Notes

Structure−Activity Relationships of Diverse Annonaceous Acetogenins against Multidrug Resistant Human Mammary Adenocarcinoma (MCF-7/Adr) Cells

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Fourteen structurally diverse Annonaceous acetogenins, representing the three main classes of bis-adjacent, bis-nonadjacent, and single-THF ring(s), were tested for their ability to inhibit the growth of adriamycin resistant human mammary adenocarcinoma (MCF-7/Adr) cells. This cell line is resistant to treatment with adriamycin, vincristine, and vinblastine and is, thus, multidrug resistant (MDR). Among a series of bis-adjacent THF ring acetogenins, those with the stereochemistry of *threo-trans-threo-trans-erythro* (from C-15 to C-24) were the most potent with as much as 250 times the potency of adriamycin. A spacing of 13 carbons between the flanking hydroxyl of the THF ring system and the γ-unsaturated lactone seems to be optimum with a spacing of 11 and 9 carbons being significantly less active. Several single-THF ring compounds were also quite potent with gigantetrocin A (**11**) being the most potent compound tested. The acetogenins may, thus, have chemotherapeutic potential, especially with regard to MDR tumors.

Introduction

A serious obstruction to the successful chemotherapeutic treatment of patients with any form of cancer is the emergence of cancerous tissues which are resistant both to the originally used antineoplastics and to new, structurally and mechanistically unrelated, compounds.[1,2] The term multidrug resistance (MDR) has been applied for this phenomenon. MDR has often been characterized by the increased expression of a 170 kDa plasma membrane glycoprotein (P-gp) which acts as a cellular "pump" by extruding the anticancer compound before antitumor efficacy can be realized. Two intracellular ATP-binding sites have been found on P-gp, and the active export of compounds requires the energy derived from this ATPase activity.[3−6]

The Annonaceous acetogenins are a relatively new class of natural products first described by Jolad *et al.*[7] in 1982. At the time of our most recent review[8] (through December 1995), over 220 of these compounds had been isolated from the natural plant (tree and shrub) sources. Less than 30 have been completely synthesized;[8−11] however, that number will certainly increase as researchers, such as Hoye *et al.*,[12,13] Naito *et al.*,[14] Sinha *et al.*,[15,16] and Yao *et al.*,[17] develop new stereoselective synthetic techniques and as large quantities of pure products are demanded for future *in vivo*, chemotherapeutic, and clinical studies.

The acetogenins typically have cytotoxic bioactivities due to their potent inhibition of NADH:ubiquinone oxidoreductase (complex I) of the mitochondrial electron transport system (ETS);[18,19] thus, they inhibit oxidative phosphorylation and lower ATP levels[19] such that cell growth is inhibited. Morré *et al.*[20] have shown that they also inhibit a ubiquinone-linked NADH oxidase, involved in substrate level phosphorylation, which is constituently expressed in the membranes of cancerous cells, while it is only transiently expressed in "normal" cells; this action also lowers ATP levels. Further, in an *in vitro* disk diffusion assay, a series of acetogenins potently inhibited the growth of various cancerous cells, including an adriamycin resistant mammary cell line, while only minimally affecting noncancerous rat GI epithelial cells.[21]

We have previously hypothesized that the acetogenins should be especially effective against MDR cells,[8−11,21] because such cells possibly have a taxed supply of ATP since it is required to "fuel" the P-gp pump.[5] In exploring this concept, we have recently demonstrated that parental human mammary adenocarcinoma (MCF-7/wt) cells respond quite differently to acetogenin treatment than their adriamycin resistant clones (MCF-7/Adr);[22] against the parental cell line, the acetogenin bullatacin (**1**) seems to initiate a cytostatic response, while against the adriamycin resistant (MDR) cell line, **1** induces a cytotoxic response. This mechanism presents an exciting potential for the acetogenins to serve as adjuvants with standard anticancer chemotherapeutic regimes, and timely utilization of appropriate acetogenins could minimize or even eliminate MDR tumor cells before they become problematic.

We have now expanded the above finding to include structure−activity relationships (SAR) among 14 structurally diverse acetogenins representing the three main structural classes of bis-adjacent, bis-nonadjacent, and single-tetrahydrofuran (THF) ring compounds (Table 1). Although two previous publications have drawn preliminary conclusions on the SAR of various acetogenins using inhibition of oxygen uptake in a cell free mitochondrial inhibition assay,[23,24] this is the first SAR study to utilize an assay which requires their cellular membrane transport. Following the example of Alfonso *et al.*,[24] we have normalized the observed data to that of

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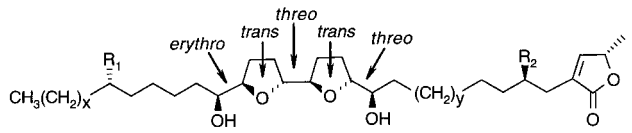
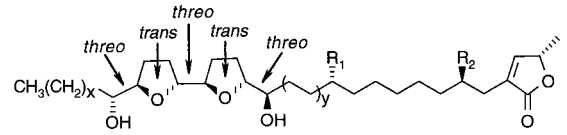
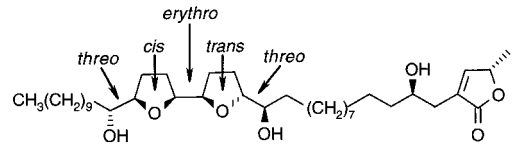
Table 1. Structures of the Annonaceous Acetogenins Tested



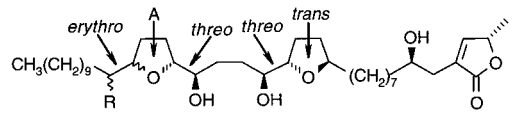
Compound	R_1	R_2	x	y
bullatacin (**1**)	H	OH	4	7
motrilin (**2**)	OH	H	4	7
squamotacin (**3**)	H	OH	6	5



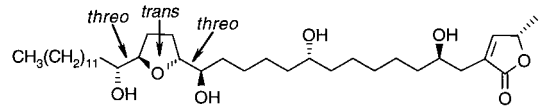
Compound	R_1	R_2	x	y
asimicin (**4**)	H	OH	9	2
longimicin B (**5**)	H	OH	11	0
longimicin D (**6**)	OH	H	11	1



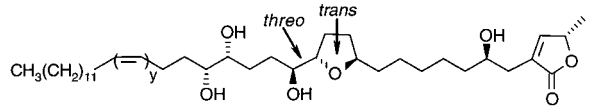
Compound
trilobacin (**7**)



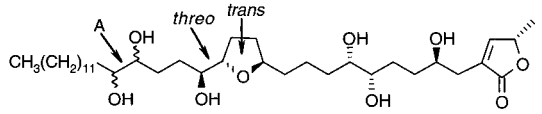
Compound	R	A
bullatalicin (**8**)	ß-OH	*trans*
sylvaticin (**9**)	α-OH	*cis*



Compound
annonacin (**10**)



Compound	y
gigantetrocin A (**11**)	0
gigantetronenin (**12**)	1



Compound	A
murihexocin A (**13**)	both OH's α, *threo*
murihexocin B (**14**)	both OH's ß, *threo*

bullatacin so that a "bullatacin index" can be used to make comparisons more easily.

Chemistry

The Annonaceous acetogenins tested (Table 1) were all isolated and characterized in our laboratory as previously described.[8–11] They were all single-compound entities as verified by HPLC separation followed by high-resolution mass spectrometry (with <5.0 ppm difference between actual and calculated molecular weights) and were thoroughly characterized using a combination of 1D and 2D NMR techniques and derivative preparations to ascertain their stereochemistries.

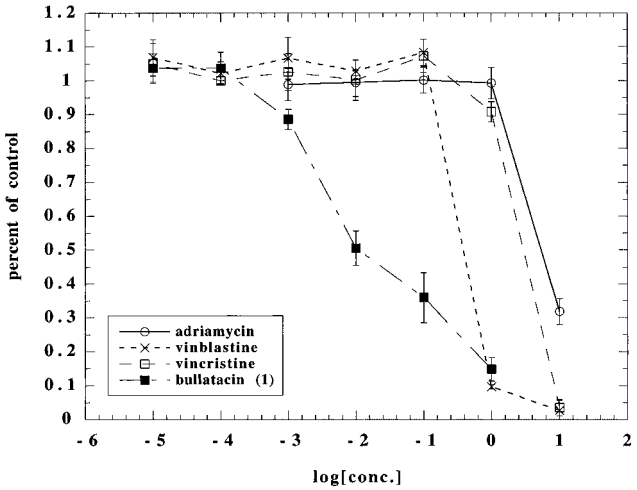


Figure 1. Comparison of the cell growth inhibition potential of bullatacin (**1**) vs the standard antineoplastic compounds adriamycin, vinblastine, and vincristine against the multidrug resistant MCF-7/Adr cells. Values are expressed as a percentage of the vehicle-treated controls with each point representing the normalized average of four values and the error bars representing the standard deviation about that average. The concentration values are log [dose] with units of μg/mL.

Table 2. Biological Activities of the Acetogenins and Standard Antineoplastic Compounds Tested against MDR MCF-7/Adr Cells

compound	IC_{50} (mg/mL)[a]	*R* value[b]	bullatacin index[c]
bullatacin (**1**)	1.08×10^{-2}	0.97	1.00
motrilin (**2**)	2.56×10^{-2}	0.99	1.23
squamotacin (**3**)	1.41×10^{-1}	1.00	6.79
asimicin (**4**)	7.04×10^{-2}	0.97	3.39
longimicin B (**5**)	>1.0	nd	nd
longimicin D (**6**)	1.40	1*	67.2
trilobacin (**7**)	5.99×10^{-2}	0.98	2.89
bullatalicin (**8**)	9.80×10^{-2}	0.98	4.72
sylvaticin (**9**)	5.72×10^{-2}	0.94	2.76
annonacin (**10**)	8.71×10^{-2}	0.99	4.19
gigantetrocin A (**11**)	1.04×10^{-2}	0.97	0.50
gigantetronenin (**12**)	1.09×10^{-1}	0.98	5.26
murihexocin A (**13**)	>1.0	nd	nd
murihexocin B (**14**)	>1.0	nd	nd
adriamycin	5.37	1*	258.5
vincristine	2.93	1*	141.3
vinblastine	3.90×10^{-1}	1*	18.8

[a] IC_{50} values are listed below for the compounds tested against the MCF-7/Adr cells. [b] *R* values are shown to give an indication of statistical significance; however, values with an asterisk (*) indicate lines in which only two points were used for the linear regression. [c] The bullatacin index relates the IC_{50} values by normalizing to the value of bullatacin so that comparisons can be made easily.

Results and Discussion

Cytotoxicity results (Figure 1 and Table 2) against the MCF-7/Adr cells using the standard antineoplastic compounds adriamycin, vinblastine, and vincristine illustrate the resilience of these MDR cells. Alternatively, these standard compounds showed impressive IC_{50} values of approximately 5×10^{-2}, 5×10^{-6}, and $<1 \times 10^{-8}$ μg/mL, respectively, against the parental MCF-7/wt cells (data not shown).[22] Bullatacin (**1**), as a representative acetogenin, however, showed an IC_{50} value against the MCF-7/Adr cells which was, surprisingly, comparable to the IC_{50} value of adriamycin

against the MCF-7/wt cells. This observation, that acetogenins such as **1** can be more effective than the standard antineoplastics against the MCF-7/Adr cells,[22] prompted this SAR study (Table 2) to evaluate the relative effectiveness of the 14 selected, chemically diverse, acetogenin compounds (Table 1).

The stereochemistry of the bis-adjacent THF rings has an effect on bioactivity in this test system (Table 2). The arrangement of *threo-trans-threo-trans-erythro* across positions C-15−C-24, for bullatacin (**1**) and motrilin (**2**), showed slightly more potency than either *threo-trans-threo-trans-threo* in asimicin (**4**) or *threo-trans-erythro-cis-threo* in trilobacin (**7**); **4** and **7** differ from **1** only in the stereochemical arrangement of their THF rings (Table 1). Also, squamotacin (**3**), an analog of **1** which has its hydroxyl-flanked THF ring system frameshifted by two carbons toward the γ-unsaturated lactone, was 10 times more active than longimicin D (**6**), its asimicin (**4**) frameshifted "cousin" (Tables 1 and 2). Receptor binding sites have not been identified for the acetogenins as their exact target within the ubiquinone-linked enzymes has yet to be discovered. In previous studies on electron transport inhibition in rat liver mitochondrial suspensions,[23,24] such differences in stereochemistries did not significantly affect potency; however, the mitochondrial assay is cell free and, therefore, does not take into consideration factors such as membrane transport, intracellular transport, metabolic inactivation, *etc.*

A hydroxyl moiety at carbon 4 does not necessarily increase potency in the bis-adjacent THF ring compounds, as motrilin (**2**), which is 29-OH-4-deoxybullatacin, showed a potency similar to **1** (Tables 1 and 2). We have previously concluded that a median polarity provided by three hydroxyls for the bis-adjacent THF ring compounds seems to be optimum in various test systems (cytotoxicity, mitochondria, mosquito larva, and brine shrimp).[8,9] This median polarity may be required for passage across lipophilic membranes, where excessive hydrophilicity might result in exclusion. Indeed, the very polar hexahydroxylated compounds murihexocins A (**13**) and B (**14**) were essentially inactive toward the MCF-7/Adr cells (Table 2). It is curious to note that the acetogenins are essentially C-35 or C-37 linear compounds, and lipid bilayers are typically 36 carbons across; this observation suggests a linear stretching of the acetogenins through the lipid membrane. Drug−membrane interactions for a series of acetogenins in liposomes are currently under investigation.

The number of carbons between the bis-adjacent THF ring system and the γ-unsaturated lactone is crucial for maximizing biological activity against the MCF-7/Adr cells. For example, the two-carbon frameshift of the hydroxyl-flanked bis-THF ring system toward the lactone in the analogs of **1** and **4**, squamotacin (**3**) and longimicin D (**6**), respectively (Table 1), resulted in significant decreases in potency, relative to the parent compounds; this is especially evident in the latter compound (Table 2). Also, in a four-carbon frameshift of the ring system of **4** (along with a shortening of the hydrocarbon chain by two methylenes) to longimicin B (**5**), all potency was eliminated. The spacing of 13 carbon atoms between the THF ring system and the γ-unsaturated lactone in **1**, **2**, **4**, and **7** appears to be optimum for cytotoxicity against the MCF-7/Adr cells. A smaller distance of 11 or 9 carbons between the two moieties may not allow for proper inhibition of the ubiquinone-linked NADH oxidases. Alternatively, the smaller spacing may complicate transport into the cancer cell or the mitochondrial membrane. A thorough probing of the active site in the mitochondrial NADH: ubiquinone oxidoreductase would assist future mechanistic studies, as would the examination of bis-adjacent THF ring acetogenin analogs which have more than 13 carbons between the THF rings and the γ-unsaturated lactone; none of the latter have ever been found in our plant extracts.[8−11]

The two bis-nonadjacent THF ring compounds tested, bullatalicin (**8**) and sylvaticin (**9**), which differ from each other only in the stereochemistry of one of the THF rings (Figure 1), have similar potencies to those of compounds **4** and **7** (Table 2). In both **8** and **9**, one of the THF rings begins at carbon 20 similar to the location of the second bis-adjacent THF rings in compounds **1**, **2**, **4**, and **7** (Table 1).

The single-THF ring acetogenins are the largest class of these natural products, with over 80 compounds known to date.[8] However, they are not often pursued because they usually have less potency than either the bis-adjacent or bis-nonadjacent THF ring compounds.[8−11] Hence, we expected that their bioactivity against the MCF-7/Adr cells would, likewise, be diminished. Surprisingly, annonacin (**10**) and gigantetronenin (**12**) both exhibited potencies which were equivalent to those of some of the bis-ring compounds such as **4** or **8**; also gigantetrocin A (**11**) was more potent than bullatacin (**1**), which had been previously one of the most potent acetogenins tested (Table 2).

Conclusion

In this study we have expanded on our earlier report of effective acetogenin bioactivities against adriamycin drug resistant tumors.[21,22] The ability of the acetogenins to lower ATP levels, *via* inhibition of complex I in the mitochondria[18,19] and inhibition of NADH oxidase at the plasma membrane,[20] would seem, logically, to inhibit the MDR transporter proteins which require ATP for energizing the P-gp efflux pump.[3−6] If expression of the MDR phenotype requires exposure to antineoplastic agents and develops over a period of time,[3−6] then acetogenin therapy may be able to assist in the early stages of cancer treatment by hampering the growth and replication of the cells which carry this phenotype.

Conclusions about the SARs of these compounds against the MDR MCF-7/Adr cell line can now be drawn as follows: 1. The stereochemistry of the bullatacin (**1**) THF ring system, *i.e.*, *threo-trans-threo-trans-erythro* (from C-15 to C-24), seems to be the most potent among those acetogenins with bis-adjacent THF ring systems. 2. A hydroxyl at carbon 4 is not required as long as there are a total of three hydroxyl moieties in the above bis-adjacent THF ring series. 3. A separation of not less than 13 carbon atoms between the THF ring system and the γ-unsaturated lactone is optimum in the bis-adjacent THF ring compounds. 4. The bis-nonadjacent THF ring compounds tested (**8**, **9**) have similar potencies to the bis-adjacent THF ring compounds, possibly because of a similar THF ring moiety beginning at carbon 20. 5. Some single-THF ring compounds were

virtually equipotent to many of the bis-ring compounds against the MCF-7/Adr cells, and gigantetrocin A (**11**) was 2 times as potent as bullatacin (**1**).

Future work with these compounds can follow many different paths. From a synthetic point of view, bis-adjacent THF ring compounds with a separation of more than 13 carbons between the THF ring system and the γ-unsaturated lactone may help to explain how the compounds bind in the active site or across lipophilic bilayers; such compounds are not known from the natural sources.[8−11] Effective acetogenin analogs which lack a hydroxyl at carbon 4 could eliminate possible contamination due to intramolecular translactonization of the γ-unsaturated lactone to the keto lactone;[25] this commonly occurs when isolating these compounds on silica gel columns or after long term storage. Trying to generate bis-nonadjacent THF ring compounds (certainly a more arduous task)[8] may not be necessary since their potency is similar to that of the bis-adjacent ring compounds. However, certain nonadjacent ring compounds, such as bullatalicin (**8**), have shown an improved therapeutic efficacy (75% tumor growth inhibition vs 67% for **1**) in an *in vivo* human tumor model.[26]

Perhaps an even more fruitful pursuit would be the development of the single-THF ring acetogenins. It has been suggested by some critics that, since the acetogenins affect such a basic cellular function as ATP formation, they are expected to be "generally" toxic and would not be good candidates for antitumor drug development. Yet, if significantly less generally cytotoxic acetogenins, such as most of the single-THF ring compounds,[21] can still potently inhibit adriamycin resistant tumors, then such acetogenins might be used efficaciously and with less general toxicity. For instance, in the initial stages of chemotherapy, standard antineoplastics will not eliminate drug resistant tumor cells. These cells may, initially, be small in number and undetectable, but they will grow and eventually overtake the patient.[27] If the acetogenins and especially the less toxic single-THF ring compounds, such as gigantetrocin A (**11**), were used in these early stages as adjuvants with standard drugs, drug resistant tumor cells might be decimated or eliminated prior to their detection. A prolongation of patient survival time might result. This idea is unique in that most previous work against MDR tumor cells has focused on using compounds which compete with or block the P-gp,[1−6,27] whereas the acetogenins have the biochemical potential to target and preferentially eliminate MDR cells right from the beginning of chemotherapy.

In conclusion, the Annonaceous acetogenins are a rapidly growing class of compounds whose true anticancer potential as ATP inhibitors is just beginning to be tested. Promise has been observed *in vivo* against P388[7] and L1210[26] murine leukemias in normal mice and against A2780[26] human ovarian carcinoma xenographs in athymic mice. Bullatacin (**1**), one of the most potent acetogenins, is effective in these *in vivo* models at only 50 μg/kg/day (which is 300 times the *in vivo* potency of taxol).[26] This current investigation illustrates that the cytotoxic effects of a range of chemically diverse acetogenin molecules are not thwarted by classical multidrug resistance in the adriamycin resistant human mammary adenocarcinoma (MCF-7/Adr) model. The semblance of the acetogenins to long-chain fatty acids may cloak their recognition by the P-gp, and they are apparently not effectively eliminated from the MDR cells. Thorough *in vivo* studies are definitely needed to support the extrapolations proposed from *in vitro* data such as this. The potent and unique activity of the acetogenins seems to be very effective, especially against resilient cell lines such as MCF-7/Adr, and merits further pharmacological evaluation *in vivo*. To meet the demand for the quantity of these compounds needed in the future, economical syntheses of structures with the optimum effectiveness, as determined by these SAR evaluations of the natural compounds, must be encouraged.

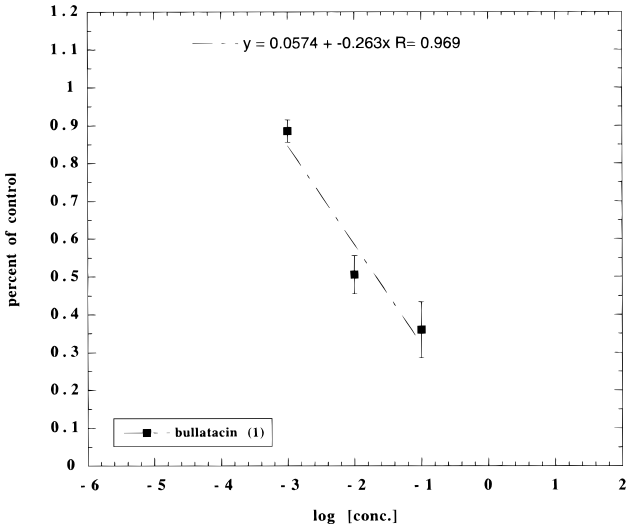


Figure 2. Representative example of how the IC_{50} values in Table 2 were calculated using the data from bullatacin (**1**). In this, linear regression is used to draw a line through the three points which span the IC_{50} value. The equation is then solved for x when $y = 0.5$. The "R" value indicates the statistical significance of the linear equation.

Experimental Section

Adriamycin, vincristine, vinblastine, penicillin, streptomycin, poly(D-lysine), and Nonidet NP-40 were purchased from Sigma, St. Louis, MO. The MCF-7/Adr cells were originally isolated from MCF-7/wt cells by growing them in the presence of 10 μM adriamycin and were kindly provided by Craig Fairchild of NIH/NCI. The cells were maintained in RPMI (Gibco, Grand Island, NY) with 10% heat-inactivated fetal calf serum (Intergen, Purchase, NY) and 1% penicillin/streptomycin; they will retain their adriamycin resistance for 6 months in its absence.

The explicit details of the *in vitro* assay used in this study have been previously described.[22,28] Briefly, MCF-7/Adr cells were plated on day 0 at 15 000 cells/mL on poly(D-lysine)-coated[29] 96-well microtiter plates, in a total volume of 200 μL of media/well, and incubated overnight in a humidified CO_2 incubator at 37 °C. We previously determined that the cells could tolerate 1% by volume of 95% ethanol without significantly affecting their growth (data not shown). This facilitated the dilution and dispersion of the test compounds. Thus, on day 1, 100 μL of fresh media was added to the test wells followed by an appropriate concentration of acetogenin in a total volume of 3 μL of 95% ethanol; the plates were then incubated for 6 more days at 37 °C in the humid atmosphere. The dilutions were tested in duplicate on each of two plates so that $n = 4$ for the entire experiment. Adriamycin was used as a positive control on every plate, while six wells were used as a standard vehicle control in order to normalize the determinations.

On day 6, the amount of cell growth inhibition was determined using a bicinchoninic acid (BCA) protein assay reagent

kit (Pierce, Rockford, IL).[22,28] In this, the cells were first carefully washed with an eight-channel plate washer (Flow Laboratories) with phosphate-buffered saline (PBS); 10 μL of nonionic detergent solution (1% by volume of Nonidet NP-40 in sterile water) was added to each well in order to solublize the cells followed by 200 μL of BCA working reagent (a 50:1 mixture of base reagent:4% copper sulfate solution). The plates were then incubated at 37 °C for approximately 30 min. Absorbancies were measured at 570 nm on a Dynatek MR 600 microplate reader.

The results were determined by first averaging the absorbance in the standard vehicle control wells. All subsequent wells were normalized to this average so that the abscissa on all of the graphs represents percent of control. The average normalized absorbance of the test wells was plotted at each respective concentration (log [dose]) with the error bars representing the standard deviation of the four determinations. Tabular IC_{50} values were calculated for the acetogenin compounds *via* linear regression through the three data points which most closely spanned the 50% inhibition range; *R* values for the line are shown in order to demonstrate the statistical significance (Table 1). The same calculation was also used for the IC_{50} values of the standard antineoplastic controls; however, in these, only two points spanned the IC_{50} value (Figure 2) so that $R = 1$. Using the data for bullatacin (**1**), a representative IC_{50} value calculation is shown in Figure 2.

The bis-adjacent THF ring acetogenin, bullatacin (**1**), is one of the most potent compounds tested against the MCF-7/Adr cells[22] and, therefore, served as a benchmark for subsequent determinations. For this, the IC_{50} values of the test compounds were simply divided by the IC_{50} value of bullatacin. This normalizes the data so that relative potencies can be compared quickly such that bullatacin index values < 1.0 indicate an activity more potent than that of bullatacin while values > 1.0 denote decreased potency.[24]

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