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Induction of allergic contact dermatitis by astigmatid mite-derived monoterpene, α-acaridial

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ABSTRACT

α-Acaridial [2(*E*)-(4-methyl-3-pentenyl)butenedial] is a novel monoterpene secreted from the house dust mites. Because of its molecular nature of a highly reactive, small lipidic compound, we addressed whether α-acaridial might function as a haptenic allergen that induced allergic contact dermatitis. Mice sensitized with α-acaridial were challenged by the same antigen on the ear skin. After 2 days, significant ear swelling with a prominent infiltration of $CD4^+$ T lymphocytes was observed. *In vitro*, α-acaridial exhibited an outstanding ability to quickly interact with and chemically modify a reference protein. Virtually all cysteine residues and a sizable fraction of lysine residues were found to be selectively modified, suggesting that α-acaridial could potentially interact with any proteins. Previously, numerous mite-derived proteinaceous allergens have been associated with contact dermatitis. Our study now emphasizes that small lipidic compounds released from mites comprise a new class of mite allergens, and therefore, is of significant medical implications.

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House dust mites are a major source of allergens associated with human allergic diseases such as atopic dermatitis, bronchial asthma, and perennial rhinitis. Allergens from diverse species of house dust mites have been extensively investigated [1], and at least 17 allergen groups have been found from *Dermatophagoides ptronyssinus* and *Dermatophagoides farinare* (Acari:Pyroglyphidae). All of these defined antigens are derived from the mite proteins. However, a few studies have suggested a new class of mite-derived allergens that are non-proteinaceous and organic solvent soluble [2,3]. Indeed, these non-proteinaceous components of the house dust mites exhibited positive patch tests in some patients with atopic dermatitis, raising the possibility that, besides proteinaceous allergens, small lipidic molecules from the house dust mites might be associated with allergic reactions.

One of these lipidic components that have been implicated in allergic reactions is α-acaridial [2(*E*)-(4-methyl-3-pentenyl) butenedial] (Fig. 1A) which we have identified recently as a novel monoterpene from the acarid mite, *Tyrophagus pernicious* [4] and also from the house dust mite, *Tyrophagus putrescentiae* (Acari:Acaridae) [5]. α-Acaridial contains the butenedial moiety, as found in 4-oxo-2(*E*)-nonenal (4ONE, Fig. 1C), a product of lipid oxidation [6,7] that is known to attack and chemically modify protein side chains [8–10]. Modification of proteins and other biochemical molecules by lipid peroxidation products is proposed to play a critical role in inflammation and tissue damage [11]. Moreover, a recent study [12] has showed that 4ONE and the related compound, 4-hydroxy-2(*E*)-nonenal (4HNE, Fig. 1D) generate nonenal-modified peptides and act as haptenic determinants for antibody responses. On the basis of these pieces of knowledge, we hypothesized that α-acaridial might be capable of chemically modifying self proteins and function as a hapten recognized by T lymphocytes, thereby inducing atopic dermatitis in house dust mite-sensitized individuals. Here we report that α-acaridial showed high reactivity to a reference protein, bovine serum albumin (BSA), via direct interactions with cysteine and lysine residues. Further, we established the murine model of α-acaridial-induced contact dermatitis with local infiltration of $CD4^+$ T lymphocytes, which experimentally represented positive patch test of patients with atopic dermatitis. To our knowledge, this is the first report to clearly define a non-protein compound derived from house dust mites as an allergen capable of inducing allergic contact dermatitis.

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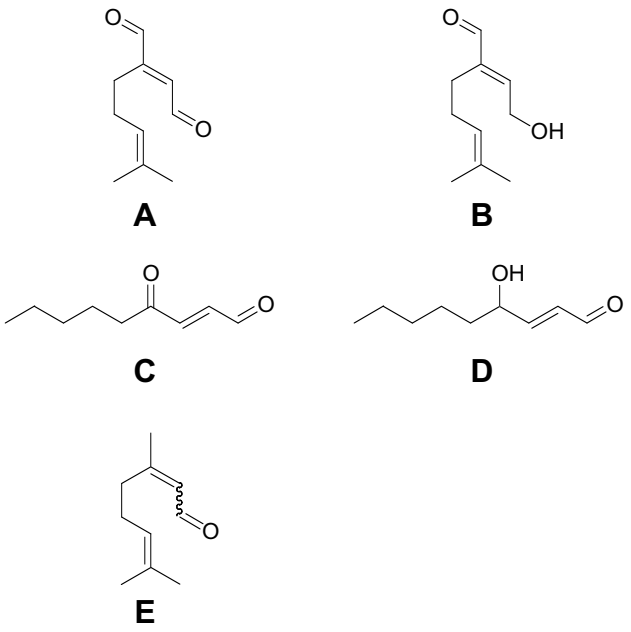


Fig. 1. Structures of α-acaridial (A), α-acariolal (B), 4-oxo-2(*E*)-nonenal (4ONE) (C), 4-hydroxy- 2(*E*)-nonenal (4HNE) (D), and citral (E).

Materials and methods

Synthesis of α-acaridial and α-acariolal. α-Acaridial [2(*E*)-(4-methyl-3-pentenyl)butenedial] was synthesized as previously reported [13] with some modifications. Briefly, a mixture of pyruvic aldehyde dimethyl acetal and cyclohexylamine in toluene was stirred at refluxing temperature in Dean-Stark apparatus. The obtained imine was reacted with lithium diisopropylamide in tetrahydrofuran at −78 °C for 1 h, then prenyl bromide was added [14]. After stirring at −50 °C for another 20 min, the mixture was warmed to room temperature within 6 h, and then the mixture was poured into chilled water, and extracted with ether. After silica-gel purification of the extracts, 1,1-dimethoxy-6-methyl-5-hepten-2-one was obtained. α-Acaridial and α-acariolal [(*E*)-2-(2-hydroxyethylidene)-6-methyl-5-heptanal] (Fig. 1B) were prepared from the ketone as previously reported, and the ^{1}H NMR spectra were in agreement with published results [13]. ^{1}H NMR (400 MHz) spectra were recorded with tetramethyl silane as an internal standard in $CDCl_3$ by using a Bruker AC400 spectrometer.

Induction of allergic contact dermatitis in mice with α-acaridial and α-acariolal. Eight-week-old female BALB/c mice (Japan SLC Inc., Shizuoka, Japan) were first sensitized by applying 25 μl of 1% α-acaridial or α-acariolal in acetone/olive oil (4:1, v/v) to the shaved mouse abdomen skin. Five days later, mice were challenged with 10 μl of the same antigen preparations in acetone/olive oil on the dorsal surface of the left ear while the corresponding area of the right ear was painted with an identical amount of the solvent alone (acetone/olive oil). Ear thickness was measured at 0, 2, 4, and 7 days after challenge, and the results were expressed as the net ear swelling, which was calculated by subtracting the thickness of the right ear from the thickness of the left ear. Mock-treated mice received the solvent alone for both sensitization and challenge. Data are shown as means ± SD ($N = 4$). Student's *t*-test was used for the statistical evaluation of the results.

Histology and immunochemistry. Skin samples were collected 2 days after challenge, and paraffin embedded for standard histological staining with hematoxylin and eosin. Separately, some skin samples were deep frozen in OCT compound, and the cryosections were fixed with 4% paraformaldehyde. The samples were then labeled with biotin-conjugated rat monoclonal antibodies to CD4 (clone GK1.5), followed by incubation with streptavidin-fluorescein isothiocyanate (both from Becton–Dickinson, Franklin Lakes, NJ). After washing, the labeled sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and viewed under fluorescence microscope.

Incubation of BSA with α-acardial. A 1.0 ml aliquot of α-acaridial (3 mM of 20 mM sodium phosphate buffer/acetonitrile, 3:1, v/v, pH 5.5) was incubated with BSA (75 μM, 1 ml) solution at 36 °C for 1, 3, 9, 24, 30, and 100 h (hereafter referred to as "mixture A"). To monitor the amount of the remaining unbound form of α-acardial, a 200-μl aliquot of the mixture was extracted with hexane (500 μl) containing 5-methyl-2-octanone as an internal standard (80 μg/ml), and then, 1 μl of the upper layer was immediately injected into a Hewlett-Packard HP-6850 gas chromatograph, using an HP-5 capillary column (30 m × 0.25 mm ID, with 0.25 μm thickness; Hewlett-Packard, USA) and helium was used as the carrier gas. The injector was kept at 240 °C and the detector at 300 °C. The temperature of the column oven was programmed to increase at 10 °C/min from 60 to 290 °C. A related, but non-allergic, compound, citral [(*E*)- and (*Z*)-3,7-dimethyl-2,6-octadienal, Fig. 1E], was also used for comparison purposes.

Reaction of α-acaridial with amino acid analogs. To determine which amino acid residues in proteins were attacked by α-acardial, amino acid analogs, imidazole, ethanol, guanidine, diethyldisulfide, 1-butylamine, and 1-butanethiol, were used. A 1.0 ml aliquot of α-acaridial (6 mM of 20 mM sodium phosphate buffer/acetonitrile, 2:1, v/v, pH 5.5) was incubated with solutions (1.0 ml) of the amino acid analogs (6 mM of 20 mM sodium phosphate buffer/acetonitrile, 2:1, v/v, pH 5.5) at 36 °C for 1, 3, 9, and 24 h (hereafter referred to as "mixture B"). The amount of the remaining free form of α-acardial was monitored as described above.

Structural analyses of α-acaridial adducts of butylamine and butanethiol. Reaction mixtures (1.0 ml, 20 mM sodium phosphate buffer/acetonitrile, 2:1, v/v, pH 5.5) containing α-acardial (30 mg, 180 μmol) and either butylamine (13.2 mg, 180 μmol) or butanethiol (16.2 mg, 180 μmol) were incubated at 36 °C for 24 h. The reaction mixtures were then extracted with ethyl acetate, and the organic layers were washed with brine, dried over anhydrous sodium sulfate, and evaporated to dryness. The crude residues were chromatographed on silica-gel, using *n*-hexane/ether (2:1, v/v) to isolate main products. The isolated α-acaridial adducts of butylamine and butanethiol were characterized by ^{1}H NMR and gas chromatograph–mass spectrometry (GC–MS) analyses. GC–MS analyses were conducted on an HP-5890 plus series II gas chromatograph with a 30 m × 0.32 mm, 0.33 μm film thickness, HP-5MS capillary column, interfaced to an HP-5989B mass spectrometer (Hewlett-Packard, USA) operated at 70 eV in the split-less mode. The temperature settings of the injector and the column are as indicated above.

Analyses of amino acid composition of the α-acaridial modified BSA. The amino acid composition was determined by GC–MS analyses based on a published methodology that readily achieved both high recovery rate and high resolution [15,16]. After 30 h incubation, an aliquot (100 μl) of mixture A was treated with 10% trichloroacetic acid (200 μl) and precipitated protein was then hydrolyzed with 6 N HCl (500 μl) at 110 °C for 72 h under nitrogen atmosphere. One portion of the hydrolyzed samples (200 μl) was evaporated to dryness, and was neutralized with 75 μl of 4 M ammonia solution and 75 μl of MeOH. After evaporation, 60 μl of H_2O and 40 μl of ethanol/pyridine (4:1, v/v) were added to the samples, and then, 5 μl of ethyl chloroformate was added. The samples were mixed gently and kept in room temperature for 30 min. Subsequently, 200 μl of chloroform containing 1% ethyl chloroformate was added, and the derivatives were extracted into

the organic layer by vigorous shaking. For qualitative and quantitative analyses of amino acids, an aliquot (1 μl) of the organic phase was analyzed by GC–MS. The temperature of the column oven was programmed as follows: 80 °C for the initial 4 min, followed by increment to 280 °C at a constant rate of 10 °C/min and 280 °C for the final 5 min. Relative intensities of each amino acid to that of valine are calculated and % decreases of each amino acid [{1 − (relative intensity of α-acaridial-treated sample)/(relative intensity of untreated sample)} × 100] are expressed as means ± SD ($N = 4$). To determine the remaining cysteine content of α-acaridial-modified BSA, the sulfhydryl assay was modified from Robyt et al. [17]. An aliquot (100 μl) of mixture A was treated with 10% trichloroacetic acid (200 μl). The precipitated protein was dissolved in 0.9 ml of 50 mM Tris buffer (pH 8.0) containing 8 M guanidine hydrochloride and 10 mM EDTA. Immediately after the addition of 0.1 ml of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) in 100 mM potassium phosphate buffer (pH 7.6), the absorbance at 412 nm was measured. Percent decrease of cysteine by treatment of α-acaridial was measured and shown as means ± SD ($N = 4$), as shown above. Citral was also used for comparison experiments.

Results and discussion

High levels of allergen-specific T lymphocyte reactions are a hallmark of contact dermatitis and are detected as a form of delayed-type hypersensitivity in the antigen-challenged skin. Therefore, to examine the capacity of α-acaridial to mediate induction of contact dermatitis, we first addressed whether α-acaridial-sensitized mice could develop delayed-type hypersensitivity reactions. Sensitization was performed by applying the antigen on the abdominal skin, followed by challenge with the same antigen preparation on the ear skin. As shown in Fig. 2A, α-acaridial-sensitized mice developed significant ear thickness 2 days after the challenge with α-acaridial ($P = 0.02$). In sharp contrast, mice that were sensitized with a related compound, α-acariolal, mounted no apparent skin reactions against the challenged antigen, underscoring a potential of α-acaridial to induce specific skin reactions. Histological studies of the α-acaridial-challenged skin detected local infiltration of mononuclear cells as well as vasodilatation and edematous tissue changes (Fig. 2C), which were absent in control mice either challenged with α-acariolal (Fig. 2D) or mock treated (Fig. 2B). Immunofluorescence microscopic analysis revealed that the infiltrating mononuclear cells were mostly $CD4^+$ T lymphocytes (Fig. 2F), and few $CD8^+$ T lymphocytes were detected (data not shown). These observations represented typical features for delayed-type hypersensitivity directed against protein antigens. Because α-acaridial was a non-proteinaceous small molecule, we reasoned that α-acaridial might chemically modify self proteins. Recent studies of lipid peroxidation products demonstrated that 4ONE which has a butenedial moiety similarly to α-acaridial, acts as a haptenic determinant [12]. In this case, however, 4HNE that is structurally similar to 4ONE, but a mono-aldehyde, also acts as a hapten that stimulates humoral immune responses. In contrast, α-acariolal, a mono-aldehyde analog of α-acaridial, did not induce any inflammatory response in the sensitized mouse, implicating that structural differences between the mite monoterpenes and linoleic acid peroxidation products could affect the mode of protein modification and the types of immune responses.

To obtain a molecular basis for the haptenic potential of α-acaridial, we examined its reactivity to proteins in a buffer system. α-Acaridial and citral were separately incubated with BSA in the phosphate buffer/acetonitrile mixture at 36 °C, and their reactivity to BSA was assessed by measuring the amount of their unconjugated free form. Whereas approximately 70% of the citral was detected as an unconjugated form even after 100 h of incubation, the free form of α-acaridial totally disappeared after 30 h (Fig. 3A), indicating that α-acaridial could much more efficiently reacted with amino acid residues of proteins than citral.

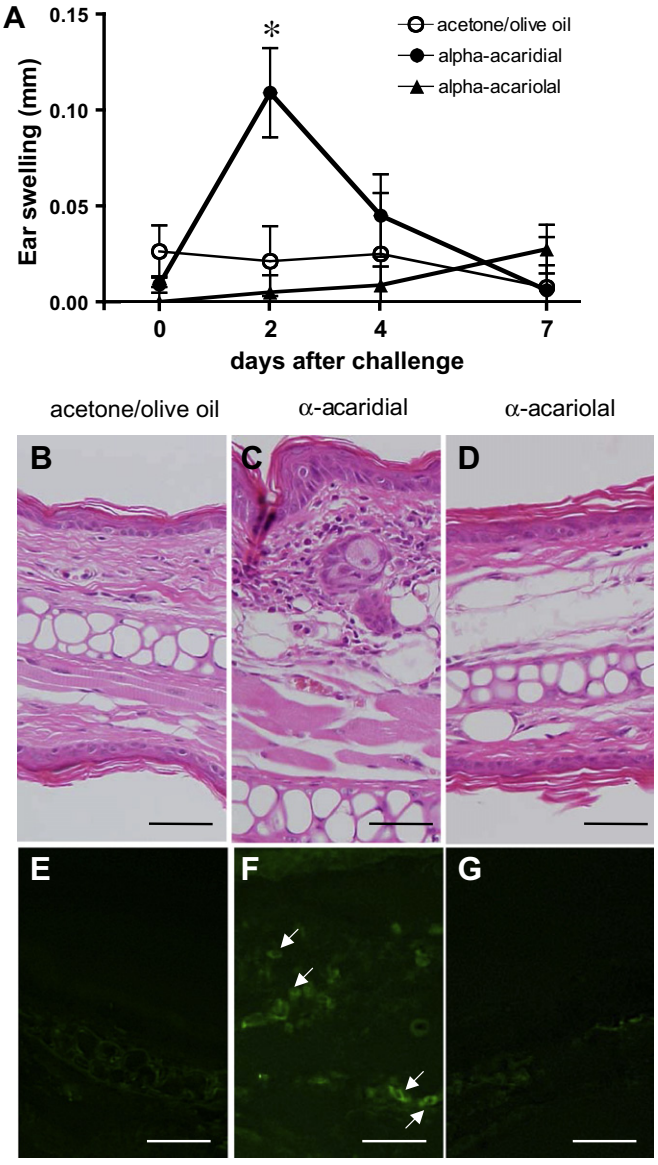


Fig. 2. Induction of allergic contact dermatitis in mice with α-acaridial. Mice (4 in each group) were sensitized with either α-acaridial (filled circles), α-acariolal (filled triangles) or vehicle alone (open circles), and challenged on the ear skin with the same preparations. The net ear swelling was monitored before and 2, 4, and 7 days after challenge. The α-acaridial sensitized and challenged mice elicited a significant ear swelling at 2 days ($^*P < 0.05$) (A). Two days after challenge with either α-acaridial (C,F), α-acariolal (D,G) or vehicle alone (B,E), the challenged skin was subjected to hematoxylin–eosin staining (B–D) and immunofluorescence microscopic analysis with anti-CD4 antibodies (E–G). A significant swelling and vasodilatation as well as infiltration of mononuclear cells were observed in the α-acaridial challenged skin (C). Labeling with specific antibodies revealed that the infiltrating cells were mostly $CD4^+$ T lymphocytes (F, indicated with arrows). Scale bars, 50 μm.

Next, we used amino acid analogs to gain insight into what amino acids reacted with α-acaridial. We used imidazole, ethanol, guanidine, diethyldisulfide, 1-buthylamine, and 1-butanethiol which mimic histidine, serine (or threonine), arginine, cystine, lysine, and cysteine side chains, respectively. When incubated for 10 h with butylamine or butanethiol, the free form of α-acaridial de-

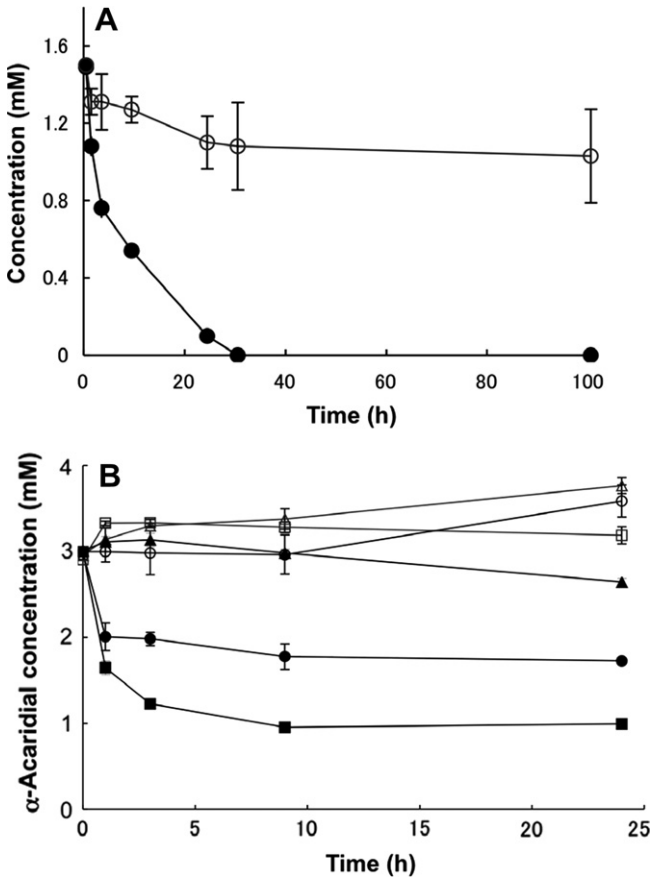


Fig. 3. Reactivity of α-acaridial to BSA and amino acid analogs. (A) α-Acaridial (filled circle) and citral (open circle) were separately incubated with BSA at 36 °C for 1, 3, 9, 24, 30, and 100 h. At each time point, the amount of the remaining unbound form was determined by GC with 5-methyl-2-octanone as an internal standard. The results are shown as means ± SD of three replications. (B) Reactivity of α-acaridial to imidazole (open triangle), ethanol (open circle), guanidine (open square), diethyldisulfide (filled triangle), 1-buthylamine (filled circle), and 1-butanethiol (filled square) was monitored similarly as in (A).

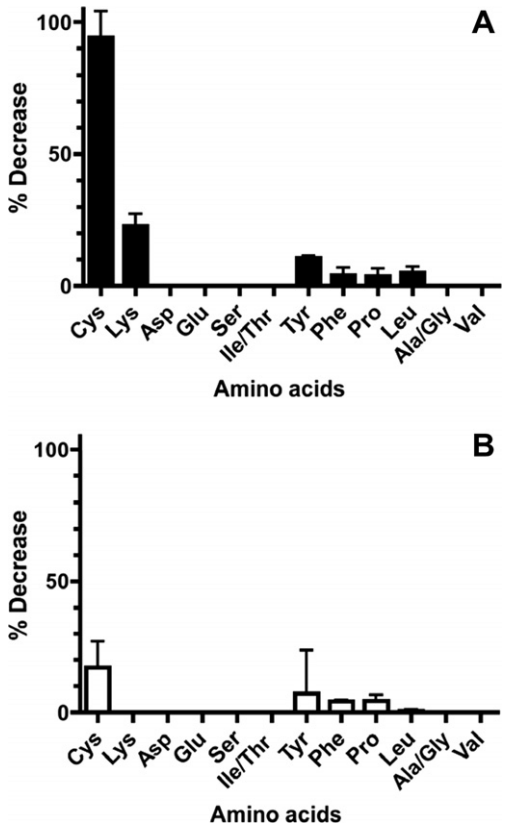


Fig. 4. Specific reactivity of α-acaridial to cysteine and lysine. (A) Amino acid compositions of the α-acaridial-modified BSA and the untreated BSA were determined by the retention time and fragmentation pattern of GC–MS analyses after ECEE derivatization. Amounts of remaining cysteine after the treatments with α-acardial were determined by the modified sulfhydryl assay. Values indicate % decrease of each amino acid as compared to untreated BSA, and are shown as means ± SD ($N = 4$). (B) Following incubation of BSA with citral, the remaining amounts of amino acids were determined similarly as in (A). Values indicate % decrease of each amino acid as compared to untreated BSA, and are shown as means ± SD ($N = 4$).

tected in the mixture was significantly diminished by 66% and 33%, respectively (Fig. 3B). On the other hand, α-acaridial showed low reactivity to imidazole, ethanol, guanidine, and diethyldisulfide. These results indicated that α-acaridial could selectively react to lysine and cysteine residues. To gain further insight into the molecular structure of the α-acaridial adducts of butylamine and butanethiol, the crude extracts were separated by thin-layer chromatography on silica-gels and the main products were isolated by column chromatography. The isolated α-acaridial adducts of butylamine and butanethiol were identified as *N*-butyl-3-(4-methyl-3-pentenyl)pyrrole and 2-butylthio-3-(4-methyl-3-pentenyl)furan, respectively. *N*-Butyl-3-(4-methyl-3-pentenyl)pyrrole: ^{1}H NMR ($CDCl_3$) δ 0.92 (3H, t, $J = 7.3$ Hz), 1.30 (4H, m), 1.57 (3H, s), 1.67 (3H, s), 2.25 (2H, m), 2.47 (2H, t, $J = 6.9$ Hz), 3.79 (2H, t, $J = 7.2$ Hz), 5.20 (1H, t, $J = 6.9$ Hz), 5.98 (1H, t-like, $J = 2.1$ Hz), 6.42 (1H, br s), 6.54 (1H, t-like, $J = 2.1$ Hz). GC–MS (t_R 13.5 min) *m/z* (%) 205 (M^+, 14.7), 136 (100), 94 (16.0), 80 (14.1). 2-Butylthio-3-(4-methyl-3-pentenyl)furan: ^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, $J = 7.3$ Hz), 1.40 (2H, m, $J = 7.5$ Hz), 1.52 (2H, m, $J = 7.5$ Hz), 1.56 (3H, s), 1.68 (3H, s), 2.20 (2H, q, $J = 7.5$ Hz), 2.50 (2H, t, $J = 7.2$ Hz), 2.68 (2H, t, $J = 7.4$ Hz), 5.15 (1H, t, $J = 7.1$ Hz), 6.31 (1H, d, $J = 1.6$ Hz), 7.41 (1H, d, $J = 1.6$ Hz). GC–MS (t_R 15.1 min) *m/z* (%) 238 (M^+, 16.1), 169 (66.1), 113 (100), 41 (25.0). The isolated yields were 5% and 10% (based on α-acaridial) for *N*-butyl-3-(4-methyl-3-pentenyl)pyrrole and 2-butylthio-3-(4-methyl-3-pentenyl)furan, respectively.

Further, we analyzed α-acaridial-modified amino acids in BSA after incubation with α-acaridial. Among amino acids examined, 94.3% of cysteine and 22.8% of lysine were markedly lost upon the treatment, as compared with the untreated BSA (Fig. 4A). This result was consistent with the results using amino acid analogs: thiol or amine groups were more reactive for α-acaridial. In contrast to α-acaridial, citral poorly reacted with BSA, in which reactivity to cysteine and lysine were much less than α-acaridial (Fig. 4B, also see Fig. 3A). The differential reactivity toward BSA between α-acaridial and citral indicated that the butenedial moiety of α-acaridial could potentially be critical in the modification of proteins as in the case of 4ONE.

When 4ONE is incubated with amino acid analogs [10] or the model peptide [9,18], the aldehyde reacts primarily via Michael addition to peptide nucleophiles to yield a covalent adduct. In this Michael reaction, the addition occurs at the central double bond, more preferentially at C3 than at C2, to generate substituted aldehydes [9,10]. Furthermore, the C1 carbonyl moiety of the C3 substituted aldehyde reacts to the ε-NH_2 groups of lysyl residues to form Schiff bases, ending up with the generation of nucleophile-substituted pyrroles [8,10,19]. Indeed, when apomyoglobin is incubated with 4ONE, four types of adducts are detected: 4ONE-histidine Michael adduct, 4ONE-lysine pyrrolinone adduct, 4ONE-histidine-lysine pyrrole cross-link, and an unidentified lysine adduct [20]. In the case of α-acaridial, however, none of the α-acaridial-nucle-

ophiles Michael adducts was found as main compounds. Instead of Michael-type adducts, *N*-butyl-3-(4-methyl-3-pentenyl)pyrrole (yield, 5%) and 2-butylthio-3-(4-methyl-3-pentenyl)furan (yield, 10%) were identified as main compounds when α-acaridial was incubated with butylamine and butanethiol, respectively. The former pyrrole compound might be formed by reactions in which the C1 or C4 carbonyl moiety of α-acaridial reacted to the ε-NH_2 groups of lysyl residues to form a Schiff base, and then the Schiff base would form a pyrrole ring, through a reaction obviously driven by aromatic stabilization in a way that was parallel to 4ONE reacting to amino acid analogs [10,19,20]. Regarding the latter furan compound, it has been suggested that thiohemiacetal is generated first, followed by the furan ring formation through cyclization and aromatization of the acetal, as previously reported for 4ONE reacting to imidazole and butylamine [10]. These results suggest that α-acaridial and 4ONE could show comparable, but substantially distinct modes of reactivity to proteins or peptide.

Difference of the biological outputs in mice by α-acaridial and α-acariolal might be explained simply by differential reactivity of these compounds to target proteins as seen in 4ONE and 4HNE, which are known to react with their target molecules, such as butanethiol and buthylamine, at significantly different kinetic constants [21]. It is also possible that the repertoire of T-cell receptors developed in mice may be substantially smaller for α-acariolal than for α-acaridial. It is noteworthy that α-acaridial is actively secreted from living mites. Therefore, unlike 4ONE that is produced via the oxidative processes from linoleic acid, the secreted α-acaridial readily makes a direct interaction with host proteins without requirements for any prior metabolic processes.

The present study has established a murine model of contact dermatitis with a mite-derived lipidic compound, α-acaridial, and provided the fine molecular basis for its biological function. Identification of a new class of non-protein mite allergens sheds light on a critical aspect of allergic contact dermatitis that has never been fully appreciated previously, and thus, is of significant medical implications.

Acknowledgments

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