Anti-inflammatory Agents III: Structure–Activity Relationships of Brusatol and Related Quassinoids

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Abstract □ A series of quassinoids were observed to be potent inhibitors of induced inflammation and arthritis in rodents. Brusatol afforded the most potent activity followed by brucein-D. A 3-hydroxy-Δ^3-2-oxo moiety in brusatol or a 1-hydroxy-Δ^3-2-oxo moiety in brucein-D, as well as a C-15 ester-bearing δ-lactone ring in brusatol and C-11 and C-12 free hydroxyl groups are required in both quassinoids for potent anti-inflammatory activity. Preliminary studies indicate that one of the modes of action of quassinoids as anti-inflammatory agents is to stabilize lysosomal membranes, reducing the release of hydrolytic enzymes that cause damage to surrounding tissues.

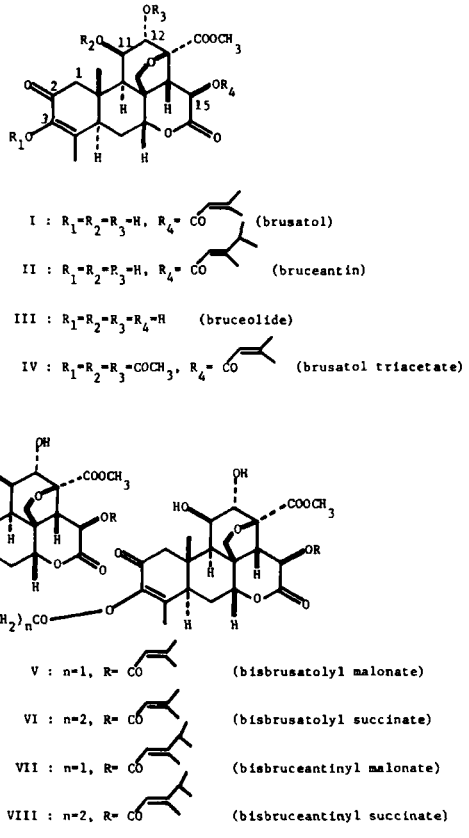
Keyphrases □ Anti-inflammatory agents—potential, brusatol and related quassinoids, structure–activity relationships □ Brusatol—related quassinoids, anti-inflammatory potential, structure–activity relationships □ Structure–activity relationships—brusatol and related quassinoids, anti-inflammatory potential

Previously, a series of pseudoguaianolides and germacranolides were reported to possess potent anti-inflammatory and antiarthritic activity in rodents (1, 2). Common to all of these compounds is the inclusion within their structure of an α-methylene-γ-lactone moiety which is a γ-lactone enone system. Those compounds containing an α-methylene-γ-lactone were ~2–4 times more potent than indomethacin in rodent screens. Since quassinoids contain a cyclohexanone enone system within their structure in addition to a δ-lactone ring, a study was undertaken to investigate a series of quassinoids and their ester derivatives for anti-inflammatory activity. Furthermore, brusatol-producing *Brucea javanica* and other quassinoid-producing species, such as *Simaba cedron*, have been observed to have anti-inflammatory activity (3, 4).

Bruceantin is an antineoplastic agent used in clinical trials in humans. Previous studies have shown that brusatol (I) and bruceantin (II) suppress oxidative phosphorylation of P-388 lymphocytic leukemia cells (5) and inhibit lysosomal hydrolytic enzymatic activities, *e.g.*, cathepsin and ribonuclease (6). Aspirin-type anti-inflammatory agents uncouple oxidative phosphorylation, and corticosteroids stabilize lysosomal membranes. Thus, both modes of action are important to anti-inflammatory activity.

EXPERIMENTAL

Source of Compounds—Bruceoside-A (X), brucein-D (XIII), and brucein-E (XIV) were originally isolated from *B. javanica* (7). Brusatol (I) was obtained either by treating bruceoside-A (X) with 3 *N* H_2SO_4–methanol (1:1) to hydrolyze the glycosidic linkage (7, 8) or directly from the chloroform extract of *B. javanica*. Bruceantin (II) and bruceolide (III) were obtained from bruceoside-A by a synthetic procedure (9). The chemical synthesis, purification, and physical characteristics of a series



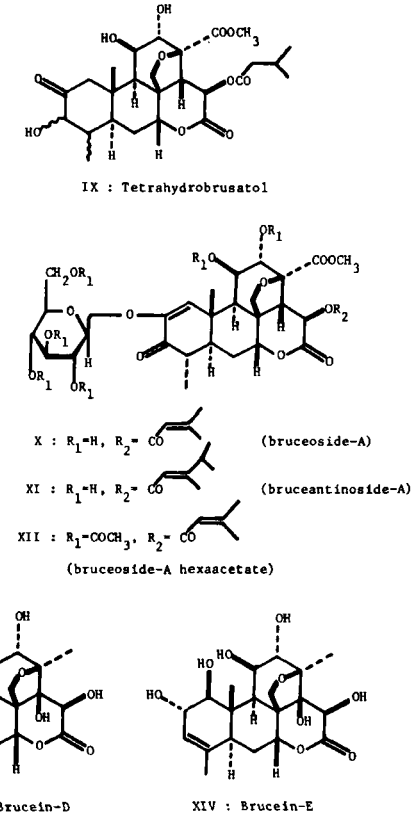


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Table I—Anti-inflammatory Activity of Quassinoids in Male Rats [a]

Compound	Percent of Control	
	0.25 mg/kg, Administered Twice	1 mg/kg, Administered Twice
I Brusatol	33 ± 4[b]	18 ± 3[b]
II Bruceantin	51 ± 6[b]	25 ± 4[b]
III Bruceolide	71 ± 6[b]	60 ± 5[b]
IV Brusatol triacetate	81 ± 7[b]	70 ± 8[b]
V Bisbrusatolyl malonate	62 ± 5[b]	60 ± 4[b]
VI Bisbrusatolyl succinate	72 ± 8[b]	70 ± 8[b]
VII Bisbruceantinyl malonate	72 ± 9[b]	62 ± 9[b]
VIII Bisbruceantinyl succinate	69 ± 8[b]	60 ± 7[b]
IX Tetrahydrobrusatol	69 ± 5[b]	54 ± 6[b]
X Bruceoside-A	83 ± 8	68 ± 7[b]
XI Bruceantinoside-A	98 ± 10	72 ± 9[b]
XII Bruceoside-A-hexacetate	73 ± 6[b]	65 ± 5[b]
XIII Brucein-D	64 ± 5[b]	37 ± 5[b]
XIV Brucein-E	78 ± 4[b]	66 ± 6[b]
Indomethacin (10 mg/kg, administered twice)	—	27 ± 6[b]
Phenylbutazone (50 mg/kg, administered twice)	—	53 ± 5[b]
Control (0.05% polysorbate 80)	100 ± 10	100 ± 9

[a] Mean ± *SD;* six animals per group. [b] Significant, $p \leq 0.001$.

of brusatolyl and bisbrusatolyl esters (IV–VI) (10), two bisbruceantinyl esters (VII and VIII) (11) and tetrahydrobrusatol (IX) (10) have been reported previously by this laboratory. Bruceantinoside-A (XI) was isolated from *Brucea antidysenterica* by a published procedure (12), and bruceoside-A hexaacetate (XII) was synthesized from bruceoside-A (8).

Anti-inflammatory Screen—Sprague–Dawley male rats (~160 g) were administered test compounds at 0.25 and 1.0 mg/kg in 0.05% polysorbate 80–water at 3 hr and again at 30 min prior to injection of 0.2 ml of 1% carrageenan in saline into the plantar surface of the right hindfoot. Isotonic saline was injected into the left hindfoot, which served as a baseline. After 3 hr, both feet were excised at the tibiotarsal (ankle) joint according to a modified method (13), resulting in an average net weight increase of 0.655 g for the control animals. Indomethacin and phenylbutazone were used as standards.

Antiarthritic Screen—Male Sprague–Dawley rats (~160 g) were injected at the base of the tail with 0.2 ml of light mineral oil containing 1 mg of dried *Mycobacterium butyricum* and 0.4 mg of digitonin. The test drugs were administered on days 3–20 at 0.25 and 0.50 mg/kg/day ip. Animals were sacrificed on day 21, and the feet were excised and weighed (14). The control animals achieved an average net weight increase of 0.830 g.

Writhing Reflex—The writhing reflex also was utilized as an analgesic test. Mice were administered the test drugs at 0.25, 0.5, and 1.0 mg/kg ip 20 min prior to the administration of 0.5 ml of 0.6% acetic acid (15). After 5 min, the number of stretches, characterized by repeated contractures, was counted for 10 min. The control mice averaged 78 stretch reflexes/10 min.

Cell Preparation—Metabolic studies were conducted on CF_1 male mouse (~30 g) liver homogenates. Homogenates (10%) were prepared using a pestle in 0.25 *M* sucrose and 0.001 *M* EDTA (ethylenediaminetetraacetic acid) at pH 7.2.

Oxidative Phosphorylation Studies—Basal and adenosine diphosphate-stimulated respirations of the 10% liver homogenates of CF_1 male mice were measured using succinate or α-ketoglutarate as the substrate (16). The reaction vessel contained sucrose (55 μmoles), potassium chloride (22 μmoles), dibasic potassium phosphate (22 μmoles), sodium succinate (90 μmoles) or α-ketoglutarate (60 μmoles), and the test compounds (brusatol, bruceantin, and bruceoside-A) at 25, 50, and 100 μ*M* in 0.05% polysorbate 80–water in a total volume of 1.8 ml.

Oxygen consumption was measured using a Clark electrode connected to an oxygraph. After the basal metabolism (state 4) level was obtained, 0.257 μmole of adenosine diphosphate was added to obtain the adenosine diphosphate-stimulated respiration rate (state 3). The respiration rate was calculated in microliters of oxygen consumed per hour per milligram of wet weight of CF_1 mouse liver homogenate. *In vivo* oxidative phosphorylation studies were conducted on male mice and rats treated with

Table II—Effects of Quassinoids on the Writhing Reflex of Male Mice [a]

Compound	Dose, mg/kg	Percent of Control
I Brusatol	0.3	61 ± 5[b]
	0.6	42 ± 4[b]
	1.2	71 ± 6[b]
II Bruceantin	0.6	63 ± 7[b]
X Bruceoside-A	0.6	65 ± 8[b]
Indomethacin	10	43 ± 7[b]
Control (0.05% polysorbate 80)	—	100 ± 6

[a] Mean ± *SD;* six animals per group. [b] Significant, $p \leq 0.001$.

brusatol at 0.25 mg/kg/day for 3 days. Animals were sacrificed on day 4, and liver homogenates were prepared for the respiration studies.

Lysosomal Hydrolytic Enzymatic Activities—Free and total acid phosphatase activities were studied using 0.1 *M* β-glycerol phosphate in pH 5.0, 0.1 *M* acetate buffer incubated with CF_1 mouse liver homogenates for 30 min (17). The test drugs were incubated *in vitro* at 25, 50, and 100 μ*M* in 0.05% polysorbate 80–water. The total enzymatic activity was obtained by treating the liver homogenates with 0.02% alkylphenoxypolyethoxyethanol 100 to release the bound hydrolytic enzymes from the lysosomal membrane. The reaction was terminated with 10% trichloroacetic acid, and the mixture was centrifuged. Inorganic phosphate was determined by the method of Chen *et al.* (18). Free, total, and percent released acid phosphatase activities were calculated after correcting for the blank values.

Free and total cathepsin activities were determined in an analogous manner on mouse liver homogenates with 2% azocasein as the substrate in pH 5.0, 0.1 *M* acetate buffer (19). The supernatant was assayed for acid-soluble peptide fragments at 366 nm. Free, total, and percent released cathepsin activities were calculated after correcting for the blank values.

Prostaglandin Synthetase Activity—The incubation medium of Tomlinson *et al.* (20) was used to determine the [^{3}H]prostaglandin formation from [^{3}H]arachidonic acid (86.2 Ci/mmole) and 10 mg of purified commercial prostaglandin synthetase from bovine seminal vesicles. After 1 hr at 37°, the reaction was terminated with 1 *N* HCl, and the mixture was extracted with ether and evaporated. The residue was dissolved in ethyl acetate and spotted on silica gel TLC plates, which were eluted with chloroform–methanol–water–acetic acid (90:8:1:0.8) (21). The plates were dried and developed in iodine vapor; with the use of prostaglandin standards, the appropriate areas were scraped and counted for tritium content. Indomethacin was used as an internal standard at 10^{-4} and 10^{-6} *M*. Quassinoids were tested at 25, 50, and 100 μ*M* final concentrations.

RESULTS AND DISCUSSION

In Tables I–VI the values are presented as percent of control ± the standard deviation. The number of animals in each group was six.

The quassinoids demonstrated significant anti-inflammatory activity at 0.25 and 1 mg/kg in Sprague–Dawley rats (Table I). When tested at 1 mg/kg, all of the quassinoids showed at least 30% reduction of inflammation. Brusatol (I) was the most potent compound, demonstrating higher activity at 1 mg/kg than indomethacin at 10 mg/kg. The hydroxy enone ring-A system (as seen in I, II, and XIII) and an ester group attached to C-15 of the δ-lactone ring of I and II are required for potent anti-inflammatory activity, as either reduction of the ring-A enone olefinic bond or removal of the C-15 ester group (as in IX and III, respectively) led to decreased activity. Esterification of the hydroxyl groups at C-3, C-11, and C-12 or glucosidation of the hydroxyl group at C-2 gave rise to less active compounds (*e.g.*, IV, V–VIII, and X–XII). In addition, a reduction of the ring-A enone carbonyl group of XIII to an α-glycol (XIV) also caused a diminution in activity. Thus, the structural requirement for the potent anti-inflammatory activity of the foregoing brusatol-related quassinoids may include either a 3-hydroxy-Δ^3-2-oxo

Table III—Effects of Quassinoids on Chronic Adjuvant-Induced Arthritis in Male Rats [a]

Compound	Dose, mg/kg	Percent of Control
I Brusatol	0.25	51 ± 6[b]
	0.50	49 ± 6[b]
Indomethacin	10	55 ± 5[b]
Control (0.05% polysorbate 80)	—	100 ± 9

[a] Mean ± *SD*, six animals per group. [b] Significant $p \leq 0.001$.

Table IV—Effects of Quassinoids on the Oxidative Phosphorylation Process of Male Mouse Liver Homogenates [a]

Compound	Percent of Control			
	Succinate		α-Ketoglutarate	
	State 4	State 3	State 4	State 3
In vitro				
I Brusatol, 100 μM	101 ± 8	93 ± 8	96 ± 7	95 ± 9
II Bruceantin, 100 μM	97 ± 7	95 ± 6	93 ± 4	97 ± 6
X Bruceoside-A, 100 μM	95 ± 4	96 ± 7	89 ± 7	93 ± 6
Control (0.05% polysorbate 80)	100 ± 12[b]	100 ± 12[c]	100 ± 9[d]	100 ± 11[e]
In vivo				
I Brusatol, 0.25 mg/kg/day	95 ± 5	98 ± 6	97 ± 5	92 ± 8
Control (0.05% polysorbate 80)	100 ± 12[b]	100 ± 8[c]	100 ± 11[d]	100 ± 13[e]

[a] Mean ± *SD*; six animals per group. [b] 5.92 μl O_2 consumed/hr/mg of wet tissue. [c] 11.31 μl O_2 consumed/hr/mg of wet tissue. [d] 3.51 μl O_2 consumed/hr/mg of wet tissue. [e] 5.21 μl O_2 consumed/hr/mg of wet tissue.

moiety in ring-A for I or a 1-hydroxy-Δ^3-2-oxo moiety in ring-A of XIII, a C-15 ester bearing δ-lactone ring-D of I, and the free hydroxyl groups at C-11 and C-12.

Brusatol at 0.6 mg/kg appeared to be as potent as indomethacin at 10 mg/kg in reducing the writhing reflex in mice, which is a test for irritant or inflammation pain (Table II). After 3 weeks administration, brusatol effectively reduced induced chronic arthritis in rats at 0.25 and 0.50 mg/kg/day, which was comparable to the activity of indomethacin at 10 mg/kg/day (Table III).

Although an in-depth mode of action study was not carried out, preliminary data (Table IV) indicates that brusatol and bruceantin did not uncouple oxidative phosphorylation of liver mitochondria with succinate as substrate (which is a flavin adenine dinucleotide dehydrogenase) or with α-ketoglutarate (a nicotinic adenine dinucleotide-linked dehydrogenase) in both the *in vitro* and *in vivo* studies. It has been noted before that quassinoids uncouple oxidative phosphorylation of P-388 lymphocytic leukemia cells, but not liver cells (3). The quassinoids tested did not inhibit prostaglandin synthesis using an isolated enzyme system (Table V); however, indomethacin did effectively inhibit prostaglandin synthesis in this system. Brusatol and bruceantin reduced the release of hydrolytic enzymes from lysosomes of the liver (Table VI). Both acid phosphatase and cathepsin release was inhibited significantly. Brusatol and bruceantin inhibited acid phosphatase release 49 and 60%, respectively, at 100 μM. Cathepsin activity was inhibited 78% by brusatol at 25 μM and 30% by bruceantin at 100 μM. Elevated cathepsin activity has previously been related to the inflammation process, and suppression of the enzymatic activity has been linked to anti-inflammatory action (22). The margin of inhibition of free lysosomal enzyme release by the quassinoid is sufficient to account for the anti-inflammatory activity observed. Quassinoids have been observed to inhibit other lysosomal enzymes in P-388 cells (4).

Other studies have established that the antineoplastic activity of quassinoids is linked directly to their ability to suppress the elongation step of protein by blocking the peptidyl synthetase reaction of P-388 cells. The inhibition of protein synthesis may be important in the rapid proliferation of lymphocytes and polymorphonuclear neutrophils during the inflammation process. Therefore, this mode of action cannot be excluded as a mode for the anti-inflammatory action of quassinoids.

Table V—*In Vitro* Effects of Quassinoids on Prostaglandin Synthetase Activity [a]

Compound	dpm of Prostaglandin Formed/hr	Percent of Control
I Brusatol		
25 μM	61377 ± 3387	102 ± 6
50 μM	63278 ± 3511	104 ± 6
100 μM	63114 ± 3109	108 ± 5
II Bruceantin, 100 μM	64392 ± 3686	107 ± 6
Indomethacin	36482 ± 2033[b]	60 ± 3[b]
Control (0.05% polysorbate 80)	60389 ± 3453	100 ± 6

[a] Mean ± *SD*; six animals per group. Conversion from [^{3}H]arachidonic acid to prostaglandins = 69.9%. [b] Significant, $p \leq 0.001$.

Table VI—*In Vitro* Effects of Quassinoids on Male Mouse Liver Lysosomal Hydrolytic Enzymes [a]

Compound	Percent of Control	
	Free Acid Phosphatase Activity	Free Cathepsin Activity
I Brusatol		
25 μM	72 ± 6[d]	22 ± 3[d]
50 μM	65 ± 6[d]	66 ± 7[d]
100 μM	51 ± 5[d]	73 ± 6[d]
II Bruceantin, 100 μM	40 ± 4[d]	64 ± 5[d]
Control (0.05% polysorbate 80)	100 ± 8[b]	100 ± 6[c]

[a] Mean ± *SD*; six animals per group. [b] 0.753 mg of phosphate released/hr/g wet tissue. [c] 2.688 mg of amino acids released/hr/g wet tissue. [d] Significant, $p \leq 0.001$.

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