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Novel Taste Enhancing 4-Amino-2-methyl-5-heteroalkypyrimidines formed from Thiamine by Maillard-type reactions

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ABSTRACT

Increasing the thiamine concentration in a respective process flavor yields in a product with a significant higher kokumi activity. S-Plot analysis of the mass spectrometric data revealed beside thiamine itself, 4-methyl-5-thiazoleethanol, *S*-((4-amino-2-methylpyrimidin-5-yl)methyl)-L-cysteine, *N*-((4-amino-2-methylpyrimidin-5-yl)methyl)-formamide, 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one and 2-methyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine as marker molecules for a process flavor with higher thiamine concentration. Sensory based targeted isolation revealed that *S*-((4-amino-2-methylpyrimidin-5-yl)methyl)-L-cysteine, 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one and 2-methyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine showed an influence on the kokumi taste activity with taste threshold concentrations between 35 to 120 µmol/L. An adapted mass spectrometric based carbon modul labeling (CAMOLA) experiment as well as quantitative studies clearly demonstrated thiamine as the only precursor and an intermolecular formation pathway for the compounds *S*-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one and 2-methyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine. Based on the knowledge that several thiamine derivatives showed taste modulating activity, selected thiamine-based binary model reactions and synthesis were carried out. This resulted in the isolation of further thiamine derived taste modulators like *S*-((4-amino-2-methylpyrimidin-5-yl)methyl)-L-cysteinylglycine, (*S*)-3-((((4-amino-2-methylpyrimidin-5-yl)methyl)thio)methyl)-piperazine-2,5-dione, 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)pentan-2-one, 5-(((furan-2-ylmethyl)thio)methyl)-2-methylpyrimidin-4-amine, (4-amino-2-

methylpyrimidin-5-yl)methanethiol, 2-methyl-5-((methylthio)methyl)pyrimidin-4-amine with taste thresholds ranging from 35 to 880 µmol/L.

KEYWORDS: *umami, kokumi, Process Flavors, thiamine, S-Plot, CAMOLA, model reactions, taste enhancer, taste modulators, meat flavor*

INTRODUCTION

The so-called process flavors (PFs) are food flavorings which are produced by heating various precursors together under controlled reaction conditions, whereby the reactions mimic kitchen-like conditions.[1,2] The aroma and the taste properties of such process flavors are similar to those found in thermally treated foodstuff like meat, chocolate, coffee, caramel, popcorn or bread.[3] Common precursors during the manufacturing of the process flavors are amino acids, reducing sugars, thiamine, ribonucleotides, phospholipids, yeast extracts or hydrolyzed vegetable proteins. The most important reactions involved in the formation of process flavors include the Maillard reaction, Strecker degradation, sugar degradation/fragmentation or thermal conversions of thiamine.[1–3]

Along with the characteristic aroma, particularly the umami and the kokumi taste perception are very important for a meaty flavor impression. The term kokumi is defined as a perceived complexity, continuity and mouth fullness. It was first described by Ueda et al. who isolated kokumi active compounds from a water extract of garlic.[4,5] Since that time many kokumi active compounds have been isolated from food stuff e.g. edible beans, thermally processed avocados and gouda cheese.[6–8] Recently, Mittermeier et al. isolated kokumi modulating octadecadien-12-ynoic acids from chanterelles.[9]

Taste modulating compounds have not only been isolated from food stuff but also from model reactions systems.[10–12] A important representative of this compound class formed during the Maillard reaction is the so called alapyridaine. It could be isolated from an alanine/hexose model reaction mixture after thermal treatment.[10] This compound is tasteless itself but enhances the sweet, salty and umami taste perception. Festring and Hofmann (2010) identified the umami modulating N^2-(1-

Carboxyethyl)guanosine-monophosphate- a nucleotide modified by the Maillard reaction.[11] By thermal treatment of creatinine and various sugars it was possible to identify *N*-(1-methyl-4-oxoimidazolidin-2-ylidene)-α-amino acids as another taste modulating substance class. Sensory evaluations of these compounds revealed that they have a kokumi modulating effect.[12] Very recently, an activity guided fractionation of a meaty process flavor system lead to the isolation and identification of a new taste modulating substance derived from thermally induced reaction of thiamine and cysteine, the *S*-((4-amino-2-methylpyrimidin-5-yl)methyl)-L-cysteine.[13]

One precursor of this compound, thiamine, plays an important role during the formation of meaty process flavors, as it is also a precursor for many (meaty) aroma compounds such as aliphatic sulphides and thiols, sulphur-containing carbonyl compounds, sulphur-substituted furans, thiophenes, thiazolees, bicyclic compounds and alicyclic sulphur.[14-16] The meaty aroma compound 2-methyl-3-furanthiol (MFT) is one of the most important aroma compounds formed by thiamine degradation.[14] However, the focus of thiamine degradation in flavor research has mostly been directed towards the volatile fraction only. Whereas for a meaty impression especially the umami and the kokumi taste perception seemed to be important as well. The isolation of the taste modulating compound *S*-((4-amino-2-methylpyrimidin-5-yl)methyl)-L-cysteine showed that thiamine also plays an important role as a precursor for taste-modulating compounds.[13] Hence, the current study focusses on the formation of non-volatile taste-modulating compounds derived from thiamine. Therefore, binary model reactions consisting of thiamine and various further compounds were performed. The reaction partner for thiamine was selected from substances, which play a key role in the flavor perception of meaty process flavors, e.g. the kokumi active glutathione or the meat aroma compounds 2-methyl-3-furanthiol (MFT) and furan-2-ylmethanethiol (FFT).

MATERIALS AND METHODS

Chemicals.

The following compounds were obtained commercially: acetonitrile, methanol (J.T. Baker, Netherlands), ethyl acetate (VWR, Darmstadt, Germany), formic acid, (Merck, Darmstadt, Germany). Solvents used for LC-MS/MS analysis were of LC-MS grade (Honeywell, Seelze, Germany). Water for HPLC separation was purified using a Milli-Q water advantage A10 water system (Millipore, Schwalbach, Germany). For sensory analysis, bottled water (Evian, Danone, Wiesbaden, Germany) was used. Yeast extract (Gistex XII LS) was obtained from Food Ingredients Distribution (Werne, Germany). Deuterated solvents, thiamine hydrochloride (**1, Figure 1**), L-Cysteine, 4-methyl-5-thiazoleethanol (**2, Figure 1**), 2-methyl-3-furanthiol (MFT, **20, Figure 4**), sodium thioacetate, thiomethoxide, methylamine (dissolved in THF), thiamine-(4-methyl-^{13}C-thiazole-5-yl-$^{13}C_3$) hydrochloride, water free Dimethylformamide (DMF) and sodium hydroxide were obtained from Sigma Aldrich (Steinheim, Germany). 3-mercapto-2-pentanone was obtained from ABCR-Chemicals (Karlsruhe, Germany). 2-furfurylthiol (FFT) was purchased from Givaudan (Dübendorf, Switzerland). *N*-((4-amino-2-methylpyrimidin-5-yl)methyl)formamide (**4, Figure 1**), (4-amino-2-methylpyrimidin-5-yl)methanol (**11, Figure 1**) and 5-(aminomethyl)-2-methylpyrimidin-4-amine (**12, Figure 1**) were obtained from Carbosynth (Berkshire, United Kingdom). Ethyl thiamine (**16, Figure 1**), 5-(bromomethyl)-2-methylpyrimidin-4-amine were obtained from Santa Cruz Biotechnology (Heidelberg, Germany).

Two different process flavors (PF1 and PF2) were kindly provided by Lucta S.A. (Barcelona, Spain).

Model Reaction Systems.

Binary Reaction Systems: For the preparation of the binary model reaction systems thiamine hydrochloride (1 mmol) or ethyl thiamine (1 mmol) and a second compound (L-cysteine, glutathione, 3-mercapto-2-pentanone, MFT or FFT; 1 mmol each) were dissolved in aqueous KH_2PO_4-buffer (0.1 M, 10 mL). The pH value was adjusted to 7 with a NaOH-solution (1 M). Afterwards the mixture was heated in an aluminum block at 120 °C for 120 minutes while stirring in a closed glass vessel.

Adapted CAMOLA Experiment: For the adapted CAMOLA experiment $^{13}C_4$ labeled thiamine hydrochloride (0.1 mmol) and ethyl thiamine (0.1 mmol) were dissolved in aqueous KH_2PO_4-buffer (0.1 M, 1 mL). After adjusting the pH-value to 7 (with a 1 M NaOH solution), the mixture was heated at 120 °C for 120 minutes in a closed glass vessel.

Fractionation of the Thiamine and Cysteine Reaction Mixture.

After cooling the thiamine cysteine reaction mixture to room temperature, the reaction mixture was extracted twice with ethyl acetate (EtOAc). The combined organic layers were freed from solvent in vacuum to obtain the ethyl acetate extractables. After lyophilization the EtOAc-extract was stored at -20 °C until further use.

Aimed at locating the targeted compounds the ethyl acetate fraction was dissolved in acetonitrile/water (50/50, v/v, membrane-filtered (0.45 µm) and separated by means of preparative RP-HPLC on a MonoChrom MS (250 x 21.2mm i.d., 5u; Varian, Darmstadt, Germany) column into 7 fractions (F1 – F7). Monitoring the effluent with an UV-detector at 254 nm, chromatography was performed at a flow rate of 20 mL/min using aqueous formic acid (0.1%) as solvent A and acetonitrile as solvent B. Starting with 0% B for 2 minutes the gradient was increased to 40% B in 12 minutes. Within another 2 minutes, the gradient was increased to 100% B and maintained for 1 minute.

Sub Fractionation and Structure Determination of Fraction F5. The lyophilized HPLC-fraction F5 was dissolved in acetonitrile/water (50/50, v/v), membrane-filtered (0.45 µm) and separated by means of preparative RP-HPLC on Luna Pentafluorphenyl (250 x 10 mm i.d., 5µm, 100 Å; Phenomenex, Aschaffenburg, Germany) column into three sub fractions F5-a - F5-c. For the separation aqueous formic acid (0.1%) was used solvent A and methanol as solvent B. The effluent was set to 20 mL/min and was monitored with an UV-detector (λ= 254 nm). The gradient started with 25% B for 2 minutes, afterwards solvent B was increased to 65% in 6 minutes. Within 1 minute solvent B was increased to 100% B. After separation the organic solvent was evaporated, and fractions were lyophilized. Based on UV-Vis, LC-MS/MS, TOF-MS and 1D/2D-NMR experiments, the structure of the compound isolated from fraction F5-c, could be identified as 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)-5-hydroxy-pentan-2-one (**5a, Figure 1**).

UV-Vis λ_{max} = 254 nm (MeOH/H_2O, 50/50, v/v), UPLC-TOF-MS: *m/z*=256.1232 $[M+H]^+$; ^{1}H NMR (500 MHz, D_2O, 300 K): δ (ppm) 8.07 [s, 1H, H-C(6)], 3.74 – 3.64 [m, 4H, H-C(5',7)], 3.54 [t, J = 7.4 Hz, 1H, H-C(3')], 2.57 [s, 3H, H-C(8)], 2.30 [s, 3H, H-C(1')], 2.12 [ddt, J = 14.3, 7.4 Hz, 5.5 Hz 1H H_a-C(4')], 1.88 [ddt, J = 14.3, 7.4, 5.5 Hz, 1H, H_b-C(4')]. ^{13}C NMR (125 MHz, D_2O, 300 K): δ (ppm) 210.66 [C(2')] , 163.09 [C(4)], 162.18 [C(2)], 143.40 [C(6)], 111.47 [C(5)], 58.53 [C(5')], 50.56 [C(3')], 31.72 [C(4')], 27.54 [C(7)], 26.01 [C(1')], 21.04[C(8)].

Structure Determination of Fraction F6. After separation, evaporation of the organic solvent and lyophilization the structure of F6 was identified by means of UV-Vis, LC-MS/MS, TOF-MS and 1D/2D-NMR experiments as 2-methyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**6, Figure 1**).

UV-Vis λ_{max} = 254 nm (MeOH/H_2O, 50/50, v/v), UPLC-TOF-MS: *m/z*=236.0974 $[M+H]^+$; ^{1}H NMR (400 MHz, MeOD-d_4, 298 K): δ (ppm) 7.35 [d, *J* = 1.9 Hz, 1H, H-C(5')], 7.29 [s, 1H, H-C(6)], 6.29 [d, *J* = 1.9 Hz, 1H, H-C(4')], 3.65 [s, 2H, H-C(7)], 2.38 [s, 3H, H-C(8)], 1.97 [s, 3H, H-C(6')]. ^{13}C NMR (100 MHz, MeOD-d_4, 298 K): δ (ppm) 166.73 [C(2)] , 163.11 [C(4)], 158.23 [C(2')], 153.99 [C(6)], 142.24 [C(5')], 116.42 [C(4')], 111.94 [C(5)], 109.92 [C(3')], 34.09 [C(7)], 24.57 [C(8)], 11.24 [C(6')].

Fractionation of Binary Model Reaction Systems. The cooled reaction mixtures were separated by means of HPLC. The exact parameters are disclosed in the supporting information. Following substances could be isolated and confirmed by means of UV-Vis, LC-MS/MS, TOF-MS and 1D/2D-NMR experiments.

S-((4-amino-2-methylpyrimidin-5-yl)methyl)cysteinylglycine (**7, Figure 1**). UV-Vis λ_{max} = 254 nm (MeOH/H_2O, 50/50, v/v), UPLC-TOF-MS: *m/z*=300.1129 $[M+H]^+$; ^{1}H NMR (500 MHz, D_2O, 300 K): δ (ppm) 8.07 [s, 1H, H-C(6)], 4.23 [t, *J* = 6.4 Hz, 1H, H-C(5')], 3.91 – 3.71 [m, 4H, H-C(7), H-C(2')], 3.03 [d, *J* = 6.4 Hz, 2H, H-C(6')], 2.58 [s, 3H. H-C(8)]. ^{13}C NMR (125 MHz, D_2O, 300 K): δ (ppm) 175.68 [C(1')], 167.94 [C(4')], 163.24 [C(4)], 161.75 [C(2)], 141.74 [C(6)], 111.20 [C(5)], 52.23 [C(5')], 43.26 [C(2')], 31.11 [C(6')], 28.38 [C(7)], 20.73 [C(8)].

3-((((4-amino-2-methylpyrimidin-5-yl)methyl)thio)methyl)piperazine-2,5-dione (**8, Figure 1**). UV-Vis λ_{max} = 254 nm (MeOH/H_2O, 50/50, v/v), UPLC-TOF-MS: *m/z*=282.1030 $[M+H]^+$; ^{1}H NMR (400 MHz, D_2O, 298 K): δ (ppm) 8.09 [s, 1H, H-C(6)], 4.52 [dd, *J* = 8.5, 5.2 Hz, 1H, H-C(6')], 3.82 [s, 2H, H-C(3')], 3.73 [m, 2H, H-C(7)], 2.58 [s, 3H, H-C(8)]. ^{13}C NMR (100 MHz, D_2O, 298 K): δ (ppm) 175.04 [C(5')], 171.82 [C(2')], 163.93 [C(4)], 161.64 [C(2)], 141.33 [C(6)], 111.75 [C(5)], 52.66 [C(6')], 42.90 [C(3')], 31.11 [C(7')], 28.48 [C(7)], 20.48 [C(8)].

3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)pentan-2-one (**9, Figure 1**). UV-Vis λ_{max} = 254 nm (MeOH/H_2O, 50/50, v/v), UPLC-TOF-MS: *m/z*=240.1170 $[M+H]^+$; ^1H NMR (400 MHz, D_2O, 298 K): δ (ppm) 7.92 [s, 1H, H-C(6)], 3.68 [d, *J* = 14.7 Hz, 1H H_a-C(7)], 3.62 [d, *J* = 14.7 Hz, 1H, H_b-C(7)], 3.32 [dd, *J*= 7.35, 7.35, 1H, H-C(3‘)], 2.41 [s, 3H, H-C(8)], 2.20 [s, 3H, H-C(1‘)], 1.81 [dq, *J* = 14.5, 7.3 Hz, 1H, H_a-C(4‘)], 1.67 [dq, *J* = 14.5, 7.3 Hz, 1H, H_b-C(4‘)], 0.88 [t, *J* = 7.3 Hz, 3H, H-C(5‘)]. ^{13}C NMR (100 MHz, D_2O, 298 K): δ (ppm) 213.25 [C(2‘)] , 167.35 [C(4)], 162.157 [C(2)], 155.11 [C(6)], 111.58 [C(5)], 56.56 [C(3‘)],29.18 [C(7)], 26.85 [C(1‘)], 24.16 [C(1‘)], 17.45[C(8)], 11.54 [C(5‘)].

5-(((furan-2-ylmethyl)thio)methyl)-2-methylpyrimidin-4-amine (**10, Figure 1**). UV-Vis λ_{max} = 254 nm (MeOH/H_2O, 50/50, v/v), UPLC-TOF-MS: *m/z*=236.0911 $[M+H]^+$; ^1H NMR (400 MHz, MeOD-d_4, 298 K): δ (ppm) 7.87 [s, 1H, H-C(6)], 7.41 [dd, J = 1.9, 0.9 Hz, 1H, H-C(5’)], 6.33 [dd, J = 3.2, 1.9 Hz, 1H, H-C(4’)], 6.20 [dd, 1H, J=3.2, 0.9 Hz, H-C(3’)], 3.67 [s, 2H, H-C(6’)], 3.61 [s, 2H, H-C(7)], 2.41 [s, 3H, H-C(8)]. ^{13}C NMR (100 MHz, MeOD-d_4, 298 K): δ (ppm) 166.61 [C(2)], 163.55 [C(4)], 153.56 [C(6)], 152.99 [C(2’)], 143.41 [C(5’)], 111.80 [C(C4’)], 111.51 [C(5)], 108.62 [C(3’)], 29.79 [C(7)], 28.32 [C(6’)], 24.43 [C(8)].

2-ethyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**17, Figure 1**), UV-Vis λ_{max} = 254 nm (MeOH/H_2O, 50/50, v/v), UPLC-TOF-MS: *m/z*= 250.1024 $[M+H]^+$*;* ^1H (400 MHz, MeOD-d_4, 298 K): δ (ppm) 7.33 [d, 1 H, *J* = 1.9 Hz, H-C(5‘)] , 7.27 [s, 1 H, H-C(6)], 6.28 (d, 1 H, *J* = 1.9 Hz, H-C(4‘)], 3.63 [s, 2 H, H-C(7)], 2.61 [q, 2 H, *J* = 7.7 Hz, H-C-(8)], 1.91 [s, 3 H, H-C(6‘)], 1.22 [t, 3 H, *J* = 7.7 Hz, H-C(9)].^{13}C (125 MHz, MeOD-d_4, 298 K): δ (ppm) 171.3 [C(2)], 163.2 [C(4)], 158.3 [C(2)], 154.3 [C(6)], 142.2 [C(5‘)], 116.4 [C(4‘)], 112.0 [C(5)], 109.9 [C(3‘)], 34.2 [C(7)], 32.5 [C(8)], 13.3 [C(9)], 11.2 [C(6‘)].

3-(((4-amino-2-ethylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one (**18,** Figure 1**)** UV-Vis λ_{max} = 254 nm (MeOH/H_2O, 50/50, v/v), UPLC-TOF-MS: *m/z*= 270.1014 $[M+H]^+$; ^1H (400 MHz, D_2O, 298 K): δ (ppm) 7.98 [s, 1H, H-C(6)], 3.71 – 3.62 [m, 4H. H-C(7), H-C(5')], 3.51 [t, 1H, H-C(3')], 2.73 [q, *J* = 7.6 Hz, 2H, H-C(8)], 2.23 [s, 2H,H-C(1')], 2.08 [ddt, 1H, H_a-C(4'), *J*=7.6, 7.6, 13.74], 1.84 [ddt, 1H, H_b-C(4'), *J* = 7.6, 7.6, 13.74 Hz], 1.26 [t, *J* = 7.6 Hz, 3H, H-C(9)].

Synthesis of further Thiamine Derivates. Further thiamine derivatives were synthesized according to Zheng et al.[17] The synthesis pathway and the isolation parameters can be found in the supporting information. Following substances could be isolated and confirmed by means of UV-Vis, LC-MS/MS, TOF-MS and ^1H-NMR experiments.

(4-amino-2-methylpyrimidin-5-yl)methanethiol (**13, Figure 1**) UV-Vis λ_{max} = 254 nm (MeOH/H_2O, 50/50, v/v), UPLC-TOF-MS: *m/z*=156.056 $[M+H]^+$; ^1H NMR (400 MHz, MeOD-d_4, 298 K): δ (ppm) 7.83 [s, 1H, H-C(6)], 3.54 [s, 2H, H-C(7)], 2.43 [s, 3H, H-C(8)].

2-methyl-5-((methylamino)methyl)pyrimidin-4-amine (**14, Figure 1**), UV-Vis λ_{max} = 254 nm (MeOH/H_2O, 50/50, v/v), UPLC-TOF-MS: *m/z*=153.1141 $[M+H]^+$; ^1H NMR (400 MHz, D_2O, 298 K): δ (ppm) 8.11 [s, 1H, H-C(6)], 4.11 [s, 2H, H-C(7)], 2.70 [s, 3 H, H-C(1')], 2.37 [s, 3 H, H-C(8)].

2-methyl-5-((methylthio)methyl)pyrimidin-4-amine (**15, Figure 1**), UV-Vis λ_{max} = 254 nm (MeOH/H_2O, 50/50, v/v), UPLC-TOF-MS: *m/z*=170.0781 $[M+H]^+$; ^1H NMR (400 MHz, MeOD-d_4, 298 K): δ (ppm) 7.96 [s, 1H, H-C(6)], 3.61 [s, 2H, H-C(7)], 2.46 [s, 3H, H-C(8)], 2.02 [s,H- C(1')].

Quantitation of Thiamine and Thiamine Derivates. For screening and quantitation of the thiamine derivatives **1-15** an LC-MS/MS method was developed on

a 5500 QTrap LC-MS/MS system (AB Sciex, Darmstadt, Germany) equipped with Luna PFP column a (150 × 2.0 mm i.d., 3 µm, Phenomenex, Aschaffenburg, Germany). Chromatography was performed with acetonitrile containing 1% formic acid and as solvent B and water containing 1% formic acid as solvent A. The flow rate was set to 0.3 mL/min. Starting with 5% B and holding it for 3 min, the ratio of solvent B was linearly increased to 40% within 8 min and held for 1 min. Thereafter starting conditions were readjusted within 0.5 min and equilibrated for 3.5 min. The compounds **1-15** were quantitated by means of multiple reaction monitoring (MRM) with defined mass transitions in an ESI^+ mode. The parameters like declustering potential (DP, in V), collision energy (CE in V) and cell exit potential (CXP in V) are disclosed in the supporting information.

Aliquots (1 mg) of the process flavors or diluted reaction mixtures were dissolved in a water/methanol-mixture (1 mL, 50/50, v/v). After adding the internal standard (**17**), the samples were membrane filtered (0.45 µm) and injected to the LC-MS/MS-system.

Sensory Analyses. *Sensory Panel Training*. Eight female and nine male panellists (23-32 years in age), who had no history of known taste disorders and who had given the informed consent to participate in the present sensory tests, were trained in weekly training sessions for at least two years in order to became familiar with the taste language and methodologies used, to evaluate the taste of aqueous reference solutions (2.0 mL; pH 5.9): sucrose (50 mmol/L) for sweet taste, *L*-lactic acid (20 mmol/L) for sour taste, NaCl (20 mmol/L) for salty taste, caffeine (1 mmol/L) for bitter taste, monosodium *L*-glutamate (3 mmol/L) for umami taste. To train the activity of mouth fullness enhancement and complexity increase, coined kokumi activity, the

panelists were requested to compare the gustatory impact of a blank model broth (control) with a solution of reduced glutathione (5 mmol/L) in model broth.[4,5]

Preparation of Model Broth. For the detection of umami modulating substances, a model broth was prepared. This model broth served as reference for all sensory analyses. Therefore sodium chloride (2.9), monosodium glutamate (MSG 1.9 g), maltodextrin (6.4 g) and yeast extract (2.1 g) were solved in 1 L of ultrapure water.

Duo-Trio-Test. To evaluate the sensory differences within between PF1 and PF2 duo-trio tests were used. Therefore, the respective process flavors were dissolved in model broth (5 g/L).

Determination of Taste Threshold. For the determination of the taste threshold, a solution with known concentration was prepared in water or in model broth and successively diluted 1:1 (v/v). The solutions were presented to the panelists in order of increasing concentrations in a duo-trio test. Water or model broth was used as blank and reference, respectively. The panelists were asked to describe the difference between the blank and the sample. The first dilution step where a difference between the blank and the sample was detectable was defined as the taste threshold. The taste threshold was calculated by the geometric mean.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a binary high-pressure HPLC pump system PU-2080 Plus, an AS-2055 Plus autosampler, a DG-2080-53 degasser, a MD-2010 Plus type diode array detector (Jasco, Gross-Umstadt, Germany) and a Rh 7725i type Rheodyne injection valve (Rheodyne, Bensheim, Germany). Data acquisition was performed by means of Chrompass Chromatography Data System, Version 1.9 (Jasco, Gross-Umstadt, Germany).

Liquid Chromatography-Mass spectrometry (LC-MS). LC-MS/MS analysis was performed on a 5500 QTrap MS/MS system (AB Sciex, Darmstadt, Germany) connected to a Shimadzu Nexera X2 system (Shimadzu, Duisburg, Germany) system running in the positive electrospray ionization (ESI^+) mode. For measurements on the QTrap system the following conditions were used. Zero grade air served as nebulizer gas (55 psi), and as turbo gas for solvent drying (65 psi, 450 °C). Nitrogen served both as curtain gas (35 psi) and collision gas (8.7×10^{-7} psi) dissociation potential (-2 V) and entrance potential (-10 V). Both quadrupoles were set at unit resolution. ESI^+ mass and product ion spectra were acquired with direct flow infusion. For ESI^+, the ion spray voltage was set at +5500 V. Energies for declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) as well as MS/MS parameters for measuring in the MRM mode were optimized for each compound individually, to detect the fragmentation of molecular ions into specific product ions after collision with nitrogen. For instrumental control and data acquisition, Sciex Analyst software v1.6 was used.

UPLC/Time-of-Flight Mass Spectrometry (UPLC/TOF-MS). An aliquot (0.1 mg - 1mg) of the sample, dissolved in methanol/water (30/70, v/v; 1mL), was injected into an Acquity UPLC core system (Waters UK Ltd., Manchester, UK) connected to a SYNAPT G2 HDMS spectrometer (Waters UK Ltd., Manchester, UK) operating in a positive electrospray (ESI^+) modus with the following parameters: capillary voltage (+2.0 kV), sampling cone (20 V), source temperature (120 °C), desolvation temperature (450 °C), cone gas (5 L/h), and de-solvation gas (850 L/h). Chromatographic separations were performed on a BEH C18 column (2.1 x 150 mm, 1.7 µm, Waters UK Ltd., Manchester, UK) operated at 45 °C with a solvent gradient (flow rate 0.4 mL/min) of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid

in acetonitrile (solvent B). Starting with 5% B the ratio was increased in 4 min to 100% B. The instrument was calibrated over a mass range from *m/z* 100 to 1200 using a solution of sodium formate (0.5 mmol/L) in 2-propanol/water (9/1, v/v). All data were lock mass corrected using leucine enkephaline as the reference (*m/z* 556.2771 for $[M+H]^+$; *m/z* 554.2615 for $[M-H]^-$). Data acquisition and analysis were done by using the MassLynx software (version 4.1; Waters).

For the calculation of the group differences the PF1 and PF2 were dissolved in methanol/water (30/70, v/v; 0.5 mg/mL) and injected 5 times. The raw data the process flavors and their replicates obtained from UPLC-ESI-TOF-MS analysis were processed with Progenesis QI. The processed data were exported to EZinfo. The group differences between the two process flavors were calculated using orthogonal partial least-squares discriminant analysis (OPLC-DA) highlighted as S-Plot.

Nuclear Magnetic Resonance Spectroscopy (NMR). 1D- and 2D-NMR experiments were performed on a Bruker 400 MHz AV III or 500 MHz AV III spectrometer (Bruker, Rheinstetten, Germany) equipped with a Z-gradient 5 mm multinuclear observe probe ($BBFO_{plus}$) or a triple resonance cryo-Probe (TCI, H/C/N) . MeOD-d_4 (600 µL) or D_2O (600 µL) were used as solvent and chemical shifts are reported in parts per million referenced to the MeOD-d_4 or D_2O solvent signals, respectively. Data processing was performed by using Topspin NMR software (version 3.2; Bruker, Rheinstetten, Germany) and MestReNova 10.0 (Mestrelab Research, Santiago de Compostela, Spain). For quantitative NMR spectroscopy (qHNMR), the spectrometer (400 MHz, $BBFO_{plus}$) was calibrated by using the ERETIC 2 tool based on the PULCON methodology as reported earlier.[18] The isolated signal at 3.96 ppm (t, J = 5.3 Hz, 1H) was used for absolute quantitation of **1-15** by using a sample of *L*-

tyrosine at a defined concentration (5.21 mmol/L) as the external standard and its specific resonance signal at 7.10 ppm (m, 2H) for analyses.

RESULTS AND DISCUSSION

Recently a reaction product of thiamine (**1, Figure 1**) and L-cysteine, *S*-((4-amino-2-methylpyrimidin-5-yl)methyl)-L-cysteine **(3, Figure 1**) was identified as a new taste modulating compound in a complex model reaction system, a so-called process flavor (PF).[13] This compound is formed during heating of thiamine (**1**, **Figure 1**) and cysteine, wherein cysteine substitutes the thiazole moiety (**2, Figure 1**) of thiamine. It is of tremendous interest for the food industry to increase the formation of taste modulating substances in savory systems like process flavors. One possible way to enhance the formation of **3** in process flavors is to increase the amount of thiamine. In order to evaluate whether an increased thiamine concentration leads to other savory products, two different process flavors were produced. One with the original thiamine concentration (PF1) and one with a ten times higher thiamine concentration (PF2). All other ingredients and the heating parameters were kept constant.

To evaluate the sensory impact of the increased thiamine concentration on the taste activity of the process flavors a sensory duo-trio test was performed. With a significance value of α=0.1 the panelists could detect a difference between PF1 and PF2. The panelists characterized PF1 as less savory and described a higher kokumi activity in PF2.

However, the composition of the process flavors is very complex and other compounds might be formed during thermal thiamine conversion as well. To identify other potential savory impact compounds which are higher abundant in PF2 an UPLC-TOF-MS

screening of the two process flavors was performed. To visualize the differences of the two process flavors a mass spectrometric based S-Plot, representing pairs of exact molecular mass to charge ratio and retention time of each compound from the two process flavors was calculated. The y-axis of the S-plot is a visualization of the confidence of a compounds' contribution to the group difference and the x-axis denotes the contribution of a compound to the group difference.[19,20] The S-Plot indicated that the ions *m/z* 122.0845, 144.0612, 167.1061, 236.0974, 243.1032 and 256.1232 are *m/z* ratios of compounds showing the highest difference in between PF1 and PF2 and were higher abundant in PF2 (**Figure 2**).

By comparing the compounds with reference substances and co-chromatography, the fragment ion with *m/z* 144.0610 (R_t=0.9 min) and the fragment ion *m/z* 122.0845 (R_t=0.9 min) could be explained by in-source fragmentation of thiamine).

Furthermore, it was possible to identify known degradation products of thiamine such as 4-methyl-5-thiazoleethanol (**2, Figure 1**) with *m/z* 144.0612 (R_t=1.6 min) and *N*-((4-amino-2-methylpyrimidin-5-yl)methyl)formamide (**4**, **Figure 1**) with *m/z* 167.1061 (R_t=1.12 min).[21–23] In addition, the already discovered taste modulating compound **3** could be identified as marker for PF2 with *m/z* 243.1032 (R_t=1.0 min).[13]

Apart from that, the compounds with *m/z* 256.1232 (R_t=1.6 min) and *m/z* 236.0974 (R_t=2.1 min) could not be identified by comparing with reference substances. To evaluate whether those highly abundant substances have an impact on the overall taste of the PF2, the two unknown substances have to be isolated.

Isolation of unknown compounds. Since the composition of the process flavors is very complex an isolation directly from the process flavors would be very laborious and time consuming. Hence, a binary model reaction system of thiamine and cysteine was prepared. Cysteine was chosen, as a second ingredient, since the

elemental composition of both compounds indicated a sulfur in the target molecule and due to the fact that it play an important role during the formation of **3**.

Through screening with UPLC-TOF-MS it was possible to confirm that the unknown targeted substances with the *m/z* ratios of 256.1232 and 236.0974 are formed during this reaction.

Aimed at locating the these compounds a sequential solvent fractionation was performed. The model reaction was extracted twice with ethyl acetate (EtOAc) to obtain a water and an organic solvent soluble fraction. UPLC-TOF-MS screening revealed that the target compounds are extractable with ethyl acetate. This organic extract was further fractionated by means of preparative HPLC into seven sub fractions (F1-F7; supporting information). UPLC-TOF-MS screening revealed that fraction F5 contained the compound with *m/z* 256.1232 whereas, the compound with *m/z* 236.0974 eluted in fraction F6.

Sub Fractionation and Structure Determination of Fraction F5. In order to isolate the compound from fraction F5 the thiamine-cysteine model reaction system was further fractionated by means of semipreparative HPLC into three sub fractions (F5-a - F5-c). The target compound eluted in fraction F5-c (supporting information).

The UPLC-TOF-MS analysis in the ESI^+ mode indicated a pseudo molecule ion of *m/z* 256.1232 $[M+H]^+$ with a calculated formula of $[C_{11}H_{17}N_3O_2S+H]^+$. The fragment ion of *m/z* 154.044 $[C_6H_8N_3S]^+$ indicated that a compound is linked via a sulfur to the aminopyrimidine moiety of the thiamine.

For accurate structure determination of the unknown compound 1D- and 2D-NMR-experiments were performed. In the ^{1}H-NMR spectrum, a strong de-shielded single aromatic proton at 8.03 ppm (H-C(6)) was observed, well in the line with the aromatic proton of the aminopyrimidine moiety of thiamine. This assumption was further

strengthened by the HMBC correlations of H-C(6) to C(5), C(2) and C(4) at 114.4 ppm, 162.18 ppm and 163.09 ppm, respectively (**Figure 3A**). With exception of the carbon C(7) resonating at 27.54 ppm the chemical shifts of the protons and the carbon atoms of the aminopyrimidine moiety were comparable to those of an intact thiamine.[24] The carbon atom C(7) at 27.54 ppm showed a correlation signal in the HMBC spectrum with H-C(3') at 3.54 ppm, which is most likely linked to a hetero-nucleus. In addition, with the results of the high-resolution mass spectrometry, this correlation confirmed the connection of the aminopyrimidine moiety via a sulfur atom to another constituent of the molecule. The correlation of H-C(3') in the HMBC spectrum with a quaternary carbon C(2') resonating at 210.7 ppm, indicated that a keto group was incorporated in the structure. This keto group showed a $^{3}J_{C,H}$ coupling in the HMBC with a methyl group H-C(1') at 2.22 ppm as well as with a diastereotopic methylene group H-C(4'). This methylene group showed a $^{3}J_{C,H}$ coupling to another another methylene group at 3.64 ppm (H-C(5')). The shift of H-C(5') to higher frequencies indicated that it is linked to a hydroxy group (**Figure 3A**). Taking all spectrometric and spectroscopic data into consideration the compound of fraction F5-c was confirmed as 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one (**5a, Figure 1**), in which the thiazole moiety (**2**) of the thiamine (**1**) is substituted by 5-hydroxy-3-mercapto-2-pentanone (**19a, Figure 4**). ^{1}H-NMR experiments revealed that this thiamine derivate does not only occur in an open chained (**5a**) but also as closed cyclic ketal form (**5b, Figure 1**). There is a clear chemical shift of the methyl group of the open-chained form (2.30 ppm) and the cyclic ketal form (1.46 ppm). By means of qHNMR it was possible to show that the ratio of the two tautomers (85 % open, 15% cyclic ketal) was favored to the open form. To the best of our knowledge, these compounds have not been described in literature before.

Cerny et al. (2008) isolated 5-hydroxy-3-mercapto-2-pentanone (**19a, Figure 4**) as an instable intermediate during thiamine degradation.[25] They also could show this intermediate occurs in two different tautomeric forms, the open chained (**19a**) and closed cyclic form (**19b, Figure 4**).[25] The ratio of the two tautomeric forms in the underivatized compound (79% open; 21 % cyclic ketal) is similar to the ratio found for compound **5a/5b**.

Since **19a** is a degradation product of thiamine, UPLC-TOF-MS analysis confirmed that this compound is also formed in a sole thiamine reaction system and the presence of cysteine is not necessary.

Identification of Fraction F6. Due to the high purity of fraction F6 isolated from the thiamine-cysteine model reaction system no further sub fraction was required. UPLC-TOF-MS analysis in the ESI^+-mode of F6 revealed *m/z* 236.0974 as the pseudo molecular ion ($[M+H]^+$), and an elemental composition of $[C_{11}H_{14}N_3OS]^+$. To elucidate the structure 1D- and 2D-NMR-experiments were performed. Compared to compound **5** the HMBC spectrum of the isolated substance showed also the typical signals of the aminopyrimidine moiety of thiamine.[24] Due to the correlation signals in the HMBC-spectrum it was possible to confirm that a MFT-motif, which is linked to the aminopyrimidine moiety at position C(7). The methylene group H-C(7) at 3.64 ppm showed a correlation signal with the aromatic carbon atom C(3') of the MFT-moiety at 109.9 ppm. This carbon atom showed further couplings to the two aromatic proton H-C(4') and H-C(5') as well as to the methyl group H-C(6') at 1.96 ppm. The two aromatic protons H-C(4') and H-C(5') showed couplings in the proton NMR as well as in the H,H COSY with typical coupling constants of 1.9 Hz for the furanyl moiety. Compared to H-C(4') the proton H-C(5') is shifted to higher frequencies, due to the adjacent oxygen atom. The long-range coupling of the methyl group H-C(6') to C(7) confirmed the

linkage of the MFT moiety to the pyrimidine moiety (**Figure 3B**). Taking all spectrometric and spectroscopic data into consideration, the compound isolated from F6 was confirmed as 2-methyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**6, Figure 1**), this compound can be classified as a derivate of thiamine where MFT (**20, Figure 4**) substitutes the thiazole moiety (**2**) of thiamine (**1**). This compound was isolated from Jhoo et al. for the first time from a heated thiamine solution, however no sensory data were reported so far.[26]

As MFT is a known degradation product of thiamine, UPLC-TOF-MS analysis confirmed, analogous to **5**, that this compound is also formed in a sole thiamine reaction system and the presence of cysteine is not necessary et all.

Sensory Evaluation of Thiamine Derived Compounds in process flavors. To evaluate the impact of these compounds in the thiamine enriched PF2. The taste thresholds of the isolated or commercially available compounds **1**-**6** were determined in water as well as in model broth (**Table 1**).

Thiamine (**1**) itself showed no modulating activity in model broth, the taste activity was only described as bitter and the taste threshold in model broth was determined at 1200 µmol/L. The astringent taste threshold of the thiamine degradation product 4-methyl-5-thiazoleethanol (**2**) was 10 times lower in water (120 µmol/L) than in model broth (1200 µmol/L). In addition, the panelists could not detect any modulating activity in model broth. Literature already describes the kokumi taste modulating effects of compound **3**.[13] With a taste threshold of 120 µmol/L in model broth and a 4-fold higher taste threshold in water this compound could be confirmed as taste modulator. Up to a concentration of 1000 µmol/L compound **4** did not show any taste activities neither in model broth nor in water. The isolated compounds **5** and **6** showed strong taste modulating effects in model broth with taste thresholds of 40 µmol/L and 50 µmol/L,

492 respectively. The panelists characterized the compounds with a strong kokumi effect,
493 which was described as long-lasting and mouth-filling. The two compounds did not
494 show any intrinsic taste activities in water up to a concentration of 710 µmol/L and 280
495 µmol/L, respectively (**Table 1**).
496 The untargeted screening of the PF1 and PF2 confirmed different thiamine conversion
497 products are higher abundant in the thiamine enriched PF2 compared to PF1.
498 However, not all conversions products contribute to the higher kokumi activity of PF2.
499 Only, the thiamine derivates **3, 5** and **6** showed a kokumi activity.
500 **Formation Pathway of 5 and 6.** In order to obtain highly kokumi active
501 products, it is essential to get a better understanding of the formation pathways of
502 these taste enhancers. The recently published reaction product of cysteine and
503 thiamine (**3**) is formed via an S_N1 reaction of the two precursors.[13] Literature describes
504 the degradation of thiamine to 5-hydroxy-3-mercapto-2-pentanone (**19a, Figure 4**).
505 Wherein, the degradation of thiamine to 5-hydroxy-3-mercapto-2-pentanone (**19a**)
506 occurs via water addition to the thiazole moiety, which leads to the intermediate **21**.
507 This intermediate then undergoes a ring opening and tautomerization to **22**. The
508 following hydrolysis of **22** leads to the targeted 5-hydroxy-3-mercapto-2-pentanone
509 (**19a**) and to the formamide derivate **4** or the amino derivate **12** (**Figure 4**).[23,22] The
510 degradation product 5-hydroxy-3-mercapto-2-pentanone (**19a**) was identified from a
511 thiamine, cysteine and xylose reaction mixture by means of NMR from Cerny et al. in
512 2008.[25] This compound is described as very reactive and an important intermediate. It
513 serves as precursor for numerous *S*-containing flavor compounds.[15,25] For example it
514 reacts via its cyclic ketal form (**19b**) after dehydration and oxidation to MFT (**20**), a very
515 important meaty aroma compound (**Figure 4**).[25]

As **19a** and **20** are constituents of **5a** and **6**, respectively. The latter substitute the thiazole part (**2**) of thiamine (**1**) and are linked to the pyrimidine motif via a sulfur atom. As **19a** and **20** are degradation products of thiamine themselves, two formation pathways of **5a** and **6** are possible. One is the intramolecular formation by a rearrangement during the thermal process (**Figure 5A**). The other possible pathway is an intermolecular pathway whereby first thiamine (**1**) degrades to the volatile compounds **19a** and **20** which than react via an S_N1-reaction mechanism with thiamine. The cationic intermediate **23** can be formed either from thiamine (**1**), the formamide derivate (**4**) or the amino derivate (**12**) with 4-methyl-5-thiazoleethanol (**2**), ammonia or formamide as leaving group, respectively (**Figure 5B**).

To elucidate the formation pathway of e.g. Maillard reaction products the so-called CAMOLA (**ca**rbon **mo**dule **la**beling) approach is widely used. Where fully labeled and unlabeled analogues are heated in an equimolar ratio.[27–30] Since in the reaction of thiamine to the target compounds both, the thiazole and aminopyrimidine moiety are involved it is necessary to obtain fully ^{13}C-labeled thiamine. However, in purchasable products only the thiazole part of thiamine is ^{13}C-labeled. Therefore, it was necessary to adapt the CAMOLA experiment. To get a different type of labeling into the pyrimidine moiety, ethyl thiamine (**16, Figure 1**) was used. Here the methyl group at position C(2) is substituted by an ethyl group. The degradation of the thiazole moiety to the sulfur compounds 5-hydroxy-3-mercapto-2-pentanone (**19a**) and MFT (**20**) should not affected by the alkyl chain in the pyrimidine moiety. Hence, they should be formed by ethyl thiamine as well as by thiamine. By heating ethyl thiamine and $^{13}C_4$ labeled thiamine in an equimolar ratio it should be possible to clarify the reaction mechanism by mass spectrometry. If the compounds **5** and **6** are formed via an intramolecular mechanism only four *m/z*-signals in the mass spectra should be observed (unlabeled

ethyl thiamine derivatives (**17**, **18**) and labeled thiamine derivatives (**5*, 6***), **Figure5A**). In contrast, if the compounds **5** and **6** are generated by an intermolecular mechanism labeled and unlabeled 5-Hydroxy-3-mercapto-2-pentanone and MFT would react with ethyl thiamine and thiamine respectively and as a consequence to eight different *m/z*-signals (ethyl thiamine-derivate unlabeled (**17,18**) and labeled (**17***,**18***), thiamine derivate unlabeled (**5,6**) and labeled (**5***, **6***), **Figure5B**) should be observed.

For this labeling experiment $^{13}C_4$-labeled thiamine and ethyl thiamine were heated in an equimolar mixture at optimized conditions. A comparison of the pseudomolecular ion $[M+H]^+$ of **5a** and **18** showed an enrichment of three ^{13}C-carbon atoms in the two derivates (**Figure 6 A1&A2**), which confirms that the reaction product of thiamine and 5-hydroxy-3-mercapto-2-pentanone, **5** is formed via an intermolecular mechanism (**Figure 5B**). The comparison of the pseudomolecular ion $[M+H]^+$ of **6** and **17** also showed an incorporation of three ^{13}C-carbon atoms (**Figure 6 B1&B2**). Concluding, that derivate of thiamine and MFT, **6** is also formed via an intermolecular mechanism (**Figure 5B**).

That new type of CAMOLA-experiments can be widely adapted to different reaction mechanism where the reactant can be divided into two different reactive parts and no fully labeled compounds are available but are required for this type of investigations. The application is not only limited to model reaction system and can also be applied for biological systems.

Formation of further Thiamine Derivates. By means of an activity guided fractionation of a process flavor and untargeted screenings followed by targeted isolation it was possible to identify several taste modulating compounds, which are derived from thiamine (**3**, **5** and **6**).[13] Aimed at isolate further thiamine derived taste modulating compounds several binary model reactions were performed. The binary

model reactions are preferred over more complex reaction systems (e.g. PFs) because it facilitates the isolation and structure elucidation step.

*Formation of **7** and **8**.* Since the thiamine derivate of cysteine, **3**, appears to be active a further model reaction of thiamine and the tripeptide glutathione (GSH) was performed. GSH degrades during heating into cysteinyl glycine and its respective diketopiperazine (DKP).[31,32] Due to this, it was not possible to isolate a direct derivate of thiamine and GSH but derivatives of thiamine and cysteinyl glycine S-((4-amino-2-methylpyrimidin-5-yl)methyl)-L-cysteinylglycine (**7, Figure 1**) and its respective DKP (S)-3-((((4-amino-2-methylpyrimidin-5-yl)methyl)thio)methyl)piperazine-2,5-dione (**8, Figure 1**). Both compounds have not been described in literature so far. Their structures where confirmed by their characteristic TOF-MS spectra and 1D- and 2D-NMR experiments.

Compound **7** showed a pseudo molecular ion ($[M+H]^+$) at *m/z* 300.113 Da. By means of HMBC-experiments it was possible to confirm the structure e.g. via coupling of the aminopyrimidine moiety with cysteinylglycine. As an example, the carbon atom C(7) at 28.4 ppm showed a coupling to the methylene group H-C(6') at 3.03 ppm. Compound **8** showed a pseudo molecular ion ($[M+H]^+$) at *m/z* 282.103 Da. The HMBC experiments confirmed the linkage of the aminopyrimidine moiety with the DKP by the connection of C(7) with H-C(7').

The isolated compounds showed a kokumi modulating effect in model broth with a taste threshold of 880 µmol/L (**7**) and 255 µmol/L (**8**) respectively (**Table 1**). Compared to the other taste modulating compounds **3**, **5,** and **8** the taste threshold of **7** was higher. Both substances were detectable in the two process flavors by LC-MS/MS-screening. Comparing PF1 and PF2 the derivates increased in the thiamine enriched

process flavor (PF2) from 44 µmol/kg to 81 µmol/kg for **7** and from 43 µmol/kg to 137 µmol/kg for **8**, respectively.

*Formation of **9**.* A further model reaction was performed with thiamine and the flavor active compound 3-mercapto-2-pentanone, which is formed during Maillard reaction from ribose with cysteine and plays an important role in the aroma profile of meat.[14,33] By means of HPLC it was possible to isolate a derivate of thiamine and 3-mercapto-2-pentanone 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)pentan-2-one (**9, Figure 1**) from a heated mixture of thiamine and 3-mercapto-2-pentanone. The structure was confirmed by its TOF-MS spectra and 1D- and 2D-NMR experiments. The UPLC-TOF-MS analysis revealed a pseudo molecular ion ($[M+H]^+$) with 240.117 Da. The NMR spectra of **9** was similar to the NMR spectra of **5a** as they only differ in the hydroxy group at position C(5'). In comparison to the ^{1}H-NMR spectra of **9** H-C(5') is shifted to lower frequencies than in **5a**.

In order to evaluate the taste enhancing effects of the compound the taste threshold was determined in water for the intrinsic taste and in model broth to evaluate the taste enhancing effects. The compound showed a kokumi effect with a taste threshold of 35 µmol/L in model broth. The taste threshold in water was nearly 7-fold higher than in model broth (220 µmol/L), confirming the compound as a taste modulating compound (**Table 1**). The new discovered taste modulator has not been described in literature so far. LC-MS/MS screening revealed that the compound was detectable in a sole thiamine reaction system as well as in PF2 (1 µmol/kg) but not in PF1.

*Formation of **10**.* A further important Maillard reaction product with a roasty aroma is Furfurylthiol (FFT). This compound is a key aroma compound in meaty dishes and in process flavors.[14,33,34] By means of HPLC it was possible to isolate the derivate of thiamine and FFT 5-(((furan-2-ylmethyl)thio)methyl)-2-methylpyrimidin-4-amine (**10,**

Figure 1) from a reaction mixture of thiamine and FFT. The structure of the compound was confirmed by its TOF-MS spectra and 1D- and 2D-NMR experiments. As **10** is an isomer of **6** it shows the same pseudo molecular ion ($[M+H]^+$) at *m/z* 236.091 Da. **10** and **6** can be differentiated by their characteristic NMR spectra. The HMBC spectrum of **10** showed a characteristic coupling of C(7) to the methylene group H-C(6)'. This proton is further connected with the carbon atoms of the furan ring.

The Sensory evaluations of the compound revealed that it has a kokumi effect in model broth (taste threshold 120 µmol/L) and is tasteless in water up to a concentration of 330 µmol/L. This new taste modulating compound has not been described in literature so far. LC-MS/MS screening revealed that the compound was detectable in PF2 (1 µmol/kg) but not in PF1.

Structure-Activity-Relationship. All thiamine derived taste modulating compounds that have been isolated so far, have in common that a thiol is linked to the aminopyrimidine moiety of the thiamine. If an amino compound like formamide instead of the thiol is linked to the aminopyrimidine moiety (**4**) no taste modulating effects were detectable. To evaluate the sensory impact of the sulfur, several structural related compounds, which only differ by the heteroatom were compared. Those derivates were either commercially available or synthesized according to Zheng et al.[17] The hydroxy derivate (4-amino-2-methylpyrimidin-5-yl)methanol (**11, Figure 1**) and the amino derivate 5-(aminomethyl)-2-methylpyrimidin-4-amine (**12**, **Figure 1**) were compared to the respective thiol derivate (4-amino-2-methylpyrimidin-5-yl)methanethiol (**13, Figure 1**). In order to o check whether the polarity might also play a role the derivate of methyl amine 2-methyl-5-((methylamino)methyl)pyrimidin-4-amine (**14, Figure 1**) was compared to the derivate of thiomethoxide 2-methyl-5-((methylthio)methyl)pyrimidin-4-amine (**15, Figure 1**). The sensory evaluation showed that all compounds which

contain a sulfur had in common that they showed a kokumi activity in model broth with a low taste threshold of 70 - 80 µmmol/L. The taste thresholds of these compounds were comparable to the taste threshold of the already identified thiamine derived kokumi substances (35 - 255 µmol/L). The analogous compounds with other heteroatoms (oxygen or nitrogen) did not show any kokumi effects (**Table 1**). Therefore, it can be concluded that the sulfur definitively plays an important role in the taste perception of the thiamine derived kokumi substances.

As many thiamine derivates show modulating effects, it seemed to be interesting to know, whether the respective ethyl thiamine derivates also show kokumi effects in model broth. Therefore, a model reaction of ethyl thiamine (**16, Figure 1**) and MFT was performed to isolate the respective ethyl thiamine derivate (**17, Figure 1).** Surprisingly, sensory evaluations of this compound revealed that is does not have any taste modulating properties (**Table 1**). This leads to the assumption, that the alkyl chain in the aminopyrimidine moiety plays an important role, during the kokumi taste perception.

Untargeted screening followed by targeted isolation of a model reaction screening lead to the identification of new thiamine derived taste modulating compounds **5** and **6** which have not been described in literature before. By a novel adaption of the classic CAMOLA-experiment it was possible to confirm the intermolecular formation pathway of the compounds. Furthermore, binary model reactions with thiamine lead to the isolation of additional taste modulating compounds. Those reactants, like glutathione, 3-mercapto-2-pentanone or FFT, chosen for the model reactions play a key role in the development of meaty process flavors. Based on those structure-activity relationships it was possible to confirm that the sulfur plays an important role in the kokumi taste perception. The discovery of the new taste modulating compounds gives several

665 possibilities to optimize the savory taste perception of thermally treated model
666 reactions systems like meaty process flavors.
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673 **Notes**

674 The authors declare no competing financial interest.

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Figure 1. Chemical structures of derivates formed by thiamine (**1**) or ethyl thiamin (**16**) during model reactions: 4-methyl-5-thiazoleethanol (**2**), *S*-((4-amino-2-methylpyrimidin-5-yl)methyl)-L-cysteine (**3**), *N*-((4-amino-2-methylpyrimidin-5-yl)methyl)formamide (**4**), 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one (**5a**) 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)-2-methyltetrahydrofuran-2-ol **(5b**), 2-methyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**6**), *S*-((4-amino-2-methylpyrimidin-5-yl)methyl)-L-cysteinylglycine (**7**), (*S*)-3-((((4-amino-2-methylpyrimidin-5-yl)methyl)thio)methyl)piperazine-2,5-dione (**8**), 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)pentan-2-one (**9**), 5-(((furan-2-ylmethyl)thio)methyl)-2-methylpyrimidin-4-amine (**10**), 4-amino-2-methylpyrimidin-5-yl)methanol (**11**), 5-(aminomethyl)-2-methylpyrimidin-4-amine (**12**), (4-amino-2-methylpyrimidin-5-yl)methanethiol (**13**) 2-methyl-5-((methylamino)methyl)pyrimidin-4-amine (**14**), 2-methyl-5-((methylthio)methyl)pyrimidin-4-amine (**15**), 2-ethyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**17**) and 3-(((4-amino-2-ethylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one (**18**)

Figure 2. S-Plot of original process flavor PF1 (-1) versus thiamine enriched process flavor PF2 (+1)

Figure 3. Excerpts of HMBC spectra (500/ 125 MHz, MeOD-d_4 or D_2O, 300 K) of 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one (**5**, **A**), 2-methyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**6**, **B**).

Figure 4. Formation pathway of MFT (**20**) from thiamine (1) via 5-hydroxy-3-mercapto-2-pentanone (**19a**).

Figure 5. Intramolecular (A) or intermolecular formation (B) pathway of 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one (**5**), 2-methyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**6**), 2-ethyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**18**) and 3-(((4-amino-2-ethylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one (**19**) with labeled* und labeled derivates.

Figure 6. UPLC-TOF/MS spectra of 3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one (**5**) and 3-(((4-amino-2-ethylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one (**19**) (A) and of 2-methyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**6**) and 2-ethyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**18**) (B) in a ^{13}C-labeled thiamine, ethyl thiamine reaction mixture.

Table 1. Taste threshold concentrations of the isolated and synthesized compounds in model broth and water.

	threshold conc.	
compound (no.)	TC [µmol/L] model broth (taste)	TC [µmol/L] water (taste)
thiamine (**1**)	1200 (bitter)	600 (astringent)
4-methyl-5-thiazoleethanol (**2**)	1200 (bitter)	120 (bitter)

S-((4-amino-2-methylpyrimidin-5-yl)methyl)-L-cysteine (**3**)	120 (kokumi)	480 (astringent)
N-((4-amino-2-methylpyrimidin-5-yl)methyl)formamide (**4**)	> 1000	>1000
3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)-5-hydroxypentan-2-one (**5**)	40 (kokumi)	710 (astringent)
2-methyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**6**)	50 (kokumi)	280 (astringent)
S-((4-amino-2-methylpyrimidin-5-yl)methyl)-L-cysteinylglycine (**7**)	880 (kokumi)	n.d.
(*S*)-3-((((4-amino-2-methylpyrimidin-5-yl)methyl)thio)methyl)piperazine-2,5-dione (**8**)	255 (kokumi)	n.d.
3-(((4-amino-2-methylpyrimidin-5-yl)methyl)thio)pentan-2-one (**9**)	35 (kokumi)	220 (astringent)
5-(((furan-2-ylmethyl)thio)methyl)-2-methylpyrimidin-4-amine (**10**)	120 (kokumi)	330 (astringent)
4-amino-2-methylpyrimidin-5-yl)methanol (**11**)	700 (bitter)	>1100
5-(aminomethyl)-2-methylpyrimidin-4-amine (**12**)	>1000	>1000
(4-amino-2-methylpyrimidin-5-yl)methanethiol (**13**)	80 (kokumi)	n.d.
2-methyl-5-((methylamino)methyl)pyrimidin-4-amine (**14**)	600 (bitter)	n.d.

2-methyl-5-((methylthio)methyl)pyrimidin-4-amine (**15**)	70 (kokumi)	200 (astringent)
2-ethyl-5-(((2-methylfuran-3-yl)thio)methyl)pyrimidin-4-amine (**17**)	>1000	n.d.

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796 **Brehm et al. (Figure 1)**

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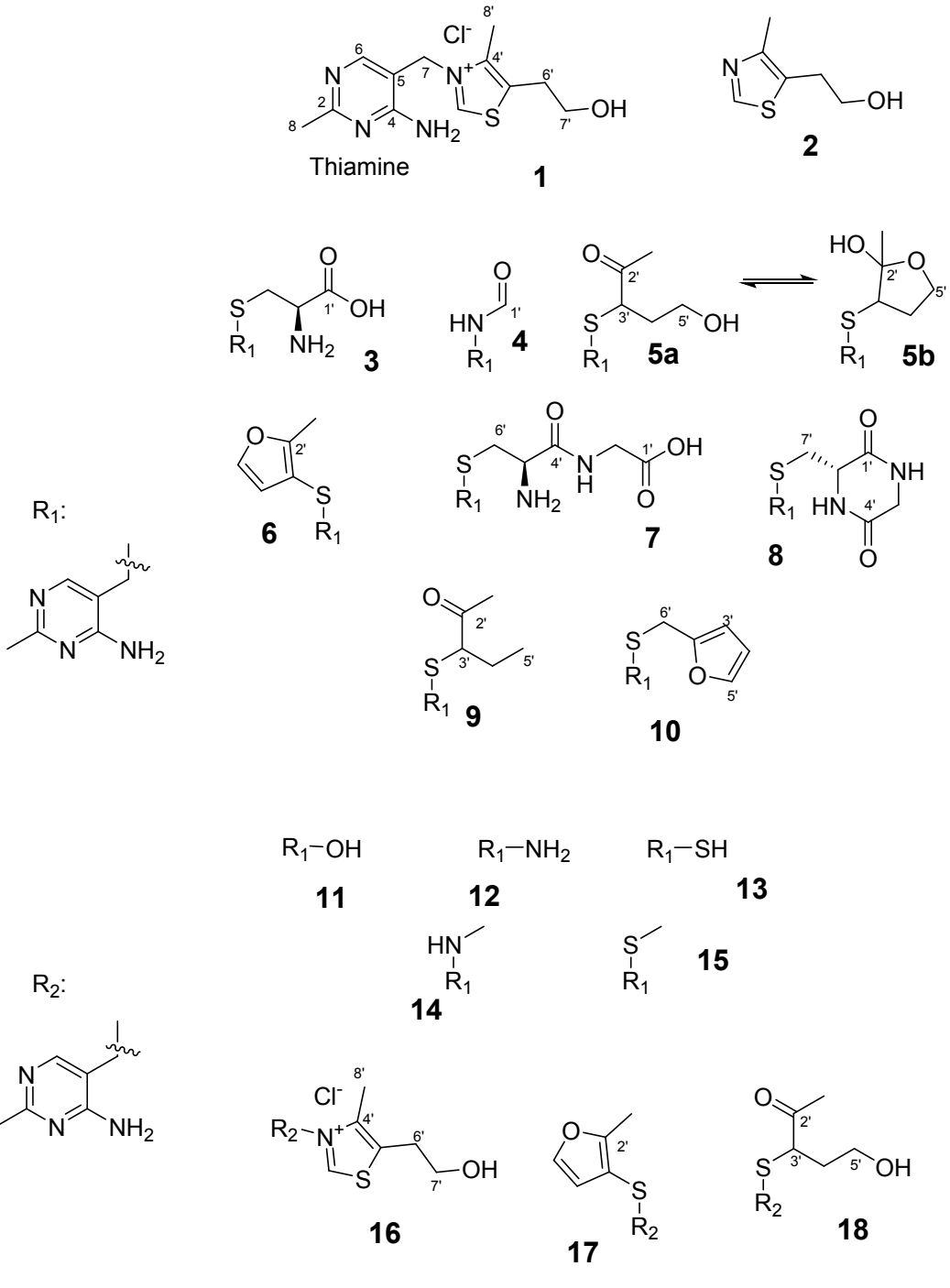

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803 **Brehm et al. (Figure 2)**

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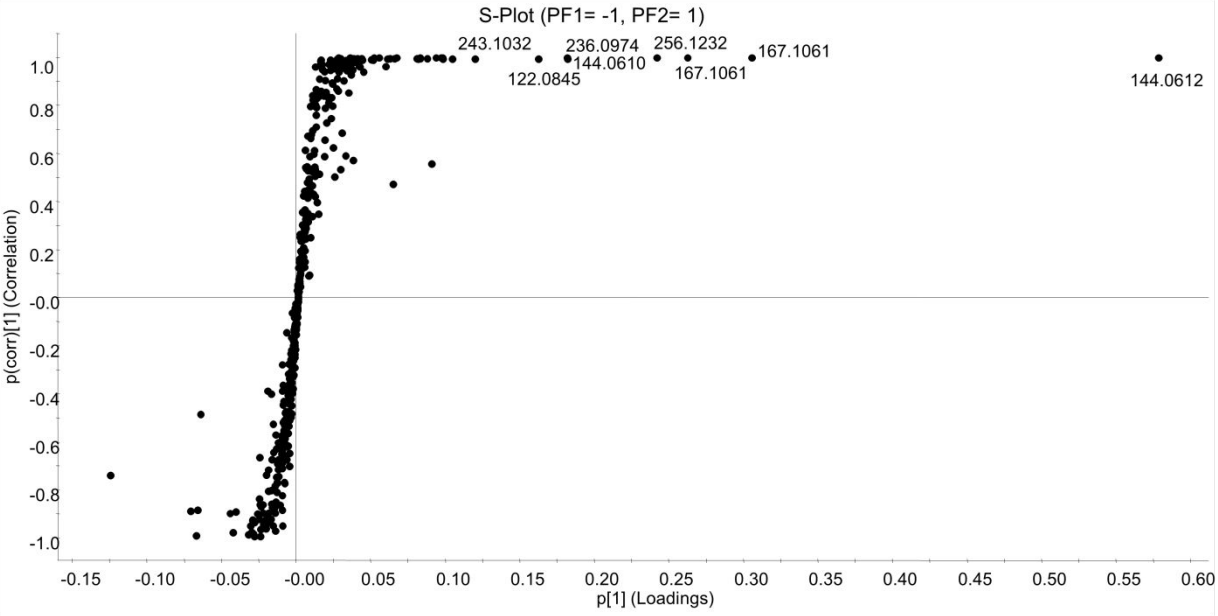

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807 **Brehm et al. (Figure 3)**

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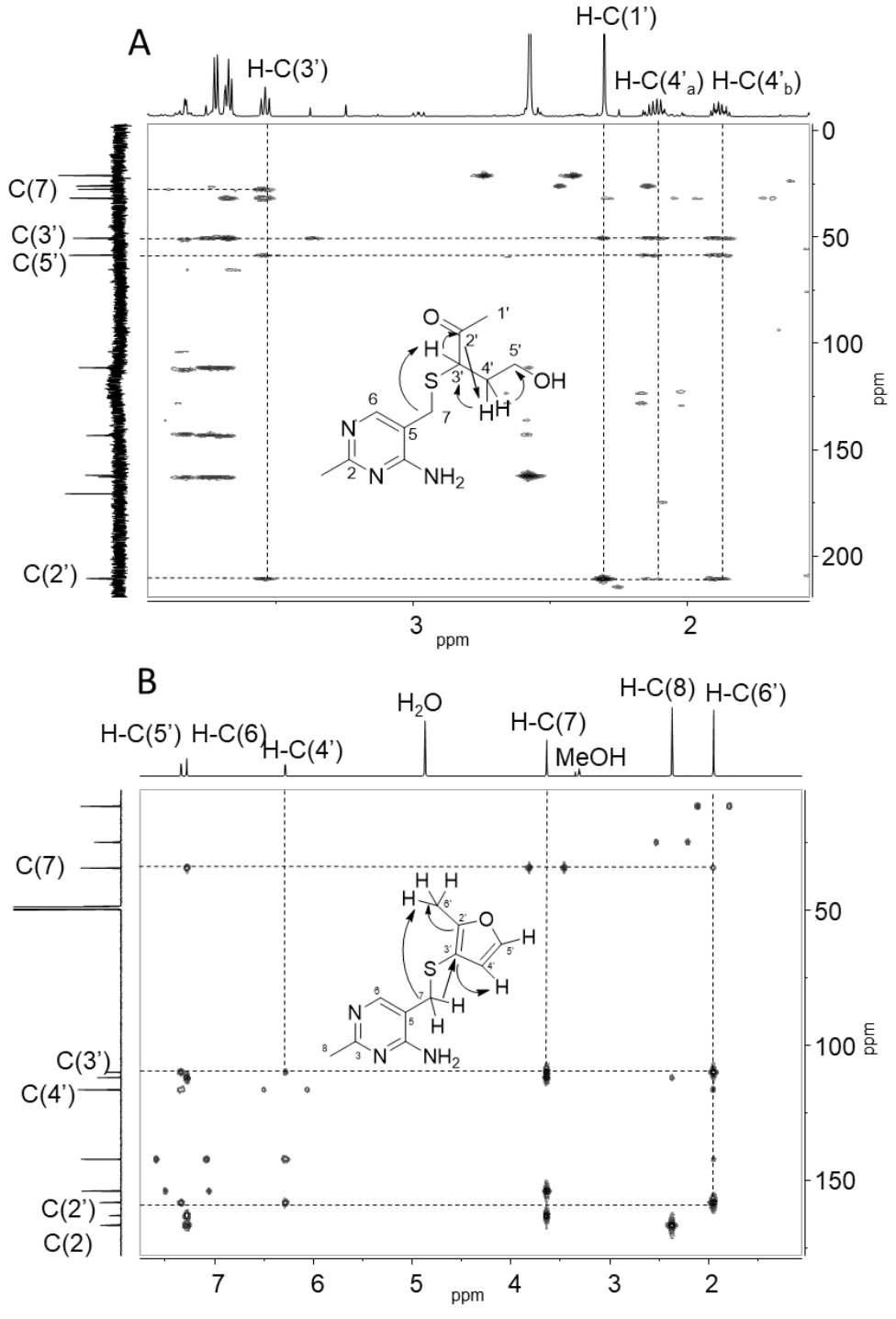

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812 **Brehm et al. (Figure 4)**

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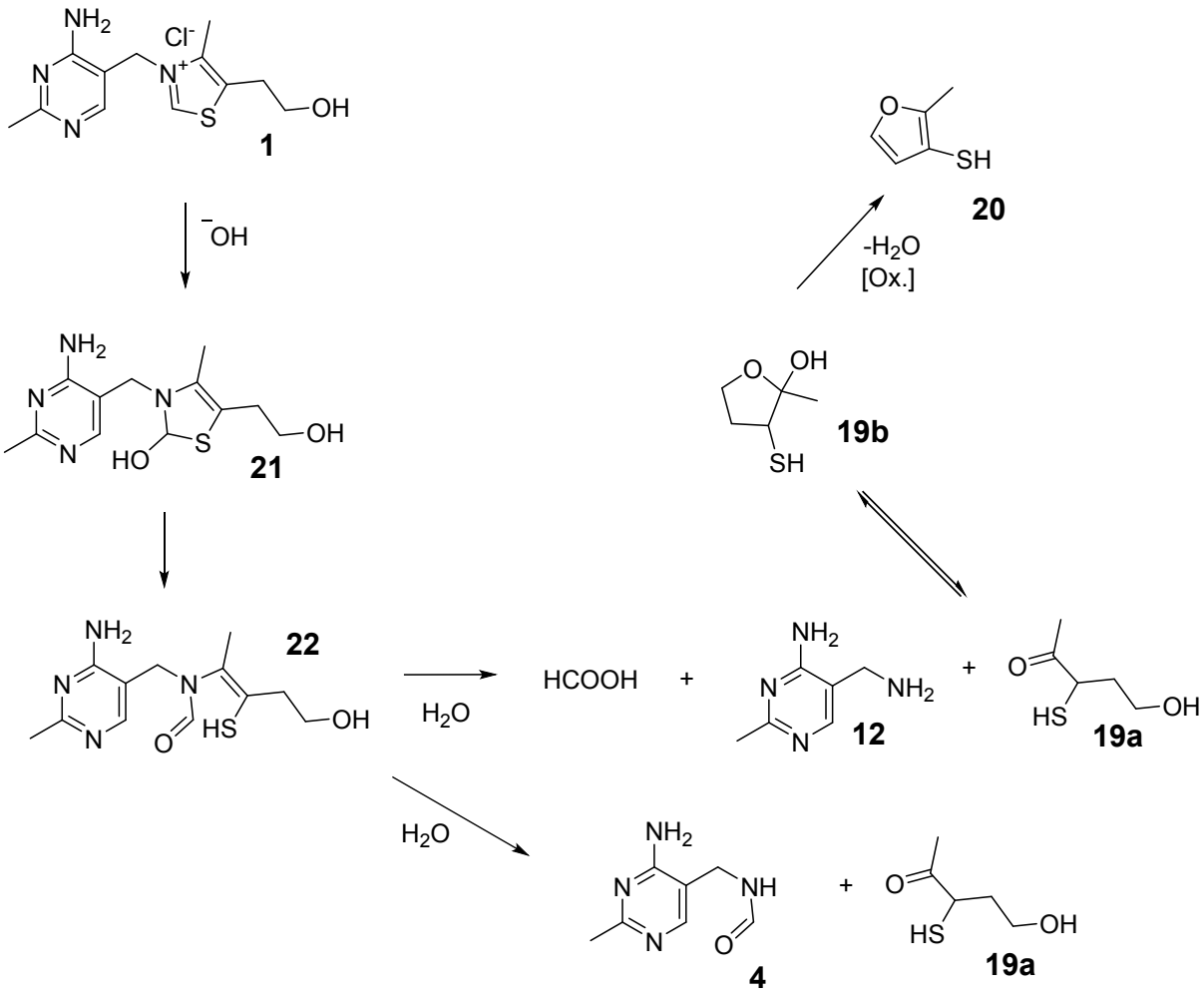

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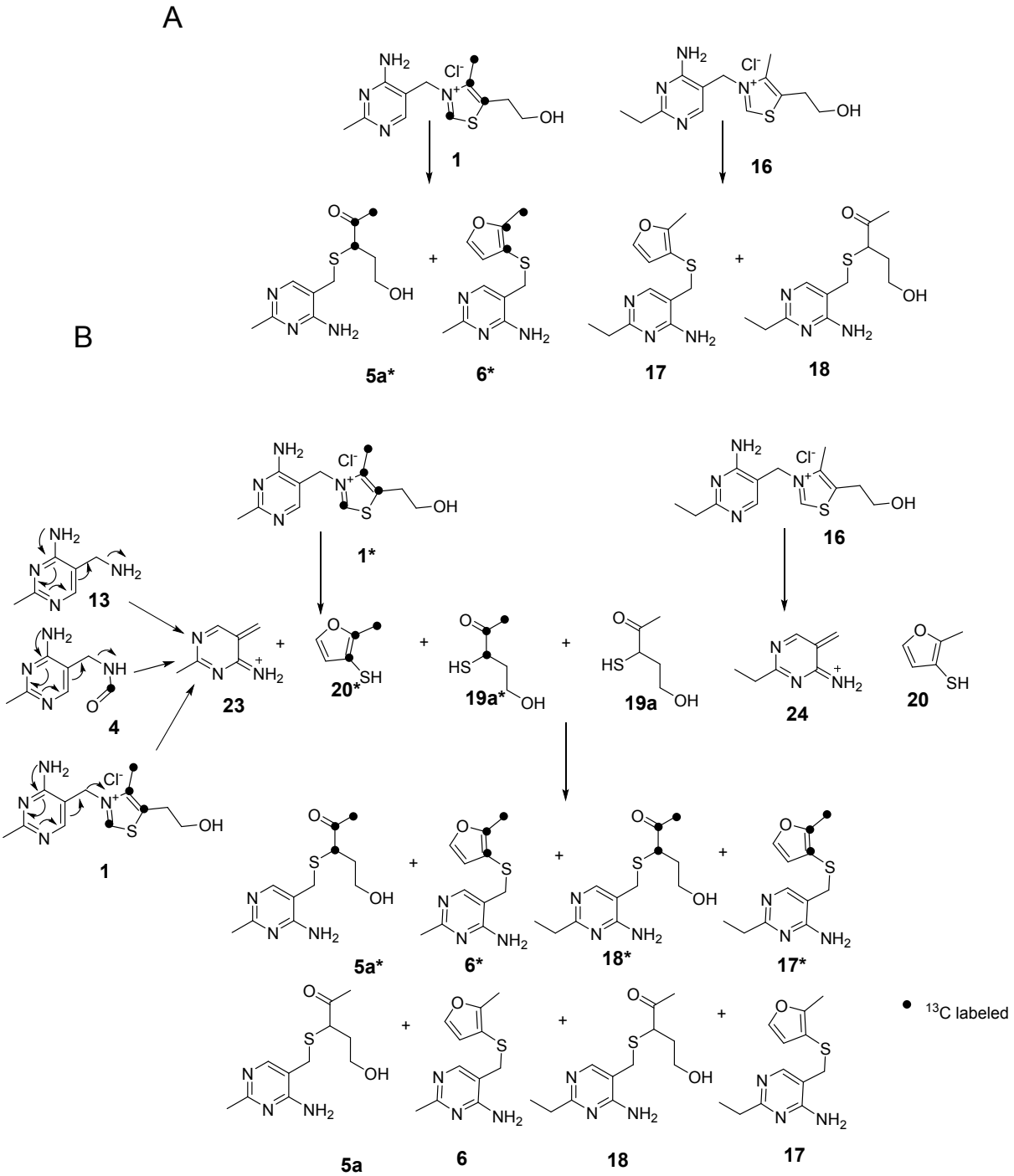
Brehm et al. (Figure 5)



822 **Brehm et al. (Figure 6)**
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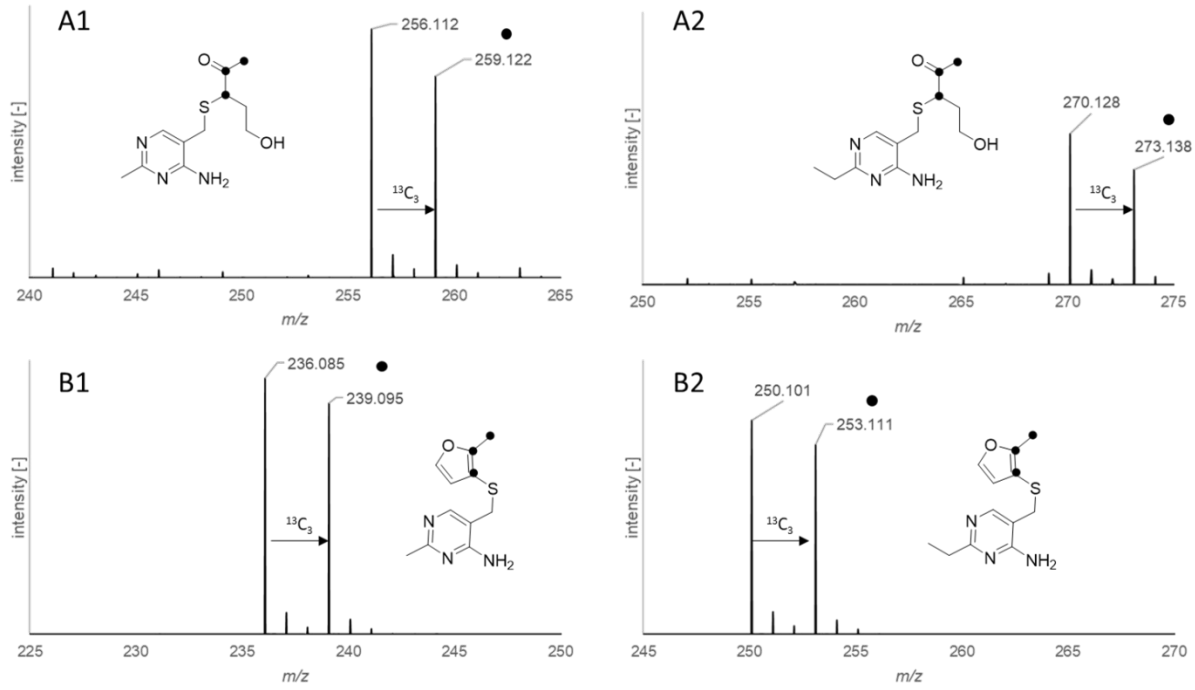

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830 **Brehm et al. (TOC picture)**
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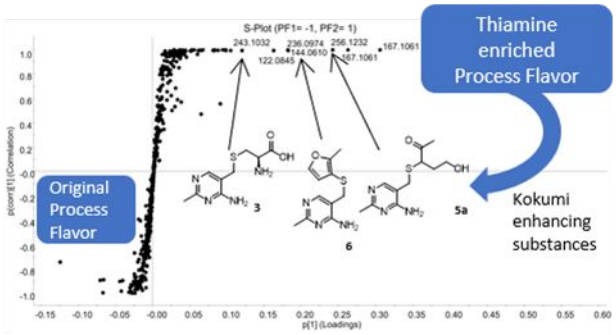

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