Total Synthesis and Cytoprotective Properties of Dykellic Acid

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Small molecule inhibitors of apoptosis hold considerable promise for the treatment of a host of diseases, including neurodegeneration, myocardial infarction, and stroke. Many compounds that delay or prevent apoptotic death either reduce the amount of cellular reactive oxygen species (ROS) or are direct inhibitors of caspases. With the goal of using small molecules to identify novel antiapoptotic targets, we have investigated the cytoprotective activity of the natural product dykellic acid. Described herein is the first total synthesis of dykellic acid, the synthesis of several dykellic acid derivatives, and the evaluation of these compounds in assays related to cell death. We have found that dykellic acid protects cells from death as induced by etoposide and rotenone. Further experiments strongly suggest that dykellic acid does not scavenge ROS or directly inhibit caspase enzymes, and analysis of synthetic derivatives establishes key functional groups of the molecule that are essential for its cytoprotective activity.

Introduction

Apoptosis is an energy-dependent pathway used by higher eukaryotes to selectively remove damaged or unwanted cells. The misregulation of this pathway has negative consequences for the organism, as abnormally low levels of apoptosis allow cells to proliferate unchecked and elevated levels of apoptosis can damage healthy tissue. As such, compounds that modulate apoptosis have great potential as therapeutics.[1] Specifically, inducers of apoptosis can be powerful anticancer agents,[2] and compounds that inhibit apoptosis could be useful in the treatment of neurodegeneration,[3] sepsis,[4] osteoarthritis,[5] myocardial infarction,[6] ischemic injury/stroke,[7] and many other disease states where cytoprotection is sought.

Apoptosis is classically believed to be initiated through one of two major pathways: (1) the intrinsic pathway that involves the mitochondria; (2) the extrinsic pathway in which apoptosis is initiated through death receptors on the cell surface. The intrinsic pathway can be stimulated by DNA damaging agents or other insults that generate reactive oxygen species (ROS[a]),[8] while death through the extrinsic pathway is initiated by a host of death receptor ligands including FasL and TNFα.[9] There is considerable cross-talk between these pathways; thus, most proapoptotic signals ultimately lead to the release of apoptogenic factors from the mitochondria.[10] Both the intrinsic and extrinsic pathways funnel to the activation of caspases, cysteine proteases that cleave a multitude of cellular substrates and execute the cell death program.[11]

Targeted approaches to the inhibition of apoptosis that have focused on the scavenging of ROS or the inhibition of caspases have revealed that these strategies can effectively halt apoptosis in certain cell culture models.[12,13] Unfortunately, large retrospective studies have shown that individuals taking high doses of the antioxidant vitamin E have no increase in life span.[14] In addition, the recent failure of free radical scavenger disufenton sodium (NXY-059) in large clinical trials for treatment of stroke has led to a rethinking of the manner in which neuroprotection clinical trials are performed,[15] although certainly some antioxidants may still hold promise as therapeutics.[16] Multiple potent caspase inhibitors are available,[17] and both caspase-1 inhibitors and pan-caspase inhibitors are being evaluated in clinical trials for arthritis, liver disease, and protection of organs during transplant.[18] However, there is some concern that inhibition of caspases may be too late in the apoptotic pathway to prevent cellular demise.[19] In this school of thought, the "point of no return" for apoptotic cell death is believed to be much earlier than caspase activation, likely depolarization of the mitochondrial membrane and the concomitant release of proapoptotic proteins.[20] By this logic, for an antiapoptotic agent to have maximal therapeutic benefit it would need to work upstream of caspase activation.

As a means to discover novel antiapoptotic targets, we sought to identify cytoprotective agents that operate through mechanisms not based on the scavenging of ROS or the direct inhibition of caspases. In this regard, dykellic acid (**1**, Scheme 1), a product of the fermentation broth of soil fungus *Westerdykella multispora*, attracted our attention because of its compact, densely functionalized structure and its reported ability to inhibit etoposide-induced apoptosis in HL-60 (human leukemia) cells.[21] Other interesting biological properties of dykellic acid have been described, including its ability to inhibit cell migration[22] and interfere with NF-κB transcriptional activity.[23] Dykellic acid has also been reported to decrease the overall caspase-3-like activity in cells treated with camptothecin,[24] though on the basis of its structure, it seems unlikely that dykellic acid is a direct caspase inhibitor. We thus set out to synthesize dykellic acid, assess its protective effects in several cell culture models of apoptosis, and conduct experiments designed to clarify its mechanism of action.

Described herein is the first total synthesis of dykellic acid, the synthesis of several dykellic acid derivatives, and the biological evaluation of these compounds. Our results show that dykellic acid strongly protects cells from apoptosis as induced by two distinct proapoptotic stimuli, etoposide and rotenone. On the basis of the evaluation of dykellic acid derivatives, it

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[a] Abbreviations: ROS, reactive oxygen species; HWE, Horner−Wadsworth−Emmons; MBH, Morita−Baylis−Hillman; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; TNFα, tumor necrosis factor α; PARP-1, poly(ADP-ribose)polymerase-1; Z-VAD-fmk, Cbz-VAD-fluoromethyl ketone; pNA, *p*-nitroaninline; H_2DCF, 2,7-dichlorodihydrofluorescein; DHE, dihydroethidium; DCF, 2,7-dichlorofluorescein; TACE, tumor necrosis factor α converting enzyme; MMP, matrix metalloproteinases; SDM, standard deviation from the mean.

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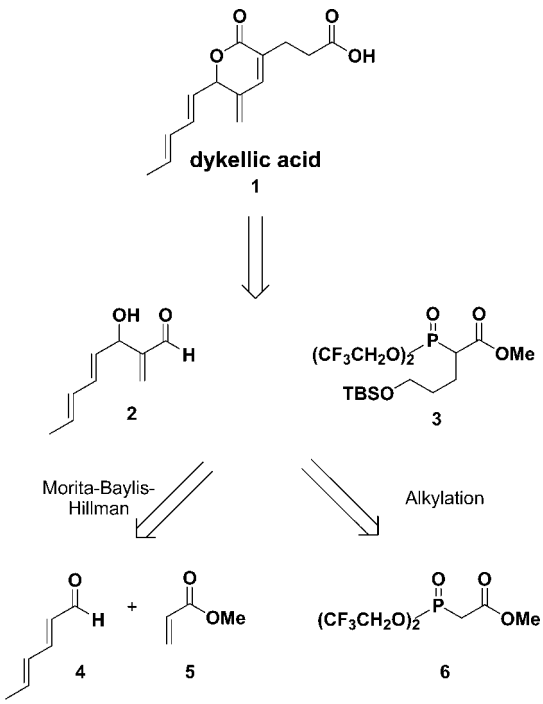
Scheme 1. Retrosynthetic Analysis of Dykellic Acid



appears that the diene side chain and the carboxylic acid moiety are critical to the cytoprotective effect of dykellic acid. Further, dykellic acid does not inhibit caspases in vitro nor does it scavenge ROS in cells. These data imply that dykellic acid exerts its antiapoptotic effect through an alternative mechanism, one that allows it to protect cells from diverse proapoptotic stimuli.

Results and Discussion

Retrosynthetic Analysis. A concise and flexible route to dykellic acid was desired to allow synthesis of both the parent natural product and a series of analogues. In the retrosynthetic analysis (Scheme 1), two key carbon−carbon bond forming reactions were envisioned: a Horner−Wadsworth−Emmons (HWE) reaction to unite aldehyde **2** with phosphonate **3** and a Morita−Baylis−Hillman (MBH) reaction between 2,4-hexadienal (**4**) and methyl acrylate (**5**). The Still modification of the HWE olefination was chosen because of the reliable *Z*-selectivity it offers in the formation of the desired trisubstituted double bond,[25] while the MBH reaction is a facile method for the formation of allylic alcohols;[26,27] as applied in Scheme 1, this reaction would allow for the assembly of a large fragment of dykellic acid in a single step. As dykellic acid was isolated from *Westerdykella multispora* as the racemate,[21] no attempt was made to synthesize the compound as a single enantiomer.

Total Synthesis of Dykellic Acid. The MBH reaction creates new carbon−carbon bonds between α,β-unsaturated esters,[28] amides,[29] and nitriles,[30] and appropriate aldehyde electrophiles. This method is most suited to alkylaldehydes and arylaldehydes, though there is some precedent for α,β-unsaturated aldehydes.[31] When α,β-unsaturated aldehydes are utilized in the MBH reaction, longer reaction times and lower yields (relative to their saturated counterparts) are typically observed.[27] As the first step toward the synthesis of dykellic acid, 2,4-hexadienal (**4**) and methyl acrylate (**5**) were used as partners for the MBH reaction (Scheme 2). After a variety of temperatures, times, and tertiary amines were screened, it was determined that the reaction proceeded best at 0 °C for 3 days in the presence of 1,4-diazabiclyclo[2.2.2]octane (DABCO), generating alcohol **7** in 70% yield; this reaction has been performed on as much as a 72 g (0.75 mol) scale. Longer reaction times did not significantly improve the yield, while temperatures above 0 °C led to increased amount of side products. To the best of our knowledge 2,4-hexadienal has not previously been used in the MBH reaction. Commercial 2,4-hexadienal is provided as a mixture of *trans,trans* and *trans,cis* isomers (about 85:15, respectively), leading to a corresponding mixture of isomers in the product. As shown in Scheme 3, independent synthesis of isomerically pure *trans,trans*-2,4-hexadienal (**4a**) from *trans,trans*-2,4-hexadienol[32] followed by the MBH reaction with methyl acrylate also provided alcohol **7** as an isomeric mixture, presumably because of unproductive 1,6 addition of DABCO to the aldehyde.

Alcohol **7** was converted to its triethylsilyl ether **8** in 95% yield before being subjected to reduction by DIBAL-H, which provided alcohol **9** in 80% yield (Scheme 2). At this time the *trans,cis* isomer of **9** was removed by chromatography with 25% $AgNO_3$-impregnated silica gel.[33] Loading 1 g of alcohol **9** onto 100 g of $AgNO_3$-impregnated silica gel yielded 35% pure isomer **9a** and 50% of isomerically impure alcohol **9**, which could be purified again through this procedure. Repeating this process several times afforded an overall yield of 55% of **9a**. After this separation, isomerically pure **9a** was oxidized using Dess−Martin periodinane[34] to provide aldehyde **10** in 79% yield (Scheme 2).

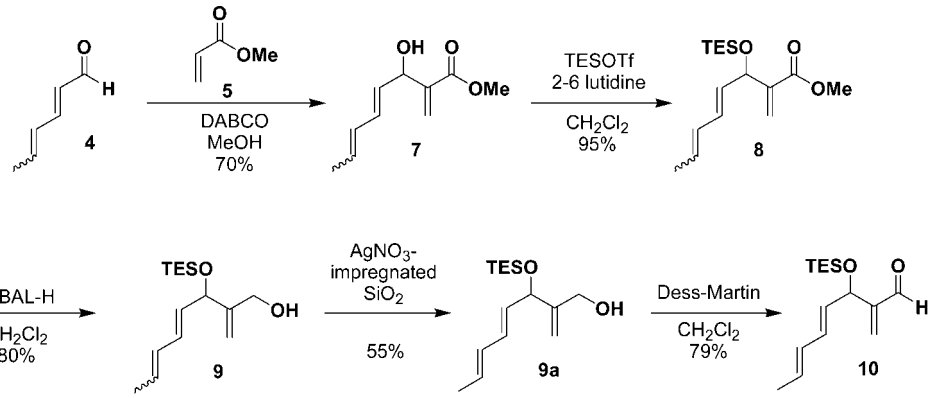
In preparation for the key olefination, phosphonate **3** was synthesized as shown in Scheme 4. Commercially available 1-bromo-3-propanol was protected as its *tert*-butyldimethylsilyl ether, which was then subjected to Finkelstein's conditions to exchange the bromide for an iodide. Iodide **11** was subsequently used to alkylate hexafluorodiethyl phosphonate **6**, providing target phosphonate **3** in 86% yield.

With both main subunits of dykellic acid in hand, attention was turned to the critical HWE reaction. In an effort to obtain the desired *Z*-olefin, Still's modification of the HWE reaction was utilized. Initially, aldehyde **2** (generated from an acid-catalyzed deprotection of **10**) was used as the substrate in the HWE reaction with phosphonate **3** (Scheme 5, eq 1). It was envisioned that olefination would be followed by rapid intramolecular cyclization to provide lactone **12**. Unfortunately, under a variety of reaction conditions (bases, solvents, additives, temperatures), the reaction between **2** and **3** produced only very low (<15%) yields of the desired product (Scheme 5, eq 1). However, utilization of the TES-protected substrate **10** in this reaction provided the trisubstituted olefin **13** in 58% yield with a *Z*/*E* ratio (around the new double bond) of 10:1 (Scheme 5, eq 2).

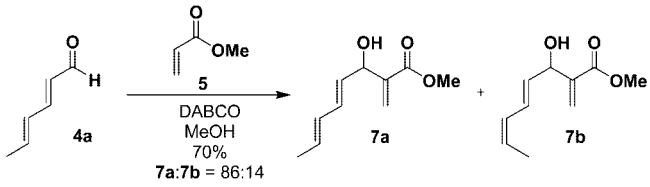
With the final carbon−carbon bond in place, the two silylether protecting groups of **13** were removed using an AcOH/H_2O mixture, which also catalyzed the spontaneous cyclization to the desired lactone **14** (Scheme 6). Lactone **14** was found to be unstable and was thus immediately oxidized to aldehyde **15** using Dess−Martin periodinane. Treatment of aldehyde **15** with sodium chlorite[35] furnished dykellic acid (**1**) in a 60% yield. The spectroscopic data for this synthetic dykellic acid match the data of the isolated natural product[21] in all respects (see Supporting Information).

Synthesis of Dykellic Acid Derivatives. With a synthetic route to dykellic acid in place, derivatives in which various functional groups were systematically deleted/altered were synthesized. These derivatives (shown in Figure 1) were designed to probe the relationship of compound structure to biological activity. Specifically, the importance of the diene side

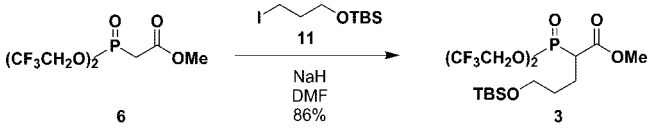
Scheme 2. Synthesis of Aldehyde **10**



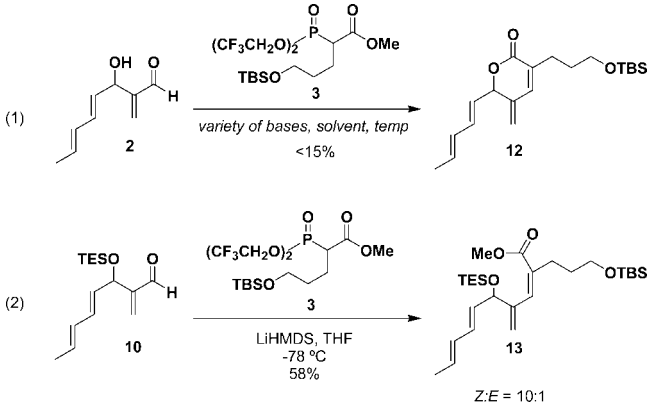
Scheme 3. Reaction of Isomerically Pure *trans,trans*-2,4-Hexadienal with Methyl Acrylate under the MBH Conditions Provides a Mixture of Product Isomers



Scheme 4. Synthesis of Phosphonate **3**



Scheme 5. Olefination of Aldehydes **2** and **10**



chain and the pendent acid were probed through the synthesis and evaluation of compounds **16**−**23**.

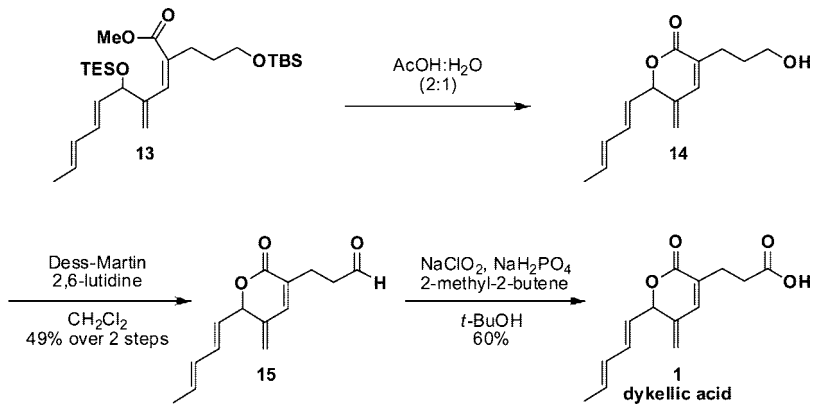
The carboxylic acids in **1** and **18** were converted to the corresponding methyl esters using trimethylsilyldiazomethane ($TMSCHN_2$), producing derivatives **16** and **22**, respectively. Derivatives **17** and **18** were synthesized by following the same route to **1** but with substitution of 2,4-hexadienal with hex-2-enal or hexanal in the first step of the synthesis. Lactones **20**, **21**, and **23** (lacking the carboxylic acid side chain) were produced through a HWE reaction with phosphonate **6** and the appropriate aldehyde (see Supporting Information for full routes and details).

Cytoprotective Effects of Dykellic Acid. To define the generality of the cytoprotective effect of dykellic acid, this compound was evaluated in several different cell culture models of cell death. These models consisted of pretreatment of U-937 (human lymphoma cell line) cells with dykellic acid for 5 h, followed by application of a cellular insult and measurement of cell death after 24 h by propidium iodide staining and cell flow cytometry. U-937 cells are a promonocytic cell line that grows rapidly as a suspension (nonadherent) in tissue culture flasks and are commonly used in cytoprotection assays.[36−38] The ability of dykellic acid to protect U-937 cells from a diverse array of cellular insults was explored through the use of rotenone (a mitochondrial complex I inhibitor that induces apoptosis[39] and is commonly used in cell culture models of Parkinson's disease[40]), etoposide (a proapoptotic topoisomerase inhibitor and anticancer chemotherapeutic utilized as a cell culture model of damage induced by chemotherapeutics),[41] H_2O_2 (induces necrotic cell death and used as a model of oxidative damage),[42] and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (a DNA alkylating agent that can induce a variety of types of cell death).[43,44]

For these experiments, U-937 cells were incubated with a range of dykellic acid concentrations for 5 h and then subjected to the various toxins. After 24 h, the viability of the U-937 cells was assessed by propidium iodide staining and cell flow cytometry. The level of protection observed is expressed as percent protection, calculated from the percent of cells alive when compared to control cells treated with either vehicle alone or toxin alone (for more details, see the Supporting Information). As shown by the graphs in Figure 2, dykellic acid is able to protect cells in a dose-dependent fashion from etoposide- and rotenone-induced cell death. Interestingly, dykellic acid is only minimally protective against MNNG-induced cell death and not protective against H_2O_2-induced cell death (see Supporting Information for graphs).

Cytoprotective Effects of Dykellic Acid Derivatives. The various dykellic acid derivatives synthesized (Figure 1) were evaluated for their ability to protect U-937 cells from etoposide-induced cell death. As shown in Figure 3, compound **19**, the isomerically impure form of dykellic acid, is as protective as **1**. Removal of the γ,δ-unsaturation from the diene chain (**17**) provides a compound that remains protective, whereas the compound with the diene side chain fully saturated (**18**) has no protective effect. All compounds in which the carboxylic acid is modified (**16** and **22**), or removed entirely (**20**, **21**, and **23**) are not protective, and in fact some of these compounds are toxic to U-937 cells (see Supporting Information). From the data, it appears that minimally the α,β-unsaturation in the diene side chain and the carboxylic acid functionality of dykellic acid are critical to its cytoprotective properties.

Scheme 6. Deprotection, Cyclization, and Oxidation Provides Dykellic Acid



Dykellic Acid Reduces Cellular Levels of Caspase-3/-7 Activity. Caspases are cysteine proteases that are key players in the cascade of events leading to apoptotic death.[45] Caspases exist in the cell as low activity zymogens (procaspases) that are activated in response to a variety of proapoptotic signals. Caspases-8 and -9 are initiator caspases that directly catalyze the hydrolysis of procaspase-3 and -7, converting them to the active executioner caspases, caspases-3 and -7.[46] Once activated, these enzymes then cleave a variety of cellular substrates. A common means to assess apoptotic death is through the measurement of caspase-3/-7 activity in the lysate of cells that have been treated with a proapoptotic agent.[47] Thus, as an initial experiment to determine the mechanism by which dykellic acid exerts its cytoprotective effect, U-937 cells were pretreated with a range of dykellic acid concentrations for 5 h and then treated with etoposide (to a final concentration of 1.5 μM) for 24 h. After 24 h the cells were lysed and the amount of caspase-3/-7 activity was determined by monitoring the cleavage of the peptidic substrate Ac-DEVD-pNA. Active caspase enzymes catalyze the hydrolytic release of *p*-nitroaniline (pNA) from this substrate, a reaction that can conveniently be monitored at 405 nm. A control with the pan-caspase inhibitor Cbz-VAD-fluoromethyl ketone (Z-VAD-fmk)[17] was also included in the experiment. As shown in Figure 4, caspase activity is not detectable in the lysates from Z-VAD-fmk treated cells, and high concentrations of dykellic acid also significantly reduce the amount of cellular caspase activity in this assay.

Dykellic Acid Is Not a Direct Inhibitor of Caspase-3, -6, -7, or -8. One means by which compounds can delay cell death is through direct inhibition of caspases. The vast majority of caspase inhibitors are peptidic in nature,[48] although small molecule caspase inhibitors are also known.[49] The data presented in Figure 4 indicate that dykellic acid exerts its cytoprotective effect either by inhibiting an upstream cascade that leads to caspase activation or through direct inhibition of caspases. To test whether dykellic acid itself directly inhibits caspases in vitro, full Michaelis−Menten (velocity vs [substrate]) curves were obtained for recombinantly expressed and purified caspases-3, -6, -7, and -8 in the presence of various concentrations of dykellic acid (see Supporting Information for full curves). The enzymes' activity was assessed by monitoring the caspase-mediated hydrolytic release of *p*-nitroaniline (pNA) from tetrameric peptide substrates specific for each enzyme.[50] Thus, the Ac-DEVD-pNA substrate was used for caspase-3 and -7, Ac-VEID-pNA was used for caspase-6, and Ac-IETD-pNA was used for caspase-8. The data from these experiments show that dykellic acid has no effect on the ability of these caspases to process their substrate in vitro, even up to dykellic acid concentrations of 100 μM. For example, we measured the k_{cat}/K_M values for caspase-3, -6, -7, -8 as 308, 245, 80, and 38 M^{-1} s^{-1}, respectively; in the presence of 100 μM dykellic acid we obtained similar values of 292, 246, 78, and 41 M^{-1} s^{-1}, respectively. Hence, dykellic acid is not an inhibitor of caspase-3, -6, -7, or -8 in vitro and is unlikely to be a direct inhibitor of any of the caspase enzymes

Effect of Dykellic Acid on Levels of Cellular ROS. General antioxidants are often cytoprotective because of the central role of ROS in mitochondria-mediated apoptosis.[13] We therefore sought to establish if dykellic acid acts directly as a ROS scavenger in cells. Two different ROS sensitive dyes were employed for this assay, 2,7-dichlorodihydrofluorescein (H_2DCF) and dihydroethidium (DHE). H_2DCF is converted to 2,7-dichlorofluorescein (DCF) in the presence of intracellular

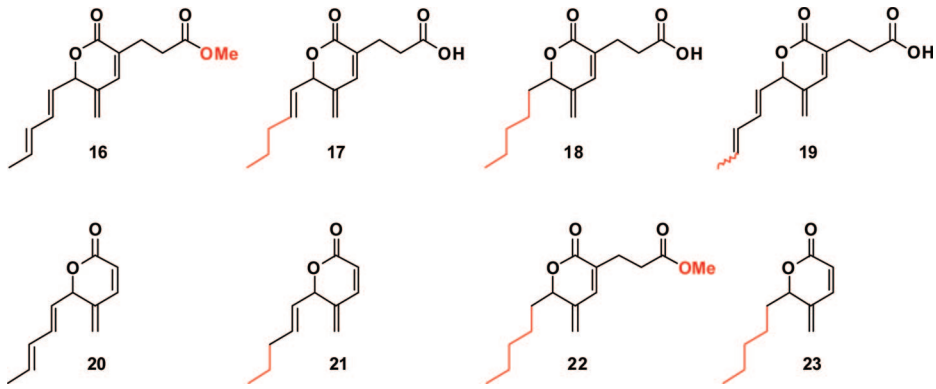


Figure 1. Derivatives of dykellic acid.

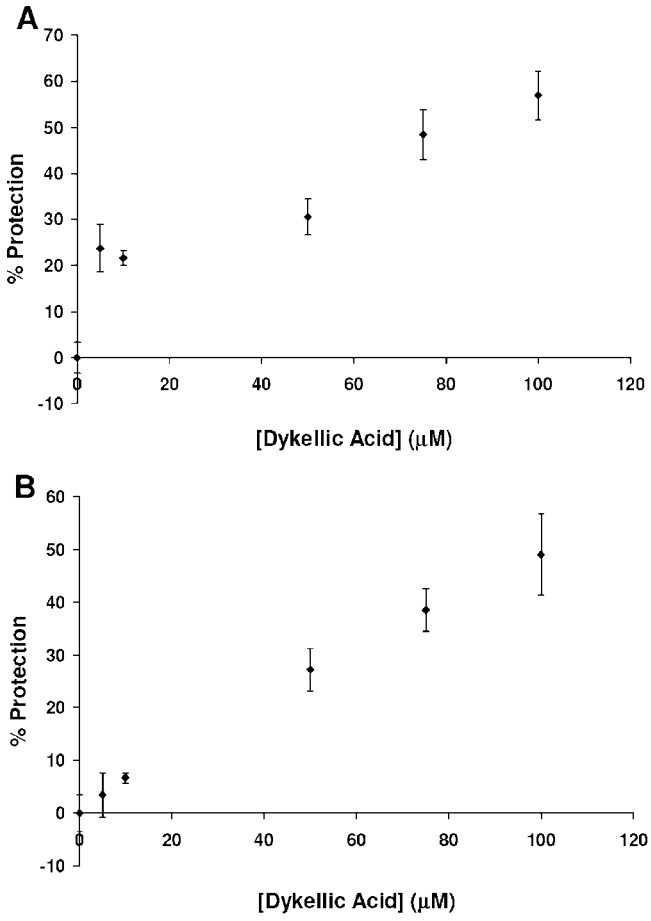


Figure 2. Dykellic acid protects cells against two different types of insult. U-937 cells were treated with dykellic acid for 5 h before (A) etoposide was added to a final concentration of 1.5 μM or (B) rotenone was added to a final concentration of 5 μM. Cell death was assessed 24 h later by propidium iodide staining and cell flow cytometry. Error bars represent SDM from $n = 3$, with each trial performed in triplicate.

peroxides,[51] whereas DHE provides a sensitive readout on intracellular levels of superoxide radicals through oxidation by superoxide anion radials to form ethiduim.[52] U-937 cells were treated with dykellic acid at varying concentrations for 5 h, after which time rotenone was added and the levels of cellular ROS were measured. α-Tocopherol, a well established antioxidant,[53] was used as a positive control. As shown in Figure 5, α-tocopherol significantly decreases ROS levels in rotenone-treated cells (as measured by the fluorescence of both DCF and ethidium). However, dykellic acid had no effect in this assay, even at concentrations of 100 μM, strongly suggesting that dykellic acid does not alter intracellular levels of ROS.

Discussion

The data presented herein show that dykellic acid protects U-937 cells from death as induced by rotenone and etoposide. Although cells treated with dykellic acid show markedly reduced levels of caspase-3/-7-like activity, our data indicate that dykellic acid is not a direct inhibitor of caspase-3, -6, -7, or -8 in vitro, suggesting that the compound acts to suppress caspase activation in some upstream portion of the apoptotic cascade. As ROS is a key player in apoptotic induction, we examined the effect of dykellic acid on ROS production. Interestingly, the levels of intracellular ROS were not altered in the presence of dykellic acid. The fact that dykellic acid does not appear to directly quench cellular ROS is also consistent with the inability of this compound to protect against H_2O_2-induced cell death, as H_2O_2-treatment results in the direct and rapid generation of cellular ROS.[54] Thus, the combined data suggest that dykellic acid exerts its cytoprotective effect on cells in culture through some less common mechanism, one not involving direct caspase inhibition or ROS-scavenging.

The protective properties of dykellic acid were evaluated against four different toxins: etoposide, rotenone, MNNG, and hydrogen peroxide. Etoposide inhibits topoisomerase II, a phosphodiesterase that catalyzes the hydrolysis of double stranded DNA, allowing relaxation of supercoiled DNA. By preventing topoisomerase II from re-ligating the double strand break, etoposide turns topoisomerase II into a destructive DNA cleaving enzyme.[55] While the pathway between etoposide-induced DNA damage and apoptotic induction is not entirely understood, caspase-2, -3, and -8 have been shown to be important links for stimulation of the mitochondrial apoptotic pathway and the execution of apoptosis.[56] The pesticide rotenone is a potent inhibitor of complex I, an enzyme in the mitochondrial electron transport chain. This inhibition in turn causes the production of ROS, which causes cellular stress and ultimately leads to death, again through an apoptotic pathway in U-937 cells.[39,57]

The other two toxins tested, MNNG and H_2O_2, are fundamentally different from etoposide and rotenone in that in U-937 cells they cause mainly caspase independent cell death or necrosis. MNNG is a DNA alkylating agent that causes caspase-independent cell death through poly(ADP-ribose)polymerase-1 (PARP-1) activation.[58] Upon DNA alkylation PARP-1 is activated, inducing synthesis of the toxic poly(ADP-ribose) biopolymer[59] and causing depletion of cellular energy stores, both of which lead to rapid death without the activation of caspase enzymes.[60] Hydrogen peroxide has been shown to induce necrotic death in U-937 cells.[61] When hydrogen peroxide is added to cells, peroxide radicals oxidize not only DNA but also proteins and lipids, starting a host of enzymatic cascades that lead to cell death.[35,37]

Comparing the mechanisms of these toxins allows some conclusions to be drawn about how dykellic acid might be protecting cells from death. Dykellic acid is most protective against the toxins that effect cell death through caspase-dependent apoptosis, specifically through the mitochondria, and is less protective against those that cause necrotic cell death/caspase-independent cell death. On the basis of the data presented herein, we postulate that dykellic acid inhibits apoptosis at some point between the ROS-mediated initiation of apoptosis and caspase activation. Importantly, by inhibiting cell death before activation of the executioner caspases, dykellic acid has the potential to protect cells before they have passed the "point of no return" down the cell death pathway, although it remains to be seen if dykellic acid acts on targets upstream or downstream of mitochondria membrane depolarization. Compounds that inhibit apoptosis by acting on targets before caspase activation have intriguing potential as therapeutic agents. Although small molecules that inhibit apoptosis without directly affecting cellular ROS levels or caspase activity are less common, they are not without precedent; for example, geldanamycin and herbimycin A inhibit death in U-937 cells through induction of Hsp70 synthesis,[36,62] and small molecule inhibitors of poly(ADP-ribose)polymerase-1 (PARP-1) are known to be protective in certain cell culture and animal models of neurodegeneration and stroke.[63]

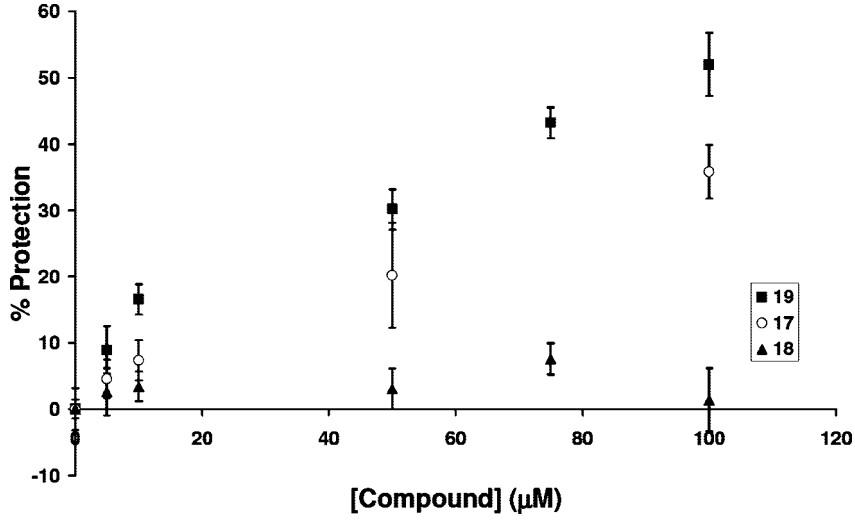


Figure 3. An intact diene side chain is essential to the full cytoprotective activity of dykellic acid. Cells were treated with compound **18**, **19**, or **20** for 5 h before etoposide was added to a final concentration of 1.5 μM. Cell death was assessed 24 h later by propidium iodide staining and cell flow cytometry. Error bars represent SDM from $n = 3$, with each trial performed in triplicate.

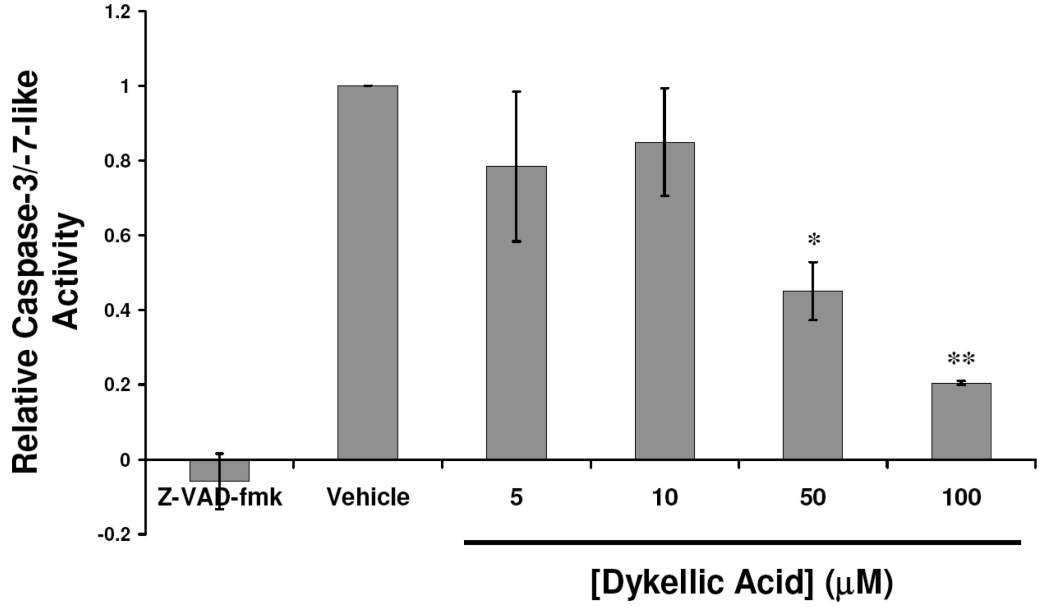


Figure 4. Dykellic acid reduces caspase-3/-7-like activity in etoposide-treated cells. U-937 cells were treated with Z-VAD-fmk (100 nM) or varying concentrations of dykellic acid for 5 h, and etoposide was then added to a final concentration of 1.5 μM. After 24 h the cells were transferred to caspase assay buffer and lysed, and the caspase substrate Ac-DEVD-pNA was added to a final concentration of 200 μM. The amount of pNA produced per minute was measured at 405 nm. All wells contained 1% of the vehicle, DMSO. The caspase-3/-7-like activity of cells treated with vehicle plus etoposide was normalized to 1.0, while the activity of cells treated with just vehicle (not shown) was normalized to 0. Error bars represent SDM from a single experiment performed in triplicate; these data are representative of three experiments. Statistical significance relative to vehicle control is indicated: (*) $P < 0.02$; (**) $P < 0.001$.

The structure−activity relationship established through synthesis and evaluation of dykellic acid derivatives provides insights into the mode of action of this compound and suggests opportunities for the enhancement of efficacy. Given the importance of the α,β-unsaturation in the diene side chain to its cytoprotective activity and the preponderance of electrophilic sites on dykellic acid, a key outstanding question is whether dykellic acid is covalently modifying its biological target. However, the δ,γ-double bond of the diene side chain can be eliminated with minimal loss of activity, suggesting that chemical modifications could be made to this side chain in an effort to improve biological activity or to identify the cellular target. With a tractable and flexible route dykellic acid in place, such studies are now feasible. Members of the gelastatin family of natural products are structurally related to dykellic acid. Gelastatins and some derivatives have been reported as inhibitors of matrix metalloproteinases (MMP) and tumor necrosis factor α converting enzyme (TACE),[64] and dykellic acid itself is known to inhibit the expression and activity of MMP-9 in cells.[23] Whether or not there is a direct connection between this MMP inhibition and dykellic acid's cytoprotective effect, at this point, is unclear.

In summary, we have achieved the first total synthesis of dykellic acid through an efficient and flexible route whose longest linear sequence is eight chemical steps from commercially available starting materials. As shown herein, dykellic acid protects U-937 cells from cellular insults that induce caspase-dependent apoptosis but is minimally protective against insults that induce other forms of cell death. Interestingly, this cytoprotection is mediated through neither the scavenging of

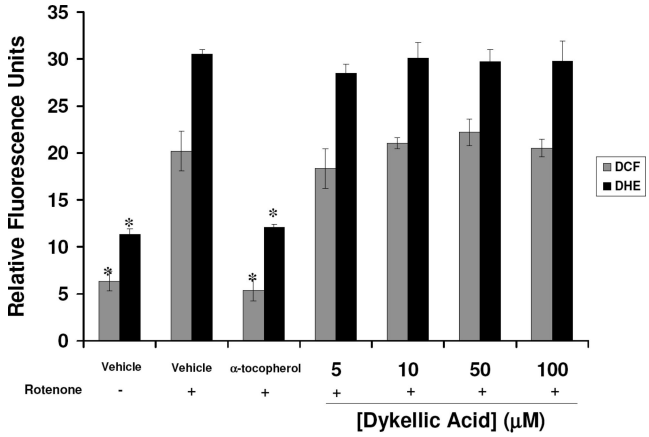


Figure 5. Dykellic acid does not quench intracellular reactive oxygen species. U-937 cells were treated with α-tocopherol (100 μM) or a range of dykellic acid concentrations for 5 h, at which point rotenone was added to a final concentration of 20 μM. After 40 min dihydroethidium or dichlorodihydrofluorescein diacetate (H_2DCF) was added and cellular fluorescence was assessed by cell flow cytometry. The 5, 10, 50, and 100 μM dykellic acid data points are not statistically different from the vehicle plus rotenone control. Statistical significance versus rotenone treated vehicle control is noted: (∗) $P < 0.016$ Error bars represent SDM from $n = 3$, with each trial performed in triplicate.

cellular ROS nor the direct inhibition of caspases. The structure−activity relationship established through the synthesis of key dykellic acid derivatives sets the stage for the optimization of dykellic acid's cytoprotective activity and for studies aimed at identifying the precise biological target of this compound. There are a multitude of disorders caused by premature cell death; dykellic acid's potency and nonstandard mechanism of cytoprotection make it an interesting candidate for evaluation in models of these disease states.

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Supporting Information Available: Materials and methods, NMR spectra of all new compounds, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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