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Structure-Pungency Relationships and TRP Channel Activation of Drimane Sesquiterpenes in Tasmanian Pepper (*Tasmannia lanceolata*)

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ABSTRACT

Sensory-guided fractionation of extracts of Tasmanian pepper berries revealed 20 drimane sesquiterpens, amongst which polygodial, warburganal, and 1β-acetoxy-9-deoxy-isomuzigadial exhibited the lowest pungency threshold concentrations on the tongue surface (0.6 - 2.8 nmol/cm^2) and elicited a dose-dependent calcium influx into mTRPA1 expressing CHO cells with the lowest EC_{50} values (4.5 ± 1.0 - 16.7 ± 7.5 µmol/L) and a good correlation to oral pungency thresholds (R^2 = 0.986, linear regression). Calcium imaging assays demonstrated these chemosensates to induce a calcium influx into cultured trigeminal neurons prepared from wildtype (TRPA1$^{+/+}$) mice, whereas no calcium influx was observed in neurons from TRPA1 knockout mice (TRPA1$^{-/-}$), thus confirming the α,β-unsaturated 1,4-dialdehyde structure to be the required structural motif for a low oral puncency thresholds and activation of the Transient Receptor Potential Channel A1 (TRPA1). Time-resolved NMR experiments confirmed the pungency mediating mechanism for electrophilic drimane sesquiterpene dialdehydes to be different from that found for other electrophilic pungent agents like isothiocyanates, which have been shown to undergo a covalent binding with cysteine residues in TRPA1. Instead, the high-impact chemosensates polygodial, warburganal, and 1β-acetoxy-9-deoxy-isomuzigadial showed immediate reactivity with the ε-amino group of lysine side chains to give pyrrole-type conjugates, thus showing evidence for TRPA1 activation by covalent lysine modification.

KEYWORDS: pungency, drimane, TRPA1, TRPV1, taste dilution analysis, half-tongue test, Tasmanian pepper*, Tasmannia lanceolata*

INTRODUCTION

Tasmanian Mountain Pepper (*Tasmannia lanceolata*) is a spice plant from the family of *Winteraceae* native to Tasmania and parts of Australia.[1] Often recognised as "bushfood", Tasmanian Pepper is part of a range of edible indigenous plants which have gained increasing popularity in modern cuisine.[2,3] Next their use as medicinal herb,[4,5] the lanceolate leaves as well as the aromatic berries of the large shrub are highly appreciated for their typical pungency and their characteristic aromatic and fresh-spicy odor,[5] which makes Tasmanian mountain pepper an appreciated ingredient for manufacturing of wasabi paste and chewing gum[6].

More than 50 years ago, the drimane sesquiterpene polygodial, **1** (Figure 1), featuring an α,β-unsaturated 1,4-dialdehyde structure, was isolated from Tasmanian pepper leaves[7] and was proposed as the key pungent principle of the berries.[8] Over the last decades, polygodial had been found in a range of botanicals, such as, e.g. water pepper (*Polygonum hydropiper*),[9] as well as in animal species,[10,11] and is widely discussed for its antifeedant[12] properties and its role as a predator deterrent.[13,14] Just as wide as the occurrence of drimane aldehydes in nature, are their biological activities, ranging from pungent taste[12] to antimicrobial,[15] antifungal,[16] antibiotic,[17] antifeedant,[18] antinociceptive,[19] and antihyperalgesic properties.[20] Several studies proposed the biological activity to be directly related to its pungent activity induced by the reactive α,β-unsaturated 1,4-dialdehyde moiety.[12,21,22]

Although chemical reactions of polygodial with nucleophiles such as, e.g. amino and mercapto groups,[23-28] have been suggested, defined reaction products could not yet be identified. More recent studies with pungent compounds from mustard and garlic revealed that isothiocyanates undergo a reversible covalent binding with the Transient Receptor Potential Channel A1 (TRPA1),[29,30] which

together with TRPV1 is a key receptor channel protein involved in the perception of pain, temperature and pungency.[31] Next to these two polymodic, non-selective cation channels, only recently TRPV1 and TRPA1 agonists, such as, e.g. piperine, capsaicin, 6-gingerol and polygodial, were found to possess a marked effect on two-pore domain (KCNK, K_{2P}) potassium channels,[32] which have been shown to "fine-tune" the cellular response to stimuli that active TRP channels.[33] Today it is well accepted that the reaction of pungent agonists, such as, e.g. isothiocyanates, sulfides, thiols and small reactive unsaturated aldehydes like acrolein, with cysteine residues plays a major role in TRPA1 activation. However, the unaltered channel activation by unsaturated dialdehydes in TRPA1 mutants in which some key cysteine residues were replaced,[31,34] suggests a fundamentally different TRPA1 activation mechanism by polygodial. Despite all recent efforts, the mechanism by which TRPA1 is activated by drimane sesquiterpenes as well as knowledge on structure-activity relationships within this class of components remain rather obscure.

Therefore, the objectives were to locate and identify the most intense pungent phytochemicals by application of a sensory-guided fractionation approach on Tasmanian mountain pepper, and to study structure-activity relationships by means of human sensory tests, TRP channel activation studies, and NMR-spectroscopic experiments on reactions between drimane sesquiterpenes and nucleophilic amino acid residues as candidate TRPA1 targets.

MATERIALS AND METHODS

Chemicals and Materials. Unless stated otherwise, all chemicals were obtained commercially from Sigma-Aldrich (Steinheim, Germany) and were of p.a. grade. *N*-

Acetyl-L-lysine, formic acid, diethyl ether, ethanol absolute, and aluminium oxide 90 (basic, 0.063-0.200 mm, activity 1, for column chromatography) were obtained from Merck (Darmstadt, Germany), deuterium oxide and chloroform (D100, 0.03% TMS) from Euriso-Top (Gif-Sur-Yvette, France), the solvents methanol, acetonitrile, ethyl acetate, and hexane used for high-performance liquid chromatography (HPLC) were HPLC grade and from J.T. Baker (Deventer, Netherlands), *N*-acetyl-L-arginine and polygodial from Santa Cruz Biotechnology (Heidelberg, Germany), nerve growth factor from Promega (Southampton, UK), penicillin-streptomycin and laminin from Invitrogen (UK), collagenase type IV and DNAse I from Worthington Biochem (US), fetal bovine serum (FBS), MEM glutamax, hygromycin B, trypsine from Gibco Life Technologies (UK), and FURA2-AM from Molecular Probes (UK). Membrane filter disks for filtration of HPLC samples (0.45 µm) were purchased from Satorius AG (Goettingen, Germany). Water used for chromatography was purified by means of a Milli-Q Advantage A10 water purification system (Millipore, France), and bottled water (Evian) was used for sensory analyses. Filter paper (Rundfilter Original 1, Melitta, Minden, Germany) was used as carrier for sensory analyses using half-tongue tests. Tasmanian pepper berries were purchased from the Australian retail (A Taste Of The Bush; Matcham, New South Wales, Australia).

Solvent Extraction of Tasmanian Pepper Berries. Tasmanian pepper berries were ground using a laboratory blender and a portion (350 g) was extracted four times with ethanol (1.8 L) at room temperature upon ultrasonification. After filtration through a Buchner funnel (filter 1291, 90 mm, 84 g/m^2, Sartorius, Germany), ethanol was removed in vacuum at 40°C, followed by freeze-drying. The dried extract was kept at -20°C until used.

Sensory Analyses. *General Conditions and Panel Training.* 12 assessors (age 22-38 years), who had given informed consent to participate the sensory tests

and had no history of known taste disorders, were recruited from the Chair of Food Chemistry and Molecular Sensory Science (Freising, Germany). All participants were trained in sensory experiments using reference compounds.[35] Sensory assessments were conducted at room temperature in a sensory panel room, equipped with individual booths.

Modified Half-Tongue Test. Sensory test were performed by means of a modified half-tongue test according to literature.[35] Briefly, test samples were prepared by dissolving extracts, fractions or purified compounds in ethanol and applying aliquots (20 µL) onto filter paper rectangles (1×2 cm). After solvent removal in a nitrogen stream at 38 °C, the participants were presented with pairs of test samples and control filters without additive, both encoded with three digit numbers and in a randomised order. Pairs of sample and control filters were presented in ascending order of application levels. The participants were asked to place both filters of a pair onto their tongue at the same time, one onto each side. After a stimulation time of 30 s, they were asked to select the sample perceived as more pungent, as well as recording any other sensations perceived. Participants had breaks of 5 min between each assessment, in which they were given water as a palate cleanser.

Taste Dilution Analysis (TDA). An aliquot (4.3 g) of the pepper extract was separated by RP-MPLC to give 10 fractions, which were separated from solvent in vacuum at 40°C, followed by freeze-drying. The residues were then dissolved in equal volumes of ethanol (2 mL) in order to maintain the “natural” concentration ratios between the fractions and diluted sequentially 1:1 with ethanol. Aliquots (20 µL) of each dilution were applied onto filter paper rectangles (1 × 2 cm) and half-tongue tests were conducted as described above. The highest dilution at which a sensory difference between the filter loaded with a pepper fraction and the control

filter could be detected was defined as taste dilution (TD) factor[36]. The TD-factors determined by the participants were averaged.

Recognition Threshold Concentration. Threshold concentrations of the purified compounds were determined in duplicate using the half-tongue test as detailed above. Individual recognition thresholds were determined by calculation of the geometric mean of the two lowest correctly identified concentrations. The highest concentration at which compounds were tested was 350 nmol on a filter vehicle.

Fractionation of Pepper Extracts by Medium Pressure Liquid Chromatography (MPLC). Ethanol extracts of Tasmanian pepper berries, as well as a hexane/ethyl acetate partition thereof, were fractionated by means of reversed phase (RP) MPLC on 150 mm x 40 mm polypropylene cartridges using 25-40 µm LichroPrep RP18 bulk material (Merck, Darmstadt, Germany) as stationary phase and a gradient of 0.1% aqueous formic acid (solvent A) and methanol (solvent B) as mobile phase (flow rate: 50 mL/min): 2 min / 20% B, 5 min / 40% B, 20 min / 80% B, 26 min / 80% B, 28 min / 100% B, 40 min / 100% B. Chromatography was monitored using UV detection at 230 nm. The MPLC system (Büchi, Flawil, Switzerland) consisted of a pump manager C-615 with three pump modules C-605, 20 mL samples loop and 6-way-injection valve, C-660 fraction collector, C-635 UV detector and was controlled by the software SepacoreControl. Collected fractions were separated from solvent in vacuum and freeze-dried twice prior to TDA and chemical analysis, respectively.

Preparation of Epipolygodial (2). Epipolygodial was obtained by conversion of polygodial according to a literature protocol with some modifcations.[35] Polygodial (36 mg) were dissolved in diethyl ether (8 mL), mixed with basic aluminium oxide (8 g; activity 1) and stirred for 30 min. After filtration, the solvent was separated in vacuum and epipolygodial isolated by preparative RP-HPLC on a 250 x 21.2 mm, 5 µm,

Varian Pursuit C18 column with a mixture (50/50, v/v) of 0.1% aqueous formic acid and acetonitrile as mobile phase (flow rate: 21.2 mL/min) and UV detection at 230 nm. Structure confirmation was performed by one- and two-dimensional nuclear resonance spectroscopy (1D/2D-NMR) and liquid mass spectrometry (LC-TOF-MS). Spectroscopic data on compound **2**, Figure 1, can be found as Supporting Information.

Isolation and Identification of Pungent Phytochemicals. Most pungent compounds in fractions judged with a high TD-factor were enriched from the ethanolic pepper extract by means of liquid-liquid-extraction using 0.1% aqueous formic acid and hexane/ethyl acetate (8/2, v/v). The aqueous fraction was discarded and the solvent of the organic fraction separated in vacuum, followed by freeze-drying. Aliquots were subjected to preparative MPLC for further fractionation under the same conditions as detailed above for the taste dilution analysis (TDA). Individual compounds were isolated from the MPLC fractions by means of preparative RP-HPLC on a 250 x 21.2 mm, 5 µm, Varian Pursuit C18 column as stationary phase using the following gradient of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B) as mobile phase (flow rate: 21.2 mL/min): 0 min / 30% B, 12 min / 40% B, 17 min / 40%B, 30 min / 45% B, 35 min / 55% B, 38 min / 55% B, 40 min / 100% B, 43 min / 100% B. Chromatography was monitored by means of an UV detector (λ=230 nm) and an evaporative light scattering detector (ELSD) using a Sedex LT-ELSD Model 80 (Sedere, Alfortville, France). The HPLC system (Jasco, Gross-Umstadt, Germany) consisted of a PU-2087 Plus pump, a DG-2080-53 degasser, a MD-2010 Plus diode array detector, and run with the Chrompass 1.9 software. Re-chromatography was conducted, if needed, to obtain sufficient purities (>98%, HPLC-ELSD) for spectroscopic structure determination. Spectroscopic data on compounds **1** and **3** - **20**, Figure 1, can be found as Supporting Information.

TRP Channel Activation Experiments. *Cell culture.* Untransfected CHO cells, CHO cells expressing mouse TRPA1 or rat TRPV1 were grown in MEM-medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), FBS (10%) and for TRPA1-transfected cells additionally with Hygromycin B (200 µg/mL).[38] TRPA1 expression was tetracycline-induced (1 µg/mL) 12-24 h before calcium imaging measurements.[39] Trigeminal ganglion (TG) neurons were dissected from adult male or female mice and cultured using methods described previously for Dorsal Root Ganglion neurons[40].

Imaging of Intracellular Calcium Levels: Calcium imaging assays were conducted as described previously.[38,41,42] Briefly, Chinese Hamster Ovary (CHO) cells and TG neurons were loaded with 2 µM FURA-2AM in the presence of 1mM probenecid for approximately 1h. The dye loading and subsequent experiments were performed in a physiological saline solution containing NaCl (140 mmol/L), KCl (5 mmol/L), glucose (10 mmol/L), Hepes (10 mmol/L), $CaCl_2$ (2 mmol/L), and $MgCl_2$ (1 mmol/L) buffered to pH 7.4 with NaOH. Changes in intracellular calcium levels in mTRPA1 or rTRPV1 expressing CHO cells were determined at 25°C using an automated fluorometric plate reader (Flexstation 3, Molecular Devices). For calcium imaging of neurons compounds were applied to cells by local continuous microperfusion of solution through a fine tube placed very close to the cells being studied. Experiments were conducted at 22±1 °C. Images of a group of cells were captured every 2 s using 340 and 380 nm excitation wavelengths with emission measured at 520 nm with a microscope-based imaging system (PTI). Analyses of emission-intensity ratios at 340 nm/380 nm excitation (R, in individual cells) were performed using the ImageMaster suite of software.

UPLC/Time-of-Flight Mass Spectrometry (UPLC/TOF-MS). High-resolution mass spectra of the target substances were measured on a SYNAPT G2S HDMS

(Waters UK Ltd., Manchester, UK) in the positive ESI and resolution modus with the following parameters: capillary voltage +2.5 kV, sampling cone 30, extraction cone 4.0, source temperature 150 °C, desolvation temperature 450 °C, cone gas 30 L/h and desolvation gas 850 L/h. The samples were introduced into the instrument via an Acquity UPLC core system (Waters, Milford, MA, USA) consisting of a binary solvent manager, a sample manager and a column oven. For chromatography, a 2 x 150 mm, 1.7 µm, BEH C18 column (Waters) was used as stationary phase with a flow rate of 0.3 mL/min at a temperature of 40 °C and following gradient of acetonitrile (solvent A) and aqueous formic acid (0.1% in water, pH 2.5; solvent B): 0 min / 50% B, 7 min / 100% B, 9 min / 100% B, 10 min / 50% B. The instrument was calibrated over a *m/z* range of 100 to 1200 using a solution of sodium formate (0.5 mM) in a 2-propanol/water mixture (9/1, v/v). All data were lock mass corrected using leucine enkephaline as the reference (*m/z* 556.2771, $[M+H]^+$). Data acquisition and interpretation were performed by using MassLynx software (version 4.1; Waters).

Nuclear Magnetic Resonance Spectroscopy (NMR). One- and two-dimensional ^{1}H and ^{13}C NMR spectra were acquired on a 400 MHz DRX and a 500 MHz Avance III Cryoprobe spectrometer (Bruker, Rheinstetten, Germany), respectively. Chemical shifts were measured either by using tetramethylsilane (TMS) as the internal standard or from residual chloroform signals ($CDCl_3$, 0.03 % TMS, Euriso-top, Gif-sur-Yvette, France). For structural elucidation and NMR signal assignment 2D-NMR experiments, like COSY-, HMQC-, and HMBC-spectroscopy were carried out using the pulse sequences taken from the Bruker software library. Data processing was performed by using Topspin 1.3 software (Bruker, Rheinstetten, Germany) and evaluated by using MestReNova 7.0.1 (Mestrelab Research, Spain).

To monitor the reaction of pungent aldehydes with amino acid residues, aliquots (0.2 mL) of individual solutions of polygodial, warburganal, 1β-acetoxy-9-deoxy-

isomuzigadial and changweikangaldehyde (2.5 mg/mL each) in deuterated methanol were mixed with aliquots (0.4 mL) of stock solutions of the *N*-acetylated amino acids N_α-acetyl-L-lysine (3.38 mg/mL), N_α-acetyl-L-arginine (1.83 mg/mL), and N_α-acetyl-L-cysteine (2.5 mg/mL) in potassium hydrogenphospate buffer (20.4 mg KH_2PO_4 mg/mL in deuterium oxide, pH 7), and placed in 5 mm NMR tubes. ^{1}H NMR spectra (500 MHz, relaxation time: 1 s) were recorded immediately after mixing and in regular time intervals thereafter whilst keeping the instrumental parameters constant. Reactivity was evaluated by integrating compound specific signals.

RESULTS AND DISCUSSION

Tasmanian pepper berries were ground and exhaustively extracted with ethanol to afford a strongly pungent powder after separating the solvent in vacuum. To locate the pungent phytochemicals in the extract, a taste dilution analysis[36] was conducted in the following.

Taste Dilution Analysis (TDA). The ethanol extract of ground pepper berries was separated into 10 fractions by means of RP-MPLC-UV (Figure 2) which, after solvent separation, were dissolved in equal volumes of ethanol and, then, 1:1 dilutions were applied onto filter paper rectangles (1x2 cm) as vehicles for sensory assessment in ascending order of concentration using the half-tongue-test. Medium to non-polar fractions were perceived as intensely pungent with fraction F9 showing the highest taste dilution (TD)-factor of 4096, followed by fractions F7, F8 and F10 judged with TD-factors of 256. As the taste impact of the polar fractions F1 to F6 was negligible, the target fractions F7-10 were enriched by liquid-liquid extraction in the

following and used for natural product isolation and chemosensory characterisation, respectively.

Isolation and Identification of Pungent Phytochemicals. A total of 19 target compounds were isolated from the most pungent tasting MPLC fractions F7 to F10 by means of preparative RP-HPLC. The major constituent polygodial (**1**, Figure 1) could be unequivocally confirmed in fraction F9 by comparison of chromatographic (RP-HPLC) and spectrometric data (NMR, LC-TOF-MS, UV) with those determined for a reference standard.[9,43] In addition, 13 compounds (**3-13**, **19-20**) were isolated from fraction F9, compound **16** was purified from fraction F8, and four compounds (**14-15**, **17-18**) from fraction F7. As fraction F10 contained mainly lipids and some residual amounts of the same phytochemicals as found in fraction F9 (data not shown), this fraction was not further used for natural product isolation. ^{13}C NMR experiments and accurate mass spectral data revealed that 10 of these additional 18 phytochemicals consist of 15 carbon atoms, thus suggesting sesquiterpene substructures as found for polygodial, while the 13, 14 or 17 carbon atoms detected in the remaining 8 phytochemicals indicate a truncated or a modified sesquiterpene skeleton. 1D/2D-NMR analyses confirmed that, with the exception of compound **10**, all phytochemicals share a polygodial-type, unsaturated trimethyl-*trans*-decaline carbon backbone differing in the decoration of ring B with functional groups. Overall, four groups of compounds, namely aldehydes, acids, lactones and alcohols, were determined in their chemical structures in the following.

The four phytochemicals **3** to **6** showed characteristic NMR signals of aldehydes and were identified by mass spectrometric and NMR spectroscopic analysis. Compound **3** showed a pseudomolecular ion of *m/z* = 249.1419, thus indicating an elemental composition of $C_{15}H_{22}O_3$ and differing by only one oxygen atom from polygodial (**1**). Two NMR signals with typical chemical shifts for aldehydes

(9.73 ppm/H-C(11), 9.41 ppm/H-C(12)) and an olefinic proton H-C(7) at 7.27 ppm were identified. Instead of the proton H-C(9) in **1**, an oxygen-bound proton signal HO-C(9) has been assigned for the resonance signal detected at 4.09 ppm. In line with this, the aldehyde proton H-C(11) appeared as singlet rather than as a doublet. Therefore, this phytochemical could be unequivocally identified as the C9-hydroxylated polygodial derivative warburganal (**3**, Figure 1). To the best of our knowldege, compound **3** has not yet been reported in Tasmanian Pepper, although it was found in other Warburgia species[42,43] as well as water pepper (*Polygonum hydropiper*).[46]

Compound **4** showed the typical NMR signal pattern of unsaturated drimane dialdehydes, however, with modifications at the A-ring when compared to **1** indicated by two additional quaternary, olefinic carbons resonating at 124.2 and 125.1 ppm and coupling with two methyl groups H_3-C(13)/H_3-C(14) at 1.61/15.4 ppm and 1.63/19.0 ppm respectively as well as a methine proton H-C(1) shifted to the lower field at 4.89 ppm. A molecular formula of $C_{17}H_{22}O_4$ (m/z = 291.1615 Da) showing a mass loss of 60 Da in the mass spectrum indicated an acetyl cleavage which, along with HMBC couplings, identified the compound as 1β-acetoxy-9-deoxy-isomuzigadial (**4**, Figure 1). While 9-deoxyisomuzigadial was isolated previously from *Canella winterana*,[47] the acetylated derivative **4** has yet not been reported in literature.

Compound **5** also showed an aldehyde substructure with a characteristic ^{1}H NMR chemical shift of 9.48 ppm, which showed HMBC connectivity to the carbon atoms C(7), C(8) and C(9), and could be assigned to proton H-C(11). The molecular formular $C_{14}H_{22}O_2$ suggested a norsesquiterpene backbone as confirmed by ^{13}C NMR. Low-field shifted signals of a methine group (H-C(9)/3.85 ppm, C(9)/70.3 ppm) and ROESY couplings between H-C(9) and the methyl group H_3-C(14) revealed the identification of the previously not reported hydroxylated drimane derivative **5** (Figure

1). Consistent with the structurally related changweikang acid A, reported as natural product in *Polygonum hydropiper* and *Daphniphyllum calcynicum*,[48] compound **5** was coined changweikang aldehyde.

Compound **6**, showing a monoaldehyde motif by its characteristic ^{1}H NMR resonance signal at 9.33 ppm, revealed a sequential mass loss of 18 and 28 Da from the molecular ion m/z 251 in LC-MS analysis, thus indicating the presence of a carboxylic acid as additional functional group. In comparison to **1**, NMR analysis showed one of the aldehyde moieties to be oxidised to the carboxylic acid. In particular, the absence of a COSY coupling to another proton leads to the assignment of the aldehyde at C(12) and confirmed the structure as polygonic acid (**6**, Figure 1), which has been reported earlier in water pepper *Polygonum hydropiper*.[49]

Next to the aldehydes **1** and **3**-**6**, NMR and LC-MS experiments led to the identification of the isomeric hydroxylated carboxylic acids **7** - **9** as well as the carboxylic acid **10** showing only a C13 carbon backbone. High resolution MS and 2D-NMR experiments demonstrated compounds **7**-**9** to be the drimane norsesquiterpenes polypiperic acid (**7**), epipolypiperic acid (**8**) and changweikang acid A (**9**) sharing the same decaline backbone and differing only in the positions and stereochemistry of their hydroxy and carboxy groups that were assigned based on specific coupling patterns in HMBC and ROESY experiments. MS analysis of compound **10** revealed a molecular formula of $C_{13}H_{20}O_2$ and showed a cleavage of water and CO_2 fragments, thus indicating the presence of a carboxylic acid function. This was confirmed by ^{13}C-NMR showing a resonance at the low field of 170.34 ppm for C(8). NMR also revealed strong similarities with other isolated drimanes and 2D-NMR experiments confirmed that the compound is an indene derivative with ring A being the identical to the other drimanes and ring B being an unsaturated 5-

membered ring. HMBC couplings from the olefinic signal H-C(2) at 6.96 ppm to the carboxylic group and all carbon signals of ring B as well as from the olefinic carbon signal C(3) at 146.6 ppm into both rings A and B led to the identification of **10** as 3a,4,5,6,7,7a-Hexahydro-3a,7,7-trimethyl-1H-inden-3-carboxylic acid (Figure 1). To the best of our knowledge, the carboxylic acids **7**-**10** are reported in Tasmanian Pepper for the first time. Changweikang acid A (**9**) was previously identified in the Chinese medicine Changweikang,[48] polypiperic acid (**7**) was previously reported as the corresponding methylester in *Drimys granadensis*[50] and *Polygonum hydropiper*,[51] whereas phytochemicals **8** and **10** have not been described in literature.

Moreover, nine drimane sesquiterpenes (**11**-**19**) were identified to share a lactone structure. Compound **11**, matching the molecular mass of polygodial (234 Da), was identified as the lactone Cinnamolide (**11**, Figure 1) with the characteristic NMR resonance signal of 170.3 ppm for the carbonyl group C(12). HMBC correlations of the carbonyl carbon to the olefinic proton H-C(7) at 6.87 ppm and to a methylene group H_2-C(11) at 4.03 / 4.37 ppm led to the unequivocal identification of the lactone structure at ring B. Although cinnamolide was first identified in *Cinnamosma fragrans*[52] and later in other species,[44,53] this is the first report on **11** as a phytochemical in Tasmanian pepper. Compounds **12**-**17** showed molecular masses of 248, 250 and 266 Da respectively and the mass differences of 14, 16, and 32 amu compared to cinnamolide (**11**) indicated keto-, mono- and dihydroxy derivatives, respectively. This was confirmed by 2D-NMR experiments which led to the identification of 7-ketoconfertifolin (**12**), dendocarbin A (**13**), dendocarbin L (**14**), dendocarbin M (**15**), ugandenial A (**16**) and fuegin (**17**), the structures of which are displayed in Figure 1. The NMR signal pattern of further additional compounds (**18**, **19**) was rather similar to the other constituents but appeared to consist of two additional carbon atoms with chemical shifts as expected for acetyl moieties. This

was susbtantiated by 2D-NMR and the cleavage of 60 amu fragments from the molecular ions and, thus, confirmed the target compounds as 3-β-acetoxy-7-ketoconfertifolin (**18**) and 3-β-acetoxydrimenin (**19**), respectively. None of the lactones 11 – 19 have been reported earlier in Tasmanian pepper, although compounds **11** – **17** and **19** have been reported as phytochemicals in other botanical.[13,54-57] 3-β-Acetoxy-7-ketoconfertifolin (**18**) has to the best of our knowledge not yet been reported in literature. Finally, the UV-inactive compound **20** could be detected by evaporative light scattering detection in fraction F9 and was identified as the 11,12-dihydroxy derivative of polygodial, named drimendiol (**20**, Figure 1), by comparison with literature data.[43] Although drimendiol was reported earlier in *Drimys winteri*,[56] this is the first report on its occurrence in Tasmanian pepper.

Human Sensory Activity. Compounds **1**, **3**-**7**, **10**, **11**, **13**-**18**, and **20** could be isolated in sufficient quantities and purities (>98% HPLC-ELSD) to determine orosensory recognition threshold concentrations by means of an half-tongue test using filter-paper vehicles as reported recently.[35] In addition, epipolygodial (**2**) was synthetically prepared from polygodial (**1**) and used for sensory studies. The lowest orosensory thresholds of 0.6, 1.9, and 2.8 nm/cm^2 were found for polygodial (**1**), warburganal (**3**), followed by 1β-acetoxy-9-deoxy-isomuzigadial (**4**), whereas epipolygodial (**2**) and 3β-acetoxy-7-ketoconfertifolin (**18**) showed 14 and 25 times higher threshold concentrations when compared to **1** (Table 1). The data found for **1**-**3** were in good agreement with the threshold concentration of 0.4 (**1**), 21.4 (**2**) and 2.0 nmol/tongue (**3**) reported earlier,[59] and are in a similar order of magnitude as found for piperine (3 nmol/cm^2),[35] the main pungent principle in black pepper. Comparatively, high threshold levels of 30-70 nmol/cm^2 were found for changweikang aldehyde (**5**) and polygonic acid (**6**), which both have only one aldehyde function, as well as for the hydroxylactones fuegin (**17**), cinnamolide (**11**), ugendenial A (**16**), and

dendocarbins A, L and M (**13**, **14**, **15**). Drimendiol (**20**) and 3a,4,5,6,7,7a-hexahydro-3a,7,7-trimethyl-1*H*-inden-3-carboxylic acid (**10**) did not induce any pungent sensation up to a maximum test concentration of 175 nm/cm^2. These findings clearly demonstrated the α,β-unsaturated 1,4-dialdehyde structure to be the required structural motif for a low orosensory threshold for pungency, thus confirming earlier proposals.[24,34,61,62] However, some of the isolated phytochemicals reported here, such as, e.g. changweikang aldehyde (**5**) and polygonic acid (**6**), were also found to induce a clear pungent orosensation, although at somewhat higher concentration levels. In order to gain some insight into the mechanisms as to how the pungency of these compounds is mediated, cell-based TRPA1 and TRPV1 receptor studies were conducted in the following as these ion channels, amongst other functions, are known to play a crucial role for the perception of temperature, pain and pungency.[63]

Activation of Transient Receptor Potential Channels A1 (TRPA1) and V1 (TRPV1). While the vanilloid receptor TRPV1 is reported to be activated by pungent compounds like capsaicin from chillies[64] and piperine from black pepper,[65] a wide range of stimuli from noxious cold via environmental irritants up to diverse natural products like polygodial are known agonists of the ion channel TRPA1.[30,34,39,66-69] Therefore, selected phytochemicals isolated from Tasmanian pepper were screened in cell-based calcium influx experiments for their ability to activate TRPA1 and TRPV1, respectively. In a second set of experiments, intracellular calcium imaging experiments were performed using murine trigeminal neurons.

Using tetracycline induced mTRPA1 expressing CHO cells[37] loaded with FURA-2AM as a calcium fluorescent dye, TRPA1 activation was investigated by FURA-2 based calcium multi-well plate assays as described earlier.[42] Among all tested compounds, the low-threshold dialdehydes polygodial (**1**), epipolygodial (**2**), warburganal (**3**), and 1β-acetoxy-9-deoxy-isomuzigadial (**4**), as well as the

monoaldehyde changweikang aldehyde (**5**) elicited a significant calcium influx into the cells in a dose-dependent fashion (Figure 3). Polygonic acid (**6**), which exhibits one aldehyde moiety additionally to its carboxylic function, and to a lesser extent the hydroxylactones dendocarbin A (**13**), dendocarbins L/M (**14**/**15**) and ugandenial A (**16**), evoked a calcium influx at the highest concentrations tested. Intringuingly, all remaining compounds (**7**, **10**, **11**, **17**, **18**) did not show any significant calcium influx at the tested concentration range to indicate a TRPA1 mediated mechanism for pungency. The calcium influx observed for drimendiol (**20**) at a concentration of 200 µmol/L was identified as being unspecific and not related to TRPA1 activation as control measurements with non-transfected CHO cells also showed a calcium response (data not shown).

EC_{50} values for the TRPA1 response were calculated for the most potent agonists from the log(concentration) response relationships fitted with a logistic function (

Table 2). The compounds with a dialdehyde structure showed EC_{50} values between 4.5 and 34.5 μM, which are significantly lower than the EC_{50} value determined for the monoaldehyde changweikang aldehyde (340 μmol/L). The EC_{50} values of the dialdehydes are highly correlated with their oral recognition thresholds (R^2 = 0.986, linear regression). This suggests that the perception of pungency of the dialdehydes is directly mediated via the TRPA1. The approximate EC_{50} value for changweikang aldehyde is outside of this linear correlation and suggests that the activation mechanism of the monoaldehyde is different.

The TRPV1 activation by the isolated drimanes was investigated in the same fashion as the TRPA1 using a calcium imaging with rTRPV1 transfected CHO cells. The majority of compounds showed no activation at the tested concentration ranges, but the dialdehydes polygodial (**1**), warburganal (**3**) and 1β-acetoxy-9-deoxy-isomuzigadial (**4**) showed some activation (data not shown). However, the amplitudes of the responses were negligible and responses were only observed at the highest concentrations tested, except for polygodial (**1**), which was the only test compound to cause a significant calcium influx. Nevertheless, this was only observed at concentrations approximately ten times higher than required for TRPA1 activation. Therefore, it can be concluded that the TRPV1 activation has a minor contribution to the perception of pungency for the investigated drimanes. However, it is possible that the TRPV1 activation contributes a small component to the overall perceived pungency *in vivo*.

In a second set of experiments, the compounds showing TRPA1 activation in the CHO model system were further studied using calcium imaging assays with trigeminal neurons prepared from wildtype ($TRPA1^{+/+}$) and TRPA1 knockout mice ($TRPA1^{-/-}$), respectively. Calcium influx into the cultured neurons loaded with FURA2-AM was monitored by fluorescence microscopy. All dialdehydes **1**-**4** triggered

calcium influx into neurons prepared from Wild-type mice (Figure 4 A, C, E, G), whereas no calcium influx was observed in neurons from TRPA1$^{-/-}$ mice (Figure 4 B, D, F, H). To further characterize the subpopulation of neurons activated by dialdehydes **1**-**4** in trigeminal neurons from TRPA1$^{+/+}$ mice, the same neurons were challenged with allyl isothiocyanate (AITC; 50 µmol/L) and capsaicin (1 µmol/L) to selectively stimulate and visualize TRPA1 and TRPV1 expressing neurons, respectively, as well as potassium chloride (50 mmol/L) to activate all neurons.[70] As expected, responses to capsaicin and potassium chloride remained unchanged, while the population of neurons activated by the aldehydes **1**-**4** was identical to that activated by the TRPA1 agonist AITC. Microperfusion with dialdehydes **1**-**4** triggered immediate calcium influx during the application of the compounds to neurons from TRPA1$^{+/+}$ mice as shown as an example for 1β-acetoxy-9-deoxy-isomuzigadial (**4**) in Figure 5 A, while no activation was found in neurons from TRPA1$^{-/-}$ mice (Figure 5 B). Again, responses to capsaicin and potassium chloride remained unchanged, while the neuron activated by dialdehyde **4** was also activated by AITC. These data unequivocally confirm that the dialdehydes not only activate heterologously expressed TRPA1 channels, but also native ion channels in murine trigeminal neurons.

Also changweikang aldehyde (**5**) activated the same neuron population in Wildtype neurons but, interestingly, evoked a different and interesting response. Whilst microperfusion with dialdehydes **1**-**4** triggered calcium influx during the application of the compounds, no calcium influx was observed during perfusion of neurons from TRPA1$^{+/+}$ mice with changweikang aldehyde (**5**) (Figure 5 C). Instead, a rapid and significant calcium influx was observed when the neurons were washed with buffer solution after perfusion of changweikang aldehyde at concentrations ≥240 µmol/L. This effect was repeatable and calcium levels decreased during subsequent

administration of changweikang aldehyde (**5**). When the aldehyde was applied at 120 µmol/L, no effect was observed, neither during perfusion, nor upon washout. In comparison, neurons from TRPA1$^{-/-}$ mice did not respond to **5**, neither during administration, nor during washout of **5** (Figure 5 D). These data for the first time suggest changweikang aldehyde (**5**) to have a bimodal activating and inhibitory function on the TRPA1 channel due to two distinct ligand interaction sites at the ion channel with the activating site having a stronger binding affinity for **5** than the inhibitory site. Such bimodal effects on the TRPA1 have been observed for other natural compounds such as menthol,[71] cinnamaldehyde and camphor.[72] The phenomenon of having both TRPA1 inhibiting and activating properties may well explain why not all drimane sesquiterpenes, which were perceived as pungent in the sensory studies, evoked significant TRPA1 or TRPV1 mediated in vitro responses.

Model Studies on the Binding of Drimane Sesquiterpenes to Nucleophilic Amino Acid Residues of TRPA1. Previous studies attributed the TRPA1-mediated pungency of electrophilic pungent compounds, such as, e.g. isothiocyanates like AITC and disulfides like diallyldisulfide, to a reversible covalent binding to cysteine residues in a complex reactive cysteine profile of the TRPA1 receptor protein,[29,30,74] Amongst others, cysteine C621 had been suggested to be most critical for electrophile-binding and activation of TRPA1 but also that lysine residues, in particular lysine K620, play a critical role for receptor activation.[75] In comparison, dialdeyhde sesquiterpenes are suggested to follow a different activation mechanism than isothiocyanates and sulfides as polygodial retained full activity on the triple cysteine-lysine TRPA1-mutant.[34] Although chemical reactions of polygodial with nucleophiles such as, e.g. amino and mercapto groups,[23-28, 61, 73] have been proposed, defined reaction products could not yet be identified.

To study potential reactions between proposed nucleophilic amino acid side chains of TRPA1 and drimane dialdehydes, time-resolved NMR-spectroscopic experiments were conducted on binary mixtures of polygodial and N_α-acetyl-cysteine, N_α-acetyl-lysine, and N_α-acetyl-arginine, respectively. The signal of the aldehyde proton H-C(11) of polygodial was integrated relative to the TMS signal at each time point. No reaction was observed between polygodial and N_α-acetyl-cysteine or N_α-acetyl-arginine (Figure 6, A), respectively, thus contradicting the hypothesized reaction of polygodial with cysteine residues of TRPA1.[12,22,27] In comparison, a rapid degradation of polygodial was observed when incubated in the presence of N_α-acetyl-lysine (Figure 6, A), e.g. the integrals of both aldehyde protons H-C(11) and H-C(12) as well as the olefinic proton H-C(7) were decreased to 50% within the first seven minutes of incubation (Figure 7). After 60 min, not even traces of polygodial were detectable anymore (Figure 6, A), while a series of new proton resonance signals corresponding to three reaction products were observed (Figure 7).

The first reaction product showed a proton singlet at 8.71 ppm for H-C(12), a multiplet at 7.27 ppm for H-C(7), and a doublet at 5.75 ppm for H-C(11) and was assigned as the cationic pyrrolinium ion **21** (Figure 7) by comparing the NMR data with those reported for the reaction product of 1,4-dialdehydes and methylamine.[23] The intermediate **21**, formed upon nucleophilic reaction of polygodial and the lysine side chain, was found to be formed very fast and, after running through a maximum after 3 min, disappeared again 40 min after incubation (Figure 6, B). With increasing incubation time and depletion of **21**, two additional reaction products were formed (Figure 6, B) and were identified as the two isomeric pyrroles **22** and **23** showing characteristic resonance signals at 6.41/6.61 ppm and 6.67/6.45 ppm for the pairs of aromatic pyrrole protons of **22** and **23**, respectively (Figure 7). In addition, H-C(7) of **22** resonated at 4.42 ppm as a multiplet with small coupling constants of 1.9 and 4.1

Hz, while the multiplet of H-C(7) of **23**, resonating at 4.54 ppm, showed coupling constants of 7.4 ($^{3}J_{H\text{-}C(6b)/H\text{-}C(7b)}$) und 9.4 Hz ($^{3}J_{H\text{-}C(6a)/H\text{-}C(7b)}$), thus indicating a 180° angle between the vicinal protons H-C(7b) and H-C(6a) in this isomer (Figure 7).

To study the influence of the drimane structure on the reactivity with the ε-amino group of lysine, N_{α}-acetyl-lysine was incubated with epipolygodial (**2**), warburganal (**3**), 1β-acetoxy-9-deoxy-isomuzigadial (**4**), and changweikang aldehyde (**5**), respectively, followed by time-resolved ^{1}H NMR-spectrocopy (Figure 8). Both, warburganal (**3**) and 1β-acetoxy-9-deoxy-isomuzigadial (**4**) reacted quickly with N_{α}-acetyl-lysine similarly to polygodial with somewhat slower reaction observed for **3**, most likely due to the additional hydroxylation at C9 (Figure , A). These data are well in line with the lowest pungency threshold concentrations found for **1**, **3** and **4** within the very narrow range of 0.6 to 2.8 nmol/cm^2. In comparison, the dialdehyde epipolygodial (**2**) and the monoaldehyde changweikangaldehyde (**5**), showing significantly higher pungency threshold concentrations of 8.6 and 27.0 nmol/cm^2, respectively, showed a much slower reaction with N_{α}-acetyl-lysine, e.g. 20% of **2** and **5** had been reacted after 300 and 5500 min of incubation, respectively (**Error! Reference source not found.** B and C). Based on these data, it may be concluded that, due to the long reaction times, a reaction with lysine residues of the TRPA1 receptor is unlikely to be relevant for the pungent properties of epipolygodial (**2**) and changweikang aldehyde (**5**). However, there is the possibility that the reaction is catalyzed *in vivo* by stabilizing intermediate reaction products by cooperativity with other amino acid side chains in TRPA1.

In summary, among the 20 drimane sesquiterpens isolated from Tasmanian pepper berries, polygodial (**1**), warburganal (**3**), and 1β-acetoxy-9-deoxy-isomuzigadial (**4**) exhibited the lowest pungency threshold concentrations within the very narrow range of 0.6 to 2.8 nmol/cm^2, elicited a dose-dependent calcium influx

into mTRPA1 expressing CHO cells with EC_{50} values between 4.5 ± 1.0 and 16.7 ± 7.5 µmol/L (correlation to oral pungency thresholds: R^2 = 0.986, linear regression) and into cultured trigeminal neurons prepared from wildtype ($TRPA1^{+/+}$) mice, whereas no calcium influx was observed in neurons from TRPA1 knockout mice ($TRPA1^{-/-}$). These findings clearly confirmed the α,β-unsaturated 1,4-dialdehyde structure to be the required structural motif for a low orosensory threshold for pungency, [24,34,61,62] and to activate TRPA1.[12,21,22,31,34] Time-resolved NMR experiments proposed the pungency mediating mechanism for electrophilic drimane sesquiterpene dialdehydes to be different from that found for other electrophilic pungent agents like isothiocyanates, which have been shown to undergo a covalent binding with cysteine residues in TRPA1.[29,30] Instead, the high-impact chemosensates polygodial (**1**), warburganal (**3**), and 1β-acetoxy-9-deoxy-isomuzigadial (**4**) showed immediate reactivity with the ε-amino group of lysine side chains to give pyrrole-type conjugates (**22**, **23**), thus showing evidence for TRPA1 activation by covalent lysine modification.

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Supporting Information Available

Spectroscopic data on compounds **1** - **20**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Table 1. Human Recognition Thresholds of Isolated Compounds

Compound	Recognition threshold for pungency ($nmol/cm^2$)
Polygodial (**1**)	0.6
Warburganal (**3**)	1.9
1β-Acetoxy-9-deoxyisomuzigadial (**4**)	2.8
Epipolygodial (**2**)	8.6
3β-Acetoxy-7-ketoconfertifolin (**18**)	15.5
Changweikang aldehyde (5)	27
Polygonic acid (**6**)	35
Fuegin (**17**)	39
Cinnamolide (**11**)	49
Ugandenial A (**16**)	50
Dendocarbins L/M (**14/15**)	50
Polypiperic acid (**7**)	69
Dendocarbin A (**13**)	152
Drimendiol (**20**)	>175[b]
HTIC (**10**)	>175b

[a] The structures of the compounds given as numbers are displayed in Figure 1; [b] Highest concentration tested.

Table 2. EC_{50} Values from mTRPA1 Activation Experiments in Transfected CHO Cells

Compound	EC_{50} in µM
Polygodial (**1**)	4.5 ± 1.0
Warburganal (**3**)	8.1 ± 3.4
1β-Acetoxy-9-deoxzisomuzigadial (**4**)	16.7 ± 7.5
Epipolygodial (**2**)	34.5 ± 11.6
Changweikang aldehyde (**5**)	340[a]

[a] approximation, as no full dose-response-function could be determined

1 # FIGURE LEGEND

2

Figure 1. Chemical structures of compounds isolated from Tasmanian Pepper berries: polygodial (**1**), epipolygodial (**2**), warburganal (**3**), 1β-acetoxy-9-deoxyisomuzigadial (**4**), changweikang aldehyde (**5**), polygonic acid (**6**), polypiperic acid (**7**), epipolypiperic acid (**8**), changweikang acid A (**9**), 3a,4,5,6,7,7a-Hexahydro-3a,7,7-trimethyl-1H-inden-3-carboxylic acid (**10**), cinnamolide (**11**), 7-ketoconfertifolin (**12**), dendocarbin A (**13**), dendocarbin L (**14**), dendocarbin M (**15**), ugandenial A (**16**), fuegin (**17**), 3β-acetoxy-7-ketoconfertifolin (**18**), 3-β-acetoxydrimenin (**19**), and drimendiol (**20**).

Figure 2. MPLC fractionation (left hand side) and taste dilution analysis (TDA, right hand side) of an ethanol extract of Tasmanian Pepper berries.

Figure 3. Calcium imaging with mTRPA1 transfected CHO cells after application of drimane sesquiterpenes: **A**: polygodial (**1**), **B**: epipolygodial (**2**), **C**: warburganal (**3**), **D**: 1β-acetoxy-9-deoxyisomuzigadial (**4**), **E**: fuegin (**17**), **F**: dendocarbines L/M (**14/15**), **G**: ugandenial A (**16**), **H**: changweikang acid A (**9**), **I**: polypiperic acid (**7**), **J**: epipolypiperic acid (**8**), **K**: changweikang aldehyde (**5**), **L**: polygonic acid (**6**), **M**: dendocarbin A (**13**), **N**: drimendiol (**20**), **O**: 3a,4,5,6,7,7a-Hexahydro-3a,7,7-trimethyl-1H-inden-3-carboxylic acid (**10**), **P**: cinnamolide (**11**), **Q**: ethanol.

Figure 4. Calcium Imaging of trigeminal neurons (false-color images) prepared from TRPA1$^{+/+}$ mice (**A, C, E, G**) and TRPA1$^{-/-}$ mice (**B, D, F, H**) after activation with: **A/B**: polygodial (**1**), **C/D**: epipolygodial (**2**), **E/F**: warburganal (**3**), **G/H**: 1β-acetoxy-9-deoxyisomuzigadial (**4**). Allylisothiocyanate (AITC), capsaicin and potassium chloride (KCl) were applied as control.

Figure 5. Representative cell responses $[Ca^{2+}]_i$ over time of neurons prepared from TRPA1$^{+/+}$ (**A, C**) and TRPA1$^{-/-}$ mice (**B, D**) for applications of 1β-acetoxy-9-deoxy-isomuzigadial (**4**; 132 µM; **A/B**) and changweikang aldehyde (**5**; 120-900 µM; **C/D**); 500 µM menthol (**A**), 50 µM AITC (**A-D**), 1 µM capsaicin (**A-D**), and potassium chloride (50 mM, **A-D**) were applied as control.

Figure 6. (**A**) Time course of the reaction of polygodial (**1**) with *N*-acetyl-arginine, *N*-acetyl-cysteine and *N*-acetyl-lysine, respectively, quantitatively monitored by signal integration of H-C(11) of **1** by means of ^{1}H NMR spectroscopy. (B) Time course of educts and products formed upon reaction of polygodial (**1**) with *N*-acetyl-lysine monitored by determination of substance specific ^{1}H-NMR-signals: polygodial (**1**; 9.50 ppm), *N*-acetyl-lysine (4.16 ppm), pyrrolinium cation **21** (8.71 ppm), pyrrole **22** (4.12 ppm), and pyrrole **23** (4.54 ppm).

Figure 7. Time-resolved NMR spectroscopy of a solution (0.2 mL) of polygodial (**1**; 2.5 mg/mL) with N_{α}-acetyl-L-lysine (3.38 mg/mL) in potassium hydrogenphospate buffer (20.4 mg KH_2PO_4 mg/mL in deuterium oxide, pH 7) placed in 5 mm NMR tubes. ^{1}H NMR spectra (500 MHz, relaxation time: 1 s) were recorded immediately after mixing and in regular time intervals thereafter whilst keeping the instrumental parameters. Relative amount of polygodial (**1**) was measured by integration of the aldehyde proton H-C(11) in the ^{1}H NMR spectrum. The conversion of polygodial (1) via the cationic pyrrolinium ion **21** into the pyrrols **22** and **23** and the distinct protons used for structural assignment is shown below.

Figure 8. Time course of the reaction of (**A**) polygodial (**1**), warburganal (**3**) and 1β-acetoxy-9-deoxyisomuzigadial (**4**) in presence of *N*-acetyl-lysine, (**B**) epipolygodial (**2**) in the absence and presence of *N*-acetyl-lysine, and (**C**) changweikang aldehyde (**5**) in the absence and presence of *N*-acetyl-lysine. Relative amount measured by integration of the corresponding aldehyde proton H-C(11) in the 500 MHz ^{1}H-NMR spectra.

Figure 1 (Mathie et al.)

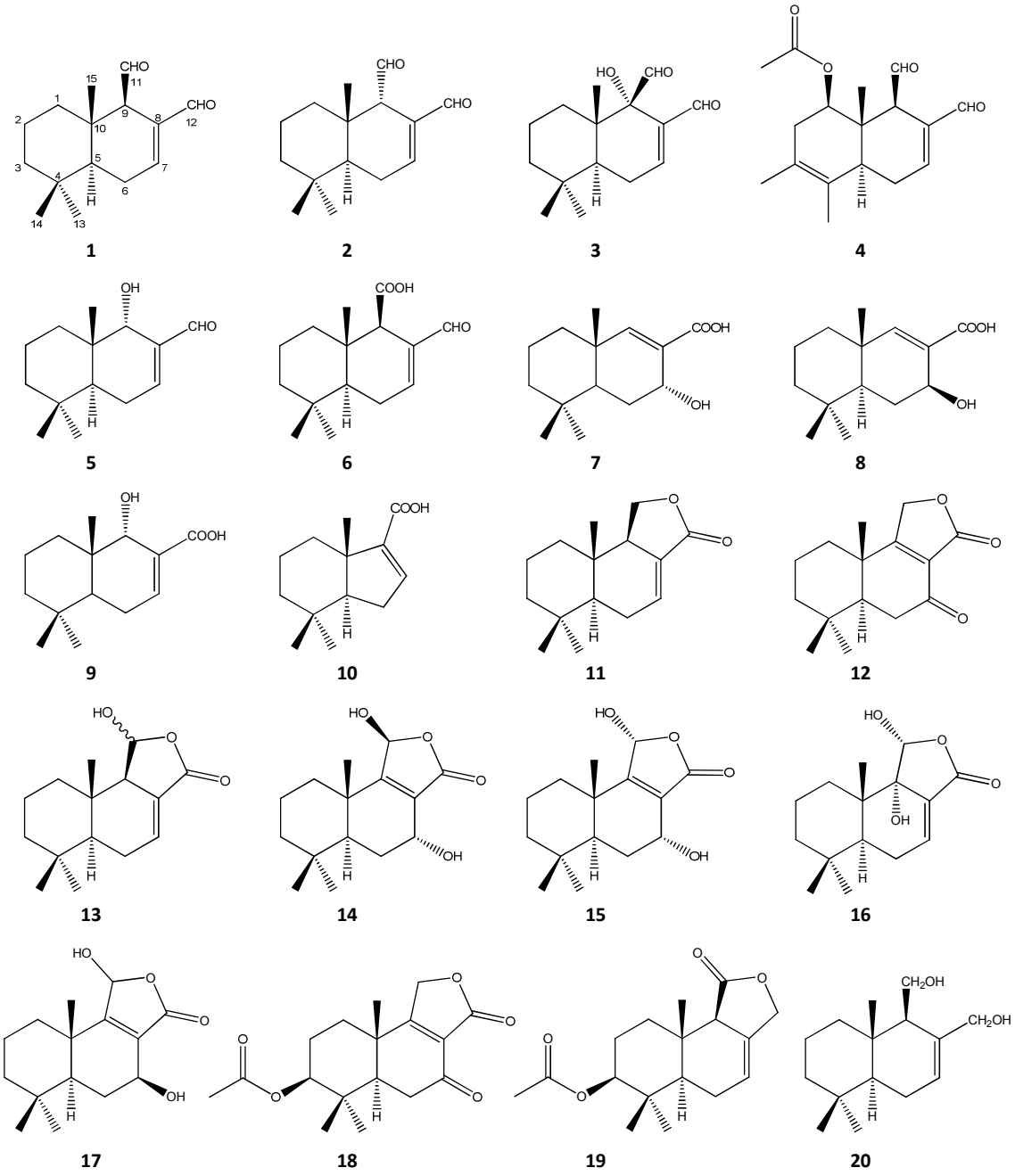


Figure 2 (Mathie et al.)

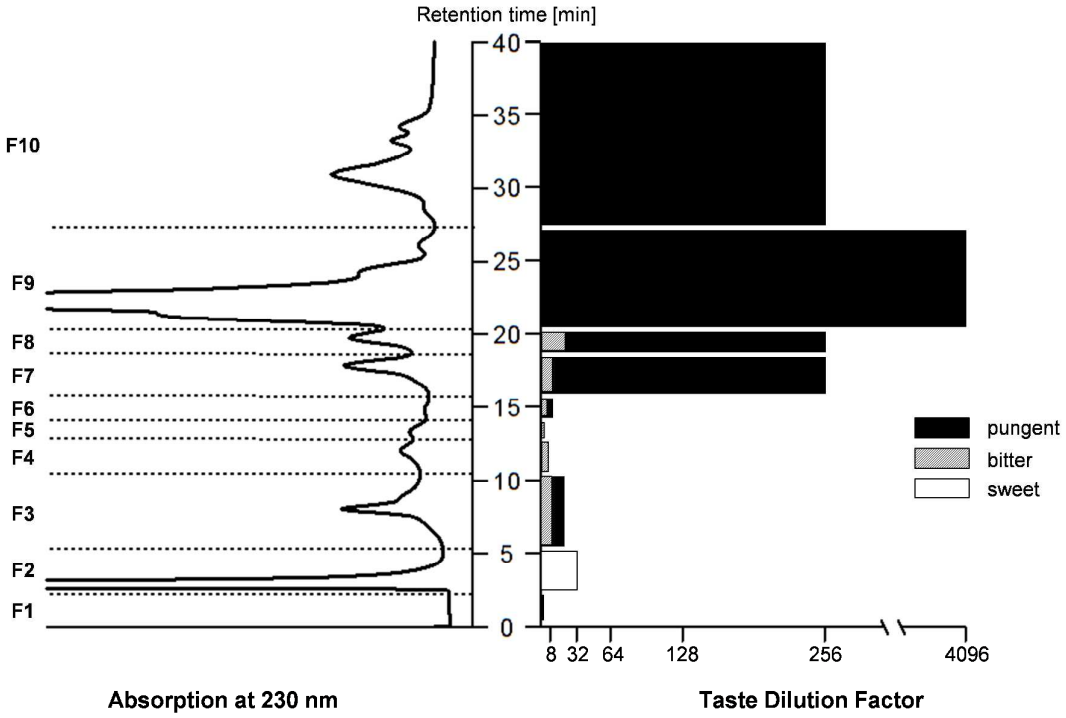


Figure 3 (Mathie et al.)

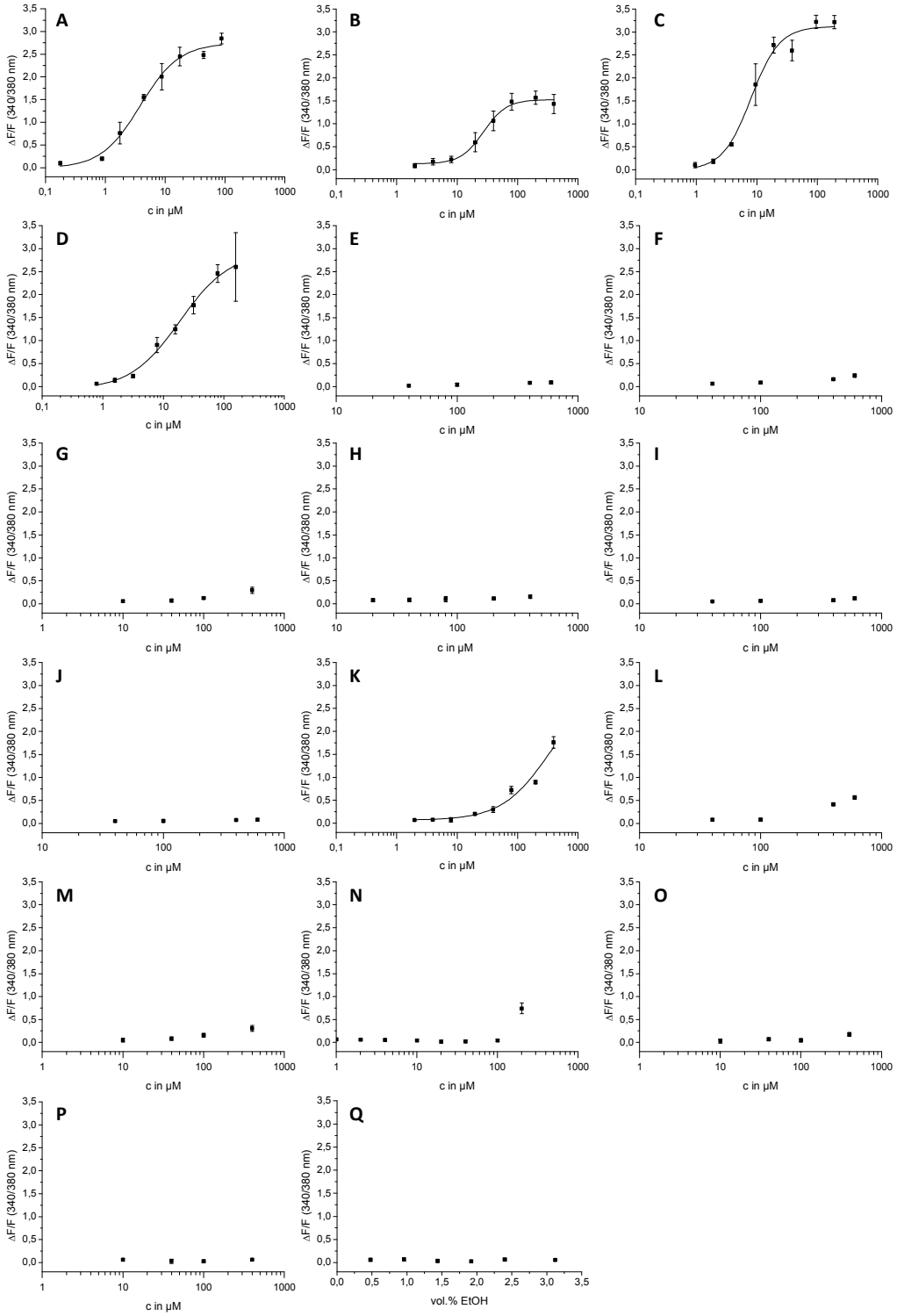
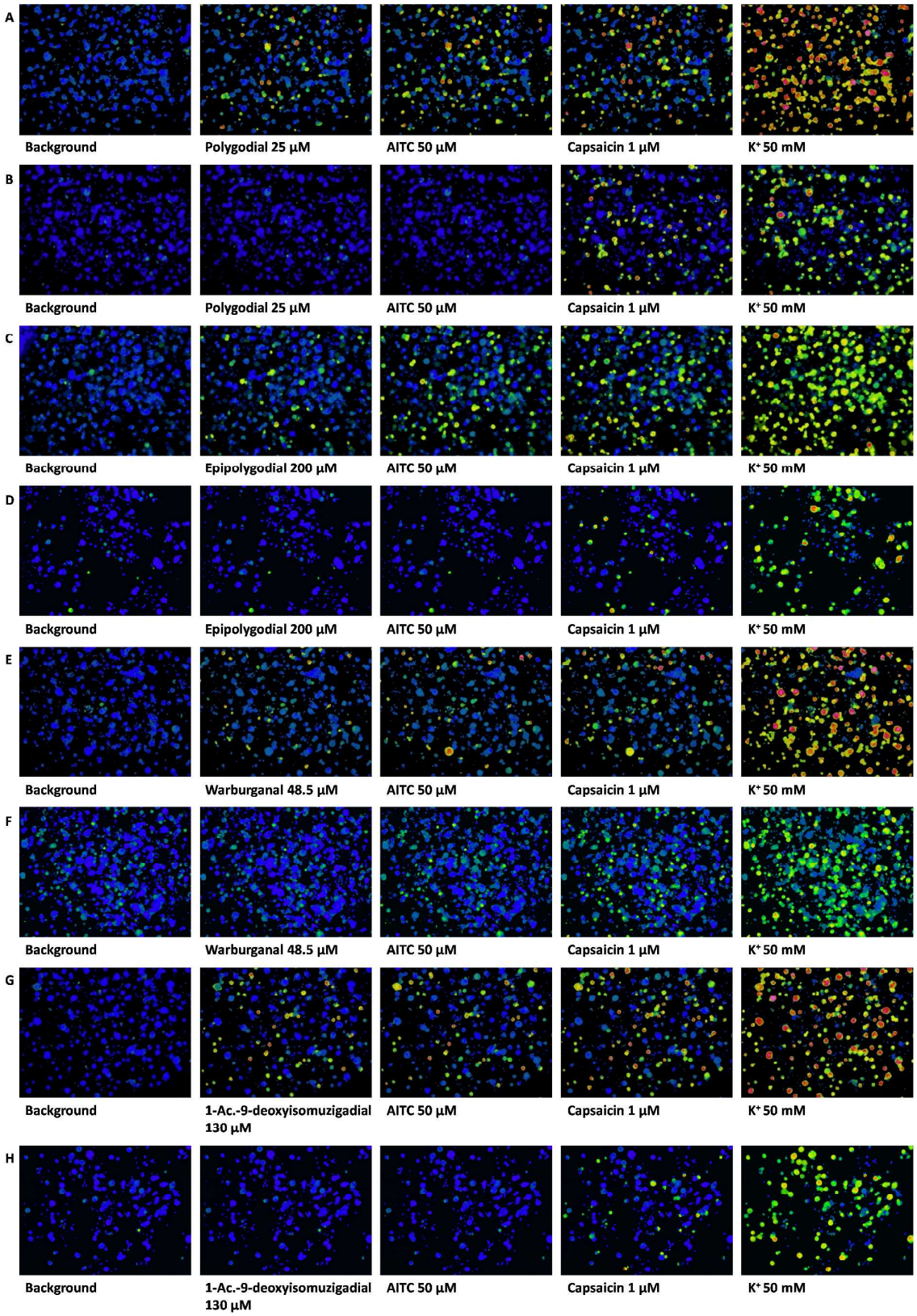


Figure 4 (Mathie et al.)



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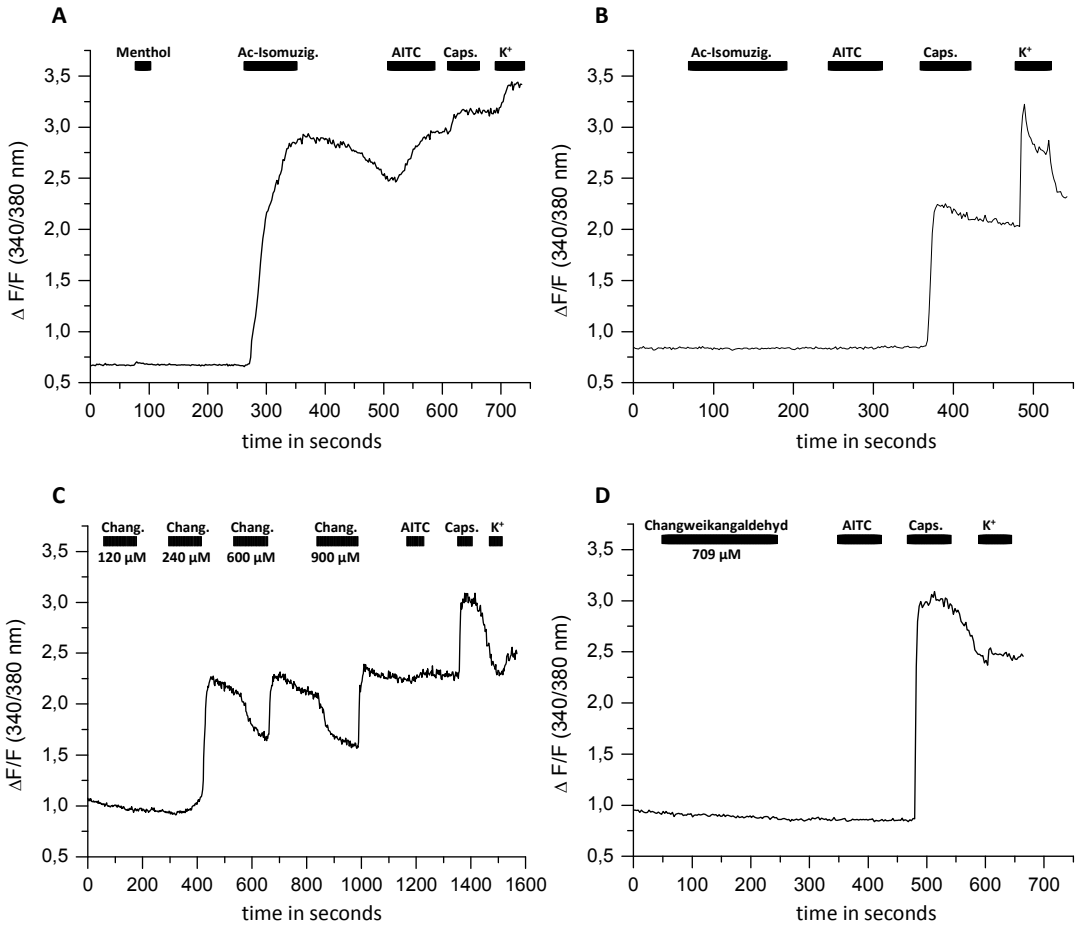


Figure 6 (Mathie et al.)

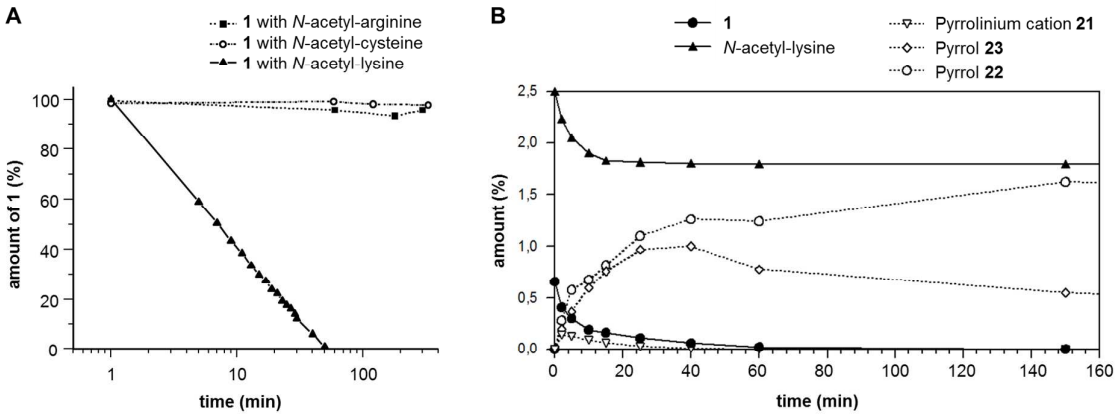


Figure 7 (Mathie et al.)

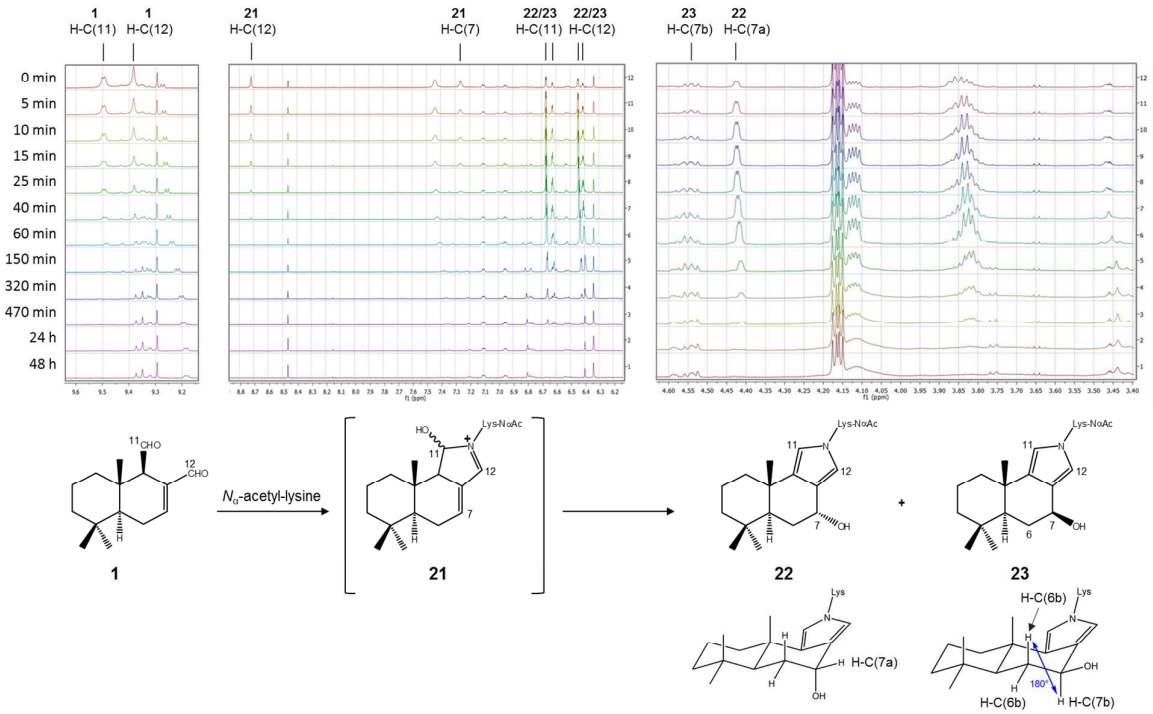
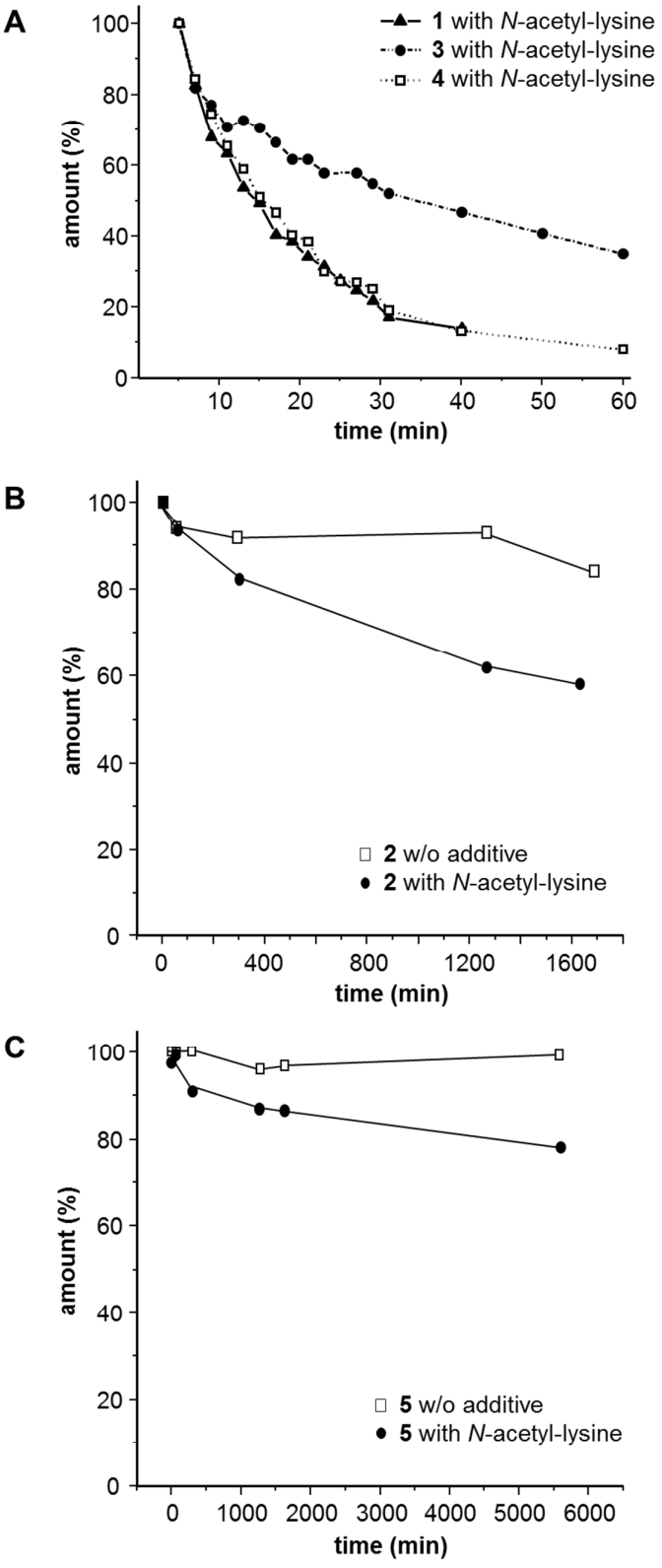
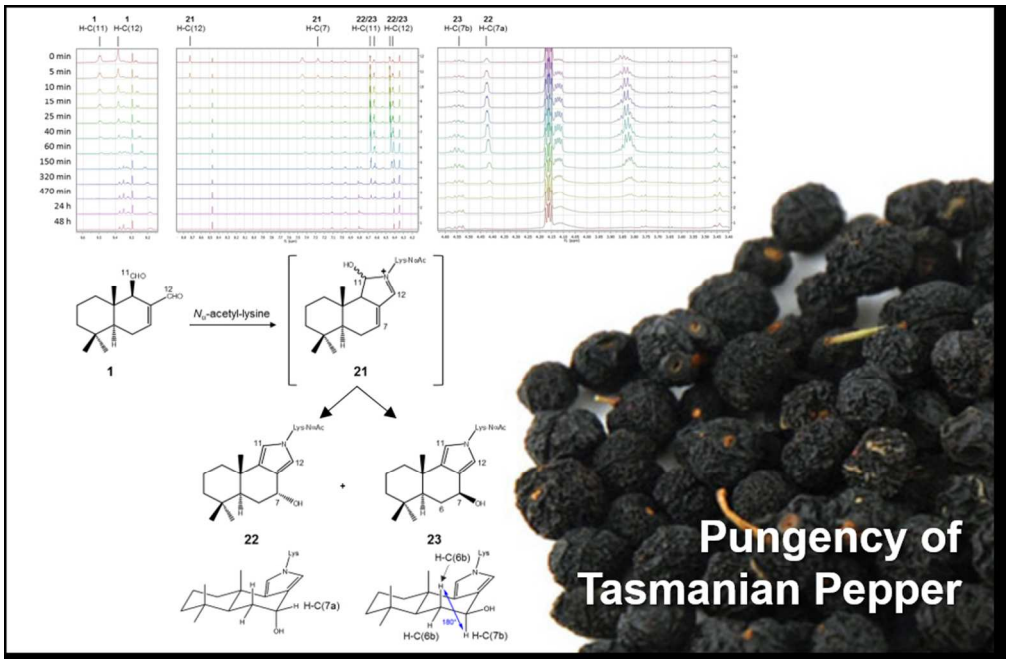


Figure 8 (Mathie et al.)





TOC graphic

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