Bruceantin | 25 | 1.64 | 90 |
| II. + Brusatol | 25 | 1.55 | 85 |
| XIV. + Bisbrusatolyl malonate | 25 | 1.51 | 83 |

mixture (0.5 ml) containing 10 m$M$ tromethamine (pH 7.6), 76 m$M$ KCl, 1 m$M$ adenosine triphosphate, 0.2 m$M$ guanosine triphosphate, 15 m$M$ creatine phosphate, 2 m$M$ $MgCl_2$, 1 m$M$ dithiothreitol, 0.1 m$M$ of each of the essential amino acids, 0.9 mg/ml creatine phosphokinase, and 20 $\mu$Ci [$^3$H]leucine (56.6 Ci/mmole). An aliquot of the reaction mixture was incubated at 30°. After 90 sec of incubation, test drugs or the standards (pyrocatechol violet or emetine) were added to a final concentration of 1, 10, and 100 $\mu M$. At 1-min intervals, 50-$\mu$l aliquots were removed from the reaction tubes and spotted on filter papers[3] which were treated for 10 min in boiling 5% trichloroacetic acid, followed by 10 min in cold 5% trichloroacetic acid and washed with cold 5% trichloroacetic acid, ether–ethanol (1:1), and ether. The filter papers were dried and counted in scintillation fluid.

The effects of bruceantin, brusatol, bisbrusatolyl malonate, pyrocatechol violet, and emetine on the ribosome profile (4) of P-388 cell lysates were assayed using the reaction medium described previously (500 $\mu$l). Following drug addition to a 100-$\mu M$ final concentration, the reaction was incubated for 4 min at 37°. The reaction was terminated in ice and gradient buffer consisting of 1 ml of tromethamine (pH 7.6), 10 m$M$ KCl, and 1.5 m$M$ $MgCl_2$-$6H_2O$ was added. The mixture was layered over 36 ml of 10–25% linear sucrose gradient (4), prepared in gradient buffer, and centrifuged for 165 min at 25,000 rpm in a swinging bucket rotor[4] at 4°. The absorbance profile at 260 nm was determined with a flow cell (light path 0.2 cm) attached to a spectrophotometer[5].

The reaction medium for the polyuridine (poly U) directed polyphenylalanine synthesis (16) contained 50 m$M$ tromethamine (pH 7.6), 12.5 m$M$ magnesium acetate, 80 m$M$ KCl, 5 m$M$ creatine phosphate, 0.05 mg/ml creatine phosphokinase, 0.36 mg/ml polyuridine[6] ($A_{280}/A_{260}$ = 0.34), 0.5 $\mu$Ci [$^{14}$C]phenylalanine (536 mCi/mmole), 75 $\mu$g uncharged P-388 cell transfer RNA, 70 $\mu$g of P-388 "pH 5" enzyme preparation, and 0.9 $A_{260}$ of P-388 cell runoff ribosomes. Test drugs I–XIX were present in 5-, 10-, and 15-$\mu M$ concentrations. Incubation was for 20 min at 30°,

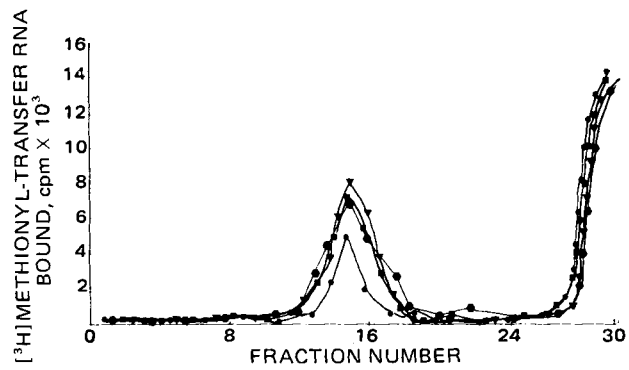



**Figure 3**—*Formation of the 80S initiation complex of P-388 cell system (linear sucrose gradient centrifugation). Key: ●, control; ■, brusatol; ●, bruceantin; and ▼, bisbrusatolyl malonate.*

[3] Whatman No. 3.
[4] Beckman. SW 27.
[5] Gilford.
[6] Miles Laboratory, Inc.

**Table IV—Inhibition by Quassinoids of Polyuridine Directed Polyphenylalanine Syntheses of 10-Day P-388 Cells**

| Drugs | Control Protein Synthesis, % Concentration, $\mu M$ 5 | 10 | 15 | $ID_{50}$ |
|---|---|---|---|---|
| I. Bruceoside A | 76 | 63 | 40 | 12.5 |
| II. Brusatol | 54 | 43 | 23 | 6.4 |
| III. Bruceantin | 75 | 58 | 34 | 11.5 |
| IV. Brusatol triacetate | 92 | 87 | 80 | 90.0 |
| V. Bruceolide | 77 | 55 | 38 | 11.6 |
| VI. 3-(3,4-Dimethyl-2-pentenoyl)bruceolide | 80 | 67 | 45 | 13.5 |
| VII. 3-(3,4-Dimethyl-2-pentenoyl)bruceantin | 44 | 36 | 19 | 2.9 |
| VIII. 12-Acetyl-3,15-disenecioyl bruceolide | 90 | 80 | 72 | 46.0 |
| IX. 3,15-Disenecioyl bruceolide | 48 | 39 | 20 | 4.4 |
| X. Bis-12,12′-diacetyl brusatolyl succinate | 84 | 71 | 60 | 21.5 |
| XI. Bisbrusatolyl adipate | 53 | 41 | 22 | 6.0 |
| XII. Bisbrusatolyl glutarate | 51 | 40 | 22 | 5.2 |
| XIII. Bisbrusatolyl succinate | 39 | 27 | 15 | 2.7 |
| XIV. Bisbrusatolyl malonate | 31 | 18 | 8 | 1.9 |
| XV. Bisdihydrobrusatolyl succinate | 45 | 36 | 21 | 3.4 |
| XVI. Bistetrahydrobrusatolyl succinate | 81 | 66 | 50 | 15.0 |
| XVII. Tetrahydrobrusatol | 83 | 70 | 48 | 14.5 |
| XVIII. Dihydrobrusatol | 79 | 65 | 42 | 13.0 |
| XIX. 2-Hydroxy-2-2-deoxotetrahydrobrusatol lactol | 80 | 72 | 65 | 36.0 |
| XX. 0.05% Polysorbate-water | 100 | 100 | 100 | |

after which a 35-$\mu$l aliquot was spotted on filter paper[3] and processed as indicated previously.

The reaction medium (200 $\mu$l) used to measure the formation of the 80S initiation complex and the methionyl puromycin reaction (17) contained 15 m$M$ tromethamine (pH 7.6), 80 m$M$ KCl, 1 m$M$ adenosine triphosphate, 0.5 m$M$ guanosine triphosphate, 20 m$M$ creatine phosphokinase, 3 m$M$ magnesium acetate, 0.1 m$M$ edetic acid, 1 m$M$ dithiothreitol, 0.1 m$M$ each of the 19 essential amino acids, 3 mg of P-388 cell lysates, 100 $\mu$g/ml chlortetracycline[7] 3 × 105 cpm [$^3$H]methionyl-transfer RNA, and 20 $\mu$g/ml (polyadenosine–uridine–guanosine); and 5 $\mu$l of bruceantin, brusatol, and bisbrusatolyl malonate (25 $\mu$moles). The incubation was carried out at 23° and aliquots were withdrawn after 2 min to analyze for 80S complex formation. Puromycin (10 $\mu$g/ml) was then added to the reaction medium. The incubation was continued for another 6 min and aliquots were withdrawn to analyze for reaction of the 80S complex with puromycin. All aliquots (50 $\mu$l) were diluted to 250 $\mu$l with 20 m$M$ tromethamine (pH 7.6), 80 m$M$ KCl, 3 m$M$ magnesium acetate, 1 m$M$ dithiothreitol, and 0.1 m$M$ edetic acid, layered on 11.8 ml of a 15–30% linear sucrose gradient, and centrifuged for 3 hr at 36,000 rpm in a swinging bucket rotor[8]. Fractions (0.4 ml) were collected and precipitated with 10% trichloroacetic acid on filter papers and counted.

The reaction mixtures (75 $\mu$l) for the ternary complex formation[1] (18) contained 21.4 m$M$ tromethamime (pH 8.0), 80 m$M$ KCl, 0.26 m$M$ gua-

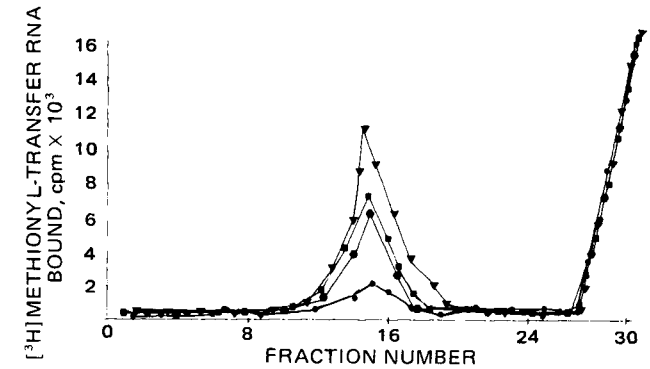



**Figure 4**—*Effects of quassinoids in the methinoyl puromycin reaction of P-388 cell system (linear sucrose gradient centrifugation). Key: ●, control; ■, brusatol; ●, bruceantin; and ▼, bisbrusatolyl malonate.*

[7] Sigma Chemical Co.
[8] Beckman SW 40.

**Table V—Effects of Bisbrusatolyl Malonate on Amino Acid tRNA Activation in P-388 Lymphocytic Leukemia Cells**

| | Inhibitor | Concentration, $\mu M$ | Amino Acid t-RNA Formation, pmole | Control, % |
|---|---|---|---|---|
| | [$^{14}$C]Phenylalanine-tRNA | | | |
| | Control | | 1.05 | 100 |
| | + Emetine | 100 | 0.97 | 92 |
| | + Pyrocatechol violet | 100 | 0.98 | 93 |
| III. | + Bruceantin | 50 | 0.96 | 91 |
| II. | + Brusatol | 50 | 0.95 | 90 |
| XIV. | + Bisbrusatolyl malonate | 50 | 0.97 | 92 |
| | [$^{3}$H]Leucyl-tRNA | | | |
| | Control | | 1.83 | 100 |
| | + Emetine | 100 | 1.74 | 95 |
| | + Pyrocatechol violet | 100 | 1.72 | 94 |
| III. | + Bruceantin | 50 | 1.68 | 92 |
| II. | + Brusatol | 50 | 1.72 | 94 |
| XIV. | + Bisbrusatolyl malonate | 50 | 1.76 | 96 |
| | [$^{3}$H]Methionyl-tRNA | | | |
| | Control | | 1.62 | 100 |
| | + Emetine | 100 | 1.54 | 95 |
| | + Pyrocatechol violet | 100 | 1.57 | 97 |
| III. | + Bruceantin | 50 | 1.59 | 96 |
| II. | + Brusatol | 50 | 1.56 | 98 |
| XIV. | + Bisbrusatolyl malonate | 50 | 1.52 | 94 |

nosine triphosphate, 2.14 m$M$ dithiothreitol, 10 $\mu$g of bovine serum albumin, 5 pmoles of P-388 cell [$^{3}$H]methionine-transfer RNA (Met-tRNA$_f$, $1 \times 10^4$ cpm), 100 $A_{260}$/ml of crude P-388 cell initiation factors and 10 $\mu$l of drug or standard. The incubation was conducted for 5 min at 37° and terminated by addition of 3 ml of cold buffer [21.4 tromethamine (pH 8.0), 80 m$M$ KCl, and 2.14 m$M$ dithiothreitol]. The samples were filtered through 0.45-$\mu$m filters, washed twice in buffer, and counted.

The reaction mixture (75 $\mu$l) for the 80S initiation complex[2] (18) formation contained, in addition to the components necessary for the ternary complex formation reaction, 1.9 m$M$ magnesium acetate, 5 $A_{260}$/ml polyadenosine–uridine–guanosine[7], and 100 $A_{260}$/ml of 80S P-388 cell ribosomes. Incubation was 10 min at 37° which was then cooled to 4° and titrated to 5 m$M$ with magnesium acetate. After 5 min at 4°, the samples were diluted with cold buffer [21.4 m$M$ tromethamine (pH 8.0), 80 m$M$ KCl, 5 m$M$ magnesium acetate, and 2.14 m$M$ dithiothreitol] and filtered as indicated for the ternary complex formation experiment.

Amino acid transfer RNA activation steps were determined by the method of Moldave (19). The reaction medium contained 0.1 m$M$ tromethamine (pH 7.4), 0.2 m$M$ adenosine triphosphate, 0.3 mg/ml "pH 5" enzyme from P-388 cells, and 2.5 $\mu$Ci/ml of [$^{14}$C]phenylalanine (536 mCi/mmole), [$^{3}$H]leucine (56.5 Ci/mmole), or [$^{3}$H]methionine (80.0 Ci/mmole) in a total volume of 1 ml. After incubation at 37° for 20 min, 2 ml of ice cold 10% trichloroacetic acid was added and the activated amino acid-transfer RNA was collected on nitrocellulose filters.

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