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Sesquiterpenoids from Common Ragweed (*Ambrosia artemisiifolia* L.), an Invasive Biological Polluter

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Dedicated to the memory of Ernesto Fattorusso

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Allergy to common ragweed (*Ambrosia artemisiifolia* L.) is increasingly important from a clinical standpoint, but surprisingly, little is known on the secondary metabolites of this plant and their potential involvement in the severity of the allergic reactions induced by its pollen. Along with known compounds, eight novel sesquiterpenoids were isolated from the aerial parts of *A. artemisiifolia*, evidencing the presence of large amounts of electrophilic α,β-unsaturated carbonyl derivatives. Because compounds of this type were also present in pollen, their reactivity with thiols was investigated and correlated to the activation of TRPA1, a polymodal sensor highly expressed in the aerial pathways and involved in airways sensory irritation, a common complication of allergy to *Ambrosia*.

Introduction

Common ragweed (*Ambrosia artemisiifolia* L.) is an invasive species native to North America and nowadays widespread in most temperate regions of the world.[1] It is a very competitive weed, capable to quickly colonize both agricultural and urban areas. Its pollen is a strong allergen, responsible for an airborne allergy unmatched in terms of severity of the symptoms and second only to grass pollen in terms of incidence in the general population of many European countries.[2] The pollen from *Ambrosia* induces respiratory symptoms ranging from rhinitis and rhinoconjunctivitis to asthma in a growing share of the population, with spiraling economic costs for the public health systems.[2]

In Europe, allergy to ragweed was first recorded in Hungary in the early 1990s, in the wake of the abandonment of communist-style collective agriculture and the presence of large areas of uncultivated and disturbed agricultural land. The plant spread rapidly throughout the continent,[3] and by 2007 it became clear that ragweed had become a pan-European problem, with a recorded sensitization rate higher than 15% in Denmark and Germany.[3] Alarmingly, one-fourth of ragweed-sensitive patients also show asthmatic symptoms, and allergic reactions to *Ambrosia* are often accompanied by a severe irritation of the airways.[1–3] A pan-European plan of ragweed containment and the adoption of a common strategy to globally limit its diffusion has not yet materialized, and the diffusion of the plant is nowadays substantially out of control.[4] This, coupled to the boosting effect on pollen output associated with increasing CO_2 concentrations[5] is turning *Ambrosia* into a major health problem in Europe, with densely populated areas like the surroundings of Lyon, France, and Milan, Italy, being particularly affected.[3]

The pollen of *A. artemisiifolia* is produced in enormous amounts (millions of grains per plant) from early August to late September. The pollen grains are small (18–22 μm) and light and are easily subjected to long distance transport, remaining above the threshold concentration for the induction of allergy responses (10–20 pollen/m^3 air) over a large area around the producing plant.[1] For the same reason, they can also deeply penetrate the aerial pathways, inducing local irritancy. As a result, and because the seeds of the plant can remain vital for decades, *A. artemisiifolia* is considered as a biological environmental polluter.[1]

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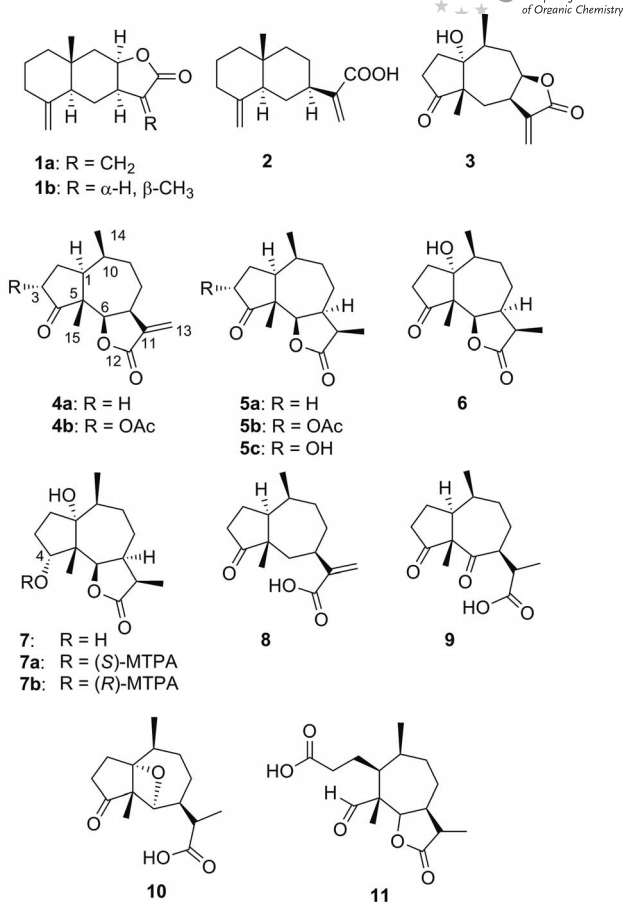
The increasing problem of sensitization to *Ambrosia* pollen has stimulated studies aimed at monitoring its concentration in urban air and curbing the diffusion of the plant by either mechanical or chemical ways.[3] Surprisingly, limited information exists on the phytochemistry of *A. artemisiifolia*, despite the possibility that specific phytochemicals from the plant could contribute to the severity of the allergic reactions to this species. Thus, we have recently shown that some exomethylene-γ-lactones, a type of compound abundant in asteraceous plants and reported from an American collection of *A. artemisiifolia*,[6] are potent activators of TRPA1,[7] a pleiotropic sensor[8] involved in the irritant action of pollutants to the airways and in the genesis of asthma[9] and chronic obstructive pulmonary disease (COPD).[10] We wondered, therefore, whether this type of compound was also present in the European population of *A. artemisiifolia* and if these compounds could activate TRPA1 and, therefore, be potentially involved in the allergic and inflammatory reactions induced by the plant. We present evidence of a surprising variation in the phytochemical profile of the European population of *A. artemisiifolia*, highlighting the presence of chemotypes containing high concentrations of exomethylene-γ-lactones endowed with a high reactivity with thiols and powerful activating properties on TRPA1.



Results and Discussion

Common ragweed was collected in an urban environment (outskirts of Novara) in an area (North-Eastern Piedmont) severely hit by *Ambrosia* pollinosis. Aerial parts of flowered plants were dried and then extracted with acetone. After solid-phase extraction on RP-18 silica gel to remove pigments and waxes, the purified extract was fractionated by gravity column chromatography to afford various mixtures of sesquiterpenoids that were further purified by crystallization or, alternatively, by a combination of flash chromatography and HPLC on stationary phases of complementary polarity (silica gel or RP-18 silica gel). Along with several known compounds, eight new sesquiterpenoids were eventually isolated and characterized. The known compound obtained belonged to three major classes, namely, sesquiterpene lactones [isoalantolactone (**1a**),[11] dihydroisoalantolactone (**1b**),[12] peruvin (**3**),[13] damsin (**4a**),[14] dihydrodamsin (**5a**),[15] acetoxydihydrodamsin (**5b**),[16] dihydrocoronopilin (**6**),[17] and secoambrosanolide (**11**)[18]], their acidic precursors [pre-isoalantolactone (**2**)[19] and damsinic acid (**8**)[20]], and daucane alcohols [lasidiol anisate **12**[21,22]]. Sesquiterpene lactones and acids are widespread in plants from the genus *Ambrosia*, but, with the exception of peruvin (**3**), none of these known compounds had been isolated from American populations of *A. artemisiifolia*, where the occurrence of sesquiterpene acids and daucanes is unreported. In addition to these compounds, eight new sesquiterpenoids (i.e., **4b**, **7**, **9**, **10**, **13**–**16a**) were also obtained, whose structure elucidation is outlined here.

The ^{1}H NMR and ^{13}C NMR spectroscopic data of **4b** [$C_{17}H_{22}O_5$, HRMS (ESI)] were similar to those of damsin (**4a**), suggesting an overall acetoxydamsin structure for this compound. Thus, the resonances of the exomethylene (δ_H = 6.27 and 5.57 ppm), the two methyl groups (δ_H = 1.10 ppm, d, J = 6.9 Hz; δ_H = 1.13 ppm, s), the lactone oxymethine (δ_H = 4.72 ppm, d, J = 8.5 Hz), and the lactone and ketone carbonyl groups (δ_C = 168.0 and 221.2 ppm, respectively) were almost identical to those of damsin (**4a**), but the presence of an additional acetoxyl group was evident from the observation of a deshielded oxymethine (δ_H = 5.22 ppm, δ_C = 72.0 ppm) and an acetyl group (δ_H = 2.08 ppm, δ_C = 170.5 and 21.0 ppm). The acetoxyl group was located at C-3 based on considerations of multiplicity and confirmed by analysis of the COSY and HMBC spectra, whereas its α-orientation was evident from the detection of a ROESY correlation between 3-H and the C-15 methyl group. Compound **7** [$C_{15}H_{24}O_4$, HRMS (ESI)], was a close analogue of dihydrocoronopilin (**6**), differing only in the reduction of the ketone carbonyl group to a 4α-hydroxy group. Thus, all the ^{1}H NMR and ^{13}C NMR resonances of the lactone ring and the seven-membered ring were practically superimposable to those of **6**, whereas the differences in the five-membered ring could be rationalized by the reduction of the keto group of **6**. The pattern of ROESY cross-peaks showed that **7** and **6** shared the same configuration at all common stereogenic centers, whereas the cross-peak 4-H/Me-15 indicated an α-orientation for the 4-hy-

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droxy group. The relative configuration of **7** was upgraded to the absolute one through application of the modified Mosher method for secondary alcohols.[23] Treatment of two aliquots of **7** with (–)- and (+)-MTPA (α-methoxy-α-trifluoromethylphenylacetic acid) chloride in dry pyridine gave the corresponding diesters **7a** and **7b**, respectively. The pattern of $\Delta\delta$ (*S*-*R*) values (see Supporting Information) established the *R* configuration at C-3.

The ^{13}C NMR spectrum of **9** [$C_{15}H_{22}O_4$, HRMS (ESI)] showed three carbonyl resonances diagnostic of two ketone carbonyl groups (δ_C = 211.0 and 210.3 ppm) and one carboxylic acid (δ_C = 178.6 ppm). The ^1H NMR spectrum of **9** was analyzed in light of the 2D COSY spectrum, which pooled all the proton multiplets into a single and extended spin system, shown in bold in Figure 1, and spanning from Me-13 to 3-H, and including also a methyl branching (Me-14). The HSQC spectrum associated all the $^1H/^{13}C$ directly linked NMR resonances, evidencing the presence of a single unprotonated sp^3 carbon atom (δ_C = 65.9 ppm) and suggesting an overall similarity to 11,13-dihydrodamsinic acid.

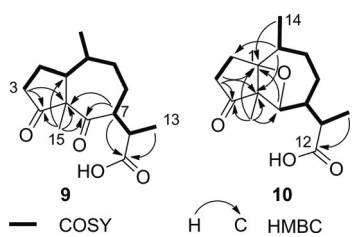
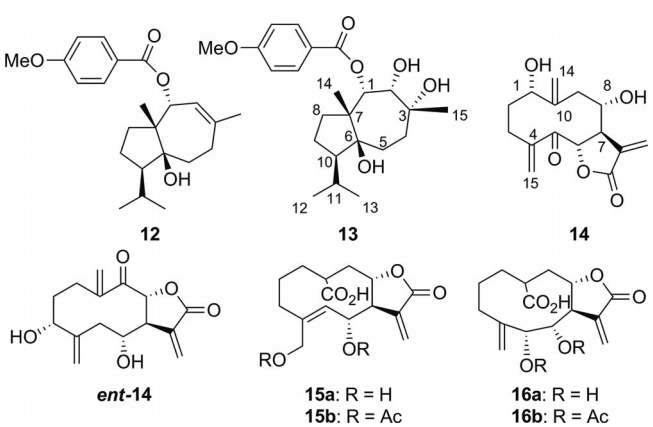


Figure 1. COSY and key HMBC cross-peaks of 6-oxodihydrodamsinic acid (**9**) and ambroxetane (**10**).

The pattern of $^{2,3}J_{C,H}$ HMBC cross-peaks exhibited by **9** (Figure 1) confirmed this insight, whereas the ROESY cross-peaks of Me-15 with Me-14 and 11-H and those of 3α-H with both 1-H and 7-H showed that **9** was indeed 6-oxo-11,13-dihydrodamsinic acid. Due to free rotation around the C-7/C-11 bond, the relative orientation of the C-11 methyl group could not be assigned.

Compound **10** (ambroxetane) had the same molecular formula of **9** [$C_{15}H_{22}O_4$, HRMS (ESI)]. Its NMR resonances could be sorted out into three methyl groups (two doublets and one singlet), four sp^3 methylenes, four sp^3 methines (one of which is an oxymethine, δ_H = 4.72 ppm, δ_C = 80.0 ppm) and four nonprotonated carbon atoms, including one ketone carbonyl group (δ_C = 218.8 ppm), one carboxylic acid group (δ_C = 178.6 ppm), and one oxygenated sp^3 carbon atom (δ_C = 86.2 ppm). The presence of a carboxylic group was confirmed by the formation of a methyl ester upon reaction with diazomethane. The COSY spectrum pooled the proton multiplets into two spin systems, shown in bold in Figure 1. These were bridged by a three non-protonated carbon link, and by an oxygen atom. Especially diagnostic were the $^{2,3}J_{C,H}$ HMBC cross-peaks of 10-H with C-1, C-2, and C-5, of Me-15 with C-1, the ketone C-4, C-5, and the oxymethine C-6, and of 3-H_2 with C-4 and C-5 for the carbon link, whereas the presence of an oxygen link was suggested by the molecular formula and the presence of two still-unassigned oxygenated carbon atoms. The marked downfield shift of C-1 (δ_C = 81.9 ppm in **6**, δ_C = 86.2 ppm in **10**) compared to data of dihydrocoronopilin (**6**)[17] further supported this assignment. The relative configuration at C-5, C-7, and C-10 of **10** was assigned on the basis of the ROESY cross-peaks of Me-15 with both Me-14 and 11-H, whereas the α-orientation of the oxygen bridge was suggested by the structural similarity of **10** with structurally related compounds **6** and **7**, where the 10-hydroxy group is α-oriented. Ambroxetane (**10**) is an oxetane-containing pseudoguaiane sesquiterpenoid, a very rare class of natural products of which only a handful of members is known.[24]

Two of the remaining novel compounds belong to structural types of daucane terpenoids, which are rare in plants from the genus *Ambrosia* and totally unprecedented for *A. artemisiifolia*. Daucane ester **13** [$C_{23}H_{34}O_6$, HRMS (ESI)] was identified as dihydroxylasidiol anisate. The ^1H NMR and ^{13}C NMR resonances of **13** were assigned on the basis of its 2D COSY and HSQC spectra, which were similar to those of **12**,[21] with significant differences only for the H/C resonances associated to the C1–C4 fragment. Thus, the olefin carbon atoms C-2 and C-3 of **12** were replaced by two oxygenated sp^3 carbon atoms, an oxymethine (C-2, δ_H = 4.32 ppm, δ_C = 62.5 ppm) and a quaternary carbon atom (C-3, δ_C = 86.0 ppm), whereas the upfield resonance of the 15-methyl and 4-methylene could be rationalized in the loss of their allylic state.



The HMBC spectrum of **13** fully confirmed this structural assignment by detecting diagnostic cross-peaks centered on Me-14 (C-7, C-8, and the oxygenated carbon atoms C-1 and C-6), 10-H (C-5), and Me-15 (C-2, C-3, and C-4), whereas the cross-peak of 1-H with the ester carbonyl (δ_C = 165.6 ppm) unambiguously located the acylation site. Despite the neopentyl location, no acyl rearrangement to the adjacent secondary hydroxy was observed. Finally, the network of ROESY couplings confirmed that compounds **12** and **13** share the same relative orientation at C-1, C-6, C-7, and C-10, whereas the cross-peaks of 2-H with both Me-14 and Me-15 was critical to establish the orientation of the hydroxy groups at C-2 and C-3 in **13**.

The remaining novel compounds were all highly oxygenated germacranolides. The ^{13}C NMR spectrum of **14** [$C_{15}H_{18}O_5$, HRMS (ESI)] showed the presence of one ali-

phatic methine, three oxymethines, three aliphatic and three olefin methylenes, and five non-protonated carbon atoms [a lactone carbonyl (δ_C = 171.7 ppm) a conjugated ketone (δ_C = 206.5 ppm), and three olefin carbon atoms]. The COSY spectrum of **14** could be rationalized in terms of two spin systems (Figure 2), the first one encompassing an oxymethine (δ_H = 4.72 ppm) and two adjacent diastereotopic methylenes, and the second one spanning from the other aliphatic methylene to the more downfied (δ_H = 4.66 ppm), and presumably lactonized, of the three oxymethines. Further details on the carbon skeleton of **14** were obtained from the HMBC spectrum. In this context, the $^{2,3}J_{C,H}$ correlations of sp^2 methylenes provided critical information to fit the two spin systems into a germacrane skeleton. Thus, the 14-exomethylene correlated with C-1, the olefin carbon at C-10 (δ_C = 145.1 ppm) and with C-9, providing a first link between the two systems. A second one was established on the basis of the correlations centered on the other exomethylene (15-H) [C-3, C-4, and the ketone carbonyl (C-5)] and on 6-H (C-5 and C-4). The site of lactonization was located at the oxymethine adjacent to the carbonyl on the basis of HMBC cross-peaks of the downfield oxymethine with C-11 and C-12 and C-7. The relative configuration of the stereogenic carbon atoms of **14** was deduced on the basis of the ROESY cross-peaks 8-H/6-H, 1-H/9β-H, and 9β-H/6-H. Given the symmetry of the germacrane skeleton through C-2 and C-7, the symmetrical oxygenation pattern adjacent to the C-7 side chain (arbitrarily drawn in β-orientation), the lack of endocyclic double bonds that could have provided a biogenetic clue, these observations could fit both enantiomeric structures **14** and *ent*-**14**. We favor **14**, as it places the oxygenated functions on carbon atoms related to the "primordial" location of the double bonds (C-1/C-10 and C-4/C5) in the putative farnesyl precursor of the germacrane skeleton, but only the determination of the absolute configuration could solve unambiguously the issue. Unfortunately, this was not attempted due to the very small amounts obtained for **14**.

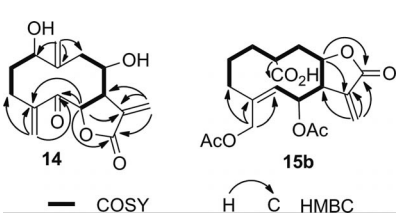


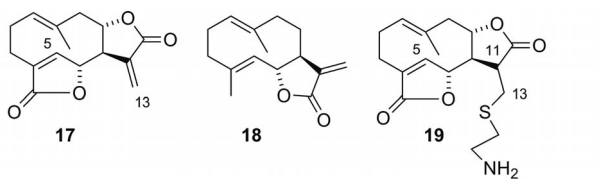
Figure 2. COSY and key HMBC cross-peaks of germacranolides **14** and **15b**.

The two last novel compounds characterized are a pair of germacranolide carboxylic acids, which we have named artemisiifolinic acid (**15a**) and isoartemisiifolinic acid (**16a**). The two compounds could be separated only after acetylation, and the structure elucidation was carried out on their semisynthetic diacetyl derivatives **15b** and **16b**. As already observed with **14**, the ^{1}H NMR spectrum of **15b** [$C_{19}H_{24}O_8$, HRMS (ESI)] also showed no methyl signal, apart from the two acetyl groups, and displayed an olefin methine (δ_H = 5.30 ppm), an olefin methylene (δ_H = 6.40 and 5.92 ppm), and four deshielded aliphatic protons in the 4–5 ppm area. The HSQC identified them as two oxymethines (δ_H = 4.30 ppm, δ_C = 77.1 ppm) and one oxymethylene (δ_H = 4.60 and 5.00 ppm, δ_C = 62.8 ppm). In addition to the acetate carbonyl groups, the ^{13}C NMR spectrum also featured four non-protonated sp^2 carbon atoms, sorted out into a lactone carbonyl (δ_C = 168.6 ppm) and a carboxylic acid carbonyl (δ_C = 178.6 ppm). The COSY spectrum of **15b** revealed that all the multiplets belonged to the same spin system, highlighted in bold in Figure 2, whereas the HMBC cross-peaks of the oxymethylene 15-H_2 eventually linked the two termini of the spin system into a 10-membered ring. The carboxylic group was placed on C-10 on the basis of the HMBC cross-peak 10-H/C-14, whereas the pattern of HMBC correlations shown by 8-H and 13-H_2 (see Figure 2) located the closure of the lactone ring. The presence of an endocyclic double bond makes it possible to tentatively formulate **15a** as a C-8 lactonized germacranolide. Finally, HMBC correlations of 15-H_2 and 6-H with the acetyl carbonyl groups secured the acetylation positions. The ROESY spectrum showed cross-peaks of 6-H with both 8-H and 13-H_2, making it possible to assign the relative configuration of the three adjacent stereogenic carbon atoms. Because 10-H showed no significant ROESY correlation, the relative configuration at C-10 could not be assigned. Artemisiifolinic acid (**15a**) is closely related to artemisiifolin, a compound isolated from an American collection of *A. artemisiifolia*,[25] from which it differs in the oxidation of Me-14 to a carboxylic acid and in the hydrogenation of the $\Delta^{1,10}$ double bond. The ^{1}H NMR spectrum of **15b** was, however, characterized by sharp and well-defined signals, showing a substantial difference from the conformational behavior of artemisifolin, a mixture of slowly interconverting rotamers at room temperature.[26]

Isoartemisiifolinic acid (**16a**) differed from **15a** only in the allylic rearrangement of the primary oxygen function. The analysis of the ^{1}H NMR and ^{13}C NMR resonances of diacetyl derivative **16b** by 2D NMR COSY and HSQC spectra evidenced two major differences, namely, the disappearance of the olefinic methine at C-5, replaced by an oxymethine group (δ_H = 5.32 ppm, δ_C = 79.5 ppm) and the disappearance of the oxymethylene at C-15, which is replaced by an exomethylene (δ_H = 4.80 ppm, 2 H; δ_C = 113.2 ppm). The pattern of HMBC cross-peaks detected for **16b** supported this structural relationship with **15b**. Finally, the ROESY coupling of the 5-OAc with 7-H was instrumental to extend the relative configuration previously determined for **15b** (and confirmed by ROESY cross-peaks of **16b**) to the stereogenic center C-5. Compound **15a** was formulated as a C-8 germacranolide to accommodate the endocyclic double bond in the location expected from its biogenetic derivation from a farnesyl precursor. The same consideration also holds for **16a**, its product of allylic rearrangement.

A. artemisiifolia forms dense populations, making it possible to collect large amounts of biomass in a single collection area. Surprisingly, a collection from a site with a dis-

tance of only ca. 100 m from the first one afforded a plant with a markedly different phytochemical profile, characterized by the presence of large amounts (ca. 0.15%) of hydroxydihydrodamsin (**5c**),[16] whereas a third collection in the surrounding of Novara gave germacranolide isabelin (**17**)[27,28] as its major constituent (ca. 0.10%). Both **5c** and **17** were absent in the first collection investigated, whose major constituents were germacrane carboxylic acids **15a** and **16a** (overall ca. 0.60%). These surprising differences in the phytochemical profile parallel the results of a recent genomic analysis of various French populations of the plant, where a remarkable genetic variation was detected.[29]



Several abundant constituents of common ragweed contain unsaturated carbonyl group(s), potentially capable to behave as Michael acceptors, induce allergic reactions, and activate TRPA1, a polymodal sensor highly expressed in the aerial pathways and involved in airways sensory irritation. Of special relevance is the occurrence in some chemotypes of large amounts of germacrane dilactone isabelin (**17**). This compound, existing in solution as a pair of slowly interconverting rotamers,[30] contains two electrophilic Michael acceptor sites, centered on C-13 and C-5. When subjected to an NMR assay for Michael reactivity,[7] exclusive formation of the adduct at C-13 was observed with 1.5 equiv. of cysteamine, obtaining an adduct characterized by a single set of signals. Under these conditions, the unsaturated endocyclic double bond was unreactive (see Supporting Information). As expected, also isoalantolactone (**1a**) readily formed an irreversible adduct with cysteamine, whereas neither its acidic version **2** nor damsinic acid (**8**) gave an adduct in the test. Interestingly, isabelin outperformed costunolide (**18**), the standard irreversible Michael acceptor,[7] in comparative assays where an equimolar mixture of the two compounds was treated with 1 equiv. of cysteamine (Figure 3). The remarkable selectivity of this reaction is puzzling and might be related to the relief of strain associated to piramidalization of C-11. This increases the flexibility of the lactone ring centered at C-8, making it possible for the strained cyclodecadiene ring to better accommodate it. Interestingly, whereas at room temperature isabelin (**17**) is a ca. 1.3:1 mixture of two interconverting rotamers, in cysteamine Michael adduct **19** the rotameric ratio was ca. 9:1.

In a previous study, an excellent correlation was found between activation of TRPA1, as measured by calcium imaging in HEK 293 cells transfected with rTRPA1, and Michael reactivity, as evaluated in the NMR cysteamine assay.[7] TRPA1, a polymodal sensor, is highly expressed in the aerial pathways and is a major player in the irritation associated to airborne contaminants[31] and in the induction of asthmatic crises.[32] In experimental animal models,

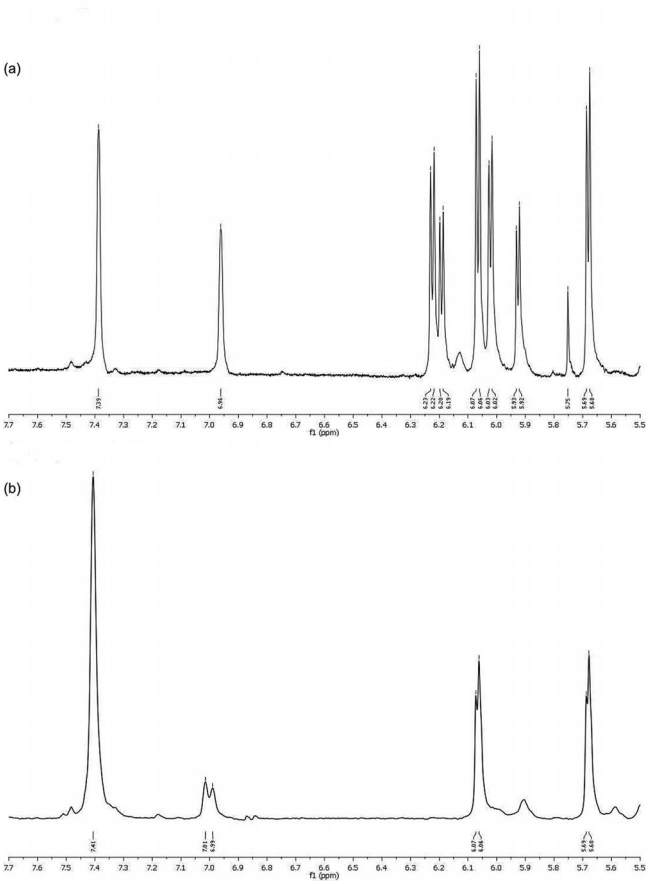


Figure 3. Comparative thiol trapping assay on a mixture of isabelin (**17**) and costunolide (**18**). A snapshot of the ^{1}H NMR spectrum shows the olefin region (δ = 5.0–7.5 ppm) of a DMSO solution of an equimolar mixture of **17** and **18** before (a) and after (b) the addition of 1.5 equiv. of cysteamine. Note the selective disappearance of the exomethylene signals of the two rotamers of **17** (δ = 6.22, 6.02 ppm and δ = 6.17, 5.92 ppm, respectively), the maintenance of the signals of the exomethylene of costunolide (δ = 6.06 and 5.68 ppm), and the substantial merging of the signals of the deshielded olefin methine 5-H (δ = 7.38 and 6.96 ppm) of isabelin into one single resonance (δ = 7.40 ppm) in adduct **19**.

TRPA1 is critically involved in allergen-induced airways neurogenic inflammatory responses,[33] and the severity of the allergenic responses to *Ambrosia* in urban environments has been associated to the chronic airways irritation induced by a series of TRPA1-activating polluters present in the city air (formaldehyde, ozone).[34] It was therefore interesting to evaluate the action of a selection of electrophilic sesquiterpenoids from *Ambrosia* on this ion channel and to compare it with the results observed in the cysteamine assay. In the event, isabelin (**17**) strongly activated TRPA1 (Table 1), outperforming costunolide (**18**), in line with the results observed with the comparative cysteamine assay. Also, isoalantolactone (**1a**), a known allergen,[35] potently activated TRPA1, as expected from the positivity of the cysteamine assay. On the other hand, damsinic acid (**8**) was unreactive both in the cysteamine assay and in TRPA1-activating experiments, whereas the acidic version of isoalantolactone (**2**), despite its inability to react with thiols, never-

theless activated TRPA1, presumably behaving as a non-covalent modulator. These results show that some electrophilic terpenoids from *A. artemisiifolia* behave as potent activators of TRPA1, being capable, in addition to induce contact dermatitis, also to potentially induce severe irritation of the aerial pathways. The unusually high reactivity of isabelin compared to costunolide is surprising and suggests that the cysteamine assay,[7] originally reported to identify reversible Michael acceptors, has a remarkable discriminatory capacity within structurally related electrophilic compounds.

Table 1. Biological evaluation of a selection of electrophilic sesquiterpenoids from *A. artemisiifolia*, with costunolide (**18**) as a control.

Compound	EC_{50} [μM]	Efficacy[a]
1a	18.4 ± 2.2	112.4 ± 33
2	26.0 ± 1.7	314.5 ± 6.9
8	7.7 ± 0.1	25.0 ± 0.1
17	9.2 ± 1.2	72.3 ± 2.7
18	15.7 ± 0.1	88.7 ± 0.1

[a] % efficacy of 100 μM allyl isothiocyanate (AITC).

The presence of plant secondary metabolites in pollen has been suggested to explain violent allergic reactions to phytochemicals,[36] as exemplified by the sudden death of the very first patient treated with the anticancer drug Taxol.[37] Although the scarcity of the material at disposal did not allow the identification of any specific constituents, a pollen extract from *A. artemisiifolia* showed the presence of a high concentration of compounds having carbonyl conjugated exomethylenes (see Supporting Information). The presence of high concentrations of allergenic terpenoids[38] in common ragweed, the potent TRPA1 activating properties of these compounds, and the occurrence of this type of compound in the pollen of the plant, make it possible that the potent sensitizing properties of the pollen proteins might be aggravated by the allergenicity of secondary metabolites from the plant, and by their potential to induce potent inflammatory responses by the activation of TRPA1, a major player in the genesis of irritative pathologies of the airways.[8]

Conclusions

Our results show that, in accordance with a previous genetic analysis,[29] the European population of *A. artemisiifolia* is heterogeneous, and, at least as regards isoprenoids, different from the native American populations investigated before.[6,25] A series of sesquiterpenoids, including compounds belonging to structural classes never identified before in this species, have been characterized, and some of them have been identified as highly reactive Michael acceptors and activators of TRPA1, a major player in the induction of inflammatory pathologies of the airways. Taken together, our results add a further layer of complexity to the pathogenesis of allergy to common ragweed, showing that, in addition to proteins, also low-molecular terpenoids might play a role. Furthermore, the metabolic plasticity of the plant in terms of terpenoid production suggests that heterogeneity in its allergenic proteins should also be taken in due consideration in the development of vaccines. No quick fix to the consequences of the proliferation of *A. artemisiifolia*, a sort of revenge of the land for its mismanagement by humans, seems clearly in sight.

Experimental Section

General: Gravity Column Chromatography (GCC): Merck Silica Gel 60 (70–230 mesh). IR: Shimadzu DR 8001 spectrophotometer. NMR: Jeol Eclipse (300 and 75 MHz for ^1H and ^{13}C, respectively) and Varian Inova (500 and 100 MHz for ^1H and ^{13}C, respectively). For ^1H NMR, $CDCl_3$ as solvent, $CHCl_3$ at δ = 7.25 ppm as reference. For ^{13}C NMR, $CDCl_3$ as solvent, $CDCl_3$ at δ = 77.0 ppm as reference. Reactions were monitored by TLC on Merck 60 F_{254} (0.25 mm) plates that were visualized by UV inspection and/or staining with 5% H_2SO_4 in ethanol and heating. Organic phases were dried with Na_2SO_4 before evaporation. Biological data are expressed as mean ±S.E. IC_{50} values are approximations determined by graphical analysis (linear interpolation between the points between 50% activity). The program Graphpad Instat (Graphpad Software Inc., San Diego, CA) was used for statistical comparisons. Statistical evaluation of the data was performed by one-way ANOVAs for independent or correlated samples followed by Tukey HSD post-hoc tests. A P value of < 0.05 (*) was considered significant.

Plant Material: *A. artemisifolia* was collected in Novara, Italy (the crossing of via Visconti and via Bovio for sample A, and via Visconti 1 for sample B) and in Galliate (NO, Italy) (in front of the mall "Il Gallo", sample C) at the time of flowering (second half of August). Samples A and B were collected twice (2010 and 2011), whereas sample C was collected only in 2011. No difference between the samples collected in different years was observed. Voucher specimens for the five collections are kept at the laboratory in Novara. Plant material was dried, and then leaves and flowers were separated from the woody stems and powdered. All manipulations were done wearing face and body protection to avoid inhalation of the plant powder. Pollen was collected from flowering plants by capping their flowerheads with a paper bag. After approximately two weeks, the powdery material that had deposited on the walls of the bag was removed.

Warning: Personnel working on common ragweed should be preventively screened allergologically for lack of sensitivity to this plant. Exomethylene-γ-lactones are potent skin allergens and the plant material and the chromatographic fractions should be handled with care, avoiding skin contact.

Extraction and Isolation of Sesquiterpenoids: Sample A as representative: 576 g of non-woody aerial parts (collected on August 22, 2010) were extracted with acetone (2 × 2 L) whilst stirring (shaker). Removal of the solvent left a black gum (34 g, 6%), which was purified by solid-phase extraction on RP-18 silica gel. To this purpose, the extract was dissolved in the minimal amount methanol at 40 °C, and then charged on a cake of RP-18 silica gel (170 g) packed into a suction funnel (9 × 15 cm) with methanol. Washing with methanol (3 L) removed the terpenoid fraction (25.7 g of a brown gum), and the cake was next washed with acetone (3.5 L) to remove apolar constituents (pigments, waxes, essential oil, 6.1 g) and regenerate the expensive solid phase. The terpenoid fraction was separated by vacuum chromatography on silica gel (315 g, 50 mL fractions) by using a petroleum ether/EtOAc gradient (from

Special Section

5:5 to 3:7). A total of 32 fractions was collected, eventually pooled after TLC analysis into four major subfractions (I–IV). Fraction I (1.71 g) was sequentially purified by gravity column chromatography on silica gel (petroleum ether/EtOAc, 6:4) and flash chromatography of R-18 silica gel (methanol/water gradient) to afford, after crystallization from ether, **1a** (10 mg), **2** (21 mg), and the crude fractions Ic (16 mg) and Id (90 mg). Fraction Ic was further purified by silica gel HPLC (*n*-hexane/EtOAc, 9:1) to give dihydroisoalantolactone (**1b**, 0.9 mg) and 2,3-dihydro,2,3-dihydroxylasidiol anisate (**13**, 2.1 mg). Fraction Id was further purified by silica gel HPLC (*n*-hexane/EtOAc, 9:1) to give lasidiol anisate (**12**, 4.5 mg), tetrahydrocoronopilin (**7**, 8.5 mg), and 5-oxo-8α-hydroxy-1-epiartemorin (**14**, 0.8 mg). Fraction II (4.01 g) was purified by gravity column chromatography on silica gel (75 g, petroleum ether/EtOAc, 6:4) to obtain a fraction (950 mg) that, crystallized from ether, afforded 500 mg of **5b**. Fraction III (4.0 g) was fractionated by gravity column chromatography into two subfractions (IIIa and IIIb, 980 mg and 2.7 g, respectively), further purified by preparative HPLC on silica gel (petroleum ether/EtOAc, 6:4) to obtain further **5b** (37 mg), **3** (12.1 mg), and fraction IIIa1 (50 mg), whose HPLC purification (*n*-hexane/EtOAc, 65:35) gave dihydrodamsin (**5a**, 2.0 mg), 3-acetoxydamsin (**4b**, 1.8 mg), damsin (**4a**, 3.5 mg), and dihydrocoronopilin (**6**, 4.5 mg). Fraction IV (3.4 g) was crystallized from ether, affording 3.44 g of a waxy solid. A portion of this (50 mg) was acetylated with Ac_2O/pyridine (0.1 mL each, overnight) to give IVa. This was purified by HPLC (*n*-hexane/EtOAc, 7:3) to give diacetyl artemisiifolinic acid (**15b**, 2.1 mg) and diacetyl isoartemisiifolinic acid (**16b**, 2.1 mg). Sample B (360 g, collected on August 22, 2010) was processed as described for A, affording hydroxydihydrodamsin (**5c**, 536 mg, 0.15%) as the major terpenoids. Damsinic acid (**8**), 6-oxodamsinic acid (**9**), ambroxetane (**10**), and secoambrosanolide **11** were also obtained from sample B. In a similar way, from sample C (141 g, collected on August 18, 2011), isabelin (**17**; 360 mg, 0.10%) was isolated. The full characterization of the phytochemical profile of these collections will be reported in a future publication.

3-Acetoxydamsin (4b): $[\alpha]_D$ = –4.2 (*c* = 0.3, $CHCl_3$). ^1H NMR (500 MHz,$CDCl_3$, 25 °C): δ = 6.27 (br. d, *J* = 3.1 Hz, 1 H, 13a-H), 5.57 (br. d, *J* = 2.7 Hz, 1 H, 13b-H), 5.22 (dd, *J* = 5.5, 2.2 Hz, 1 H, 3-H), 4.72 (d, *J* = 8.5 Hz, 1 H, 6-H), 3.36 (m, 1 H, 7-H), 2.51 (ddd, *J* = 12.2, 8.1, 2.2 Hz, 1 H, 2a-H), 2.38 (m, 1 H, 2b-H), 2.16 (m, 1 H, 1-H), 2.08 (s, 3 H, OAc), 1.97 (m, 1 H, 10-H), 1.86 (overlapped, 1 H, 8a-H), 1.81 (overlapped, 1 H, 9a-H), 1.66 (m, 1 H, 8b-H), 1.47 (m, 1 H, 1b-H), 1.13 (s, 3 H, 15-H), 1.10 (d, *J* = 6.9 Hz, 3 H, 14-H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 221.2 (C-4), 171.5 (3-OAc), 168.0 (C-12), 135.1 (C-11), 122.3 (C-13), 81.0 (C-6), 72.0 (C-3), 48.9 (C-5), 42.2 (C-7), 41.1 (C-1), 38.6 (C-10), 35.4 (C-9), 31.2 (C-8), 20.3 (C-2), 21.5 (3-OAc), 19.1 (C-14), 14.2 (C-15) ppm. MS (ESI): *m*/*z* = 329 $[M + Na]^+$. HRMS (ESI): calcd. for $C_{17}H_{22}NaO_5$ $[M + Na]^+$ 329.1365; found 329.1358.

Tetrahydrocoropilin (7): $[\alpha]_D$ = –27.2 (*c* = 0.6, $CHCl_3$). ^1H NMR (500 MHz,$CDCl_3$, 25 °C): δ = 5.06 (d, *J* = 8.5 Hz, 1 H, 6-H), 4.35 (m, 1 H, 4-H), 3.63 (d, *J* = 4.5 Hz, 1 H, 4-OH), 2.87 (overlapped, 1 H, 7-H), 2.86 (overlapped, 1 H, 11-H), 2.51 (ddd, *J* = 9.5, 9.5, 2.2 Hz, 1 H, 2a-H), 2.16 (m, 1 H, 3a-H), 2.04 (m, 1 H, 10-H), 1.80 (m, 1 H, 9a-H), 1.68 (overlapped, 1 H, 8a-H), 1.61 (overlapped, 1 H, 3b-H), 1.46 (overlapped, 1 H, 8b-H), 1.45 (overlapped, 1 H, 2b-H), 1.17 (d, *J* = 6.9 Hz, 3 H, 13-H), 0.97 (d, *J* = 6.9 Hz, 3 H, 14-H), 0.92 (s, 3 H, 15-H) ppm. ^{13}C NMR (125 MHz,$CDCl_3$, 25 °C): δ = 170.0 (C-12), 83.7 (C-1), 82.1 (C-6), 70.7 (C-4), 51.3 (C-5), 43.9 (C-7), 43.0 (C-11), 38.6 (C-10), 35.8 (C-9), 35.7 (C-3), 33.0 (C-8), 20.0 (C-2), 18.0 (C-14), 15.4 (C-13), 11.9 (C-15) ppm. MS (ESI): *m*/*z* = 291 $[M + Na]^+$. HRMS (ESI): calcd. for $C_{15}H_{24}NaO_4$ $[M + Na]^+$ 291.1572; found 291.1577.

Preparation of MTPA Ester Derivatives of Tetrahydrocoronopilin (7): Tetrahydrocoronopilin (**7**) (1.0 mg) was dissolved in dry pyridine (500 μL), treated with an excess amount of (–)-(*R*)-MTPA chloride (20 μL), and then stirred at room temperature overnight. After removal of the solvent, the reaction mixture was purified by HPLC on SI-60 column (*n*-hexane/EtOAc, 85:15) to afford (*S*)-MTPA ester **7a** in the pure state (0.8 mg). Using (+)-(*S*)-MTPA chloride, the same procedure afforded (*R*)-MTPA diester **7b** in the same yield. Data for (*S*)-MTPA ester (**7a**): ^1H NMR (500 MHz,$CDCl_3$, 25 °C): δ = 7.35 and 7.45 (MTPA phenyl protons), 5.52 (m, 1 H, 4-H), 5.09 (d, *J* = 8.5 Hz, 1 H, 6-H), 3.53 (MTPA OCH_3, s), 2.85 (overlapped, 1 H, 7-H), 2.85 (overlapped, 1 H, 11-H), 2.40 (m, 1 H, 3a-H), 2.22 (ddd, *J* = 9.5, 9.5, 2.2 Hz, 1 H, 2a-H), 2.02 (m, 1 H, 10-H), 1.81 (m, 1 H, 3b-H), 1.80 (overlapped, 1 H, 2b-H), 1.77 (m, 1 H, 9a-H), 1.70 (m, 1 H, 8a-H), 1.46 (overlapped, 1 H, 8b-H), 1.36 (m, 1 H, 9a-H), 1.19 (d, *J* = 6.9 Hz, 3 H, 13-H), 1.03 (s, 3 H, 15-H), 0.96 (d, *J* = 6.9 Hz, 3 H, 14-H) ppm. MS (ESI): *m*/*z* = 479 $[M + Na]^+$. Data for (*R*)-MTPA ester (**7a**): ^1H NMR (500 MHz,$CDCl_3$, 25 °C): δ = 7.32 and 7.55 (MTPA phenyl protons), 5.52 (m, 1 H, 4-H), 5.23 (d, *J* = 8.5 Hz, 1 H, 6-H), 3.49 (MTPA OCH_3, s), 2.87 (overlapped, 1 H, 7-H), 2.85 (overlapped, 1 H, 11-H), 2.40 (m, 1 H, 3a-H), 2.19 (ddd, *J* = 9.5, 9.5, 2.2 Hz, 1 H, 2a-H), 2.04 (m, 1 H, 10-H), 1.81 (m, 1 H, 9a-H), 1.70 (m, 1 H, 8a-H), 1.68 (overlapped, 1 H, 2b-H), 1.55 (m, 1 H, 3b-H), 1.46 (overlapped, 1 H, 8b-H), 1.36 (m, 1 H, 9a-H), 1.17 (d, *J* = 6.9 Hz, 3 H, 13-H), 1.14 (s, 3 H, 15-H), 1.03 (d, *J* = 6.9 Hz, 3 H, 14-H) ppm. MS (ESI): *m*/*z* = 479 $[M + Na]^+$.

6-Oxodamsinic Acid (9): $[\alpha]_D$ = –23.9 (*c* = 0.35, $CHCl_3$). ^1H NMR (500 MHz,$CDCl_3$, 25 °C): δ = 3.12 (q, *J* = 6.5 Hz, 1 H, 7-H), 3.02 (m, 1 H, 1-H), 2.92 (q, *J* = 6.5 Hz, 1 H, 11-H), 2.55 (ddd, *J* = 9.5, 4.5, 2.2 Hz, 1 H, 3a-H), 2.38 (ddd, *J* = 9.5, 65, 4.2 Hz, 1 H, 3b-H), 2.12 (m, 1 H, 10-H), 2.03 (overlapped, 1 H, 2a-H), 2.02 (overlapped, 1 H, 8a-H), 2.00 (overlapped, 1 H, 2b-H), 1.82 (m, 1 H, 9a-H), 1.56 (m, 1 H, 8b-H), 1.22 (m, 1 H, 9b-H), 1.27 (s, 3 H, 15-H), 1.15 (d, *J* = 6.9 Hz, 3 H, 13-H), 1.01 (d, *J* = 6.9 Hz, 3 H, 14-H) ppm. ^{13}C NMR (125 MHz,$CDCl_3$, 25 °C): δ = 211.0 (C-4), 210.3 (C-6), 178.6 (C-12), 65.9 (C-5), 51.1 (C-7), 45.6 (C-1), 41.4 (C-11), 37.0 (C-3), 34.2 (C-9), 34.0 (C-10), 27.3 (C-8), 22.6 (C-2), 17.4 (C-14), 16.5 (C-13), 15.8 (C-15) ppm. MS (ESI): *m*/*z* = 289 $[M + Na]^+$. HRMS (ESI): calcd. for $C_{15}H_{22}NaO_4$ $[M + Na]^+$ 289.1416; found 289.1422.

Ambroxetane (10): $[\alpha]_D$ = +5.2 (*c* = 0.1, $CHCl_3$). ^1H NMR (500 MHz,$CDCl_3$, 25 °C): δ = 4.72 (d, *J* = 3.8 Hz, 1 H, 6-H), 2.85 (overlapped, 1 H, 11-H), 2.82 (overlapped, 1 H, 7-H), 2.70 (ddd, *J* = 9.5, 6.5, 4.2 Hz, 1 H, 3a-H), 2.47 (overlapped, 1 H, 3b-H), 2.47 (overlapped, 1 H, 2a-H), 2.09 (m, 1 H, 10-H), 2.02 (m, 1 H, 8a-H), 1.69 (m, 1 H, 9a-H), 1.60 (m, 1 H, 2b-H), 1.55 (overlapped, 1 H, 9b-H), 1.52 (overlapped, 1 H, 8b-H), 1.21 (d, *J* = 6.9 Hz, 3 H, 14-H), 1.18 (s, 3 H, 15-H), 1.16 (d, *J* = 6.9 Hz, 3 H, 13-H) ppm. ^{13}C NMR (125 MHz,$CDCl_3$, 25 °C): δ = 218.8 (C-4), 178.6 (C-12), 86.2 (C-1), 80.3 (C-6), 58.3 (C-5), 47.4 (C-7), 43.1 (C-10), 40.0 (C-11), 34.1 (C-3), 32.2 (C-9), 28.5 (C-8), 26.0 (C-2), 18.8 (C-15), 18.1 (C-14), 16.1 (C-13) ppm. MS (ESI): *m*/*z* = 289 $[M + Na]^+$. HRMS (ESI): calcd. for $C_{15}H_{22}NaO_4$ $[M + Na]^+$ 289.1416; found 289.1412.

Methylation of Ambroxetane: To compound **10** (2.0 mg) was added a saturated solution of CH_2N_2 in Et_2O dropwise. After removal of the organic solvent the residue was purified by HPLC (*n*-hexane/EtOAc, 95:5,;flow 0.8 mL/min) to afford pure ambroxetane methylester (2.1 mg). Data for methylambroxetane: $[\alpha]_D$ = +4.2 (*c* = 0.1, $CHCl_3$). ^1H NMR (500 MHz,$CDCl_3$, 25 °C): δ = 4.72 (d, *J* =

3.8 Hz, 1 H, 6-H), 3.69 (s, 3 H, 12-OMe), 2.77 (overlapped, 1 H, 11-H), 2.82 (overlapped, 1 H, 7-H), 2.70 (ddd, *J* = 9.5, 65, 4.2 Hz, 1 H, 3a-H), 2.47 (overlapped, 1 H, 3b-H), 2.47 (overlapped, 1 H, 2a-H), 2.09 (m, 1 H, 10-H), 2.02 (m, 1 H, 8a-H), 1.69 (m, 1 H, 9a-H), 1.60 (m, 1 H, 2b-H), 1.55 (overlapped, 1 H, 9b-H), 1.52 (overlapped, 1 H, 8b-H), 1.21 (d, *J* = 6.9 Hz, 3 H, 14-H), 1.18 (s, 3 H, 15-H), 1.18 (d, *J* = 6.9 Hz, 3 H, 13-H) ppm. MS (ESI): *m*/*z* = 303 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{16}H_{24}NaO_4$ [M + Na]$^+$ 303.1572; found 303.1573.

2,3-Dihydro,2,3-dihydroxylasidiol Anisate (13): $[\alpha]_D$ = +17.0 (*c* = 0.1, $CHCl_3$). ^{1}H NMR (500 MHz,$CDCl_3$, 25 °C): δ = 8.00 (d, *J* = 8.7 Hz, 2 H, 4′-H, 6′-H), 6.97 (d, *J* = 8.7 Hz, 2 H, 3′-H, 7′-H), 5.33 (d, *J* = 4.6 Hz, 1 H, 1-H), 4.32 (d, *J* = 4.6 Hz, 1 H, 2-H), 3.88 (s, 3 H, 5′-OMe), 2.78 (m, 1 H, 5a-H), 2.49 (m, 1 H, 4a-H), 1.82 (m, 1 H, 11-H), 1.74 (overlapped, 2 H, 9-H), 1.72 (overlapped, 1 H, 5b-H), 1.57 (overlapped, 1 H, 4b-H), 1.53 (overlapped, 1 H, 10-H), 1.50 (s, 3 H, 15-H), 1.37 (overlapped, 1 H, 8a-H), 1.32 (overlapped, 1 H, 8b-H), 1.23 (s, 3 H, 14-H), 1.07 (d, *J* = 6.9 Hz, 3 H, 12-H), 0.82 (d, *J* = 6.9 Hz, 3 H, 13-H) ppm. ^{13}C NMR (125 MHz,$CDCl_3$, 25 °C): δ = 165.6 (C-1′), 163.5 (C-5′), 131.7 (C-4′, C-6′), 122.0 (C-2′), 113.8 (C-3′, C-7′), 90.1 (C-6), 86.0 (C-3), 76.1 (C-1), 62.5 (C-2), 55.5 (5′-OMe), 52.5 (C-10), 49.1 (C-7), 35.5 (C-5), 30.3 (C-4), 28.6 (C-8), 26.6 (C-11), 25.4 (C-9), 24.7 (C-15), 23.3 (C-12), 22.2 (C-14), 21.6 (C-13) ppm. MS (ESI): *m*/*z* = 429 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{23}H_{34}NaO_6$ [M + Na]$^+$ 429.2253; found 429.2249.

5-Oxo-8α-hydroxy-1-epiartemorin (14): $[\alpha]_D$ = +7.2 (*c* = 0.1, $CHCl_3$). ^{1}H NMR (500 MHz,$CDCl_3$, 25 °C): δ = 6.30 (br. d, *J* = 3.1 Hz, 1 H, 13a-H), 6.05 (br. d, *J* = 2.7 Hz, 1 H, 13b-H), 5.82 (br. s, 1 H, 15a-H), 5.63 (br. s, 1 H, 15b-H), 5.43 (br. s, 1 H, 14a-H), 5.38 (br. s, 1 H, 14b-H), 4.72 (dd, *J* = 5.5, 5.0 Hz, 1 H, 1-H), 4.66 (d, *J* = 7.8 Hz, 1 H, 6-H), 4.33 (m, 1 H, 8-H), 3.15 (m, 1 H, 7-H), 3.08 (dd, *J* = 6.2, 2.0 Hz, 1 H, 9a-H), 2.60 (m, 1 H, 3a-H), 2.39 (overlapped, 1 H, 2a-H), 2.35 (overlapped, 1 H, 2b-H), 2.33 (overlapped, 1 H, 3b-H), 2.32 (overlapped, 1 H, 9b-H) ppm. ^{13}C NMR (125 MHz,$CDCl_3$, 25 °C): δ = 206.5 (C-5), 171.7 (C-12), 145.1 (C-10), 144.1 (C-4), 136.5 (C-11), 125.8 (C-13), 123.2 (C-15), 115.8 (C-14), 86.8 (C-6), 82.1 (C-1), 71.1 (C-8), 49.3 (C-7), 40.3 (C-9), 32.5 (C-2), 22.8 (C-3) ppm. MS (ESI): *m*/*z* = 301 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{15}H_{18}NaO_5$ [M + Na]$^+$ 301.1052; found 301.1049.

Diacetyl Artemisiifolinic Acid (15b): $[\alpha]_D$ = +32.2 (*c* = 0.15, $CHCl_3$). ^{1}H NMR (500 MHz,$CDCl_3$, 25 °C): δ = 6.40 (br. s, 1 H, 13a-H), 5.92 (br. s, 1 H, 13b-H), 5.30 (overlapped, 1 H, 5-H), 5.29 (overlapped, 1 H, 6-H), 5.00 (d, *J* = 11.5 Hz, 1 H, 15a-H), 4.60 (d, *J* = 11.5 Hz, 1 H, 15b-H), 4.30 (m, 1 H, 8-H), 3.18 (dd, *J* = 4.5, 1.5 Hz, 1 H, 7-H), 2.70 (m, 1 H, 10-H), 2.46 (m, 1 H, 3a-H), 2.33 (m, 1 H, 3b-H), 2.18 (m, 1 H, 9a-H), 2.09 (s, 6 H, 6-OAc, 15-OAc), 1.78 (m, 1 H, 9b-H), 1.65 (m, 1 H, 1a-H), 1.50 (overlapped, 1 H, 2a-H), 1.46 (overlapped, 1 H, 1b-H), 1.43 (overlapped, 1 H, 2b-H) ppm. ^{13}C NMR (125 MHz,$CDCl_3$, 25 °C): δ = 178.6 (C-14), 172.0 (6-OAc), 171.6 (15-OAc), 168.6 (C-12), 139.9 (C-4), 135.3 (C-11), 128.5 (C-5), 126.5 (C-13), 77.1 (C-8), 72.4 (C-6), 62.8 (C-15), 46.8 (C-7), 40.2 (C-10), 33.5 (C-3), 25.5 (C-2), 21.8 (C-1), 21.6 (6-OAc), 21.5 (15-OAc), 19.5 (C-9) ppm. MS (ESI): *m*/*z* = 403 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{19}H_{24}NaO_8$ [M + Na]$^+$ 403.1369; found 403.1373.

Diacetyl Isoartemisiifolinic Acid (16b): $[\alpha]_D$ = –4.1 (*c* = 0.2, $CHCl_3$). ^{1}H NMR (500 MHz,$CDCl_3$, 25 °C): δ = 6.39 (br. s, 1 H, 13a-H), 5.93 (br. s, 1 H, 13b-H), 5.40 (m, 1 H, 6-H), 5.32 (m, 1 H, 5-H), 4.80 (br. s, 2 H, 15-H), 4.13 (m, 1 H, 8-H), 3.07 (dd, *J* = 4.5, 1.5 Hz, 1 H, 7-H), 2.72 (m, 1 H, 10-H), 2.46 (m, 1 H, 3a-H), 2.33 (m, 1 H, 3b-H), 2.09 (m, 1 H, 9a-H), 2.08 (s, 3 H, 6-OAc), 2.07 (s, 3 H, 15-OAc), 1.65 (m, 1 H, 1a-H), 1.59 (m, 1 H, 9b-H), 1.50 (overlapped, 1 H, 2a-H), 1.46 (overlapped, 1 H, 1b-H), 1.43 (overlapped, 1 H, 2b-H) ppm. ^{13}C NMR (125 MHz,$CDCl_3$, 25 °C): δ = 178.6 (C-14), 172.0 (6-OAc), 171.6 (15-OAc), 168.6 (C-12), 145.5 (C-4), 135.3 (C-11), 126.6 (C-13), 113.2 (C-15), 79.5 (C-5), 78.0 (C-8), 72.0 (C-6), 46.3 (C-7), 40.2 (C-10), 34.5 (C-3), 25.5 (C-2), 21.8 (C-1), 21.8 (5-OAc), 21.4 (6-OAc), 19.9 (C-9) ppm. MS (ESI): *m*/*z* = 403 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{19}H_{24}NaO_8$ [M + Na]$^+$ 403.1369; found 403.1366.

Cysteamine Assay: In a standard 5 mm NMR tube (Armar Chemicals), the substrate (or an equimolecular mixture of substrates for the comparative trapping experiments) was dissolved in [D_6]DMSO (500 μL), and the spectrum was registered. Cysteamine (1.5 mol. equiv.) was next added, and the spectrum was re-registered 5 min after the addition. A positive assay was evidenced by the disappearance of the peculiar AB-olefin system of the carbonyl-conjugated exomethylene of the substrate in the δ = 5.50–6.20 ppm region.[7]

TRPA1 Receptor Assay: HEK293 embryonic kidney cells were grown as monolayers in minimum essential medium supplemented with nonessential amino acids, 10% fetal calf serum, and 2 mM glutamine, maintained under 5% CO_2 at 37 °C plated on 100-mm diameter Petri dishes. The cells were transfected at approximately 80% confluence with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) by using a plasmid pcDNA3 (Invitrogen) containing the rat TRPA1-cDNA according to the manufacturer's protocol. Stably transfected clones were selected by G-418 (Geneticin; 600 μg/mL). The effect of the substances on $[Ca^{2+}]_i$ was determined by using Fluo-4, a selective intracellular fluorescent probe for Ca^{2+}. At this aim, on the day of the experiment, cells overexpressing TRPA1 channels were loaded for 1 h at room temperature with the methyl ester Fluo4-AM (4 μM; Invitrogen) in minimum essential medium without foetal bovine serum. After the loading, cells were washed twice in Tyrode's buffer (145 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES, pH 7.4), resuspended in Tyrode's buffer, and transferred (50–60000 cells) to the quartz cuvette of the spectrofluorimeter (Perkin–Elmer LS50B; PerkinElmer Life and Analytical Sciences, Waltham, MA) under continuous stirring. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined before and after the addition of various concentrations of test compounds by measuring cell fluorescence at 25 °C (λ_{EX} = 488 nm, λ_{EM} = 516 nm). Curve fitting (sigmoidal dose-response variable slope) and parameter estimation were performed with GraphPad Prism® (GraphPad Software Inc., San Diego, CA). Potency was expressed as the concentration of test substances exerting a half-maximal agonist effect (i.e. half-maximal increases in $[Ca^{2+}]_i$) (EC_{50}), calculated by using GraphPad®. The efficacy of the agonists was first determined by normalizing their effect to the maximum Ca^{2+} influx effect on $[Ca^{2+}]_i$ observed with application of 4 μM ionomycin (Sigma). The effects of TRPA1 agonists are expressed as a percentage of the effect obtained with 100 μM allyl isothiocyanate. When significant, the values of the effect on $[Ca^{2+}]_i$ in wild-type (i.e. not transfected with any construct) HEK293 cells were taken as baseline and subtracted from the values obtained from transfected cells. All determinations were at least performed in triplicate. Statistical analysis of the data was performed by analysis of variance at each point using ANOVA followed by Bonferroni's test.

Supporting Information (see footnote on the first page of this article): Original NMR spectra for all the new compounds and for thiol trapping experiments.

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