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New Monocyclic, Bicyclic, and Tricyclic Ethynylcyanodienones as Activators of the Keap1/Nrf2/ARE Pathway and Inhibitors of Inducible Nitric Oxide Synthase

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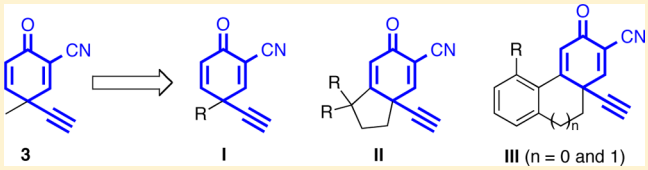
Supporting Information

ABSTRACT: A monocyclic compound **3** (3-ethynyl-3-methyl-6-oxocyclohexa-1,4-dienecarbonitrile) is a highly reactive Michael acceptor leading to reversible adducts with nucleophiles, which displays equal or greater potency than the pentacyclic triterpenoid CDDO in inflammation and carcinogenesis related assays. Recently, reversible covalent drugs, which bind with protein targets but not permanently, have been gaining attention because of their unique features. To explore such reversible covalent drugs, we have synthesized monocyclic, bicyclic, and tricyclic compounds containing **3** as an electrophilic fragment and evaluated them as activators of the Keap1/Nrf2/ARE pathway and inhibitors of iNOS. Notably, these compounds maintain the unique features of the chemical reactivity and biological potency of **3**. Among them, a monocyclic compound **5** is the most potent in these assays while a tricyclic compound **14** displays a more robust and specific activation profile compared to **5**. In conclusion, we demonstrate that **3** is a useful electrophilic fragment for exploring reversible covalent drugs.



1. INTRODUCTION

Tricyclic compound **1** [TBE-31, (±)-(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, Figure 1] is one of the most potent activators of the Keap1/Nrf2/ARE pathway known to date.[1−4] Compound **1** suppresses pro-inflammatory responses and induces heme oxygenase-1 (HO-1) in RAW264.7 cells, upregulates NAD(P)H:quinone oxidoreductase 1 (NQO1) in Hepa1c1c murine hepatoma cells through the Keap1/Nrf2/ARE pathway, and protects animals against liver carcinogenesis induced by aflatoxin.[3] Incorporation of small quantities (9.2 mg per kg of food) of **1** in the diet of mice profoundly and dose dependently induces NQO1 and glutathione *S*-transferases in the stomach, skin, and liver.[4] Long-term (5 days per week for 4 weeks) topical daily applications of 200 nmol of **1** causes a robust systemic induction of the Keap1/Nrf2/ARE pathway and decreases the 6-thioguanine incorporation into DNA of skin, blood, and liver of azathioprine-treated mice, indicating extraordinary bioavailability and efficacy.[5]

Tricyclic compound **1** has two different monocyclic non-enolizable cyanoenones **2** and **3** (Figure 1) in rings A and C. We chemically demonstrated by UV spectroscopy that the A and C rings of TBE-31 produce reversible Michael adducts with the sulfhydryl groups of Keap1 and dithiothreitol (DTT).[3,4] We

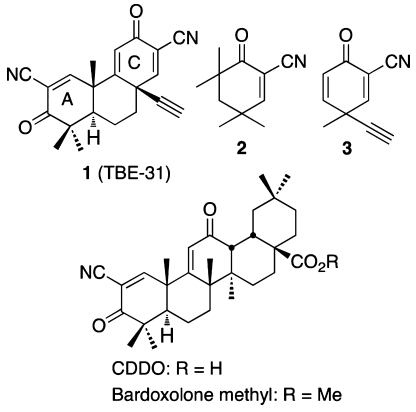


Figure 1. Structures of **1** (TBE-31), monocyclic cyanoenones **2** and **3**, CDDO, and bardoxolone methyl.

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speculated that **1** regulates proteins affecting inflammation, oxidative stress, differentiation, apoptosis, and proliferation, including Keap1, IKKβ, and JAK1, to name a few, by reversible Michael addition between the cyanoenone functionalities and the sulfhydryl groups of cysteine moieties on these proteins. More recently, we have focused on monocyclic cyanoenones, which are considered to be the phamacophores of **1**. We designed and synthesized a preliminary set of eight monocylic cyanoenones, including **2** and **3**, and then evaluated the chemical reactivity as Michael acceptors and biological potency.[6] Among monocyclic cyanoenones, ethynylcyanodienone **3** is a highly reactive Michael acceptor with thiol nucleophiles. Furthermore, an important feature of **3** is that its Michael addition is reversible. For the induction of NQO1, **3** demonstrates the highest potency of this series.[6] For the inhibition of nitric oxide (NO) production in RAW264.7 cells (murine macrophage-like cell line) stimulated with IFN-γ, **3** also shows the highest potency.[6] Remarkably, in this assay, the ethynylcyanodienone **3**, which has such a simple structure, is about three times more potent than a pentacyclic triterpenoid, CDDO, whose methyl ester (bardoxolone methyl) is presently being evaluated in phase 2 clinical trials for the treatment of pulmonary arterial hypertension (PAH) in the United States and diabetic nephropathy in Japan. Overall, we found that **3** displays unique features regarding chemical reactivity and biological potency.

Generally, there are three categories of drugs: (1) irreversible covalent drugs, which permanently bind to their protein targets through covalent bonds. This category includes alkylating agents (e.g., cyclophosphamide, mitomycin C), β-lactam antibiotics (e.g., penicillin, cephalosporin) and irreversible enzyme inhibitors (e.g., acetylsalicylic acid, omeprazole); (2) reversible noncovalent drugs, which noncovalently (e.g., hydrogen bond, hydrophobic effects, and van der Waals forces) bind with protein targets; this category contains receptor antagonists (for example, angiotensin II receptor blocker, β-blockers, and histamine H_2-receptor antagonists and many others); (3) reversible covalent drugs, which covalently bind but not permanently with protein targets. This final category is the newest and gaining the most interest because of the resulting enhanced properties. Currently, dimethyl fumarate (DMF),[7] a Michael acceptor and an activator of the Keap1/Nrf2/ARE pathway, is the only drug that is clinically used for the treatment of multiple sclerosis. CDDO and bardoxolone methyl also belong to this category. Recently, the development of a series of reversible covalent inhibitors of MSK/RSK-family kinases has been reported.[8]

Although irreversible covalent drugs have long duration of action, high potency, and high ligand efficiency because they irreversibly bind to both on- and off-protein targets, there is a potential for immune-mediated hypersensitivity and therefore such drugs are not suitable for chronic dosing. Reversible noncovalent drugs do not form permanent adducts and are therefore suitable for chronic dosing. However, their selectivity and potency are moderate because their ligand efficiency is usually poor. In sharp contrast, reversible covalent drugs have high potency, high ligand efficiency, and long duration of action and because they do not form permanent adducts, they are suitable for chronic dosing. Overall, reversible covalent drugs combine the advantages and circumvent the disadvantages of irreversible covalent and reversible noncovalent drugs.

Nevertheless, reversible covalent drugs have been largely ignored because of the lack of reactive compounds to produce the reversible covalent adducts with protein targets. On the basis of the evidence described above, ethynylcyanodienone **3** is considered to be one of the best fragments that we can use for exploring reversible covalent drugs which are targeting on the Keap1/Nrf2/ARE pathway. Thus, we have designed and synthesized new monocyclic, bicyclic, and tricyclic compounds **I**–**III** containing **3** as the electrophilic fragment (Figure 2, specifically Table 1). Because several monocyclic cyanoenones[6] have been synthesized and biologically evaluated and consequently they gave interesting SARs, we have designed **I**–**II**. Also, we have designed **III** because tricyclic compounds which do not contain aromatic rings in the mother nuclei have never been synthesized although more than 40 tricyclic compounds have been synthesized so far.[2] Then we measured the reactivity of ethynylcyanodienone moiety as a Michael acceptor in these compounds **I**–**III** by UV spectroscopy and evaluated their potency for the induction of NQO1 in Hepa1c1c murine hepatoma cells and the inhibition of iNOS in RAW264.7 cells stimulated with LPS. We herein describe the full account of our synthetic work with these new compounds, their unique and interesting features with respect to the chemical reactivity as Michael acceptors, and biological potency.

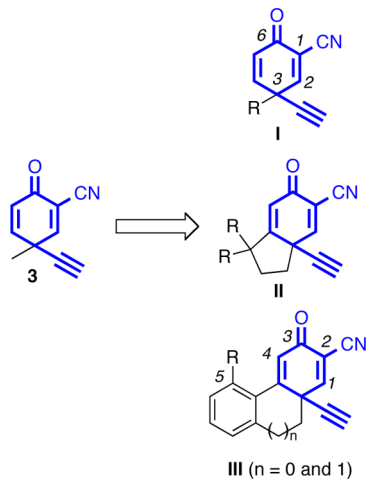

Figure 2. Monocyclic, bicyclic, and tricyclic compounds containing ethynylcyanodienone **3**.

2. CHEMISTRY

2.1. Monocyclic Ethynylcyanodienones. Monocyclic diethynylcyanodienone **4** was synthesized in nine steps from 2,2-dimethyl-1,3-dioxan-5-one by the sequence as shown in Scheme 1. Known dienophile precursor **16**[9] was synthesized in 42% yield through Sonogashira coupling between triisopropylsilylacetylene (TIPS acetylene) and the triflate derived from 2,2-dimethyl-1,3-dioxan-5-one. Diels–Alder reaction between the Danishefsky's diene and **16** gave previously reported adduct **17**.[9] Without purification of the crude adduct, **17** was treated with (chloromethyl)triphenylphosphonium chloride[10] in the presence of *n*-BuLi in THF to afford **18**. Dehydrochlorination of **18** with LDA in THF, followed by quenching the acetylide with TMSCl, produced **19**.[11] Monocyclic enone **20** was obtained in pure form by the treatment of **19** with TFA in 1,2-dichloroethane, followed by flash column chromatography purification (33% yield from **16**). Cyanation of the enolate of **20** generated using LDA in THF, with *p*-TsCN, gave **21** in 77%

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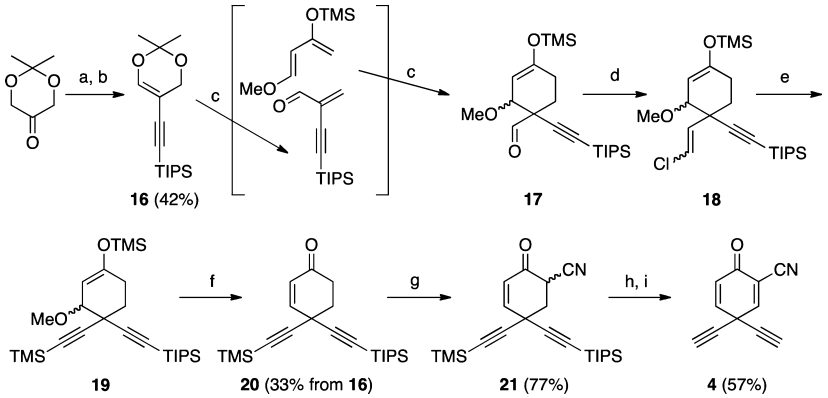
Scheme 1[a]

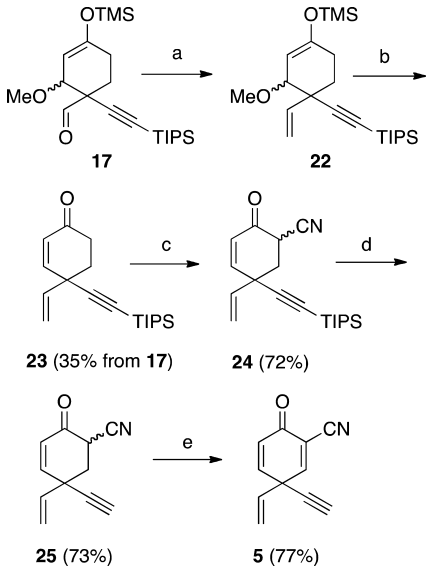


[a]Reagents: (a) triflic anhydride, DMAP, pyridine; (b) TIPS acetylene, $Pd(PPh_3)_4$, CuI, Et_2NH; (c) toluene; (d) $Ph_3PCH_2Cl_2$, *n*-BuLi, THF; (e) TMSCl, LDA, THF; (f) TFA, $ClCH_2CH_2Cl$; (g) *p*-TsCN, LDA, THF; (h) TBAF, THF; (i) PhSeCl, pyridine, CH_2Cl_2, 30% aqueous H_2O_2, CH_2Cl_2.

yield.[12] Deprotection of **21** with TBAF in THF, followed by addition of PhSeCl in the presence of pyridine and subsequent oxidation/elimination of the selenated intermediate with 30% aqueous H_2O_2 solution afforded **4** in 57% yield.[13]

Monocyclic ethynylvinylcyanodienone **5** was synthesized in five steps starting from adduct **17** (Scheme 2). A Wittig

Scheme 2[a]



[a]Reagents: (a) Ph_3PCH_3I, *n*-BuLi, THF; (b) D-(+)-CSA, dioxane; (c) *p*-TsCN, LDA, THF; (d) TBAF, THF; (e) PhSeCl, pyridine, CH_2Cl_2, 30% aqueous H_2O_2, CH_2Cl_2.

reaction on **17** with methyltriphenylphosphonium iodide in the presence of *n*-BuLi in THF provided **22**. Enone **23** was obtained by the treatment of **22** with D-(+)-camphor sulfonic acid [D-(+)-CSA] in 1,4-dioxane under reflux conditions (35% yield from **17**). The desired compound **5** was prepared in three steps in 40% yield via **24** and **25** from **23** by the same sequence (cyanation, deprotection, and insertion of a double bond) as for **4**.

Monocyclic ethylethynylcyanodienone **6** was synthesized with six steps from the Diels–Alder adduct **27**, which was obtained from the Danishefsky's diene and 2-ethylacrolein (**26**) under microwave conditions (Scheme 3).[14] Enone **30** was produced in three steps from **27** by the same sequence (Wittig reaction, conversion of a chlorovinyl group to a TMS protected ethynyl group, and deprotection) as for **20** (21% yield from **26**). Cyanation of the enolate of **30** with *p*-TsCN, followed by deprotection with TBAF, gave **32** in 29% yield. Cyanodienone **6** was prepared in 79% yield from **32** by addition of PhSeCl in the presence of pyridine and subsequent oxidation/elimination of the selenated intermediate with 30% aqueous H_2O_2 solution.

2.2. Bicyclic Ethynylcyanodienones. Bicyclic ethynylcyanodienone **7** was synthesized in 10 steps from the previously reported compound **33**,[15] which was prepared by Michael addition between methyl 2-oxocyclopentanecarboxylate and but-3-en-2-one (Scheme 4). Intramolecular aldol condensation of **33** in the presence of pyrrolidine (1 equiv) and acetic acid (1 equiv) in EtOAc gave enone **34** in 58% yield. Enone **34** was protected with ethylene glycol in the presence of PPTS in toluene to afford **35** in 95% yield. Reduction of **35** with LAH in Et_2O, followed by Swern oxidation, produced **36**. Without purification, **36** was treated with Ohira reagent (dimethyl(1-diazo-2-oxopropyl)phosphonate)[16] to give **37** (48% yield from **35**). The lithium acetylide, which was derived from **37** with MeLi, was protected with TBSCl to afford **38** in 92% yield. Removal of a ketal of **38** under acidic conditions, followed by cyanation of the lithium enolate of **39** with *p*-TsCN, provided **40** (47% yield from **38**). The desired compound **7** was obtained by deprotection of **40** with TBAF in THF, followed by DDQ oxidation in PhH (22% yield from **40**).

Bicyclic ethynylcyanodienone **8**, which has a nonenolizable cyanodienone while **7** has an enolizable cyanodienone, was synthesized in 11 steps from the known compound **42**,[17] which was obtained by methoxycarbonylation of 2,2-dimethylcyclopentanone (Scheme 5). Enone **43** was prepared by intramolecular aldol condensation of the adduct obtained by Michael addition between **42** and but-3-en-2-one (53% yield). Conversion from **43** to **46** was achieved by the same sequence (ketalization, reduction, oxidation, and introduction of an ethynyl group) as for **37** (23% yield from **43**). A cyano group was introduced using Johnson isoxazole method[18] instead of *p*-TsCN. Removal of a ketal of **46**, followed by formylation with ethyl formate in the presence of NaH in THF, gave **47**. Without purification, **47** was treated with hydroxylamine hydrochloride in aqueous EtOH to afford isoxazole **48** (46% yield from **46**).[18] The desired compound **8** was obtained

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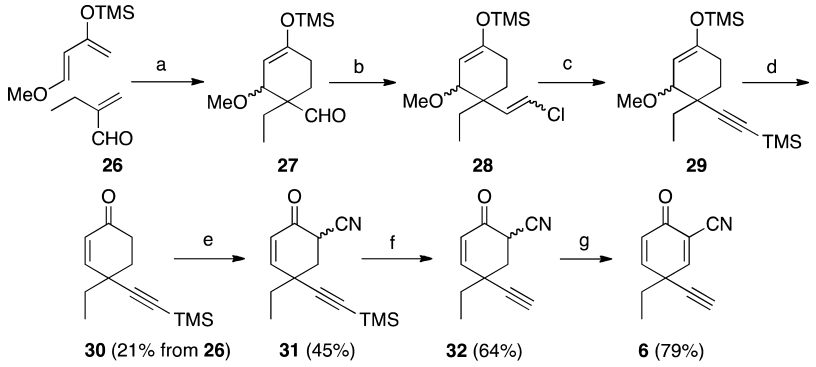
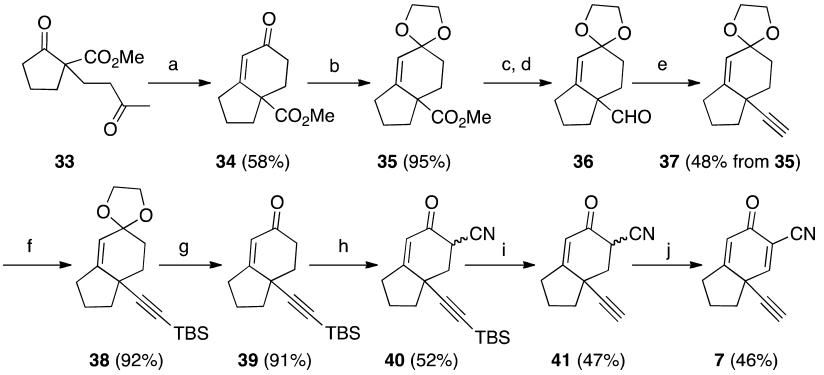
Scheme 3[a]



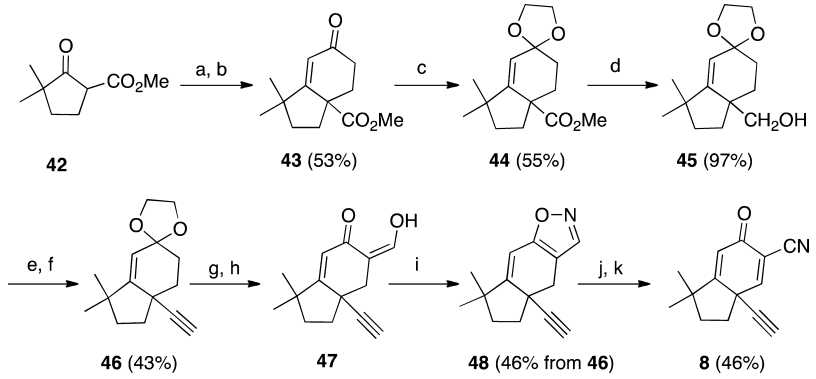
[a]Reagents: (a) microwave at 120 °C; (b) $Ph_3PCH_2Cl_2$, *n*-BuLi, THF; (c) TMSCl, LDA, THF; (d) TFA, $CHCl_3$; (e) *p*-TsCN, LDA, THF; (f) TBAF, THF; (g) PhSeCl, pyridine, CH_2Cl_2, 30% aqueous H_2O_2, CH_2Cl_2.

Scheme 4[a]



[a]Reagents: (a) pyrrolidine, AcOH, EtOAc; (b) ethylene glycol, PPTS, toluene; (c) LAH, Et_2O; (d) $(COCl)_2$, DMSO, CH_2Cl_2; (e) Ohira reagent, K_2CO_3, MeOH; (f) MeLi, TBSCl, THF; (g) 10% aqueous HCl, MeOH; (h) *p*-TsCN, LDA, THF; (i) TBAF, THF; (j) DDQ, PhH.

Scheme 5[a]



[a]Reagents: (a) methyl vinyl ketone, Et_3N; (b) pyrrolidine, AcOH, toluene; (c) ethylene glycol, PPTS, toluene; (d) LAH, Et_2O; (e) $(COCl)_2$, DMSO, CH_2Cl_2; (f) Ohira reagent, K_2CO_3, MeOH; (g) 10% aqueous HCl, MeOH; (h) HCO_2Et, NaH, THF; (i) $NH_2OH{\cdot}HCl$, aqueous EtOH; (j) NaOMe, MeOH; (k) DDQ, PhH.

by cleavage of the isoxazole moiety of **48** with NaOMe in MeOH,[18] followed by DDQ oxidation in PhH (46% yield).

Bicyclic ethynylcyanodienone **9**, which has a naphthalene skeleton, was synthesized via the known compound **51** from 1,2,3,4-tetrahydronaphthalene-1-carboxylic acid (**49**) as shown in Scheme 6. We have obtained racemic **51** by a new sequence that is different from the previously reported synthesis[19,20] of optically active **51**. Methyl ester **50**[21] was prepared by methylation of **49**, followed by insertion of a methyl group (79% yield). Reduction of **50** with LAH and subsequent Swern oxidation gave **51** (100% yield). A formyl group of **51** was converted to an ethynyl group of **52** with Ohira reagent (87% yield). Chromium-mediated allylic oxidation[22] of **52** provided ketone **53** in 52% yield. Formylation of **53** with ethyl formate in the presence of NaOMe in PhH,[23] followed by isoxazole formation with hydroxylamine hydrochloride in aqueous EtOH, afforded **55** in 84% yield. The desired compound **9** was obtained by cleavage of the isoxazole moiety of **55** with

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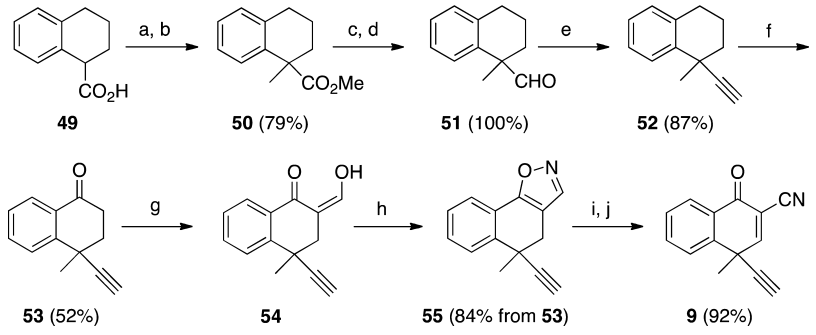
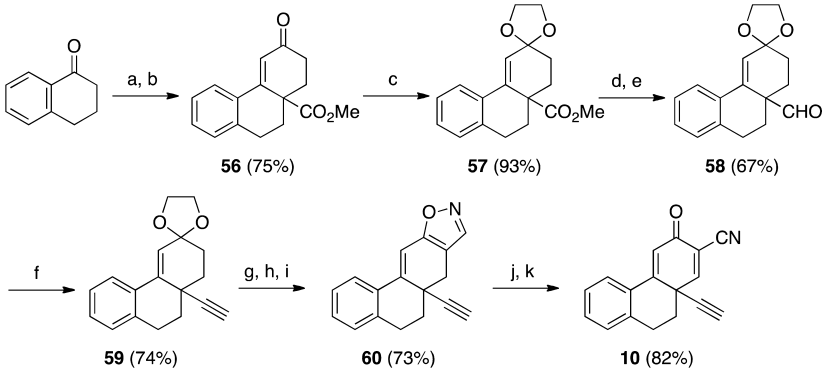
Scheme 6[a]



[a]Reagents: (a) *p*-TsOH, MeOH; (b) MeLi, LDA, THF; (c) LAH, THF; (d) $(COCl)_2$, DMSO, CH_2Cl_2; (e) Ohira reagent, K_2CO_3, MeOH; (f) CrO_3, *t*-BuO_2H, CH_2Cl_2; (g) HCO_2Et, NaOMe, PhH; (h) $NH_2OH{\cdot}HCl$, aqueous EtOH; (i) NaOMe, MeOH, Et_2O; (j) PhSeCl, pyridine, CH_2Cl_2, 30% aqueous H_2O_2, CH_2Cl_2.

Scheme 7[a]



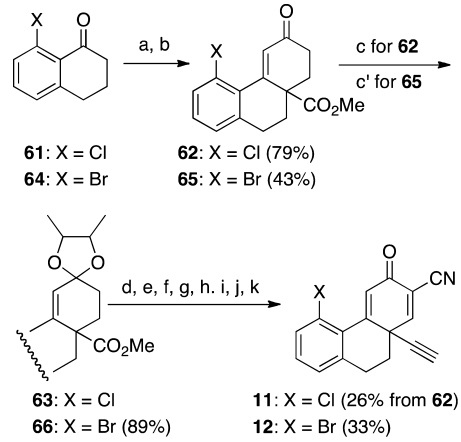
[a]Reagents: (a) NaH, Me_2CO_3; (b) methyl vinyl ketone, NaOMe, MeOH; (c) ethylene glycol, *p*-TsOH, toluene; (d) LAH, Et_2O; (e) $(COCl)_2$, DMSO, CH_2Cl_2; (f) Ohira reagent, K_2CO_3, MeOH; (g) 10% aqueous HCl, MeOH; (h) HCO_2Et, NaOMe, PhH; (i) $NH_2OH{\cdot}HCl$, aqueous EtOH; (j) NaOMe, MeOH, Et_2O; (k) PhSeCl, pyridine, CH_2Cl_2, 30% aqueous H_2O_2, CH_2Cl_2.

NaOMe in a mixture of MeOH and Et_2O, followed by addition of PhSeCl in the presence of pyridine and subsequent oxidation/elimination of the selenated intermediate with 30% aqueous H_2O_2 solution (92% yield).

2.3. Tricyclic Ethynylcyanodienones. Tricyclic ethynylcyanodienone **10**, which has a phenanthrene skeleton, was synthesized in 11 steps from 1-tetralone (Scheme 7). The known compound **56**[24] was prepared by an improved methodology than that previously reported. Methoxycarbonylation of 1-tetralone, followed by Robinson annulation with ethyl vinyl ketone, gave **56** in 75% yield. After protection of an enone of **56** with ethylene glycol (93% yield), a methoxycarbonyl group of **57** was converted to a formyl group of **58** by reduction with LAH and subsequent Swern oxidation (67% yield). Transformation of a formyl group of **58** to an ethynyl group with Ohira reagent afforded **59** in 74% yield. Isoxazole **60** was prepared in 73% yield from **59** by deketalization under acidic conditions, followed by formylation with ethyl formate and subsequent isoxazole formation with hydroxylamine hydrochloride. The desired compound **10** was obtained by isoxazole cleavage of **60** with NaOMe, followed by addition of PhSeCl in the presence of pyridine and subsequent oxidation/elimination of the selenated intermediate with 30% aqueous H_2O_2 solution (82% yield).

Chloro- and bromotricyclic ethynylcyanodienones **11** and **12** were synthesized in 11 steps from 8-chloro- and 8-bromo-1-tetralone (**61** and **64**)[25] as shown in Scheme 8. Tricyclic

Scheme 8[a]



[a]Reagents: (a) NaH, Me_2CO_3; (b) methyl vinyl ketone, NaOMe, MeOH; (c) 2,3-butanediol, *p*-TsOH, toluene; (c′) 2,3-butanediol, PPTS, toluene; (d) LAH, Et_2O; (e) $(COCl)_2$, DMSO, CH_2Cl_2; (f) Ohira reagent, K_2CO_3, MeOH; (g) 10% aqueous HCl, MeOH; (h) HCO_2Et, NaOMe, PhH; (i) $NH_2OH{\cdot}HCl$, aqueous EtOH; (j) NaOMe, MeOH, Et_2O; (k) PhSeCl, pyridine, CH_2Cl_2, 30% aqueous H_2O_2, CH_2Cl_2.

precursors **62** and **65** were obtained by methoxycarbonylation of **61** and **64** with Me_2CO, followed by Robinson annulation with methyl vinyl ketone, respectively. Initially, although an

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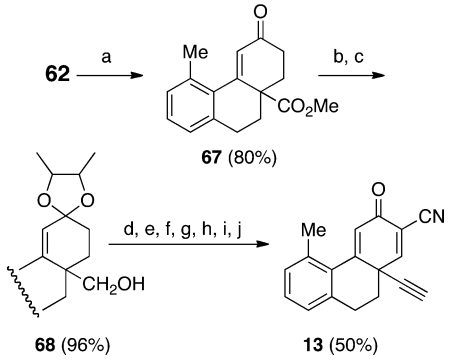
enone of **62** was protected with ethylene glycol, because the ketal was easily converted to the enone, the desired ketal was not isolated. Consequently, we attempted to protect the enone of **62** with a bulky diol, 2,3-butandiol in the presence of *p*-TsOH in toluene, because it is known that such a bulky ketal resists hydrolysis under acidic conditions.[1] As we expected, the more stable ketal **63** was obtained. Similarly, ketalization of **65** with 2,3-butandiol in the presence of PPTS in toluene gave **66** in 89% yield. The desired chloro- and bromotricyclic compounds **11** and **12** were prepared in eight steps from **63** and **66** by the same sequence as for **10**, respectively (**11**, 26% yield from **62**; **12**, 33% yield from **66**).

Methyltricyclic ethynylcyanodienone **13** was synthesized in 10 steps from **62** (Scheme 9). We have employed a Buchwald

Scheme 9[a]

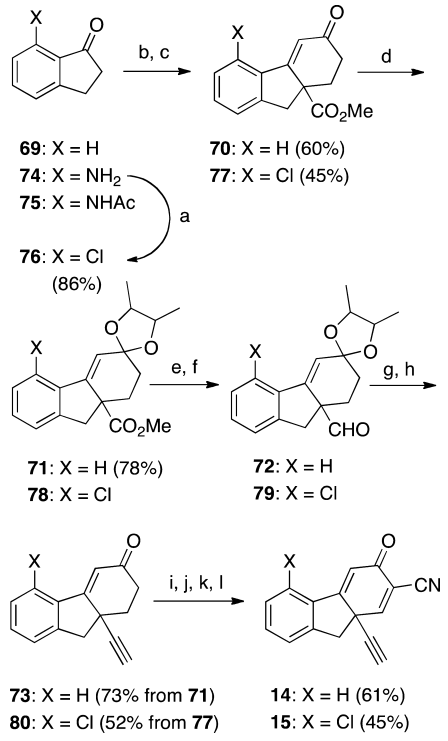


[a]Reagents: (a) $Pd(OAc)_2$, RuPhos, $MeB(OH)_2$, K_3PO_4, toluene; (b) 2,3-butanediol, PPTS, toluene; (c) LAH, Et_2O; (d) $(COCl)_2$, DMSO, CH_2Cl_2; (e) Ohira reagent, K_2CO_3, MeOH; (f) 10% aqueous HCl, MeOH; (g) HCO_2Et, NaOMe, PhH; (h) $NH_2OH{\cdot}HCl$, aqueous EtOH; (i) NaOMe, MeOH; (j) PhSeCl, pyridine, CH_2Cl_2, 30% aqueous H_2O_2, CH_2Cl_2.

ligand,[26,27] RuPhos (2-dicyclohexyl-phosphino-2′,6′-diisopropoxybiphenyl), $Pd(OAc)_2$, and K_3PO_4 for the Suzuki coupling between **62** and $MeB(OH)_2$. The desired methyltricyclic compound **67** was obtained from **62** in 80% yield. Ketalization of **67** with 2,3-butandiol, followed by LAH reduction, gave **68** in 96% yield. The target compound **13** was prepared in 7 steps from **68** by the same sequence as for **11** and **12** (50% yield).

Tricyclic ethynylcyanodienones **14** and **15**, which have fluorene skeletons, were synthesized in 11 steps from 1-indanone (**69**) and 7-chloroindanone (**76**), respectively (Scheme 10). Unexpectedly, a previously reported method[25] for conversion of 7-amino-1-indanone (**74**) to **76** using $CuCl_2$ and *t*-$BuNO_2$ in CH_3CN gave **76** in much lower yield (15%) than the reported yield (64%), with *N*-(3-oxo-2,3-dihydro-1*H*-inden-4-yl)acetamide (**75**) as the major (side) product.[28] However, we could obtain **76** in 86% yield by the traditional Sandmeyer reaction conditions (diazotization and subsequent chlorination with CuCl under acidic conditions). Methoxycarbonylation and subsequent Robinson annulation of **69** and **76** provided the known compound **70**[25,29] and the new compound 77 in 60% and 45% yield, respectively. The ketal of fluorene **70** with ethylene glycol was easily converted to the original **70**, while the ketal of phenanthrene **56** with ethylene glycol was stable (see Scheme 7). Enones **70** and 77 were protected with 2,3-butandiol to give **71** and **78**, respectively. Ethynylenones **73** and **80** were prepared via **72** and **79** from **71** and **78** in four steps (LAH reduction, Swern oxidation,

Scheme 10[a]



[a]Reagents: (a) $NaNO_2$, 15% aqueous HCl, CuCl, 37% aqueous HCl; (b) NaH, Me_2CO_3; (c) methyl vinyl ketone, NaOMe, MeOH; (d) 2,3-butanediol, PPTS, toluene; (e) LAH, Et_2O; (f) $(COCl)_2$, DMSO, CH_2Cl_2; (g) Ohira reagent, K_2CO_3, MeOH; (h) 10% aqueous HCl, MeOH; (i) HCO_2Et, NaOMe, PhH; (j) $NH_2OH{\cdot}HCl$, aqueous EtOH; (k) NaOMe, MeOH, Et_2O; (l) PhSeCl, pyridine, CH_2Cl_2, 30% aqueous H_2O_2, CH_2Cl_2.

ethynylation with Ohira reagent, and deprotection under acidic conditions), respectively. The desired compounds **14** and **15** were obtained in 61% and 45% yield from **73** and **80** in four steps (formylation, isoxazole formation, introduction of a cyano group, and insertion of a double bond), respectively.

3. RESULTS AND DISCUSSION

3.1. Chemical Reactivity of Ethynylcyanodienones as Michael Acceptors. We have evaluated the chemical reactivity of monocyclic, bicyclic, and tricyclic compounds **4**–**15** as Michael acceptors using UV spectroscopy. Michael reaction between ethynylcyanodienones with DTT give adducts **IV** (Scheme 11). Because adducts **IV** have different UV absorption spectra (λ_{max} 330–380 nm) from those of the corresponding ethynylcyanodienones (λ_{max} 230–320 nm), UV spectroscopy can clarify whether or not the Michael adducts are produced.[6]

Scheme 11

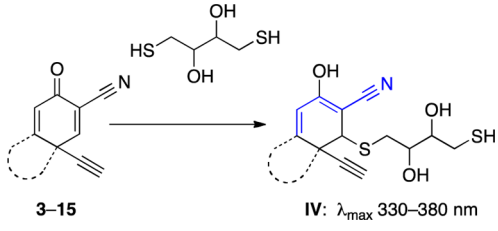


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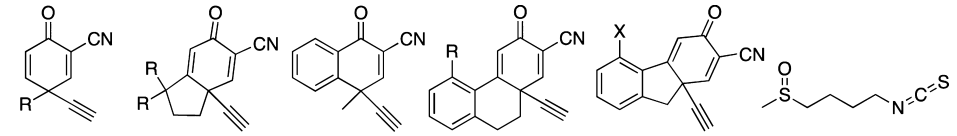
Table 1. UV Spectra[a] and Biological Potency of Ethynylcyanodienones 3−15



3: R = CH_3 **7**: R = H **9** **10**: R = H **14**: X = H Sulforaphane
4: R = C≡CH **8**: R = Me **11**: R = Cl **15**: X = Cl
5: R = $CH{=}CH_2$ **12**: R = Br
6: R = CH_2CH_3 **13**: R = Me

	with DTT (10 equiv)		with DTT (1 equiv)		without DTT[b] (with Cl^-)				
compd	λ_{max} (nm)	A^d	λ_{max} (nm)	A^d	λ_{max} (nm)	A^d	$K^c \times 10^3$ L/mol	NQO1[h] CD (nM)	iNOS[i] IC_{50} (nM)
3[e]	334	0.406	334	0.227	322	0.083	2.0	21	18
4	333	0.510	330	0.614	328	0.635	10.6	180	210
	250	0.439							
5	338	0.481	335	0.369	326	0.197	4.0	20	16
6	335	0.353	332	0.135	320	0.036	1.0	42	29
7	337	0.355	333	0.281	324	0.151	2.7	56	160
8	337	0.472	333	0.376	324	0.279	4.2	100	200
9	333	0.872	331	0.848	321	0.831	NT[g]	220	NT
10	382	0.723	380	shoulder			NT[g]	150	230
11	380	0.262	380	shoulder			NT[g]	900	500
12	NT[f]	NT[f]	NT[f]	NT[f]			NT[g]	1300	650
13	374	0.279					NT[g]	1800	940
14	382	0.820	377	0.808	364	0.867	NT[g]	41	380
15	383	0.803	378	0.819	369	1.025	NT[g]	130	800
TBE-31 (**1**)								0.9	1.0
CDDO								2.3	23
sulforaphane								200	400

[a]UV spectra of the Michael adducts between ethynylcyanodienones (0.1 mM) and DTT (1 mM and 0.1 mM) in phosphate buffer saline−1% ethanol (pH 7.4) at rt. The full UV spectra of the reaction mixtures are shown in Figure S1 in the Supporting Information. [b]Some ethynylcyanodienones gave Michael adducts with Cl^- in phosphate buffer saline−1% ethanol (pH 7.4). [c]Equilibrium constant at 0.1 mM of compound with 0.1 mM of DTT. [d]*A*: absorbance. [e]See ref 6. [f]Not observed. Compound **12** was not soluble in phosphate buffer saline−1% ethanol (pH 7.4). [g]Equilibrium constant at 0.1 mM of compound with 0.1 mM of DTT could not be obtained because the UV spectrum of the compound is not as simple as that of **3**. [h]Hepa1c1c7 cells (10000 per well) were grown in 96-well plates for 24 h and then treated with increasing concentrations of compounds for 48 h. Cells were lysed, and the protein concentration of the lysates was determined by the bicinchoninic acid (BCA) assay (Thermo Scientific). The concentration required to double (CD) the specific enzyme activity of NQO1 was used to quantity 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%. [i]RAW 264.7 cells (20,000 per well) were grown in 96-well plates for 24 h. Cells were then cotreated with LPS (10 ng/mL) and increasing concentrations of compounds for a further 48 h. The concentration of NO in the cell culture medium was determined using the Griess reagent. The cells were lysed, and the protein concentration of the lysates was determined by the BCA assay. IC_{50} values were determined based on suppression of LPS-induced NO production normalized to the protein concentration. The value is based on determinations from eight replicate wells at each concentration. Results shown are the average of three independent experiments.

3.1.1. UV Studies on Monocyclic and Bicyclic Compounds 4−9 with DTT. The UV spectra of compounds **5**−**8** with DTT are similar to those of **3**.[6] Compounds **5**−**8** have local maximum absorptions at 332−338 nm upon the addition of DTT (1 and 10 equiv) under dilute (0.1 mM of the compounds) and neutral aqueous conditions (pH 7.4 phosphate buffered saline−1% ethanol containing 1 mM KH_2PO_4, 5.6 mM Na_2HPO_4, and 154 mM NaCl) (Table 1 and Supporting Information Figure S1). Additionally, these compounds react with a chloride anion (a much weaker nucleophile than a sulfhydryl group of DTT), which is contained in the buffer solution, to give Michael adducts whose local maximum absorptions are observed at 320−326 nm. We have calculated approximate equilibrium constants (*K*) of these Michael reactions in the solutions of **5**−**8** with DTT (each initial concentration, 0.1 mM; see the calculation of the equilibrium constants in the Supporting Information). The values of *K* are approximately 1.0−4.2 × 10^3 (L/mol), implying that these additions are very strongly favored.

Compound **4** has two local maximum absorptions at 333 and 250 nm (absorbance (*A*) = 0.510 and 0.439, Table 1 and Supporting Information Figure S1) with 10 equiv of DTT. We have never observed such a spectrum with DTT. The wavelengths of the local maximum absorbance (λ_{max}) at 333 and 250 nm are assigned to those of **81** (cyanodiene) and **82** (cyanoene), respectively (Scheme 12). Similarly, **4** reacts with a chloride anion, producing a high concentration of the adduct; a similar high concentration of the adduct was observed with DTT (1 equiv). Interestingly, these findings indicate that the two ethynyl groups at C3 increase the reactivity of both cyanoenone and enone without a cyano group. The approximate *K* of the Michael reactions of **4** with DTT (each initial concentration, 0.1 mM) is 10.6 × 10^3 (L/mol), and the value is much higher than those of **3** and **5**−**8**. This means that the addition of **4** with DTT is much more favored than those of **3** and **5**−**8**.

These *K* values demonstrate that the order of the reactivity as Michael acceptors is **4** (ethynyl group) ≫ **8** (dimethylcyclo-

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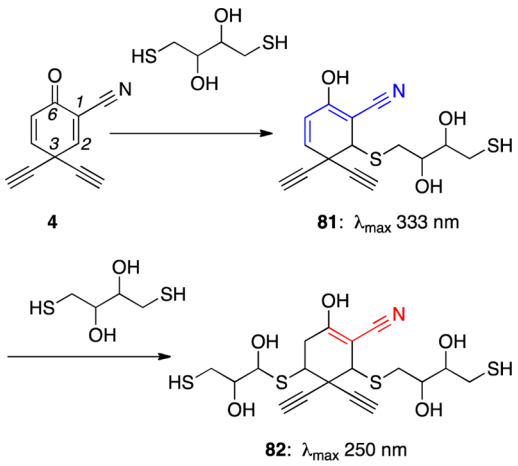
Scheme 12. Michael Reaction between Compound 4 and 10 equiv of DTT



pentane ring) ≥ **5** (vinyl group) > **7** (cyclopentane ring) > **3** (methyl group) > **6** (ethyl group). We have observed a tendency that electron-donating groups at the C3 position decrease the reactivity. Notably, an ethynyl group, a moderate electron-withdrawing group, enhances the reactivity of enone without a cyano group. Importantly, the enolizable cyanodienone **7** is less reactive than the corresponding nonenolizable cyanodienone **8**.

The naphthalene derivative **9** also reacts with a chloride anion to give a similar high concentration of the adduct to those of the adducts with DTT (1 and 10 equiv). The reactivity of **9** is similar to that of **4**.

*3.1.2. UV Studies on Tricyclic Compounds **10**–**15** with DTT.* These tricyclic compounds **10**–**15** react with DTT to give the adducts whose local maximum absorptions are observed at 374–383 nm range (Table 1 and Supporting Information Figure S1). Interestingly, in this series, phenanthrene derivatives **10**–**13** are much less reactive than fluorene derivatives **14** and **15**. Phenanthrene derivatives do not react with a chloride anion, while fluorene derivatives react with a chloride anion to give similar concentrations of the adducts to those of the adducts with DTT. Among phenanthrene derivatives, substituents at C5 decrease the reactivity. Overall, UV spectra demonstrate that the order of the reactivity as Michael acceptors is **14** and **15** (fluorene) ≫ **10** (phenanthrene) > **11** (5-chlorophenanthrene) > **13** (5-methylphenanthrene).

3.2. Biological Results. *3.2.1. Compounds **4**–**15** Induce NQO1 in Hepa1c1c7 Murine Hepatoma Cells.* To determine the ability to activate the Keap1/Nrf2/ARE pathway, compounds **4**–**15** were evaluated for the induction of the classical Nrf2 target, the phase 2 cytoprotective enzyme NQO1 in Hepa1c1c7 murine hepatoma cells.[30,31] In this assay, the concentration required to double (CD value) the specific enzyme activity of NQO1 is used to quantify inducer potency. The CD values (nM) of **3**–**15**, TBE-31, and CDDO are shown in Table 1. The CD values of these compounds are observed in the 20–2000 nM concentration range. Notably, many of these compounds are more potent than sulforaphane,[32] which is a widely used activator of the Keap1/Nrf2/ARE pathway and has demonstrated protective effects in many disease models related to inflammation and cancer.[32,33] Among these compounds, **5** is the most potent and the potency is similar to that of **3**. Compounds **6** and **14** are next in the rank order of potency. Interestingly, the bicyclic compound **7** and tricyclic compounds **10**, **11**, and **14** induce NQO1 more effectively than monocyclic compounds **3**–**6** at the higher concentrations (Figure 3) although these compounds are less potent than the monocyclic compounds according to their CD values. These compounds may be more robust inducers than monocyclic compounds.

As previously reported,[6] we have found a correlation between reactivity of monocyclic cyanoenones as Michael acceptors and biological potency. In this series of monocyclic and bicyclic compounds **3**–**9**, we found a similar correlation. Compounds **4** and **9**, which have the highest reactivity with a sulfhydryl group and a chloride anion in this series, and compound **8**, which has the third highest reactivity, are less potent than other compounds which have much lower reactivity with a chloride anion than **4**, **8**, and **9**. On the basis of these results and our previous observations,[6] we speculate that (i) due to its exceedingly high chemical reactivity, a large portion of **4**, **8**, and **9** could be "quenched" by abundant cellular thiols, such as the cysteine residue of glutathione, which is present at millimolar concentrations, (ii) **4**, **8**, and **9** 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 determining the biological potency. Among the compounds whose reactivity with a chloride anion is relatively low, interestingly, compound **5**, which has the highest reactivity with a sulfhydryl group, is the most biologically potent.

On the other hand, a series of tricyclic compounds with phenanthrene and fluorene skeletons, **10**–**15**, show a different correlation between reactivity and potency. The fluorene derivatives **14** and **15** are more potent than the phenanthrene derivatives **10**–**13**, while **14** and **15** show much higher reactivity with a chloride anion and a sulfhydryl group than **10**, **11**, and **13**. Notably, among these monocyclic, bicyclic, and

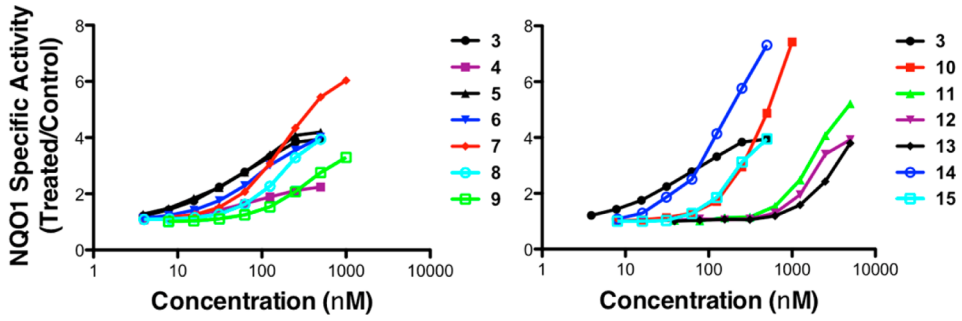


Figure 3. Dose response curves of compounds **3**–**15**.

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tricyclic compounds **3**–**15**, **14** shows not only the third lowest CD value but also induces NQO1 to a greater magnitude than **3** and **5** at higher concentrations. In a series of phenanthrene derivatives, the potency is well correlated to the reactivity.

3.2.2. Compounds 4–15 Inhibit NO Production Induced by LPS Stimulation in RAW 264.7 Cells. We evaluated the inhibitory activities of **3**–**15** on NO production in RAW 264.7 cells stimulated with LPS.[34] The inhibitory activities [IC_{50} (nM) values] of **3**–**15**, TBE-31, and CDDO are shown in Table 1. Among these new compounds, **5** has the highest potency, which is similar to that of **3**. Notably, both **3** and **5**, which are monocyclic compounds, are as potent as or even slightly more potent than a pentacyclic tritepenenoid, CDDO.

We previously demonstrated a linear correlation between NQO1 inducer potency (CD) and inhibitory activity against NO production (IC_{50}) of semisynthetic triterpenoids[35,36] and synthetic tricyclic compounds.[2] However, we did not observe a linear correlation in monocyclic cyanoenones which have been previously reported, although there was a general correlation between the rank order of potencies in the two assays.[6] Remarkably, in this series of compounds **3**–**13**, we observed an even more striking linear correlation (r^2 = 0.96) than the previous reported correlations (r^2 = 0.91)[2,35] (Figure 4).

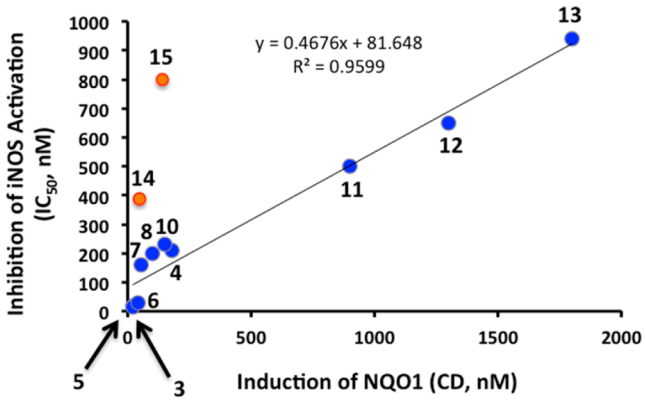

Figure 4. Correlation of potencies of ethynylcyanodienones **3**–**8**, **10**–**13** (as shown in blue circle), and **14**–**15** (shown as orange circles) as inducers of NQO1 in Hepa1c1c7 murine hepatoma cells, expressed as CD values, and for suppression of iNOS induction by LPS in RAW 264.7 cells, expressed as IC_{50} values. The linear correlation coefficient for the blue circles is r^2 = 0.96.

Interestingly, the fluorene derivatives **14** and **15** are not correlated to this line. While **14** has high potency in the NQO1 induction assay, **14** shows only moderate inhibitory activity in the iNOS assay.

4. CONCLUSION

To explore reversible covalent drugs, we have designed, synthesized, and biologically evaluated new monocyclic, bicyclic, and tricyclic ethynylcyanodienones **4**–**15** containing **3** as the electrophilic fragment.

The designed compounds have been synthesized in 7–11 steps and relatively good yields from the starting materials. We employed the Diels–Alder reaction and Robinson annulation for the key cyclization steps of these syntheses.

In this series of monocyclic, bicyclic, and tricyclic compounds excluding a fluorene derivative **14**, biological potency of the compounds, which have high reactivity with a chloride anion, are weak as inducers of NQO1 and as inhibitors of iNOS. The compounds, which have low reactivity with a chloride anion but high reactivity with a sulfhydryl group, are highly potent as the inducers and the inhibitors. A monocyclic compound **5** is the most potent among them and is as potent as the electrophilic fragment **3**. Exceptionally, while **14** has high reactivity with a chloride anion, **14** has high potency as the inducer of NQO1. However, potency of **14** as the inhibitor of iNOS is weak.

Notably, we observed a striking linear correlation (r^2 = 0.96) between the potency of the compounds **3**–**13** as inducers of NQO1 (CD values) and as inhibitors of iNOS (IC_{50} values), but compounds **14** and **15** do not fit this line. As the iNOS inhibitory activity is only partially dependent on Nrf2,[37] this finding suggests that **14** and **15** may be more specific as Nrf2 activators than as iNOS inhibitors. Although the potency of **14** is lower than that of **3** and **5** according to the CD values, the magnitude of NQO1 induction by **14** is greater than that by **3** and **5** at concentrations higher than the CD values. Thus, **14** may be a more robust inducer than **3** and **5**. Overall, interestingly and notably, **14** is clearly different from the other compounds in this series.

We have clarified that the designed compounds containing the fragment **3** maintain the features of the reactivity and biological potency of **3**. Thus, **3** is a useful electrophilic fragment for exploring reversible covalent drugs. Indeed, fragment **3**-conatining compound **1** (TBE-31) is highly bioavailable and extremely potent in protecting against tumor development in a preclinical model of solar-simulated UV radiation-mediated cutaneous squamous cell carcinoma.[38] Currently, further design and synthesis of reversible covalent drugs based on the electrophilic fragment **3** are underway.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures and characterization data for new compounds **1**–**80**, UV spectra of compounds **3**–**11** and **13**–**15** with DTT, and list of elemental analyses for specifying the purity of **4**–**15**. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00393.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ARE, antioxidant response element; CDDO, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid; IFN-γ, interferon-γ; IKKβ, inhibitor of nuclear factor κB kinase β; iNOS, inducible nitric oxide synthase; JAK1, Janus kinase 1; Keap1, Kelch-like ECH-associated protein 1; LPS, lipopolysaccharide; MSK, mitogen- and stress-activated kinase; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; *p*-TsCN, *p*-toluenesulfonyl cyanide; RSK, ribosomal s6 kinase

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