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Michael Acceptors

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An NMR Spectroscopic Method to Identify and Classify Thiol-Trapping Agents: Revival of Michael Acceptors for Drug Discovery?**

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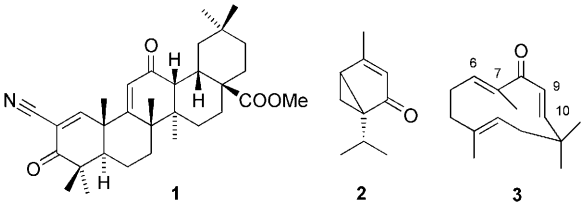
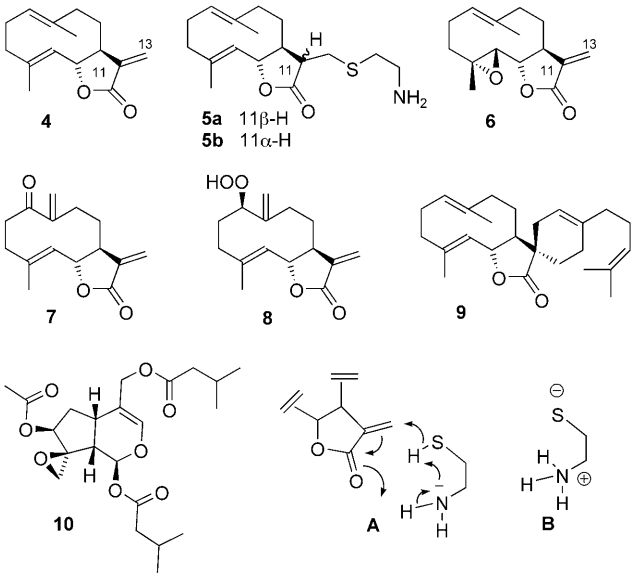
Although Michael acceptors are traditionally shunned in modern drug discovery,[1] trapping of thiols by covalent coupling represents an important mechanism of bioactivity, and many biologically relevant and druggable pathways are targeted by thiol-reactive compounds.[2] Research on Michael acceptors, long confined to the realm of toxicology,[3] was rekindled by the development of the antioxidant inflammation modulator (AIM) homo-triterpenoid bardoxolone methyl (RTA402, **1**).[4] This orally bioavailable biological



mimic of prostaglandin enones was given orphan drug status by the FDA for the treatment of pancreatic cancer,[5] and is also in late clinical development for the treatment of diabetes-associated chronic kidney disease.[5] The hallmark of the biological profile of **1** is its reversible reaction with thiol groups.[6] Thus, **1** and related analogues react quickly with thiols, but the resulting adducts, although spectroscopically characterizable, revert to the starting compounds upon attempted isolation.[6] No satisfactory mechanistic explanation has so far been proposed for this behavior, thereby hampering a systematic investigation of this class of agents.[6] Spurred by these observations, we have developed, and validated biologically in a thiol-sensitive assay, an expeditious NMR method to identify Michael acceptors and sort them into reversible and irreversible thiol sinks.[7] We believe that our method warrants disclosure because of its mechanistic implications and its utility for bioorganic studies, as exemplified by the identification of some new chemotypes of transient receptor potential ankyrin 1 (TRPA1) agonists, and of the monoterpene umbellulone (**2**) and the sesquiterpene zerumbone (**3**) as reversible Michael acceptors.

The exomethylene-γ-lactone costunolide (**4**), a known sink for biological thiols,[8,9] was used as a probe in a series of exploratory experiments. When **4** was treated with two



equivalents of the odorless thiol dodecanethiol in deuterated solvents of various polarity (toluene, chloroform, methanol, acetonitrile, DMSO), no reaction was observed within a time frame (24 h) considered suitable for the assay. These results are in accordance with the general requirement of harsh conditions and long reaction times for uncatalyzed thia-Michael additions.[10] On the other hand, treatment with two equivalents of cysteamine (2-aminoethanethiol), a more biologically relevant model thiol,[11] in DMSO led to the instantaneous formation of an approximate 6:1 mixture of the diastereomeric Michael adducts **5a** and **5b** (Figure 1). No

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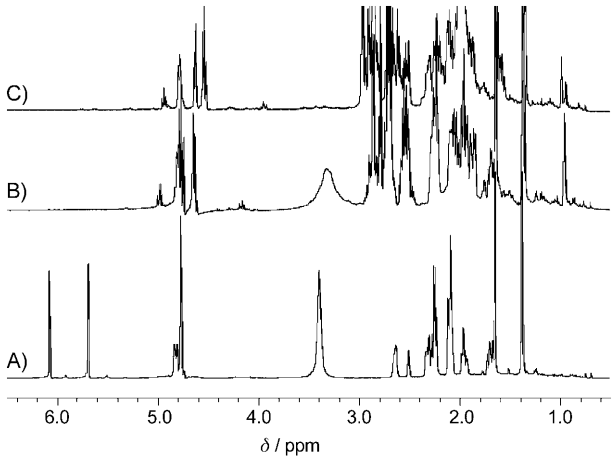


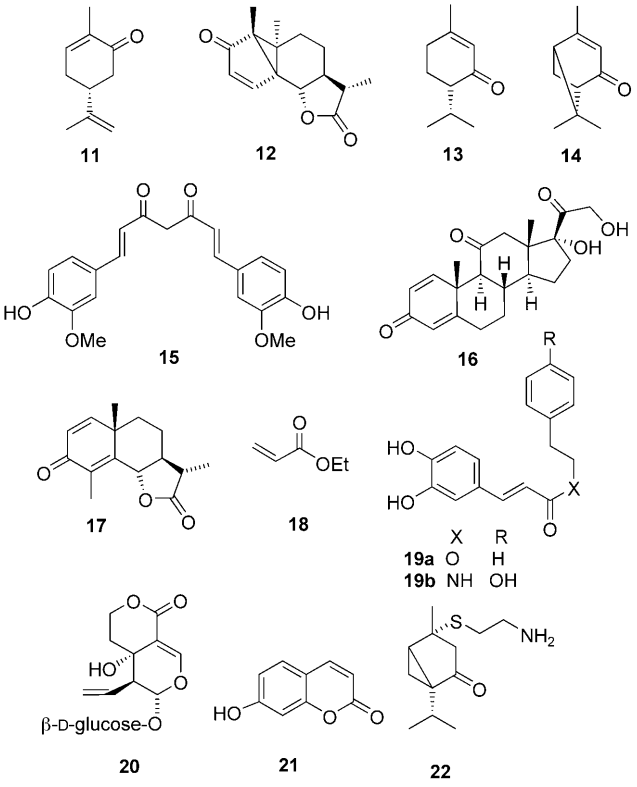
Figure 1. Reaction of costunolide (**4**) with cysteamine. A) ^{1}H NMR spectrum of **4** in [D_6]DMSO. B) Spectrum recorded 5 min after the addition of 2 mol equiv cysteamine. C) Spectrum recorded 5 min after dilution (1:20) of the reaction mixture with $CDCl_3$. All spectra were taken at 500 MHz. *Note the complete and irreversible disappearance of the olefin signals at* $\delta = 6.06$ and 5.65 ppm (H-13a and H-13b) upon addition of cysteamine.

reaction occurred in apolar solvents, while only a sluggish and incomplete reaction took place in other polar solvents; a solution of costunolide and cysteamine in $CDCl_3$ failed to show any evidence of reaction after two weeks at room temperature.

The related and more functionalized exomethylene-γ-lactones parthenolide (**6**),[9] anhydroverlotorin (**7**),[9] and verlotorin (**8**)[9] reacted in the same way, thus showing the potential generality of the reaction. Interestingly, while addition to the exomethylene ketone group of **7** also took place, neither the reactive epoxide group of **6** nor the hydroperoxy group of **8** reacted,[12] and lactone thiolysis was not observed with the related sesterterpene lactone genepolide (**9**).[9] These observations suggest a selectivity for Michael addition versus other reaction modes in substrates containing multiple electrophilic sites. The stability toward cysteamine of the reactive epoxide ring of **6** provides support for the view that epoxides are generally stable toward thiol opening under biological conditions,[13] as confirmed by the stability of dihydrovaltrate (**10**),[9] an exceedingly reactive epoxide,[14] in the conditions of the assay.

Under these conditions, no reaction occurred with *N*-acetylcysteamine, which suggests that simultaneous activation of the thiol group and the carbonyl oxygen through intermolecular proton transfer, as depicted in **A**, might underlie the mechanism of the rate acceleration observed with cysteamine. Alternatively, the reaction might involve the zwitterionic form of cysteamine (**B**), the ammonium group of which can activate the carbonyl oxygen by proton transfer and, at the same time, position the activated thiolate in proximity to the electrophilic β-carbon atom. The mechanism depicted in **A** is reminiscent of the catalytic triad of cysteine hydrolases,[13] and is similar to the one evoked to explain the promoting effect of α-hydroxy groups in thia-Michael additions to enones.[15] In general, Michael additions are known to be highly sensitive to solvent polarity,[16] and DMSO, because of its polar aprotic nature, might either favor the intermolecular proton-transfer activation mechanism depicted in **A** or, alternatively, stabilize the zwitterionic form of cysteamine **B**.[17]

The experimental protocol developed for costunolide was then applied to a range of electrophilic type-2 alkenes encompassing enones (**2**, **11**–**15**),[9] dienones (**3**, **16, 17**),[9] and α,β-unsaturated esters (**18**, **19a**),[9] amides (**19b**),[9] and lactones (**20**, **21**).[9] While far from comprehensive and



basically reflecting ongoing research lines in our laboratories, this selection encompasses all major types of electrophilic alkenes encountered in natural products. The enones (*R*)-carvone (**11**)[9] and lumisantonin (**12**)[9] gave the expected Michael adducts and the bis-enone curcumin (**15**) its corresponding bis-adduct, while, in accordance with the detrimental steric and electron effects of β disubstitution for conjugate additions,[18] both (+)-piperitone (**13**)[9] and (+)-verbenone (**14**) gave a negative thiol-trapping test. However, the related enone umbellulone (**2**) gave in a diastereoselective way the adduct **22**, which confirmed the surprising reactivity of this compound in conjugate additions previously observed with malonates,[19] as well as the reliability of our thiol-trapping assay.

The cross-conjugated dienones prednisone (**16**) and α-santonin (**17**) were unreactive, thus showing that cross-conjugation can quench Michael acceptor behavior, a surprising observation since cross-conjugation is known to increase the thiol affinity of prostaglandin dienones[20] and their chlorinated marine analogues punaglandins.[21] The medium-

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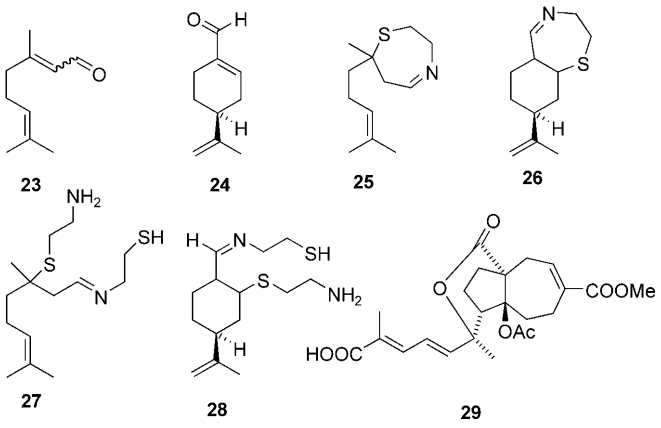
sized dienone zerumbone (**3**)[9] behaved as a "doubled enone" rather than a cross-conjugated dienone, and gave a bis-adduct, presumably because the noncoplanarity of the two strained double bonds prevents cross-conjugation.[22] Ethyl acrylate (**18**) gave a positive assay, but the β-phenyl acrylates phenethylcaffeate (**19a**, CAPE)[9] from propolis and *trans*-caffeoyltyramine (**19b**)[9] gave no reaction, as did the enoyl lactone swertiamarin (**20**). The coumarin umbelliferone (**21**) gave a sluggish reaction that required long reaction times (ca. 24 h) or a large excess (8 equiv) of cysteamine to go to completion.[23] While ambiguous in terms of biological implications, the data on umbelliferone nevertheless support an organocatalyzed mechanism for the rapid addition of cysteamine to conjugated carbonyl compounds. Because of the constrained s-*trans* conformation of the unsaturated system, a concerted proton-transfer process as in **A** would, in fact, be impossible for coumarins.

Under the conditions of the assay, aldehydes gave thiazoline derivatives,[24] and the α,β-unsaturated aldehydes citral (**23**) and perillaldehyde (**24**)[9] gave a mixture of the corresponding 1,4-thiazepines (**25** and **26**, respectively) and the bis-adducts **27** and **28.**[25] In the conditions of the assay,



α,β-unsaturated carboxylic acids were deprotonated, and no reaction took place. Remarkably, the diterpene pseudolaric acid B[9] (**29**) was totally unreactive with cysteamine, despite the presence of two unsaturated carbonyl groups and one strained lactone ring.

Having established a quick and straightforward method to identify Michael acceptors, we validated its extension to biological systems using the activation of TRPA1, a thiol-sensitive assay, as an end point.[26] TRPA1 has an intracellular thiol-rich ankyrin domain, the alkylation of which by Michael acceptors triggers opening of the channel pore and the development of an ion current, easily detectable by calcium imaging.[26] Various classes of thiol traps (sulfinates, isocyanates, enals)[27] are known to activate TRPA1,[28] and we validated the extension of our assay to biological systems with the exomethylene-γ-lactones **4** and **6**–**8**, which gave a clear activation of TRPA1 in HEK 293 cells transfected with rTRPA1 ($EC_{50} = (15.75 \pm 0.01)$, (25.9 ± 4.9), (63.9 ± 6.0), and (45.2 ± 3.4) μM for **4**, **6**, **7**, and **8**, respectively), with 11,13-dihydrocostunolide and 11,13-dihydroparthenolide[29] being inactive. Given the potent anti-inflammatory activity of parthenolide,[30] it is surprising that TRPA1 activation/desensitization has so far been overlooked as a possible mechanistic rationale, in addition to the inhibition of transcription factors like NF-κB,[30] for this action. As predicted by the NMR spectroscopic assay, the cross-conjugated dienone santonin was inactive, while its lumi-derivative (**12**) gave a positive TRPA1-activating response ($EC_{50} = (36.6 \pm 0.02)$ μM), as did zerumbone (**3**; $EC_{50} = (14.8 \pm 4.5)$ μM), while umbelliferone (**21**), despite its slow kinetics of thiol addition, was a good activator of TRPA1 ($EC_{50} = (6.0 \pm 0.8)$ μM). The potent activity of curcumin ($EC_{50} = (3.0 \pm 0.5)$ μM) adds TRPA1 to the almost 100 biological end points[31] addressed by this dietary diarylheptanoid.[32]

Reversible thia-Michael adducts decompose during workup, and attempts to isolate them from the reaction medium fail.[7,20] With the exception of enals, all compounds that gave Michael adducts in [D_6]DMSO were unreactive with cysteamine in deuterochloroform. Therefore, dilution with this solvent could be used as a test for reversibility since, in the presence of an equilibrium, the change of polarity would reverse the reaction, with reappearance of the olefin resonance(s) lost during the addition reaction in DMSO. Adduct stability is a critical determinant for the biological profile of thiol-trapping agents, with rapid reversibility being associated with low toxicity.[33] Therefore, the availability of a rapid method to identify these compounds could be of interest. In the event, an aliquot (25 μL) of the [D_6]DMSO solution of the in-situ-generated Michael adducts was diluted 1:20 with $CDCl_3$, and the ^{1}H NMR spectrum was recorded. Within the set of compounds investigated, only the cysteamine adducts of umbellulone (**2**) and zerumbone (**3**) showed solvent-induced reversibility. Interestingly, reversal of Michael addition was only observed for the disubstituted Δ^9 double bond of zerumbone (see the Supporting Information). Remarkably, the Michael adducts of umbellulone and zerumbone were thermally stable in DMSO, with no degradation being detected upon heating to 50 °C, a critical threshold for reversibility with the thiol adduct of bardoxolone methyl.[7]

The reversibility of the thia-Michael addition of prostaglandin dienones has been attributed to cross-conjugation.[20] While endocyclic cyclohexadienones such as santonin and prednisone do not behave as Michael acceptors, it is nevertheless interesting that the UV spectrum of umbellulone is similar to that of cross-conjugated dienones,[34] which suggests that this compound behaves as the cyclopropylogous version of a reactive cyclopentadienone (Scheme 1, **A′**). A very unusual electronic structure for umbellulone is also suggested by the dramatic upfield shift (ca. 25 ppm) of the cyclopropane methylene (C5) upon hydrogenation of the double bond (δ C5 = 38.1 in **2**, and 13.7 in its dihydro derivative, see the Supporting Information). The inactivity of piperitone and verbenone in the thiol assay shows that the presence of a cyclopropane ring is critical for the Michael addition. Since a cyclopropane ring has a strong stabilizing effect on an adjacent cationic center,[35] it is tempting to speculate that

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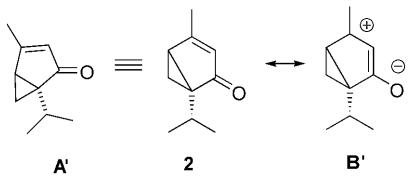

Scheme 1. Umbellulone (**2**) as a cyclopropylogous cyclopentadienone (**A′**), and the polarizing effect of the cyclopropane ring (**B′**).

the positive partial charge associated with the dipolar resonance form of the enone system (Scheme 1, **B′**) would be equally stabilized.[36]

The reason for the reversibility of the thia-Michael reactions of certain cross-conjugated compounds, such as bardoxolone methyl (**1**), umbellulone (**2**), zerumbone (**3**), and prostaglandin dienones, is still elusive but the availability of a quick (minutes) assay to identify further examples could foster a systematic investigation of this issue and elucidate the role, if any, played by cross-conjugation.

In conclusion, we have developed a simple and quick NMR spectroscopic method to identify Michael acceptor sites in complex multifunctional compounds. The method sorts them out in reversible and irreversible thiol sinks, and predicts their potential to modify proteins, as validated by the identification of several new chemotypes of TRPA1 activators. These include well-known anti-inflammatory agents, such as curcumin and parthenolide, which had been overlooked as ligands for this druggable end point of inflammation.[27,28] The simplicity of the reaction conditions stands in sharp contrast to the complexity of the cellular milieu, and it would be pretentious to claim a straightforward general transfer of the results to biological systems. Nevertheless, the validation of the results in a bioassay sensitive to Michael acceptors suggests the possibility of using our rapid NMR spectroscopic test as a prescreen for more complex assays like ALARM NMR spectroscopy, which requires a labeled protein substrate and 2D NMR spectroscopic measurements,[37] or UV-based glutathione trapping experiments.[38] Whether Michael acceptors only *pollute* screening libraries or, alternatively, represent interesting opportunities for drug discovery is an interesting issue of debate, to which our assay will provide new food for thought.

Experimental Section

Cysteamine assay with costunolide (**4**) as example: Costunolide (22.0 mg, 0.96 mmol) was dissolved in [D_6]DMSO (500 μL) in a standard 5 mm NMR tube (Armar Chemicals), and the spectrum was recorded (Figure 1 A). Cysteamine (14.8 mg, 0.19 mmol, 2 mol equiv) was then added, and the spectrum was recorded 5 min after the addition (Figure 1 B). An aliquot (25 μL) of the solution was then transferred into a second NMR tube containing $CDCl_3$ (500 μL) and a new spectrum was recorded (Figure 1 C). A positive assay was evidenced by the disappearance of a particular olefin system of the substrate, and the reversibility of the Michael addition by its reappearance upon dilution 1:20 with $CDCl_3$ (see the Supporting Information). All the original spectra are presented in the Supporting Information.

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