Reviews

Paw Paw and Cancer: Annonaceous Acetogenins from Discovery to Commercial Products[⊥]

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Extracts of paw paw (*Asimina triloba*, Annonaceae) are among the most potent of the 3500 species of higher plants screened for bioactive compounds in our laboratories at Purdue University. The paw paw is a small tree native to eastern North America; its edible fruits (sometimes referred to as "Indiana Bananas") have nurtured mankind for centuries. Activity-directed fractionation of the paw paw extracts, using the brine shrimp lethality bioassay, led to the isolation and molecular characterization of over 50 unique annonaceous acetogenins. Fractionation of extracts from related species resulted in the identification of over 150 additional acetogenins. The annonaceous acetogenins are derivatives of long-chain (C_{32} or C_{34}) fatty acids. They are potent inhibitors of mitochondrial (complex I) as well as cytoplasmic (anaerobic) production of adenosine triphosphate (ATP) and the related nucleotides. The powerful cytotoxicity, in vivo antitumor, pesticidal, antimalarial, anthelmintic, piscicidal, antiviral, and antimicrobial effects indicated a myriad of potentially useful applications. Commercial development of these compounds uses natural mixtures of active components, incorporated into pesticidal, topical, and dietary supplement products. Successful applications and commercial products include a shampoo, highly effective in treating infestations of head lice, fleas, and ticks; a series of pesticidal sprays, which protects host plants against a diversity of pests; and an ointment for treatment of oral herpes (HSV-1) and other skin afflictions. The extract (in capsule form) enhances a mixture of natural anthelmintics. In addition, an encapsulated extract has been effectively used by certain cancer patients as a botanical supplement product.

Introduction

The American Cancer Society estimates that some 10.5 million Americans have been diagnosed with cancer and that 34% of these patients will succumb to this disease within five years. About 1 444 920 new cancer cases were diagnosed and 559 650 victims died of cancer in 2007. More than 1500 Americans per day (approximately one per minute) die of cancer. With more attention being given recently to cancer prevention and early detection, the incidences of certain cancer types (stomach, colon, breast) have decreased in the past 18 years, but other cancer types (pancreas, ovary, leukemia) have stubbornly taken a consistent and heavy toll, year after year, since 1930. Cancer is now the second leading cause of death in the United States—exceeded only by heart disease—and accounts for one in every four deaths. American men have slightly less than a 1 in 2 lifetime risk of developing cancer; for women, the risk is a little more than 1 in 3. The U.S. National Institutes of Health (NIH) estimated overall costs for cancer in 2006 to be $206.3 billion.[1] There is little doubt that cancer is a major health problem today; its immediate social significance supersedes those of infectious diseases, diabetes, drug abuse, crime, immigration, and, perhaps, even global warming.

As scientists, we are duty bound to help solve the problems of society. It has been my privilege to have known, personally, the discoverers of the anticancer effects of the vinca alkaloids, paclitaxel, camptothecin, homoharringtonine, and podophyllotoxin. As pharmacognosists and natural product chemists, we are experts at detecting, isolating, and characterizing useful molecules found in nature. The chemical diversity of higher plants continues to intrigue us. Certainly, many additional, novel, molecules that can specifically interrupt the biochemistry of cancer cells, alleviate suffering, and prolong life are awaiting discovery. Our past success in finding clinically useful antitumor botanicals, the promise of a host of new active natural compounds found from all natural sources in the past 25 years,[2] and the diversity of natural compounds being reported in the scientific literature would certainly seem to justify research funding for the search for new antitumor botanicals. Unfortunately, the winds of good fortune for funding have been blowing in other directions. I feel fortunate, indeed, to have received 13 years of R01 support before the apparent "no grants for plants" era was initiated by some of the NIH study sections. What follows is a narrative of my successful results in a lifetime's quest for new antitumor botanicals.

Early Work at Purdue University

As a young professor of pharmacognosy in 1971, I was lured away from the University of Washington to Purdue University to work with a growing, critical mass of young natural product enthusiasts that was being assembled by Varro E. (Tip) Tyler and Heinz G. Floss. My early work on the alkaloids of cacti had led to a few dozen new compounds,[3] but such phytochemical work was considered "ho hum" by an NIH study section, and this project was soon abandoned. Fortunately, President Nixon had recently declared war on cancer, and new possibilities for funding from the United States National Cancer Institute (NCI) opened up. After receiving some start-up support for work on antitumor botanicals, from the fledgling Purdue Cancer Center, I joined forces with John M. Cassady and C.-J. Chang at Purdue; we soon were awarded two successive NCI plant antitumor contracts (1976−1982).

The purpose of the NCI contracts was to extract plant accessions that were supplied through the United States Department of

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Agriculture (USDA, Beltsville, MD) from collectors worldwide. The extracts were sent to screening contractors for biological evaluation. Usually the 9KB (human nasopharyngeal carcinoma) in vitro cytotoxicity assay and the 3PS (P388) (methylcholanthrene-induced murine leukemia) in vivo assay were used. Turn-around times for these bioassays were lengthy; 9KB required about one month, while 3PS required up to six months, and likely as not, the 3PS results were ambiguous and required retesting, which consumed precious time and materials. Nonetheless, the 3PS system, when it worked, provided leads that translated into actives in animal solid tumor systems. Most of the antitumor botanicals in clinical use today were found by following in vivo activities in such murine leukemias. The contract work provided us with several actives that were processed using cytotoxicities and 3PS for activity-directed fractionation;[4] we eventually established our own cytotoxicity panel through the Cell Culture Laboratory of the Purdue Cancer Center. The turn-around times were, thus, reduced to one week, but the costs were high (about $120 per sample for six cell lines). These costs would soon deplete our research budget if this were the only available assay.

Cytotoxicity results often do not translate into in vivo actives. Millions of dollars have been spent detecting and isolating in vitro active compounds that are found later to be inactive when tested in vivo. Anticancer research may have been set back 30 years by the decision in the early 1980s to abandon in vivo screening at NCI and replace it with the in vitro panel of 60 human tumor cell lines. Even the notion that selectivity of susceptible tumor types, as suggested by such in vitro tests, has any meaning in predicting in vivo activity in the same tumor type dogmatically persists and remains unproven. Consequently, the use of so many cell lines is probably a waste of money, and, indeed, this has more recently been reduced to only three cell lines for initial screening. A satisfactory replacement for the in vivo murine leukemias has still not emerged. The high cost of using athymic mice, bearing xenografts of human solid tumors, precludes their use in academic research in monitoring the dozens of fractions encountered in natural product, bioactivity-directed, isolation work, so we felt a proven bioassay that could predict in vivo activity was sorely needed.

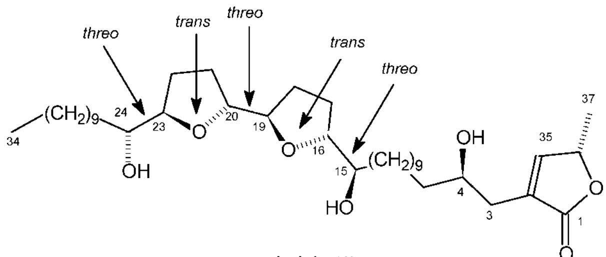
Murine toxicity is a frequent problem encountered when using the 3PS assay itself. Extracts that killed the mice were sometimes abandoned when a little more work with further dilutions would have led to doses within a therapeutic index. Such was the case with several of our most potent plant species, e.g., *Goniothalamus giganteus* Hook. f. and Thomas (Annonaceae), *Asimina triloba* (L.) Dunal (Annonaceae), and *Kalanchoe tubiflora* (Harv.) Hamet. (Crassulaceae). Consequently, these "toxic" species from the contract work were abandoned by NCI and turned over to my laboratory by the late Matt Suffness, the NCI contract officer, for fractionation to identify possible new pesticidal components. At this time (1980), I had just completed a sabbatical leave at the USDA laboratory (Peoria, IL); while there I had struggled with a laborious bioassay employing European corn borers to isolate some pesticidal cardenolides from *Thevetia thevetioides* (HBK.) K. Schum. (Apocynaceae).[5] I had also waited patiently for 3PS assay results to guide the first isolation of 10-deacetylbaccatin III, 10-deacetyltaxol, and 10-deacetylcephalomannine from *Taxus wallichiana* Zucc. (Taxaceae).[6]

These experiences convinced me that the bioassays, as being employed by the NCI contractors, were the major road block in such fractionation work. Consequently, I directed my small research team to explore a number of alternatives. Brian Meyer developed the brine shrimp lethality bioassay,[7] and Nelson Ferrigni perfected the potato disk bioassay (inhibiting crown gall tumors).[8] Matt Suffness provided us with a blind set of test compounds, and Jon Anderson quickly demonstrated that these tests were statistically predictive of 3PS activity.[9] These two benchtop methods soon began leading us to a diversity of 3PS active compounds. For example, the stilbene piceatannol,[10] isolated from *Euphorbia lagascae* Spreng. (Euphorbiaceae), is now being studied as a protein tyrosine kinase inhibitor.[11] Pamela Boner, with Nelson Ferrigni as her laboratory mentor, isolated two new bufodienolides from the murine toxic extracts of *Kalanchoe tubiflora.* The success of these bioassays eventually led to R01 grant support (1984–1997) from NCI, and I subsequently taught these procedures[12] in 22 workshops worldwide sponsored by UNESCO, IOCD, CONICET, and other agencies. The brine shrimp method has now been cited thousands of times in the natural product literature. Using these simple bioassays, we were able to isolate over 350 significantly cytotoxic compounds, which are described in four successive review papers.[13–16] In addition to the several hundred plant accessions that were accumulated during the NCI contracts, we were able to obtain through Burroughs Welcome and Co. (Tuckahoe, NY) a part of the plant collection of the late Morris Kupchan. From the USDA (Peoria, IL) and FMC Corporation (Princeton, NJ), we were given hundreds of additional, previously unscreened, species that had originally been collected by the USDA (Beltsville, MD) for the NCI. Eventually, over 4000 accessions were accumulated and housed in the Purdue warehouses. About 3500 of these were extracted, screened for bioactivity, and submitted under contracts to Eli Lilly and Company (Greenfield, IN), Merck Pharmaceuticals (Princeton, NJ), FMC Corporation (Princeton, NJ), and/or Xenova Limited (Berkshire, UK), where they were incorporated into libraries for high-throughput drug and pesticidal screens. At the time of my retirement (1999), Tom McCloud came from NCI (Frederick, MD) and salvaged some 500 species that had never been screened. The remainder of this huge plant collection was unfortunately later destroyed. At least 56 uninvestigated, active, species were among the accessions lost.

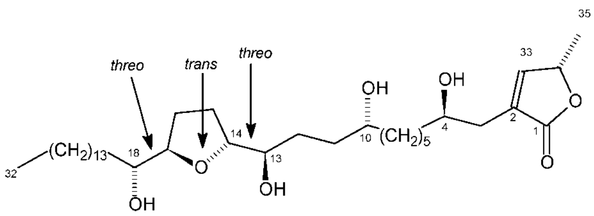
Initial Work with the Annonaceae

The extracts of two annonaceous plant species, *Asimina triloba* (paw paw) and *Goniothalamus giganteus*, that had been toxic to the injected leukemic mice in the 3PS assay were sent in 1982 to Eli Lilly and Company (Greenfield, IN) for screening in a panel of seven indicator agricultural pests. The extracts of paw paw were more potent and were surprisingly very active against five of the seven pest species.[17] Kent Rupprecht, with Tom McCloud as his laboratory mentor, used the brine shrimp assay to isolate asimicin[18] (**1**), our first annonaceous acetogenin, as one of the major bioactive components of the bark. At the same time, Mike Mikolajczak at the USDA (Peoria, IL) isolated **1** from the seeds of paw paw rather than from the bark. Selective ^1H decoupling using 200 MHz NMR helped to determine the regiochemistry by locating the molecule's third hydroxyl at C-4 and ultimately solving or confirming the molecular structure. Compound **1** was 3PS and L1210 (murine leukemia) active, very active (with ED_{50} values $<10^{-12}$ μg/mL) in cytotoxicity tests, immunosuppressive, antimalarial, and strongly pesticidal. Two U.S. patents were issued protecting the use of the acetogenins as pesticides and the composition of matter of **1**.[19,20]

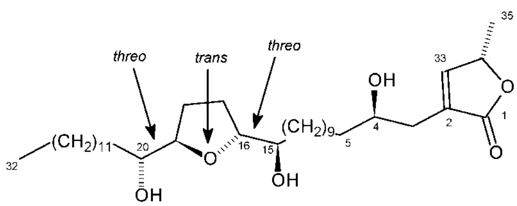
Ahmad Alkofahi fractionated the toxic extracts of *Goniothalamus giganteus* using the brine shrimp assay and quickly isolated goniothalamicin (**2**) and annonacin (**3**), two single tetrahydrofuran (THF) ring acetogenins, each, like **1**, bearing a hydroxyl at C-4.[21] John Cassady's group, following cytotoxicity assays, had previously found **3** in *Annona densicoma* Mart.[22] Compounds **1**–**3** were all cytotoxic, pesticidal, and antimalarial (Walter Reed Army Hospital, Washington, DC), and **1** and **3** were active in vivo in 3PS. We next solicited bark samples of annonaceous trees and shrubs from all over the tropical world; brine shrimp tests revealed that over 50 of the 80 some species evaluated were active and worthy of future fractionation. Some of these materials yielded active substances that were, surprisingly, not acetogenins.[23] However, the acetogenins were found to be the most potent of all the annonaceous components. Currently the Annonaceae remains a "hot" family



asimicin (**1**)



goniothalamicin (**2**)



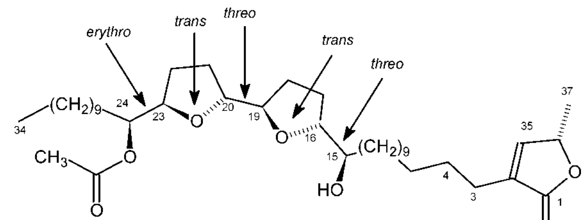
annonacin (**3**)

representing numerous uninvestigated genera and hundreds of uninvestigated species.[24]

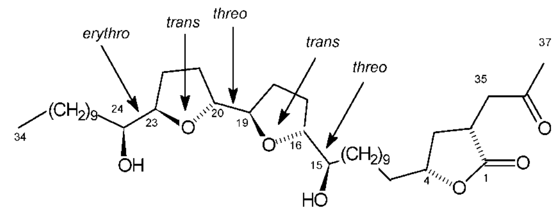
The Annonaceae

The Annonaceae is comprised of some 120 genera and includes over 2100 species. *Asimina triloba* (paw paw) is the only temperate species; the rest of the family is tropical or subtropical. The fruits of paw paw have nourished wild animals and mankind in eastern North America for thousands of years, and paw paw festivals are to be found in September throughout the Midwestern United States. Many of the tropical species also bear edible fruits and have been naturalized from central and South America to other warm climates in Asia and Africa. The late Julia Morton has summarized the economic potential of the annonaceous fruits and noted that certain parts of several species are poisonous and/or pesticidal.[25] Sour sop (guanabana) and cherimolia are two of the best known annonaceous fruits and are sold either fresh or in processed forms; thousands of pounds of the seeds of these commercial species, with their rich concentrations of acetogenins, are discarded during the processing.

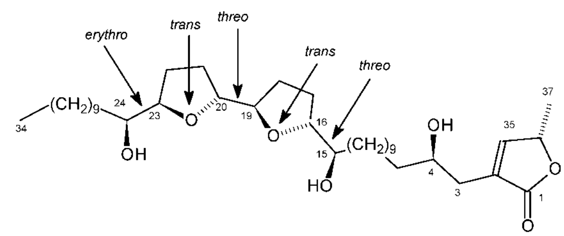
The research group of Andre Cavé in France is well known for their phytochemical studies of the Annonaeae,[26] but the annonaceous acetogenins were overlooked until the discovery in 1982 of uvaricin (**4**) by the group of Jack Cole at the University of Arizona; **4** was isolated from *Uvaria accuminata* following 3PS activity and was significantly active.[27] Desacetyluvaricin was found subsequently.[28] Our work eventually focused on 14 annonaceous species that yielded acetogenins; these were *Asimina triloba* (paw paw), *Goniothalamus giganteus, Annona squamosa* L. (sugar apple), *A. muricata* L. (sour sop, graviola, guanabana), *A. bullata* Rich., *Asimina parviflora* (Michx.) Dunal (dwarf paw paw), *A. longifolia* Kral. (long-leafed dwarf paw paw), *Annona reticulata* L. (custard apple), *A. glabra* L. (pond apple), *A. jahnii* Saff., *A. cherimolia* Mill. (cherimolia), *Xylopia aromatica* (Mart.) Lam., *Rollinia mucosa* (Jacq.) Baill. (biriba), and *R. emarginata* Schlecht. From the paw paw and these additional species, my research group isolated and characterized over 200 new annonaceous acetogenins, which represents approximately one-half of these compounds that have been reported to date. We published, in sequence, five comprehensive reviews that consolidated the chemical and biological findings published in this field of work up to 1999.[29–33] Cavé's group has also reviewed the acetogenins,[34–36] and Cortes has published a more recent review.[37]



uvaricin (**4**)



bullatacinones (**5**)

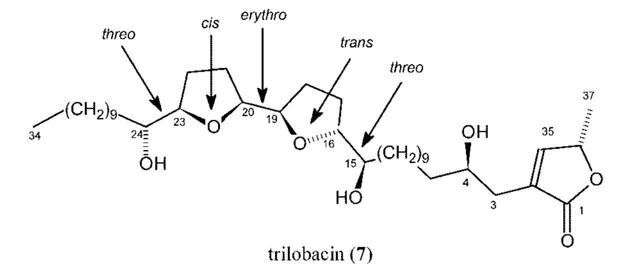


bullatacin (**6**)

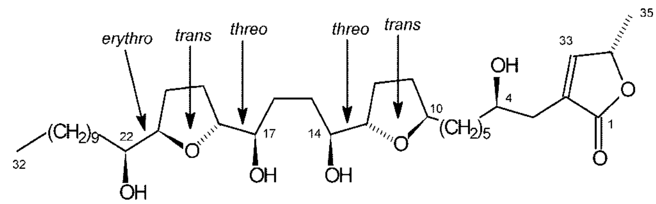
Structures of the Annonaceous Acetogenins

Chemically, the annonaceous acetogenins are white, waxy, derivatives of long-chain (C_{32} or C_{34}) fatty acids that have been combined with a 2-propanol unit at C-2 to form a methyl-substituted α,β-unsaturated γ-lactone; sometimes the lactone is rearranged with a hydroxyl at C-4 to create a mixture of 2,4-*cis*- and 2,4-*trans*-ketolactones such as the bullatacinones (**5**). Biogenetically, epoxidation of strategically placed double bonds, followed by cyclizations, gives rise to one to three tetrahydrofuran (THF) or tetrahydropyran (THP) rings. The ring systems can be single, adjacent, or nonadjacent types, and these systems, with their flanking hydroxyls, create a number of chiral centers. A complex mixture of diastereomers is the usual result. The stereochemistry of the ring systems leads to subclasses of acetogenins, and these subclasses are subsequently named after the first compound within that subclass to have its relative configuration determined. For example, extracts of paw paw contain single THF, nonadjacent bis-THF, and adjacent bis-THF classes (or types) of acetogenins. Most of the acetogenins in paw paw are of the adjacent bis-THF class. However, when observing the relative stereochemistry from C-15 to C-24, three major subclasses are revealed; these are named for asimicin (**1**), which is *threo, trans, threo, trans, threo*; bullatacin (**6**), which is *threo, trans, threo, trans, erythro*; and trilobacin (**7**), which is *threo, trans, erythro, cis, threo*. The chain length of the fatty acid (C_{32} or C_{34}) adds an additional variable in naming and classifying these compounds. Most of the acetogenins isolated from the paw paw are of the asimicin subclass and contain a total of 37 carbon atoms, which includes the three carbons of the 2-propanol subunit.

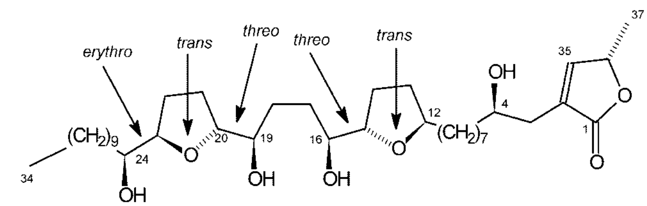
Separation of these complex mixtures of acetogenins in annonaceous plant extracts, for their isolation, characterization, and biological evaluation, has been facilitated by various high-performance liquid chromatographic methods (HPLC). In addition, countercurrent chromatography has been useful in the isolation of



trilobacin (**7**)



trilobalicin (**8**)



bullatalicin (**9**)

new acetogenins.[38] Their quantitative estimation is now routine using HPLC coupled with tandem mass spectrometry (HPLC/MS/MS), and these methods are quite successful in screening studies; for example, 44 acetogenins, including four new ones, were detectable in 2 μg samples of crude methanolic extracts of *Rollinia mucosa*.[39] Structural determinations of the acetogenins have been facilitated by carefully studying the fragments produced during electron impact-mass spectrometry (EI-MS); for example, by analyzing the trimethylsilyl (TMS) and deutero-TMS derivatives, using EIMS and fast atom bombardment mass spectrometry (FABMS), the positions of the hydroxyl groups along the hydrocarbon chain can be determined.

The acetogenins are waxy, do not crystallize readily, and, thus, are not readily amenable to X-ray crystallographic analyses, so a combination of ^{1}H and ^{13}C nuclear magnetic resonance (NMR) methods has evolved to aid in their structural elucidations. Synthetic single-THF and adjacent bis-THF models[40,41] have been indispensible. Three of my graduate students (Kent Rupprecht, Matt Reiser, and Yu-Hua Hui) worked closely with the group of Thomas Hoye at the University of Minnesota to apply Mosher ester methods to solve the absolute stereochemistry of the carbinol centers, especially those flanking the THF subunits.[42] Zhe-Ming Gu devised micromethods for preparing formaldehyde acetals that, upon ^{1}H NMR analyses, identify *erythro/threo* relationships among vicinal and nearby diols.[43] Such strategies for structural elucidation, the revisions of erroneous and incomplete structures published in the literature, a brief mention of several synthesis methods, and the biological effects are covered in our review papers previously cited. New methods for the syntheses of the acetogenins and their congeners continue to be developed,[44] and it is reassuring that our structures, as proposed from the spectral methods employed, have been confirmed, so far. Asimicin (**1**), bullatacin (**6**), and trilobacin (**7**) have all been synthesized.[45,46] Composition of matter patents have been assigned to Purdue University protecting the rights to commercialization of many of the acetogenins that we have found, especially those that show cytotoxic selectivity toward pancreatic and prostate carcinomas.[47–51]

Compounds Found in Paw Paw

Previous phytochemical studies of *Asimina triloba* (paw paw) have led to the isolation of oil, lipids, fatty acids, and proteins from the fruits and seeds, tannins, sitosterol, caffeic acid, several flavonoids (procyanidin, quercetin, quercetin 3-glycoside, quercetin 3-rutinoside, and quercetin 3-glycoside-7-glucoside), and a number of alkaloids (asiminine, which was reported to be emetic, analobine, which was once used as a medicine, coreximine, anolobine, asimilobine, isocorydine, liriodenine, and norushinsunine).[52] During our bioactivity-directed work, we isolated four nonacetogenin compounds that were bioactive; these were *N-p*-coumaroyltyramine, *N-trans*-feruloyltyramine, (+)-syringaresinol, and squamolone,[52,53] but the acetogenins are, without a doubt, the major bioactive components of paw paw.

Following our isolation of asimicin (**1**) from the bark and seeds of paw paw,[18] continued work by Geng-Xian Zhao,[52–58] Kan He,[59,60] Mi-Hee Woo,[61–65] and Eun-Jung Kim[66,67] led to the isolation and characterization of 49 additional acetogenins from the extracts, monitoring the fractionations with the brine shrimp test. The majority (29) of the paw paw acetogenins represent the adjacent bis-THF type of compounds and can be organized into three major subtypes. The asimicin (**1**) subtype (asimicin, asimin, asiminacin, asiminecin, asiminocin, asimilobin, parviflorin, 2,4-*cis*- and *trans*-asimicinones, asimitrin, asitribin, asimenins A and B, and 10-hydroxyasimicin) includes 14 compounds. The bullatacin (**6**) subtype (bullatacin, 2,4-*cis*- and 2,4-*trans*-bullatacinones, bullatetrocin, bullatin, 30*R*- and 30*S*-bullanin, squamocin, and motrilin) includes nine compounds. The trilobacin (**7**) subtype (trilobacin, trilobin, 10-hydroxytrilobacin, 2,4-*cis*- and 2,4-*trans*-trilobacinones, and 4-hydroxytrilobin) includes six compounds. Most of these compounds within the subtypes differ from the parent compounds only in the addition of another hydroxyl group or a repositioning of the hydroxyl from C-4 to another position further down the chain. Only one nonadjacent bis-THF acetogenin, trilobalicin (**8**), has been reported, but bullatalicin (**9**), a nonadjacent bis-THF acetogenin originally found in *Annona bullata*,[68] is apparent upon HPLC/MS/MS analyses of the extracts of the fruit and twigs and in the extracts of zebra swallowtail butterflies, which eat the leaves of paw paw and sequester the acetogenins as a defense against predation.[69] Some 24 mono-THF (single ring) acetogenins have been found in the paw paw extracts; these are 2,4-*cis*- and 2,4-*trans*-annonacin-A-ones, 2,4-*cis*- and 2,4-*trans*-gigantetracinones, annonacin A, annonacin (**3**), 16,19-*cis*-murisolin, murisolin A, 2,4-*cis*- and 2,4-*trans*-murisolinones, gigantetrocin A, 2,4-*cis*- and 2,4-*trans*-gigantetrocin A-ones, 2,4-*cis*- and 2,4-*trans*-isoannonacins, asitrilobins A–D, annomontacin, xylomatacin, asitricin, and 2,4-*cis*- and 2,4-*trans*-asitricinones. Several additional acetogenins can be detected using HPLC/MS/MS; for example, previously unidentified peaks at *m/z* 620 are probably dehydro analogues of the several C_{37} bis-THF compounds that carry three hydroxyls. The roots have not been fractionated so far, and their potent bioactivity suggests that they might yield something new.

Biological Studies

Our initial work with Eli Lilly and Company (Greenfield, IN) and the USDA (Peoria, IL) demonstrated that the paw paw acetogenins are potent in inhibiting a number of agricultural pests: mosquito larvae, two-spotted spider mites, Mexican bean beetles, striped cucumber beetles, European corn borers, melon or cotton aphids, blowfly larvae, and a nematode (*Caenorhabditis elegans*).[17–20] More recently the group of Cortes[70] evaluated the antifeedant and insecticidal effects of squamocin and annonacin (**3**) against three additional insect species. A number of experiments at AgriDyne Inc. (Salt Lake City, UT) are worthy of mention.[71] With Colorado potato beetles, foliar sprays of paw paw extract showed excellent results, with concentrations as low as 250 ppm being quite effective. Against white flies on cotton leaves, the paw paw extract and

Table 1. Highest and Lowest Brine Shrimp Test LC_{50} Values of Extracts from 135 Individual Paw Paw Trees Growing at the WMREC Plantation[a,b]

high producers		low producers	
tree sample	BST-LC_{50} (ppm)	tree sample	BST-LC_{50} (ppm)
4-11	0.033 ± 0.0010	2-32	32 ± 12
5-82	0.053 ± 0.015	1-19	12 ± 4.7
4-70	0.056 ± 0.0354	4-46	10 ± 0.55
2-88	0.063 ± 0.020	5-14	8.4 ± 2.5

[a] Grand mean LC_{50} value of 1.2 ppm $\pm$ 0.30 ppm. [b] Twig samples were collected, September 26, 1995, from trees planted in the spring of 1984.

pyroside (a natural pyrethrum extract) showed synergism with the mixture giving higher than the additive kill rate. Similarly, against Colorado potato beetles the paw paw extract synergized well with a standardized neem (azadirachtin) extract. Such experiments clearly demonstrated that the acetogenins need not be purified, beyond a crude level of concentration, to produce effective pesticidal products. Furthermore, the application levels can be reduced, using synergistic mixtures, saving money and reducing the environmental loads of individual components.

Evaluation of various parts of the paw paw tree, using the brine shrimp test, identified the small twigs as the optimum plant part for commercial harvest of biomass for extraction;[72] thus, by pollarding the trees, the collection of biomass is renewable through regrowth, and the trees are not killed. A subsequent study of paw paw twigs collected from the same tree every month for a year used the brine shrimp test and HPLC/MS/MS to determine that the bioactivity is highest in the month of May, and the concentrations of the major bioactive acetogenins, asimicin (**1**), bullatacin (**6**), and trilobacin (**7**), peak concurrently in May/June;[73] thus, seasonal variations can affect the concentrations of phytochemicals, and paw paw biomass is collected in May for commercial purposes.

Neal Peterson has established a beautiful plantation of over 600 paw paw trees at the Western Maryland Regional Education Center (Keedysville, MD); to study the infraspecific variations in biological activity from tree to tree, we used the brine shrimp test to evaluate twig samples collected at Keedysville on the same day from 135 individual trees. Table 1 lists the highest and lowest acetogenin producers and illustrates that the trees can vary up to 1000 times in twig potency.[74] These results are important to keep in mind when making plant collections for phytochemical work: one should collect materials from as many individual plants as possible to avoid the collection of those genotypes that are low producers. The highest producing genotypes of paw paw are, thus, available for grafting and/or clonal reproduction through plant tissue cultures.

Mechanisms of Action

Londerhausen et al.[75] initially observed that the toxicities caused by the annonaceous acetogenins in insects resulted in lethargy and decreased mobility prior to death; treated insects had substantially lower levels of adenosine triphosphate (ATP), similar to the effects of antimycin A, a known inhibitor of the mitochondrial electron transport system (ETS). Mitochondrial enzymes were tested, and the acetogenins were 2.5 to 5 times as potent as rotenone in inhibiting complex I (NADH:ubiquinone oxidoreductase). Concurrently, at Thor Arnason's laboratory at the University of Ottawa, Lewis et al.[76] observed a lower level of oxygen consumption in treated European corn borer larvae and located the site of action of asimicin (**1**) and paw paw extract at mitochondrial complex I. Meanwhile, at Bob Hollingworth's laboratory at Michigan State University, bullatacin (**6**) was tested in SF9 insect cells, with mitochondria from rat liver and *Manduca sexta,* and with complex I isolated from beef heart and arrived at the same conclusion.[77] This group later determined that **6** is among the most potent of the known inhibitors of complex I.[78]

Other workers have found that the acetogenins bind competitively with respect to the ubiquinone site at complex I.[79,80] Hiroko Shimada, in my laboratory, prepared deuterated liposomes and used intermolecular nuclear Overhauser effects with ^{1}H NMR spectroscopy to investigate and possibly predict the orientation and positioning of the acetogenins in biological membranes.[81,82] Jennifer Landolt, Trina Colman, and Dorothe Alfonso isolated rat liver mitochondria to determine the structure−activity relationships (SAR) of the acetogenins at the subcellular level.[83,84]

Kan He et al.[85] determined the SARs among 44 of the acetogenins in yellow fever mosquito larvae and in the brine shrimp test. In general, the optimum activities, in most of the SAR bioassay test systems, reside in the C_{37} acetogenins bearing bis-THF rings with flanking hydroxyls, usually at the C-15 to C-24 positions, and a third hydroxyl somewhere along the hydrocarbon chain; the stereochemistry of the ring systems was not as important as one might think. We supplied a set of 22 acetogenins to Hideto Miyoshi at Kyoto University for SAR determinations in submitochondrial preparations of complex I; to our surprise, most of the compounds were similarly potent, suggesting that the intact mitochondrial membrane plays a crucial role in determining the SARs.[86] Molecular pharmacologists who attempt to design drug screens using isolated enzymes that are naturally embedded in complex lipoid membranes should, perhaps, take note. Useful SARs are determined only when the membranes are intact.

Using bullatacin (**6**) supplied by us, Schuler et al.[87] at the University of California (Berkeley) localized the acetogenin binding site in complex I to the 23-kDa PSST subunit. Miyoshi et al.[88] synthesized a photoreactive acetogenin analogue that binds to a 30 kDa protein that is in the ND1 subunit of complex I; other complex I inhibitors, such as piericidin A and rotenone, efficiently suppressed the binding of the acetogenin analogue, indicating that they all share a common binding domain.

Jim Morré at Purdue University has shown that bullatacin (**6**) potently inhibits the NADH oxidase that is found in the plasma membranes of tumor cells;[89] this enzyme permits the tumor cell to produce ATP under anaerobic conditions by restoring NAD levels and permitting glycolysis and substrate level phosphorylation to continue. Thus, inhibition of ATP production is the result of both of the proposed actions of the acetogenins, and both aerobic (oxidative) and anaerobic (substrate level) phosphorylations are being inhibited. ATP depletion, as seen in the early studies with insects,[75] occurs, and apoptosis (programmed cell death) would be expected to be the result.[90] Indeed, Bob Geahlen at Purdue University has demonstrated DNA laddering (apoptosis) in cancerous human B-lymphocytes that had been treated with bullatacin (**6**). and **6** has now been reported to induce apoptosis.[91]

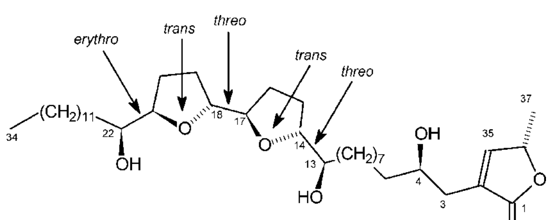
In Vivo Anticancer Testing with the Acetogenins

As mentioned above, uvaricin (**4**), the first acetogenin to be reported, was isolated by monitoring the fractionation with the 3PS in vivo murine leukemia assay (157% T/C at 1.4 mg/kg).[27] Asimicin (**1**), rollinone, and rolliniastatin, other early isolated acetogenins, were also determined to be 3PS actives (124% T/C at 25 μg/kg, 147% T/C at 1.4 mg/kg, and 128% T/C at 250 μg/kg).[29] However, with the demise of in vivo testing at the NCI in the 1980s, we decided to seek help at the pharmaceutical companies to secure additional in vivo results. Pat McGovren at the Upjohn Corporation (Kalamazoo, MI) determined that asimicin (**1**), bullatacin (**6**), and the bullatacinones (**5**) were all active in conventional mice bearing ip implanted L-1210 leukemia (131% T/C at 200 μg/kg, 138% T/C at 50 μg/kg, and 144% T/C at 400 μg/kg).[77] Taxol (paclitaxel) gives 139% T/C at 15 000 μg/kg against L-1210, so bullatacin (**6**) was 300 times as potent as taxol in this in vivo test system. Also, the mice treated with taxol lost 10% of their body weight during the 10-day test period, while the bullatacinone-treated mice gained 5%, suggesting, potentially, less toxicity than taxol.

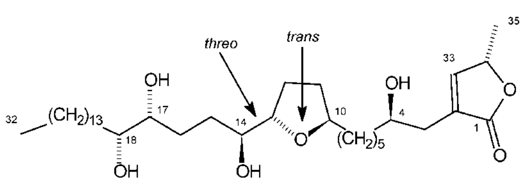
We had noted that bullatacin (**6**) was selectively cytotoxic for the A-2780 (human ovarian carcinoma) cell line in the NCI tumor panel, so at Upjohn xenografts of A-2780 were prepared and implanted into athymic mice. As a positive control, cisplatin, in a single dose of 5000 μg/kg, caused, after 10 days, 78% tumor growth inhibition (TGI); bullatalicin (**9**) at 1000 μg/kg/day for 10 days caused 75% tumor growth inhibition; bullatacin (**6**) at 50 μg/kg/day caused 67% TGI; and the bullatacinones (**5**) at 125 μg/kg/day caused 52% TGI.[77] Subsequently, at Eli Lilly and Company (Indianapolis), the late Gerry Grindey showed that bullatacin (**6**) at 50 μg/kg/day caused 66% TGI against X-5563 plasma cell myeloma implants in athymic mice; he also confirmed our observations that the acetogenins are extremely potent in their cytotoxic effects by determining that bullatacin (**6**) gave IC_{50} values of $<10^{-13}$ μg/mL against human CCRF-CEM leukemic cells. Other companies tested our acetogenins, but, in our naive absence of formal testing agreements, some refused to inform us of the results.

The Enhanced ATP Demand of Cancer Cells

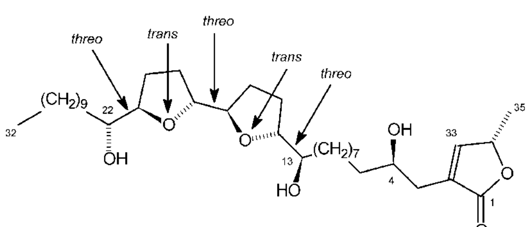
In spite of the success of the in vivo studies presented above, the inhibition of ATP production was deemed, at the pharmaceutical companies and NCI, as too general a mechanism to make possible any systemic application of the acetogenins in cancer chemotherapy. It was argued that all cells require ATP, and, thus, ATP inhibitors would be simultaneously cytotoxic to essential tissues as well as cancer cells. However, it is clearly obvious that the acetogenin-treated mice in the above in vivo studies would have all died if this argument were true. Tom Corbett at Wayne State University, with his disk diffusion assay, demonstrated that members of a series of acetogenins were all less toxic than adriamycin to normal cells, but very toxic to cancer cells.[92] Indeed, certain acetogenins are often selectively cytotoxic to one or only a few cancer cell lines. For example, squamotacin (**10**) is selective for PC-3 prostate cells,[93] and the 9-keto acetogenins are selective for PACA-2 pancreatic cells.[94] Over 30 of our acetogenins have been repeatedly evaluated by David Newman in the NCI (Frederick, MD) human tumor panel and typically show selectivity.



squamotacin (**10**)



gigantetrocin A (**11**)



parviflorin (**12**)

It seems logical to conclude that, with their constant need to undergo mitosis, cancer cells versus normal cells must have a greater demand for ATP. Not only is the hydrolysis of ATP needed to supply the biochemical energy required for cell division, but, as the key nucleotide, ATP is a basic building block of the nucleic acids that are needed for chromosomal construction for new mitochondria and new nuclei. Cancer cells must produce ATP as rapidly as possible, and any interruption of ATP production would be expected to upset the timing of cell division and have apoptotic consequences.

Table 2. Ribonucleotide Depletion in Human CEM Leukemic Cells Induced by Bullatacin (**6**) at 100 ng/mL[a,b]

	exposure time	
	2 days	3 days
ribonucleotide	% of control	% of control
uridine 5′ triphosphate (UTP)	74%	24%
cytidine 5′ triphosphate (CTP)	75%	36%
adenosine 5′ triphosphate (ATP)	98%	46%
guanosine 5′ triphosphate (GTP)	125%	69%

[a] Nucleotide pools determined initially in units of pmol/106 cells. [b] Average of two experiments.

It is now understood that the endogenous molecular biology of cancer cells involves the autocrine and paracrine secretion of insulin and insulin-like growth factors (IGF I and II), which facilitate the enhanced energy production and growth stimulation, respectively, required by these cells. A practical consequence of this is insulin-potentiated chemotherapy in which a small amount of insulin is coadministered with chemotherapeutic agents and causes, along with increased glucose uptake, a more rapid uptake of the agent into the cancer cells.[95] Breast cancer cells, for example, have an average of 7 times more insulin receptors[96] and 10 times more IGF receptors[97] than normal breast cells. Thus, these cells can take up glucose 17 times faster than normal cells, and it is logical that they must be able to metabolize glucose 17 times faster than normal cells. The increased glucose in cancer cells must be metabolized, either aerobically (utilizing mitochondria and the NADH:oxidoreductase of complex I) or anaerobically (utilizing the NADH oxidase of the plasma membrane). Inhibitors of these enzymes would be expected to show a selection for cancer cells, and the resulting inhibition of glucose metabolism would deplete the levels of ATP and the related nucleotides, leading the cancer cell to apoptosis. Sophia Fotopoulos, at Clinical Reference Laboratory (Lenexa, KS), kindly determined for us the nucleotide levels in human CEM leukemic cells as they were being killed with bullatacin (**6**); after three days the levels of all of the ribonucleotides were decreased significantly just as one would expect (Table 2). Supporting this conclusion is the evidence for constituently high levels of NADH oxidase in cancer cells versus normal cells; the enzyme is markedly elevated in the plasma membrane vesicles of cancerous HeLa cells and HL-60 cells, but not in vesicles isolated from normal hepatocytes.[89]

When I took my first course in biochemistry at the University of Michigan, the professor told us,"If we can discover a biochemical difference between cancer cells and normal cells, we will be able to control cancer." The enhanced demand of cancer cells for ATP seems to be such a biochemical difference.

Thwarting Resistance with the Annonaceous Acetogenins

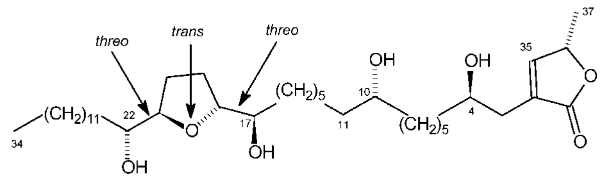
In 1988, before we understood the ATP-inhibiting mechanisms of action of the acetogenins, the mean bar graphs of cytotoxicity data from the NCI tumor panel revealed some surprising results. The data for bullatacin (**6**), for example, showed that, for pairs of cell lines that were normal (wild-type) versus adriamycin-resistant, the resistant cells were inhibited at equal or lower, instead of higher, doses. The IC_{50} for bullatacin (**6**) against the P388 (9PS) leukemic cell line was $>9.24 \times 10^{-2}$ μg/mL, while against the P388/Adr (adriamycin-resistant) cell line the IC_{50} for bullatacin (**6**) was 100 times lower, at $<9.26 \times 10^{-4}$ μg/mL.[47] Adriamycin typically gave IC_{50} values that were 10 or more times higher in the resistant cell lines.

Nick Oberlies, one of my graduate students, observed that at the Purdue Cell Culture Laboratory the parental nonresistant wild-type (MCF-7/wt) human mammary adenocarcinoma cells and multidrug-resistant (MDR) MCF-7/Adr cells exposed to bullatacin (**6**) yielded the same sort of result, wherein **6** inhibited the MDR cells at lower doses than were required to inhibit the wild-type cells. After completing cell refeeding experiments, it was concluded that **6** is cytotoxic to MCF-7/Adr cells but is cytostatic to the MCF-7/wt (wild-type) cells.[98] Thus, such MDR cells are more susceptible to ATP depletion than their parental cells; this is another important biochemical difference between parenteral cancer cells and their MDR descendants and should be exploitable by the chemotherapeutic use of the acetogenins to prevent, and even combat, multiple drug resistance.

With most other anticancer agents, a higher dose is required to inhibit resistant cells than normal (wild-type) cells. It is known that MDR cells have a P-170-kDa glycoprotein (P-gp),[99] which forms a channel or pore in the plasma membrane and pumps out the intracellular xenobiotics. This mechanism is very efficient at keeping the resistant cells functioning and explains why chemotherapy fails as the tumor becomes populated with MDR cells. The P-gp has two ATP-binding sites, and, to provide energy to drive the pump, ATP is cleaved through its ATPase action. Being ATP-dependent, the P-gp causes the MDR cells to be more susceptible to compounds that inhibit ATP production. Hence, when the acetogenins, as potent inhibitors of complex I and NADH oxidase, decrease intracellular levels of ATP, they, therefore, decrease the effectiveness of the P-gp efflux pump, and they should even synergize with other chemotherapeutic agents. Oberlies et al.[100] evaluated 14 acetogenins (seven adjacent bis-THF, two nonadjacent bis-THF, and five mono-THF ring compounds) against the MCF-7/Adr cell line to establish their SARs. All compounds were tested with adriamycin, vincristine, and vinblastine as standard chemotherapeutic agents. Of the 14 acetogenins, 13 were generally more potent than all three of the standard drugs. Bullatacin (**6**) was 258 times more cytotoxic against the MCF-7/Adr cell line than adriamycin. Acetogenins with the stereochemistry *threo-trans-threo-trans-erythro* from C-15 to C-24 were the most potent among those having adjacent bis-THF rings. A bullatacin index permitted comparisons of activities, and gigantetrocin A (**11**), a mono-THF compound, was the most potent, being about twice as potent as bullatacin (**6**).

A part of our research effort has been directed toward the development of the annonaceous acetogenins as new, environmentally friendly, organic, pesticides.[17,71] Reasoning that pesticide resistance in insects may, as with drug resistance in cancer, also involve ATP-driven efflux mechanisms, we demonstrated that the acetogenins are equipotent or even more potent against insecticide-resistant versus insecticide-susceptible German cockroaches.[101] The speed of kill values (LT_{50}) for six acetogenins and five standard synthetic pesticides were determined against second and fifth instar stages of the insecticide-resistant and insecticide-susceptible roaches. The bis-THF acetogenins showed the highest potency among the three structural classes of acetogenins. Parviflorin (**12**), a C_{35} acetogenin of the asimicin subtype and originally found in *Asimina parviflora* (Michx.) Dunal,[102] was the most potent, followed closely by asimicin (**1**). Both compounds showed more potent activities than the five synthetic pesticides, with the exception of cypermethrin, against both stages of both strains. Chlorpyrifos showed the highest resistance ratio at 8.0. Annomontacin (**13**), a mono-THF acetogenin, showed particular effectiveness against the resistant strain, acting 5 times faster than against the nonresistant strain. Low resistance ratios for the acetogenins and for hydramethylnon (an inhibitor of ETS complex III rather than complex I) strongly suggest that the inhibition of ATP production, indeed, thwarts insect, as well as tumor cell, resistance.

Two review papers summarize our work with the acetogenins in attempting to thwart ATP-dependent resistance with anticancer agents as well as with pesticides.[103,104] Since these reviews were written, Fu et al.[105] confirmed our observations using bullatacin (**6**) and resistant KB cells, and Raynaud et al.[106] confirmed our observations using squamocin and resistant MCF-7 cells. Fu et al.[105] also found that **6** induced an increase of intracellular adriamycin in the treated MCF-7/Adr cells. This group worked also with an acetogenin named 89-2 (which seems to be a 4-deoxy-28,29-*erythro*-dihydroxyasimicin); compound 89-2 increased, by 4.3-fold, the concentration of a fluorescent xenobiotic (Fura-2) in KBv200 (MDR) cells but not in parental (wild-type) KB cells. Such results again suggest that the acetogenins block the P-gp efflux pump and imply that coadministration of acetogenins, with other anticancer agents, should help to avoid and even circumvent MDR. Thus, the acetogenins are promising in the treatment of both nonresistant and MDR types of tumors.



annomontacin (**13**)

In further experiments, Fu et al.[107] demonstrated that compound 89-2 exhibited similar but potent cytotoxicities against the KBv200 (MDR) and parental (drug sensitive) KB cell lines. They next studied xenografts of the resistant and parental KB cell lines in nude mice. As predicted, the KBv200 (MDR) xenografts were refractory to vincristine, while vincristine successfully inhibited the parental KB xenografts. Compound 89-2, when administered as a treatment of 900 μg/kg every two days for six days, caused significant inhibition, 52.3% and 56.5%, respectively, of both types of xenografts with no significant weight losses or deaths in the treated mice. Thus, the acetogenins have been demonstrated to be effective in vivo against MDR tumors.

Safety and Toxicology of Annonaceous Acetogenins

Using a modified guinea pig maximization test, a paw paw extract was found to be only a weak skin sensitizer and asimicin (**1**) was found to be only a weak skin irritant; neither produced the vesication or ulceration typical of urushiol (poison ivy) components.[108] In our extensive work with the Annonaceae over the past 30 years, our researchers have never experienced any form of dermatitis during plant collection, drying, milling, extraction, or isolation of the acetogenins. One researcher rubbed one eye after his finger came in contact with a concentrated solution of mixed acetogenins, and he experienced severe eye irritation and the loss of the outer layer of cells from the cornea but with complete recovery. The Paw Paw Lice Remover Shampoo (described below), which contains 0.5% of standardized paw paw extract, passed the Draize test for eye irritation.[109]

Ames test results for mutagenicity were obtained at Sitek Research Laboratories (Rockville, MD) using a paw paw extract. The tests were negative in nine out of 10 determinations and only slightly positive (2.5% above background reversions) on one histidine mutant of *Salmonella typhimurium* after enzyme activation of the extract.[110] These results have been confirmed using the purified acetogenins squamocin and annonacin (**3**); neither acetogenin was mutagenic in three different strains of *S. typhimurium,* although both were toxic to the bacteria in the absence of a metabolic activation system.[70]

In feeding experiments,[71] mice tolerated paw paw extract mixed in their diet at 1% (a no choice diet); the mice ate this for 4 days without lethal effects. However, at 5% and above in their diets, they succumbed after 3 days, showing lethargy but with their internal organs appearing normal. At Asta Laboratories (Germany), bullatacin (**6**) was emetic, after injection at 185 μg/kg, to pigs; this was our first proof that the acetogenins are the emetic principles of paw paw, and this explains the effectiveness of the old fluid extract of paw paw seeds as sold by Eli Lilly and Company at the end of the 1800s as a fast-acting emetic.[111] Thus, emesis is a safety factor should someone ingest excessive amounts of any paw paw product; indeed, nausea and vomiting can occur if too many of the paw paw fruits are eaten. The question remained that the emetic dose might be less than the therapeutic dose and, thus, prevent clinical usefulness. In such a case, antinausea drugs, as commonly employed with anticancer agents, might be needed.

Using the brine shrimp test, a biologically standardized (LC_{50} 0.5 ppm) extract of paw paw twigs was prepared, and capsules (containing the standardized extract) were tested in male beagle dogs in an ascending oral dosing schedule. The testing was performed at White Eagle Toxicology Laboratories (Doylestown, PA).[112] There was a gradual increase in signs of emesis and loose stools as the doses were increased, but it was impossible to reach a harmful or a fatal dose due to the emetic effect. There were no effects on alertness, appetite, or weight. The doses ranged from 50 mg four times a day (qid) to 800 mg qid. Five to seven resting days were permitted between doses. At the maximum dose (800 mg qid/dog), there were no severe effects (other than emesis and loose stools). Thus, any acutely toxic effects are conveniently avoided by emesis. Following oral dosage, emesis is always a safety valve to prevent any life-threatening systemic effects. Since the previous murine studies involved ip injections and the mice, which could not eliminate the injected materials by vomiting, still survived at the effective doses, it was obvious that favorable therapeutic indexes for the acetogenins must exist and that oral dosing would be safe, provided no chronic toxicities would occur.

Epidemiological reports, from the island of Guadeloupe in the West Indies, have associated the dietary consumption of the fruits and teas (made from the leaves) of *Annona muricata, A. reticulata*, and *A. squamosa* with an atypical form of Parkinsonism.[113–115] Postural instability with early falls, prominent frontal lobe dysfunction, and pseudobulbar palsy were common, and 75% of the patients were unresponsive to treatment with L-dopa. All three patients, on whom postmortem studies were performed, showed, upon neuropathological examination, an accumulation of tau proteins, predominantly in the midbrain. These neurological symptoms were similar to a Parkinsonism-dementia complex previously observed on the island of Guam in the Pacific, and this complex is now proposed to be associated with consumption of the Annonaceae.[116] Also, atypical Parkinsonism, associated with the Annonaceae, was observed on New Caledonia in the Pacific,[117,118] and in Afro-Caribbean and Indian populations now living in England.[119] The disease appears to be chronic, with the average age in the first Guadeloupe study of 74 years (range 42−84).[113] Especially in the younger patients, the symptoms show a regression after stopping the consumption of the annonaceous foods. Patients with severe and rapid progression of the disease often ate the fruits together with the seeds.

At first the atypical Parkinsonism was attributed to the benzylisoquinoline alkaloids that are well known as phytochemical components of the Annonaceae;[26] some of these alkaloids are known to cause neurotoxicity including Parkinsonism.[120,121] However, toxic levels of rotenone, an inhibitor of ETS complex I, also can induce degeneration of multiple neuronal systems,[122,123] and the annonaceous acetogenins, as new complex I inhibitors, thus, became suspect as potential neurotoxins. Annonacin (**3**) is the most abundant acetogenin in *A. muricata*;[124] it comprises about 70% of a mixture of over 30 acetogenins, most of which, like **3**, are of the mono-THF type.[33,34] Compound **3** was found to induce nigral and striatal neurodegeneration after subacute administration employing iv infusion in rats,[125] and this study followed in vitro studies[126,127] in which **3** was cytotoxic to dopaminergic and nondopaminergic neurons by impairment of energy production. In primary cultures of rat striatal neurons, treated for 48 h, there was a concentration-dependent decrease in ATP levels, a redistribution of tau protein from the axons to the cell body, and cell death; the ATP depletion caused by **3** resulted in a transport of mitochondria to the cell soma and induced changes in the intracellular distribution of tau that are reminiscent of neurogenic diseases.[128]

Kirk Pomper is studying paw paws at Kentucky State University with the intention of making the fruits into a better commercial product.[129] He has reviewed the data concerning the consumption of the tropical Annonaceae and the association with atypical Parkinsonism, in view of the currently increasing consumption of paw paw fruits in the Midwestern states of the U.S. Might paw paws cause a similar problem? The seasonal consumption of paw paws has never been connected with any neurotoxic effects, but the frozen pulp is becoming available, more and more, for continuous marketing and year-long consumption. In addition, the sales of paw paw extract as a dietary supplement (see the discussion below) are increasing and, with overuse, might have the potential of exposing the public to the possible danger of neurotoxicity. Using the brine shrimp test, the ripe fruits of several paw paw cultivars showed bioactivity in the pulp that, surprisingly, was almost equipotent to that of the twigs; Bill Keller at Nature's Sunshine Products (NSP, Spanish Fork, UT) then used HPLC/MS/MS analyses to show that the bioactivity in the pulp was due to acetogenins, and the highly potent bis-THF acetogenins, bullatacin (**6**), bullatalicin (**9**), and asimicin (**1**)/trilobacin (**7**), were predominant.[130] One cultivar (Sunflower), however, was lower than the others in acetogenins, showing that the fruit of paw paw, as well as the twigs,[73,74] can vary considerably in acetogenin content and that cultivars with lower acetogenin content in the fruit could be selected for human consumption.

One wonders why atypical Parkinsonism has never been reported in people who eat paw paws. Perhaps the bis-THF acetogenins (which predominate in paw paw) are better emetics, and neurotoxic levels are not achievable due to emesis with overconsumption of paw paw. There seem to be no reports of emesis caused by the suspect tropical species. Perhaps the limited consumption of paw paw, which is primarily a short-term seasonal event, avoids the toxicity, which seems to be a cumulative, chronic, problem caused by day to day consumption of the tropical species over a period of several years. Perhaps the neurotoxicity is caused by a synergism between the neurotoxic benzyltetrahydroisoquinoline alkaloids and the mono-THF acetogenins that are peculiar to *A. muricata*. Perhaps there are genetic factors that predispose some people to atypical Parkinsonism. It is interesting that not everyone is susceptible to the condition; a high percentage (60%) of the Parkinsonism patients in Guadeloupe who consume the suspect foods do not display atypical Parkinsonism.[113] Whether it is the vanillin in our ice cream cones, the allyl isothiocyanate and allyl cyanide in our cole slaw, the lycopene and tomatine from the tomatoes on our pizzas, the solanine in our French fried potatoes, the cyanogenic glycosides in our apple seeds, the prussic acid in our tapioca, the acetogenins in our annonaceous fruits, or whatever, our bodies must, each day, detoxify and excrete a whole host of undesirable food chemicals.[131] Individuals differ from each other in their capacities to do this. In the meantime, in 2006, Kentucky State University asked the U.S. Food and Drug Administration (FDA) for an opinion on this topic, and their conclusion was that paw paw has a long history of food use and the FDA does not currently have any evidence that paw paw is unsafe to eat.

There is an old adage that toxicology is simply pharmacology at a higher dose. The relationship between the desired effects and the undesired effects of a drug is defined as the therapeutic index, which, consequently, attempts to quantitate the margin of safety. All drugs are two-edged swords, and few drugs are completely selective at eliciting only the desired effects. If the neurotoxicity studies discussed above are accepted as valid, it can be concluded that the energy requirements for certain neurons in the brain are higher than those of other somatic cells and, perhaps, may be as high as those of cancer cells. If the annonaceous acetogenins are to be useful systemically against cancerous cells, their therapeutic indexes must be favorable. The data currently available are limited, but still some comparisons can be presented. The work cited above using iv infusions of annonacin (**3**) in rats[125] showed neurotoxic effects at 3.8 and 7.6 mg/kg/day for 28 days. In the 3PS assay using mice, **3** was active (124% T/C) at 0.95 mg/kg/day for 10 days.[29] Thus, disregarding the difference in species, the therapeutic index for **3** would be between 4 and 8. Asimicin (**1**) in the murine 3PS assay was active (124% T/C) at 0.025 mg/kg/day for 10 days but toxic at 0.22 mg/kg/day.[29] Accordingly, the therapeutic index for **1** in mice is about 10. These numbers are tolerable for anticancer agents, although, especially for long-term therapy, it might be wise to monitor the patient for adverse neurological effects. The observation that the neurologic symptoms improve and stabilize after stopping daily consumption[113] suggests that the condition need not be life-threatening. Given the choice between dying of cancer and experiencing some symptoms of Parkinsonism after years of effective treatment with acetogenins, most cancer patients would choose the latter.

Commercial Products Containing Acetogenins

In 1999, I took an early retirement from Purdue University and was hired as Vice President for Research and Development and Chief Scientific Officer at Nature's Sunshine Products (NSP) in Spanish Fork, UT. This position gave me the unique opportunity to develop some useful commercial products containing the annonaceous acetogenins. Paw paw (*Asimina triloba*) was selected as the best source of biomass because it is abundant in the eastern United States and its collection and commercial development would not be encumbered by having to interact with customs officials, listen to claims of "biopiracy", and deal with potential embargos, as can be encountered when importing botanicals. The utility of the acetogenin-containing extracts in pest control seemed to be a good, practical, application.[71] Market analysis revealed that some 16 million people annually (primarily school children) in the United States are infested with pyrethrin-resistant head lice, and a new head lice shampoo that circumvented pesticide resistance[101] might gain success as an innovative health-care product.

It took two years to secure sources of supply of the paw paw twigs (collected in May), contract for and secure large-scale extraction, standardize the extract (using brine shrimp and HPLC/MS/MS), formulate the best shampoo base, select synergistic additives (thymol and tea tree oil), and ensure stability. In vitro tests with head lice were performed to determine the optimum concentration, treatment time, and dosing schedule. The final product passed the Draize test in rabbits for eye irritation,[132] and the product, named Paw Paw Lice Remover Shampoo, was subjected to a clinical trial in school children, some of whom had harbored head lice for up to three years while unsuccessfully using the ineffective pyrethrin-based products that are on the market. The school nurses involved were happy to report that the paw paw shampoo was 100% effective, and we published a clinical report describing the product and the trial results.[133] The product was introduced in 2001. Subsequently, we worked with Carroll-Loye Biological Research (Davis, CA) to demonstrate that the shampoo is 100% effective at killing fleas in vitro[134] and on dogs.[135] The shampoo was also effective at killing ticks in vitro.[136] Unfortunately, the product was discontinued after encountering insufficient sales.

In the early 1990s, biological testing at Merck and Company had determined that a series of our acetogenins was active against the parasite *Hemonchus contortus*, a nematode that infects sheep, goats, and other animals. Earlier tests at Eli Lilly and Company (Greenfield, IN) had shown the effectiveness of our acetogenins and paw paw extracts against *Caenorhabditis elegans*, a free-living nematode.[17,19] Consequently, we added a capsule containing 12.5 mg of standardized paw paw extract to an established NSP combination product called Paracleanse. Three additional new potential paw paw products were prepared in the NSP Pilot Plant Laboratory under my direction. These include a paw paw ointment (that controls skin cancers, herpes simplex, herpes zoster, athletes foot, the pain of bee stings, etc.), a paw paw lotion (that controls acne, skin infections, etc.), and a paw paw spray (that controls most plant pests). At Purdue we had shown, using flat head minnows, that paw paw extracts are very potent as natural piscicidal agents and would be much less expensive than rotenone. Unfortunately, the high costs involved in obtaining EPA registrations and FDA approvals for these new pest control and drug uses will probably prevent these products from being sold in the United States.

After determining that the standardized paw paw extract was apparently safe acutely due to emesis upon overdosage, we prepared it for oral administration in capsular form (12.5 mg/capsule to be administered qid) for human testing. The centuries-old tradition of human consumption as an edible fruit, the fact that the fruits contain appreciable levels of the acetogenins and are, apparently, eaten with impunity, the previous marketing by Eli Lilly and Company of the extract as an emetic, and the lack of any adverse reports about paw paw at the FDA provided compelling evidence that this plant has a historical record of being generally recognized as being safe. FDA consultants assured us that, for development as a dietary supplement, testing in human subjects could proceed. A law in Nevada permits terminal cancer patients, under the direction of their physician, to try new treatments. James Forsythe, M.D., Director of the Cancer Screening and Treatment Center of Nevada, in Reno, agreed to recruit test subjects for us among his stage 4 cancer patients.

Dr. Forsythe found that the paw paw capsules, named Paw Paw Cell-Reg, when given one capsule qid, stabilized a number of patients with advanced breast, lung, prostate, lymphatic, and colorectal cancers as well as with Waldenstrom's macroglobulinemia; furthermore, the patients showed no abnormalities in liver, kidney, electrolyte, blood sugar, or bone marrow functions.[137] The product was effective whether used alone or as an adjuvant with other treatments including IGF-I and insulin-potentiation. Evidence of effectiveness included reductions in the blood levels of tumor antigens, measurable decreases in tumor sizes, inhibition of further metastases, weight gain, increased mobility, enhanced energy, and increased duration of survival. We expanded the number of case studies, with similar encouraging results, and introduced the product to the market, as a dietary supplement, in the spring of 2003. The small number (26) of adverse events reported, through March 2008, and the success of the product suggest that the inhibition of cellular energy (ATP) with the mixture of annonaceous acetogenins from paw paw offers a novel, safe, and effective mechanism for the alleviation of cancer. As a dietary supplement, however, the paw paw product cannot be advertised as a treatment in the United States, and the company (NSP) makes no such claims for the product. A U.S. patent, assigned to NSP, is pending[138] and protects the extract and its antitumor use in animals and humans.

Acknowledgment. This work is dedicated to the memory of my sister, Janean A. (McLaughlin) Kistel, whose courageous battle with breast cancer inspired me to never give up in the search for a cure. Financial support is acknowledged from Nature's Sunshine Products, Spanish Fork, UT; R01 Grant No. 30909, from NCI, NIH; the Purdue Cancer Research Center; the Trask Fund from the Purdue Research Foundation; and Marilyn Cochran, San Angelo, TX (in memory of Shiela). Thanks are due to all of the students, postdoctoral fellows, technicians, visiting scholars, and colleagues who each devoted a part of their working life to the various acetogenin projects. Special thanks go to John F. Kozlowski for his help in the preparation of the manuscript.

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