New Umami Amides: Structure-Taste Relationship Studies of Cinnamic Acid-Derived Amides and Natural Occurrence of an Intense Umami Amide in *Zanthoxylum piperitum*

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ABSTRACT

A series of aromatic amides was synthesized from various acids and amines selected from naturally occurring structural frameworks. These synthetic amides were evaluated for umami taste in comparison with monosodium glutamate. The effect of the substitution pattern of both the acid and the amine parts on umami taste was investigated. The only intense umami-tasting amides were those made from 3,4-dimethoxycinnamic acid. The amine part was more tolerant to structural changes. Amides bearing an alkyl- or alkoxy-substituted phenylethylamine residue displayed a clean umami taste as 20 ppm solutions in water. Ultra-performance liquid chromatography coupled with a high quadrupole-Orbitrap mass spectrometer (UPLC/MS) was subsequently used to show the natural occurrence of these amides. (*E*)-3-(3,4-dimethoxyphenyl)-N-(4-methoxyphenethyl)acrylamide was shown to occur in the roots and stems of *Zanthoxylum piperitum,* a plant of the family Rutaceae growing in Korea, Japan, and China.

KEYWORDS

Aromatic amides, umami taste, UPLC/MS, *Zanthoxylum piperitum*

INTRODUCTION

Amides are ubiquitous compounds in nature, especially in plants of the genus *Piperaceae*.[1] Many of the amides occurring in foods elicit a trigeminal sensation. Thus, capsaicin is responsible for the hot sensation of red pepper (*Capsicum annuum* L.)[2] and piperine for that of black pepper (*Piper nigrum* L.)[3]. Red Sichuan pepper (*Zanthoxylum bungeanum* Maxim.)[4], jambu oleoresin (extract from *Spilanthes acmella* L. flowers)[5] and pellitory roots (*Anacyclus pyrethrum* L.)[6] provide another trigeminal effect, called tingling. This effect is mostly attributed to the presence of unsaturated amides: hydroxy-α-sanshool in red Sichuan pepper, spilanthol in jambu oleoresin and pellitorin in pellitory. There is no mention in the peer-reviewed literature of naturally occurring alkyl- or aromatic amides that provide umami taste.

The interest in new umami molecules was reviewed by Winkel and colleagues.[7] In addition to amino acid derivatives, small peptides, and nucleotide derivatives, recent years have seen the emergence of umami compounds that are active at very low levels. Tachdjian *et al.* of Senomyx used receptor-based assays to screen libraries of thousands of compounds, among which amides such as FEMA 4233 and FEMA 4232 (Figure 1) were found to possess umami taste at concentrations as low as 1 μM.[8,9]

Dewis *et al.* of IFF (International Flavors and Fragrances) patented many unsaturated alkamides as umami or more general taste enhancers.[10-11] These amides have structural similarities to naturally occurring amides. One example of a polyunsaturated isobutylamide similar to spilanthol, is presented in Figure 1.[11] In addition, Backes *et al.* of Symrise proposed umami amides that are based on neomenthylamine.[12]

The work of Adesina showed the occurrence of various cinnamic acid-related amides in Nigerian plants of the Rutaceae family, in particular *Zanthoxylum rubescens* Planch. ex Hook. (Figure 2).[13,14] Plants of this genus are generally used as spice or as phytomedicine to cure various diseases.[15,16] At the beginning of the present work, the taste of the amides occurring in *Z. rubescens*[13] had not been described. When we tasted Rubemamine **1**, we noticed its umami taste and this prompted us to study other cinnamic acid-derived amides for their potential umami taste. Rubemamine **1** was included in a patent of 2003 from Symrise related to the pungent taste of ferulic acid amides[17] and in 2012 in another patent claiming the umami taste of cinnamic acid related amides.[18]

The subject of this work includes the synthesis of many cinnamic acid-related amides, their sensory evaluation for umami taste, the necessary structural features for umami taste,[19] and the subsequent analysis of *Zanthoxylum* piperitum to show the natural occurrence of these umami amides.

MATERIALS AND METHODS

General. Unless otherwise specified, commercially available reagents and solvents were purchased from Fluka-Sigma-Aldrich, Buchs, Switzerland; Acros Organics, Geel, Belgium; and Carlo-Erba, Val de Reuil, France.

NMR Spectra. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a DPX 400 spectrometer (Bruker, Rheinstetten, Germany) at 25 °C operated at 400 MHz (^{1}H) or 100 MHz (^{13}C), with tetramethylsilane as the internal standard. The ^{13}C signal assignments were obtained from standard gradient-selected correlated spectroscopy, heteronuclear single quantum coherence, and heteronuclear multiple bond correlation experiments performed on a Bruker Avance 500 spectrometer. NMR spectra were processed with Bruker TopSpin 2.0 software (s, singlet; d, doublet; t, triplet; m, multiplet).

UPLC-MS Method. Analyses of samples were performed on a Acquity I-Class UPLC system (Waters, Milford, USA) coupled with a Q-Exactive Plus mass spectrometer (Thermo, San Jose, CA, USA). The column was an Acquity HSS-T3, 100 $\times$ 2.1 mm i.d. (Waters) operated at 30 °C. Solvent A was UPLC/MS grade water (BioSolve, Valkenswaard, The Netherlands) containing 0.1% MS grade formic acid (Sigma-Aldrich, Steinheim, Germany). Solvent B was acetonitrile (Biosolve) containing 0.1% formic acid at 0.3 mL/mL. The gradient elution was as follows: 40%, 1 min; 40-80% B for 9 min; 80% B, 1 min; and re-equilibration at 40% B for 1 min.

The mass spectrometer was equipped with an H-ESI II electrospray source operated in the positive mode: spray 3500 V, probe heater 400 °C, heated capillary 300 °C, sheath gas 50 arb units, auxiliary gas 10. The mass calibration was performed each week. The "full scan/ddMS^2" combined with the "tMS^2" acquisition modes were used. The full scan/ddMS^2 triggered the recording of the MS^2 spectra for the three most intense ions. The targeted MS^2 mode (tMS^2) recorded the MS^2 spectra of 15 amides (see Results and Discussion) on the

basis of an inclusion list containing their molecular formulae. The full-scan spectra were acquired with a resolution of 70,000 from 100 to 1000 Da and the MS^2 spectra with a resolution of 17,500 and a higher energy collisional dissociation cell energy of 30 V. Automated gain control was targeted to 3.10^6 and 1.10^5 ions with a maximum injection time of 100 ms for full scan and 50 ms for $ddMS^2$.

Synthesis of Amides with Ethyl Chloroformate, General Procedure. The acid component (typically 33 mmol) and diisopropyl ethyl amine (2 equiv) were diluted in 200 mL of EtOAc and 50 mL of dichloromethane. The solution was cooled to 15 °C, and ethyl chloroformate (1 molar equiv) was added drop wise. The reaction was stirred for 1 h before the primary amine component (1 molar equiv, diluted 2-3 times in EtOAc) was added. The reaction was stirred overnight at room temperature. It was washed with aqueous 5% $KHSO_4$, brine, aqueous 5% $NaHCO_3$, and brine, and then dried over Na_2SO_4 and evaporated under high vacuum for 3 h. The crude product was purified by flash chromatography (silica gel; cyclohexane/EtOAc, e.g. 2:8). Yields were between 50 and 80% of the purified product. MS and UV data only are given below. The description of ^1H and ^{13}C NMR spectra can be found as supporting information.

Amide **1**, Rubemamine**.** (*E*)-N-(3,4-dimethoxyphenethyl)-3-(3,4-dimethoxyphenyl)acrylamide

ESI (+): 372.1798 ($C_{21}H_{26}O_5N$, -2.1 ppm). UV max (nm): 316, 287, 222.

Amide **2**. (*E*)-N-(3,4-dimethoxyphenethyl)-3-(4-methoxyphenyl)acrylamide

ESI (+): 342.1690 ($C_{20}H_{24}O_4N$, -2.8 ppm). UV max (nm): 310, 288, 224.

Amide **3**. (*E*)-3-(benzo[d][1,3]dioxol-5-yl)-N-(3,4-dimethoxyphenethyl)acrylamide

ESI (+): 356.1483 ($C_{20}H_{22}O_5N$, -4.2 ppm). UV max (nm): 322, 286, 228, 219.

Amide **4**. (*E*)-3-(3,4-dimethoxyphenyl)-N-(4-methoxyphenethyl)acrylamide

ESI (+): 342.1693 ($C_{20}H_{24}O_4N$, -2.0 ppm). UV max (nm): 316, 291, 223.

Amide **5**. (*Z*)-3-(3,4-dimethoxyphenyl)-N-(4-methoxyphenethyl)acrylamide. Amide **4** (500 mg) in 50 mL of ethanol was irradiated for 16 h by using a water-cooled 125 W Hg lamp. UPLC analysis showed that the crude product contained 50% of the (*Z*)-isomer. The product was chromatographed over a Puriflash 50 C18 cartridge (Interchim¸ Montluçon, France) eluted with water:ethanol (60:40). A mixture of amide **5** and **4** (90:10) was obtained (96 mg). ESI (+): 342.1693 ($C_{20}H_{24}O_4N$, -2.1 ppm). UV max (nm): 310, 276, 224.

Amide **6**. 3-(3,4-dimethoxyphenyl)-N-(4-methoxyphenethyl)propanamide

ESI (+): 344.1849 ($C_{20}H_{26}O_4N$, -2.0 ppm). UV max (nm): 277, 225.

Amide **7**. N-(4-methoxyphenethyl)cinnamamide

ESI (+): 282.1481 ($C_{18}H_{20}O_2N$, -2.8 ppm). UV max (nm): 275, 222.

Amide **8**. N-(3-methoxyphenethyl)cinnamamide

ESI (+): 282.1491 ($C_{18}H_{20}O_2N$, -2.6 ppm). UV max (nm): 274, 223.

Amide **9**. (E)-N-(4-methoxyphenethyl)-3-(4-methoxyphenyl)acrylamide

ESI (+): 312.1585 ($C_{19}H_{22}O_3N$, -2.9 ppm). UV max (nm): 310, 290, 224.

Amide **10**. (E)-N-(4-methoxyphenethyl)-3-(2-methoxyphenyl)acrylamide

ESI (+): 312.1586 ($C_{19}H_{22}O_3N$, -2.7 ppm). UV max (nm): 317, 274, 225.

Amide **11**. (E)-N-(4-methoxyphenethyl)-3-(3-methoxyphenyl)acrylamide

ESI (+): 312.1587 ($C_{19}H_{22}O_3N$, -2.4 ppm). UV max (nm): 310, 277, 224.

Amide **12**. (E)-3-(2,5-dimethoxyphenyl)-N-(4-methoxyphenethyl)acrylamide

ESI (+): 342.1690 ($C_{20}H_{24}O_4N$, -2.8 ppm). UV max (nm): 342, 276, 223.

Amide **13**. (E)-N-(4-methoxyphenethyl)-3-(3,4,5-trimethoxyphenyl)acrylamide

ESI (+): 372.1797 ($C_{21}H_{26}O_5N$, -2.4 ppm). UV max (nm): 299, 228.

Amide **14**. (E)-2-methoxy-4-(3-((4-methoxyphenethyl)amino)-3-oxoprop-1-en-1-yl)phenyl acetate. ESI (+): 370.1639 ($C_{21}H_{24}O_5N$, -2.6 ppm). UV max (nm): 310, 277, 224.

Amide **15**. (E)-3-(4-hydroxy-3-methoxyphenyl)-N-(4-methoxyphenethyl)acrylamide. Amide **14** was deprotected in MeOH/Na_2CO_3. The reaction was acidified and extracted from EtOAc. The product was recrystallized from EtOAc (yield 75%). ESI (+): 328.1535 ($C_{19}H_{22}O_4N$, -2.5 ppm). UV max (nm): 317, 293, 222.

Amide **16**. (E)-3-(benzo[d][1,3]dioxol-5-yl)-N-(4-methoxyphenethyl)acrylamide

ESI (+): 326.1379 ($C_{19}H_{20}O_4N$, -2.4 ppm). UV max (nm): 322, 284, 219.

Amide **17**. (E)-3-(3,4-dimethylphenyl)-N-(4-methoxyphenethyl)acrylamide

ESI (+): 310.1794 ($C_{20}H_{24}O_2N$, -2.6 ppm). UV max (nm): 285, 224.

Amide **18**. (E)-3-(3,4-dimethoxyphenyl)-N-phenethylacrylamide

ESI (+): 312.1588 ($C_{19}H_{22}O_3N$, -2.0 ppm). UV max (nm): 317, 291, 235, 211.

Amide **19**. (E)-3-(3,4-dimethoxyphenyl)-N-(3-methoxyphenethyl)acrylamide

ESI (+): 342.1693 ($C_{20}H_{24}O_4N$, -2.1 ppm). UV max (nm): 317, 291, 218.

Amide **20**. (E)-3-(3,4-dimethoxyphenyl)-N-(2-methoxyphenethyl)acrylamide

ESI (+): 342.1691 ($C_{20}H_{24}O_4N$, -2.7 ppm). UV max (nm): 317, 291, 220.

Amide **21**. (E)-N-(3,5-dimethoxyphenethyl)-3-(3,4-dimethoxyphenyl)acrylamide

ESI (+): 372.1797 ($C_{21}H_{26}O_5N$, -2.4 ppm). UV max (nm): 317, 292, 219.

Amide **22**. (E)-3-(3,4-dimethoxyphenyl)-N-(3-ethoxyphenethyl)acrylamide

ESI (+): 356.1848 ($C_{21}H_{26}O_4N$, -2.4 ppm). UV max (nm): 316, 292, 219.

Amide **23**. (E)-3-(3,4-dimethoxyphenyl)-N-(3-propoxyphenethyl)acrylamide

ESI (+): 370.2005 ($C_{22}H_{28}O_4N$, -2.2 ppm). UV max (nm): 316, 290, 226.

Amide **24**. (E)-3-(3,4-dimethoxyphenyl)-N-(4-isopropoxyphenethyl)acrylamide

ESI (+): 370.2005 ($C_{21}H_{28}O_4N$, -2.1 ppm). UV max (nm): 316, 290, 226.

Amide **25**. (E)-3-(3,4-dimethoxyphenyl)-N-(4-ethylphenethyl)acrylamide

ESI (+): 340.1899 ($C_{21}H_{26}O_3N$, -2.3 ppm). UV max (nm): 316, 292, 220.

Amide **26**. (E)-3-(3,4-dimethoxyphenyl)-N-(4-isopropylphenethyl)acrylamide

ESI (+): 354.2055 ($C_{22}H_{28}O_3N$, -2.4 ppm). UV max (nm): 316, 292, 219.

Amide **27**. (E)-3-(3,4-dimethoxyphenyl)-N-(3,4-dimethylphenethyl)acrylamide

ESI (+): 340.1899 ($C_{21}H_{26}O_3N$, -2.6 ppm). UV max (nm): 316, 292, 218.

Amide **28**. (E)-3-(3,4-dimethoxyphenyl)-N-(4-methoxybenzyl)acrylamide

ESI (+): 328.1537 ($C_{19}H_{22}O_4N$, -2.6 ppm). UV max (nm): 317, 292, 223.

Amide **29**. (E)-N-(benzo[d][1,3]dioxol-5-ylmethyl)-3-(3,4-dimethoxyphenyl)acrylamide ESI (+): 342.1327 ($C_{19}H_{20}O_5N$, -2.7 ppm). UV max (nm): 317, 292, 235, 218.

Amide **30**: Beecheyamide. (E)-3-(3,4-dimethoxyphenyl)-N-(4-methoxyphenethyl)-N-methylacrylamide. A solution of amide **4** (478 mg, 1.4 mmol) in 14 mL of tetrahydrofuran (THF) was added to a suspension of sodium hydride (120 mg, 5.0 mmol) in 9 mL of THF at 0 °C under argon atmosphere. The reaction was stirred for 30 min at 0 °C before 240 mg of methyl iodide was added. The reaction was stirred at room temperature overnight, the mixture poured into 50 mL of cold water, and the pH adjusted to 7 with 5% $KHSO_4$. Extraction from EtOAc, followed by flash chromatography (pure EtOAc), gave 402 mg of **20** (yield 81%). The product was fully in agreement with the NMR data (showing two rotamers of the amide bond) described by Cheng et al.[20] ESI (+): 356.1847 ($C_{21}H_{26}O_4N$, -2.6 ppm). UV max (nm): 320, 285, 224.

Amide **31**. Podocarpamide. (E)-N-(2-(benzo[d][1,3]dioxol-5-yl)ethyl)-3-(3,4-dimethoxyphenyl)-N-methylacrylamide. Amide **16** (1.70 g, 5.2 mmol) in 50 mL of THF was added drop wise to a suspension of sodium hydride (150 mg, 1.2 equiv) in 34 mL of THF at 0 °C under argon atmosphere. The reaction was stirred for 30 min at 0 °C. Iodomethane (390 μL, 1.2 equiv) was added and the reaction was stirred overnight at room temperature. After workup and flash chromatography (cyclohexane:EtOAc, 60:40), 1.37 g was obtained (77% yield). NMR data (showing two rotamers of the amide bond) were compliant with those reported by Delle Monache et al.[21] ESI (+): 340.1535 ($C_{20}H_{22}O_4N$, -2.4 ppm). UV max (nm): 321, 285, 232.

Amide **32**. (E)-3-(4-hydroxy-3-methoxyphenyl)-N-(4-methoxyphenethyl)-N-methylacrylamide

4-Acetoxy-3-methoxycinnamic acid (360 mg, 1.5 mmol, Alfa Aesar) was condensed on [2-(4-methoxy-phenyl)-ethyl]methylamine (1 equiv, Aldrich), as described above. The product was purified by flash chromatography (cyclohexane:EtOAc, 50:50) and subsequently deprotected (MeOH:1M KOH, 1/1 v:v). The reaction was neutralized by addition of 5% $KHSO_4$ and the product was extracted 3 x with EtOAc, then with CH_2Cl_2. After drying over Na_2SO_4 and evaporation, 132 mg of pale yellow oil was obtained (26%). NMR showed the presence of two rotamers (as described for amide **30**[20]) in a 55:45 ratio. ESI (+): 342.1691 ($C_{20}H_{24}O_4N$, -2.6 ppm). UV max (nm): 321, 285, 232.

Preparation of the Extracts from *Zanthoxylum piperitum* (L.) DC. The stems and roots were separately cut from the fresh plants, and 207.15 g stems and 110.44 g of roots were obtained. The stems were ground into a fine powder, poured into 4 L of dichloromethane, stirred for 6 h, and left standing over the weekend. The suspension was filtered and the solvent was evaporated to yield 1.06 g of brownish oil (0.51% w/w). Similarly, the roots gave 1.6 g of extract (1.45% w/w). The botanical identification of the species was confirmed by a professional botanist (Y.-M. Yuan). Voucher specimens were prepared and kept in the herbarium of the company.

Sensory Evaluation.

All of the compounds were assessed by a toxicologist and many of them classified as Cramer class III. Therefore a daily intake of 90 µg/day was allowed. Only one compound of the cinnamic amides family was tasted per session using the “sip and spit” method for 20 ml of solution at 20 ppm therefore minimizing the amount of ingested product. All panelists had signed a consent form informing them about the risk associated with such tasting of new compounds.

Umami Score (small panel). Most of the amides were evaluated by a panel of four to nine trained people. Each amide was evaluated at 20 ppm in Henniez water against 0.05% monosodium glutamate (MSG) in a blind pair test. The panelists were asked to score the umami taste on a scale of 0-10 for the sample in each cup. The *relative umami score* was calculated as follows: *Relative umami score* = (amide score/MSG score) $\times$ 10

Umami Score (large panel). To assess the sensory performance of the selected amides with a larger panel, we asked 15 panelists to evaluate them at 20 ppm or 100 ppm in water (Henniez mineral water) alone and in a 0.05% MSG solution. They were asked to taste the sample while using nose clips in order to focus on taste and trigeminal sensations in the mouth, and then without nose clips in order to describe the olfactive notes. The panelists evaluated the samples (20 mL served in a black cup) blind by sipping and spitting the sample out after 5 s before answering the questions. For the pure water system, the panelists had to select the most appropriate attributes from attributes lists (see below) when they were and were not using nose clips. The attributes were ranked by decreasing order as a function of attribute number cited by the panelists. In the MSG model system, all solutions tasted contained 0.05% MSG and the panelists had to evaluate the perceived umami intensity in the reference solution and in the samples by using a linear scale score from 0 (not intense) to 10 (very intense). The potential umami-enhancing effect of the amide was then evaluated by comparing the perceived intensity of the samples “amide in MSG” vs “MSG alone” (“delta” score corresponding to the intensities of “amide in MSG” minus “MSG alone”). A student test (paired test, one tailed) was performed to evaluate the significance of the observed umami difference. The following is the list of attributes: with nose clips: astringent, bitter, cooling, fatty, hot, licorice taste, metallic, mouthfeel, pungent, salivating, salty, sour,

sweet, tingling, umami; without nose clips: earthy, fruity, green, herbal, mushroom, nutty, tea, woody.

RESULTS AND DISCUSSION

Structure Taste Relationship Study of Nature-Inspired Amides. Most of the amides occurring in *Z. rubescens* consist of amides of substituted cinnamic acid and phenethylamine.[13] Throughout this study, 29 such amides (1-29) bearing different substituents were synthesized (figure 3 and table 1). A few amides shown in table 1 were already identified in plants[13,22,23] or microorganisms[24]. We limited the study to amides formed from acids and amines whose structural scaffolds were close to those already known to occur in nature. We thought it might increase the chances of showing the natural occurrence of these amides *afterwards* by targeted analysis. Thus, for example, 4-methoxyphenethylamine clearly derives from the amino acid tyrosine, but 3-methoxyphenethylamine could also form from the uncommon but naturally occurring *meta*-tyrosine.[25] The amide bond was formed from the mixed carbonic anhydride generated by reaction of the acid with ethyl chloroformate, followed by addition of the amine. The synthetic amides were subsequently evaluated for their umami taste by a panel of four to nine trained people. A *relative umami score* was calculated as follows: (amide score/MSG score) $\times$ 10. For a *umami score* of 10, the umami taste intensity of the amide at 20 ppm was perceived as being equal to that of 0.05% MSG. Thus, rubemamine **1**, which was perceived as umami with a score of 3.3, was used as a lead compound in this study. The structural optimization was performed step by step by varying the substituents independently.

The comparison of the relative umami score for these amides shown in Table 1 led to the following conclusions. The double bond of the (*E*) configuration in the acid part was needed because compound **5** of the (*Z*)-configuration and the saturated compound **6** did not have a umami taste. Two methoxy groups were the most favorable (**4** vs **7**, **9-11**, and **13**) and they should be in the *meta* and *para* position (**4** better than **12**). With substituents other than methoxy groups (**14**-**17**), the umami taste intensity decreased significantly. Thus, it appeared that 3,4-dimethoxycinnamic acid as the acid part of the amide was needed. The phenethylamine part (n = 1) tolerated much more variation on the aromatic ring. Thus, compounds **18**-**19** and **20**-**27**, bearing different R_5 and R_6 groups, were perceived as umami, either moderately (**18**, **21**, **22**) or strongly (**19**, **22**-**27**). In particular, compounds having one alkoxy or one alkyl group in *meta* position were all very strong (**19** > **4** > **20**). Benzylamides (n = 0) **29** and **30** were only very weakly umami.

In the large panel study, all but two of the evaluated amides significantly enhanced the umami taste (see Table 2). The delta scores represent the average difference between the perceived umami intensity of the model solution (MSG) and that with the amide added. A positive value denotes an increase of umami taste intensity when the amide was present. The student test (paired test, one tailed) indicates whether this difference is significant at different levels of confidence (*, **, ***, at 95%, 99%, and 99.9%, respectively) between these two samples. The best-performing amides were **1**, **4**, **19**, and **23-27**. One of the most interesting compounds was **4**, which increased the perceived intensity of umami by 1.3, with a significance level of 99.9% with and without the nose clip. Furthermore, the comments regarding the water system with the nose clips were quite positive, except for the astringency; there was no olfactive off-note (no comment without the nose clips).

Targeted UPLC/MS/MS Analysis and Occurrence of Amides 1, 4, 15, 16 and 30-32 in *Z. piperitum*. There are more than 500 species in the genus *Zanthoxylum* (Rutaceae),[15] often used as medicinal plants, as ornamentals, or in culinary applications. Cheng et al. analyzed *Z. beecheyanum* K. Koch, which K. Koch purchased in Taiwan, and it contained many aromatic amides.[20] The description of this plant as an evergreen shrub originating from Japan and introduced as an ornamental plant in Taiwan is very similar to *Z. piperitum*. The latter plant is used as ornamental plant in China and Japan as bonsai, whereas the pericarp of the species is commonly used as a spice in Japan.[15] We decided to focus our study on this plant. Dichloromethane was selected as the solvent for the extraction because the synthetic amides described earlier were all very soluble in it. The stems and the roots were extracted separately and analyzed by UPLC/MS operated in positive electrospray ionization mode. For this study, we used a high-resolution hybrid quadrupole-Orbitrap mass spectrometer in full scan/data dependent MS^2 experiment followed by a targeted MS^2 experiment (t-MS^2) within the same run. The t-MS^2 used an inclusion list comprising the molecular formulae of the 15 selected amides (see Table 3) to record the corresponding product ion spectra. Figure 4 shows the UPLC/MS profile of the root extract of *Z. piperitum*. It was not the aim of this study to fully characterize the extract, but some major products already known to occur in *Zanthoxylum* species could be tentatively identified on the basis of their accurate masses. The major products were amides such as α-sanshool (Figure 4, peak 11), beecheyamide **30** (peak 4), podocarpamide **31** (peak 6), alkaloids such as skimmianine (peak 3) and zanthoxyline (peak 2); and probably lignan-related products (peaks 9-10). Amides **30**, **31** and also **32** (Figure 5) were synthesized to be used in the study.

According to the International Organization of the Flavor Industry guidelines for the positive identification of chemicals by high resolution LC/MS/MS,[26] an undisputed identification of new compounds in plants "requires six points of identification when comparing the reference synthetic material with the analyte in the plant: 1 precursor in low resolution + 2 product ions in high resolution + 1 ratio → 6 IPs". A mixture of 15 amides (Table 3) was injected in concentrations ranging from 0.1 to 1000 ppb. The t-MS^2 data were then compared with those obtained from the injection of the extract at 1000 ppm after blank runs were performed. The comparison of the chromatographic and mass spectral data (Table 3 and Supporting Information) between the synthetic compounds and the natural extract showed the unambiguous occurrence of amides **1**, **4**, **15**, **16**, **30**, **31**, and **32** in *Zanthoxylum piperitum* root extract. Quantitative results presented below should be considered with care since we did not check for ion suppression effects. Also, the most abundant amides were in concentration higher than the highest concentration of the calibration curves.

Amides **1** (rubemamine), **16**, **20** (beecheyamide), and **31** (podocarpamide) were already reported to occur in *Zanthoxylum* species (see Table 1). Amides **30** and **31** were abundant compounds in our extract, accounting for 0.57 and 1.87% w/w, respectively. Figure 6 shows the t-MS^2 trace of ion 342.17. The peak at 3.816 min in the plant extract (trace A) and the synthetic amide **4** eluting at 3.820 min in the reference mixture (trace B) injected separately showed identical MS/MS spectra (C and D, same fragments, same ratio). As amide **4** was well separated from its isomers **5** and **19**, its occurrence is thus unambiguously supported. The comparisons between the UPLC/MS/MS data of the synthetic products and the natural products for the other amides are available as Supporting Information.

This work illustrates how compounds already known to occur in plants served as a starting point for umami taste relationship studies to find more intense ingredients. Targeted UPLC/MS/MS analyses then unambiguously supported the natural occurrence of a few of these molecules in a plant extract. The combination of plant knowledge, chemical synthesis, and modern LC/MS techniques thus provided an efficient way to discover new taste-active and naturally occurring ingredients. A similar approach was used by Süss *et al.* who synthesized a series of "kitchen-like" amides by reaction of lactones and amines, evaluated them for their umami taste and finally showed the natural occurrence of one of them in blue cheese.[27]

ABBREVIATIONS USED

MSG, monosodium glutamate; THF, tetrahydrofuran; UPLC/MS, ultra-performance liquid chromatography mass spectrometry; ESI, electrospray ionization.

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338 **Supporting Information**

339 ^{1}H and ^{13}C NMR descriptions of products **1**-**32**.

340 Chromatograms and mass spectra comparison of synthetic amides vs natural amides in
341 *Z.piperitum*. This material is available free of charge via the Internet at http://pubs.acs.org.

342

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418 Figure captions

Figure 1. High-intensity umami ingredients.

Figure 2. Amides from *Zanthoxylum rubescens*.[13]

Figure 3. Structure of cinnamic acid-derived amides synthesized for umami taste evaluation.

Figure 4. UPLC/MS chromatogram (Full scan mode) of *Zanthoxylum piperitum* root extract.

Figure 5. Amides **30-32** synthesized for targeted UPLC/MS analysis if *Z.piperitum*

Figure 6. UPLC/MS trace in targeted-MS^2 mode (see Material & Methods) of ion 342.17 in *Z. piperitum* root extract (A) and in a mixture of synthetic products (B). MS/MS spectra of peak at 3.827 min in *Z.piperitum* (C) and peak at 3.820 min in the synthetic mixture (D), showing the natural occurrence of amide **4**

419

Table 1. Cinnamic Acid-Derived Amides and Their Umami Taste Score

product no.	natural occurrence	number of panelists	rel. umami score	product no.	natural occurrence	number of panelists	rel. umami score
1	*Z. rubescens*[13]	4	3.3	**17**		3	2.6
2		5	3.9	**18**	*Chloranthus serratus*[24]	7	3.8
3	*Z. rubescens*[14]	[b]		**19**		9	10.2
4	*Z. piperitum*[a]	6	5.6	**20**		7	3.2
5		4	1.3	**21**		7	3.7
6		5	0.9	**22**		6	9.8
7	*Pisonia aculeata*[22]	4	1.6	**23**		7	9.9
8		4	1.5	**24**		5	6.5
9		6	2.4	**25**		5	11.5
10		5	1.0	**26**		5	5.9
11		4	2.2	**27**		4	13.3
12		9	1.8	**28**		6	1.4
13		4	0.3	**29**		6	0.8
14		6	2.2	**30**	*Z. beecheyanum*[20]	[b]	
15	*Z. piperitum*[a]	4	1.1	**31**	*Z. piperitum*[a]	[b]	
16	*Z. armatum*[23]	9	2.1	**32**	*Z. piperitum*[a]	[c]	

[a]This work

[b]Evaluated in large panel only (table 2)

[c]Not evaluated

Table 2. Sensory Evaluation of Selected Amides by a Large Panel

compound no.	conc. (ppm)[a]	delta[b] with NC	delta[b] without NC	*t*-test with NC	*t*-test without NC	comments for amides in water evaluated with NC[c]	comments for amides in water evaluated w/o NC[c]
1	20	0.9	0.8	***	**	umami (4), astringent (3)	no
4	20	1.1	1.3	***	***	umami (9), mouthfeel (6), salty (5), astringent (4)	no
19	20	1.7	1.7	**	**	umami (10), mouthfeel (4), salty (4), sour (3)	green (3), herbal (3)
23	20	1.3	1.3	**	**	umami (11), astringent (3), salty (3)	no
24	20	1	1.2	**	**	umami (4), sweet (3)	no
25	20	0.9	0.9	**	**	umami (12)	no
26	20	1	1.1	**	**	umami (7), salty (4), sweet (4), metallic (3)	no
27	20	0.8	1.0	NS	NS	umami (11)	green (3), herbal (3)
30	20	0.7	0.6	NS	*	no	no
31	100	0.1	0.0	NS	NS	bitter (10), astringent (4)	no

[a]The amides were diluted to 10% in ethanol. The quantity of ethanol added to the solution was therefore 0.02% for the compounds tasted at 20 ppm and 0.1% for compound **31** tasted at 100 ppm.

[b]Difference in perceived intensity between the amide+MSG vs MSG alone

[c]The numbers in parentheses indicate the number of panelists among the 15 who made this comment.

*, **, ***: levels of confidence at 95%, 99%, and 99.9%, respectively

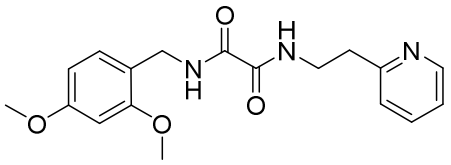
Abbreviations: NC, nose clip; NS, not significant; No, no comments or comments given by less than 20% of the panel (fewer than three panelists).

Table 3. Identification of Selected Amides in *Zanthoxylum piperitum* Root Extract

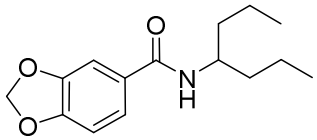
no.	RT (min)	accurate mass	MS/MS fragment 1	MS/MS fragment 2	MS/MS fragment 3	quantity [mg/kg of extract]
1	2.94	372.1805	191.0702	163.0753		41
		$C_{21}H_{26}NO_5$ (-0.2)	$C_{11}H_{11}O_3$ (-0.2)	$C_{10}H_{11}O_2$ (-0.2)		
4	3.82	342.1699	191.0702	135.0846		29
		$C_{20}H_{24}NO_4$ (-0.2)	$C_{11}H_{11}O_3$ (-0.5)	$C_9H_{11}O$, (+0.1)		
5	3.62	n.d.	n.d.	n.d.		
15	2.85	n.d.	177.0546	145.0284	135.0805	6
			$C_{10}H_9O_3$ (-0.1)	$C_9H_5O_2$ (+0.0)	$C_9H_{11}O$ (+0.4)	
16	4.47	326.1387	175.0389	145.0284	135.0847	527
		$C_{19}H_{20}NO_4$ (-0.0)	$C_{10}H_7O_3$ (-0.2)	$C_9H_5O_2$ (-0.2)	$C_9H_{11}O$ (+0.2)	
19	3.92	n.d.	n.d.	n.d.		
22	4.79	n.d.	n.d.	n.d.		
23	5.92	n.d.	n.d.	n.d.		
24	5.44	n.d.	n.d.	n.d.		
25	5.80	n.d.	n.d.	n.d.		
26	6.63	n.d.	n.d.	n.d.		
27	5.64	n.d.	n.d.	n.d.		
30	4.26	356.1854	191.0703	135.0805		5784[a]
		$C_{21}H_{26}NO_4$ (-0.6)	$C_{11}H_{11}O_3$ (-0.1)	$C_9H_{11}O$ (+0.5)		
31	5.01	340.1541	175.0389	145.0284	135.0805	18723[a]
		$C_{20}H_{22}NO_4$ (-0.6)	$C_{10}H_7O_3$ (+0.0)	$C_9H_5O_2$ (+0.0)	$C_9H_{11}O$ (+0.5)	
32	3.18	342.1700	175.0390	145.0284	135.0805	845
		$C_{20}H_{24}NO_4$ (+0.2)	$C_{10}H_9O_3$ (-0.2)	$C_9H_5O_2$ (-0.2)	$C_9H_{11}O$ (+0.1)	

[a]above the highest point of calibration

420



Senomyx, FEMA 4233



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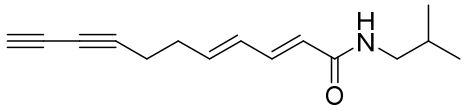

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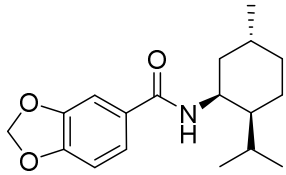
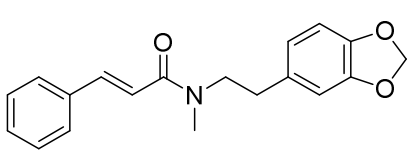
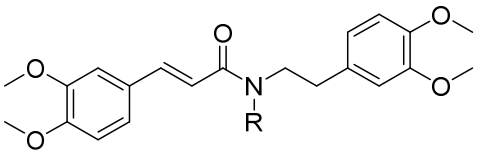


Symrise

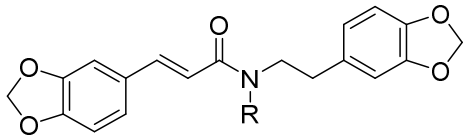
Figure 1.



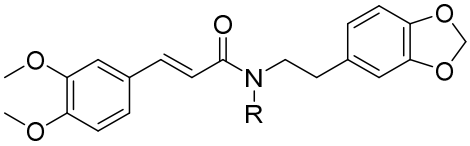
Zanthomamide



Rubemamine **1** (R = H)
Rubemamide (R = CH_3)

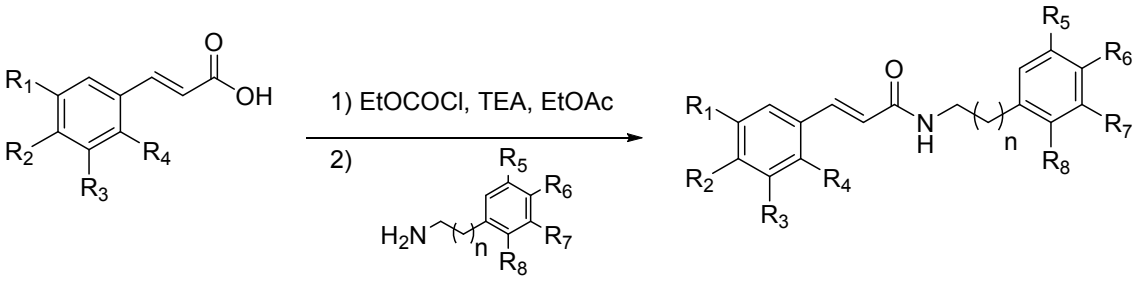


Dioxamin (R = H)
Dioxamide (R = CH_3)



Rubescenamine (R = H)
Rubescenamide (R = CH_3)

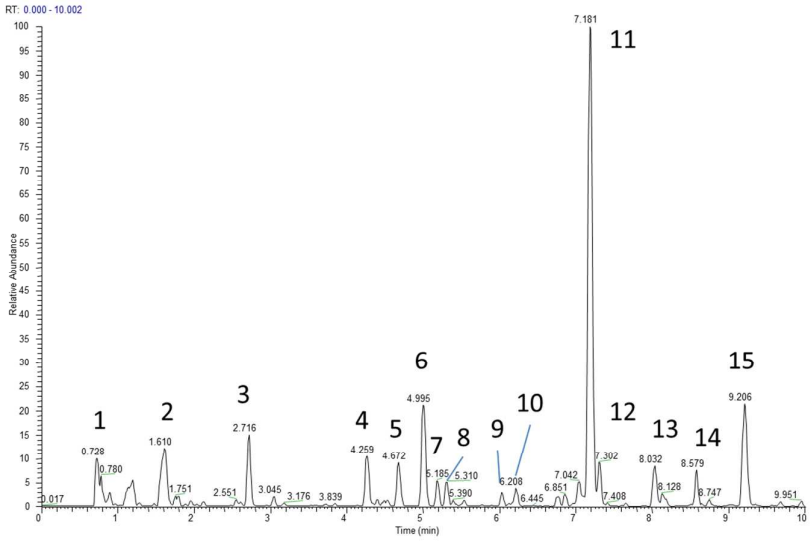
Figure 2.



N°	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	n	double bond
1	OMe	OMe	H	H	H	OMe	OMe	H		
2	H	OMe	H	H	H	OMe	OMe	H		
3	a	a	H	H	H	OMe	OMe	H		
4	OMe	OMe	H	H	H	OMe	H	H		
5	OMe	OMe	H	H	H	OMe	H	H		(*Z*)
6	OMe	OMe	H	H	H	OMe	H	H		single bond
7	H	H	H	H	H	OMe	H	H		
8	H	H	H	H	H	H	OMe	H		
9	H	OMe	H	H	H	OMe	H	H		
10	H	H	H	OMe	H	OMe	H	H		
11	OMe	H	H	H	H	OMe	H	H		
12	OMe	H	H	OMe	H	OMe	H	H		
13	OMe	OMe	OMe	H	H	OMe	H	H		
14	OMe	OAc	H	H	H	OMe	H	H		
15	OMe	OH	H	H	H	OMe	H	H		
16	a	a	H	H	H	OMe	H	H		
17	Me	Me	H	H	H	OMe	H	H		
18	OMe	OMe	H	H	H	H	H	H		
19	OMe	OMe	H	H	H	H	OMe	H		
20	OMe	OMe	H	H	H	H	H	OMe		
21	OMe	OMe	H	H	OMe	H	OMe	H		
22	OMe	OMe	H	H	H	H	OEt	H		
23	OMe	OMe	H	H	H	H	O*n*Pr	H		
24	OMe	OMe	H	H	H	O*i*Pr	H	H		
25	OMe	OMe	H	H	H	Et	H	H		
26	OMe	OMe	H	H	H	*i*Pr	H	H		
27	OMe	OMe	H	H	H	Me	Me	H		
28	OMe	OMe	H	H	H	OMe	H	H	0	
29	OMe	OMe	H	H	H	a	a	H	0	

double bond of (*E*)-configuration except otherwise stated
[a] R_1-R_2 or R_6-R_7 = O-CH_2-O (methylenedioxy)
n = 1 (phenethylamine series) except 0 stated (benzylamine series)

Figure 3.


RT: 0.000 - 10.002
Relative Abundance
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
0.728
0.780
1.610
1.751
2.551
2.716
3.045
3.176
3.839
4.259
4.672
4.995
5.185
5.310
5.390
6.208
6.445
6.851
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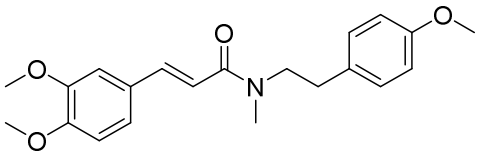
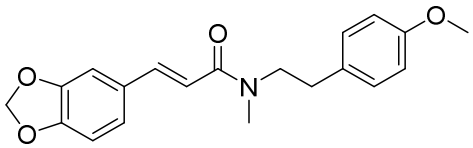
8.579
8.747
9.206
9.951

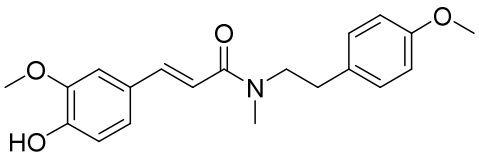
Figure 4.



Beecheyamide **30**



Podocarpamide **31**



32

Figure 5.

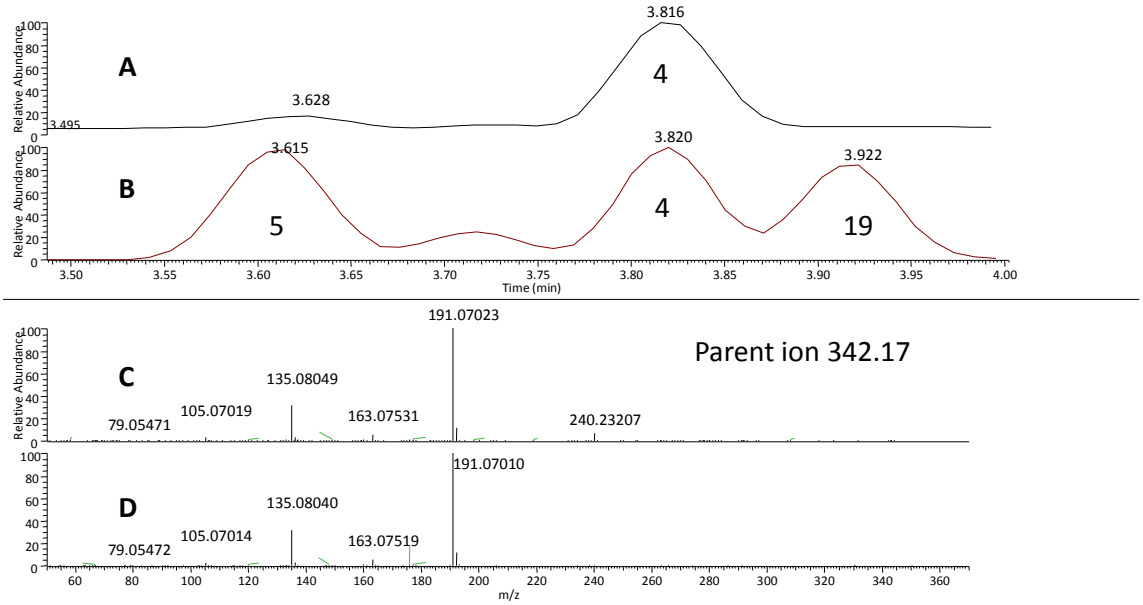
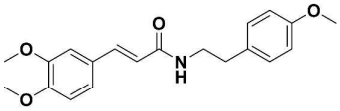

A
3.816
4
3.628
3.495
B
3.615
5
3.820
4
3.922
19
Relative Abundance
Time (min)
C
191.07023
Parent ion 342.17
135.08049
105.07019
79.05471
163.07531
240.23207
D
191.07010
135.08040
105.07014
79.05472
163.07519
m/z

Figure 6.



An intense umami tasting amide (compound **4**) found in Zanthoxylum piperitum

TOC graphic