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Structural and Sensory Characterization of Key Pungent and Tingling Compounds from Black Pepper (*Piper nigrum* L.)

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Supporting Information

ABSTRACT: To gain a more comprehensive knowledge on whether, besides the well-known piperine, other compounds are responsible for the pungent and tingling oral impression imparted by black pepper, an ethanol extract prepared from black pepper (*Piper nigrum* L.) was screened for its key sensory-active nonvolatiles by application of taste dilution analysis (TDA). Purification of the compounds perceived with the highest sensory impact, followed by LC-MS and 1D/2D NMR experiments as well as synthesis, led to the structure determination of 25 key pungent and tingling phytochemicals, among which the eight amides 1-(octadeca-2*E*,4*E*,13*Z*-trienyl)piperidine, 1-(octadeca-2*E*,4*E*,13*Z*-trienyl)pyrrolidine, (2*E*,4*E*,13*Z*)-*N*-isobutyl-octadeca-2,4,13-trienamide, 1-(octadeca-2*E*,4*E*,12*Z*-trienoyl)-pyrrolidine, 1-(eicosa-2*E*,4*E*,15*Z*-trienyl)piperidine, 1-(eicosa-2*E*,4*E*,15*Z*-trienyl)pyrrolidine, (2*E*,4*E*,15*Z*)-*N*-isobutyl-eicosa-2,4,15-trienamide, and 1-(eicosa-2*E*,4*E*,14*Z*-trienoyl)-pyrrolidine were not yet reported in literature. Sensory studies by means of a modified half-tongue test revealed recognition thresholds ranging from 3.0 to 1150.2 nmol/cm^2 for pungency and from 520.6 to 2162.1 nmol/cm^2 for the tingling orosensation depending on their chemical structure.

KEYWORDS: *pepper, pungency, piperine, tingling, taste, taste dilution analysis, half-tongue test, Piper nigrum L.*

INTRODUCTION

Judged as the king of spices, pepper shares a place on most dinner tables together with salt. Both black and white pepper are produced from the unripened berries of the shrub *Piper nigrum* L.. After they are dried in the sun, the green berries turn dark to become black pepper. If white pepper is to be produced, the peppercorns are either boiled and then stored in heaps or are packed in large sacks that are maintained in a running water stream for up to 15 days. Bacterial action induces the separation of the outer pericarp from the remainder of the peppercorn.

Black pepper is highly appreciated by the consumer for its alluring aroma as well as the typical pungent and tingling orosensory impression. Inspired by the attractive aroma of fresh black pepper, various studies have been performed to identify the key aroma compounds by means of GC-olfactometry.[1−3] Early research on the pungent principles of black pepper was performed already more than a hundred years ago with Oersted[4] and Landenburg[5] being the first to isolate and identify piperine (**1a**, Figure 1) as the major chemosensate and quantitatively predominating amide present in concentrations of 40−50 mg/100 g.[6−8] Over the years, a series of additional amides were reported to contribute to the pungent sensation imparted by black pepper.[7−10] In general, these amides can be grouped into two chemical classes, namely, the amides decorated with a piperonal moiety as found in piperlonguminine (**2a**, Figure 1),[11] piperyline (**2b**),[12] piperettine (**4c**),[13] and retrofractamide A (**5a**),[14] respectively, and the unsaturated, long-chain fatty acid amides such as (2*E*,4*E*,12*Z*)-*N*-isobutyloctadeca-2,4,12-trienamide (**9a**).[13] Both classes of amides share isobutylamine, pyrrolidine, and piperidine, respectively, as the common amine moiety, and depending on their chemical structure, some of these pepper amides are reported to exhibit biological activities including anticarcinogenic,[15−20] antidepressant,[21−23] antidiarrheal,[24] antimicrobial,[25−32] antioxidative,[33,34] antiulcer,[35−37] immunomodulatory,[15,18] insecticidal,[38−42] and larvicidal[43] activities.

Although the pungent impact of the amides present in *P. nigrum* has been known for many decades and, along with capsaicin and ethanol, has been reported to be mediated by the activation of the vanilloid receptor TRPV1,[44−47] the data published on the sensory attributes of the purified individual amides and their contribution to the pungent and tingling effect of black pepper are rather fragmentary and sometimes even contradictory.

The objective of the present investigation was, therefore, to reinvestigate the pungent and tingling key molecules in black pepper by application of a taste dilution (TD) approach,[48] which was already successfully used for the discovery of cooling compounds in dark malt,[49] bitter phytochemicals in carrot products,[50] the taste enhancer (*S*)-alapyridaine in beef bouillon,[50,51] and astringent key taste compounds in black tea infusions,[52] roasted cocoa nibs,[53] red current juice,[54] and spinach,[55] respectively.

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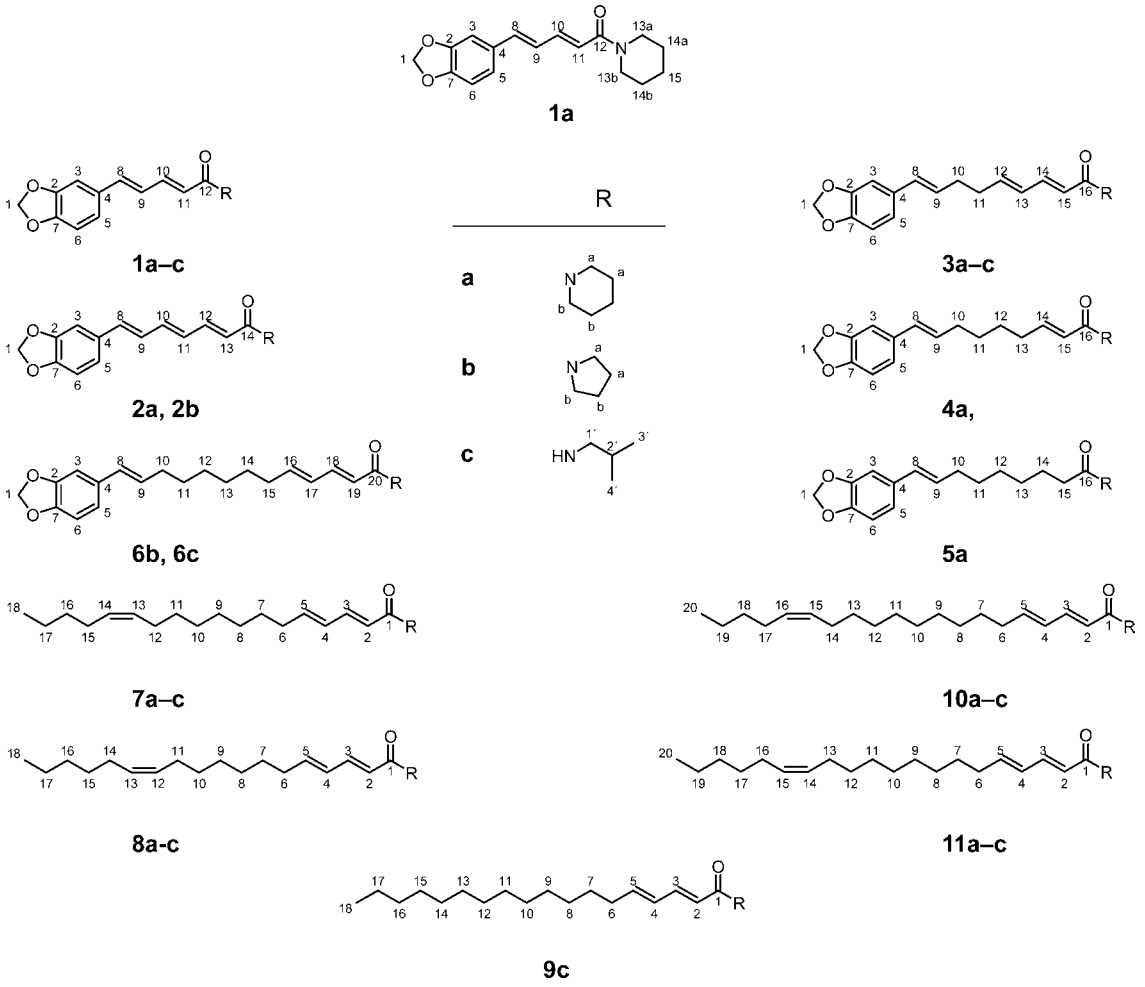

Figure 1. Chemical structures of pungent and tingling compounds **1**−**11c** in black pepper: piperine (**1a**), piperyline (**1b**), piperlonguminine (**1c**), piperettine (**2a**), piperoleine (**2b**), dehydropipernonaline (**3a**), 1-[1-oxo-9(3,4-methylenedioxyphenyl)-2*E*,4*E*,8*E*-nonatrienyl]-pyrrolidine (**3b**), retrofractamide A (**3c**), pipernonaline (**4a**), piperoleine B (**5a**), brachyamide A (**6b**), guineensine (**6c**), 1-(octadeca-2*E*,4*E*,13*Z*-trienyl)piperidine (**7a**), 1-(octadeca-2*E*,4*E*,13*Z*-trienyl)pyrrolidine (**7b**), (2*E*,4*E*,13*Z*)-*N*-isobutyl-octadeca-2,4,13-trienamide (**7c**), 1-(octadeca-2*E*,4*E*,12*Z*-trienyl)-piperidine (**8a**), 1-(octadeca-2*E*,4*E*,12*Z*-trienyl)pyrrolidine (**8b**), (2*E*,4*E*,12*Z*)-*N*-isobutyl-octadeca-2,4,12-trienamide (**8c**), (2*E*,4*E*)-*N*-isobutyl-octadeca-2,4-dienamide (**9c**), 1-(eicosa-2*E*,4*E*,15*Z*-trienyl)piperidine (**10a**), 1-(eicosa-2*E*,4*E*,15*Z*-trienyl)pyrrolidine (**10b**), (2*E*,4*E*,15*Z*)-*N*-isobutyl-eicosa-2,4,15-trienamide (**10c**), 1-(eicosa-2*E*,4*E*,14*Z*-trienyl)piperidine (**11a**), 1-(eicosa-2*E*,4*E*,14*Z*-trienyl)pyrrolidine (**11b**), and (2*E*,4*E*,14*Z*)-*N*-isobutyl-eicosa-2,4,14-trienamide (**11c**).

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: Aliquat 336, formic acid, hydrochloric acid, ethanol, potassium hydroxide, potassium carbonate, piperonal, starch, and triphenylphosphine (Merck, Darmstadt, Germany); hydrochloric acid, sodium hydroxide, and sorbic acid (Riedel-de-Haen, Seelze, Germany); DMSO-d_6 (Euriso-Top, Saarbrücken, Germany); isobutylamine, capsaicine, piperine, pyrrolidine, thionyl chloride, and sodium hydride (Sigma-Aldrich, Steinheim, Germany); potassium iodide (VWR, Leuven, Belgium); and sodium chloride (Grüssing, Filsum, Germany). Water for high-performance liquid chromatography (HPLC) separation was purified by means of a Milli-Q water advantage A 10 water system (Millipore, Molsheim, France). Solvents were of HPLC grade (Merck). Filter paper (Rundfilter classic, 94 mm; Melitta, Minden, Germany), cut into a rectangular shape (1 × 2 cm^2), was used as a vehicle for the sensory evaluation of pungent and tingling amides. Black peppercorn samples were purchased from the German retail market.

Solvent Extraction of Black Pepper. A portion of black peppercorn (200 g) was frozen in liquid nitrogen, ground in a laboratory blender (Analysenmühle A10, Ika Labortechnik, Germany), and then extracted with ethanol (6 × 250 mL) at room temperature upon ultrasonification. After filtration (MN 6151/4, 150 mm), the combined organic extract was separated from ethanol in vacuum at 30 °C, freeze-dried for 48 h, and then kept at −20 °C until used.

Sensory Analyses. *General Conditions and Panel Training.* Twelve assessors (seven women and five men, ages 22−38 years), who had given informed consent to participate in the sensory tests of the present investigation and had no history of known taste disorders, were recruited from the Chair of Food Chemistry and Molecular Sensory Science (Freising, Germany) and from the Institute of Food Chemistry (Münster, Germany). All panelists were trained in sensory experiments with purified reference compounds at regular intervals for at least 2 years and were experienced to evaluate the orosensation of a tingling szechuan pepper extract (40 μg of the extract was applied on filter paper rectangles in a dimension of 1 × 2 cm^2; Synthite, Kerala, India), isolated from *Zanthoxylum piperitum* by means of carbon dioxide extraction and containing hydroxy-α-sanshool and hydroxy-β-sanshool in amounts of 2.7 and 9.1% (HPLC-UV/vis), and the pungent compounds capsaicine (3 μg/1 × 2 cm^2) and piperine (20 μg/1 × 2 cm^2), respectively, by means of a half-tongue test[53] using filter paper rectangles (1 × 2 cm^2) as the vehicle. Panelists were instructed to rinse their mouth after each dilution step with a 3% sucrose solution (15 mL), followed by water (15 mL), and to wait

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for 2 min between each sample. Sensory analyses were performed at 19−22 °C in a sensory panel room, equipped with single booths under yellow light conditions.

Precaution Taken for Sensory Analysis of Food Fractions and Taste Compounds. To remove solvent traces and buffer compounds from all fractions and compounds isolated from pepper, the individual fractions were suspended in water and, after the volatiles were removed in high vacuum (<5 mPa), were freeze-dried twice. ^{1}H NMR spectroscopic, GC/MS, and ion chromatographic analysis of an aliquot revealed that food fractions treated by that procedure were essentially free of the solvents and buffer compounds used.

Modified Half-Tongue Test. Sensory studies with pungent and/or tingling compounds were performed by means of the previously reported half-tongue test[52] with some modifications. Serial 1:1 dilutions of HPLC fractions or purified compounds in ethanol were applied on filter paper rectangles (1 × 2 cm^2), and the solvent was removed in a laboratory oven at 38 °C. As the control vehicle, filter paper rectangles were loaded with ethanol (blank), followed by evaporation at 38 °C. Using a 3% sucrose solution (15 mL) and water (15 mL) as cleansing solutions and an interstimulus interval length of 5 min between the sample dilutions, the vehicles loaded with the stimulus in order of ascending concentration were presented to the sensory panel in three independent sessions and were randomly placed on the right or the left side of the anterior tongue together with an anonymized control vehicle (blank) on the other side. For each dilution, the subjects were asked to locate the side of the tongue where a pungent and/or a tingling sensation was perceivable.

Taste Dilution Analysis (TDA). An aliquot of the ethanol extract prepared from black pepper as described above was separated by RP-HPLC to give 41 fractions, which were freeze-dried twice and dissolved in "natural" concentration ratios matching the amounts present in 30 mg of peppercorns. After each fraction had been sequentially diluted 1:1 with ethanol and applied on filter paper rectangles (1 × 2 cm^2), solvent traces were removed at 38 °C, and the serial dilutions of each of these fractions were presented to the sensory panel in order of ascending concentration. Each dilution was evaluated for pungency, tingling, and bitter taste by means of the half-tongue test detailed above. The dilution at which a sensory difference between the paper rectangle loaded with a pepper fraction and a blank vehicle (control) could just be detected was defined as TD factor.[48] The TD factors evaluated by six different assessors in two different sessions were averaged. The TD factors between the trained individuals and the separate sessions did not differ by more than plus/minus one dilution step.

Recognition Threshold Concentration. Threshold concentrations of the purified compounds were determined by 12 panelists using the half-tongue test and the filter paper rectangles as detailed above. To prevent excessive fatigue, the trials began at a concentration level two steps below the threshold concentration that had been evaluated in preliminary sensory experiments. Whenever the panelist selected incorrectly, the next trial took place at the next higher concentration step. Whenever the panelist selected correctly, the same dilution of the stimulus was evaluated again as a proof of the correctness of the data. The threshold value of the sensory group was approximated by averaging the threshold values of the individuals in three independent sessions. The geometric mean of the two lowest correctly identified concentrations was calculated and taken as the individual recognition threshold. Values between individuals and separate sessions differed not more than plus or minus two dilution steps; that is, a threshold value of 3.0 nmol/cm^2 for the reference compound piperine represents a range of 0.75−9.0 nmol/cm^2.

Influence of the Paper Rectangle Dimension on the Perceived Pungency of Piperine. To investigate the influence of the dimension of the filter paper vehicle on the perceived pungency, the human recognition threshold as well as dose−response functions of piperine were placed on filter paper rectangles, varying in the dimension (0.5 × 1, 1 × 1, 1 × 2, and 1 × 3 cm^2). For the human dose−response functions, paper vehicles of various dimensions (0.5 × 0.5, 0.5 × 1, 1 × 1, 1 × 2, 1 × 3, and 1 × 4 cm^2) were loaded with ethanolic solutions of piperine to afford concentrations of 0.21, 0.43, 0.87, 1.74, 3.48, 6.96, 13.92, 27.84, 55.68, 111.36, and 222.72 μg/cm^2 after removal of the solvent in a laboratory oven at 38 °C. First, the panel was asked to compare the taste intensity of the individual test compounds, each at the highest concentration level, on a five-point scale from 0 (not detectable) up to 5 (intensely perceived) by means of the half-tongue test. By definition, the sensory impression induced by piperine at its maximum concentration, that is, 222.72 μg/1 × 3 cm^2 in the present study, was set to a score of 5.0. After the sensory intensity of the paper carriers, loaded with piperine in its maximum concentration per cm^2, had been evaluated, the intensity of the pungency perceived for the remaining dilution steps was evaluated in comparison by means of the half-tongue test. Human dose−response functions with concentrations per cm^2 on the *x*-axis and the intensity of the pungent impression on the *y*-axis were averaged in three independent sessions.

Sensory Comparison of Piperine and Black Pepper Extracts in "Natural" Ratios. Aliquots of purified piperine and the total ethanolic pepper isolate, respectively, were dissolved in ethanol and placed on filter paper rectangles (1 × 2 cm^2) to adjust the concentration corresponding to 25 mg of ground pepper corns per filter paper after evaporation of the solvent at 38 °C. These loaded filter paper rectangles were then presented to the trained sensory panelists, who were asked to rate the intensity of the pungent and tingling sensation on a linear scale from 0 (not detectable) to 5 (very intense) by means of the half-tongue test detailed above.

Quantitative Analysis of Piperine. An aliquot of the ethanol extract prepared from black pepper as described above was diluted with methanol/water (70/30; v/v), membrane filtered, and then analyzed by means of HPLC-DAD on an analytical 250 × 4.6 mm^2 i.d, 5 μm, Microsorb-MV C18 column (Varian, Darmstadt, Germany). Monitoring the effluent at 260 nm, chromatography was performed at a flow rate of 0.8 mL/min with methanol (solvent A) and aqueous formic acid (0.1% in water, pH 3.5; solvent B) using the following gradient: 0 min/70% B, 5 min/70% B, 22 min/80% B, 50 min/80% B, 60 min/100% B, 65 min/100% B, and 70 min/30% B. For quantitation of piperine, a 12-point external standard calibration was used. Calibration curves were prepared by plotting the peak area against the reference concentration, showing correlation coefficients of >0.99. Analyte concentrations in pepper extracts were calculated from the corresponding linear equation.

Identification of Chemosensates. Aliquots of the ethanolic pepper extract were dissolved in methanol/water (70/30, v/v) and, after membrane filtration, were fractionated by means of preparative RP-HPLC. The peaks were collected individually in several runs, and the eluates of the corresponding fractions were combined. After the purity of each fraction was checked by means of analytical RP-HPLC, single compounds were directly analyzed by LC-MS and NMR spectroscopy, while the fractions containing mixtures of individual substances were purified by rechromatography using semipreparative HPLC. Compounds **1a−c**, **2a**, **2b**, **3a−c**, **4a**, **5a**, **6b**, **6c**, **7a−c**, **8a−c**, **9c**, **10a−c**, and **11a−c** were isolated from HPLC fractions given in Table 2 in a purity of more than 98% and, after the solvent was removed in vacuum and freeze-dried twice, were used for sensory experiments as well as for structure determination by means of UV−vis, LC-MS/MS, UPLC-TOF-MS, and 1D/2D NMR. Their spectroscopic data are given in the Supporting Information.

Determination of the Position of the (*Z*)-Configured Double Bond in Alkyl Amides. The positions of (*Z*)-configured double bonds in the alkyl amides **7a−c**, **8a−c**, **10a−c**, and **11a−c** were determined by ozonolysis, followed by reductive cleavage and analysis of the generated aldehyde by means of GC-MS.[56] Ozone was generated by means of an ozone generator model 502 (Fischer, Bonn-Bad Godesberg, Germany), operating with an oxygen flow of 200 L/h (purity, 5.0; Westfalen, Germany) and 76 W and was bubbled through the stirred solution of alkyl amides (3 mg) in methanol/methylene chloride (18 mL; 1/1; v/v) cooled to −78 °C. Ozonization was stopped as soon as the starch−potassium iodide solution (a mixture of 5 g of starch cooked in 45 mL of water and 10 g of potassium iodide in 90 mL of water) in a wash bottle turned blue. After the excess of ozone was removed by a stream of nitrogen, triphenylphosphine (100 mg) was added, and the mixture was stirred for 15 min at room temperature.

An aliquot of the solution was used for GC-MS analysis. Comparison of the retention times (DB-5 column) and mass spectrometric data with those of the reference compounds, followed by cochromatography, led to the identification of pentanal and hexanal as the volatile aldehydes released from **7a−c**, **8a−c**, **10a−c**, and **11a−c**, upon ozonolysis.

Synthesis of Piperlonguminine (1c) and Piperyline (1b). *Preparation of Piperic Acid.* Following a procedure described in literature[57,58] with some modifications, piperine (7.1 mmol) was added to a solution of potassium hydroxide (5.6 g) in diethylene glycol (20 mL) and then stirred for 4 h at 98 °C, followed by 20 h at 110 °C. After it was cooled to room temperature, the resulting suspension was diluted with methanol (140 mL) and water (250 mL), and the aqueous layer was adjusted to pH 11 with aqueous hydrochloric acid (1 mol/L) and then extracted with ethyl acetate (4 × 200 mL). The combined organic layers were discarded, the aqueous phase was acidified with aqueous hydrochloric acid (1 mol/L) to pH 3 and extracted with ethyl acetate (4 × 200 mL), the organic phase was dried over anhydrous Na_2SO_4, and after filtration, the solvent was removed in vacuum. Crystallization from methanol afforded piperic acid (4.7 mmol; 66% in yield) as an amorphous powder.

Piperic Acid. UV/vis (MeOH/Wasser; pH 3.5): λ_{max} = 340. LC-MS (ESI^+): *m/z* 241 (100, $[M + Na]^+$), 219 (98, $[M + H]^+$), 459 (89, $[2M + Na]^+$), 437 (21, $[2M + H]^+$). ^1H NMR (400 MHz, DMSO-d_6; COSY): δ/ppm: δ 5.93 [d, 1H, *J* = 15.5 Hz, H−C(11)]; δ 6.05 [s, 2H, H−C(1)]; δ 6.91−6.98 [m, 3H, H−C(6,8,9)]; δ 7.01 [dd, 1H, *J* = 1.5 Hz, *J* = 8.0 Hz, H−C(5)]; δ 7.23 [d, 1H, *J* = 1.5 Hz, H−C(3)]; δ 7.27−7.34 [m, 1H, H−C(10)]. ^{13}C NMR (100 MHz, DMSO-d_6; HMQC, HMBC): δ 101.8 [C(1)]; 106.2 [C(3)]; 108.9 [C(6)]; 121.6 [C(11)]; 123.5 [C(9)]; 125.3 [C(8)]; 130.9 [C(4)]; 140.1 [C(8)]; 145.1 [C(10)]; 148.4 [C(7)]; 148.5 [C(2)]; 167.2 [C(12)].

*Piperlonguminine (**1c**) and Piperyline (**1b**).* Following a procedure described in literature[57,59] with some modifications, thionyl chloride (6 mmol) was added dropwise to a solution of the piperic acid (3 mmol) in dry tetrahydrofurane (4 mL). Thereafter, pyridine (100 μL) was added, and the reaction mixture was kept stirring continuously under an argon atmosphere for 4 h at room temperature. After the solvent was removed under a steam of nitrogen, piperic acid chloride was taken up in dry tetrahydrofurane (10 mL) under an atmosphere of argon and isobutyl amine (6 mmol; for synthesis of **1c**) and pyrrolidine (6 mmol; for synthesis of **1b**), respectively, solved in tetrahydrofurane (1 mL), and was added dropwise over about 30 min. After the reaction mixture was stirred for 5 h at room temperature, the sample was diluted with *tert*-butyl methyl ether (200 mL) and washed with water (3 × 200 mL), followed by aqueous hydrochloric acid (0.5 N, 200 mL) and brine (200 mL). The organic layer was dried over Na_2SO_4, and after filtration, the solvent was removed in vacuum. Crystallization from a mixture of hexane and *tert*-butyl methyl ether (50/50; v/v; 10 mL), followed by preparative RP-HPLC, afforded the target amides piperlonguminine (**1c**; 1.73 mmol; 58% in yield) and piperyline (**1b**; 2.2 mmol; 73% in yield) as white powders in a purity of >98%. Spectroscopic (NMR, LC-MS, UV−vis) and chromatographic data (RP-HPLC) as well as the sensory threshold concentrations of the synthetic compounds were identical to the data obtained for **1c** and **1b** isolated from black pepper. Spectroscopic data are given in the Supporting Information.

Synthesis of Piperoleine (2b) and Piperettine (2a). Following a literature procedure[60] with some modifications, thionyl chloride (12 mmol) was added dropwise to a solution of sorbic acid (6 mmol) in dry tetrahydrofurane (7 mL) in a brown glass reaction vessel under an atmosphere of argon. Then, pyridine (100 μL) was added, and the reaction mixture was kept stirring for 4 h at room temperature. After the solvent was removed under a stream of nitrogen, an orange oil was obtained that was taken up in tetrahydrofurane (7 mL) and divided into two equal aliquots. A solution of pyrrolidine (3 mmol, for synthesis of **2b**) and piperidine (3 mmol, for synthesis of **2a**), respectively, in tetrahydrofurane (1 mL) was added dropwise to either one of the aliquots over a periode of 30 min. Each mixture was heated for 2 h at 60 °C under an atmosphere of argon and, after it was cooled to room temperature, was diluted with dichloromethane (200 mL), followed by an extraction with water (3 × 200 mL), aqueous hydrochloric acid (0.5 N, 200 mL), and brine (200 mL). The organic layer was dried over Na_2SO_4 and, after filtration, separated from solvent in vacuum. The residue was crystallized from a mixture (50/50; v/v; 5 mL) of ethyl acetate and diethyl ether to afford 1-[(2*E*,4*E*)-hexadienoyl]-pyrrolidine (2.21 mmol; 73% in yield) and 1-[(2*E*,4*E*)-hexadienoyl]-piperidine (1.47 mmol; 49% in yield) as white powders.

A portion of 1-[(2*E*,4*E*)-hexadienoyl]-pyrrolidine (1.5 mmol) or 1-[(2*E*,4*E*)-hexadienoyl]-piperidine (1.3 mmol), respectively, piperonal (1.5 mmol), and Aliquat 336 (2 mL) were added to a suspension of anhydrous potassium carbonate (55 mg) in toluene (35 mL). Then, sodium hydride (11 mg) was added carefully, and after it was stirred for 6 h at 90 °C under an atmosphere of argon, the reaction mixture was poured onto crushed ice. The aqueous phase was extracted with dichloromethane (3 × 200 mL), the combined organic extracts were dried over Na_2SO_4, and after filtration, the solvent was separated in vacuum. The residue was dissolved in a mixture (90/10, v/v; 20 mL) of ethyl acetate and hexane and applied onto the top of a water-cooled 2.5 × 25 cm^2 glass column filled with a slurry of 0.063−0.200 mm silica gel 60 (Merck) in ethyl acetate/hexane (90/10, v/v). After the target compounds were eluted with ethyl acetate/hexane (90/10, v/v; 500 mL) and the solvent was separated in vacuum, the isolate was purified by means of solid phase extraction using C18-E SPE cartridges (10 g/60 mL; Phenomenex, Aschaffenburg, Germany), conditioned with methanol (60 mL), followed by methanol/water (70/30, v/v; 100 mL). Clean-up was performed by flushing the cartridge with three portions of a mixture of methanol/water (70/30; v/v; 30 mL). Elution with the last portion (30 mL) and separation of the solvent in vacuum revealed the target compounds piperoleine (**2b**, 1.32 mmol; 89% in yield) and piperettine (**2a**, 0.77 mmol; 59% in yield) in a purity of >98%. Spectroscopic (NMR, LC-MS, UV−vis) and chromatographic data (RP-HPLC) of the synthetic compounds were identical to the data obtained for **2b** and **2a** isolated from black pepper. Spectroscopic data are given in the Supporting Information.

HPLC. The HPLC system (Jasco, Groß-Umstadt, Germany) consisted of two PU-2087 Plus pumps, an AS-2055 Plus autosampler, a 7725i type Rheodyne injection valve (Rheodyne, Bensheim, Germany), and a MD-2010 Plus diode array detector monitoring the effluent in a range between 220 and 500 nm. Data acquisition was performed by means of Chrompass 1.8.6.1 (Jasco, Groß-Umstadt, Germany). For chromatography, aliquots of 25 μL or 0.4 or 1 mL, respectively, were injected onto a 250 × 4.6, a 250 × 10, or a 250 × 21.2 mm i.d., 5 μm, Microsorb-MV C18 column (Varian) operated with a flow rate of 0.8, 3.0, and 18 mL/min, respectively. The solvent system consisted of methanol (A) and aqueous formic acid (0.1% in water, pH 3.5; B), and a gradient was used as follows: 0 min/70% B, 5 min/80% B, 22 min/80% B, 50 min/80% B, 60 min/100% B, 65 min/100% B, and 70 min/30% B. Monitoring the effluent at 260 nm, chromatography was performed.

Liquid Chromatography/Mass Spectrometry (LC-MS/MS). For compound identification, mass and product ion spectra were acquired on an API 4000 Q Trap triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Darmstadt, Germany). The isolated fractions were dissolved in a mixture of methanol/water (70/30, v/v) and directly introduced into the mass spectrometer by flow infusion using a syringe pump. Data were aquired in full-scan mode with positive electrospray ionization (+5500 V). Both quadrupoles operated at unit mass resolution, and nitrogen served as a curtain gas (25 psi) and as a turbo gas (425 °C). Fragmentation of the pseudo molecular ions $[M + H]^+$ into specific product ions was induced by collision with nitrogen (4.5×10^{-5} Torr). Data acquisition and instrumental control were performed with Analyst 1.4.2 software (Applied Biosystems).

UPLC/Time-of-Flight Mass Spectrometry (UPLC/TOF-MS). High-resolution mass spectra of the target substances **1−11c** were measured on a SYNAPT G2 HDMS (Waters UK Ltd., Manchester, United Kingdom) 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. All chemosensates were dissolved in 1 mL of methanol, and aliquots (1−5 μL) were injected into the UPLC-TOF-MS system. The samples were introduced into the instrument via an Acquity UPLC core system (Waters, Milford, MA) consisting of a binary solvent manager, a sample manager, and a column oven. For chromatography, a 2 × 150 mm^2 i.d., 1.7 μm, and a BEH C18 column (Waters) was operated with a flow rate of 0.3 mL/min at a temperature of 40 °C. The solvent system consisted of acetonitrile (A) and aqueous formic acid (0.1% in water, pH 2.5; B). The following gradient was used: 0 min/50% B, 7 min/100% B, 9 min/100% B, and 10 min/50% B. The instrument was calibrated over a m/z range of 100−1200 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) and the tool "elemental composition".

High-Resolution Gas Chromatography−Mass Spectrometry (HRGC-MS). Electron impact (EI) GC-MS data were acquired on a 5890 series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) connected to a sector field mass spectrometer type MAT 95 S (Finnigan, Bremen, Germany). Data acquisition was carried out with the ICIS software (Finnigan). Chromatographic separation was performed on a 30 m × 0.25 mm, 0.25 μm DB-5 capillary (J&W Scientific, Agilent Technologies, Santa Clara, CA). The initial oven temperature was set to 40 °C, then raised at a rate of 6 °C/min to 120 °C, thereafter with 15 °C/min to 240 °C, and, finally, held isothermally for 10 min at 240 °C. Mass spectra in the electron ionization mode (MS-EI) were recorded at 70 eV ionization energy and mass spectra in the chemical ionization mode (MS-CI) at 115 eV using isobutane as reactant gas.

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 spectrometer (Bruker, Rheinstetten, Germany), respectively. DMSO-d_6 was used as the solvent, and chemical shifts are reported in parts per million relative to the solvent signal. For structural elucidation and NMR signal assignment 2D NMR experiments, COSY, HMQC, and HMBC spectroscopy were carried out using the pulse sequences taken from the Bruker software library. Data processing was performed by using XWin-NMR software (version 3.5; Bruker, Rheinstetten, Germany) as well as Mestre-C (Mestrelab Research, La Coruña, Spain).

RESULTS AND DISCUSSION

To study the pungent and tingling key molecules in black pepper by application of a TD approach, a modified half-tongue test was developed to overcome the limited water solubility of pepper extracts and compounds isolated thereof for the sensory evaluation. Although 5% ethanolic solutions would partially overcome the limited solubility of the pungent amides, literature reports showed ethanol to cause potentiation of vanilloid receptor-1 activity.[47] As synergistic activities of ethanol on the perception of pungent pepper compounds could not be excluded, an ethanol-free sensory assay needed to be developed first.

Half-Tongue Test Using Filter Paper Rectangles. The recently developed half-tongue test[52] was modified in a way that filter paper rectangles were used as the vehicle instead of aqueous solutions. To investigate the influence of the dimension of the filter paper carrier on the sensory perception of pungent and tingling molecules, the recognition threshold concentrations of purified piperine placed in serial dilutions onto the top of the filter paper vehicles of varying dimensions (0.5 × 0.5, 0.5 × 1, 1 × 1, 1 × 2, 1 × 3, and 1 × 4 cm) were determined using a half-mouth strategy with corresponding blank filters of the same dimension as the control (Table 1).

Table 1. Influence of the Dimension of the Filter Paper Vehicle on the Human Oral Recognition Thresholds of Piperine (1a)

	recognition threshold for pungency[a]	
vehicle dimension	nmol	nmol/cm^2
0.5 × 0.5 cm	0.2	0.8
0.5 × 1.0 cm	0.3	0.6
1.0 × 1.0 cm	0.3	0.3
1.0 × 2.0 cm	0.6	0.3
1.0 × 3.0 cm	2.1	0.7
1.0 × 4.0 cm	4.0	1.0

[a]The recognition thresholds were determined by 12 panelists using the half-tongue test and filter paper vehicles of varying dimensions loaded with serial dilutions of piperine.

While application of 0.5 × 0.5 cm piperine-loaded filter papers did not allow us to clearly locate pungency, the 1 × 4 cm vehicles were too big to place them onto the tongue surface. Best results were found for the paper carrier dimension 1 × 2 cm. Comparison of the threshold concentrations of piperine-loaded vehicles with the dimensions 0.5 × 1, 1 × 1, 1 × 2, and 1 × 3 cm revealed rather similar values ranging between 0.3 and 0.7 nmol/cm^2. In consequence, all sensory tests were accomplished using 1 × 2 cm paper vehicles, and the recognition threshold concentrations were given in nmol/cm^2.

In a second set of experiments, human psychometric functions were recorded for rectangular paper vehicles of various dimensions loaded with piperine in increasing concentrations of 0.21 up to 222.72 μg/cm^2. To fit the dose−response functions into a five-point numeric scale, first, the panel was asked to compare the taste intensity of the vehicles loaded with the highest piperine concentrations on a five-point scale from 0 (not detectable) up to 5 (intensely perceived) by means of the half-tongue test. By definition, the sensory impression induced by piperine at its maximum concentration, that is, 222.72 μg/1 × 3 cm in the present study, was set to a score of 5.0. After the sensory intensity of the paper vehicles loaded with piperine in its maximum concentration of 222.72 μg/cm^2 had been evaluated, the intensity of the pungency perceived for the remaining dilution steps was evaluated in comparison by means of the half-tongue test. Oral response functions with concentrations per cm^2 on the x-axis and pungent intensities on the y-axis (Figure 2) showed that the panelists were able to

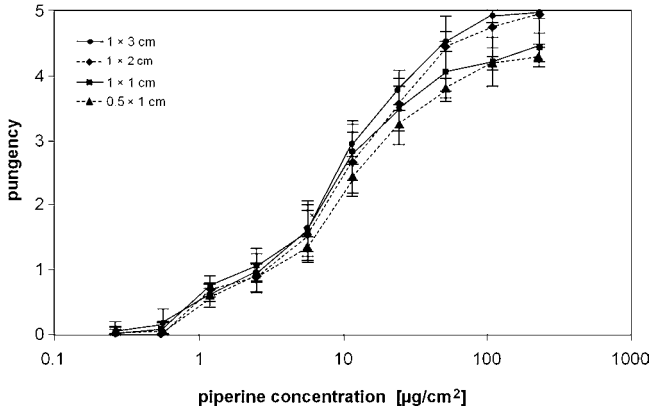

Figure 2. Influence of the vehicle dimension on the human dose−response function of piperine (**1a**) using the half-mouth procedure. The data are given as the means of three replicates, and error bars indicate the confidence interval (P = 95%) of the arithmetical mean.

differentiate between the concentrations on the individual vehicle dimensions. In particular, the dimension of the vehicles strongly influenced the orosensory response following rather different dose−response functions. Depending on the dimension of the paper vehicles, panelists perceived different pungent intensities for piperine, although present in the same amount per cm^2, thus demonstrating that the size of the activation surface on the tongue is influencing the sensory impression of pungency. Larger paper vehicles (1 × 2 and 1 × 3 cm), presenting the same amount of stimulus per surface area but higher amounts in total, were found to induce higher pungency intensities reaching the maximum score of 5.0 in concentrations above 6.96 $\mu g/cm^2$, whereas significantly lower intensities were found for smaller filter papers with dimensions of 0.5 × 1 and 1 × 1 cm, respectively, loaded with the same piperine amount per cm^2. In conclusion, the intensity of the perceived pungency depends on the size of the filter paper carrier and, therefore, on the activated surface size of the tongue as well as on the concentration of the stimulus. Because of the user friendly handling and the ability of the 1 × 2 cm carriers to evoke rather high pungent intensities, this dimension was chosen for further half-mouth tests with the other pepper ingredients.

Sensory Comparison of Black Pepper Extract vs. Purified Piperine. To estimate the sensory contribution of piperine and that of additional candidate molecules to the pungency of black pepper, an ethanol extract of black pepper corns was prepared, and after quantitative analysis of the piperine content by means of HPLC/DAD, an aliquot of the ethanol extract as well as a sample of purified piperine, respectively, were placed on rectangular filter paper vehicles (1 × 2 cm) to match the concentration corresponding to 25 mg of ground pepper corns per vehicle. These stimulus-loaded vehicles were then presented to the trained sensory panelists, who were asked to rate the intensity of the pungent and tingling sensation on a linear scale from 0 (not detectable) to 5 (very intense) by means of the half-tongue test described above. The trained sensory panel perceived a clear difference between the vehicle loaded with the black pepper extract and the piperine. While the pepper extract was rated with the highest score of 5.0 for pungency and, in addition, some tingling activity (0.5), the purified piperine exhibited a less strong pungent sensation scored with a value of 2.5 only (data not shown). Although piperine is an important sensory contributor, these data allowed the conclusion that additional compounds account for the pungency of black pepper.

Taste Dilution Analysis (TDA). To sort out the pungent and tingling compounds from the bulk of less taste-active or tasteless substances, the pepper extract was separated by means of preparative RP-HPLC. Monitoring the absorption at 260 nm (Figure 3), a total of 41 HPLC subfractions were collected, separated from solvent using vacuum, and taken up in ethanol, and serial 1:1 dilutions were placed on top of filter paper vehicles (1 × 2 cm), which were then freed from solvent at 38 °C. The serial dilutions of each of these fractions were presented to the sensory panel in order of ascending concentration and used to determine TD factors by means of the half-tongue test (Figure 3). The highest TD factor of 1024 was found for the pungent fraction no. 7, followed by fractions no. 5, 6, 11, and 37, all of which exhibited a pungent orosensation up to a dilution of 1:128. In addition, fractions no. 10, 13, 33, 35, and 36−40 induced, besides a pungent sensation, also a pronounced tingling effect on the tongue evaluated with TD factors between 4 and 32. All other fractions showed comparatively lower taste impacts. Aimed at characterizing the molecular structure of the compounds imparting the most intense pungent and tingling sensation of black pepper, LC-MS and NMR experiments as well as

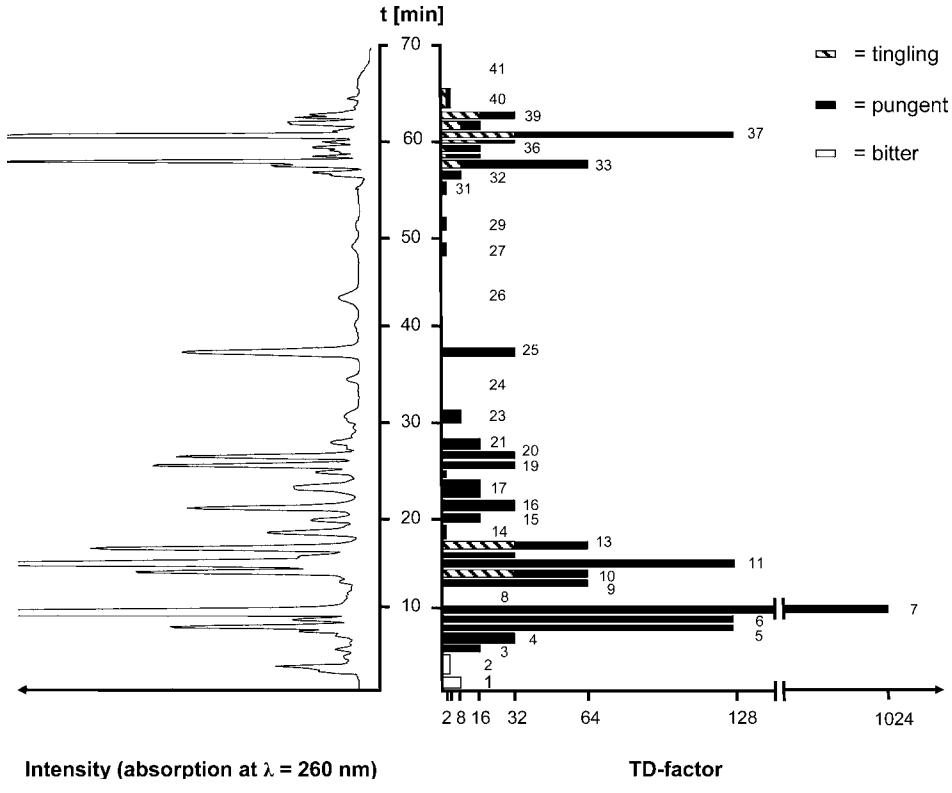

Figure 3. RP-HPLC chromatogram (A) and TD chromatogram (B) of the ethanol extract prepared from black pepper.

synthetic studies were focused on the HPLC fractions no. 3−20, 27, 29, and 33−38.

Identification of Pungent Chemosensates in HPLC Fraction 3−20, 27, and 29. Comparison of chromatographic (RP-HPLC) and spectroscopic data (NMR, LC-MS, UV/vis) with those of the reference compound, followed by cochromatography, led to the identification of piperine (**1a**) as the pungent principle of fraction 7 judged with the highest TD factor of 1024 (Table 2).

Separation of fractions no. 5 and 6 by means of semipreparative HPLC revealed two pungent compounds (**1b**, **1c**), both in a purity of >98%, which showed pseudomolecular ions ($[M + H]^+$) with *m/z* 274 (**1c**) and *m/z* 272 (**1b**), respectively, in the LC-MS spectrum recorded by means of electrospray ionization. The MS/MS fragmentation patterns of both compounds were rather similar to that found for piperine (**1a**) exhibiting the daughter ion *m/z* 201 of the piperonal unit upon cleavage of the piperic acid amide structure. The loss of 73, 71, and 85 amu, detected in the MS/MS spectrum of **1c**, **1b**, and **1a**, respectively, indicated the cleavage of the amine moieties of isobutylamine, pyrrolidine, and piperidine as typically found in pepper amides.[61] Finally, the identity of **1b** and **1c** was confirmed by independent synthesis to be piperlonguminine (**1c**) and piperyline (**1b**) (Figure 1), thus confirming literature data.[11−13]

Isolation of fractions nos. 10 and 11 by means of preparative RP-18 column chromatography, followed by 1D/2D NMR analysis, revealed a 3,4-methylenedioxyphenyl structure as well as a 2,4,6-heptatrienoyl moiety for both molecules, but differences in the amine moiety, for example, a pyrrolidine ring and a piperidine ring, were identified in compound **2a** and **2b** isolated from fractions 10 and 11, respectively. Independent synthesis, comparison of the spectroscopic and chromatographic data, as well as cochromatography led to the unequivocal identification of piperettine (**2a**) and piperoleine (**2b**) (Figure 1), thus confirming data reported earlier.[13,62,63]

The pungent compounds **3a−3c** isolated from HPLC fraction nos. 13, 14, and 16 showed the typical absorption maximum at 264 nm as reported for piperine analogues. LC-MS analysis showed a pseudomolecular ion ($[M + H]^+$) of *m/z* 328 for **3c**, *m/z* 326 for **3b**, and *m/z* 340 for **3a**, respectively. LC-MS/MS fragmentation experiments revealed the loss of isobutylamine, pyrrolidine, or piperidine moieties as found for **1c**, **1b**, and **1a**, respectively. The ^{1}H NMR spectrum of each compound showed six olefinic and four aliphatic protons, which were attributed to a *trans*-configured diene system H−C(12−15), a *trans*-configured double bond H−C(8,9) next to the benzodioxol moiety, and two methylene groups H−C(10) and H−C(11) as confirmed by couplings observed in a COSY experiment. The accurate geometry of each double bond was unambiguously identified by comparing the ^3J coupling constants of the olefinic protons. Additional 1D/2D NMR experiments enabled the unequivocal identification of the chemosensates in fractions 13, 14, and 16 as retrofractamide A (**3c**, Figure 1), 1-[1-oxo-9(3,4-methylenedioxyphenyl)-2*E*,4*E*,8*E*-nonatrienyl] pyrrolidine (**3b**), and dehydropipernonaline (**3a**), thus confirming previously reported findings.[13,14,64,65]

After HPLC purification of the key molecules in fractions 19 and 20, LC-MS/MS as well as 1D/2D NMR experiments led to the unequivocal structure determination of pipernonaline (**4a**) and piperolleine B (**5a**) as additional pungent compounds and confirmed data reported earlier.[13,64]

Table 2. Orosensory Quality and TD Factors of HPLC Fractions Isolated from an Ethanol Extract of Black Pepper

fraction no.[a]	sensory quality[b]	TD factor	chemosensate identified[c]
1	bitter	8	
2	bitter	4	
3	pungent	16	
4	pungent	32	
5	pungent	128	**1c**
6	pungent	128	**1b**
7	pungent	1024	**1a**
8			
9	pungent	64	
10	pungent	64	**2b**
	tingling	32	
11	pungent	128	**2a**
12	pungent	32	
13	pungent	64	**3c**
	tingling	32	
14	pungent	2	**3b**
15	pungent	16	
16	pungent	32	**3a**
17	pungent	16	
18	pungent	2	
19	pungent	32	**4a**
20	pungent	32	**5a**
21	pungent	16	
22			
23	pungent	8	
24			
25	pungent	32	**6c**
26			**6b**
27	pungent	4	
28			
29	pungent	4	
30			
31	pungent	4	
32	pungent	8	
33	pungent	64	**7c**, **8c**
	tingling	8	
34	pungent	16	
	tingling	2	
35	pungent	16	**7b**, **8b**
	tingling	2	
36	pungent	32	**7a**, **8a**
	tingling	16	
37	pungent	128	**9**, **10c**, **11c**
	tingling	32	
38	pungent	16	**10b**, **11b**
	tingling	8	
39	pungent	32	**10a**, **11a**
	tingling	16	
40	pungent	8	
	tingling	4	
41			

[a]Number of HPLC fractions referring to Figure 3. [b]The sensory quality and the TD factor were determined by using the half-tongue test. [c]The structures of the compounds given as numbers are displayed in Figure 1.

LC-MS and LC-TOF-MS analysis of compound **6c** isolated from fraction no. 25 showed a molecular mass of 383 Da and indicated an elemental composition of $C_{24}H_{33}NO_3$. Careful assignment of all 1D/2D NMR data confirmed the structure of

6c as the recently reported guineensine.[66,67] The LC-MS and the NMR spectra of the compound **6b** isolated from fraction 26 indicated the molecular mass of 381 Da and revealed that the structure of this compound differed from that of **6c** only in the amide moiety representing a pyrrolidine ring instead of an isobutylamine residue. Comparison of spectroscopic data and 1D/2D NMR experiments as well as comparison with data found in literature[68] allowed the identification of compound **6b** as brachyamide A (Figure 1).

Identification of Pungent and/or Tingling Chemosensates in HPLC Fractions 33 and 35−39. Application of UV/vis spectroscopy on the compounds detected in fractions 33−39 revealed typical absorption maxima at 260 nm as expected for long-chain conjugated 2,4-dienoic acid amides. LC-MS analysis of the pungent compounds **7c** and **8c** both detected in HPLC fraction 33 showed the same pseudomolecular ion ($[M + H]^+$) with m/z 334, thus confirming the presence of one nitrogen atom in each molecule. On the basis of the 22 carbon atoms detected in the ^{13}C NMR spectrum and the ^1H NMR resonance signals integrating for 39 protons for each substance, an elemental composition of $C_{22}H_{39}NO$ was proposed for compounds **7c** and **8c**. Comparison of MS as well as 1D/2D NMR data with those reported in literature confirmed the structure of **8c** (Figure 1) as (2*E*,4*E*,12*Z*)-*N*-isobutyloctadeca-2,4,12-trienamide.[13,69,70] To determine the location of the *cis*-double bond, an ozonolysis was performed, followed by reductive cleavage and analysis of the released volatile aldehyde by means of GC-MS (Figure 4). Besides the expected hexanal generated from **8c**, pentanal could be identified in a ratio of 1:4.5, thus suggesting the structure of **7c** to be the previously not reported (2*E*,4*E*,13*Z*)-*N*-isobutyloctadeca-2,4,12-trienamide (Figure 1). This structure is well in line with the two additional ^{13}C NMR signals resonating at 31.3 and 21.6 ppm. Additional integration of the ^{13}C NMR resonance signal of C(16) at 31.1 (**8c**) and 31.3 ppm (**7c**) in the fully relaxed spectrum confirmed the same ratio of **8c**:**7c** to be 1:4.5 (Figure 5).

LC-TOF-MS of the two amides isolated from HPLC fraction 35 revealed an exact mass of m/z 332.2983 Da for both alky amides, fitting well with the molecular formula of $C_{22}H_{38}NO_3$. 1D/2D NMR studies revealed rather similar spectroscopic data as found for **8c**/**7c**. The geometries of the double bonds were unambiguously determined by analyzing the ^3J proton coupling constants of the six olefinic protons. The protons at H−C(3) and H−C(4) showed double doublets at 7.06 and 6.27 ppm and coupling constants of 10.5 and 15.1 Hz, while the protons H−C(2) and H−C(5) were detected at 6.49 and 6.09−6.16 ppm. Their characteristic coupling constant of 15.1 Hz indicated the presence of a *trans*,*trans*-configured diene system conjugated with the carbonyl group C(1) resonating at 164.2 ppm in the ^{13}C NMR spectrum. Furthermore, the signals of a third double bond C(12,13) for **8b** and C(13,14) for **7b** were observed at 5.28−5.34 ppm showing the typical *cis*-olefinic coupling constant of 11 Hz. The location of the isolated *cis*-double bond in both compounds was unequivocally determined by ozonolysis, followed by reductive cleavage, thus demonstrating the release of hexanal and pentanal in a ratio of 1:3. The signal intensities of the carbon atoms C(16A/16B) of the fully relaxed spectrum reflected the ion intensity of hexanal and pentanal found by GC/MS. While **8c**/**7c** showed typical resonances of isobutylamine moieties, the structures of compounds **7b**/**8b** were decorated with a pyrrolidine moiety as deduced from the typical coupling pattern of the protons H−C(19a/b) and H-(20a/b) in the COSY spectrum. Taking all of these data into consideration, the pungent and tingling compounds isolated from fraction 35 were identified as 1-(octadeca-2*E*,4*E*,13*Z*-trienyl) pyrrolidine (**7b**, Figure 1) and 1-(octadeca-2*E*,4*E*,12*Z*-trienyl) pyrrolidine (**8b**), which to the best of our knowledge have not been reported earlier in literature.

The pungent and tingling compounds **7a** and **8a**, isolated from fraction 36 (Table 2), exhibited a pseudomolecular ion of m/z 368 ($[M + Na]^+$), fitting well with a molecular formula of $C_{23}H_{39}NO$. Homo- and heteronuclear correlation experiments (COSY, HMQC, HMBC) gave a comprehensive picture on the type of the fatty acid amide, which was structurally related to **7b** and **8b** and led to the identification of **7a** as 1-(octadeca-2*E*,4*E*,13*Z*-trienoyl) piperidine and **8a** as 1-(octadeca-2*E*,4*E*,12*Z*-trienoyl) piperidine (Figure 4) in a ratio of 1.8 to 1. Although **8a** was previously identified in *Piper retrofractum*,[70] this is the first report of its natural occurrence in *P. nigrum* L.

Rechromatography of fraction 37 by means of isocratic RP-18 HPLC revealed three pungent and tingling compounds (**9c**, **10c**, **11c**). Compound **9c** showed an identical UV/vis absorption maximum at 260 nm as well as a rather similar mass spectrometric fragmentation as found for **7**/**8a**−**7**/**8c**, thus suggesting the presence of another alkylamide. LC-MS experiments, ^1H, ^{13}C, and DEPT NMR analysis revealed a molecular formula of $C_{22}H_{41}NO$, the presence of two conjugated, *trans*-configured double bonds, the absence of another isolated double, and an isobutylamine moiety in the structure of **9c**. Taking all of the spectroscopic data into consideration, the structure of **9c** was identified as (2*E*,4*E*)-*N*-isobutyl-octadeca-2,4-dienamide (Figure 1), which has been reported earlier.[37,61,66] LC-MS analysis of amides **10c** and **11c** showed a pseudomolecular ion with m/z 362 ($[M + H]^+$), thus suggesting the presence of two further methylene groups when compared to **7c**/**8c**. NMR spectroscopic analysis revealed three terminal methyl groups H−C(3′), H−C(4′), and H−C(20), six olefinic protons assigned as H−C(2−5) and H−C(14/15) or H−C(15/16), and 13 methylene groups assigned as H−C(1′), H−C(6−13), or H−C(6−14) and H−C(16−19) or H−C(17−19) for each compound. On the basis of the signal assignment, compounds **10c** and **11c** were identified as (2*E*,4*E*,15*Z*)- and (2*E*,4*E*,14*Z*)-*N*-isobutyl-eicosa-2,4,14-trienamide (Figure 1). Although compound no. **11c** was earlier reported in *P. retrofractum*, *Piper cheba*, and *Piper longum*,[37,69,70] this is the first report in black pepper.

The spectroscopic data obtained for the pungent amides **10b**/**11b** and **10a**/**11a**, isolated from subfractions 38 and 39, indicated them as the corresponding pyrrolidine and piperidine analogues of **10c**/**11c**. On the basis of LC-MS and 1D/2D NMR data, compounds **10b** and **11b** were identified as 1-(eicosa-2*E*,4*E*,14*Z*-trienoyl) pyrrolidine (**10b**) as well as 1-(eicosa-2*E*,4*E*,15*Z*-trienoyl) pyrrolidine (**11b**, Figure 1). Moreover, compounds **10a** and **11a** were identified as 1-(eicosa-2*E*,4*E*,15*Z*-trienoyl) piperidine (**10a**) as well as 1-(eicosa-2*E*,4*E*,14*Z*-trienoyl) piperidine (Figure 1). While **10a**/**11a** showed the typical coupling pattern in the COSY spectrum of piperidine moieties, the structures of compounds **10a**/**11a** were decorated with a pyrrolidine moiety as deduced from the typical coupling pattern of the protons H−C(21a/b) and H-(22a/b) in the COSY spectrum. For an unequivocal determination of the location of the isolated *cis*-double bonds, each fraction was treated by ozonolysis to give hexanal and pentanal in a ratio of 1:5.7 for **10b**/**11b** and 1:8 for **10a**/**11a**, respectively. Although

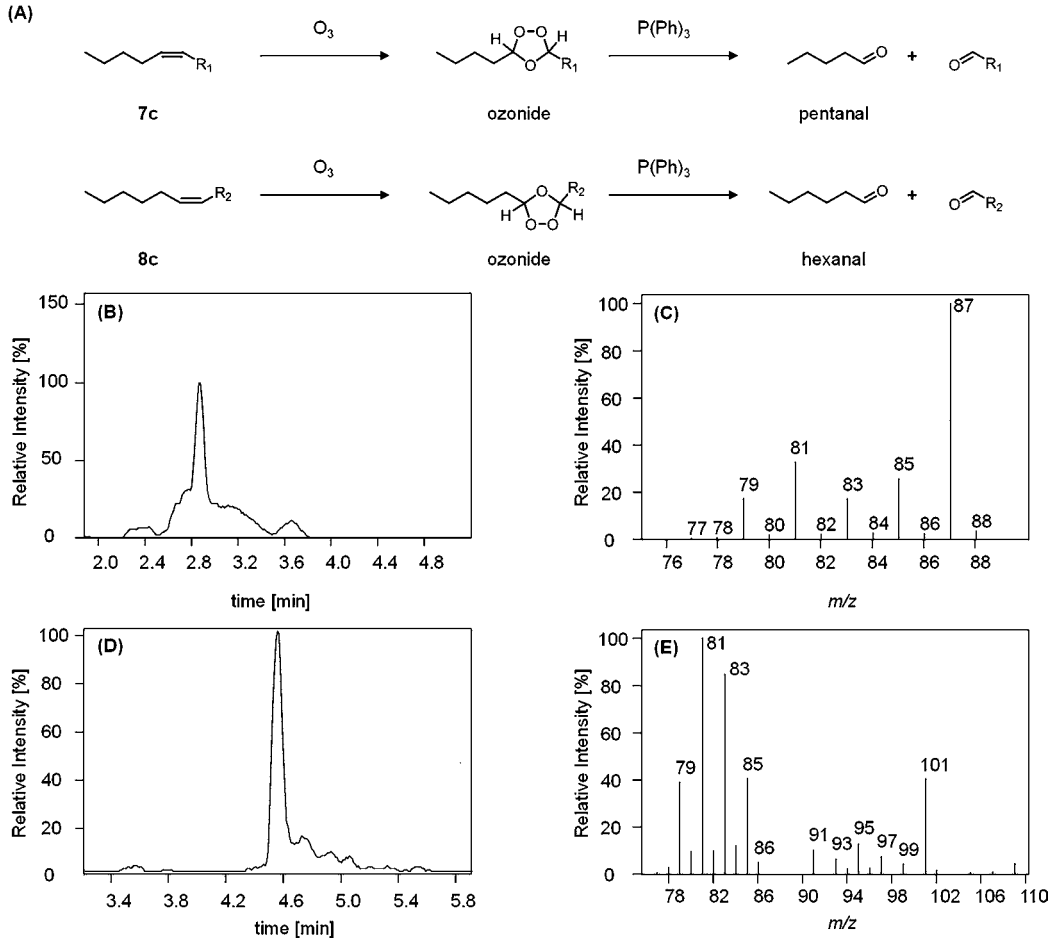

Figure 4. Ozonolysis of (2*E*,4*E*,13*Z*)-*N*-isobutyl-octadeca-2,4,13-trienamide (**7c**) and (2*E*,4*E*,12*Z*)-*N*-isobutyl-octadeca-2,4,12-trienamide (**8c**) (A) as well as the gas chromatograms (B, **7c**; D, **8c**) and mass spectra (MS-CI; isobutane) of the resulting aldehydes (C, **7c**; E, **8c**).

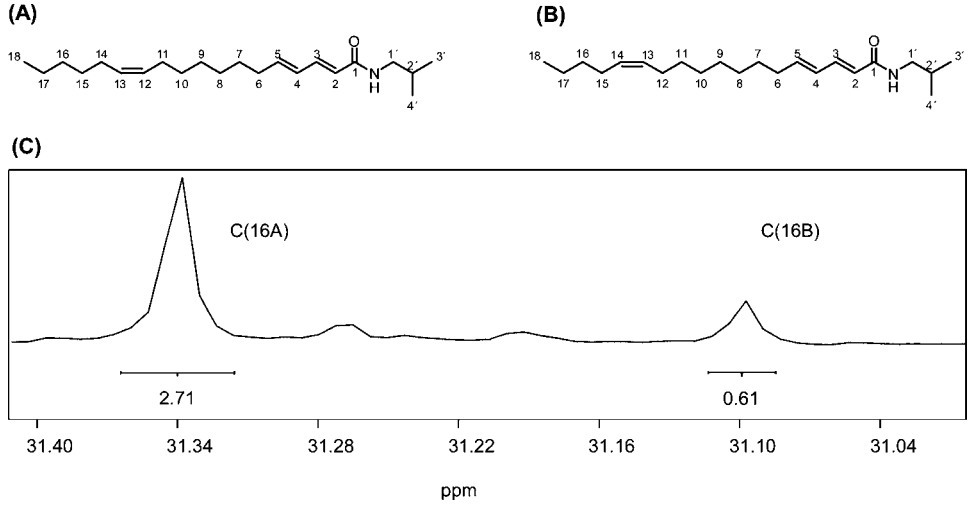

Figure 5. Structures of (2*E*,4*E*,12*Z*)-*N*-isobutyl-octadeca-2,4,13-trienamide (A, **8c**) and (2*E*,4*E*,13*Z*)-*N*-isobutyl-octadeca-2,4,12-trienamide (B, **7c**) as well as a selected region of the integrated ^{13}C NMR spectrum of **8c** and **7c** (C). The signal intensities of the carbon atoms C(16A/16B) of the fully relaxed spectrum reflect the intensity found by GC/MS.

11a was already identified in *P. retrofractum*,[70] **10a**, **10b**, **11a**, and **11b** were previously not reported in *P. nigrum* L.

Sensory Evaluation of Pungent and/or Tingling Chemosensates. Prior to sensory analysis, the purity of all compounds was confirmed by HPLC-MS as well as ^{1}H NMR spectroscopy to be more than 98%. To determine the human threshold concentrations for the pungent and tingling sensation of the selected chemosensates, paper vehicles loaded with the test compounds in serial dilutions were evaluated by means of an half-tongue test. Depending on their chemical structure, the oral sensations induced by the amides were described as pungent, tingling, or both. Whereas all of the piperine

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analogues (**1b**–**6c**) exhibited a clear pungent sensory profile, the group of conjugated 2,4-dienoic acid amides with an additional *Z*-configured double bound as found in **7a**–**c**, **8a**–**c**, **10a**–**c**, and **11a**–**c** induced a pungent impression as well as a long-lasting tingling sensation. While the human threshold concentrations determined for pungency ranged from 3.0 to 1150.2 nmol/cm^2, the oral threshold concentrations for the tingling sensation ranged from 520.6 to 2162.1 nmol/cm^2 and, for a given compound, was always above the recognition threshold determined for pungency (Table 3).

Table 3. Human Recognition Threshold Concentrations (RTC) of Selected Pungent and Tingling Chemosensates Isolated from Black Pepper

chemosensate (no.[a])	RTC[b] (nmol/cm^2) for pungency[p] and tingling[t]
piperine (**1a**)	3.0[p]
piperyline (**1b**)	5.1[p]
piperlonguminine (**1c**)	10.4[p]
piperettine (**2a**)	5.2[p]
piperoleine (**2b**)	10.3[p]
dehydropipernonaline (**3a**)	152.1[p]
retrofractamide A (**3c**)	25.3[p]
piperroleine B (**5a**)	1150.2[p]
guineensine (**6c**)	810.1[p]
(2*E*,4*E*,12/13*Z*)-*N*-isobutyl-octadeca-2,4,12/13-trienamide (**8c**/**7c**)	540.5[p] 2162.1[t]
(2*E*,4*E*)-*N*-isobutyl-octadeca-2,4-dienamide (**9**)	763.0[p]
(1-(eicosa-2*E*,4*E*,14/15*Z*-trienyl)piperidine (**11a**/**10a**)	260.2[p] 520.6[t]
(1-(eicosa-2*E*,4*E*,14/15*Z*-trienyl)pyrrolidine (**11b**/**10b**)	405.8[p] 811.6[t]
(2*E*,4*E*,14/15*Z*)-*N*-isobutyl-eicosa-2,4,14/15-trienamide (**11c**/**10c**)	741.2[p] 1482.3[t]

[a]The structures of the numbered compounds are given in Figure 1. [b]Orosensory recognition threshold concentrations were determined by means of a half-tongue test using filter paper vehicles (1 × 2 cm).

Among the pepper amides, the lowest threshold concentration for pungency was found for piperine (**1a**), followed by piperyline (**1b**) and piperettine (**2a**), with 2-fold higher values (Table 3). Comparison of the threshold data of **2a**/**b**, **3a**–**c**, and **6b**/**c** confirmed that the piperidine analogues showed always lower thresholds when compared to the isobutylamine and the pyrrolidine derivatives, respectively.

Besides the amino structure of the amide, also the alkyl moiety within the group of piperine analogues **1a**–**6c** influenced the perception of pungency. Whereas an additional double bond did not have much influence on the threshold concentration, the saturation of the two conjugated double bonds in α- and β-positions of the carbonyl group, as found in **5a**, resulted in a 8-fold increase of the threshold from 152.1 to 1150.2 nmol/cm^2 (Table 3).

When compared to the piperine type analogues **1a**–**6c**, the fatty acid amides **7a**–**11c** showed increased threshold concentrations for pungency. In addition, the unsaturated alkyl amides decorated with three double bonds induced a tingling sensation at higher concentrations, for example, compounds **7a**/**8a**, **7b**/**8b**, **7c**/**8c**, **10a**/**11a**, **10b**/**11b**, and **10c**/**11c** exhibited a lower recognition threshold for pungency and a higher threshold concentration for its tingling effect (Table 3). As shown in Table 3, the threshold concentration 2162.1 nmol/cm^2 found for the tingling effect of **8c**/**7c** was higher than the threshold concentration found for the pungency (540.5 nmol/cm^2). Among the polyunsaturated alkyl amides, in particular, the *cis*-configured double bond in the fatty acid chain was a key element for the tingling effect, for example, saturation of the double bond in the tingling compound **9c** induced a complete loss of the tingling activity of compounds **7c**/**8c** (Table 3). These findings are well in line with previous reports on synthetic sanshool and bungeanool derivatives.[71] Similar to the piperidine analogues **1a**–**6c**, isobutylamine and pyrrolidine derivatives of the polyunsaturated alkyl amides were less pungent and tingling than the corresponding piperidine derivatives. For example, **10a**/**11a** showed lowest threshold concentrations for pungency when compared to **10b**/**11b** and **10c**/**11c**. To answer the question as to which of the amides contribute to the pungent and tingling effect of *P. nigrum* L., quantitative and dose–activity studies are currently under investigation and will be published separately.

■ ASSOCIATED CONTENT

Supporting Information

Compound spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Jagella, T.; Grosch, W. Flavour and off-flavour compounds of black and white pepper (*Piper nigrum* L.), I. Evaluation of potent odorants of black pepper by dilution and concentration techniques. *Eur. Food Res. Technol.* **1999**, *209*, 16–21.

(2) Jagella, T.; Grosch, W. Flavour and off-flavour compounds of black and white pepper (*Piper nigrum* L.), II. Odor active values of desirable and undesirable odorants of black pepper. *Eur. Food Res. Technol.* **1999**, *209*, 22–26.

(3) Jagella, T.; Grosch, W. Flavour and off-flavour compounds of black and white pepper (*Piper nigrum* L.), III. Desirable and undesirable odorants of white pepper. *Eur. Food Res. Technol.* **1999**, *209*, 27–31.

(4) Oersted, H. C. Über das Piperin, ein neues Pflanzenalkaloid. Schweigers. *J. Chem. Phys.* **1820**, *29*, 80–82.

(5) Landenburg, A.; Schaltz, M. Synthese der Piperinsäure und des Piperins. *Ber Deut. Chem. Ges.* **1894**, *27*, 2958.

(6) Friedman, M.; Levin, C. E.; Lee, S.-U.; Lee, J.-S.; Ohinisi-Kameyama, M.; Kozukue, N. Analysis by HPLC and LC/MS of Pungent Piperamides in Commercial Black, White, Green, and Red Whole and Grounded Peppercorns. *Agric. Food Chem.* **2008**, *56*, 3028–3036.

(7) Freist, W. Der scharfe Geschmack des Pfeffers, Ein Rätsel, nur teilweise gelöst. *Chem. Unserer Zeit* **1991**, *3*, 135–142.

(8) Srinivasan, K. Black Pepper and its Pungent Principle-Pipetrine: A Review of Diverse Physiological Effects. *Crit. Rev. Food Sci. Nutr.* **2007**, *47* (8), 735–748.

(9) Traxler, J. T. Piperanine, a Pungent Component of Black Pepper. *J. Agric. Food Chem.* **1971**, *19* (6), 1135–1138.

(10) Labruyère, B. Detremination of Pungent Constituents of *Piper nigrum*. *J. Agric. Food Chem.* **1966**, *14* (5), 469−472.
(11) Lin, Z.; Liao, Y.; Venkatasamy, R.; Hider, R.; Soumyanath, A. Amides from *Piper nigrum* L. with dissimilar effects on melanocyte proliferation in-vitro. *J. Pharm. Pharmacol.* **2007**, *59*, 529−536.
(12) Grewe, R.; Freist, W.; Meumann, H.; Kersten, S. Über die Inhaltstsoffe des schwarzen Pfeffers. *Chem. Ber.* **1970**, *103*, 3752−3770.
(13) Wei, K.; Li, W.; Koike, K.; Pei, Y.; Chen, Y.; Nikaido, T. New amide alkaloids from the roots of *Piper nigrum*. *J. Nat. Prod.* **2004**, *67* (6), 1005−1009.
(14) Park, I.-K.; Lee, S.-G.; Shin, S.-C.; Park, J.-D.; Ahn, Y.-J. Larvividal Activity of Isobutylamides Identified in *Piper nigrum* Fruits against Three Mosquito Species. *J. Agric. Food Chem.* **2002**, *50*, 1866−1870.
(15) Pradeep, C. R.; Kuttan, G. Effect of piperine on the inhibition of lung metastasis induced B16F-10 melanoma cells in mice. *Clin. Exp. Metastasis* **2002**, *19*, 703−708.
(16) Bezerra, D. P.; Castro, F. O.; Alves, A. P.; Pessoa, C.; Moraes, M. O.; Silveira, E. R.; Lima, M. A.; Elmiro, F. J.; Costa-Lotufo, L. V. In vivo growth-inhibition of sarcoma 180 by piplartine and piperine, two alkaloid amides from *Piper*. *Braz. J. Med. Biol. Res.* **2006**, *39*, 801−807.
(17) Selvendiran, K.; Banu, S. M.; Sakthisekaran, D. Protective effect of piperine on benzo(a)pyrene-induced lung carcinogenesis in Swiss albino mice. *Clin. Chim. Acta* **2004**, *350*, 73−78.
(18) Sunila, E. S.; Kuttan, G. Immunomodulatory and antitumor activity of *Piper longum* Linn. and piperine. *J. Ethnopharmacol.* **2004**, *90*, 339−346.
(19) Reen, R. K.; Wiebe, F. J.; Singh, J. Piperine inhibits aflatoxin B1-induced cytotoxicity and genotoxicity in V79 Chinese hamster cells genetically engineered to express rat cytochrome P4502B1. *J. Ethnopharmacol.* **1997**, *58* (3), 165−173.
(20) Das, B.; Kundu, J.; Bachar, S. C.; Uddin, M. A.; Kundu, J. K. Antitumor and antibacterial activity of ethylacetate extract of *Ludwigia hyssopifolia* Linn. and its active principle piperine. *Pak. J. Pharm. Sci.* **2007**, *20*, 128−131.
(21) Li, S.; Wang, C.; Li, W.; Koike, K.; Nikaido, T.; Wang, M. W. Antidepressant-like effects of piperine and its derivative, antiepilepsirine. *J. Asian Nat. Prod. Res.* **2007**, *9*, 421−430.
(22) Li, S.; Wang, C.; Wang, M.; Li, W.; Matsumoto, K.; Tang, Y. Antidepressant like effects of piperine in chronic mild stress treated mice and its possible mechanisms. *Life Sci.* **2007**, *80*, 1373−1381.
(23) Lee, S. A.; Hong, S. S.; Han, X. H.; Hwang, J. S.; Oh, G. J.; Lee, K. S.; Lee, M. K.; Hwang, B. Y.; Ro, J. S. Piperine from the fruits of *Piper longum* with inhibitory effect on monoamine oxidase and antidepressant-like activity. *Chem. Pharm. Bull. (Tokyo)* **2005**, *53*, 832−835.
(24) Bajad, S.; Bedi, K.; Singla, A. K.; Johri, R. K. Antidiarrhoeal activity of piperine in mice. *Plant Med.* **2001**, *67*, 284−287.
(25) Khan, I. A.; Mirza, Z. M.; Kumar, A.; Verma, V.; Qazi, G. N. Piperine, a phytochemical potentiator of ciprofloxacin against *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2006**, *50*, 810−812.
(26) Huhtanen, C. N. Inhibition of *Clostridium botulinum* by spice extracts and aliphatic alcohols. *J. Food Prot.* **1980**, *43* (195−196), 200.
(27) Ghoshal, S.; Prasad, B. N. K.; Lakshmi, V. Antiamoebic activity of *Piper longum* fruits against *Entamoeba histolytica* in vitro and in vivo. *J. Ethnopharmacol.* **1996**, *50*, 167−170.
(28) Hiwale, A. R.; Dhuley, J. N.; Naik, S. R. Effect of coadministration of piperine on pharmacokinetics of beta-lactam antibiotics in rats. *Indian J. Exp. Biol.* **2002**, *40*, 277−281.
(29) Veerareddy, P. R.; Vobalaboina, V.; Nahid, A. Formulation and evaluation of oil-in-water emulsions of piperine in visceral leishmaniasis. *Pharmazie* **2004**, *59*, 194−197.
(30) Ghoshal, S.; Prasad, B. N.; Lakshmi, V. Antiamoebic activity of *Piper longum* fruits against *Entamoeba histolytica* in vitro and in vivo. *J. Ethnopharmacol.* **1996**, *50*, 167−170.
(31) Ghoshal, S.; Lakshmi, V. Potential antiamoebic property of the roots of *Piper longum* Linn. *Phytother. Res.* **2002**, *16*, 689−691.
(32) Ribeiro, T. S.; Freire-de-Lima, L.; Previato, J. O.; Mendonca-Previato, L.; Heise, N.; de Lima, M. E. Toxic effects of natural piperine and its derivatives on epimastigotes and amastigotes of *Trypanosoma cruzi*. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3555−3558.
(33) Vijayakumar, R. S.; Surya, D.; Nalini, N. Antioxidant efficacy of black pepper (*Piper nigrum* L.) and piperine in rats with high fat diet induced oxidative stress. *Redox Rep.* **2004**, *9*, 105−110.
(34) Vijayakumar, R. S.; Nalini, N. Efficacy of piperine, an alkaloidal constituent from *Piper nigrum* on erythrocyte antioxidant status in high fat diet and antithyroid drug induced hyperlipidemic rats. *Cell Biochem. Funct.* **2006**, *24*, 491−498.
(35) Bai, Y. F.; Xu, H. Protective action of piperine against experimental gastric ulcer. *Acta Pharmacol. Sin.* **2000**, *21*, 357−359.
(36) Bajad, S.; Bedi, K. L.; Singla, A. K.; Johri, R. K. Piperine inhibits gastric emptying and gastrointestinal transit in rats and mice. *Planta Med.* **2001**, *67*, 176−179.
(37) Morikawa, T.; Matsuda, H.; Yamaguchi, I.; Pongpiriyadacha, Y.; Yoshikawa, M. New amides and gastroprotective constituents from the fruit of *Piper chaba*. *Planta Med.* **2004**, *70*, 152−159.
(38) Miyakado, M.; Nakayama, I.; Yoshioka, H.; Nakatani, N. The Piperaceae amides I: Structure of pipercide, a new insecticidal amide from *Piper nigrum* L. *Agric. Biol. Chem.* **1979**, *43*, 1609−1611.
(39) Miyakado, M.; Nakayama, I.; Yoshioka, H.; Nakatani, N. Insecticidal joint action of pipercide and co-occurring compounds isolated from *Piper nigrum* L. *Agric. Biol. Chem.* **1980**, *44*, 1701−1703.
(40) Scott, I. M.; Gagnon, N.; Lesage, L.; Philogene, B. J.; Arnason, J. T. Efficacy of botanical insecticides from *Piper* species (Piperaceae) extracts for control of European chafer (Coleoptera: Scarabaeidae). *J. Econ. Entomol.* **2005**, *98*, 845−855.
(41) Yang, Y. C.; Lee, S. G.; Lee, H. K.; Kim, M. K.; Lee, S. H.; Lee, H. S. A piperidine amide extracted from *Piper longum* L. fruit shows activity against *Aedes aegypti* mosquito larvae. *J. Agric. Food Chem.* **2002**, *50*, 3765−3767.
(42) Siddiqui, B. S.; Gulzar, T.; Begum, S.; Afshan, F.; Sattar, F. A. Two New Insecticidal Dimers from Fruits of *Piper nigrum* LINN. *Helv. Chem. Acta* **2004**, *87*, 660−666.
(43) Park, I.-K.; Lee, S.-G.; Shin, S.-C.; Park, J.-D.; Ahn, Y.-J. Larvicidal Activity of Isobutylamides Identified in *Piper nigrum* Fruits against Three Mosquito Species. *J. Agric. Food Chem.* **2002**, *50*, 1866−1870.
(44) Caterina, M. J.; Schumacher, M. A.; Tominaga, M.; Rosen, T. A.; Levine, J. D.; Julius, D. The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature* **1997**, *389*, 816−824.
(45) McNamara, F. N.; Randall, A.; Gunthorpe, M. J. Effects of piperine, the pungent componente of black pepper, at the human vanilloid receptor (TRPV1). *Br. J. Pharmacol.* **2005**, *144*, 781−790.
(46) Dedov, V. N.; Tran, V. H.; Duke, C. C.; Condor, M.; Christie, MD. J.; Mandadi, S.; Roufogalis, B. D. Gingerols: A novel class of vanilloid receptor (VR1) agonists. *Br. J. Pharmacol.* **2002**, *137*, 793−798.
(47) Trevisani, M.; Smart, D.; Gunthorpe, M. J.; Tognetto, M.; Barbieri, M.; Campi, B.; Amatesi, S.; Gray, J.; Jerman, J. C.; Brough, S. J.; Owen, D.; Smith, G. D.; Randall, A. D.; Harrison, S.; Bianchi, A.; Davis, J. B.; Geppetti, P. Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. *Nature Neurosci.* **2002**, *5* (No. 6), 546−551.
(48) Frank, O.; Ottinger, H.; Hofmann, T. Characterization of an intense bitter-tasting 1*H*,4*H*-quinolizinium-7-olate by application of the taste dilution analysis, a novel bioassay for the screening and identification of taste-active compounds in foods. *J. Agric. Food Chem.* **2001**, *49*, 231−238.
(49) Ottinger, H.; Bareth, A.; Hofmann, T. Characterization of natural "cooling" compounds formed from glucose and L-proline in dark malt by application of taste dilution analysis. *J. Agric. Food Chem.* **2001**, *49*, 1336−1344.
(50) Czepa, A.; Hofmann, T. Structural and sensory characterization of compounds contributing to the bitter off-taste of carrots (*Daucus carota* L.) and carrot puree. *J. Agric. Food Chem.* **2003**, *51*, 3865−3873.

(51) Ottinger, H.; Hofmann, T. Identification of the taste enhancer alapyridaine in beef broth and evaluation of its sensory impact by taste reconstitution experiments. *J. Agric. Food Chem.* **2003**, *51*, 6791−6796.
(52) Scharbert, S.; Holzmann, N.; Hofmann, T. Identification of the astringent taste compounds in black tea infusions by combining instrumental analysis and human bioresponse. *J. Agric. Food Chem.* **2004**, *52*, 3498−3508.
(53) Stark, T.; Hofmann, T. Isolation, structure determination, synthesis, and sensory activity of *N*-phenylpropenoyl-L-amino acids from cocoa (*Theobroma cacao*). *J. Agric. Food Chem.* **2005**, *53*, 5419−5428.
(54) Schwarz, B.; Hofmann, T. Sensory-Guided Decomposition of Red Current Juice (*Ribes rubrum*) and Structure Determination of Key Astringent Compounds. *J. Agric. Food Chem.* **2007**, *55*, 1394−1404.
(55) Brock, A.; Hofmann, T. Identificatioion of the Key Astringent Compounds in Spinach (*Spinacia oleracea*) by Means of the Taste Dilution Analysis. *Chem. Percept.* **2008**, *1*, 268−281.
(56) Bauer, S. Die Zusammensetzung der Oberflächenwachse von Tomaten, Paprika und Aubergine. Dissertation, Westfälische Wilhelms-Universität Münster: Germany, 2002.
(57) Venkatasamy, R.; Faas, L.; Young, A. R.; Raman, A.; Hider, R. C. Effects of piperine analogues on stimulation of melanocyte proliferation and melanocyte differtiartion. *Bioorg. Med. Chem.* **2004**, *12*, 1905−1920.
(58) Becker, H. G. O.; Berger, W.; Domschke, G.; Fanghänel, E.; Faust, J.; Fischer, M.; Gentz, F.; Gewald, K.; Gluch, R.; Mayer, R.; Müller, K.; Pavel, D.; Schmidt, H.; Schollberg, K.; Schwetlick, K.; Seiler, E.; Zeppenfeld, G.; Beckert, R.; Fangnägel, E.; Habicher, W. D.; Knölker, H.-J.; Metz, P.; Schwetlick, K. *Organikum, 23*; Auflage, Wiley-VCH Verlag: Weinheim, 2009; pp 495−500.
(59) de Paula, V. F.; Barbosa, de A, L. C.; Demuner, A. J.; Piló-Veloso, D.; Picancp, M. C. Synthesis and insecticidal activity of new amide derivatives of piperine. *Pest Manage. Sci.* **2000**, *56*, 168−174.
(60) Venkatasamy, R.; Faas, L.; Young, A. R.; Raman, A.; Hider, R. C. Effects of piperine analogues on stimulation of melanocyte proliferation and melanocyte differtiartion. *Bioorg. Med. Chem.* **2004**, *12*, 1905−1920.
(61) Sun, C.; Pei, S.; Pan, Y.; Shen, Z. Rapid structural determination of amides in *Piper longum* by high-performance liquid chromatography combined with ion trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 1497−1503.
(62) Spring, F. S.; Stark, J. Piperettine from *Piper nigrum*; ist Isolation, Identification, and Synthesis. *J. Chem. Sci.* **1950**, *241*, 1177−1180.
(63) Li, Z. Y.; Huang, Z.; Cao, X.; Gao, K. Stereoselective synthesis of naturally occurring unsaturated amide alkaloids by a modified Ramberg-Bäcklund reaction. *Can. J. Chem.* **2004**, *82*, 622−630.
(64) Lee, S., W.; Rho, M.-C.; Park, H., R.; Choi, J.-H.; Kang, J., Y.; Lee, J., W.; Kim, K.; Lee, H., S.; Kim, Y., K. Inhibition of Diacylglycerol Acyltransferase by Alkamides Isolated from the Fruits of *Piper longum* and *Piper nigrum*. *J. Agric. Food Chem.* **2006**, *54* (26), 9759−9763.
(65) Okumura, Y.; Narukawa, M.; Iwasaki, Y.; Ishikawa, A.; Matsuda, H.; Yoshikawa, M.; Watanabe, T. Activation of TRPV1 and TRPA1 by Black Pepper Components. *Biosci., Biotechnol., Biochem.* **2010**, *5*, 1068−1072.
(66) Nakatani, N.; Inatani, R. Isobutyl Amides from Pepper (*Piper nigrum* L.). *Agric. Biol. Chem.* **1981**, *45* (6), 1473−1476.
(67) Park, I.-K.; Lee, S.-G.; Shin, S.-C.; Park, J.-D.; Ahn, Y.-J. Larvicidal Activity of Isobutylamides Identified in *Piper nigrum* Fruits against Three Mosquito Species. *J. Agric. Food Chem.* **2002**, *50*, 1866−1870.
(68) Tsukamoto, S.; Tomise, K.; Miyakawa, K.; Cha, B.-C.; Abe, T.; Hamada, T.; Hirota, H.; Ohta, T. CYP3A4 Inhibitory Activity of New Bisalkaloids, Dipiperamides D and E, and Cognates from White Pepper. *Bioorg. Med. Chem.* **2002**, *10* (9), 2981−2985.
(69) Wu, S.; Sun, C.; Pei, S.; Lu, Y.; Pan, Y. Preperative isolation of amides from the fruits of *Piper longum* L. by upright counter-current chromatography and reversed-phase liquid chromatograpy. *J. Chromatogr.* **2004**, *1040*, 193−204.
(70) Kikuzaki, H.; Kawabata, M.; Ishida, E.; Akazawa, Y.; Takei, Y.; Nakatani, N. Constituents of Piperaceae. Part V. LC-MS analysis and structural determination of new amides from Javanese long pepper (*Piper retrofractum*). *Biosci., Biotechnol., Biochem.* **1993**, *57* (8), 1329−33.
(71) Galopin, C. C.; Furrer, S. M.; Goeke, A. Pungent and Tingling Compounds in Asian Cuisine. In Hofamnn, T., Ho, C. T., Pickenhagen, W., Eds.; *Challenges in Taste Chemistry and Biology*; ACS Symposium Series 876; American Chemical Sosiety: Washington, DC, 2004; pp 139−152.