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Tricyclic Compounds Containing Nonenolizable Cyano Enones. A Novel Class of Highly Potent Anti-Inflammatory and Cytoprotective Agents[79]

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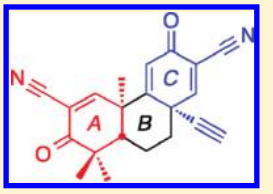
Supporting Information

ABSTRACT: Forty-four novel tricycles containing nonenolizable cyano enones (TCEs) were designed and synthesized on the basis of a semisynthetic pentacyclic triterpenoid, bardoxolone methyl, which is currently being developed in phase II clinical trials for the treatment of severe chronic kidney disease in diabetic patients. Most of the TCEs having two different kinds of nonenolizable cyano enones in rings A and C are highly potent suppressors of induction of inducible nitric oxide synthase stimulated with interferon-γ and are highly potent inducers of the cytoprotective enzymes heme oxygenase-1 and NAD(P)H:quinone oxidoreductase-1. Among these compounds, (±)-(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 ((±)-**31**) is the most potent in these bioassays in our pool of drug candidates including semisynthetic triterpenoids and synthetic tricycles. These facts strongly suggest that an essential factor for potency is not a triterpenoid skeleton but the cyano enone functionality. Notably, TCE **31** reduces hepatic tumorigenesis induced with aflatoxin in rats. Further preclinical studies and detailed mechanism studies on **31** are in progress.



1. INTRODUCTION

The concept that inflammation and carcinogenesis are related phenomena has been the subject of many studies that have attempted to link these two processes in a mechanistic fashion.[1,2] The enzyme that mediates the constitutive synthesis of nitric oxide (NO) from arginine has relatively little significance for either inflammation or carcinogenesis. In contrast, inducible nitric oxide synthase (iNOS) has critical roles in the response of tissues to injury or infectious agents and is an essential component of the inflammatory response, the ultimate repair of injury, and carcinogenesis.[3−5] Although the physiological activity of iNOS may provide a definite benefit to the organism, aberrant or excessive expression of iNOS has been implicated in the pathogenesis of many disease processes, particularly in Alzheimer's disease, Parkinson's disease, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, chronic kidney disease, and carcinogenesis.[6−10] Therefore, we adopted inhibition of NO production in primary mouse macrophages[11] and/or RAW 264.7 cells stimulated with interferon-γ (IFN-γ) (the iNOS assay) as initial screening systems for the development and evaluation of our potential anti-inflammatory and cytoprotective agents that we have designed and synthesized.

Heme oxygenase is the rate-limiting phase 2 enzyme in the catabolism of heme, producing biliverdin, iron, and carbon monoxide. Three heme oxygenase isoforms have been described:[12−14] HO-1, HO-2, and HO-3 [the last two recently described as a pseudogene[15] are constitutively expressed]. HO-1 is induced by a variety of stimuli, including growth factors, cytokines, NO, and oxidants such as heme, hydrogen peroxide, oxidized lipids, and heavy metals.[16] HO-1 and its breakdown products possess potent anti-inflammatory and cytoprotective properties.[17−19] Currently, there is major interest in stimulating HO-1 as a protective enzyme in many chronic disease conditions in which inflammation and oxidative stress play an important role. Thus, we have evaluated our compounds, whose potency in the iNOS assay is sufficient, for induction of HO-1 in RAW cells (the HO-1 assay).

NAD(P)H:quinone oxidoreductase (NQO1) is also a representative phase 2 enzyme. It is a widely distributed FAD-dependent flavoprotein that catalyzes the obligatory two-electron reduction of a broad range of substrates, including quinones, quinoneimines, and nitro compounds by using either NADPH or NADH as the hydride donor.[20] In addition and independent of its catalytic mechanism, NQO1 also has a "gatekeeping" role in regulating the proteasomal degradation of specific proteins, and this function appears to be important in the stabilization of p53, a broadly functioning tumor suppressor gene. NQO1 is transcriptionally induced in response to various agents, including xenobiotics, oxidants, antioxidants, as well as ultraviolet and ionizing radiation.[21−24] NQO1, therefore, is important as a cytoprotective enzyme. We have evaluated the potency of our compounds for induction of NQO1 in Hepa1c1c7 murine hepatoma cells (the NQO1 assay).

Over the past decade, we have been engaged in the improvement of the anti-inflammatory and cytoprotective activity of

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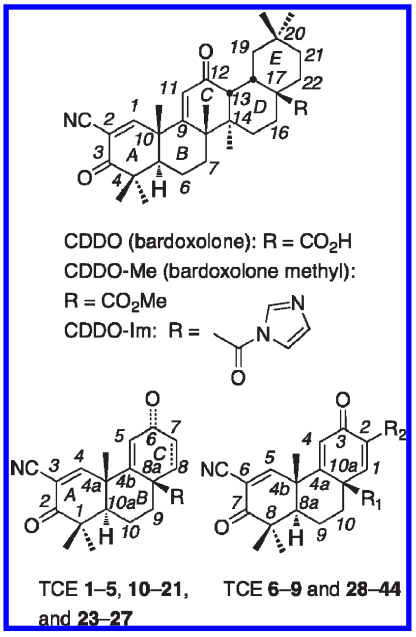


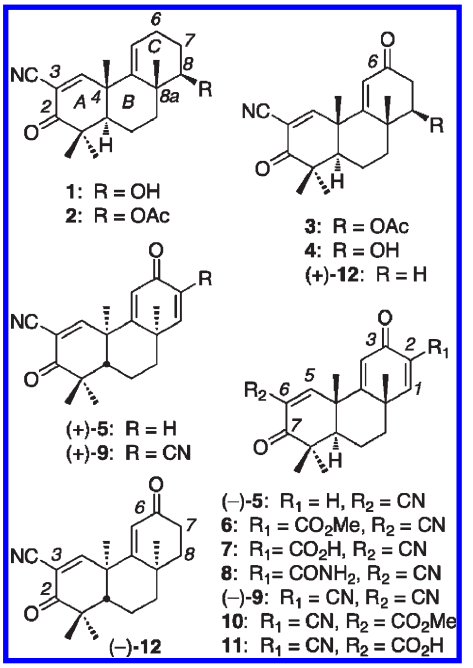
Figure 1. Structures of CDDO, CDDO-Me, and CDDO-Im and general structures of TCEs.

oleanolic acid, a ubiquitous naturally occurring triterpenoid. This led to the discovery of CDDO (Figure 1).[25−27] CDDO and its related compounds are multifunctional agents. CDDO shows high inhibitory activity in the iNOS assay[25,26] and induces HO-1 in vitro[28] and in vivo and NQO1 in vitro.[29] CDDO induces differentiation of human myeloid leukemia cells and mouse 3T3-L1 fibroblasts and enhances nerve-growth-factor-induced neuronal differentiation of rat PC12 cells,[27,30] inhibits the proliferation of human myeloid leukemia and carcinoma cell lines,[27] blocks de novo synthesis of iNOS and inducible cyclooxygenase (COX-2) in mouse macrophages, microglia, and fibroblasts,[27] and induces apoptosis of human myeloid leukemia,[31−33] osteosarcoma,[34] lung cancer,[35] and CLL cells.[36] The acylimidazole of CDDO (CDDO-Im, Figure 1) inhibits aflatoxin-induced tumorigenesis in rats.[37] The methyl ester of CDDO (CDDO-Me, bardoxolone methyl, Figure 1) prevents lung cancer induced by vinyl carbamate in A/J mice.[38] Presently, bardoxolone methyl is being developed in late phase II clinical trials for the treatment of severe chronic kidney disease in type 2 diabetes mellitus patients. It significantly increases the estimated glomerular filtration rate in more than 90% of diabetic patients.[39]

We have chemically demonstrated that the nonenolizable cyano enone in ring A of CDDO gives reversible Michael adducts with the SH group of DTT by UV and NMR studies.[40] We speculate that this characteristic reactivity may imply a molecular mechanism of action. Indeed, our mechanism studies suggest that CDDO and its related compounds regulate proteins affecting inflammation, oxidative stress, differentiation, apoptosis, and proliferation, including Keap1,[29] IKKβ, and JAK1, to name a few, by reversible Michael addition between their nonenolizable cyano enone functionality in ring A and the SH groups of cysteine moieties on these proteins. Recently, Cys179 in the kinase domain on IKKβ was identified as a target of CDDO-Me and CDDO-Im.[41,42] By binding to this site on IKKβ, CDDO-Me inactivates the kinase and ultimately results in blocking of the binding of NF-κB to DNA and thus inhibits transcriptional activation of NF-κB-dependent target genes. It has also been reported that CDDO-Me inhibits the JAK1 → STAT3 pathway by directly binding to JAK1 at Cys1077 and STAT3 at Cys259.[43] Small molecule inhibitors of the STAT3 pathway are known to be effective as anticancer agents in vitro and in animal models.

During the development of CDDO, we found the very important structure−activity relationships (SARs) that the nonenolizable cyano enone in ring A and the enone in ring C are essential for the extremely high potency of CDDO [see Figure S1 in the Supporting Information].[26] Therefore, we reasoned that the entire oleanane skeleton might not be necessary for potency. Consequently, we have designed *t*ricycles containing nonenolizable *c*yano *e*nones (TCEs, i.e., compounds **1**−**44**, Figure 1). A literature survey revealed that these compounds were previously unknown.

Table 1. Inhibitory Activity of New TCEs 1−12 on NO Production Induced by IFN-γ in Primary Mouse Macrophages[a]



compd	IC_{50} (nM)	compd	IC_{50} (nM)
(±)-**1**	310	(±)-**9**	2.1
(±)-**2**	480	(−)-**9**	14
(±)-**3**	53	(+)-**9**	1.3
(±)-**4**	75	(±)-**10**	>60
(±)-**5**	61	(±)-**11**	>600
(−)-**5**	64	(±)-**12**	19
(+)-**5**	58	(−)-**12**	26
(±)-**6**	91	(+)-**12**	19
(±)-**7**	1600	CDDO	0.5
(±)-**8**	61	CDDO-Me	0.2
		hydrocortisone	10

[a] IC_{50} values of TCEs, CDDO, CDDO-Me, and hydrocortisone were determined in the range 0.1 pM to 10 μM (10-fold dilutions). Values are an average of two separate experiments. None of the compounds were toxic to primary mouse macrophages at 10 μM. The experimental protocol is in the Supporting Information. These data have been published and presented in refs 79a−79d.

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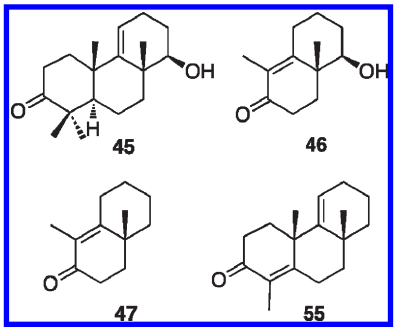


Figure 2. Structures of **45**−**47** and **55**.

Our rationale for pursuing the synthesis of these compounds is as follows. Since they could be synthesized from commercially available small molecules, these compounds with various functionalities at different positions would be easily obtained. Such diversity-oriented synthesis could lead to new potential anti-inflammatory and cytoprotective agents that have high oral potency and high water solubility for ease of administration and formulation, as well as high biological selectivity for avoiding possible side effects. Accordingly, we have synthesized various new TCEs and evaluated them in the iNOS, HO-1, and NQO1 assays. As a result, we have found that TCE **31** (code number in house: 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) is the most potent in our collection of our semisynthetic triterpenoids and synthetic tricycles. We herein describe the full account of our synthetic work with these tricycles and their interesting biological results.

2. CHEMISTRY

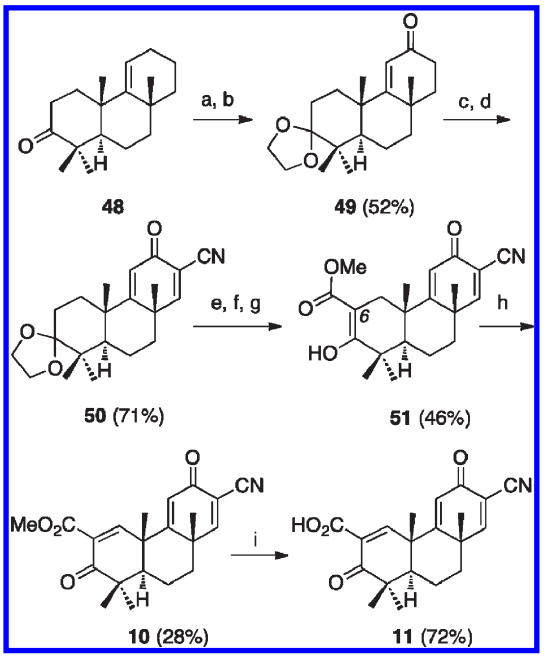
2.1. Initial Set of Tricycles. Our initial targets **1**−**5** (structures, see Table 1) were synthesized in racemic form from known compound **45**[44] (structure, see Figure 2) by synthetic sequence that has been published (syntheses, see Scheme S1 in the Supporting Information).[79a]

Because **5** in racemic form shows good potency in the iNOS assay among the initial set of the tricycles (Table 1), we have synthesized both (−)-**5**, with the same configuration as CDDO, and its antipode (+)-**5** from the known bicyclic enones (−)-**46** and (+)-**46**[45] (structure of (−)-**46**, see Figure 2), respectively, according to the improved synthetic route that is specifically directed toward (−)-**5** and (+)-**5** (syntheses, see Scheme S2 in the Supporting Information).[79c]

2.2. Insertion of Electron-Withdrawing Groups at C2 Position of Tricycles. Since **5** shows good potency in the iNOS assay, this compound was thought to be a good scaffold from which to discover new, more potent tricycles. Thus, we targeted **6**−**9** (structures, see Table 1), analogues of **5** with electron-withdrawing groups at the C2 position, to discern the influence of substituents at the C2 position on biological activity, because we previously found that substitution at the α position of an α,β-unsaturated ketone strongly affects the potency of semisynthetic triterpenoids.[46]

Racemic **6**−**9** were synthesized from **45** according to the synthetic route that has been published (syntheses, see Scheme S3 in the Supporting Information).[79a] Of these TCEs, **9** showed high potency (IC_{50} = 1 nM level) and it is approaching the potency of CDDO in the iNOS assay (see Table 1).

Scheme 1. Synthesis of TCEs 10 and 11[a]



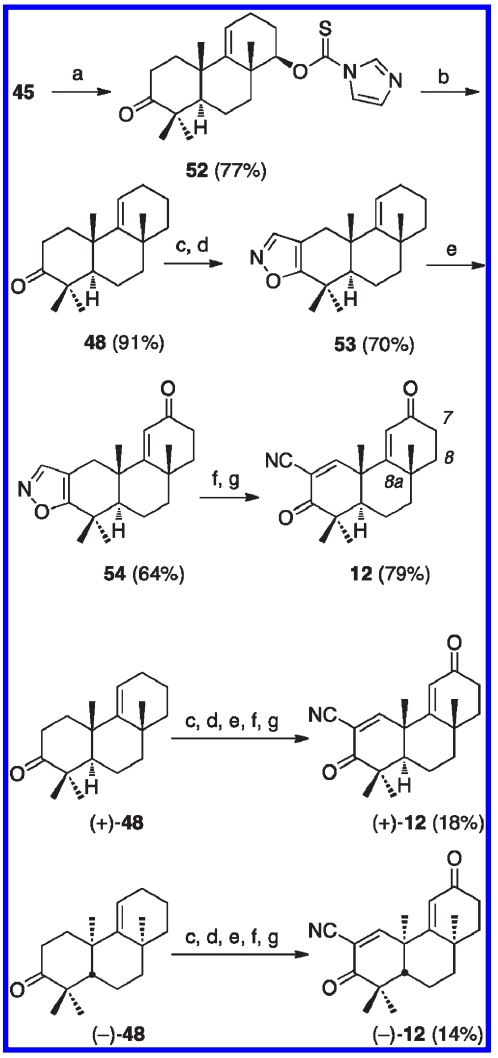
[a] Reagents and yields: (a) ethylene glycol, PPTS, PhH, 94%; (b) CrO_3, *t*-BuOOH, CH_2Cl_2, 55%; (c) *p*-TsCN, LDA, THF; (d) DDQ, PhH, 71% from **49**; (e) aqueous HCl, MeOH, 65%; (f) MMC, DMF, 100%; (g) CH_2N_2, Et_2O, 71%; (h) PhSeCl, pyridine, CH_2Cl_2, 30% H_2O_2, 28%; (i) aqueous KOH, MeOH, 72%.

We efficiently synthesized (−)-**9**, with the same configuration as the naturally occurring oleanolic acid, and its antipode (+)-**9** in seven steps from known bicyclic enones (−)-**47** and (+)-**47**,[47] respectively (structure of (−)-**47**, see Figure 2; syntheses, see Scheme S4 in the Supporting Information).[79c]

2.3. Synthesis of TCEs 10 and 11. We designed **10** and **11** in racemic form as analogues of **9** because **11** and its salts would be soluble in water. TCEs **10** and **11** were synthesized from **48**[79a,c] by the sequence shown in Scheme 1 (also see Scheme S5 in the Supporting Information). Tricycle **49** was prepared by ketalization of **48**, followed by a chromium-mediated allylic oxidation[48] with CrO_3 and *tert*-butyl hydroperoxide (*t*-BuOOH) in CH_2Cl_2 (52% yield). Cyanation of the enolate of **49**, generated using LDA in THF, with *p*-toluenesulfonyl cyanide (*p*-TsCN),[49] followed by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation, gave **50** in 71% yield. Methyl ester **51** was obtained by removal of the ketal of **50**, followed by carboxylation at the C6 position with Stiles' reagent[50] and subsequent methylation with diazomethane (46% yield). TCE **10** was prepared in 28% yield from **51** by the addition of PhSeCl in the presence of pyridine and subsequent oxidation/elimination with H_2O_2.[51] Cleavage of the methyl ester under basic conditions gave **11** in 72% yield.

2.4. Synthesis of TCE 12. We have designed and synthesized **12**, which has the same A, B, and C ring system as that of CDDO (Schemes 2 and S6 in the Supporting Information). Imidazole 1-thiocarboxylate **52** was prepared in 77% yield from **45** with 1,1′-thiocarbonyldiimidazole in THF. Reduction of **52** with tri(*n*-butyl)tin hydride in toluene gave **48** in 91% yield.[52] This tricycle **48**, which is derived from **45** (whose structure has already been confirmed),[44] is identical with that obtained by reductive methylation of **55**[53] (structure, see Figure 2; preparation, see Scheme S4

Scheme 2. Synthesis of Racemic and Optically Active TCE 12[a]

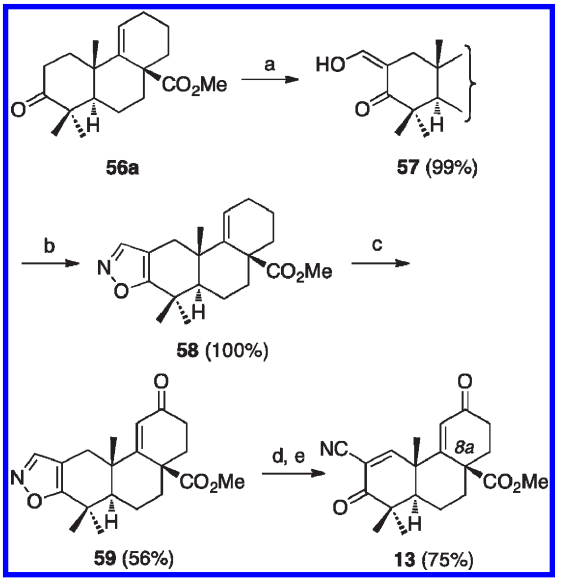


[a] Reagents: (a) 1,1′-thiocarbonyldiimidazole, THF; (b) $(n\text{-Bu})_3SnH$, toluene; (c) HCO_2Et, NaOMe, PhH; (d) $NH_2OH\cdot HCl$, aqueous EtOH; (e) CrO_3, *t*-BuOOH, CH_2Cl_2; (f) NaOMe, MeOH, Et_2O; (g) DDQ, PhH.

in the Supporting Information). Consequently, it was proven that the reductive methylation product **48** has the thermodynamically stable trans-A/B ring juncture. Isoxazole **53** was obtained in 70% yield by formylation of **48** with ethyl formate in the presence of NaOMe in benzene,[54] followed by treatment with hydroxylamine hydrochloride in aqueous EtOH.[55] A chromium-mediated allylic oxidation of **53** with *t*-BuOOH produced **54** in 64% yield. The desired TCE **12** was prepared by cleavage of the isoxazole moiety of **54** with NaOMe,[55] followed by DDQ oxidation (79% yield). We have also synthesized optically active (+)-**12** ($[\alpha]^{25}_D$ +58° (*c* 1.3, $CHCl_3$), 18% yield in five steps) and (−)-**12** ($[\alpha]^{25}_D$ −66° (*c* 2.0, $CHCl_3$), 14% yield in five steps), from (+)-**48**[79c] and (−)-**48**,[79c] respectively, by the same sequence as for (±)-**12**.

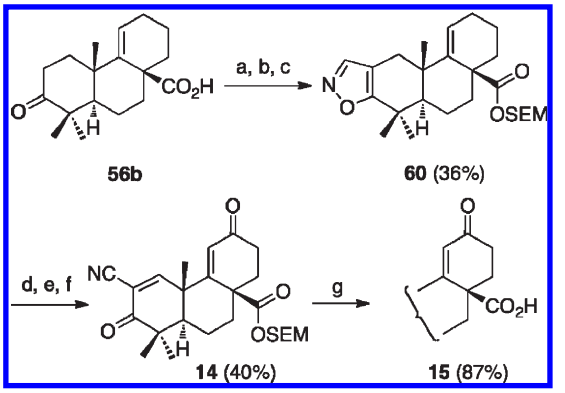
Since **12** is about 3 times more potent than TCE **5** in the iNOS assay (Table 1), we considered that the 7,8-double bond in ring C is not always necessary for potency. Therefore, we designed and synthesized various C8a functionalized analogues of **12**.

Scheme 3. Synthesis of TCE 13[a]



[a] Reagents: (a) HCO_2Et, NaOMe, PhH; (b) $NH_2OH\cdot HCl$, aqueous EtOH; (c) CrO_3, *t*-BuOOH, CH_2Cl_2; (d) NaOMe, MeOH, Et_2O; (e) DDQ, PhH.

Scheme 4. Synthesis of TCE 15[a]



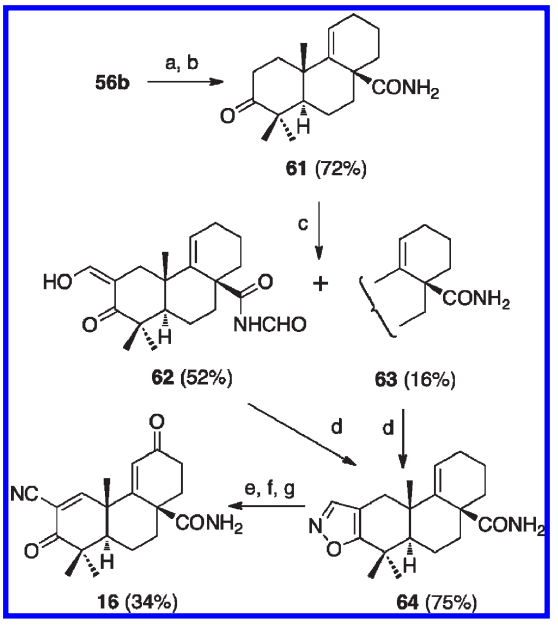
[a] Reagents and yields: (a) HCO_2Et, NaOMe, PhH, 45%; (b) $NH_2OH\cdot HCl$, aqueous EtOH, 100%; (c) SEMCl, EDIA, CH_2Cl_2, 80%; (d) CrO_3, *t*-BuOOH, CH_2Cl_2, 67%; (e) NaOMe, MeOH, Et_2O, 93%; (f) DDQ, PhH, 64%; (g) 48% aqueous HF−CH_3CN (1:9), 87%.

Substitution of functionalities at the C8a position of **12** would be expected to improve the potency and pharmacokinetics because the balance between hydrophilicity and hydrophobicity is shifted.

2.5. Functionality Substitutions at the C8a Postion of TCE 12. We synthesized various C8a functionalized TCE analogues using tricycles **56a**−**c** (structures, see Schemes 3, 4, and 6) as starting materials, whose efficient synthesis we have already established for our projected synthesis.[56] These analogues include typical electron-withdrawing, electron-releasing, hydrophilic, hydrophobic, and bulky groups.

TCE **13** with a methoxycarbonyl group at C8a was synthesized in 42% overall yield via **57**, **58**, and **59** from **56a** by the same sequence as for **12** from **48** (Scheme 3 and Scheme S7 in the Supporting Information). We attempted several methods [KOH, aqueous MeOH (reflux, overnight); LiI, DMF (reflux, 30 min);[57] $KOSiMe_3$, THF (room temp, overnight);[58] $AlBr_3$, Me_2S (room

Scheme 5. Synthesis of TCE 16[a]



[a] Reagents and yields: (a) $(COCl)_2$, CH_2Cl_2, 90%; (b) NH_3, PhH, 80%; (c) HCO_2Et, NaOMe, PhH; (d) $NH_2OH \cdot HCl$, aqueous EtOH; (e) CrO_3, *t*-BuOOH, CH_2Cl_2, 50%; (f) NaOMe, MeOH, Et_2O; (g) DDQ, 1,4-dioxane, 68% in two steps (f) and (g).

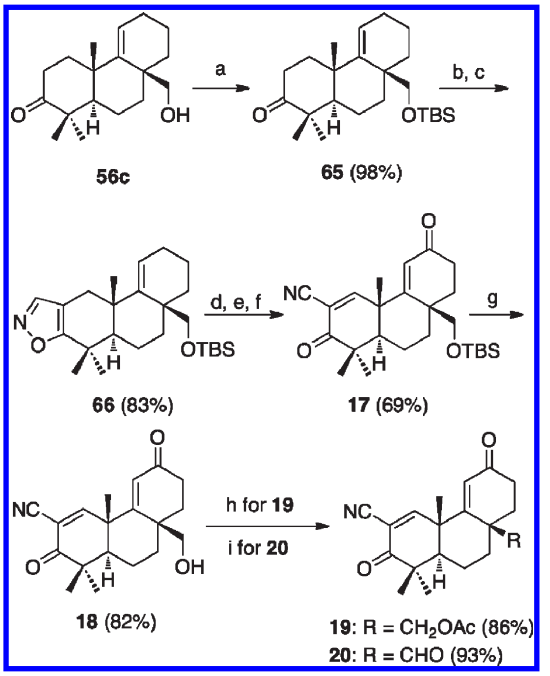
temp, overnight);[59] LiS(*n*-Pr), HMPA (room temp, 1 h)[60]] for the cleavage of methyl ester **13** to the corresponding acid **15**, but these methods failed to give **15**.

Therefore, we synthesized acid **15** according to the alternative sequence shown in Scheme 4 (Scheme S8 in the Supporting Information). Isoxazole **60** was prepared by formylation of **56b** and subsequent isoxazole ring construction, followed by protection of the hydroxyl group with 2-((chloromethoxy)ethyl)-trimethylsilane (SEMCl) in the presence of EDIA in CH_2Cl_2 (36% yield). TCE **14** was obtained in 40% yield from **60** by the same sequence as for **13** from **58**. The (trimethylsilyl)-ethoxymethyl (SEM) group was removed from **14** with 48% aqueous HF and CH_3CN (1:9) to give **15** (87% yield). Although we were concerned that **15** might be unstable toward decarboxylation in a medium used for biological testing, in fact, **15** is both soluble and stable in Dulbecco's modified Eagle's medium (10 mM) at room temperature for 4 days at least.

TCE **16** with an aminocarbonyl group was synthesized from **56b** (Scheme 5 and Scheme S9 in the Supporting Information). Amide **61** was prepared in 72% yield from **56b** by chlorination with oxalyl chloride, followed by the treatment with NH_3 in benzene. Unexpectedly, the formylation of **61** with ethyl formate in the presence of NaOMe gave **62** (52% yield) as a major product and the desired compound **63** (16% yield) as a minor one. However, treatment of **62** with hydroxylamine hydrochloride cleaved the *N*-formyl group to give the desired isoxazole **64** in 75% yield. Under the same conditions, **63** gave **64** in 75% yield. TCE **16** was obtained by allylic oxidation of **64**, followed by the cleavage of the isoxazole under basic conditions and subsequent DDQ oxidation in 1,4-dioxane (34% yield). For the DDQ oxidation step, because the precursor of **16** was not soluble in benzene, 1,4-dioxane was used as the solvent.

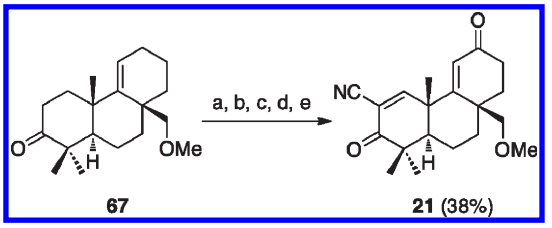
TCE **18** with a hydroxymethyl group and its derivatives **17** and **19**−**21** were synthesized according to the sequence shown in

Scheme 6. Synthesis of TCEs 17−20[a]



[a] Reagents and yields: (a) TBSCl, imidazole, DMF, 98%; (b) HCO_2Et, NaOMe, PhH, 98%; (c) $NH_2OH \cdot HCl$, NaOAc, aqueous AcOH, 85%; (d) CrO_3, *t*-BuOOH, CH_2Cl_2, 69% (e) NaOMe, MeOH, Et_2O; (f) DDQ, 1,4-dioxane, 100% in two steps (e) and (f); (g) 48% aqueous HF, CH_3CN, 82%; (h) Ac_2O, pyridine, 86%; (i) CrO_3, pyridine, CH_2Cl_2, 93%.

Scheme 7. Synthesis of TCE 21[a]



[a] Reagents and yields: (a) HCO_2Et, NaOMe, PhH, 100%; (b) $NH_2OH \cdot HCl$, aqueous EtOH, 100%; (c) CrO_3, *t*-BuOOH, CH_2Cl_2, 68%; (d) NaOMe, MeOH, Et_2O, 97%; (e) DDQ, 1,4-dioxane, 58%.

Schemes 6 and 7 (Schemes S10 and S11 in the Supporting Information). The starting material **56c** was protected with *tert*-butylchlorodimethylsilane (TBSCl) to give **65** in 98% yield. Isoxazole **66** was prepared by formylation of **65**, followed by treatment with hydroxylamine hydrochloride in the presence of NaOAc in aqueous AcOH (83% yield). At the isoxazole construction step, NaOAc and aqueous AcOH were used instead of aqueous EtOH because the latter conditions gave **66** in low yield. We considered that 1 equiv of HCl, which is produced from the latter conditions, would disrupt this conversion. TCE **17** was obtained in 69% yield from **66** by the same sequence as for **16** from **64**. The TBS group of **17** was removed by 48% aqueous HF and CH_3CN (1:9) to give **18** in 82% yield. Acetylation of **18** with acetic anhydride in pyridine afforded **19** in 86% yield. Aldehyde **20** was obtained in 93% yield by Ratcliffe oxidation[61] of **18** with CrO_3 and pyridine in CH_2Cl_2. Methyl ether **21** was synthesized in five steps from a known compound **67**[62] by the same sequence as for **16** from **61** (38% yield, Scheme 7).

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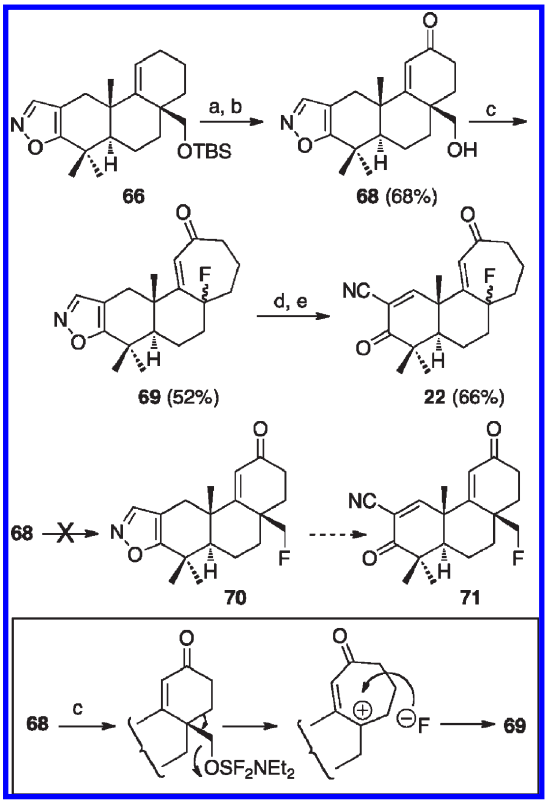
We tried to synthesize fluoro derivative **71** from **66** (Scheme 8 and Scheme S12 in the Supporting Information). Tricycle **68** was obtained in 68% yield by allylic oxidation of **66**, followed by removal of the TBS group with 48% aqueous HF and CH_3CN (1:9). However, fluorination of **68** with DAST did not give **70** but a ring expansion product **69** in 52% yield. TCE **22** was prepared in 66% yield by the cleavage of the isoxazole of **69**, followed by DDQ oxidation. The structures of **69** and **22** were fully characterized by ^1H and ^{13}C NMR, high and low MS, and elemental analysis. We speculate that **69** is produced from **68** according to the mechanisms shown in the box in Scheme 8. The configuration of the fluorine atom has not been determined.

Scheme 8. Synthesis of TCE 22[a]



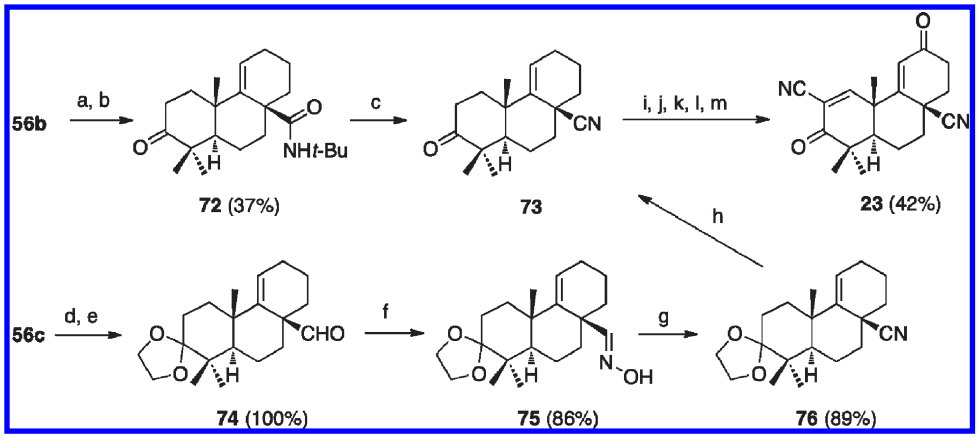
[a] Reagents: (a) CrO_3, *t*-BuOOH, CH_2Cl_2; (b) HF, CH_3CN; (c) DAST, CH_2Cl_2; (d) NaOMe, MeOH, Et_2O; (e) DDQ, 1,4-dioxane.

Dicarbonitrile **23** was synthesized from **56b** and **56c** by the different sequence shown in Scheme 9 (Scheme S13 in the Supporting Information). Amide **72** was prepared by chlorination of **56b** with oxalyl chloride, followed by the treatment with *tert*-butylamine (37% yield). Carbonitrile **73** was obtained by the treatment of **72** with $POCl_3$ (100% yield, 37% overall yield from **56b**).[63] However, since this step requires vigorous conditions, the yield varies. Thus, we explored an improved synthesis of **73** from alcohol **56c**. Ketalization of **56c**, followed by Swern oxidation[64] gave aldehyde **74** (100% yield). Oxime **75** was obtained in 86% yield by the condensation between hydroxylamine and **74** in CH_2Cl_2. Dehydration of **75** with 1,1-carbonyldiimidazole in CH_2Cl_2 provided carbonitrile **76** in 89% yield.[65] The ketal of **76** was removed under acidic conditions to afford **73** in 100% yield (77% overall yield from **56c**). The overall yield of this sequence is about twice that of **73** from **56b**. Moreover, importantly, the yield of this sequence is reproducible. Dicarbonitrile **23** was synthesized in five steps from **73** by the same sequence as for **16** from **61** (42% yield).

Amine hydrochloride **25** was synthesized from **76** by the sequence shown in Scheme 10 (Scheme S14 in the Supporting Information). Reduction of the cyano group of **76** with $LiAlH_4$ gave only a complex mixture. In contrast, reduction of **76** with a mixture of $NaBH_4$ and $CoCl_2$ (5:1),[66] followed by workup with 10% aqueous HCl solution and subsequent protection of the amino group with Boc_2O gave **77** in 54% yield. TCE **24** was obtained in five steps from **77** by the same sequence as for **16** from **61** (41% yield). Removal of the Boc group of **24** with 4 M HCl in 1,4-dioxane provided **25** in 95% yield.

TCE **26** with an ethyl group was synthesized from **74** (Scheme 11 and Scheme S15 in the Supporting Information). A Grignard reaction of **74** with MeMgBr in THF gave **78** in 87% yield as a mixture of diastereomers. Ratcliffe oxidation of **78** afforded **79** in 73% yield. A forced Wolff−Kishner reduction[67] of **79** followed by deketalization under acidic conditions provided

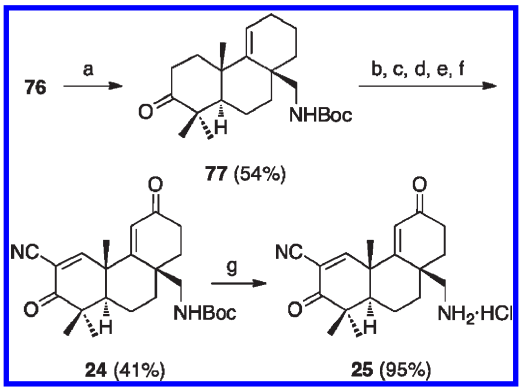
Scheme 9. Synthesis of TCE 23[a]



[a] Reagents and yields: (a) $(COCl)_2$, CH_2Cl_2; (b) *t*-$BuNH_2$, Et_3N, CH_2Cl_2, 37% from **56b**; (c) $POCl_3$, 100%; (d) ethylene glycol, PPTS, PhH, 100%; (e) Swern oxidation, 100%; (f) $NH_2OH\cdot HCl$, NaOAc, MeOH, CH_2Cl_2, 86%; (g) 1,1-carbonyldiimidazole, CH_2Cl_2, 89%; (h) aqueous HCl, MeOH, 100%; (i) HCO_2Et, NaOMe, PhH, 72%; (j) $NH_2OH\cdot HCl$, aqueous EtOH, 100%; (k) CrO_3, *t*-BuOOH, CH_2Cl_2, 83%; (l) NaOMe, MeOH, Et_2O, 88%; (m) DDQ, 1,4-dioxane, 79%.

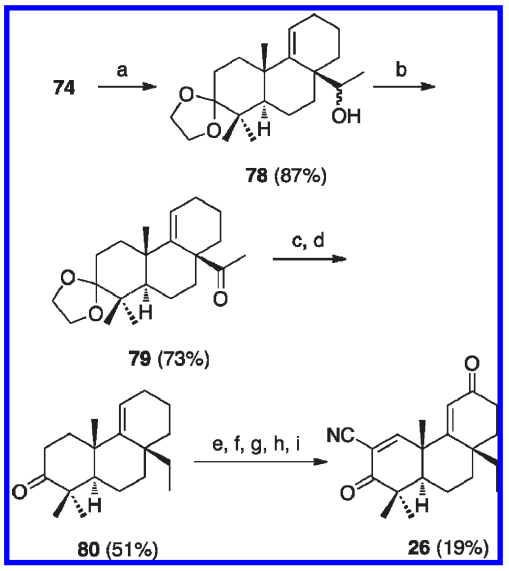
dx.doi.org/10.1021/jm101445p |*J. Med. Chem.* 2011, 54, 1762–1778

Scheme 10. Synthesis of TCEs 24 and 25[a]



[a] Reagents and yields: (a) $NaBH_4$, $CoCl_2$, MeOH, 71%; 10% aqueous HCl, Boc_2O, THF, 76%; (b) HCO_2Et, NaOMe, PhH, 100%; (c) $NH_2OH \cdot HCl$, aqueous EtOH, 76%; (d) CrO_3, *t*-BuOOH, CH_2Cl_2, 62%; (e) NaOMe, MeOH, Et_2O, 100%; (f) DDQ, 1,4-dioxane, 86%; (g) 4 M HCl in 1,4-dioxane, 95%.

Scheme 11. Synthesis of TCE 26[a]



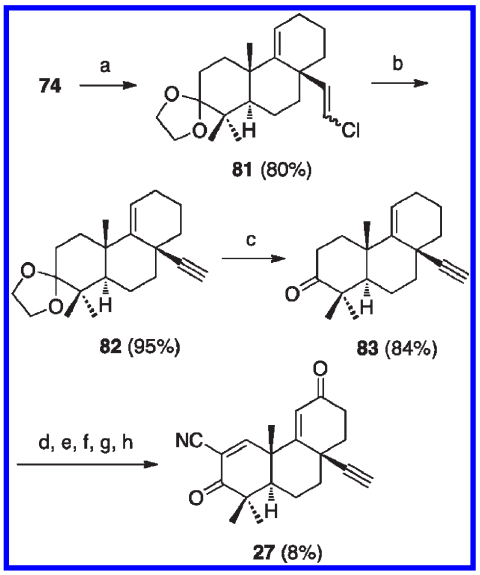
[a] Reagents and yields: (a) MeMgBr, THF, 87%; (b) CrO_3, pyridine, CH_2Cl_2, 73%; (c) NH_2NH_2, KOH, diethylene glycol, 51%; (d) aqueous HCl, MeOH, 100%; (e) HCO_2Et, NaOMe, PhH, 75%; (f) $NH_2OH \cdot HCl$, aqueous EtOH, 95%; (g) CrO_3, *t*-BuOOH, CH_2Cl_2, 69%; (h) NaOMe, MeOH, Et_2O, 100%; (i) DDQ, 1,4-dioxane, 39%.

80 in 51% yield. TCE **26** was obtained in five steps from **80** by the same sequence as for **16** from **61** (19% yield).

TCE **27** with an ethynyl group was synthesized from **74** (Scheme 12 and Scheme S16 in the Supporting Information). A Wittig reaction on **74** with (chloromethyl)triphenylphosphonium chloride[68] gave **81** as a mixture of *E*/*Z* chlorovinyl isomers (*E*/*Z* = 4:1) in 80% yield. Dehydrochlorination of **81** with MeLi followed by quenching of the acetylide with aqueous NH_4Cl solution provided **82** in 95% yield.[68] The ketal of **82** was removed under acidic conditions to afford **83** in 84% yield. TCE **27** was obtained in 8% yield from **83** by the same sequence as for **16** from **61**.

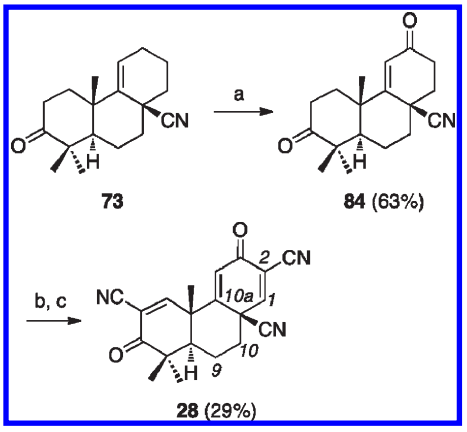
2.6. Functionality Substitutions at the C10a position of TCE 9. TCE **28** with a cyano group at C10a, which is a C10a

Scheme 12. Synthesis of TCE 27[a]



[a] Reagents and yields: (a) $Ph_3PCH_2Cl_2$, *n*-BuLi, THF, HMPA, 80%; (b) MeLi, THF; aq NH_4Cl, 95%; (c) 10% aqueous HCl, MeOH, 84%; (d) HCO_2Et, NaOMe, PhH, 76%; (e) $NH_2OH \cdot HCl$, aqueous EtOH, 70%; (f) CrO_3, *t*-BuOOH, CH_2Cl_2, 46%; (g) NaOMe, MeOH, Et_2O, 93%; (h) DDQ, 1,4-dioxane, 37%.

Scheme 13. Synthesis of TCE 28[a]

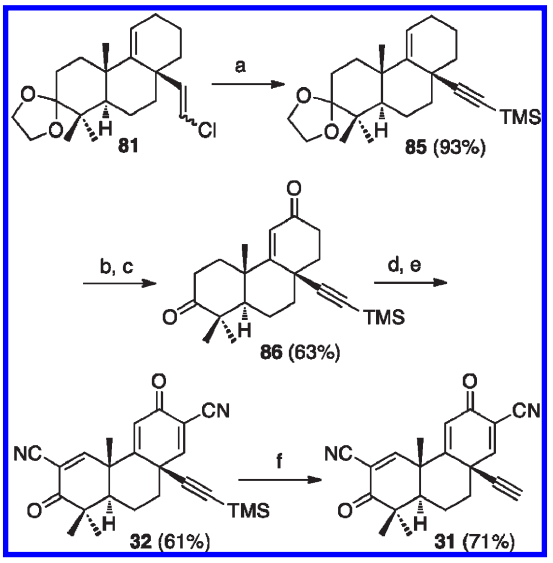


[a] Reagents: (a) CrO_3, *t*-BuOOH, CH_2Cl_2; (b) *p*-TsCN, LDA, THF; (c) DDQ, 1,4-dioxane.

functionalized derivative of TCE **9**, was synthesized by the sequence shown in Scheme 13 (Scheme S17 in the Supporting Information). A chromium-mediated allylic oxidation of **73** with CrO_3 and *t*-BuOOH in CH_2Cl_2 gave **84** in 63% yield. TCE **28** was obtained by double cyanation of **84** with LDA and *p*-TsCN, followed by DDQ oxidation in 1,4-dioxane (29% yield). TCE **29** with an ethyl group (structure, see Table 3) was synthesized in three steps from **80** by the same sequence as for **28** from **73** (preparation, see Scheme S18 in the Supporting Information).[79e] TCE **30** with a vinyl group (structure, see Table 3) was obtained in five steps from **74** (preparation, see Scheme S19 in the Supporting Information).[79e]

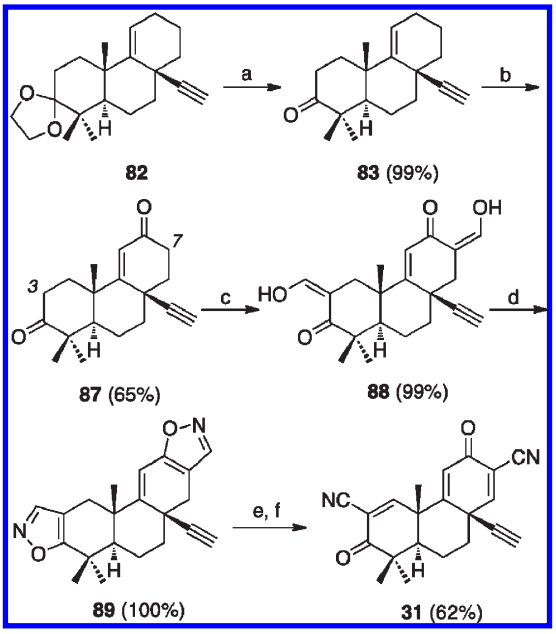
TCE **31** with an ethynyl group was synthesized in six steps from **81** (Scheme 14 and Scheme S20 in the Supporting Information). Dehydrochlorination of **81** with MeLi, followed

Scheme 14. Synthesis of TCEs 31 and 32[a]



[a] Reagents: (a) MeLi, THF; TMSCl; (b) aqueous HCl, MeOH; (c) CrO_3, *t*-BuOOH, CH_2Cl_2; (d) *p*-TsCN, LDA, THF; (e) DDQ, PhH; (f) TBAF, THF.

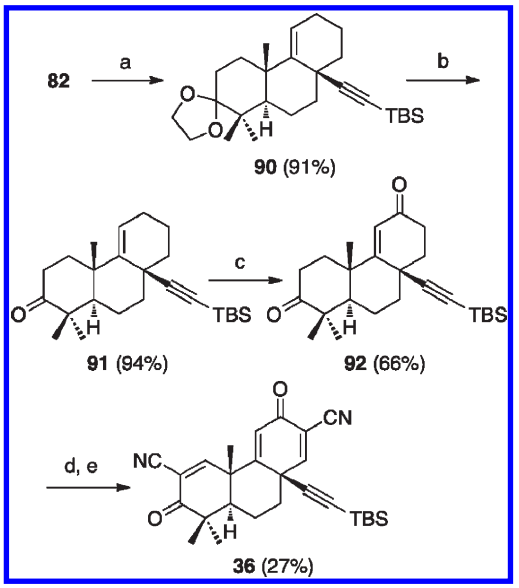
Scheme 15. Improved synthesis of TCE 31[a]



[a] Reagents: (a) 10% aqueous HCl, MeOH; (b) CrO_3, *t*-BuOOH, CH_2Cl_2; (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.

by trapping of the acetylide with chlorotrimethylsilane (TMSCl) gave **85** in 93% yield.[69] Deketalization of **85** under acidic conditions followed by a chromium-mediated allylic oxidation provided **86** in 63% yield. TCE **32** was obtained by double cyanation of **86** with LDA and *p*-TsCN, followed by DDQ oxidation in benzene (61% yield). The trimethylsilyl (TMS) group was removed by tetra(*n*-butyl)ammonium fluoride (TBAF)[70] to afford **31** in 71% yield (nine steps from **56c**, 21% overall yield).

Scheme 16. Synthesis of TCE 36[a]



[a] Reagents: (a) MeLi, THF, TBSCl; (b) aqueous HCl, MeOH; (c) CrO_3, *t*-BuOOH, CH_2Cl_2; (d) *p*-TsCN, LDA, THF; (e) DDQ, PhH.

Optically active (−)- and (+)-**31** and **32** (structures, see Table 3) were synthesized by the sequence that has been published (syntheses, see Scheme S21 in the Supporting Information).[79e]

2.7. Improved Synthesis of TCE 31. Currently, since TCE **31** is the most potent compound in our pool of semisynthetic triterpenoids and synthetic tricycles that we have evaluated in our bioassays (see Biological Results and Discussion), it is essential for further evaluation to prepare at least 1 g of **31** in a single batch. Although the sequence shown in Scheme 14 is acceptable for a small-scale synthesis (10−200 mg), it is not adequate for a medium-scale synthesis (500 mg to 2 g). Particularly, the double cyanation step using *p*-TsCN is not feasible because *p*-TsCN is a very expensive reagent and the yield of this reaction drastically decreases on a large scale. Thus, we have developed an improved synthesis of **31** (Scheme 15 and Scheme S22 in the Supporting Information) by adopting Johnson's isoxazole method[55] for the double cyanation, which we used for the synthesis of TCE **12** analogues. With this sequence, **31** is consistently obtained in 30% yield in 10 steps from **56c**.

Removal of the ketal of **82** under acidic conditions gave **83** in 99% yield. Enone **87** was prepared in 65% yield from **83** by allylic oxidation. Formyl groups were successfully inserted into the C3 and C7 positions of **87** simultaneously to afford **88** (99% yield) using twice the amount of ethyl formate and NaOMe (11 equiv each) than was used for monoformylation (5.5 equiv each). Treatment of **88** with hydroxylamine hydrochloride provided diisoxazole **89** in quantitative yield. Each isoxazole ring of **89** was converted to a cyano group under basic conditions, which gave **31** upon the addition of PhSeCl and subsequent oxidation/elimination with H_2O_2 (62% yield). This oxidation method was better than the DDQ method for this oxidation with an unprotected acetylene group.

2.8. Synthesis of TCE 31 Analogues. The high potency of **31** and **32** in the iNOS assay (see Table 3) encouraged us to explore the modifications of the ethyne group of TCE **31**. TCEs **33**−**35** (structures, see Table 3) were synthesized in five steps from **82** (syntheses, see Schemes S23−S25 in the Supporting Information).[79e]

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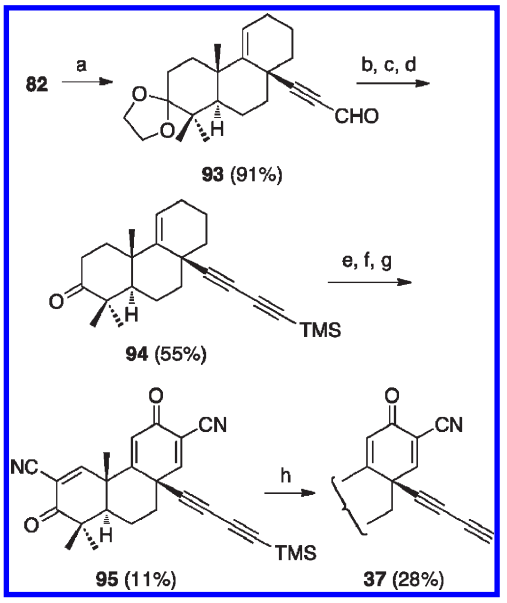
Scheme 17. Synthesis of TCE 37[a]



[a] Reagents and yields: (a) *n*-BuLi, $BF_3 \cdot Et_2O$, DMF, THF, 91%; (b) $Ph_3PCH_2Cl_2$, *n*-BuLi, HMPA, THF, 76%; (c) MeLi, THF, TMSCl, 97%; (d) 10% aqueous HCl, MeOH, 74%; (e) CrO_3, *t*-BuOOH, CH_2Cl_2, 50%; (f) *p*-TsCN, LDA, THF; (g) DDQ, PhH, 22% in two steps (f) and (g); (h) TBAF, THF, 28%.

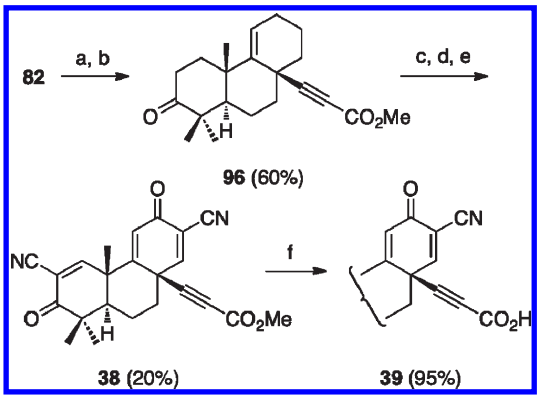
TCE **36** was synthesized in five steps from **82** (Scheme 16 and Scheme S26 in the Supporting Information). Insertion of the TBS group into the acetylene moiety was achieved by treating **82** with MeLi and trapping the resulting anion with TBSCl, to give **90** in 91% yield. The ketal **90** was subjected to acidic conditions to give **91** in 94% yield. Allylic oxidation of **91** afforded **92** in 66% yield. Double cyanation of **92**, followed by DDQ oxidation in benzene, gave **36** in 27% yield.

TCE **37** with a buta-1,3-diynyl group was synthesized in eight steps from **82** (Scheme 17 and Scheme S27 in the Supporting Information). The treatment of acetylide **82** with DMF in the presence of $BF_3 \cdot Et_2O$ in THF provided aldehyde **93** in 91% yield. Tricycle **94** was prepared by a Wittig reaction of **93** with (chloromethyl)triphenylphosphonium chloride and subsequent treatment with MeLi and TMSCl, followed by deketalization (55% yield). Tricycle **95** was obtained in three steps from **94** by the same sequence as for **36** from **91** (11% yield). The TMS group of **95** was removed with TBAF to give **37** in 28% yield.

TCE **38** with a methoxycarbonyl group and the corresponding acid **39** were synthesized from **82** (Scheme 18 and Scheme S28 in the Supporting Information). Methyl ester **96** was obtained by the treatment of acetylide **82** with methyl chloroformate, followed by deketalization (60% yield). TCE **38** was prepared in three steps from **96** by the same sequence as for **36** from **91** (20% yield). Alkaline hydrolysis of **38** gave **39** in 95% yield.

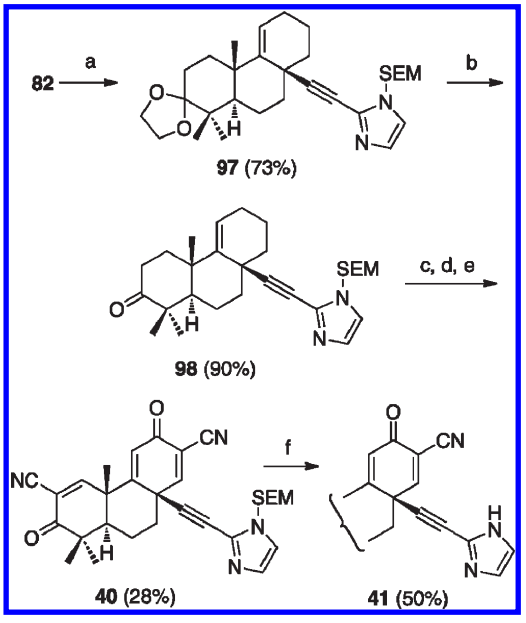
Acetylenic imidazoles **40** and **41** were synthesized in six steps from **82** (Scheme 19 and Scheme S29 in the Supporting Information). A Sonogashira coupling[71] between 2-iodo-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-imidazole[72] and **82** in the presence of $Pd(PPh_3)_2Cl_2$ and CuI in Et_3N gave **97** in 73% yield. Deketalization of **97** provided **98** in 90% yield. TCE **40** was obtained by allylic oxidation of **98** and subsequent isoxazole opening under basic conditions, followed by addition of PhSeCl in the presence of pyridine, and subsequent oxidation/

Scheme 18. Synthesis of TCEs 38 and 39[a]



[a] Reagents and yields: (a) $ClCO_2Me$, MeLi, THF, 64%; (b) 10% aqueous HCl, MeOH, 93%; (c) CrO_3, *t*-BuOOH, CH_2Cl_2, 60%; (d) *p*-TsCN, LDA, THF; (e) DDQ, PhH, 26% in two steps (d) and (e); (f) aqueous KOH, MeOH, 95%.

Scheme 19. Synthesis of TCEs 40 and 41[a]

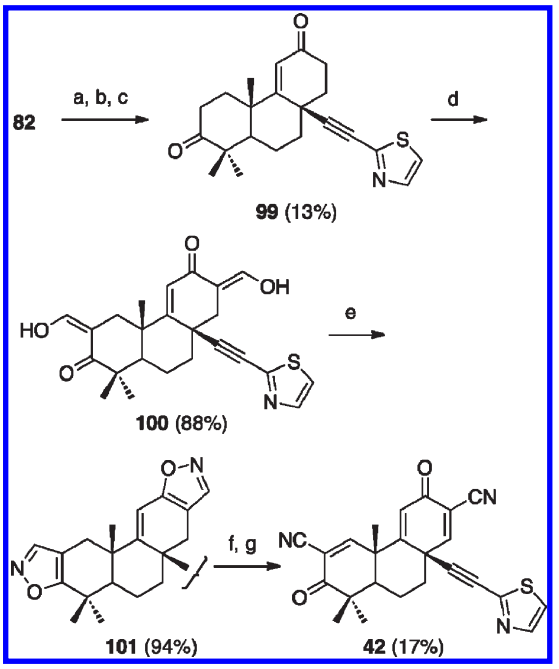


[a] Reagents and yields: (a) 2-iodo-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-imidazole, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, 73%; (b) 10% aqueous HCl, MeOH, 90%; (c) CrO_3, *t*-BuOOH, CH_2Cl_2, 43% (d) *p*-TsCN, LDA, THF, 88%; (e) PhSeCl, pyridine, CH_2Cl_2; 30% H_2O_2, CH_2Cl_2, 75%; (f) CF_3CO_2H−THF (10:1).

elimination with H_2O_2 (28% yield). In this conversion, the PhSeCl and H_2O_2 were used instead of DDQ because the DDQ oxidation gave **40** in a very low yield. Removal of the SEM group of **40** was successfully achieved by CF_3CO_2H−THF (10:1) to afford **41** in 50% yield, after the deprotection using several well-known methods (TBAF, HF−CH_3CN, HF−pyridine, $BF_3 \cdot Et_2O$ etc.) was unsuccessful.

Acetylenic thiazole **42** was synthesized in seven steps from **82** (Scheme 20 and Scheme S30 in the Supporting Information). Tricycle **99** was obtained by a Sonogashira coupling between 2-bromothiazole and **82**, followed by removal of the ketal and subsequent allylic oxidation (13% yield). Formylation of **99** gave

Scheme 20. Synthesis of TCE 42[a]



[a] Reagents and yields: (a) 2-bromothiazole, $Pd(PPh_3)_2Cl_2$, PPh_3, CuI, Et_3N, PhH, 32%; (b) 10% aqueous HCl, MeOH, 100%; (c) CrO_3, *t*-BuOOH, CH_2Cl_2, 41%; (d) HCO_2Et, NaOMe, PhH, 88%; (e) $NH_2OH \cdot HCl$, aqueous EtOH, 94%; (f) NaOMe, MeOH, Et_2O; (g) PhSeCl, pyridine, CH_2Cl_2, 30% H_2O_2, CH_2Cl_2, 17% in two steps (f) and (g).

bis(hydroxymethylene) **100** in 88% yield. Treatment of **100** with hydroxylamine provided diisoxazole **101** in 94% yield. TCE **42** was prepared in 17% yield from **101** by the same methods as for **31** from **89** (see Scheme 15). Because double cyanation of **99** with *p*-TsCN followed by DDQ oxidation gave impure **42**, which we could not sufficiently purify, we adopted the Johnson's isoxazole method for this conversion.

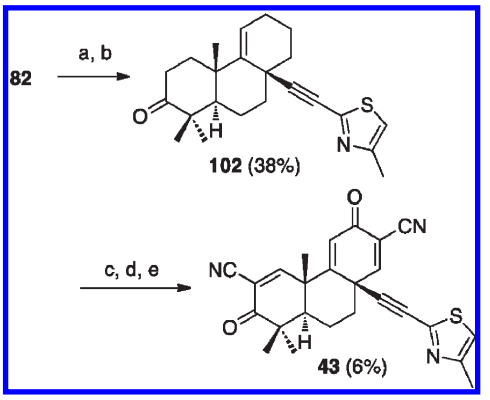
Acetylenic methylthiazole **43** was synthesized by a Sonogashira coupling between 2-iodo-4-methylthiazole and **82** and subsequent deketalization, followed by the same sequence from **102** as for **40** from **98** (2% overall yield, Scheme 21 and Scheme S31 in the Supporting Information).

TCE **44** with a phenylethynyl group was synthesized in seven steps from **82** (Scheme 22 and Scheme S32 in the Supporting Information). Enone **103** was obtained by a Sonogashira coupling between iodobenzene and **82** and subsequent deketalization, followed by allylic oxidation (40% yield). TCE **44** was prepared in four steps from **103** by the same sequence as for **42** from **99** (14% yield).

3. BIOLOGICAL RESULTS AND DISCUSSION

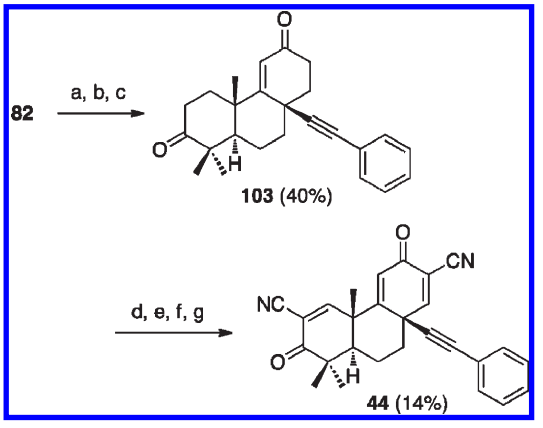
3.1. TCEs 1−12 Inhibit NO Production Induced by IFN-γ in Primary Mouse Macrophages. We have evaluated inhibitory activities of the initial set of TCEs on NO production induced by IFN-γ in mouse macrophages. The inhibitory activities [IC_{50} (nM)] of racemic **1**−**12**, optically active **5**, **9**, and **12**, triterpenoids [CDDO and CDDO-Me], and hydrocortisone (a positive control) are shown in Table 1. Important results, which were obtained from these compounds, are as follows: (1) Among all of the synthetic TCEs, **9** has the highest potency, and next is **12**. TCE **9** approaches the potency of CDDO in this assay; it is only about 4 times less potent than CDDO. A nitrile group at C2 enhances potency among the typical electron-withdrawing groups surveyed at C2. (2) Since **3** and **4** are more potent than **2** and **1**, respectively, the importance of the bis(enone) structure for high potency, even in tricycles, is confirmed. (3) A nitrile group at C6 is essential for high potency (**9** vs **10** and **11**). (4) Both enantiomers of **5** and **12** show similar potency, while (+)-**9** having the same configuration as the CDDO antipode is about 10 times more potent than (−)-**9** having the same configuration as CDDO.

Scheme 21. Synthesis of TCE 43[a]



[a] Reagents and yields: (a) 2-iodo-4-methylthiazole, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, PhH, 48%; (b) 10% aqueous HCl, MeOH, 80%; (c) CrO_3, *t*-BuOOH, CH_2Cl_2, 49%; (d) *p*-TsCN, LDA, THF; (e) PhSeCl, pyridine, CH_2Cl_2, 30% H_2O_2, CH_2Cl_2, 13% in two steps (d) and (e).

Scheme 22. Synthesis of TCE 44[a]



[a] Reagents and yields: (a) iodobenzene, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, THF, 64%; (b) 10% aqueous HCl, MeOH, 77%; (c) CrO_3, *t*-BuOOH, CH_2Cl_2, 81%; (d) HCO_2Et, NaOMe, PhH, 92%; (e) $NH_2OH \cdot HCl$, aqueous EtOH, 97%; (f) NaOMe, MeOH, Et_2O, 32%; (g) PhSeCl, pyridine, CH_2Cl_2, 30% H_2O_2, CH_2Cl_2, 50%.

The inhibitory potency of **9** in this assay was not blocked by the glucocorticoid antagonist mifepristone (RU486, at 1 μM), which reverses the action of hydrocortisone (see Figure S2 in the Supporting Information). This strongly implies that the actions of TCEs in this assay are not mediated by their interaction with the glucocorticoid receptor.

3.2. TCEs 13−44 Inhibit NO Production Induced by IFN-γ in RAW 264.7 Cells. Our results from the initial set of TCEs encouraged us to explore additional TCEs. Thus, we first designed

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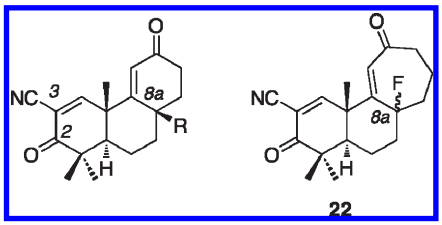
Table 2. Inhibitory Activity of New TCEs 13−27 on NO Production Induced by IFN-γ in RAW Cells[a]



compd (racemic)	R	IC_{50} (nM)	compd (racemic)	R	IC_{50} (nM)
13	CO_2Me	290	**23**	CN	64
14	CO_2SEM	150	**24**	CH_2NHBoc	64
15	CO_2H	83	**25**	$CH_2NH_2 \cdot HCl$	240
16	$CONH_2$	>10000	**26**	Et	80
17	CH_2OTBS	480	**27**	C≡CH	83
18	CH_2OH	350	**12**	Me	57
19	CH_2OAc	85	**5**	Table *1*	87
20	CHO	83	**9**	Table *1*	23
21	CH_2OMe	40	CDDO	Table *1*	17
22		360	hydrocortisone		61

[a] RAW 264.7 cells were treated with various concentrations of compounds and IFN-γ (10 ng/mL) for 24 h. Supernatants were analyzed for NO by the Griess reaction.[11] IC_{50} values are an average of two separate experiments. These data have been published and presented in refs 79f and 79h.

and synthesized the new TCEs **13**−**27**, which have various functionalities at C8a on the basis of **12**. These analogues include typical electron-withdrawing, electron-releasing, hydrophilic, hydrophobic, and bulky groups (Table 2). Among them, the lead compound **12** having a methyl group at C8a was still the best. However, we found that hydrocarbon groups are slightly better than functional groups containing heteroatoms.

Second, we designed new TCEs **28**−**32** on the basis of TCE **9**. From the SARs above, we mainly synthesized compounds with hydrocarbon groups at C10a. Also, we prepared optically active **31** and **32** to compare the differences in potency between the enantiomers. Notably and importantly, these compounds have two different nonenolizable cyano enones in rings A and C, which represent two theoretically possible monocyclic nonenolizable cyano enones.

The IC_{50} (nM) values of these TCEs are shown in Table 3. Remarkably, TCEs **29**−**32** having hydrocarbon groups are more potent than the lead compound **9**, as well as the positive controls CDDO and dexamethasone. In particular, acetylene groups dramatically enhance potency. TCE **31** is much more potent than **9**, CDDO, and dexamethasone and is as potent as CDDO-Im, which is the most potent CDDO analogue. Importantly, TCEs having two cyano enones are much more potent than TCEs having only one cyano enone (**9** vs **12**, **29** vs **26**, **31** vs **27**). These results suggest that the cyano enone is a very important factor for potency. TCE **28** having a nitrile group at C10a is much less potent than others. The fact that an electron-withdrawing group at C10a decreases potency is identical with the previous results shown in Table 2.

Interestingly, the racemic form and the (+)- and (−)-enantiomers of **32** are slightly less potent than those of **31**, although **32** has a much more bulky group than **31**.[73] Notably, (+)-enantiomers of **31** and **32**, having the same configuration as the CDDO antipode, are more potent than (−)-enantiomers of **31** and **32**, having the same configuration as CDDO, respectively. These results are identical with those of optically active **9** (Table 1).

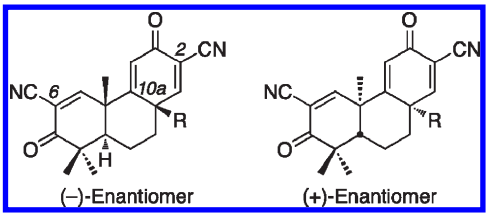
We have evaluated **31** and CDDO in the iNOS assay using primary mouse macrophages which are much more sensitive against inhibitors than RAW cells. The IC_{50} values of **31** and CDDO are 0.056 and 4.3 nM, respectively. Thus, TCE **31** is about 80 times more potent than CDDO in primary mouse macrophages, whereas **31** is about 20 times more potent than CDDO in RAW cells.

Next, we designed and synthesized acetylenic TCEs **33**−**44** and observed that **31** is still the most potent compound in this series (Table 3). However, TCEs **33**, **35**, and **37** are nearly equivalent to **31** in potency. Moreover, TCEs **34**, **38**, **42**, and **43** are still more potent than or at least as potent as **9**, CDDO, and dexamethasone. TCEs containing a hydrophilic group are much less potent than those with a hydrophobic group (**39** vs **38** and **41** vs **40**). A TCE with a bulky TBS group is much less potent than with a TMS group (**36** vs **32**).

3.3. TCEs Induce NQO1 in Hepa1c1c7 Murine Hepatoma Cells. We evaluated some TCEs for induction of the phase 2 cytoprotective enzyme NQO1 in Hepa1c1c7 murine hepatoma cells. TCEs induce NQO1 at 1−100 nM (Table 3). Notably, (+)- and (±)-**31** double the specific enzyme activity of NQO1 at 0.9 nM. The potency is higher than the potencies of CDDO and CDDO-Im. Remarkably, even in this assay, (+)-enantiomers of **9**, **31**, and **32**, having the same configuration as the CDDO antipode are more potent than (−)-enantiomers of **9**, **31**, and **32**, having the same configuration as CDDO, respectively. We previously demonstrated a linear correlation between NQO1 inducer potency (CD) and inhibitory activity on NO production (IC_{50}) of oleanolic acid derivatives.[29] In this series of TCEs, we also observed a similar correlation (Figure 3), and **31** is the most potent compound in both assays. Most recently, we have demonstrated that incorporation of **31** into the diet of SKH-1 hairless mice dose-dependently induces NQO1 enzyme activity in liver, skin, and stomach.[74]

3.4. TCEs Inhibit the Induction of iNOS in RAW Cells Stimulated with IFN-γ. CDDO blocks de novo synthesis of iNOS protein. We have confirmed that TCEs also inhibit de novo synthesis of iNOS protein (Figure 4). TCEs **31** and **32** significantly inhibit the induction of iNOS at 30 nM. These abilities are

Table 3. Inhibitory Activity of New TCEs 28−44 on NO Production Induced by IFN-γ in RAW Cells and NQO1-Inducing Potency of Some TCEs in Hepa1c1c7 Cells



Compd	R	IC_{50} (nM)[a,b]	CD (nM)[c]	Compd	R	IC_{50} (nM)[a,b]	CD (nM)[c]
(±)-**5**	Table 1	87	160	(±)-**33**	C≡C-Me	5	7.5
(−)-**5**	Table 1		170	(±)-**34**	C≡C-Et	20	
(+)-**5**	Table 1		140	(±)-**35**	C≡C-CN	4	
(±)-**9**	Me	23	18	(±)-**36**	C≡C-TBS	80	150
(−)-**9**	Me		150	(±)-**37**	C≡C-C≡CH	3	
(+)-**9**	Me		19	(±)-**38**	C≡C-CO_2Me	20	85
(±)-**28**	CN	115		(±)-**39**	C≡C-CO_2H	100	150
(±)-**29**	Et	10	18	(±)-**40**		35	
(±)-**30**	$CH=CH_2$	10	30	(±)-**41**		400	
(±)-**31**	C≡CH	1	0.9	(±)-**42**		10	35
(−)-**31**	C≡CH	3	2.7	(±)-**43**		20	
(+)-**31**	C≡CH	1	0.9	(±)-**44**		50	
(±)-**32**	C≡C-TMS	3	3.5	CDDO	Table 1	23	2.3
(−)-**32**	C≡C-TMS	3	3.0	CDDO-Im	Fig 1	1	3.3
(+)-**32**	C≡C-TMS	2	2.3	DXM		20	

[a] RAW 264.7 cells were treated with various concentrations of compounds and IFN-γ (10 ng/mL) for 24 h. Supernatants were analyzed for NO by the Griess reaction.[11] IC_{50} values are an average of two separate experiments. [b] These data have been published and presented in refs 79e−79h. [c] Hepa1c1c7 cells were grown 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 quantity inducer potency.

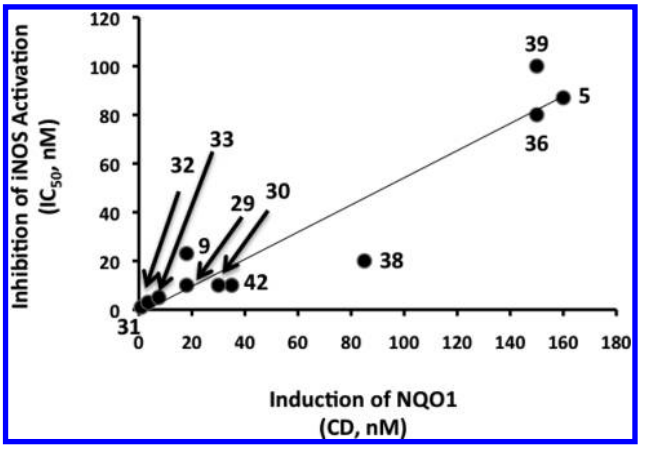


Figure 3. Correlation of potencies of TCEs as inducers of NQO1 in Hepa1c1c7 murine hepatoma cells, expressed as CD values, and for suppression of iNOS induction by IFN-γ in RAW cells, expressed as IC_{50} values. The linear correlation coefficient is $r^2 = 0.91$.

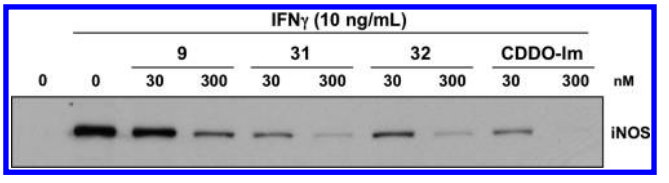


Figure 4. TCEs **9**, **31**, and **34** inhibit the induction of iNOS in RAW cells stimulated with IFN-γ. Cells were incubated with compounds (30−300 nM) and IFN-γ (10 ng/mL) for 24 h. Total cell lysates were analyzed by Western blot for iNOS. These data have been published in ref 79h.

equivalent to that of CDDO-Im. TCE **9** also inhibits the induction of iNOS at 300 nM.

3.5. TCE 31 Directly and Reversibly Reacts with Cysteine Residues of Keap1. The sensor for inducers of NQO1 and of a network of more than 100 other cytoprotective genes is Keap1, a protein endowed with highly reactive cysteine residues and an essential component of the Keap1/Nrf2/ARE signaling pathway.[75]

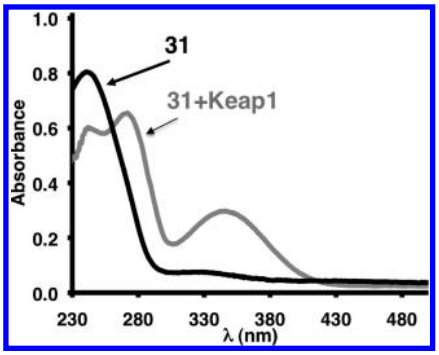


Figure 5. TCE **31** reacts with cysteine residues of Keap1. Absorption spectra of 50 μM **31** (**31**, black line) and the reaction mixture of 50 μM **31** and 10 μM Keap1 (**31** + Keap1, gray line) in 20 mM Tris-HCl/0.005% Tween 20 (pH 8.0) at 25 °C against Keap1 blank.

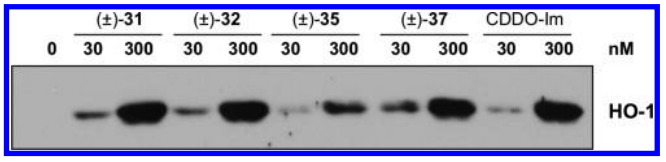


Figure 6. SARs of acetylenic TCEs in the HO-1 assay. Cells were incubated with compounds (30−300 nM) for 6 h. Total cell lysates were analyzed by Western blot for HO-1. These data have been presented and published in refs 79f and 79h.

Recently, we have found that both cyano enones in rings A and C of **31** react with cysteine residues of Keap1 to give Michael adducts.[74] The absorptions at 265 and 345 nm in the UV spectrum of **31** with Keap1 correspond to a Michael adduct of ring A and ring C with Keap1, respectively (Figure 5).[74] Also, we have demonstrated by NMR variable temperature studies[40,76] that the Michael additions of ring A and ring C with DTT are both reversible. The chemical reversibility of these reactions has very significant biological implications: (1) it enhances inducer bioavailability; (2) it allows reversible cysteine modifications of the protein sensor Keap1, which does not need to be permanently inactivated (and possibly subsequently destroyed) but could be easily regenerated without requiring de novo protein synthesis; (3) it leads to a pulse of activation rather than constitutive up-regulation of the pathway; (4) it may explain, at least in part, some of the reasons why TCE **31** is such a potent inducer in vivo, given its ability to react with Keap1 at nanomolar concentrations despite of the presence of millimolar concentrations of glutathione.

3.6. TCEs Induce HO-1 in RAW Cells. We have evaluated tricycles with two cyano enones in rings A and C (structures, see Table 3) for induction of the anti-inflammatory and cytoprotective enzyme, HO-1 in RAW cells. These results are shown in Figures 6 and 7. All compounds induce HO-1 at 30 nM. TCEs **31**, **32**, **37**, **38**, **42**, **43**, and **44** are higher inducers at 30 nM. They are superior to CDDO-Im in potency at 30 nM. Although **38**, **42**, **43**, and **44** are about 10−50 times less potent than **31** in the iNOS assay, they are similar to **31** in potency in this HO-1 assay. TCEs **29**, **30**, **35**, and **40** are moderate inducers, and TCEs **36**, **39**, and **41** are weak inducers. A hydrophilic group and a bulky silyl group decrease the potency. The SARs seen here correlate nicely with the SARs obtained in the iNOS assay.

We have also compared optically active **31** and **32** in this assay (Figure 8), but we do not observe any significant difference between the two enantiomers. Even in this experiment, racemic and optically active **31** are more potent inducers than CDDO-Im at 10 nM.

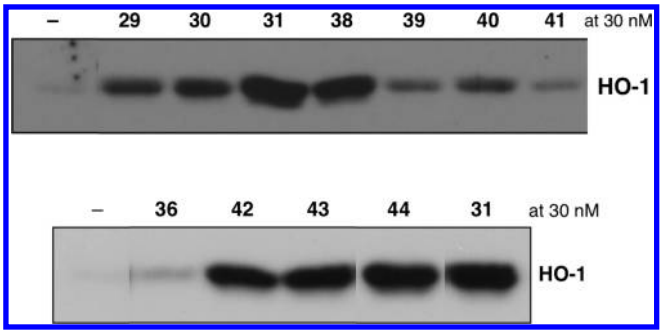


Figure 7. SARs of TCEs **29**, **30**, acetylenic TCEs **36** and **38**−**44** in the HO-1 assay. Cells were incubated with compounds (30 nM) for 6 h. Total cell lysates were analyzed by Western blot for HO-1. These data have been presented in ref 79g.

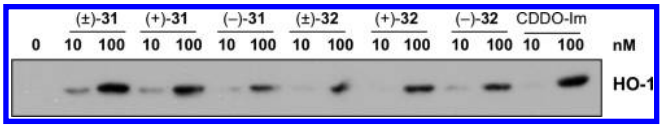


Figure 8. Racemic and optically active **31** and **32** induce HO-1 in RAW cells. Cells were incubated with compounds (10−100 nM) for 6 h. Total cell lysates were analyzed by Western blot for HO-1. These data have been presented in ref 79f.

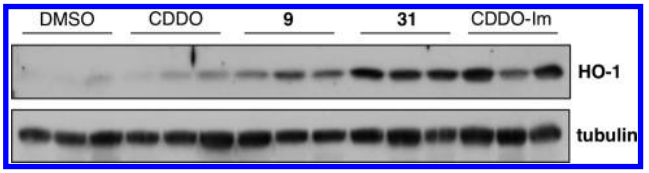


Figure 9. TCE **31** induces HO-1 in liver when given by gavage. Male CD-1 mice (three mice per group) were gavaged with 1 μmol of the following: TCE **9** or **31**, CDDO or CDDO-Im in DMSO. After 6 h, the livers were collected and analyzed by Western blot for HO-1. The tubulin blot is a loading control. These data have been published and presented in refs 79e, 79f, and 79h.

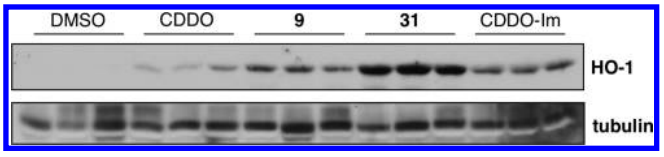


Figure 10. TCE **31** induces HO-1 in stomach when given by gavage. Male CD-1 mice (three mice per group) were gavaged with 1 μmol of the following: TCE **9** or **31**, CDDO or CDDO-Im in DMSO. After 6 h, the stomachs were collected and analyzed by Western blot for HO-1. The tubulin blot is a loading control. These data have been published and presented in refs 79e, 79f, and 79h.

3.7. TCE 31 Induces HO-1 in the Liver and Stomach of CD-1 Mouse When Given by Gavage. Subsequent to the HO-1 assay in vitro, we evaluated the potency of **9** and **31** for induction of HO-1 in the liver and stomach using male CD-1 mice by gavage. As shown in Figures 9 and 10, oral dosing of 1 μmol of **9** and **31** causes significant induction of HO-1 in the liver and stomach while CDDO is markedly less potent at this low dose.

Notably, TCE **31** is as potent as CDDO-Im in the liver but clearly more potent than CDDO-Im in the stomach, which is the most potent semisynthetic triterpenoid we have developed for induction of HO-1.[28] We have also examined optically active **31** in the same in vivo assay, but we did not find a significant difference between both enantiomers in the liver and stomach (see Figures S3 and S4 in the Supporting Information).

3.8. TCE 31 Reduces the Formation of Preneoplastic Foci in the Livers of Rats Challenged with Aflatoxin B_1 (AFB_1). We have evaluated **31** in the short-term model of hepatic tumorigenesis induced with AFB_1 in rats.[37] Prior to this experiment, we confirmed that **31** inhibits the formation of hepatic AFB_1−DNA adducts in a dose-dependent manner.[77] In the model of hepatic tumorigenesis, both **31** and CDDO-Im were well-tolerated, as indicated by the growth rate and final body weight being the same for dosed rats as the controls which were not treated with AFB_1. Both the number ($p = 0.001$) and diameter ($p = 0.024$) of the glutathione *S*-transferase P (GST-P) positive lesions in the liver were significantly decreased with increasing doses of **31**. The focal volume percent of foci (analogous to tumor burden) was also significantly ($p < 0.005$) reduced in a dose-dependent manner. There were no foci detected in the AFB_1-treated rats at the highest doses of **31** (i.e., 30 and 60 μmol/kg). Even the lowest dose of **31** (0.3 μmol/kg) reduced the tumor burden by more than 90% compared to the AFB_1-treated positive control group. TCE **31** and CDDO-Im showed the same potency ($p = 0.6$) at 10 μmol/kg. At these doses the focal burden was reduced by greater than 99% (see Figure S5 and S6 in the Supporting Information).

TCE **31** significantly reduced formation of both AFB_1−DNA adducts and preneoplastic foci in the livers of rats challenged with AFB_1. The potency of **31** is similar to or better than that of CDDO-Im for inhibiting hepatic tumorigenesis induced by AFB_1, and this chemoprevention is dependent on activation of Nrf2.[37] Notably, both CDDO-Im and **31** are 100 times more potent than what has been reported for oltipraz, which inhibits activation of aflatoxin in humans.[78]

In conclusion, although a series of TCEs was designed based on the ring A and C structures of CDDO initially, the ring C structures of analogues of **9** are clearly different from that of CDDO. It is noteworthy that the analogues of **9** have two different kinds of nonenolizable cyano enones in rings A and C, which work as strong Michael acceptors, while CDDO and its analogues have one nonenolizable cyano enone in ring A. Indeed, most of analogues of **9** are more potent than CDDO in the iNOS assay. Conversely, the triterpenoid oleanolic acid, lacking any cyano enone functional groups, is inactive. These facts strongly suggest that the essential factor for potency is not the triterpenoid or the tricycle skeleton but the functional monocyclic cyano enones that are positioned at a specific orientation relative to each other. TCE **31**, an analogue of **9** having an acetylene group at C10a, is the most bioactive compound in both in vitro and in vivo bioassays in our pool of drug candidates, including semisynthetic triterpenoids and synthetic tricycles. Tricyclic compounds, diterpenoids, and triterpenoids with an acetylene group at C10a (C8 in terpenoid nomenclature) have not been reported prior to our synthesis of **31**. Therefore, **31** may represent a new class of potential drug candidates having an entirely new structure for prevention and/or treatment of inflammatory diseases and cancers.

Further preclinical studies and detailed mechanism studies including identification of the protein targets of **31** are in progress.

■ ASSOCIATED CONTENT

Supporting Information. Synthetic procedures and characterization data for new compounds **1**−**103**, experimental procedures for biological evaluation, and Figures S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CDDO, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid; CDDO-Me, methyl 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate; CDDO-Im, 1-(2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl)-imidazole; DAST, *N*,*N*-diethylaminosulfur trifluoride; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DTT, 1,4-dithiothreitol; DXM, dexamethasone; EDIA, ethyldiisopropylamine; HMPA, hexamethylphosphoric triamide; IKKβ, inhibitor of nuclear factor κB kinase β; JAK1, Janus kinase 1; Keap1, kelchlike ECH-associated protein 1; MTPA, α-methoxy-α-(trifluoromethyl)phenylacetyl; Nf-κB, nuclear factor κB; Nrf2, nuclear factor (erythroid-derived 2) related factor 2; STAT3, signal transducer and activator of transcription 3

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