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Electrophilic Triterpenoid Enones: A Comparative Thiol-Trapping and Bioactivity Study

Danilo Del Prete,[†] Orazio Taglialatela-Scafati,[‡] Alberto Minassi,[†] Carmina Sirignano,[‡] Cristina Cruz,[§] Maria L. Bellido,[§] Eduardo Muñoz,*[,#] and Giovanni Appendino*[,†]

[†]Dipartimento di Scienze del Farmaco, Università del Piemonte Orientale, Largo Donegani 2, 28100 Novara, Italy

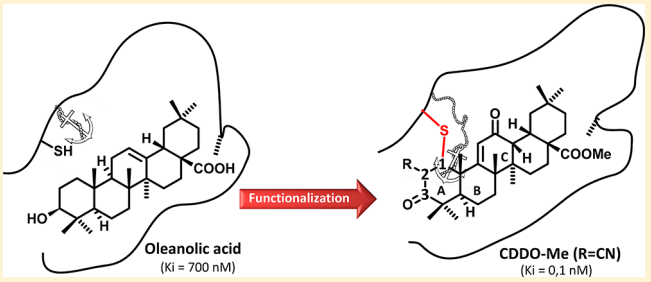
[‡]Dipartimento di Farmacia, Università di Napoli Federico II, Via Montesano 49, 80131 Napoli, Italy

[§]VivaCell Biotechnology España, Parque Científico Tecnológico de Córdoba, 14014 Córdoba, Spain

[#]Maimonides Biomedical Research Institute of Córdoba, Reina Sofía University Hospital, Department of Cell Biology, Physiology and Immunology, University of Córdoba, Avenida Menéndez Pidal s/n, 14004 Córdoba, Spain

Supporting Information

ABSTRACT: Bardoxolone methyl (**1**) is the quintessential member of triterpenoid cyanoacrylates, an emerging class of bioactive compounds capable of transient covalent binding to thiols. The mechanistic basis for this unusual "pulsed reactivity" profile and the mode of its biological translation are unknown. To provide clues on these issues, a series of Δ^1-dehydrooleanolates bearing an electron-withdrawing group at C-2 (**7a**–**m**) were prepared from oleanolic acid (**3a**) and comparatively investigated in terms of reactivity with thiols and bioactivity against a series of electrophile-sensitive transcription factors (Nrf2, NF-κB, STAT3). The emerging picture suggests that the triterpenoid scaffold sharply decreases the reactivity of the enone system by steric encumbrance and that only strongly electrophilic and sterically undemanding substituents such as a cyanide or a carboxylate group can re-establish Michael reactivity, albeit in a transient way for the cyanide group. In general, a substantial dissection between the thiol-trapping ability and the modulation of biological end-points sensitive to thiol alkylation was observed, highlighting the role of shape complementarity for the activity of triterpenoid thia-Michael acceptors.



Bardoxolone methyl (CDDO-Me, RTA402, **1**) is the quintessential member of triterpenoid cyanoenones,[1,2] an interesting class of bioactive compounds that has revitalized interest in pentacyclic triterpenoids, a major group of plant phytochemicals.[3] After failing a phase-3 study for chronic kidney disease because of cardiovascular side-effects,[4] bardoxolone methyl is currently undergoing phase-2 clinical studies for the management of pulmonary arterial hypertension, a severe condition difficult to manage with existing drugs.[4] The mechanism of action of triterpenoid cyanoenones is seemingly related to their capacity to transiently trap reactive cysteine residues,[2] with the triterpenoid scaffold providing shape complementarity and the cyanoenone moiety covalently reinforcing this interaction.[1,2] This dock-and-lock mechanism is well precedented within bioactive compounds, underlying, for instance, the mechanism of action of penicillin and aspirin.[5,6] On the other hand, although all thia-Michael reactions are in principle reversible, the interaction of triterpenoid cyanoenones with thiols is transient and expected to translate into a pulsed, rather than permanent target modulation, not unlike the one associated with noncovalent ligands.[2,6] Thus, triterpenoid cyanoenones do not form stable and isolable adducts with thiols, and their reaction with nucleophilic species is "virtual", only detectable by spectroscopic measurements (NMR, UV) and not backed up by actual isolation of the adducts.[2] Since cyanoenones are strongly electrophilic agents and the parent system (2-cyano-2-cyclohexenone, **2**) forms stable adducts with thiols (vide infra), the transient nature of the reaction when the electrophilic system is embedded into a triterpenoid scaffold is seemingly related to a *Gestalt* (shape) effect, as suggested by the presence of overall five substituents on the three tetrahedral carbons of the cyclohexenone A-ring and the steric size[7] of the C-1 sulfur substituent in the adduct. To shed light on this issue and identify alternative groups capable of inducing Michael reactivity in ring A triterpenoid enones, we have investigated the reaction of thiols with a series of 2-substituted Δ^1-oleanane triterpenoids related to bardoxolone methyl using the cysteamine assay, an NMR method to study thia-Michael reactivity based on the reversal of transient addition upon a solvent switch between DMSO and $CHCl_3$.[8] General acid–base catalysis from the adjacent amino group renders the thiol group of cysteamine a good mimic of a cysteine residue in a

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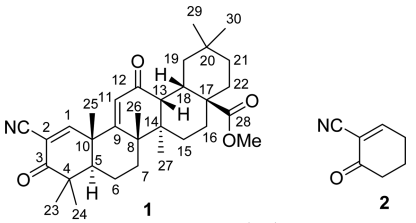
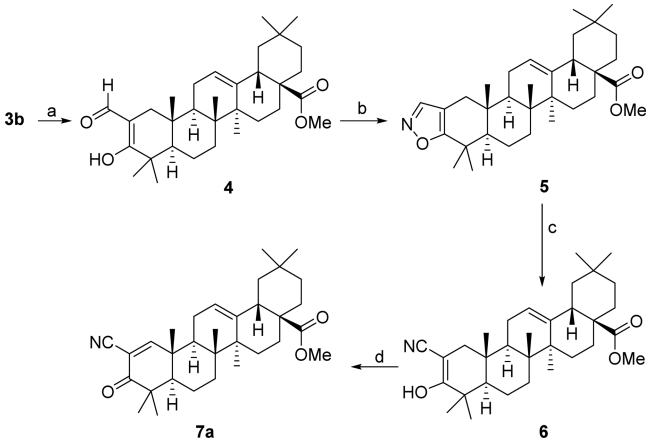
protein functional site,[9] resulting in a rapid addition in dipolar solvents such as DMSO. On the other hand, virtually no reaction occurs in apolar solvents such as $CHCl_3$, where the zwitterionic form of cysteamine and the delicate network of hydrogen bonding required by the organo-catalytic mechanism do not benefit from solvent stabilization.[8] To evaluate if the reactivity data from the cysteamine assay could be translated in terms of bioactivity modulation, we have complemented this chemical end-point with biological assays on thiol-sensitive important medicinal chemistry targets such as the transcription factors Nrf2, NF-κB, and STAT3.[1]



RESULTS AND DISCUSSION

Oleanolic acid (**3a**) was used as a triterpenoid scaffold for this study. After conversion to its more soluble methyl ester and oxidation to **3b**, it was subjected to the classic four-step sequence for the synthesis of triterpenoid cyanoenones (formylation, reaction with hydroxylamine, isoxazole fragmentation, and dehydrogenation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, DDQ),[10] eventually affording **7a** (Scheme 1). In terms of cyanoacrylate-associated bioactivity, the ring C functionalization of oleanolic acid (**3a**) leads to biologically

Scheme 1. Synthesis of the Oleanolylcyanoenone 7a[a]

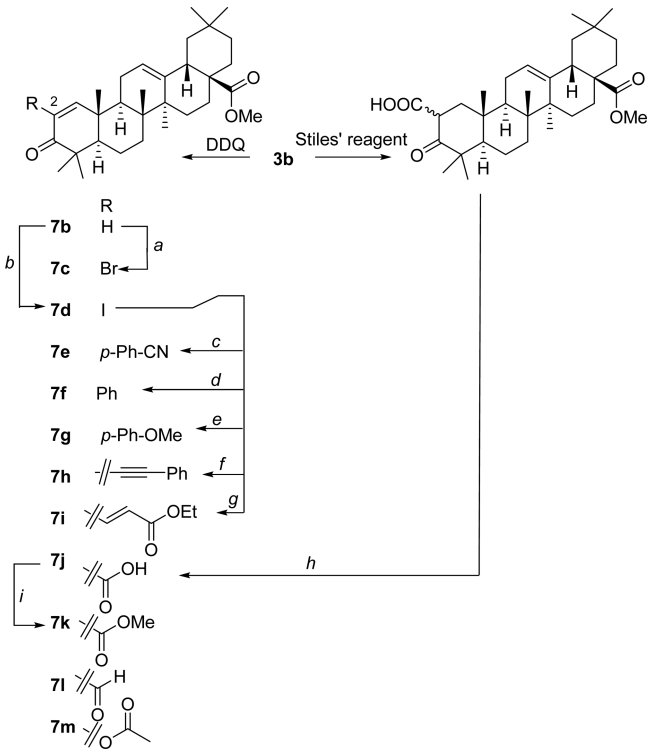


[a]Conditions: (*a*) HCOOMe, NaOMe, benzene, 95%; (*b*) NH_2OH·HCl, aq EtOH; (*c*) NaOMe, Et_2O, MeOH (85% from **5**); (*d*) DDQ, benzene, 60%.

lower potency than the triterpenoid scaffold of bardoxolone methyl (**1**), which carries a C-12 carbonyl and $\Delta^{9(11)}$-unsaturation.[2] Nevertheless, the cyanoacrylates from the two series (**1** and **7a**) show the same profile of thia-Michael reaction,[2] and comparative, rather than absolute, bioactivity data were relevant for this study.

Starting from methyl dehydrooleanolate (**3b**),[10] the Δ^1-dehydro derivative **7b** was obtained by dehydrogenation with DDQ (Scheme 2). This enone was separately reacted with

Scheme 2. Synthesis of the 2-Substituted Oleanolylenones 7b–k from 3b[a]



[a]Conditions: (*a*) Br_2, HBr, HOAc, 35°C, 68%; (*b*) I_2, DMAP, Pyr, $CHCl_3$, 90 °C, 79%; (*c*) $Pd(PPh_3)_4$, NaOMe, *p*-cyanophenylboronic acid, toluene, 80 °C, 10%; (*d*) $Pd(PPh_3)_4$, NaOMe, phenylboronic acid, toluene, 80 °C, quant.; (*e*) $Pd(PPh_3)_4$, NaOMe, *p*-methoxyphenylboronic acid, toluene, 80 °C, quant.; (*f*) $Pd(PPh_3)_4$, TEA, CuI, phenylacetylene, EtOAc, 60 °C, 73%; (*g*) ethyl acrylate, $Pd(OAc)_2$, TPP, TEA,CH_3CN, 80 °C, quant.; (*h*) DDQ, toluene, quant.; (*i*) EDC, DMAP, MeOH, 98%.

bromine and iodine to afford, by an addition–elimination mechanism, the corresponding 2-halo derivatives **7c** and **7d**, respectively. The 2-iodoenone (**7d**) served as a good substrate for palladium-mediated coupling. Suzuki reaction with *p*-cyanophenylboronic acid afforded the phenylogous cyanoenone **7e**, and the 2-phenyl- and 2-(*p*-methoxyphenyl)enones **7f** and **7g** were similarly prepared from the corresponding boronic acids. Sonogashira coupling with 2-phenylacetylene yielded the acetylenic enone **7h**, while Heck coupling with ethyl acrylate afforded the cross-conjugated keto-ester **7i**.

Alternatively, methyl dehydrooleanolate (**3b**) was carboxylated with the Stiles reagent (methyl magnesium carbonate)[11] to afford an unstable β-ketoacid, which was immediately dehydrogenated with DDQ[12] to afford the stable enone **7j** and subsequently esterified to the methyl ester **7k**. The 2-formyl derivative **7l** was prepared via **4** by DDQ-mediated

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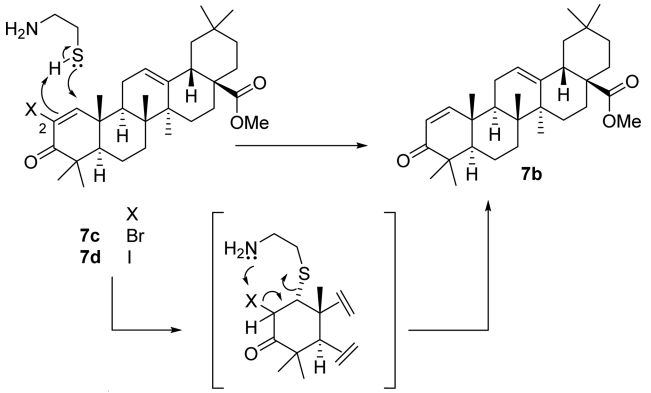
dehydrogenation, while the enol ester **7m** was prepared from chromic oxidation of maslinic acid (**3c**) followed by acetylation.

To investigate their reactivity with thiols, the 2-substituted triterpenoid enones were then subjected to the cysteamine assay.[8] This NMR-based assay capitalizes on solvent-related differences in the reactivity of cysteamine with electron-poor double bonds to identify transient acceptors. Since a rapid reaction takes place in DMSO-d_6 but no reaction occurs in $CDCl_3$, dilution of the DMSO-d_6 reaction mixture with $CDCl_3$ will regenerate the starting olefin in the case of a reversible addition, but will leave the adduct unscathed with an irreversible addition.[8] 2-Cyclohexenone gave a nontransient addition with cysteamine, but no reaction occurred in **7b**, where this structural element is part of the triterpenoid framework. Also 2-cyano-2-cyclohexenone (**2**) gave a nontransient addition with cysteamine, but the corresponding triterpenoid analogue **7a** reacted only transiently. These observations suggest that the bulky triterpenoid scaffold interferes with the thia-Michael addition, completely deactivating the parent enone **7b** and downgrading the reactivity of the cyanoenone **7a** to the realm of transiency. No reactivity was also observed in the phenylogous cyanoenone **7e**, in the two other 2-phenylsubstituted enones **7f** and **7g**, and in the 2-phenylethynyl derivative **7h**. The 2-acryloyl derivative **7i** reacted with cysteamine in a nontransient way, but at the acryloyl double bond and not at the Δ^1-double bond of the triterpenoid scaffold. An interesting behavior was observed with the 2-halosubstituted enones, since their thia-Michael adducts underwent reductive dehalogenation to the Michael-unreactive unsubstituted Δ^1-enone **7b**. The 2-halo derivatives did not react with the simple odorless thiol 1-dodecanethiol, suggesting the involvement of the amino group of cysteamine, possibly via the mechanism outlined in Scheme 3. Finally, the 2-formyl enone

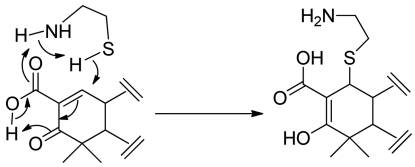
Scheme 3. Possible Mechanism for the Cysteamine-Induced Dehalogenation of 7c (X = Br) and 7d (X = I)



7l reacted with cysteamine to give, in a nontransient way, a mixture of mono- and bis-thiol adducts, in accordance with previous observations of α,β-unsaturated aldehydes,[8] while the enol acetate **7m** behaved as an acylating reagent for the amino group of cysteamine, affording the starting enolyzed α-dicarbonyl (diosphenol).

Competition experiments where equimolar amounts of two substrates were reacted with a substoichiometric amount of cysteamine were carried out to establish a reactivity scale between the compounds positive in the cysteamine assay. Since all reactions were complete within the time frame of the assay (ca. 5 min), a leveling effect was observed. On the other hand, by running competition experiments, we could establish that the 2-carboxylate **7j** was more reactive than the cyanoenone **7a**, which showed similar reactivity in the assay to the 2-haloenones **7b** and **7c**, while the acrylate **7i** was significantly less reactive. The high reactivity of the 2-carboxylate **7j** might be related to a facilitation of the addition by a network of hydrogen bondings, as depicted in Scheme 4. The role of general acid catalysis in the reaction is consistent with the observation that methylation of the carboxylate quenched the Michael reactivity, as shown by **7k**.

Scheme 4. General Acid Catalysis in the Addition of Cysteamine to the 2-Carboxyenone Moiety of 7j



The Δ^1-enones **7a**–**l** were comparatively evaluated with bardoxolone methyl (**1**) for their capacity to activate the transcription factor Nrf2, a major target of this compound,[1,2] and for the inhibition of two further transcription factors sensitive to thiol trapping (NF-κB and STAT3) (Table 1). The

Table 1. Biological Activities of Oleanolic Acid Derivatives on Specific Targets

compound	efficacy NRF2 (IRA coefficient)[a]	potency EC_{50} Nrf2 (μM)	IC_{50} STAT3 (μM)	IC_{50} NF-κB (μM)
1	40.94	0.06	2.38	1.20
3a		−(>50)	−(>50)	−(>50)
3b		−(>25)	−(>25)	−(>25)
4		−(>25)	−(>25)	−(>25)
5		−(>25)	−(>25)	−(>25)
6		−(>25)	−(>25)	−(>25)
7a	1.69	1.097	−(>25)	15.27
7b		−(>25)	−(>25)	−(>25)
7c		11.48	−(>25)	−(>25)
7d		−(>25)	−(>25)	−(>25)
7e		−(>25)	−(>25)	−(>25)
7f		−(>25)	−(>25)	−(>25)
7g		−(>25)	−(>25)	−(>25)
7h		−(>25)	−(>25)	−(>25)
7i		−(>25)	−(>25)	−(>25)
7l		ND[b]	ND[b]	7.69
7m		−(>25)	−(>25)	−(>25)
7j	0.41	2.45	25.74	−(>25)
7k		−(>25)	−(>25)	−(>25)

[a]The IRA (intrinsic relative activity) was calculated relative to *tert*-butylhydroquinone (TBHQ) (see Experimental Section). [b]Toxic at concentrations above 5 and 10 μM in the cell lines used in the assay.

function of these regulatory proteins is critically dependent on the presence of a free cysteine that works as a veritable on/off switch. For instance, inactive Nrf2 is retained in the cytoplasm by Keap-1, which contains two critical cysteine moieties. Upon interaction with reactive molecules, Keap-1 is degraded and Nrf2 is translocated to the nucleus, where it activates Nrf2-dependent genes.[13] The NF-κB pathway is critically dependent on the upstream kinase IKKβ, which hosts a loop-exposed

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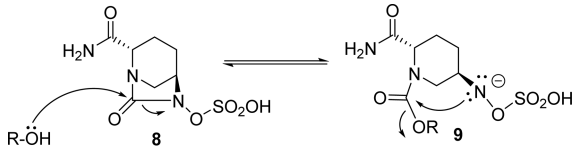
cytsteine (Cys-179) sensitive to electrophilic compounds that inhibit its kinase activity and the activation of the NF-κB pathway.[14] Finally, STAT3 also contains critical cysteine units, whose alkylation prevents binding to DNA.[15] The assays were done on cultured cells transfected with the appropriate protein gene [human keratynocytes (HaCaT-ARE-Luc) for Nrf2, mouse fibroblasts (NIH-3T3-KBF-Luc) for NF-κB, and human cervix carcinoma (HeLa-STGAT-3-Luc) for STAT3] using luciferase-based protocols (see Experimental Section for further details). The cyanoenone **7a** showed potency at low micromolar concentration in the activation of Nrf2 (EC_{50} = 1.10 μM), more than 1 order of magnitude lower, however, than bardoxolone methyl, an ultrapotent activator (EC_{50} = 0.060 μM).[2] Within the compounds investigated, only the 2-bromoenone **7c** and the 2-carboxyenone **7j** were active in assays of Nrf2 activation. Overall, a substantial dissection between thiol trapping and activation of Nrf2 was observed in terms of thiophilicy and mode of addition of the substrates. Thus, both the 2-bromoenone **7c** and the 2-iodoenone **7d** could covalently modify thiol groups, but only the former was active, while the 2-carboxyenone **7j** and its vinylogous ester **7i** were both nontransient Michael acceptors, but only **7j** could activate Nrf2. All other substrates were unable to react in vitro with thiol groups and to modulate Nrf2 activity. Regarding NF-κB inhibition, only bardoxolone methyl (**1**) was significantly active (IC_{50} = 2.38 μM), and this compound was also the most potent inhibitor of γ-IFN-induced STAT3 activation (IC_{50} = 1.20 μM), although also the cyanoenone **7a** and the 2-formylenone **7l** showed activity in this assay (IC_{50} = 15.27 and 7.69 μM, respectively). Collectively, the bioactivity data support the view that, within electrophilic triterpenoids, shape complementarity has a critical role for the biological translation of covalent reactivity, with thiol-trapping capacity being necessary but not sufficient for bioactivity against a specific target. This is in accordance with the observation that bardoxolone methyl (**1**) is at least one order of magnitude more potent than its ring A analogue **7a** for the modulation of the three thiol-trapping-sensitive end-points investigated. Oleanolic acid (**3a**) is the archetypal TGR5 dietary agonist,[16] and the activity of compounds **7a**−**m** and the intermediates for their synthesis (**3b**,**c**−**6**) was also investigated for the activation of this end-point. While confirming the activity of oleanolic acid in the assay (EC_{50} = 18.90 μM), our data also evidenced the detrimental effect of changes on ring A and the C-28 carboxylate, since all compounds, including maslinic acid (**3b**), were inactive in the assay. Similar results have been reported in the betulinic acid series.[17]

Cysteine is one of the least abundant protein residues, and, due to a unique combination of metal affinity, redox properties, and reactivity with electrophiles, it is often located at functional sites, acting as a veritable sensor of the local environment.[9] The selective manipulation of these "active" cysteine residues has therefore a profound effect on the homeostatic response, although its phenotypical translation is difficult to predict, as is the distinction between its pulsed rather than continuous modulation. Reversible covalent modification is not limited to thia-Michael acceptors, but has been reported also for the β-lactamases inhibitor avibactam (**8**), a hydroxy-trapping agent.[18] Unlike the adducts from irreversible inhibitors of these serine proteases (clavulanic acid, tazobactam, sulbactam), which are cleaved by hydrolytic turnover and deactivated, the β-lactamase-avibactam adduct (**9**) undergoes spontaneous lactamization, with regeneration of the inhibitor after an estimated "residence time" of ca. 14 min (Scheme 5).[18] This iterative and pulsed cycle of activation and inhibition is

Scheme 5. Transient Covalent Trapping of the Serine Residue of Lactamase (ROH) by Avibactam



fundamentally distinct from the kinetically irreversible profile of the other inhibitors[9] and might be involved in the superior clinical activity of avibactam compared to the other lactamase inhibitors.[19]

The reversibility of action of avibactam has been related to the lower strain, and therefore easier regeneration, of its imidazolinone-active moiety compared to the β-lactam moiety of the other inhibitors (Scheme 5).[18] The data on triterpenoid cyanoenones suggest that the reversibility of their thia-Michael addition is mainly due to an increased steric congestion on the triterpenoid A-ring. Interestingly, replacement of the 2-cyano group with a 2-halo group changes the reactivity mode, overall resulting in the reductive dehalogenation of the triterpenoid probe and the irreversible covalent modification of the nucleophilic partner. This peculiar reactivity profile is worth investigating also in a different molecular framework aimed at the modulation of macromolecules according to a "lock and dock" strategy.[20]

Finally, by highlighting the critical relevance of shape complementarity and pulse reactivity for bioactivity, our observations on triterpenoid cyanoacrylates emphasize the difficulty of sorting out compounds into good leads and PAINS (pan-assay interference compounds) based solely on test tube data on reactivity and tendency to aggregate,[21] which can both be critical for binding to biological macromolecules and elicit activity of clinical relevance.[20,21]

EXPERIMENTAL SECTION

General Experimental Procedures. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on a Varian INOVA spectrometer. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H = 7.26, δ_C = 77.0; DMSO-d_6: δ_H = 2.50). Homonuclear ^1H connectivities were determined by the COSY experiment. One-bond heteronuclear $^1H-^{13}C$ connectivities were determined with the HSQC experiment. Two- and three-bond $^1H-^{13}C$ connectivities were determined by 2D HMBC experiments optimized for $^{2,3}J$ = 9 Hz. Spectra were obtained on an Avatar 370 FT-IR Thermo Nicolet. Low- and high-resolution ESIMS spectra were obtained on an LTQ OrbitrapXL (Thermo Scientific) mass spectrometer. Silica gel 60 (63−200 mesh) used for gravity column chromatography (GCC) was purchased from Merck. Reactions were monitored by TLC on Merck 60 F254 silica gel (0.25 mm) plates and Macherey-Nagel ALUGRAM neutral alumina (0.20 mm) plates that were visualized by UV inspection (254 and 365 nm) and/or staining with 5% H_2SO_4 in EtOH and heating. Organic phases were dried with Na_2SO_4 before evaporation. Chemical reagents and solvents were from Aldrich. Oleanolic acid and maslinic acid were provided by Vivacell Spain. Petroleum ether with a boiling point of 40−60 °C was used.

Cysteamine Assay. In an NMR tube, an exact amount of substrate (ca. 5 mg) was dissolved in 500 μL of dry DMSO-d_6, and the ^1H NMR spectrum was recorded. Two equivalents of cysteamine was then added, and the spectrum was immediately recorded, with acquisition

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finishing within 5 min from the addition. The reaction was typically monitored by observing the disappearance of the deshielded (>7 ppm) signal of H-1. To test the reversibility of the addition, the sample was diluted 1:10 with $CDCl_3$, and the spectrum was recorded again. Reversion was evaluated by comparing this spectrum with an original spectrum of the product under investigation in $CDCl_3$. All competition assays were carried out by reacting a ca. equimolar solution of a pair of Michael acceptors with a substoichiometric amount (60−80% of the theoretical amount) of cysteamine and registering the spectrum within 5 min from the addition.

*Methyl 3-Dehydrooleanoate (**3b**).* To a solution of oleanolic acid (**3a**, 6.00 g, 13.13 mmol) in dimethylformamide (100 mL) were slowly added K_2CO_3 (3.61 g, 26.27 mmol; 2 molar equiv) and iodomethane (817 μL, 13.13 mmol; 1 molar equiv) under stirring at 0 °C (ice bath). The reaction mixture was stirred at room temperature for 24 h and then worked up by dilution with brine and extraction with EtOAc. The organic phase was dried, filtered, and evaporated, affording a white powder (6.00 g, 97%) of methyl oleanolate. The latter (6.00 g, 12.75 mmol) was dissolved in acetone (100 mL), and Jones reagent was added dropwise at 0 °C. The reaction mixture was stirred for 1 h, until its color had turned from orange to persistent green, and was then worked up by dilution with brine, addition of a few drops of EtOH, and extraction with EtOAc. The organic phase was dried, filtered, and evaporated, affording a semicrystalline residue that was purified by GCC on silica gel (petroleum ether/EtOAc, 9:1, → petroleum ether/EtOAc, 8:2, as eluent) to yield **3b** (6.0 g) as a white powder.[10]

*Methyl 2-Formyl-3-dehydrooleanoate (**4**).* To a stirred solution of **3b** (250 mg, 0.426 mmol) in benzene (6 mL) were sequentially added NaOMe (138 mg, 2.5 mmol; 6 molar equiv) and ethyl formate (796 μL, 3.41 mmol; 8 molar equiv) at room temperature. After 1 h, the reaction was worked up by dilution with 5% HCl and extraction with petroleum ether/Et_2O, 2:1. The organic phase was dried, filtered, and evaporated to give a white crystalline product (250 mg, quantitative yield).[22]

*Isoxazole **5**.* To a solution of **4** (250 mg, 0.50 mmol) in H_2O/EtOH (10:1, 15 mL) was added hydroxylamine hydrocloride (313 mg, 4.512 mmol, 9 molar equiv) at room temperature and under continuous stirring. The mixture was stirred at room temperature for 1 h and then worked up by dilution with brine and extraction with EtOAc. The organic phase was dried with Na_2SO_4 and filtered, and the solvent evaporated to give **5** as a white powder (250 mg, ca. quantitative).[23]

Base-Induced Fragmentation of 5. To a cooled (ice bath) and stirred 5% solution of **5** (300 mg, 0.61 mmol) in Et_2O/MeOH (2:1, 30 mL) was added NaOMe (328 mg, 6.08 mmol; 10 molar equiv). After stirring at room temperature for 1 h, the reaction was worked up by dilution with HCl 5% and extraction with Et_2O/CH_2Cl_2 (2:1). The organic phase was dried, filtered, and evaporated to afford **6** as a white powder (260 mg, 87%).[24]

*Methyl 2-Cyano-1,3-bisdehydrooleanoate (**7a**).* To a solution of **6** (100 mg, 0.20 mmol) in dry benzene (4 mL) was added DDQ (92 mg, 0.40 mmol, 2 molar equiv). After stirring at room temperature for 30 min, the reaction was worked up by dilution with brine, extracted with EtOAc, dried, and evaporated. The residue was purified by GCC on silica gel (petroleum ether/EtOAc, 9:1, → petroleum ether/EtOAc, 7:3, as eluent) to give 70 mg of **7a** (70%) as a white powder: IR ν_{max} (KBr) 3528, 3281, 1723, 1684, 1455, 1386, 1362, 1262, 1191 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 7.74 (1H, s, H-1), 5.36 (1H, t, J = 3.5 Hz, H-12), 3.64 (3H, s, 28-OMe), 2.91 (1H, dd, J = 14.0, 4.0 Hz, H-18), 2.11 (1H, dd, J = 8.9, 3.5 Hz, H-11), 2.01−1.09 (series of multiplets), 1.22 (3H, s, H-25), 1.21 (3H, s, H-24), 1.15 (3H, s, H-27), 1.14 (3H, s, H-23), 0.94 (3H, s, H-30), 0.92 (3H, s, H-29), 0.83 (3H, s, H-26); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 198.3 (C-3), 178.3 (C-28), 170.2 (C-1), 144.8 (C-13), 121.1 (C-12), 115.2 (CN-2), 114.0 (C-2), 52.8 (C-5), 51.8 (OCH_3-28), 46.9 (C-17), 45.8 (C-19), 45.1 (C-4), 42.3 (C-14), 41.7 (C-18), 41.3 (C-9), 40.8 (C-10), 40.5 (C-8), 34.0 (C-21), 32.4 (C-7), 32.3 (C-22), 30.9 (C-20), 27.9 (C-24), 27.8 (C-15), 27.0 (C-29), 26.0 (C-27), 23.8 (C-30), 23.4 (C-11), 23.1 (C-16), 21.8 (C-23), 18.9 (C-6), 18.1 (C-26), 17.6 (C-25); ESIMS (positive ions): m/z 514 $[M + Na]^+$, 1005 $[2\ M + Na]^+$; HR-ESIMS m/z 514.3299, calcd for $C_{32}H_{45}NNaO_3$ 514.3297.

*Methyl 1,3-Bisdehydrooleanolate (**7b**).* To a solution of **3b** (2.0 g, 4.27 mmol) in THF (100 mL) was added DDQ (970 mg, 4.27 mmol; 1 molar equiv) at room temperature After stirring at 60 °C for 24 h, the reaction was worked up by dilution with brine and extraction with EtOAc. After drying and evaporation, the residue was purified by GCC on silica gel (petroleum ether/EtOAc, 9:1, as eluent) to give **7b** (white solid, 1.79 g, 90%): IR ν_{max} (KBr) 1726, 1672, 1463, 1379, 1262, 1199, 1125 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 7.03 (1H, d, J = 10.1 Hz, H-1), 5.80 (1H, d, J = 10.1 Hz, H-2), 5.36 (1H, t, J = 3.5 Hz, H-12), 3.64 (3H, s, 28-OMe), 2.90 (1H, dd, J = 14.0, 4.0 Hz, H-18), 2.10−1.10 (series of multiplets), 1.16 (3H, s, H-25), 1.15 (3H, s, H-24), 1.15 (3H, s, H-27), 1.09 (3H, s, H-23), 0.94 (3H, s, H-30), 0.91 (3H, s, H-29), 0.82 (3H, s, H-26); ^{13}C NMR ($CDCl_3$) δ_C 205.5 (C-3), 178.4 (C-28), 159.3 (C-1), 144.5 (C-13), 125.2 (C-2), 121.9 (C-12), 53.6 (C-5), 51.8 (OCH_3-28), 47.0 (C-17), 45.9 (C-19), 44.7 (C-4), 42.2 (C-14), 42.0 (C-18), 41.7 (C-9), 40.3 (C-10), 39.7 (C-8), 34.1 (C-21), 33.3 (C-7), 32.7 (C-22), 32.5 (C-20), 30.9 (C-24), 28.0 (C-15), 27.9 (C-29), 26.0 (C-27), 23.8 (C-30), 23.5 (C-11), 23.2 (C-16), 21.8 (C-23), 19.1, (C-6) 18.8 (C-26), 17.5 (C-25); ESIMS (positive ions) m/z 489 $[M + Na]^+$, 955 $[2\ M + Na]^+$; HR-ESIMS m/z 514.3341, calcd for $C_{31}H_{46}NaO_3$ 489.3345.

*Methyl 2-Bromo-1,3-bisdehydrooleanolate (**7c**).* To a solution of **7b** (50 mg, 0.107 mmol) in HOAc (2 mL) were added dropwise HBr (33%, 9 μL, 0.047 mmol, 0.44 equiv) and bromine (14 μL, 0.257 mmol, 2.4 equiv). After stirring at 35 °C for 3 h, the reaction was worked up by dilution with 20% aqueous sodium thiosulfate (20 mL) and extracted with EtOAc. The combined organic phases were washed with saturated $NaHCO_3$ (2 × 10 mL) and brine (1 × 10 mL) and dried. After evaporation, the residue was purified by GCC (petroleum ether/EtOAc, 99:1, as eluant) to give **7c** (40 mg, 68%) as a yellowish powder: IR ν_{max} (KBr) 1783, 1724, 1685, 1460, 1432, 1386, 1312, 1259 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 7.41 (1H, s, H-1), 5.29 (1H, t, J = 3.5 Hz, H-12), 3.68 (3H, s, 28-OMe), 2.66−1.17 (series of multiplets), 1.38 (3H, s, H-27), 1.22 (3H, s, H-24), 1.21 (3H, s, H-25), 1.15 (3H, s, H-23), 1.01 (3H, s, H-30), 0.96 (3H, s, H-29), 0.86 (3H, s, H-26); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 196.6 (C-3), 176.8 (C-28), 157.5 (C-1), 122.3 (C-2), 55.0, 52.4, 52.1, 49.5, 47.4, 46.5, 46.1 (C-19), 43.0, 42.9, 42.8, 41.0, 40.5 (C-8), 40.2, 37.2, 35.9, 35.2, 33.5, 32.8, 31.6, 30.9 (C-20), 28.4 (C-24), 25.7, 23.1 (C-16), 22.0 (C-23), 19.0 (C-6), 18.4 (C-26), 17.7 (C-25); ESIMS (positive ions) m/z 567 and 569 $[M + Na]^+$ in 1:1 ratio; HR-ESIMS m/z 567.2443, calcd for $C_{31}H_{45}{}^{79}BrNaO_3$ 567.2450.

*Methyl 2-Iodo-1,3-bisdehydrooleanolate (**7d**).* To a solution of **7b** (100 mg, 0.21 mmol) were added pyridine/$CHCl_3$ (1:1 v/v, 2 mL), 4-dimethylaminopyridine (DMAP, 6 mg, 0.043 mmol, 0.20 molar equiv), and I_2 (163 mg, 0.64 mmol, 3.0 molar equiv). After heating to 90 °C under N_2 and in the dark for 24 h, the reaction was worked up by dilution with EtOAc (50 mL). and sequential washing with 20% sodium thiosulfate (10 mL), 1 N HCl (3 × 10 mL), saturated $NaHCO_3$ (10 mL), and brine (10 mL). After drying and evaporation, **7d** was obtained as a pale yellow powder (100 mg, 79%): IR ν_{max} (KBr) 1727, 1680, 1462, 1384, 1321, 1261, 1195, 1164 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 7.81 (1H, s, H-1), 5.36 (1H, t, J = 3.5 Hz, H-12), 3.64 (3H, s, 28-OMe), 2.91 (1H, dd, J = 14.0, 4.0 Hz, H-18), 2.20−1.09 (series of multiplets), 1.19 (3H, s, H-25), 1.18 (3H, s, H-24), 1.16 (3H, s, H-27), 1.15 (3H, s, H-23), 0.94 (3H, s, H-30), 0.91 (3H, s, H-29), 0.81 (3H, s, H-26); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 198.1 (C-3), 178.2 (C-28), 167.6 (C-1), 144.4 (C-13), 121.4 (C-12), 101.5 (C-2), 53.2 (C-5), 51.6 (OCH_3-28), 46.9 (C-17), 45.8 (C-19), 45.5 (C-4), 42.1 (C-14), 41.7 (C-18), 41.5 (C-9), 41.3 (C-10), 40.1 (C-8), 34.2 (C-21), 32.7 (C-7), 32.2 (C-22), 30.7 (C-20), 28.9 (C-24), 27.6 (C-15), 27.0 (C-29), 25.8 (C-27), 23.6 (C-30), 23.4 (C-11), 23.0 (C-16), 22.1 (C-23), 18.5 (C-6), 18.1 (C-26), 17.3 (C-25); ESIMS (positive ions) m/z 615 $[M + Na]^+$, 1207 $[2\ M + Na]^+$; HR-ESIMS m/z 615.2307, calcd for $C_{31}H_{45}INaO_3$ 615.2311.

*Methyl 2-(4-Cyanophenyl)-1,3-dehydrooleanolate (**7e**).* To a stirred solution of **7d** (200 mg, 0.34 mmol) in toluene (8 mL) were added NaOMe (18 mg, 0.337 mmol, 1 molar equiv), *p*-

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cyanophenylboronic acid (50 mg, 0.34 mmol, 1 molar equiv), and a catalytic amount of $Pd(PPh_3)_4$. After stirring under N_2 at 80 °C for 24 h, the mixture was worked up by dilution with brine and extraction with EtOAc. After drying and evaporation, the residue was purified by GCC on neutral alumina (petroleum ether/EtOAc, 99:1 → 9:1, as eluant) to give 20 mg (10%) of **7e** as a white powder: IR ν_{max} (KBr) 1726, 1672, 1604, 1511, 1462, 1388, 1361, 1247, 1178 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 7.61 (2H, d, J = 8.2 Hz, H-3′/5′), 7.41 (2H, d, J = 8.2 Hz, H-2′/6′), 7.16 (1H, s, H-1), 5.37 (1H, t, J = 3.5 Hz, H-12), 3.64 (3H, s, 28-OMe), 2.91 (1H, dd, J = 14.0, 4.0 Hz, H-18), 2.20−1.09 (series of multiplets), 1.21 (3H, s, H-25), 1.21 (3H, s, H-24), 1.21 (3H, s, H-27), 1.17 (3H, s, H-23), 0.94 (3H, s, H-30), 0.90 (3H, s, H-29), 0.86 (3H, s, H-26); ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.3, 178.2, 144.2, 141.3, 140.1, 138.1, 132.3, 127.6, 123.2, 118.6, 111.8, 56.0, 48.3, 47.3, 46.6, 42.3, 41.9, 40.0, 39.4, 39.1, 37.7, 35.8, 34.6, 33.8, 33.3, 33.1, 31.4, 28.8, 28.3, 27.9, 26.6, 24.3, 24.1, 23.7, 19.0, 17.6; ESIMS (positive ions) m/z 590 $[M + Na]^+$; HR-ESIMS m/z 590.3615, calcd for $C_{38}H_{49}NNaO_3$ 590.3610.

*Methyl 2-Phenyl-1,3-bisdehydrooleanolate (**7f**).* To a stirred solution of **7d** (50 mg, 0.084 mmol) in toluene (4 mL) were added NaOMe (4 mg, 0.084 mmol, 1 equiv), phenylboronic acid (11 mg, 0.084 mmol, 1 equiv), and catalytic $Pd(PPh_3)_4$. After stirring at 80 °C for 2 h, the reaction was worked up by dilution with brine and extraction with EtOAc. After drying and evaporation, the residue was purified by GCC on silica gel (petroleum ether/EtOAc, 9:1, as eluant) to give 50 mg (95%) of **7d** as a white powder: IR ν_{max} (KBr) 1726, 1671, 1459, 1362, 1302, 1195, 1163 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 7.34−7.29 (5H, phenyl signals), 7.10 (1H, s, H-1), 5.36 (1H, t, J = 3.5 Hz, H-12), 3.64 (3H, s, 28-OMe), 2.90 (1H, dd, J = 14.0, 4.0 Hz, H-18), 2.20−1.09 (series of multiplets), 1.21 (3H, s, H-25), 1.21 (3H, s, H-24), 1.20 (3H, s, H-23), 1.17 (3H, s, H-27), 0.94 (3H, s, H-30), 0.90 (3H, s, H-29), 0.85 (3H, s, H-26); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 204.2 (C-3), 178.2 (C-28), 155.5, 144.3 (C-13), 137.2, 136.2, 128.3 (C-2′/6′), 128.1 (C-3′/5′), 127.5 (C-4′), 121.8 (C-12), 52.8 (C-5), 51.6 (OCH_3-28), 46.8 (C-17), 45.6 (C-19), 45.2 (C-4), 42.1 (C-14), 42.0 (C-18), 41.6 (C-9), 40.0 (C-10), 38.9 (C-8), 33.9 (C-21), 33.1 (C-7), 32.3 (C-22), 32.4 (C-20), 30.7 (C-24), 28.7 (C-15), 27.7 (C-29), 25.8 (C-27), 23.6 (C-30), 23.5 (C-11),23.0 (C-16), 21.6 (C-23), 19.3 (C-6), 18.8 (C-26), 17.3 (C-25); ESIMS (positive ions) m/z 565 $[M + Na]^+$, 1107 $[2 M + Na]^+$; HR-ESIMS m/z 565.3657, calcd for $C_{37}H_{50}NaO_3$ 565.3658.

*Methyl 2-(4-Methoxyphenyl)-1,3-bisdehydrooleanolate (**7g**).* To a stirred solution of **7d** (50 mg, 0.084 mmol) in toluene (4 mL) were added NaOMe (4 mg, 0.084 mmol, 1 molar equiv), 4-methoxyphenylboronic acid (13 mg, 084 mmol, 1 molar equiv), and catalytic $Pd(PPh_3)_4$. After stirring at 80 °C for 2 h, the reaction was worked up by dilution with brine and extraction with EtOAc. After drying and evaporation, the residue was purified by GCC (petroleum ether/EtOAc, 9:1, as eluant) to give 50 mg (95%) of **7g** as a white powder: IR ν_{max} (KBr) 3307, 1726, 1672, 1604, 1511, 1462, 1388, 1361, 1247, 1178 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 7.24 (2H, d, J = 9.0 Hz, H-2′/6′), 7.03 (1H, s, H-1), 6.86 (2H, d, J = 9.0 Hz, H-3′/5′), 5.37 (1H, t, J = 3.5 Hz, H-12), 3.81 (3H, s, 4′-OMe), 3.64 (3H, s, 28-OMe), 2.90 (1H, dd, J = 14.0, 4.0 Hz, H-18), 2.20−1.09 (series of multiplets), 1.20 (3H, s, H-25), 1.19 (3H, s, H-24), 1.18 (3H, s, H-23), 1.16 (3H, s, H-27), 0.94 (3H, s, H-30), 0.91 (3H, s, H-29), 0.85 (3H, s, H-26); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 204.8 (C-3), 178.6 (C-28), 159.3 (C-4′), 144.5 (C-13), 139.9 (C-2), 135.7 (C-1), 129.7 (C-2′/6′), 129.6 (C-1′), 122.1 (C-12), 113.8 (C-3′/5′), 55.6 (OCH_3-4′), 53.0 (C-5), 52.0 (OCH_3-28), 47.1 (C-17), 45.8 (C-19), 45.4 (C-4), 42.3 (C-14), 42.2 (C-18), 41.8 (C-9), 40.3 (C-10), 39.1 (C-8), 33.4 (C-21), 33.3 (C-7), 32.6 (C-22), 32.4 (C-20), 31.0 (C-24), 28.9 (C-15), 27.8 (C-29), 26.0 (C-27), 23.9 (C-30), 23.8 (C-11),23.7 (C-16), 21.8 (C-23), 19.6 (C-6), 19.1 (C-26), 17.5 (C-25); ESIMS (positive ions) m/z 595 $[M + Na]^+$, 1167 $[2 M + Na]^+$; HR-ESIMS m/z 595.3767, calcd for $C_{38}H_{52}NaO_4$ 595.3763.

*Methyl 2-(2-Phenylethynyl)-1,3-bisdehydrooleanolate (**7h**).* To a stirred solution of **7d** (150 mg, 0.25 mmol) in EtOAc (6 mL) were added phenylacetylene (41 μL, 0.379 mmol, 1.5 molar equiv), triethylamine (TEA) (500 μL), CuI (5 mg, 0.025 mmol, 0.1 molar equiv), and $Pd(PPh_3)_4$ (29 mg, 0.025 mmol, 0.1 molar equiv). After refluxing for 12 h, the reaction was worked up by dilution with brine and extraction with EtOAc. The organic phase was dried and evaporated, and the residue was purified by column chromatography on alumina (petroleum ether/EtOAc, 9:1, as eluant) to give 105 mg (73%) of **7h** as a white powder: IR ν_{max} (KBr) 1726, 1676, 1560, 1459, 1363, 1260, 1164, 1124 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 7.50−7.35 (phenyl protons), 7.27 (1H, s, H-1), 5.37 (1H, t, J = 3.5 Hz, H-12), 3.64 (3H, s, 28-OMe), 2.91 (1H, dd, J = 14.0, 4.0 Hz, H-18), 2.20−1.09 (series of multiplets), 1.21 (3H, s, H-25), 1.20 (3H, s, H-24), 1.16 (3H, s, H-27), 1.16 (3H, s, H-23), 0.94 (3H, s, H-30), 0.91 (3H, s, H-29), 0.84 (3H, s, H-26); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 201.5 (C-3), 178.2 (C-28), 162.5 (C-1), 144.3 (C-13), 137.3 (C-2), 131.8 (Ph), 128.3 (Ph), 128.2 (Ph), 121.6 (C-12), 120.9 (Ph), 91.3 (C-2′), 85.0 (C-1′), 52.8 (C-5), 51.6 (OCH_3-28), 46.8 (C-17), 45.7 (C-19), 44.9 (C-4), 42.1 (C-14), 41.6 (C-18), 41.5 (C-9), 40.1 (C-10), 39.8 (C-8), 33.9 (C-21), 33.1 (C-7), 32.3 (C-22), 32.1 (C-20), 30.7 (C-24), 28.4 (C-15), 27.6 (C-29), 25.8 (C-27), 23.6 (C-30), 23.4 (C-11), 23.0 (C-16), 21.8 (C-23), 19.0 (C-6), 18.5 (C-26), 17.4 (C-25); ESIMS (positive ions) m/z 589 $[M + Na]^+$, 1155 $[2 M + Na]^+$; HR-ESIMS m/z 589.3657, calcd for $C_{39}H_{50}NaO_3$ 589.3658.

*Methyl (E)-2-(2-Ethoxycarbonylethenyl)-1,3-bisdehydrooleanolate (**7i**).* To a stirred solution of **7d** (20 mg, 0.034 mmol, 1 molar equiv) in CH_3CN (10 mL) were sequenatially added ethyl acrylate (17 μL, 0.17 mmol, 5 molar equiv), TEA (7 μL, 0.051 mmol, 1.5 molar equiv), $Pd(OAc)_2$ (0.76 mg, 0.0030 mmol, 0.1 molar equiv), and Ph_3P (1 mg, 0.003 mmol, 0.1 molar equiv). After stirring at 80 °C for 2 h, the reaction was worked up by dilution with brine and extraction with EtOAc. After drying and evaporation, the residue was purified by GCC (petroleum ether/EtOAc, 9:1, as eluant) to give 20 mg (95%) of **7i** as a white powder: IR (KBr) ν_{max} 1720, 1679, 1629, 1462, 1303, 1261, 1164, 1031 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 7.28 (1H, d, J = 16.0 Hz, H-1′), 7.16 (1H, s, H-1), 6.51 (1H, d, J = 16 Hz, H-2′), 5.37 (1H, t, J = 3.5 Hz, H-12), 4.22 (2H, q, J = 14.2, 7.1 Hz, 3′-OCH_2), 3.49 (3H, s, 28-OMe), 2.78 (1H, dd, J = 14.0, 4.0 Hz, H-18), 2.45−1.10 (series of multiplets), 1.30 (3H, t, J = 7.1 Hz, 3′-$OCH_2\underline{CH_3}$), 1.30 (3H, s, H-25), 1.18 (3H, s, H-24), 1.11 (3H, s, H-27), 1.08 (3H, s, H-23), 1.01 (3H, s, H-30), 0.93 (3H, s, H-29), 0.92 (3H, s, H-26); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 200.3, 178.2, 162.5, 149.4, 143.1, 142.3, 123.6, 115.3, 88.6, 61.4, 56.0, 48.3, 47.3, 46.6, 42.3, 41.9, 40.0, 39.4, 39.1, 37.7, 35.8, 34.6, 33.8, 33.3, 33.1, 31.4, 28.8, 28.3, 27.9, 26.6, 24.3, 24.1, 23.7, 19.0, 17.6, 14.2; ESIMS (positive ions) m/z 587 $[M + Na]^+$, 1151 $[2 M + Na]^+$; HR-ESIMS m/z 587.3717, calcd for $C_{36}H_{52}NaO_5$ 587.3712.

*Methyl 2-Carboxy-1,3-bisdehydrooleanolate (**7j**).* Under a N_2 atmosphere, a mixture of **3a** (100 mg, 0.21 mmol) and a 2.0 M solution of Stiles's reagent (methyl magnesium carbonate, 8 mL, 16 mmol) was heated at 110 °C. After 1 h, the reaction was worked up by dilution with 5% HCl and extraction with EtOAc. The organic phase was washed with brine, dried, and evaporated. The residue, a white powder, was dissolved in toluene (8 mL) and treated with DDQ (90 mg, 0.39 mmol, 2 molar equiv) under stirring at room temperature After 1 h, the reaction was worked up by dilution with brine and extraction with EtOAc. After drying and evaporation, the residue was purified by GCC on silica gel (10 g, petroleum ether/EtOAc, 9:1, as eluant) to afford **7j** as a white powder (70 mg, 66% from **3a**): IR ν_{max} (KBr) 1720, 1667, 1455, 1387, 1300, 1260, 1164 cm^{-1}; for ^1H and ^{13}C NMR data, see ref 10; ESIMS (positive ions) m/z 533 $[M + Na]^+$; HR-ESIMS m/z 533.3239, calcd for $C_{32}H_{46}NaO_5$ 533.3243.

*Methyl 2-Methoxycarbonyl-1,3-bisdehydrooleanolate (**7k**).* To a solution of **7j** (100 mg, 0.19 mmol) in MeOH (8 mL) were added ethylene dichloride (EDC) (38 mg, 0.195 mmol, 1 molar equiv) and catalytic DMAP. After stirring at room temperature for 1 h, the reaction was worked up by dilution with brine and extraction with EtOAc. After washing with brine, drying, and evaporation, the residue was purified by GCC (10 g silica gel, petroleum ether/EtOAc, 9:1, as eluant) to give 100 mg (98%) of **7k** as a white powder: IR ν_{max} (KBr) 1730, 1661, 1730, 1661, 1455, 1387, 1300, 1260, 1164, 1098 cm^{-1}; for ^1H and ^{13}C NMR data, see ref 10; ESIMS (positive ions) m/z 547 [M

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+ Na]$^+$, 1071 [2 M + Na]$^+$; HR-ESIMS m/z 547.3333, calcd for $C_{33}H_{48}NaO_5$ 587.3339.

*Methyl 2-Formyl-1,3-dehydrooleanoate (**7l**).* To a solution of **4** (200 mg, 0.40 mmol) in toluene (8 mL) was added DDQ (182 mg, 0.802 mmol, 2 molar equiv), and the reaction was stirred at room temperature After 30 min, the reaction was worked up by dilution with brine and extraction with EtOAc. Afte drying and evaporation, 200 mg (quant.) of **7l** was obtained as a white powder. The same reaction could be obtained by treatment with PhSeCl (2 molar equiv) and pyridine (6 molar equiv) in CH_2Cl_2: IR ν_{max} (KBr) 1727, 1667, 1458, 1389, 1302, 1260, 1164, 1098 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.01 (s, 1H), 7.79 (1H, s, H-1), 5.37 (1H, t, J = 3.5 Hz, H-12), 3.63 (s, 3H), 2.78 (1H, dd, J = 14.0, 4.0 Hz, H-18), 2.45−1.10 (series of multiplets), 1.19 (3H, s, H-25), 1.17 (3H, s, H-24), 1.16 (3H, s, H-23), 1.14 (3H, s, H-27), 0.94 (3H, s, H-30), 0.91 (3H, s, H-29), 0.85 (3H, s, H-26); ^{13}C NMR (75 MHz, $CDCl_3$) δ 203.7, 190.7, 178.3, 165.2, 144.5, 131.2, 121.6, 52.8, 51.8, 47.0, 45.8, 45.1, 42.3, 41.7, 41.3, 40.5, 39.8, 34.0, 33.3, 32.44, 32.38, 30.9, 28.2, 27.8, 26.0, 23.8, 23.5, 23.2,21.7, 19.2, 18.2, 17.6; ESIMS (positive ions) m/z 517 [M + Na]$^+$, 1011 [2 M + Na]$^+$; HR-ESIMS m/z 517.3296, calcd for $C_{32}H_{46}NaO_4$ 517.3294.

*Methyl 2-Acetoxy-1,3-bisdehydrooleanolate (**7m**).* To a stirred and cooled (ice bath) solution of of maslinic acid (**3c**) (200 mg, 0.41 mmol) in acetone (5 mL) was added dropwise Jones' reagent. After 1 h, the reaction was worked up by the addition of a few drops of EtOH and then brine and subsequently extracted with EtOAc. After drying and evaporation, the residue was purified by GCC on silica gel (petroleum ether/EtOAc, 9:1, as eluent) to afford the enol tautomer of methyl 2-oxo-3-dehydrooleanoate,[20] which was dissolved in THF (10 mL) and treated with pyridine (500 μL) and Ac_2O (27 μL 0.29 mmol, 1 molar equiv). After stirring at room temperature for 1 h, the reaction was worked up by dilution with brine and extraction with EtOAc. After drying and evaporation, the residue was purified by GCC on silica gel (petroleum ether/EtOAc, 9:1, → petroleum ether/EtOAc, 9:1, as eluent) to afford **7m** as a white solid (80 mg, 52%): IR ν_{max} (KBr) 1607, 1475, 1238, 998, 846, 774 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H 6.68 (1H, s, H-1), 5.34 (1H, t, J = 3.5 Hz, H-12), 3.63 (3H, s, 28-OMe), 2.90 (1H, dd, J = 14.0, 4.0 Hz, H-18), 2.20 (3H, s, Ac), 2.05−1.10 (series of multiplets), 1.23 (3H, s, H-25), 1.21 (3H, s, H-24), 1.15 (3H, s, H-27), 1.13 (3H, s, H-23), 0.93 (3H, s, H-30), 0.91 (3H, s, H-29), 0.81 (3H, s, H-26); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 197.9 (C-3), 178.2 (C-28), 169.0 (C-1′), 144.4 (C-13), 144.0, 135.0, 121.5 (C-12), 52.9 (C-5), 51.6 (OCH_3-28), 46.8 (C-17), 46.7 (C-19), 45.7 (C-4), 42.1 (C-14), 41.9 (C-18), 41.5 (C-9), 40.0 (C-10), 39.4 (C-8), 33.9 (C-21), 33.1 (C-7), 32.3 (C-22), 30.7 (C-20), 27.8 (C-24), 27.6 (C-15), 25.9 (C-29), 24.5 (C-27), 23.6 (C-30), 23.3 (C-11), 23.0 (C-16), 21.5 (C-23), 20.4 (C-2′), 19.2 (C-6), 18.9 (C-26), 17.3 (C-25); ESIMS (positive ions) m/z 547 [M + Na]$^+$, 1071 [2 M + Na]$^+$; HR-ESIMS m/z 517.3402, calcd for $C_{33}H_{48}NaO_5$ 547.3399.

Cell Cultures. HaCaT-ARE-Luc (human keratinocytes), NIH-3T3-KBF-Luc (mouse fibroblasts), and HeLa-STAT3-Luc (human cervix carcinoma) cell lines were constructed in our lab and characterized as previously described.[25] All the cell lines were grown in supplemented Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics, at 37 °C in a humidified atmosphere of 5% CO_2.

Luciferase Assays. For the anti-NF-κB activity NIH-3T3-KBF-Luc cells were stimulated with TNFα (20 ng/mL) in the presence or the absence of the compounds for 6 h. For the activation of the antioxidant response element (ARE) that is activated by Nrf2 HaCaTARE-Luc cells were stimulated with the compounds for 6 h. For the anti-STAT3 activity HeLa-STAT-3-Luc cells were stimulated with γIFN (20 IU/mL) in the presence or the absence of the compounds for 6 h. After treatment, the cells were washed twice in phosphate-buffered saline and lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol during 15 min at room temperature in a horizontal shaker. After centrifugation, luciferase activity in the supernatant was measured using a GloMax 96 microplate luminometer (Promega, Madison, WI, USA) following the instructions of the luciferase assay kit (Promega). For NF-κB inhibition the RLU (relative light units) was calculated, and the results were expressed as percentage of inhibition of NF-κB activity induced by TNFα (100% activation). For STAT3 inhibition the RLU was calculated, and results were expressed as percentage of inhibition of STAT3 activity induced by IFN-γ (100% activation). For Nrf2 activation the RLU was calculated, and the EC_{50} and IRA (intrinsic relative activity) values were determined relative to 20 μM *tert*-butylhydroquinone (TBHQ) using the following equation: IRA coefficient = $(EC_{50\text{-TBHQ}} \times E_{max})/(EC_{50} \times E_{max\text{-TBHQ}})$, where EC_{50} and E_{max} denote EC_{50} and E_{max} of the agonist, and $EC_{50\text{-TBHQ}}$ and $E_{max\text{-TBHQ}}$ denote EC_{50} and E_{max} values of the standard agonist TBHQ.[26] The results represent the mean of at least five independent experiments, and the SD was always lower than 15%.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00271.

Selected MS and NMR spectra for the synthetic compounds (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Tel: +34-957-213766. E-mail: e.munoz@uco.es (E. Muñoz).
*Tel: +39-0321-375744. Fax: +39-0321375744. E-mail: giovanni.appendino@uniupo.it (G. Appendino).

ORCID

Giovanni Appendino: 0000-0002-4170-9919

Notes

The authors declare no competing financial interest.

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