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Formation of Amines and Aldehydes from Parent Amino Acids during Thermal Processing of Cocoa and Model Systems: New Insights into Pathways of the Strecker Reaction

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A method based on a derivatization with dansyl chloride and LC-MS-MS determination was developed for the quantitation of 2-methylbutyl-, 3-methylbutyl-, 2-phenylethyl-, 3-(methylthio)propyl-, and 2-methylpropylamine. Its application on unfermented, fermented, and roasted cocoas from Ghana and Sulawesi revealed an increase of all amines, except the 3-(methylthio)propylamine, during cocoa fermentation, suggesting an enzymic formation from the parent amino acids isoleucine, leucine, phenylalanine, and valine. However, a much more pronounced formation of most of the amines was measured after roasting of the cocoa, leading to concentrations in the milligrams per kilogram range. This result suggested a new "thermogenic" formation pathway of "biogenic amines". A comparison of the amounts of the amines and the aldehydes in roasted cocoa revealed similar concentrations, for example, for 2- and 3-methylbutanal and the respective amines, whereas the amounts of 2-phenylethylamine were much higher as compared to the amounts of phenylacetaldehyde. Strecker-type model systems, in which each parent amino acid was reacted with 2-oxopropanal, revealed the formation of both the amine and the aldehyde; however, in contrast to cocoa, the concentrations of the aldehydes were always much higher as compared to the amines. The results showed for the first time the thermally induced generation of "biogenic amines" from amino acids. Possible reasons for the different ratios of amines versus aldehydes formed during the roasting of cocoa or the model systems, respectively, are discussed.

KEYWORDS: Biogenic amines; odor thresholds; cocoa; Strecker reaction; 2-phenylethylamine; 2-methylpropylamine; 2-methylbutylamine; 3-methylbutylamine

INTRODUCTION

Amines generated in foods by an enzymic decarboxylation of free amino acids are usually assigned as "biogenic amines". Because, in particular, microorganisms frequently use this pathway of amino acid metabolization, biogenic amines mainly occur in foods, which undergo a microbial fermentation during processing, such as cheese, wine, dry sausage, or cocoa. Furthermore, in some foods, such as fish (*1*), an increased level of biogenic amines is also correlated with food spoilage. Besides being considered as quality indicators of foods, earlier studies have also focused on their quantitation in foods because of their influence on the human physiology, for example, causing migraine attacks (*2*, *3*). The formation of biogenic amines is known to involve decarboxylases, which initiate either a decarboxylation or the elimination of a proton from the respective amino acid by a Schiff base formation with pyridoxal-5-phosphate as the cosubstrate. From the respective intermediate either the biogenic amine or an α-keto acid can be formed depending on the metabolic pathway (**Figure 1**).

Most studies reporting on the occurrence of biogenic amines in cocoa or chocolate, respectively, preferentially report on the amounts of tyramine and 2-phenylethylamine (*2*−*7*). However, in particular, literature data on the latter amine in cocoa are quite contradictory, because concentrations between 1.8 mg/kg (*7*) and 22.0 mg/kg (*6*) have been reported, whereas others (*3*) were not even able to detect this amine in cocoa. One reason for these differences might be the fact that, in particular, the isolation of such primary amines from complex food matrices is not an easy task, and usually a derivatization procedure has to be applied to enable HPLC or GC determinations (*2*, *6*, *7*).

Besides the amines, amino acid related aldehydes, such as 3-methylbutanal and phenylacetaldehyde, have also been reported to occur in roasted cocoa; for example, concentrations between 20 and 60 mg/kg have been found for 3-methylbutanal (*8*). These aldehydes, also bearing a part of the amino acid structure, are formed in foods by a Strecker-type reaction via thermally generated intermediates (*9*).

However, whereas many data are available on the occurrence and formation of such Strecker aldehydes in foods, little is

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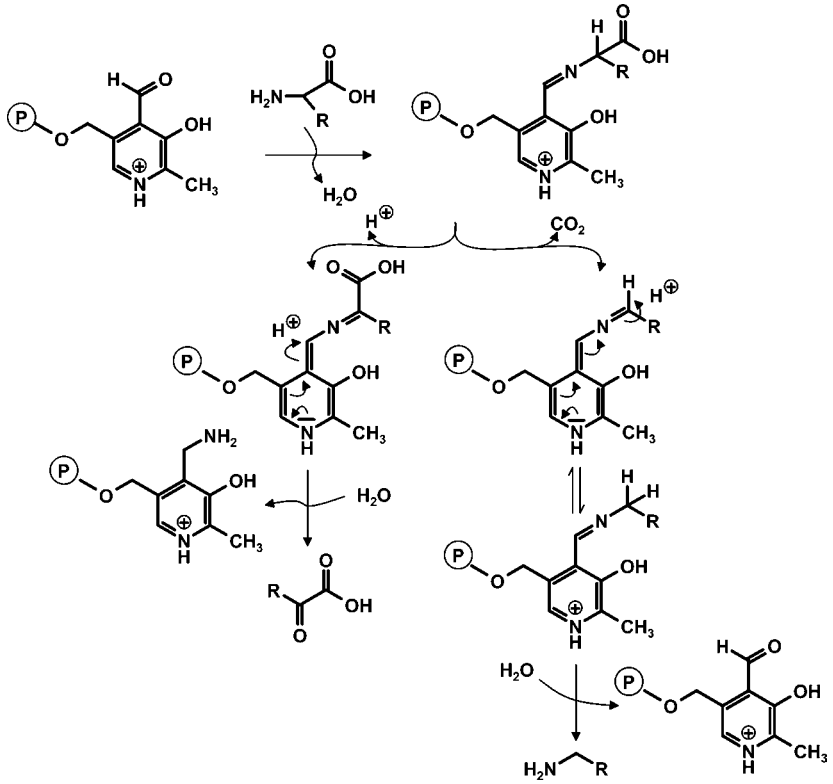

Figure 1. Formation of biogenic amines by decarboxylases as catalyzed by pyridoxal-5-phosphate.

known about the amounts of the respective amines and, in particular, their formation and aroma contribution.

In a recent investigation on acrylamide formation (*10*), we have proposed a reaction pathway leading to the formation of 3-aminopropionamide by a Strecker-type degradation of the amino acid asparagine. Because 3-aminopropionamide, the biogenic amine of this amino acid, was shown to be formed in significant amounts by a thermal degradation of asparagine in a model reaction, also other "biogenic amines", such as 2-phenylethylamine, might be formed in foods by a Strecker-type reaction.

However, except for 2-phenylethylamine and tyramine, no data are available in the literature on the occurrence of further amino acid derived amines in cocoa, or foods in general, such as 2-methylpropylamine, 2-methylbutylamine, 3-methylbutylamine, or 3-(methylthio)propylamine, which might be formed by a Strecker degradation of the parent amino acid valine, isoleucine, leucine, or methionine according to the pathway recently proposed by us for asparagine (*10*).

The purpose of the following investigation was, therefore, first, to develop an LC-MS-MS method for the quantitation of the above-mentioned amines in cocoa and, second, to shed more light on their formation during cocoa processing by comparing the concentrations of both the aldehydes and the amines in unfermented and fermented cocoa beans before and after roasting. Furthermore, model studies were performed to compare the amounts of the amino acid derived aldehydes and amines formed from the single parent amino acid in model reactions with 2-oxopropanal.

MATERIALS AND METHODS

Cocoa Beans. On-site trials on cocoa bean fermentation were performed in Sulawesi (SU) and Ghana (GH). To get unfermented beans (UF), the fruits were harvested, then washed to remove the pulp, and immediately sun-dried. These samples were designated UF-SU and UF-GH. Aliquots of the same batch of beans were fermented for 7 days and, finally, sun-dried. These samples were designated 7F-SU and 7F-GH. Aliquots of these four cocoa bean samples were roasted in a pilot plant roaster (Barth) and designated UF-R-SU, UF-R-GH, 7F-R-SU, and 7F-R-GH, respectively. Roasting was performed as follows: The cocoa nibs were kept for 15 min at 95 °C, and then the temperature was raised within 20 min to 115 °C at a rate of 1 °C/min. The nibs were cooled to room temperature and frozen at −20 °C prior to analysis. Supervision of the trial and the roasting process was done by a scientist of a chocolate manufacturer.

Chemicals. [$^2H_4$]-2-Phenylethylamine and [$^2H_9$]-*n*-butylamine hydrochloride were purchased from Dr. Ehrenstorfer (Augsburg, Germany). 2-Oxopropanal (methylglyoxal), 5-(dimethylamino)-1-naphthalene sulfonyl chloride (dansyl chloride), 2-methylpropylamine, 2-methylbutylamine, 3-methylbutylamine, 2-phenylethylamine, 3-(methylthio)-propylamine, L-isoleucine, L-leucine, L-methionine, and L-phenylalanine were from Aldrich (Sigma-Aldrich, Steinheim, Germany). The isotopically labeled internal standards [$^2H_2$]-2-methylbutanal (*11*), [$^2H_2$]-3-methylbutanal (*12*), [$^2H_3$]-3-(methylthio)propanal (*13*), and [$^{13}C_2$]-phenylacetaldehyde (*14*) were synthesized according to the literature cited in parentheses.

Quantitation of Amines. *Preparation of the Reference Derivatives.* 2-Methylpropylamine, 2-methylbutylamine, 3-methylbutylamine, 2-phenylethylamine, and 3-(methylthio)propylamine and the two internal standards [$^2H_4$]-2-phenylethylamine and [$^2H_9$]-*n*-butylamine (150 nmol each) were singly dissolved in aqueous sodium hydrogencarbonate (20 mL; 0.2 mol/L), and the pH was adjusted to 10 ± 0.2 using aqueous sodium hydroxide (1 mol/L). Dansyl chloride (10 μmol dissolved in 5 mL of acetone) was added, and the mixture was purged with argon for 30 s, stirred overnight at room temperature in the dark, and subsequently extracted with dichloromethane (three times; total volume = 45 mL). In preliminary studies, a total time of 3 h was found to be sufficient for complete derivatization. The organic phases were combined, dried over anhydrous sodium sulfate, and, finally, evaporated to dryness at about 20 kPa and 35 °C. The residue was taken up in acetonitrile (2.0 mL), the suspension was filtered (0.45 μm; Spartan 13/0.45RC) (Schleicher & Schuell, Dassel, Germany), and an aliquot (200 μL) of the solution was made up to 1.0 mL with aqueous formic acid (0.1% by volume) for LC-MS measurements.

HPLC-MS-MS. Mass spectra were recorded by means of a triple-

Table 1. Mass Spectrometric Conditions Used for Quantitation of the Amines

amine	collision energy (V)	first transition *m/z* (mother ion) to *m/z* (daughter ion)	collision energy (V)	second transition *m/z* (mother ion) to *m/z* (daughter ion)	RF[a]
[$^2H_9$]-*n*-butylamine[b]	46	316–156	33	316–158	
[$^2H_4$]-2-phenylethylamine[b]	43	359–156	32	359–158	
2-methylpropylamine	43	307–156	32	307–157	0.54
2-methylbutylamine	43	321–156	32	321–157	0.80
3-methylbutylamine	49	321–156	34	321–157	0.90
2-phenylethylamine	45	355–156	33	355–157	0.92[c]
3-(methylthio)propylamine	50	339–156	41	339–157	4.90

[a] Response factors versus [$^2H_9$]-*n*-butylamine. [b] Internal standards. [c] Response factor versus [$^2H_4$]-2-phenylethylamine.

quadrupole tandem mass spectrometer (TSQ Quantum Discovery; Thermo Electron, Dreieich, Germany) coupled to a Surveyor high-performance liquid chromatography (HPLC) system (Thermo Finnigan, Dreieich, Germany) equipped with a thermostated (20 °C) autosampler and a 150 × 2.0 mm i.d., 4 μm Synergi Polar RP 80 Å HPLC column (Phenomenex, Aschaffenburg, Germany) kept at 30 °C and connected to a 4 × 2.0 mm i.d. Polar RP precolumn (Phenomenex). The sample (10 μL) was separated at a flow rate of 0.2 mL/min. The solvent system was composed of (A) formic acid in water (0.1%, w/v) and (B) formic acid in acetonitrile (0.1%, w/v). A linear gradient was applied by increasing the concentration of B from 50 to 100% within 15 min. The mass spectrometer was operated in the positive electrospray ionization mode (ESI^+) with a spray needle voltage of 3.5 kV and a spray current of 5 μA. The temperature of the capillary was 300 °C, and the capillary voltage was 35 V. The sheath and auxiliary gas (nitrogen) were adjusted to 40 and 10 arbitrary units, respectively. The collision cell was operated at a collision gas (argon) pressure of 0.13 Pa.

The derivatives were first characterized by means of their molecular masses obtained in the full scan mode. The following molecular ions were obtained as base peaks ($M^+ + 1$) for the dansylated amines: *m/z* 355 for 2-phenylethylamine; *m/z* 359 for [$^2H_4$]-2-phenylethylamine; *m/z* 321 for 2-methyl- and 3-methylbutylamine; *m/z* 307 for 2-methylpropylamine; *m/z* 339 for 3-(methylthio)propylamine; and *m/z* 316 for [$^2H_9$]-*n*-butylamine. These were then subjected to MS-MS. First, the most intense transitions of the precursor ions (collision energy = 35 V) were determined, and, second, the yields of the product ions were optimized by performing a series of runs with different collision energies and flow rates of the sheath and auxiliary gas. The mass transitions with the highest intensities from both the internal standard and the analyte were used for quantitation (**Table 1**). On both mass filter quadrupoles, resolution settings were 0.7 fwhm, the scan time for each transition and single reaction monitoring was 0.20 s, and the scan width was 0.6 amu.

Single reaction monitoring (SRM) using the first ion transitions given in **Table 1** for each amine was then applied on seven mixtures of the analyte and the internal standard in defined concentrations (molar ratios 10+1 to 1+10), and the response factor was calculated from the results obtained as described recently (*15*). [$^2H_4$]-2-Phenylethylamine was used for the quantitation of 2-phenylethylamine. All other amines were quantified using [$^2H_9$]-*n*-butylamine as the internal standard. This labeled amine was used because cocoa itself contained the unlabeled butylamine as well as other alkylamines (data not shown).

Quantitation of Amines in Cocoa. To cocoa beans powdered in liquid nitrogen by means of a laboratory mill (type A 10) (Jahnke & Kunkel, IKA-Labortechnik, Staufen, Germany) were added tap water (50 mL) and defined amounts of both labeled amines (0.1−15 μg; depending on the amounts of the analytes determined in a preliminary experiment). For equilibration, the sample was stirred for 60 s, then homogenized using an Ultraturrax (Jahnke & Kunkel, IKA-Labortechnik) for 90 s, and ultrasonified for a further 2 min. After precipitation of the proteins with 2.5 mL of an aqueous solution of $K_4[Fe(CN)_6]\cdot 3H_2O$ (15 wt %), followed by 2.5 mL of an aqueous solution of $Zn(CH_3COO)_2\cdot 2H_2O$ (23 wt %), the suspension was centrifuged (15000 rpm) for 10 min at 10 °C. The sample (10 °C) was filtered and extracted with *n*-hexane (10 mL). To an aliquot of the aqueous phase (40 mL) was added sodium hydrogencarbonate (0.5 mol/L, 40 mL), and the pH was adjusted to 10 ± 0.2 using sodium hydroxide (2.5 mol/L). After the addition of dansyl chloride in acetone (10.8 mg in 20 mL), the reaction mixture was stirred for 3 h at room temperature in the dark. The solution was then extracted four times with dichloromethane (total volume = 100 mL), the organic phases were combined, centrifuged (4000 rpm, 5 min at 10 °C) to separate the water, and finally dried over anhydrous sodium sulfate. The solvent was removed at about 20 kPa and 35 °C, and the residue was dissolved in a mixture of acetonitrile and aqueous formic acid (0.1%; 0.35 mL; 30:70, v/v). After filtration (0.45 μm; Spartan 13/0.45RC) (Schleicher & Schuell), the sample was analyzed by LC-MS-MS as described above.

A control run without the addition of [$^2H_4$]-2-phenylethylamine and [$^2H_9$]-*n*-butylamine hydrochloride was performed to ensure the absence of the respective mass fragments in the cocoa extract.

Quantitation of Aldehydes in Cocoa. Cocoa beans (20 g) were frozen in liquid nitrogen and then ground using a laboratory mill. The powder (10 g) was stirred with diethyl ether (100 mL) for 1 h, and the three isotopically labeled aldehydes, [$^2H_2$]-2-methylbutanal, [$^2H_2$]-3-methylbutanal, and [$^{13}C_2$]-2-phenylacetaldehyde, were added. After 1 h of vigorous stirring, the mixture was filtered and the filtrate dried over anhydrous sodium sulfate. The volatile fraction was separated from the nonvolatile material using the solvent-assisted flavor evaporation (SAFE) distillation method (*16*), and the distillate extract was concentrated to 1 mL using a Vigreux column (60 × 1 cm).

For mass spectral analysis, a Trace 2000 series gas chromatograph (Thermo Finnigan, Egelsbach, Germany) equipped with an MCSS system (moving capillary stream switching) was coupled to a CP 3800 gas chromatograph (Varian, Darmstadt, Germany) and an ion trap detector Saturn 2000 (Varian) running in the chemical ionization mode (70 eV ionization energy) with methanol as reagent gas. The distillate was separated on a 30 m × 0.32 mm, film thickness = 0.25 μm, FFAP column (J&W Scientific, Folsom, CA) in the first dimension, and mass traces of the respective odorant and the labeled internal standard were recorded after transfer of the analyte and the respective internal standard on the second column (*14*). The results were corrected using calibration factors determined by the analysis of three mixtures containing known amounts of the analyte and the isotopically labeled standard (*12*).

Quantitation of Free Amino Acids in Cocoa. Aqueous buffer (20 mL; see below) containing the internal standard norleucine (30−500 μg, depending on the amounts of the analytes determined in a preliminary experiment) was added to powdered, defatted cocoa beans (2 g), and the suspension was homogenized with an Ultraturrax for 3 min. After centrifugation (10000 rpm, 30 min at 20 °C), the supernatant was filtered (0.45 μm; Spartan 13/0.45RC) and diluted 1:10 with a buffer solution prepared as follows: Lithium acetate dihydrate (16.3 g), formic acid (7.5 mL), aqueous thiodiglycol (20 mL; 25%), and octanoic acid (0.1 mL) were dissolved in water (900 mL), and the pH was adjusted to 2.20 with trifluoroacetic acid and made up to 1000 mL with water. The amino acid concentrations were analyzed by means of an amino acid analyzer LC 3000 (Onken, Gründau, Germany). For calibration, defined mixtures of the five amino acids and norleucine were analyzed (molar ratios 3:1 to 1:3).

Model Systems. *Model A.* The amino acids L-isoleucine, L-leucine, L-methionine, and L-phenylalanine (50 μmol) were singly reacted with 2-oxopropanal (50 μmol) by homogenizing both reactants with silica gel (3 g, silica gel 60, 0.063−0.200 mm) (VWR International, Darmstadt, Germany) (containing 10% of water) and heating at 170 °C in closed glass vessels. After the reaction times given in the tables, water (15 mL), the respective labeled amine, and the labeled aldehyde were added. The samples were stirred for 10 min for equilibration. After ultrasonification (4 min), the samples were centrifuged at 4000 rpm for 10 min at 10 °C.

Model B. The four amino acids (50 μmol each) were singly dissolved in phosphate buffer (0.1 mol/L; pH 7.0; 10 mL) and were heated at 170 °C in closed glass vessels after the addition of 2-oxopropanal (50 μmol). After cooling, the respective labeled internal standards were added, and the sample was stirred for 10 min for equilibration.

Quantitation of Amines. After the addition of sodium hydrogencarbonate (20 mL; 0.25 mol/L) and dansyl chloride (8.1 mg in 10 mL of acetone) to an aliquot of the reaction mixture (5 mL), the solution was stirred for 3 h at room temperature in the dark. After extraction four times with dichloromethane (total volume = 100 mL), the organic phases were combined, centrifuged for 5 min at 4000 rpm (10 °C) to separate the water, and finally dried over anhydrous sodium sulfate. The solvent was removed at about 20 kPa and 35 °C, and the residue was dissolved in a mixture of acetonitrile and aqueous formic acid (0.1%; 3+7, v/v). After filtration (0.45 μm; Spartan 13/0.45RC), the sample was analyzed by LC-MS-MS as described above.

Quantitation of Aldehydes. An aliquot of the reaction mixture (5 mL) was applied onto an Extrelut NT 20 column (VWR International) and, after equilibration for 15 min, elution of the analyte and the internal standard was performed using *n*-pentane/diethyl ether (100 mL; 9:1, v/v). The solution was dried over anhydrous sodium sulfate and concentrated to ~10 mL at a Vigreux column (60 × 1 cm) at 40 °C. This extract was directly used for GC-MS analysis.

Determination of Odor Qualities and Odor Thresholds. A defined amount of each amine was singly dissolved in 100 μL of ethanol, and either tap water or sunflower oil, respectively, was added (1 L). After stirring for 2 h, these stock solutions were diluted stepwise with tap water or sunflower oil (1:1, v/v), respectively. Odor thresholds were determined by means of the triangle test as described recently (*17*). Odor description was done by the sensory panel using a solution of the corresponding amine in a concentration adjusted to 10-fold above the odor threshold.

RESULTS AND DISCUSSION

Method Development for Amine Quantitation. As indicated in the Introduction, surprisingly little is known, in particular, on the occurrence and, also, the aroma properties of certain amino acid related amines in foods, such as 2-methylpropylamine or 2- and 3-methylbutylamine. According to our observations, only low yields of such amines can be obtained by the extraction/distillation processes commonly used in volatile analysis and, furthermore, the free amines, if at all, are eluted as very broad peaks from GC capillary columns. To overcome such analytical problems, the free amines are usually derivatized before GC (*7*) or HPLC analysis (*18*−*20*). However, for this reason, the aroma contributions of amines cannot directly be determined by GC-O and, thus, these might have been overlooked in foods so far.

On the basis of a method previously developed by us for the quantitation of 3-aminopropionamide (*15*), a new procedure for the quantitation of 2-methylpropyl-, 2- and 3-methylbutyl-, 3-(methylthio)propyl-, and 2-phenylethylamine was developed. These amines were selected because the corresponding aldehydes are well-known, potent food odorants.

First, the derivatives were synthesized by reacting the amines singly with dansyl chloride. Most of the derivatives showed a clear molecular ion as well as a *m*/*z* 156 fragment (**Table 1**), probably caused by the *N*-methylnaphthyl ion. To optimize the intensities of the mother and daughter ions, the collision energies of the first and second transitions were then adjusted to the optimized values given in **Table 1**. With these data at hand, seven mixtures containing different concentrations of both isotopically labeled internal standards ([$^2H_4$]-2-phenylethylamine and [$^2H_9$]-*n*-butylamine) as well as the five analytes were analyzed. On the basis of the calibration curves obtained, the MS response factors given in **Table 1** were calculated. Because the derivatives of 2-methylbutyl- and 3-methylbutylamine could not be separated on the HPLC stationary phase, their response factors were determined in two separate experiments. The limit of quantitation (LoQ) was estimated to be 0.2 μg/kg on the basis of a correlation between the intensity of the respective ions and the background noise.

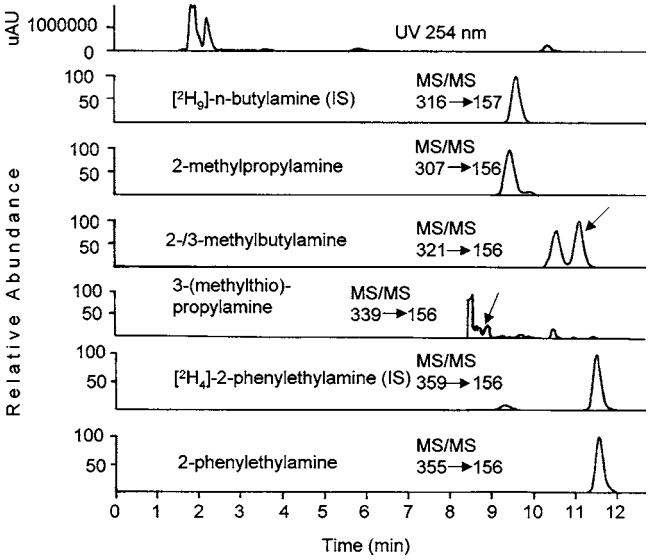

Figure 2. Mass chromatograms obtained by dansylation and LC-MS-MS of an extract prepared from fermented, roasted cocoa. IS, internal standard. UV trace is shown for comparison.

Table 2. Influence of Processing and Origin on the Concentrations of Four Amines in Cocoa Beans from Sulawesi (SU) and Ghana (GH)

	SU		GH	
amine in sample[a]	concn (μg/kg)	RSD[b] (%)	concn (μg/kg)	RSD[b] (%)
2-phenylethylamine				
UF	371	8.5	227	14.4
7F	723	17.7	1168	2.7
UF-R	779	8.2	624	6.7
7F-R	7746	3.4	10216	1.9
2-methylpropylamine				
UF	57	10.5	56	15.6
7F	191	22.9	331	16.1
UF-R	369	2.0	303	5.0
7F-R	1988	4.5	2793	3.1
2- and 3-methylbutylamine				
UF	256	20.3	129	6.1
7F	498	26.6	1219	5.2
UF-R	914	8.6	966	10.9
7F-R	9865	15.1	17070	6.0

[a] For sample assignment see Materials and Methods. [b] Relative standard deviation based on the analysis of triplicates.

Identification and Quantitation of Amines in Cocoa. The following studies were aimed at identifying the respective amines in cocoa, determining their concentrations in different cocoa samples, and at elucidating the influence of the cocoa fermentation process on amine formation. In **Figure 2**, chromatograms obtained by dansylation and LC-MS-MS of an extract prepared from fermented, roasted cocoa (7F-R-GH) are displayed. By comparing the respective mass spectra, 2-methylpropyl- and 2-phenylethylamine as well as a mixture of 2- and 3-methylbutylamine could be identified. 3-(Methylthio)-propylamine was not detectable.

The quantitative results obtained for the four amines in the eight different samples analyzed are summarized in **Table 2**. The amounts of 2- and 3-methylbutylamine are given as sum, because their derivatives could not be separated by HPLC. Interestingly, all amines were already present in the unfermented, dried cocoa beans (UF) from Ghana as well as from Sulawesi. As expected, the amounts increased after 7 days of fermentation (7F) and, for example, the amounts of 2-phenylethylamine

Table 3. Aroma Properties of Selected Amines

amine	odor quality	odor threshold (mg/L) water	oil
2-methylpropyl	fishy, amine-like, malty	8.0	48.3
2-methylbutyl	fishy, amine-like, malty	4.9	69.7
3-methylbutyl	fishy, amine-like, malty	3.2	13.7
2-phenylethyl	fishy, amine-like, honey-like	55.6	89.7
3-(methylthio)-propyl	fishy, amine-like, cooked potato	0.4	0.3

increased by a factor of 2 in the Sulawesian sample and by a factor of 5 in the Ghanian sample. This increase can undoubtedly be attributed to the decarboxylase activity in the beans forming the respective "biogenic amine" as proposed in **Figure 1**.

Already, roasting of the unfermented samples (UF-R-SU and UF-R-GH) led to an increase in the amounts of 2-phenylethylamine, thereby indicating that biogenic amines might also be formed from precursors during thermal cocoa processing. This observation was clearly corroborated by the data obtained for the fermented and then roasted cocoa beans, because the thermal treatment led to a significant increase by a factor of ~10 as compared to the fermented sample (**Table 2**), yielding 7.7 mg (7F-R-SU) or 10.2 mg (7F-R-GH), respectively, of 2-phenylethylamine per kilogram. These results clearly indicated that 2-phenylethylamine is more effectively formed in cocoa beans by a thermally induced reaction rather than by a biochemical formation. However, these data also propose that the fermentation process generates a yet unknown precursor, which, after roasting, yields the amine.

The quantitation of 2-methylpropylamine and 2- and 3-methylbutylamine (**Table 2**) in the same eight cocoa samples indicated a similar result with respect to amine formation: All amines were already present in the unfermented, dried cocoa samples (UF) from both origins and increased during the 7 day fermentation process by factors of 2 (7F-SU) to 10 (7F-GH), respectively. Such differences are possibly caused by the different activities of the decarboxylases present in the respective beans. However, as already found for 2-phenylethylamine, the most significant increases were observed in the beans fermented for 7 days and roasted, leading to maximum concentrations of 2.8 mg/kg 2-methylpropylamine (7F-R-GH) and 17 mg/kg 2- and 3-methylbutylamine in Ghanian cocoa beans (7F-R-GH; **Table 2**).

As compared to the fermented samples, the roasting process was always much more effective in amine generation than the fermentation. However, a biochemical formation of an amine precursor was always needed to receive high yields of the amine, because roasting of the unfermented samples generally yielded much lower amounts (**Table 2**). Therefore, the term "thermogenic amine" might be more appropriate than "biogenic amine" to better assign the source of such amines in cocoa or, probably, foods in general. 3-(Methylthio)propylamine could not be detected in either unfermented or fermented cocoa samples (data not shown). Thus, its concentration was presumably below the limit of detection of the method (0.1 μg/kg).

Odor Activity Values (OAVs). OAVs (ratio of concentration to odor threshold) are a useful tool to reveal whether a certain odorant might contribute to a given aroma (*21*). To reveal whether any of the amines present might contribute to cocoa aroma, the odor qualities and odor thresholds of the five amines under consideration were determined by a sensory panel. As shown in **Table 3**, solutions of all amines in tap water as well as in sunflower oil elicited a fishy, amine-like aroma, and the odor quality elicited by the respective aldehyde was used as an additional attribute to describe the overall aroma impression.

To calculate the OAVs, first, the odor thresholds of the five amines were determined in water or oil, respectively. Among the five compounds, 3-(methylthio)propylamine showed the lowest odor threshold in oil as well as in water. The highest odor threshold was determined for 2-phenylethylamine in oil (**Table 3**). Applying the odor activity concept on the concentrations of the amines present in the 7 days fermented and roasted samples (7F-R; **Table 2**), in which the highest concentrations of the respective amines were determined, suggested a rather low contribution of the amines to the aroma of cocoa, because only 2- and 3-methylbutylamine were present in concentrations above their odor thresholds in water and, thus, might contribute to the overall cocoa aroma. However, further studies involving aroma reconstitution of the overall cocoa aroma and omission experiments (*21*) would be necessary to evaluate the influence of the amines on the cocoa aroma. In addition, it must be kept in mind that the odor thresholds of amines significantly depend on the pH of the food matrix.

Nevertheless, the aliphatic amines might also be responsible for other effects on the human physiology as previously reported for 2-phenylethylamine (*2*).

On the basis of our previous findings indicating that 3-aminopropionamide, the biogenic amine of asparagine, is formed in significant amounts by a thermally induced reaction in the presence of carbohydrates or α-dicarbonyl compounds, respectively (*10*), the results obtained so far suggest that also the amines considered in this study might be formed by a Strecker-type reaction as exemplified for L-phenylalanine in **Figure 3**. After the formation of an imine by a reaction of the amino acid with an α-dicarbonyl compound, followed by a decarboxylation, the hydrolysis of tautomer **1** should yield the Strecker aldehyde, whereas hydrolysis of tautomer **2** should yield 2-phenylethylamine. To test this hypothesis, two series of further experiments were performed: First, the respective Strecker aldehydes were quantified in the cocoa samples, and their concentrations were compared to those of the amines. Second, model experiments simulating the Strecker reaction were performed to get a first idea of the concentration ratio of aldehydes and amines suggested to be thermally generated from the same parent amino acid.

Quantitation of Aldehydes in Cocoa. Quantitation of 3-methylbutanal in the eight cocoa samples (**Table 4**) revealed that the Strecker aldehyde was already present in the unfermented, dried cocoa beans from Sulawesi (UF-SU) as well as from Ghana (UF-GH). Surprisingly, already during fermentation of either the Sulawesian or the Ghanian cocoa, the concentrations of 3-methylbutanal significantly increased by factors of nearly 20 or 14, respectively (**Table 4**), suggesting that also a biochemical formation of the "Strecker" aldehyde might be possible.

Roasting of the unfermented samples yielded only small amounts of the aldehyde, whereas roasting of the fermented samples resulted in high amounts of 6.8 mg/kg (7F-R-SU) or 8.5 mg/kg (7F-R-GH) of 3-methylbutanal, respectively, suggesting that the fermentation is also needed in the formation of precursors for 3-methylbutanal generation.

A similar behavior was found for 2-methylbutanal, which was also highest in the fermented, roasted cocoa beans (7F-R; **Table 4**). Therefore, as found for the respective amines (**Table 2**), the cocoa fermentation is also an important process in supplying precursors for Strecker aldehyde formation. A comparison of

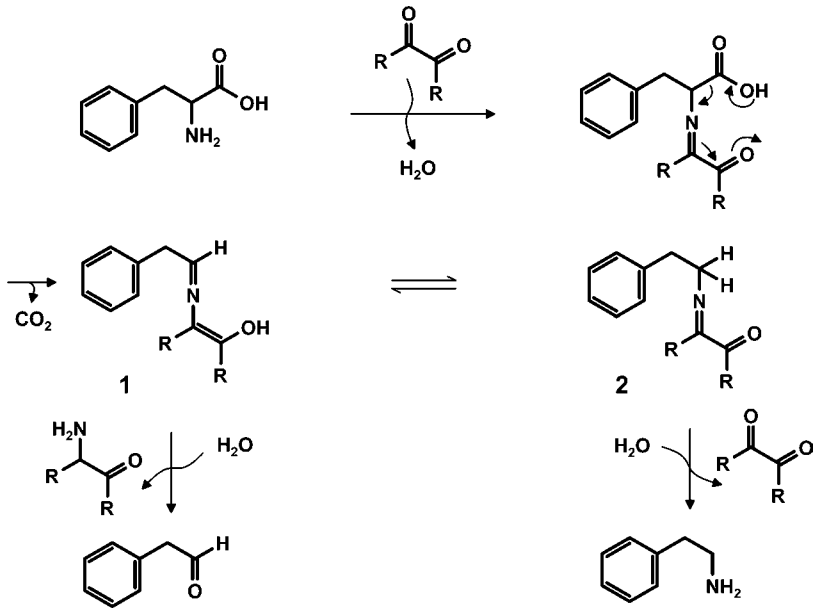

Figure 3. Pathway of the Strecker degradation of L-phenylalanine leading to phenylacetaldehyde or 2-phenylethylamine.

Table 4. Influence of Processing and Origin on the Concentrations of Three Strecker Aldehydes in Cocoa Beans from Sulawesi (SU) and Ghana (GH)

	SU		GH	
aldehyde in sample[a]	concn (μg/kg)	RSD[b] (%)	concn (μg/kg)	RSD[b] (%)
3-methylbutanal				
UF	58	21.1	116	9.5
7F	1152	2.5	1636	2.4
UF-R	565	5.0	511	20.2
7F-R	6841	2.4	8470	1.2
2- methylbutanal				
UF	109	29.9	143	6.7
7F	1132	14.9	2075	4.3
UF-R	665	7.0	504	24.0
7F-R	5718	8.5	3791	1.2
phenylacetaldehyde				
UF	42	2.4	16	2.2
7F	73	1.2	34	1.7
UF-R	43	0.5	91	1.2
7F-R	63	6.3	202	1.5

[a] For sample assignment see Materials and Methods. [b] Relative standard deviation calculated on the basis of the analysis of triplicates.

the sum of the amounts of 2- and 3-methylbutanal with the sum of 2- and 3-methylbutylamine (compare **Tables 4** and **2**) revealed that the sum of both the aldehydes and the amines in the fermented and roasted cocoa were on the same order of magnitude, thus corroborating the idea of their formation from the same parent amino acid as suggested in **Figure 3**.

The quantitation of phenylacetaldehyde in the eight cocoa samples, however, gave quite unexpected results: Although already low amounts of the aldehyde were present in the unfermented, dried cocoa samples (UF; **Table 4**), neither the fermentation nor the roasting process nor a combination of both led to concentrations of the aldehyde in the same concentration range as found for 2-phenylethylamine (compare **Tables 4** and **2**). Because high amounts of the amine were formed during the roasting of the fermented cocoa beans (**Table 2**), and assuming the Strecker reaction to be involved in its formation, obviously the routes of the degradation pathway (**Figure 3**) are significantly affected by the amino acid structure.

Table 5. Changes in the Concentrations of Five Free Amino Acids during Fermentation and Roasting of Cocoa Beans from Sulawesi (SU) and Ghana (GH)

	concn[a] (mg/kg)							
	SU				GH			
amino acid	UF	7F	UF-R	7F-R	UF	7F	UF-R	7F-R
L-phenylalanine	66	1100	83	700	190	1120	120	700
L-valine	95	510	120	470	190	600	130	460
L-leucine	81	1100	97	800	170	1240	100	760
L-isoleucine	87	320	100	290	140	390	100	280
L-methionine	9	190	9	nd[b]	15	220	15	87

[a] Mean values of triplicates. [b] Not detectable.

To clarify the influence of the fermentation and roasting steps on the concentrations of free amino acids in the cocoa samples, the concentrations of the free amino acids under consideration (**Table 5**) were measured. A clear increase of the amino acids during the 7 days of fermentation of cocoa samples from both origins was observed, and, in particular, L-phenylalanine and L-leucine showed the largest amounts after fermentation. Roasting of the beans then reduced the concentrations of the amino acids. The latter result was in good agreement with earlier data of Reineccius et al. (*22*). However, because no differences were observed between the degradation rate of L-phenylalanine and L-leucine during roasting, a lack in L-phenylalanine can be excluded as a reason for the low amounts of phenylacetaldehyde formed during the roasting of both cocoa samples.

Quantitation of Amines and Aldehydes in Model Systems. Because foods consist of a complex mixture of different compounds, model studies using binary mixtures of possible precursors are a useful tool to get first insights into possible routes of volatile generation. Therefore, in another series of experiments, the amino acids L-phenylalanine, L-isoleucine, L-leucine, and L-methionine were singly reacted in the presence of 2-oxopropanal to simulate Strecker-type reaction conditions. The amounts of the respective aldehyde and the amine, formed under either low water conditions (model A) or aqueous conditions (model B), were monitored with increasing reaction time. However, it should be mentioned that the reaction conditions and the reaction temperature used were not meant

Table 6. Amounts of 3-Methylbutanal and 3-Methylbutylamine Formed from L-Leucine in the Presence of 2-Oxopropanal

	3-methylbutanal				3-methylbutylamine			
	model A		model B		model A		model B	
reaction time (min)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)
2.5	27.7	10.2	18.0	22.5	0.02	18.1	0.02	18.2
5	36.8	14.1	28.5	2.2	0.04	2.6	0.03	22.6
10	57.6	12.4	48.7	10.7	0.20	9.2	0.08	9.4
20	149.4	6.5	64.6	2.6	0.93	8.4	0.12	24.3
30	178.8	4.5	84.6	3.3	3.44	1.8	0.26	14.4

[a] Relative standard deviation calculated on the basis of triplicates.

Table 7. Amounts of 2-Methylbutanal and 2-Methylbutylamine Formed from L-Isoleucine in the Presence of 2-Oxopropanal

	2-methylbutanal				2-methylbutylamine			
	model A		model B		model A		model B	
reaction time (min)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)
2.5	67.8	15.2	31.8	12.7	0.04	21.6	0.02	7.4
5	78.0	12.2	41.2	9.6	0.12	31.8	0.03	1.2
10	142.5	14.1	68.5	19.9	0.40	31.3	0.05	22.6
20	294.9	2.4	117.6	3.1	1.44	21.9	0.15	15.0
30	402.0	4.1	122.1	11.2	2.43	6.6	0.13	12.9

[a] Relative standard deviation calculated on the basis of triplicates.

Table 8. Amounts of Phenylacetaldehyde and 2-Phenylethylamine Formed from L-Phenylalanine in the Presence of 2-Oxopropanal

	phenylacetaldehyde				2-phenylethylamine			
	model A		model B		model A		model B	
reaction time (min)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)
2.5	19.4	13.4	9.0	4.7	0.28	30.2	0.02	5.9
5	23.2	0.5	22.8	5.4	0.51	16.6	0.09	2.7
10	28.4	2.7	43.6	1.7	1.95	14.8	0.26	4.8
20	26.4	3.5	63.0	5.9	6.53	6.9	0.54	7.4
30	26.2	8.8	72.8	3.2	6.13	11.2	0.78	15.4

[a] Relative standard deviation calculated on the basis of triplicates.

to simulate the roasting conditions of cocoa. The general purpose was to study whether the amines under consideration are unequivocally formed in a Strecker-type reaction.

Reacting L-leucine with 2-oxopropanal under low water conditions (model A, **Table 6**) yielded remarkable amounts of 3-methylbutanal after only 2.5 min. The amounts of the aldehyde then increased constantly with time, yielding ~17.9 mol % after 30 min. The generation of 3-methylbutylamine followed the same time course, but its concentrations were always much lower as compared to the aldehyde, amounting to only ~0.3 mol % after 30 min. Performing the same reaction under aqueous conditions (model B, **Table 6**) lowered the yields of both the aldehyde and the amine as compared to model A, but the amine formation was much more reduced as compared to the aldehyde. Nevertheless, the results showed for the first time that 3-methylbutylamine is formed in a Strecker-type degradation of L-leucine.

When L-leucine was replaced by L-isoleucine in both model reactions (**Table 7**), similar results were observed: Also a clear formation of 2-methylbutanal and 2-methylbutylamine was observed with time, but, for instance, after 30 min, the formation of 2-methylbutylamine was much lower in both model systems.

Next, L-phenylalanine was reacted with 2-oxopropanal, and the respective aldehyde and the amine were quantified along with the reaction time (**Table 8**). As compared to L-leucine and L-isoleucine, L-phenylalanine showed a clearly different behavior: In particular, at increased reaction times, significant amounts of 2-phenylethylamine were formed, reaching ~0.6 mol % after 30 min. Although the formation of phenylacetaldehyde was still favored, the ratio of aldehyde to amine finally reached a value of 4:1 in model A.

In the last experiment of this series, L-methionine was reacted with 2-oxopropanal. The highest amount of 3-(methylthio)propanal was obtained after 10 min in model A, but obviously this aldehyde was degraded with increasing reaction time, because the amounts present after 30 min were much lower as compared to those measured after 10 min (**Table 9**). By contrast, the formation of 3-(methylthio)propylamine was favored with increasing reaction time, and no such transient maximum was observed. Thus, the amine reached a concentration of ~0.5 mol % after 30 min (model A).

In summary, the data obtained for the four amino acids in the model experiments clearly revealed that the degradation of amino acids in Strecker-type reactions leads to the formation of the respective "Strecker amines" as suggested in **Figure 3**, yielding amounts of 0.2−0.6 mol %. However, under the

Table 9. Amounts of 3-(Methylthio)propanal and 3-(Methylthio)propylamine Formed from L-Methionine in the Presence of 2-Oxopropanal

	3-(methylthio)propanal				3-(methylthio)propylamine			
	model A		model B		model A		model B	
reaction time (min)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)	concn (mmol/mol)	RSD[a] (%)
2.5	15.7	5.4	9.1	23.2	0.02	27.6	0.01	37.4
5	21.6	13.4	15.3	3.1	0.08	12.3	0.09	11.1
10	33.3	10.7	19.3	1.0	0.51	20.9	0.19	10.6
20	19.6	8.7	25.3	9.9	2.25	0.4	0.45	13.1
30	12.6	15.4	22.5	2.1	4.71	18.9	0.72	18.8

[a] Relative standard deviation calculated on the basis of triplicates.

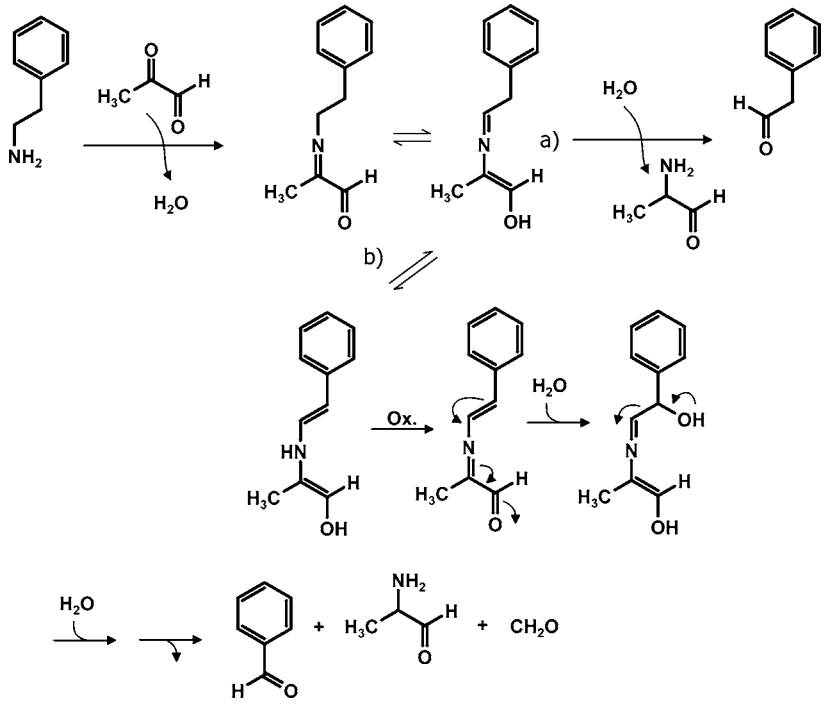

Figure 4. Hypothetical formation pathway leading to phenylacetaldehyde and benzaldehyde in a reaction of 2-phenylethylamine with 2-oxopropanal.

reaction conditions considered in the model studies, the formation of the respective Strecker aldehyde was always clearly favored, in particular from L-leucine and L-isoleucine.

Assuming that the different reaction conditions used in cocoa roasting and the model experiments did not much influence the general reaction pathway, a comparison of the results obtained in the model experiments with the data obtained for the cocoa samples revealed a clear difference. Although roasting of the cocoa beans led to nearly identical concentrations of 2- and 3-methylbutylamine and the respective Strecker aldehydes 2- and 3-methylbutanal and 2-phenylethylamine was even higher in cocoa as compared to the amounts of phenylacetaldehyde, in the model studies, the aldehyde formation was always favored.

One explanation for this difference might be the presence of the quite high concentrations of the α-dicarbonyl compound 2-oxopropanal in the model system. It may be assumed that in the model system, the amine is formed as suggested in **Figure 3**, but may then immediately be trapped as a Schiff base by 2-oxopropanal as exemplified for 2-phenylethylamine in **Figure 4**. After formation of the Schiff base, a tautomerization (route a in **Figure 4**) followed by hydrolysis may generate phenylacetaldehyde. This reaction, thus, would further promote the aldehyde formation.

To test whether the reaction between 2-phenylethylamine and 2-oxopropanal yields phenylacetaldehyde, a model reaction was performed using the reaction conditions of model A. About 0.8 mol % of phenylacetaldehyde was generated (data not shown), which can easily be explained by route a shown in **Figure 4**.

Unexpectedly, however, 7.1 mol % of benzaldehyde was generated as the main reaction product from 2-phenylethylamine and 2-oxopropanal (data not shown). A hypothesis for the formation pathway leading to benzaldehyde is given in **Figure 4** (route b). An imine−enamine tautomerization, followed by an oxidation of the enaminol structure formed, may lead to an intermediate, which, after hydrolysis, may undergo an aza-vinylogous retro-Aldol reaction to finally generate benzaldehyde and formaldehyde. Also, this reaction, which has not yet been reported for benzaldehyde formation in foods, may explain the lower amounts of 2-phenylethylamine formed in the model systems as compared to phenylacetaldehyde. Furthermore, in an earlier study Rizzi (*23*) has shown that certain aldimines formed in a reaction of furfural and leucine were quite resistant to hydrolysis. This might be another reason for the lower amounts of the amine as compared to the aldehydes.

As an explanation for the generally much higher amounts of amines in cocoa as compared to the model systems, it might be assumed that during fermentation of cocoa yet unknown precursors are formed from the amino acids, which cannot be

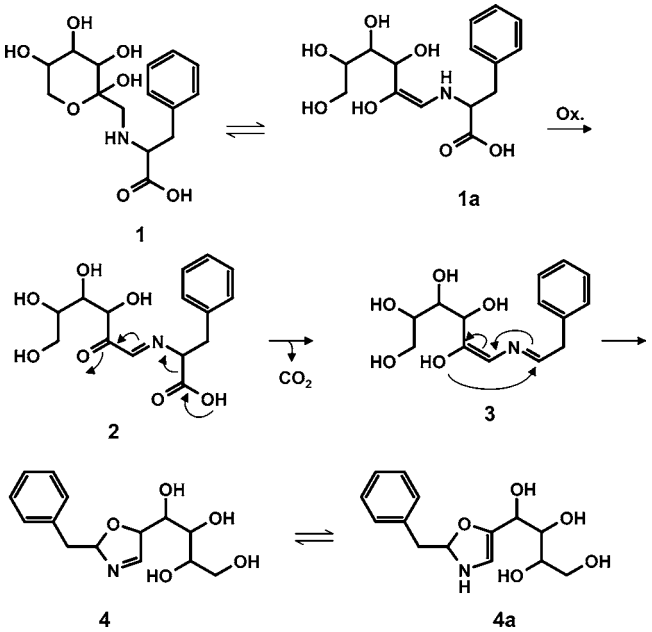

Figure 5. Formation of two oxazoline derivatives (**4** and **4a**) from the Amadori reaction product (**1**) by oxidation, decarboxylation, and ring closure.

formed, for example, from 2-oxopropanal. Cocoa beans contain rather low amounts of water, because this is more or less completely lost during roasting. It can, thus, be assumed that the Strecker degradation in water-free systems may follow a different route. Because it is known that fermented cocoa already contains quite high amounts of Amadori reaction products (*24*), their degradation during roasting might occur without the intermediate formation of "free" α-dicarbonyls as exemplified for the degradation of the Amadori product of L-phenylalanine (**1** in **Figure 5**). Ring opening of the cyclic Amadori compound followed by an oxidation may result in an oxidized compound (**2** in **Figure 5**), which will easily undergo a decarboxylation. Evidence for the importance of such oxidation steps in the Strecker reaction have recently been shown by us in the generation of "Strecker acids" (*9*, *25*). If water is limited, intermediate **3** might undergo a cyclization into the respective 3-oxazoline **4**. A differently substituted 3-oxazoline has earlier been identified in model reactions of 2,3-butanedione and valine (*26*). Compound **4** (**Figure 5**) might exist in two tautomeric forms, and it can be assumed that, upon heating and depending on the position of the double bond in the oxazoline ring, such intermediates are able to release either the aldehyde or the amine. However, because the side chains of such oxazolines might vary significantly depending on whether their formation has started from a carbohydrate or an α-dicarbonyl compound, their identification in foods, such as cocoa, is a challenge. Nevertheless, attempts to prove this hypothesis based on the respective synthesized intermediates are in progress.

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