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Synthesis, Chemical Reactivity as Michael Acceptors, and Biological Potency of Monocyclic Cyanoenones, Novel and Highly Potent Anti-inflammatory and Cytoprotective Agents[1]

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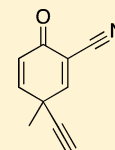
Supporting Information

ABSTRACT: Novel monocyclic cyanoenones examined to date display unique features regarding chemical reactivity as Michael acceptors and biological potency. Remarkably, in some biological assays, the simple structure is more potent than pentacyclic triterpenoids (e.g., CDDO and bardoxolone methyl) and tricycles (e.g., TBE-31). Among monocyclic cyanoenones, **1** is a highly reactive Michael acceptor with thiol nucleophiles. Furthermore, an important feature of **1** is that its Michael addition is reversible. For the inhibition of NO production, **1** shows the highest potency. Notably, its potency is about three times higher than CDDO, whose methyl ester (bardoxolone methyl) is presently in phase III clinical trials. For the induction of NQO1, **1** also demonstrated the highest potency. These results suggest that the reactivity of these Michael acceptors is closely related to their biological potency. Interestingly, in LPS-stimulated macrophages, **1** causes apoptosis and inhibits secretion of TNF-α and IL-1β with potencies that are higher than those of bardoxolone methyl and TBE-31.



1. INTRODUCTION

Over the past decade, we have been engaged in the modifications of oleanolic acid, a ubiquitous naturally occurring triterpenoid, which is almost inactive in our bioassays related to inhibition of inflammation and carcinogenesis.[2−9] Our intensive efforts led to the discovery of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO, Figure 1), which has high anti-inflammatory and cytoprotective potency.[3,4,6,10] CDDO and its related compounds are multifunctional agents. CDDO blocks the de novo synthesis of inducible nitric oxide synthase[3,4,6] and inducible cyclooxygenase (COX-2) in mouse macrophages, microglia, and fibroblasts.[10] CDDO induces the expression of the cytoprotective enzymes such as HO-1 in U937 cells[11] and NQO1 in Hepa1c1c7 murine hepatoma cells.[12] Presently, the methyl ester of CDDO (bardoxolone methyl, BARD, Figure 1) is in phase III clinical trials for the treatment of severe chronic kidney disease in type 2 diabetes mellitus patients. In a multicenter, double-blind, placebo-controlled phase IIb clinical trial, patients treated with bardoxolone methyl experienced a significant increase in estimated glomerular filtration rate (eGFR) compared with no change in the placebo group.[13]

During the development of CDDO, we found that the tricyclic compound, (±)-(4b*S*,8a*R*,10a*S*)-10a-ethynyl-4b,8,8-trimethyl-3,7-dioxo-3,4b,7,8,8a,9,10,10a-octahydrophenanthrene-2,6-dicarbonitrile (TBE-31, Figure 1) having the nonenolizable cyanoenones in rings A and C is also a novel class of potential anti-inflammatory, growth suppressive, and proapoptotic compound. TBE-31 inhibits nitric oxide (NO) production at low nanomolar concentrations in RAW cells and mouse primary macrophages stimulated with IFN-γ.[14,15] TBE-31 induces cytoprotective enzymes HO-1 in RAW cells[15] and in mice[14] and NQO1 in Hepa1c1c7 murine hepatoma cells.[1b,15] These potencies are much higher than those of CDDO and bardoxolone methyl. Incorporation of small quantities of TBE-31 in the diet robustly induces NQO1 and GST in the liver, skin, and stomach in mice.[1b] TBE-31 is orally highly active against aflatoxin-induced liver cancer in rats.[15,16]

We have chemically demonstrated that the nonenolizable cyanoenones in ring A of CDDO and in rings A and C of TBE-31 give reversible Michael adducts with the SH group of DTT

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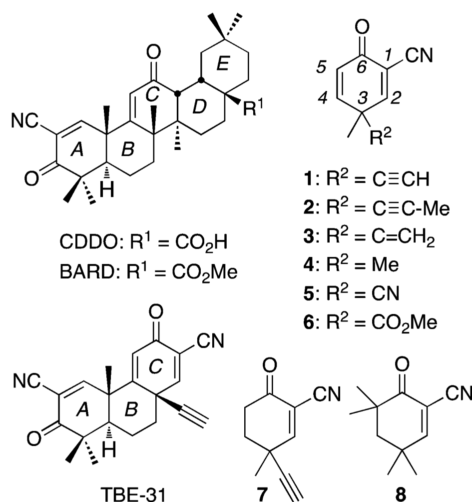


Figure 1. Structures of CDDO, bardoxolone methyl (BARD), TBE-31, and monocyclic cyanoenones **1**−**8**.

by UV and NMR studies.[1b,16,17] We speculate that this characteristic reactivity may imply a molecular mechanism of action. Indeed, our mechanism studies suggest that bardoxolone methyl and TBE-31 regulate proteins affecting inflammation, oxidative stress, differentiation, apoptosis, and proliferation, including Keap1, IKKβ, and JAK1, to name a few, by reversible Michael addition between the nonenolizable cyanoenone functionalities and the SH groups of cysteine moieties on these proteins. Keap1 is the electrophile sensor protein in the cellular Keap1/Nrf2/ARE pathway that controls a network of cytoprotective genes which defend against the damaging effects of oxidative and electrophilic stress and inflammation.[1b,12,18] Murine Keap1 is a thiol-rich protein possessing 25 cysteine residues, some of which are highly reactive.[18] Briefly, bardoxolone methyl induces Nrf2-dependent transcription by binding to the SH groups of Keap1 in the cytoplasm. Consequently, Keap1 loses its ability to target Nrf2 for ubiquitination and proteosomal degradation, leading to Nrf2 nuclear accumulation and transcriptional activation of ARE-dependent genes, including HO-1, NQO1, and GST. The Keap1/Nrf2/ARE pathway is considered to be of major importance for suppressing kidney disease progression.[13] Recently, Cys179 in the activation domain of the kinase IKKβ was identified as a target of bardoxolone methyl.[19,20] By binding to this site of IKKβ, bardoxolone methyl inactivates the kinase and ultimately results in blocking of the binding of NF-κB transcription factors to DNA and thus inhibits transcriptional activation of NF-κB-dependent target genes. It has also been reported that bardoxolone methyl inhibits the JAK1 → STAT3 pathway by directly binding to JAK1 at Cys1077 and STAT3 at Cys259.[21] Small molecule inhibitors of the STAT3 pathway are known to be effective as anticancer agents in vitro and in animal models.

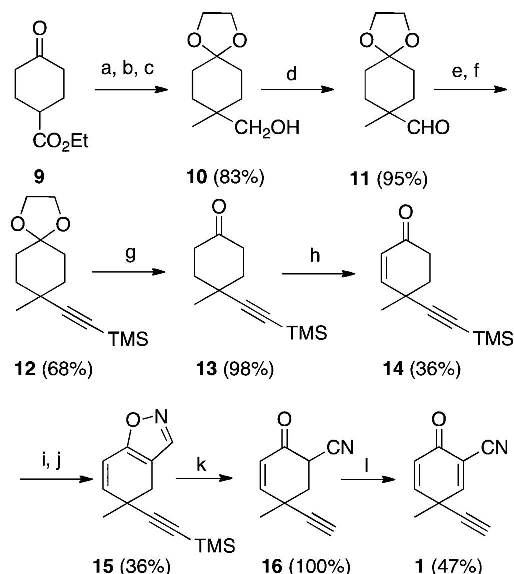
Therefore, on the basis of the mechanism studies, we concluded that the pharmacophores in semisynthetic pentacyclic triterpenoids and synthetic tricycles are nonenolizable cyanoenones which work as Michael acceptors. Thus, we were interested in the chemical reactivity as Michael acceptors and biological potency of monocyclic cyanoenones, which represent the reactive moiety in more complex tri- and pentacycles. We have synthesized a series of monocyclic cyanoenones **1**−**8** (Figure 1) to delineate the structural features important for the unique reactive properties. For example, **1** has the same structure as that of ring C in TBE-31, **8** has the same structure as that of ring A in bardoxolone methyl and TBE-31, and **5** and **6** have typical electron-withdrawing groups at quaternary carbon.

We have evaluated the chemical reactivity of monocyclic cyanoenones using UV and ^{1}H NMR spectroscopy and their biological potency in cell-based assays related to inhibition of inflammation and carcinogenesis. Monocyclic cyanoenones **1** and **5** are very reactive Michael acceptors, but more importantly, the addition is reversible. For the inhibition of NO production induced by IFN-γ in RAW cells, **1** shows the highest potency, most notably, **1** is three times more potent than CDDO. For the induction of the cytoprotective enzyme NQO1 in Hepa1c1c7 murine hepatoma cells, **1** also demonstrates the highest potency. These results suggest that the reactivity of these Michael acceptors is closely related to the biological potency. Monocyclic cyanoenone **1** causes apoptosis and inhibits secretion of IL-1β and TNF-α in LPS-stimulated macrophages, and these potencies are higher than those of bardoxolone methyl and TBE-31. Remarkably, in this assay, the simple monocyclic structure is more potent than penta- and tricycles. We herein describe the full account of our synthetic work with monocyclic cyanoenones and their unique and interesting features with respect to the chemical reactivity as Michael acceptors and biological potency.

2. CHEMISTRY

Monocyclic cyanoenone **1** was synthesized from commercially available ethyl 4-oxocyclohexanone-carboxylate (**9**) by the sequence shown in Scheme 1. Alcohol **10**[22] was prepared in

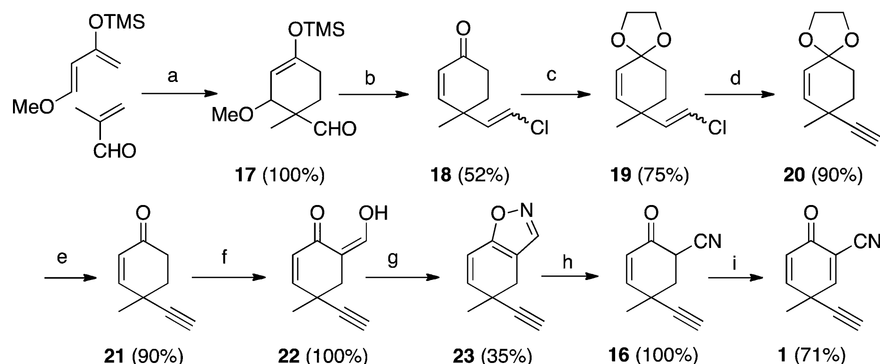
Scheme 1[a]



[a]Reagents: (a) ethylene glycol, (+)-CSA, PhH; (b) LDA, MeI; (c) $LiAlH_4$, Et_2O; (d) CrO_3, pyridine, CH_2Cl_2; (e) $Ph_3PCH_2Cl(Cl)$, *n*-BuLi, THF; (f) MeLi, THF; TMSCl; (g) aqueous HCl, acetone; (h) LDA, PhSeCl; 30% H_2O_2, CH_2Cl_2; (i) HCO_2Et, NaOMe, PhH; (j) $NH_2OH{\cdot}HCl$, aqueous EtOH; (k) NaOMe, MeOH, Et_2O; (l) DDQ, PhH.

83% yield from **9** by ketalization with ethylene glycol in the presence of (+)-CSA in benzene and subsequent methylation, followed by reduction with $LiAlH_4$. Ratcliffe oxidation of **10** with CrO_3[23] gave aldehyde **11** in 95% yield. Acethylene **12** was

Scheme 2[a]

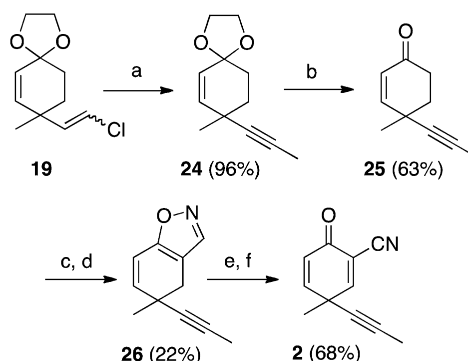


[a]Reagents: (a) toluene; (b) $Ph_3PCH_2Cl(Cl)$, *n*-BuLi, THF; (+)-CSA, Et_2O; (c) ethylene glycol, PPTS, PhH; (d) MeLi, THF; (e) aqueous HCl, acetone; (f) HCO_2Et, NaOMe, PhH; (g) $NH_2OH{\cdot}HCl$, aqueous EtOH; (h) NaOMe, MeOH, Et_2O; (i) PhSeCl, pyridine, CH_2Cl_2, 30% H_2O_2, CH_2Cl_2.

obtained in 68% yield from **11** by Wittig reaction with (chloromethyl)triphenylphosphonium chloride,[24] followed by dehydrochlorination with MeLi and subsequent treatment with TMSCl.[25] The ketal of **12** was removed under acidic conditions to afford **13** in 98% yield. Conversion of **13** into enone **14** was achieved by addition of phenylselenyl group to lithium enolate of **13** and subsequent oxidation/elimination with 30% aqueous H_2O_2 solution (36% yield) after several oxidation methods failed (for example, Br_2−HBr in acetic acid, PTAB and DBU,[26] PhSeCl in ethyl acetate and subsequent oxidation/elimination with 30% H_2O_2,[27] PhSeCl−pyridine in CH_2Cl_2 and subsequent oxidation/elimination with 30% H_2O_2[28]). Formylation of **14** with ethyl formate in the presence of NaOMe in benzene,[29] followed by the condensation with hydroxylamine hydrochloride in aqueous EtOH,[30] gave isoxazole **15** in 36% yield. The cleavage of the isoxazole ring of **15** with NaOMe in MeOH and Et_2O[30] produced **16** in quantitative yield. Monocyclic cyanoenone **1** was obtained in 47% yield by oxidation of **16** with DDQ in benzene under reflux (3.2% overall yield from **9**). Although we could synthesize 10 mg of **1** in a single batch by this sequence, it was quite difficult to provide more than 100 mg of **1** in a single batch (the amount necessary for further biological evaluation) because this synthesis requires 12 steps and the yield of some steps is pretty low. In particular, conversion of **13** into **14** (insertion of a double bond into a cyclohexanone ring) was problematic. The yield decreased according to the increasing amount of **13**.

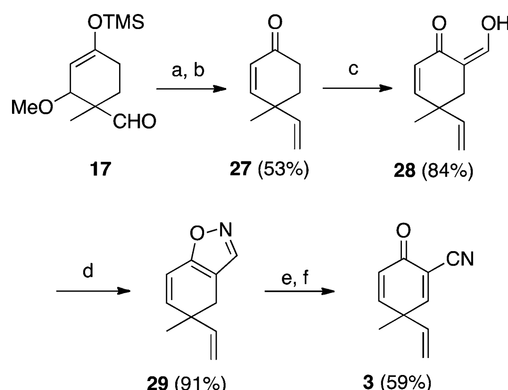
Thus, we have explored an entirely new synthesis as shown in Scheme 2. To avoid insertion of a double bond to a cyclohexanone ring, we envisioned that a cyclohexenone ring should be constructed by Diels−Alder reaction initially. Known Diels−Alder adduct **17**[31] was obtained as a mixture of two diasteromers (6.5:1.0) in quantitative yield from Danishefsky's diene and methacrolein (2.1 equiv) in toluene in a sealed tube at 110 °C. Wittig reaction on **17** with (chloromethyl)triphenylphosphonium chloride, followed by deprotection with (+)-CSA in Et_2O, gave **18** as a mixture of *E*/*Z* chlorovinyl isomers (*E*:*Z* = 2:1) in 52% yield. Ketalization of **18** with ethylene glycol in the presence of PPTS in benzene afforded ketal **19** in 75% yield. Dehydrochlorination of **19** with MeLi in THF, followed by quenching of the acetylide with aqueous NH_4Cl solution, provided **20** in 90% yield.[24] The ketal of **20** was removed under acidic conditions to produce **21** in 90% yield. Formylation of **21** with ethyl formate gave **22** in quantitative yield. Isoxazole **23** was obtained in 35% yield by condensation of **22** with

Scheme 3[a]



[a]Reagents: (a) MeI, *n*-BuLi, THF; (b) aqueous HCl, acetone; (c) HCO_2Et, NaOMe, PhH; (d) $NH_2OH{\cdot}HCl$, aqueous EtOH; (e) NaOMe, MeOH, Et_2O; (f) PhSeCl, pyridine, CH_2Cl_2; 30% H_2O_2, CH_2Cl_2.

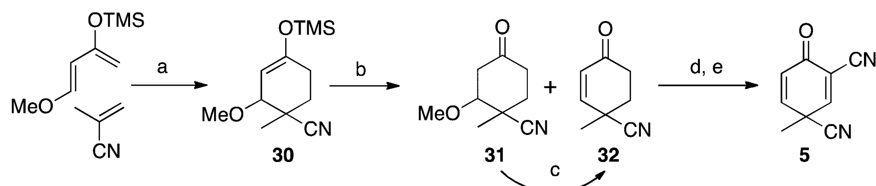
Scheme 4[a]



[a]Reagents: (a) Ph_3PCH_3I, *n*-BuLi, THF; (b) (+)-CSA, Et_2O; (c) HCO_2Et, NaOMe, PhH; (d) $NH_2OH{\cdot}HCl$, aqueous EtOH; (e) NaOMe, MeOH, Et_2O; (f) DDQ, PhH.

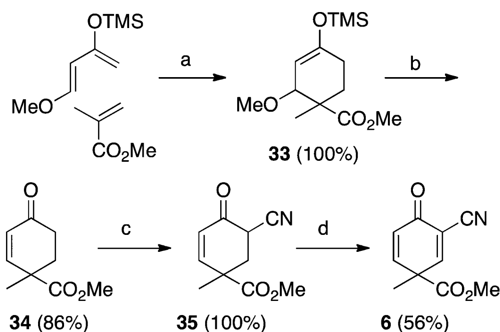
hydroxylamine hydrochloride. The cleavage of the isoxazole ring of **23** with NaOMe afforded nitrile **16** in quantitative yield. Monocyclic cyanoenone **1** was prepared in 71% yield from **16** by addition of PhSeCl in the presence of pyridine and subsequent oxidation/elimination of the selenated intermediate with 30% aqueous H_2O_2 solution.[28] This oxidation method (71% yield) was better than the DDQ method (47% yield) for

Scheme 5[a]



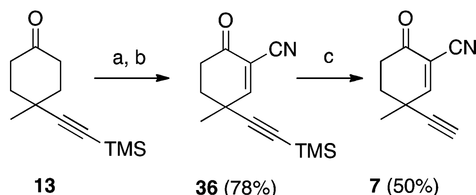
[a]Reagents and yields: (a) toluene, 93%; (b) (+)-CSA, Et_2O, **31**, 19%, **32**, 46%; (c) (+)-CSA, THF, 86%; (d) *p*-TsCN, LDA, THF, 55%; (e) DDQ, PhH, 38%.

Scheme 6[a]



[a]Reagents: (a) toluene; (b) (+)-CSA, Et_2O; (c) *p*-TsCN, LDA, THF; (d) DDQ, PhH.

Scheme 7[a]



[a]Reagents: (a) *p*-TsCN, LDA, THF; (b) DDQ, PhH; (c) TBAF, THF.

this oxidation with an unprotected acetylene group. Overall, **1** was synthesized in 7.9% yield in nine steps from Danishefsky's diene. The scheme results in three less steps and an overall yield of two times more than the previous scheme. Accordingly, we were able to synthesize 300 mg of **1** in a single batch (see Supporting Information).

New monocyclic cyanoenone **2** was synthesized from **19** by the sequence shown in Scheme 3. Ketal **24** was prepared in 96% yield by the treatment of **19** with MeI in the presence of *n*-BuLi in THF. Removal of the ketal of **24** under acidic conditions gave enone **25** in 63% yield. Monocyclic cyanoenone **2** was obtained in 15% yield via isoxazole **26** from **25** by the same sequence as for **1** from **21**.

New monocyclic cyanoenone **3** was synthesized from Diels–Alder adduct **17** by the sequence shown in Scheme 4. Wittig reaction on **17** with methyltriphenylphosphonium iodide in the presence of *n*-BuLi in THF, followed by deprotection with (+)-CSA, gave **27** in 53% yield. Monocyclic cyanoenone **3** was prepared in 45% yield via **28** and **29** from **27** by the same sequence as for **1** from **14** (see Scheme 1).

Known monocyclic cyanoenone **4** (crystalline solid, mp 105–106 °C) was prepared according to the methods in the literature.[32]

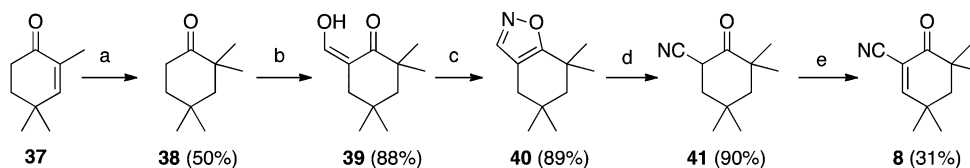
New monocyclic cyanoenone **5** was synthesized from the known Diels–Alder adduct **30**[33] by the sequence as shown in Scheme 5. Diels–Alder adduct **30** was obtained as a mixture of two separable diastereomers in 93% yield from Danishefsky's diene and acrylonitrile (3 equiv) in toluene in a sealed tube at 120 °C. Deprotection of **30** with (+)-CSA in Et_2O at rt gave **31** (19% yield as a mixture of two separable diastereomers) and **32** (46% yield). The treatment of **31** with (+)-CSA in THF at 60 °C produced **32** in 86% yield. Overall, **32** was obtained in 62% yield from **30**. Since Johnson's isoxazole method,[30] which was used for the synthesis of **1**, **2**, and **3**, followed by DDQ oxidation, afforded **5** from **32** in very low yield (<5%), we attempted to introduce a cyano group to **32** using *p*-TsCN.[34] Cyanation of the enolate of **32** generated using LDA in THF, with *p*-TsCN, followed by DDQ oxidation, gave **5** in 21% yield.

Monocyclic cyanoenone **6** was previously reported,[35] but we have explored a new synthesis of **6** as shown in Scheme 6. Known compound **34**[36] was prepared by Diels–Alder reaction between Danishefsky's diene and methyl acrylate, followed by deprotection with (+)-CSA (86% yield). New compound **35** was obtained in quantitative yield from **34** by cyanation of the enolate with *p*-TsCN. DDQ oxidation of **35** gave **6** in 56% yield.

New monocyclic cyanoenone **7** was synthesized from **13** as shown in Scheme 7. Cyanoenone **36** was prepared in 78% yield from **13** by cyanation with *p*-TsCN, followed by DDQ oxidation. Removal of TMS group from **36** gave **7** in 50% yield.

Monocyclic cyanoenone **8** was synthesized in five steps from **37** (Scheme 8), which was prepared by Robinson annulation with isobutyraldehyde and ethyl vinyl ketone.[37] Known compound **38** was prepared in 50% yield by reductive methylation of **37**.[38] Formylation of **38**, followed by the treatment with

Scheme 8[a]



[a]Reagents: (a) MeI, NH_3, Li, *t*-BuOH; (b) HCO_2Et, NaOMe, PhH; (c) $NH_2OH{\cdot}HCl$, aqueous EtOH; (d) NaOMe, Et_2O, MeOH; (e) DDQ, PhH.

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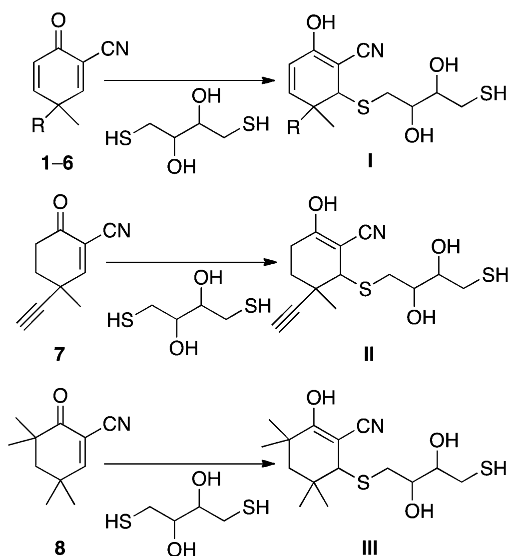
hydroxylamine, gave isoxazole **40** in 78% yield. The cleavage of the isoxazole ring of **40** with NaOMe afforded **41** in 90% yield. Monocyclic cyanoenone **8** was obtained by DDQ oxidation of **41** in 31% yield.

Scheme 9



3. RESULTS AND DISCUSSION

3.1. Chemical Reactivity of Monocyclic Cyanoenones as Michael Acceptors. *3.1.1. UV Studies on Monocyclic Cyanoenones.* We have evaluated the chemical reactivity of **1–8** as Michael acceptors using UV spectroscopy. Monocyclic cyanoenones **1–6**, **7**, and **8** with DTT give Michael adducts **I**, **II**, and **III**, respectively (Scheme 9). Because adducts **I**, **II**, and **III** have different UV absorption spectra from those of the corresponding monocyclic cyanoenones, UV spectroscopy can clarify whether or not the Michael adducts are produced.

*1. UV Studies on **1**, **2**, and **6** with DTT.* Monocyclic cyanoenone **1** has a UV absorption at 334 nm (absorbance (A) = 0.227 with 1 equiv of DTT and 0.406 with 10 equiv of DTT) upon the addition of DTT under dilute (0.1 mM of **1**) and neutral aqueous conditions (pH 7.4 phosphate buffered saline–1% ethanol), which are similar to physiological conditions (Figure 2A). The absorption at 334 nm, which increases with the addition of DTT, is identical to the absorption of **16** at 330–332 nm (Figure 2B), which increases with the addition of NaOH (presumably due to enhanced H-bonding to the enol hydroxyl). This observation strongly indicates that the absorption at 334 nm comes from the Michael adduct of DTT with **1** because the chromophore of the Michael adduct is the same as the enol form of **16**, which shows the absorption at higher wavelength than the keto form of **16**.

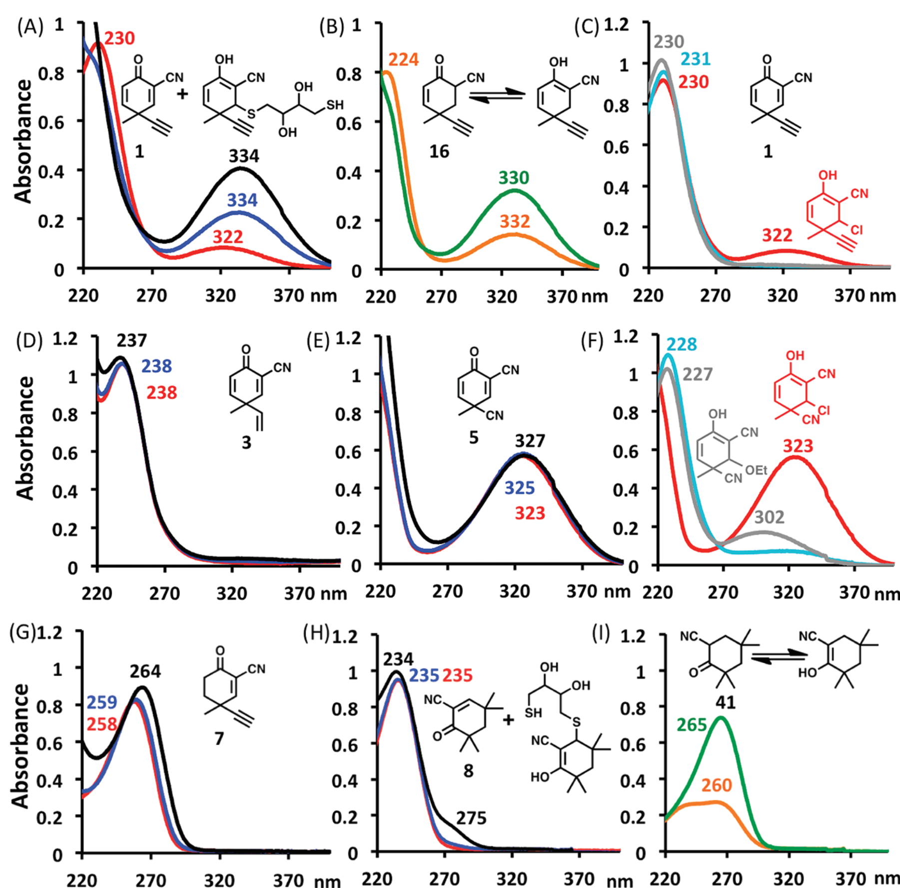


Figure 2. UV spectra of monocyclic cyanoenones with DTT at rt. (A,D,E,G,H) UV spectra of 0.1 mM monocyclic cyanoenones (red lines) in phosphate buffer saline–1% ethanol (pH 7.4) and their corresponding reaction mixtures with 0.1 mM DTT (blue lines) and 1 mM DTT (black lines). (B,I) UV spectra of **16** (0.1 mM) and **41** (0.16 mM) (orange lines) in phosphate buffer saline–1% ethanol (pH 7.4) and their corresponding reaction mixtures with 10 mM NaOH (green lines). (C,F) UV spectra of 0.1 mM monocyclic cyanoenones (red lines) in phosphate buffer saline–1% ethanol (pH 7.4), in water (light-blue lines), and in ethanol (gray lines).

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Interestingly, while the UV spectrum of **1** (c = 0.1 mM) in the phosphate buffer shows two absorption bands at 230 nm (A = 0.915) and 322 nm (A = 0.083), the one at the higher wavelength (322 nm) is not observed in ethanol and deionized water (Figure 2C). The phosphate buffer saline−1% ethanol (pH 7.4) solution includes 1 mM KH_2PO_4, 5.6 mM Na_2HPO_4, and 154 mM NaCl. Although the phosphate and chloride anions are much weaker nucleophiles than the SH group because the concentration of NaCl is 1540 times more than the concentration of DTT (0.1 mM), we expect that **1** reacts with chloride anion to give a Michael adduct.[39]

We have calculated an approximate equilibrium constant (K) of this Michael reaction in the solution of **1** and DTT (each initial concentration, 0.1 mM). The value of the constant is approximately 2.0 × 10^3 (L/mol, see the calculation in the Supporting Information), implying that this addition is very strongly favored.

While **2** with DTT in the same phosphate buffer gives similar spectra at 333 nm (A = 0.237 with 1 equiv of DTT and 0.395 with 10 equiv of DTT) to those of **1** with DTT, the spectrum of **2** without DTT gives a much smaller absorption at 319 nm (A = 0.042) than that of **1** (see Figure S1(A) in the Supporting Information). These results indicate that **2** has the similar reactivity with DTT to that of **1** but is less reactive with the chloride anion than **1**.

Monocyclic cyanoenone **6** with DTT in the phosphate buffer gives absorptions at 328 nm (A = 0.171 with 1 equiv of DTT) and 330 nm (A = 0.360 with 10 equiv of DTT), respectively. Monocyclic cyanoenone **6** without DTT in the same buffer does not show clear absorption at the higher wavelength. These spectra are almost identical to those of **2** (see Figure S1(B) in the Supporting Information).

*2. UV Studies on **3** and **4** with DTT.* Neither **3** (Figure 2D) nor **4** (Figure S1 (C) in the Supporting Information) gives the absorption at around 333 nm upon the addition of DTT in the phosphate buffer. These spectra indicate that both monocyclic cyanoenones do not react with DTT under these conditions.

*3. UV Studies on **5** with DTT.* Monocyclic cyanoenone **5** without DTT in the phosphate buffer gives the strong absorption at 323 nm (A = 0.561), which is considered to come from an adduct of **5** with chloride anion analogous to that observed for **1**.[39] Upon the addition of DTT, the UV spectra are similar [325 nm (A = 0.571 with 1 equiv of DTT) and 327 nm (A = 0.569 with 10 equiv of DTT)] to that of **5** without DTT. These observations indicate that **5** is highly reactive with both chloride anion and DTT (Figure 2E). Monocyclic cyanoenone **5** in ethanol also shows the absorption at 302 nm (A = 0.171), which comes from a Michael adduct of **5** with ethanol. The molecular ion peak [m/z 203 $(M - H)^-$] of the adduct is observed in the ethanolic solution of **5** by negative MS (ESI−). Surprisingly, **5** can react with ethanol although ethanol is a weaker nucleophile than chloride anion. In deionized water, an absorption band at the higher wavelength is not observed (Figure 2F).

*4. UV Studies on **7** with DTT.* Monocyclic cyanoenone **7** (an acetylenic enolizable cyanoenone) with 1 equiv of DTT in the phosphate buffer gives the same spectrum as that of **7** without DTT. The small red-shift, which is considered to come from a small amount of the adduct, is observed when 10 equiv of DTT is added (Figure 2G).

*5. UV Studies on **8** with DTT.* The UV spectrum of **8** shows weak absorption at 275 nm upon the addition of 10 equiv of DTT while any changes are not observed upon the addition of 1 equiv of DTT (Figure 2H). The absorption at 275 nm is identical with the absorption of **41** at 260−265 nm (Figure 2I), which increases with the addition of NaOH. This observation strongly indicates that the absorption at 275 nm comes from the Michael adduct of **8** with DTT because the chromophore of the Michael adduct is the same as the enol form of **41**, which shows the absorption at the higher wavelength than the keto form of **41**.

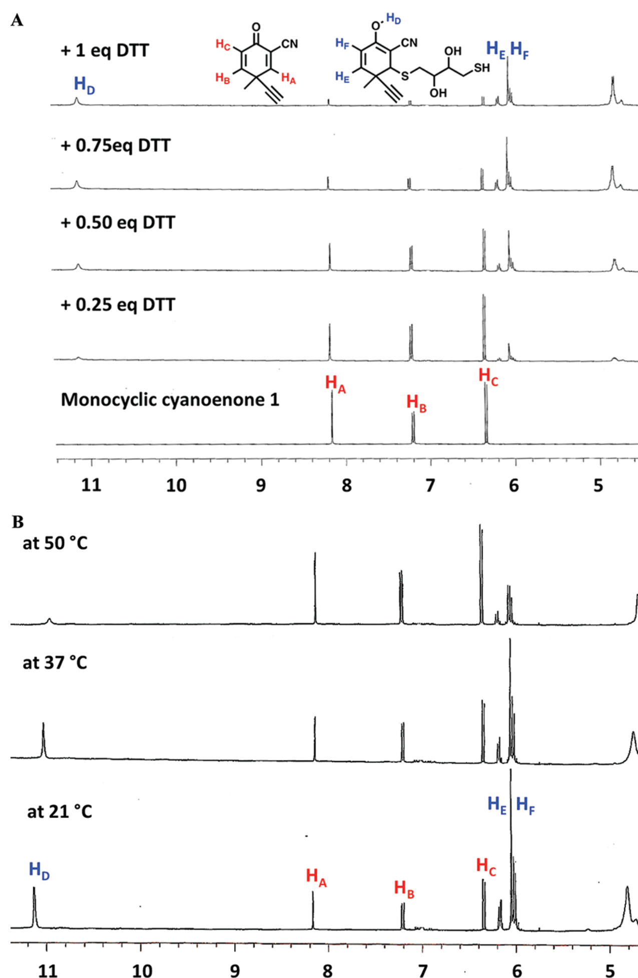
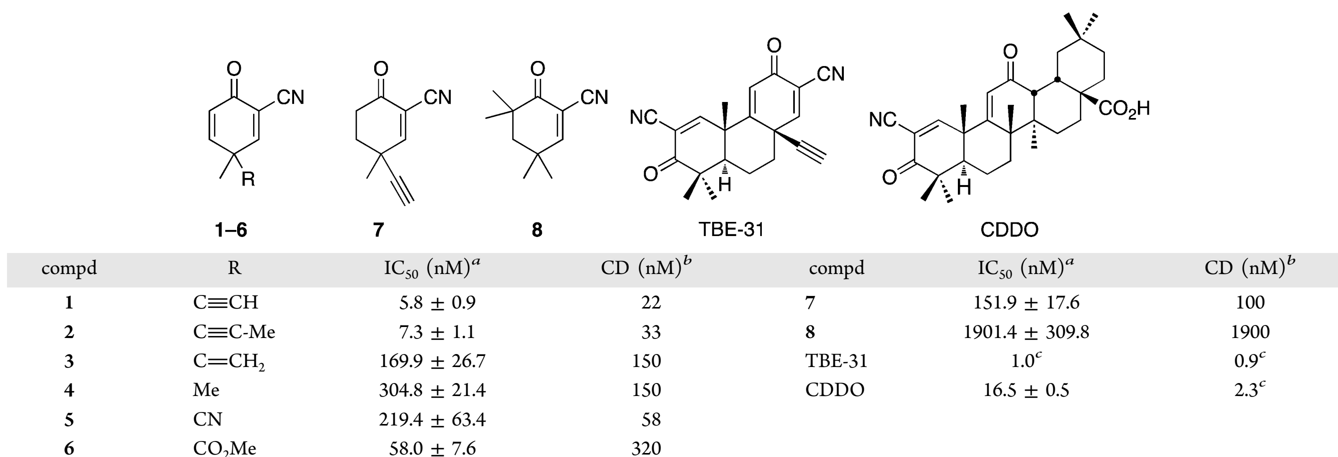


Figure 3. ^{1}H NMR studies on **1** with DTT (A) ^{1}H NMR changes upon increasing amounts of DTT in a 30 mM DMSO-d_6 solution of **1**. (B) ^{1}H NMR changes of 30 mM DMSO-d_6 solution of **1** with 1 equiv of DTT at elevated and decreased temperatures. The δ 2.50 signal of CD_3SOCD_2H was used as an internal standard.

6. UV Studies on Monocyclic Cyanoenones with Keap1. Murine Keap1 is a thiol-rich protein possessing 25 cysteine residues, some of which are highly reactive.[18] UV studies on **1**, **4**, **7**, and **8** with Keap1 are completely consistent with those with DTT (Figure S2 in the Supporting Information). Importantly, **1** is very reactive with both cysteine moieties on Keap1 as well as DTT, a small thiol nucleophile. To the contrary, **4**, **7**, and **8** have greatly reduced reactivity toward Keap1.

Overall, our UV studies demonstrate that the order of the reactivity as Michael acceptors with nucleophiles is **5** >**1** > **2** = **6** > **7** > **8** > **3** = **4**. We have observed a tendency that electron-withdrawing groups at C3 position increase the reactivity while electron-donating groups do not. Monocyclic cyanoenone **5** with a nitrile group, a strong electron-withdrawing group, is the most reactive Michael acceptor among monocyclic cyanoenones

Table 1. Inhibitory Activity of Monocyclic Cyanoenones on IFN-γ Induced NO Production in RAW 264.7 Cells and NQO1-Inducing Potency in Hepa1c1c7 Cells



compd	R	IC_{50} (nM)[a]	CD (nM)[b]	compd	IC_{50} (nM)[a]	CD (nM)[b]
1	C≡CH	5.8 ± 0.9	22	**7**	151.9 ± 17.6	100
2	C≡C-Me	7.3 ± 1.1	33	**8**	1901.4 ± 309.8	1900
3	C=CH_2	169.9 ± 26.7	150	TBE-31	1.0[c]	0.9[c]
4	Me	304.8 ± 21.4	150	CDDO	16.5 ± 0.5	2.3[c]
5	CN	219.4 ± 63.4	58			
6	CO_2Me	58.0 ± 7.6	320			

[a]RAW 264.7 mouse macrophages were plated in 96-well plates at 30000 cells/well in triplicate. On the next day, cells were pretreated with DMSO or test compounds, using a 2-fold serial dilution (0−200 nM or 0−10 μM, as appropriate) for 2 h, followed by IFN-γ (20 ng/mL) treatment for an additional 24 h. NO concentration in media was determined using the Griess reagent system (Promega). Cell viability was assessed using WST-1 reagent (Roche). IC_{50} values were determined based on suppression of IFN-γ-induced NO production normalized to cell viability. Results shown are the average of three independent experiments. Error represents standard deviation. [b]Hepa1c1c7 cells (10000 cells/well) were grown in 96-well plates for 24 h and then treated with serial dilutions of compounds for 48 h. The concentration required to double (CD) the specific enzyme activity of NQO1 was used to quantify inducer potency. The value is based on the activity from eight replicate wells at each concentration. The standard deviation in each case was between 5 and 10%. [c]These data have been published in ref 15.

evaluated. The reactivity of **5** with chloride anion is almost the same as that with DTT. Only **5** can react with ethanol, a weaker nucleophile, to produce an adduct. Notably and interestingly, moderate electron-withdrawing groups, alkyne groups enhance the reactivity equal to or slightly more than a methoxycarbonyl group. Importantly, the enolizable cyanoenone **7** is less reactive than the corresponding nonenolizable cyanoenone **1**.

3.1.2. NMR Studies on 1 and 5. ^{1}H NMR (500 MHz) studies confirmed that **1** reacts in a dose-dependent fashion with DTT to give the Michael adduct (Figure 3A). In a 30 mM DMSO-d_6 solution of **1**, three olefinic protons, H_A, H_B, and H_C, are observed at δ 8.17 ppm (d, J = 3 Hz), 7.21 ppm (dd, J = 3 and 10 Hz), and 6.35 ppm (d, J = 10 Hz), respectively. Upon increasing amounts of DTT (0.25 equiv → 1.0 equiv), H_A, H_B, and H_C decrease and an enol proton H_D and the new olefinic protons H_E and H_F appear and increase, which are derived from the Michael adduct of **1** with DTT. The adduct formation was also confirmed by the detection of molecular ion peaks at m/z 310 (ESI−) and 312 (ESI+ with formic acid) upon treating **1** with 1 equiv of DTT. ^{1}H NMR (300 MHz) studies confirmed that **5** also reacts in a dose-dependent fashion with DTT to give the Michael adduct (data not shown).

In a 30 mM DMSO-d_6 solution of **1** with 1.0 equiv of DTT at 21 °C, three olefinic protons, H_A, H_B, and H_C, which are derived from **1**, and the enol proton H_D and two olefinic protons H_E and H_F, which are derived from the adduct, are observed. At elevated temperatures (21 → 37 → 50 °C), the enol proton H_D and olefinic protons H_E and H_F of the adduct decrease and the olefinic protons H_A, H_B, and H_C of **1** increase. At 37 °C, the ratio of the adduct and **1** is about 1:1. Upon decreasing the temperature (50 → 37 → 21 °C), the reverse reaction occurs and the protons of the adduct increase while the protons of **1** decrease. This observation shows that the Michael addition is reversible (Figure 3B). Although the Michael adduct of **1** with DTT is observed by UV and ^{1}H NMR, the adduct cannot be isolated. These results indicate that the conversion of the Michael adduct to **1** is very fast.

Such chemical reversibility has very significant biological implications: (1) it enhances the bioavailability of **1**, (2) it allows reversible cysteine modifications of the protein targets, which do not need to be permanently inactivated (and possibly subsequently destroyed) but could be easily regenerated without requiring de novo protein synthesis, and (3) it leads to a pulse of inhibitory action, rather than constitutive modulation of the target signaling pathways. Taken together, these properties may explain, at least in part, why TBE-31 containing structure of **1** in ring C has extraordinary high biological potency in vivo,[1b,14−16] given their ability to react with protein targets at nanomolar concentrations despite the presence of millimolar concentrations of glutathione.

3.2. Biological Results. *3.2.1. Monocyclic Cyanoenones 1−8 Inhibit NO Production Induced by IFN-γ in RAW 264.7 Cells.* We evaluated the inhibitory activities of **1**−**8** on NO production induced by IFN-γ in RAW 264.7 cells. The inhibitory activities [IC_{50} (nM) values] of **1**−**8**, TBE-31, and CDDO are shown in Table 1. Among monocyclic cyanoenones, **1** has the highest potency, followed by **2**. Notably, both **1** and **2**, which are acetylenic monocyclic nonenolizable cyanoenones, are 2−3 times more potent than CDDO, which is a pentacyclic tritepenenoid. Remarkably, **1** approaches the potency of TBE-31 in this assay, which is currently the most potent among semisynthetic pentacyclic triterpenoids and synthetic tricycles; **1** is only about six times less potent than TBE-31.

3.2.2. Monocyclic Cyanoenones 1−8 Induce NQO1 in Hepa1c1c7 Murine Hepatoma Cells. Monocyclic cyanoenones **1**−**8** were next evaluated for the induction of the phase 2 cytoprotective enzyme NQO1 in Hepa1c1c7 murine hepatoma cells. Monocyclic cyanoenones double the specific enzyme activity of NQO1 in the 20−2000 nM concentration range (Table 1). Notably, even in this assay, **1** is the most potent and **2** is the next.

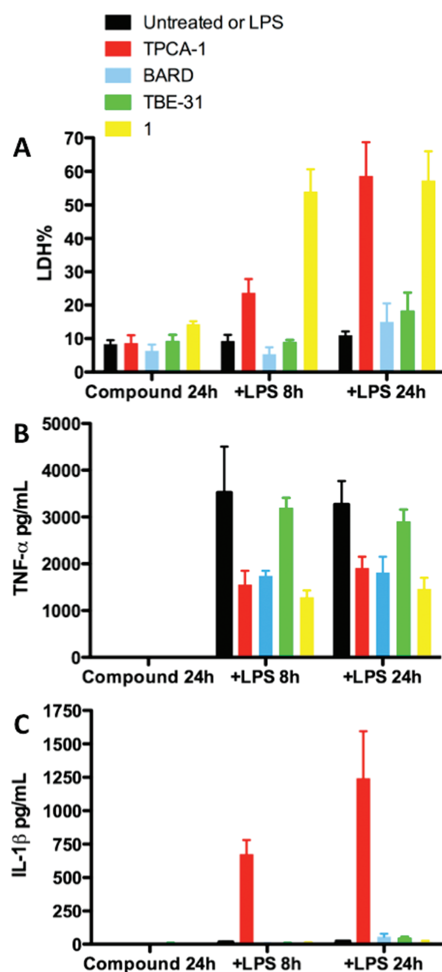


Figure 4. Monocyclic cyanoenone **1** causes apoptosis and inhibits secretion of TNF-α and IL-1β in LPS-induced macrophages. Macrophages were left untreated or treated with TPCA-1 (0.5 μM), bardoxolone methyl (BARD, 0.5 μM), TBE-31 (0.1 μM), or **1** (0.5 μM). Some of the macrophages were not exposed to LPS (compound 24 h), while other macrophages were exposed to LPS (100 ng/mL, 1 h after the start of compound treatment) for 8 h (+LPS 8 h) or 24 h (+LPS 24 h). Culture supernatants collected at 8 or 24 h were assayed for (A) lactate dehydrogenase (LDH, % LDH released) to measure cell death or (B) TNF-α (pg/mL) or (C) IL-1β (pg/mL) to measure levels of secreted cytokines. Results shown are averages from two independent experiments. Error bars represent standard deviation.

As described earlier, a strong correlation between reactivity of monocyclic cyanoenones toward DTT and interaction with Keap1 has been established. Monocyclic cyanoenone **1** is very reactive toward DTT, much higher than that observed for **8** and CDDO,[17] which has the same structure in ring A as that of **8**. Monocyclic cyanoenone **1** is also very reactive toward cysteine moieties of Keap1 (Figure S2 in the Supporting Information). This reactivity with Keap1 as monitored by UV absorption is a unique property of **1**, as the related compound **8** has greatly reduced reactivity toward Keap1. Nevertheless, **1** is about 10 times less potent than CDDO in this assay although **1** is much more potent than **8**. It is possible that **1** would be as potent as CDDO or more potent with respect to NQO1 inducing potency in other cell lines, for example, macrophages, because there may be differences between macrophages and hepatoma cells with respect to their response to monocyclic cyanoenones.

We previously demonstrated a linear correlation between NQO1 inducer potency (CD) and inhibitory activity on NO production (IC_{50}) of semisynthetic triterpenoids[9,12] and synthetic tricycles.[15] In this series of monocyclic cyanoenones, although we observed a similar general trend that the lower IC_{50} values (nM), the lower CD values (nM), the correlation was not linear.

We have found that the reactivity of these Michael acceptors is closely related to the biological potency except for **5** and that **1** has the highest biological potency among all of the monocyclic cyanoenones so far. It is very interesting that an ethynyl group at C3 position plays an important role for enhancing the reactivity as a Michael acceptor and the biological potency in both bioassays. Curiously, although **5** has the highest reactivity as a Michael acceptor, the biological potency is not so high. The reasons for this can include: (i) due to its exceedingly high chemical reactivity, a large portion of **5** could be "quenched" by abundant cellular thiols, such as the cysteine residue of glutathione, which is present at millimolar concentrations, (ii) monocyclic cyanoenone **5** could be inactivated by chloride anion in the cell culture medium used for biological testing, and/or (iii) in addition to the chemical reactivity, other factors such as cellular uptake and export mechanisms may play a role in biological potency.

3.2.3. Monocyclic Cyanoenone 1 Strongly Causes Apoptosis and Inhibits Secretion of TNF-α and IL-1β in LPS-Stimulated Macrophages. Previous results show that inhibition of IKKβ with the ATP-competitive inhibitor ML120B in LPS-stimulated macrophages results in apoptosis, caspase-1 activation, and IL-1β secretion.[40] We evaluated **1** as a representative of monocyclic cyanoenones, TBE-31, bardoxolone methyl, and TPCA-1,[41] which is an ATP-competitive IKKβ inhibitor in LPS-stimulated macrophages.[42] To measure LPS-stimulated production of cytokines in macrophages after pretreatment with these compounds, it is important to use maximal concentrations of the compounds that are not directly toxic to the cells (maximum nontoxic dose; MNTD), otherwise lack of cytokine production could be an artifact from rapid cell death. We determined the MNTDs for **1** (0.5 μM), TBE-31 (0.1 μM), bardoxolone methyl (0.5 μM), and TPCA-1 (0.5 μM). In preliminary experiments, **1** and TPCA-1 strongly caused LPS-stimulated cell death but bardoxolone methyl and TBE-31 did not (Figure 4A). Monocyclic cyanoenone **1** was more potent than TPCA-1 after macrophages were exposed to LPS for 8 h. Even 24 h after LPS exposure, **1** was as potent as TPCA-1. Monocyclic cyanoenone **1** was also more effective than TPCA-1, bardoxolone methyl, and TBE-31 at inhibiting secretion of TNF-α from LPS stimulated macrophages (Figure 4B). Importantly, unlike TPCA-1, **1** did not induce high levels of secretion of IL-1β (Figure 4C) despite the fact that both inhibitors caused LPS-stimulated cell death. Remarkably, in this assay, the simple monocyclic structure is more potent than pentacycles and tricycles. These results suggest that **1** may be a new class of IKKβ inhibitor for the treatment of inflammation and cancer.

4. CONCLUSION

In summary, through these investigations, we have demonstrated that the essential factor for potency is not the pentacyclic triterpenoid or the tricyclic skeleton but the functional monocyclic cyanoenones that are positioned at a specific orientation relative to each other. We have clarified that the reactivity as Michael acceptors of monocyclic cyanoenones is closely related to the biological potency. Among monocyclic cyanoenones

evaluated, **1** is a very reactive Michael acceptor with thiol nucleophiles and, more importantly, the addition is reversible. Monocyclic cyanoenone **1** has the highest potency for the inhibition of NO production induced by IFN-γ in RAW cells and the induction of the cytoprotective enzyme NQO1 in Hepa1c1c7 murine hepatoma cells. In LPS-stimulated macrophages, **1** causes apoptosis and inhibits secretion of TNF-α and IL-1β. These potencies are higher than those of bardoxolone methyl, TBE-31, and TPCA-1, which is an ATP-competitive IKKβ inhibitor. Importantly and interestingly, a very small molecule such as **1** has high biological potency against inflammation and carcinogenesis. Not only further biological in vivo studies on **1** but also drug design of new compounds using monocyclic cyanoenones as fragments are very interesting and promising. These investigations are in progress.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures and characterization data for new compounds **1−41**, materials and methods for LPS-induced macrophage assay, UV spectra of monocyclic cyanoenones with DTT and Keap1 (Figures S1 and S2), and list of elemental analyses for specifying the purity of **1−8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ARE, antioxidant response element; (+)-CSA, (+)-10-camphersulfonic acid; TMSCl, chlorotrimethylsilane; DDQ, 2,6-dichloro-3,5-dicyano-1,4-benzoquinone; DTT, dithiothreitol; GST, glutathione *S*-transferase; HO-1, heme oxygenase 1; IKKβ, inhibitor of nuclear factor κB kinase β; IFN-γ, interferon-γ; IL-1β, interleukin-1β; JAK1, Janus kinase 1; Keap1, kelch-like ECH-associated protein 1; LPS, lipopolysaccharide; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor (erythroid-derived 2) related factor 2; NF-κB, nuclear factor κB; PhSeCl, phenylselenyl chloride; PTAB, phenyltrimethylammonium tribromide; PPTS, pyridinium *p*-toluenesulfonate; *p*-TsCN, *p*-toluenesulfonyl cyanide; TNF-α, tumor necrosis factor-α

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