JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 5419−5428 **5419**

Isolation, Structure Determination, Synthesis, and Sensory Activity of *N*-Phenylpropenoyl-L-amino Acids from Cocoa (*Theobroma cacao*)

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Application of chromatographic separation and taste dilution analyses recently revealed besides procyanidins a series of *N*-phenylpropenoyl amino acids as the key contributors to the astringent taste of nonfermented cocoa beans as well as roasted cocoa nibs. Because these amides have as yet not been reported as key taste compounds, this paper presents the isolation, structure determination, and sensory activity of these amino acid amides. Besides the previously reported (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (clovamide), (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (deoxyclovamide), and (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine, seven additional amides, namely, (+)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid, (+)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid, (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid, (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid, (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine, (+)-*N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-aspartic acid, and (+)-*N*-[(*E*)-cinnamoyl]-L-aspartic acid, were identified for the first time in cocoa products by means of LC-MS/MS, 1D/2D-NMR, UV−vis, CD spectroscopy, and polarimetry, as well as independent enantiopure synthesis. Using the recently developed half-tongue test, human recognition thresholds for the astringent and mouth-drying oral sensation were determined to be between 26 and 220 μmol/L (water) depending on the amino acid moiety. In addition, exposure to light rapidly converted these [*E*]-configured *N*-phenylpropenoyl amino acids into the corresponding [*Z*]-isomers, thus indicating that analysis of these compounds in food and plant materials needs to be performed very carefully in the absence of light to prevent artifact formation.

KEYWORDS: Cocoa; astringency; taste dilution analysis; (+)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid; (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid; clovamide; deoxyclovamide; half-tongue test

INTRODUCTION

Besides the attractive aroma, the sensory quality of the fermented and roasted seeds of *Theobroma cacao* is driven by its desirable bitterness and its slight sour taste as well as its typical astringent mouthfeel, which is perceived as a long-lasting puckering, shrinking, and drying sensation in the oral cavity and can enhance the complexity and palate length of cocoa beverages and chocolate confectionary.

To answer the question as to which nonvolatile, key taste compounds are responsible for the typical taste of roasted cocoa, we recently applied the so-called taste dilution analysis (*1*) on fractions isolated from ground roasted cocoa nibs by means of gel permeation chromatography (*2*). This bioassay-guided fractionation led to the detection of a series of monomeric and oligomeric procyanidins, flavonol and flavanon glycosides, and the *N*-phenylpropenoyl amino acids **1**−**10** (**Figure 1**) as the key players imparting the astringent oral taste sensation during consumption of cocoa products (*2*). Because these *N*-phenylpropenoyl amino acids have as yet not been reported as key taste compounds in foods, the details on their isolation, structure determination, stereochemistry, and sensory activity are presented.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: Amberlyst 15 (H^+, 20−50 mesh) and (dimethylamino)pyridine (Fluka, Neu-Ulm, Germany); acetic anhydride, (*E*)-4-hydroxycinnamic acid, thionyl chloride, (*E*)-4-hydroxy-3-methoxycinnamic acid, and (*E*)-cinnamic acid (Sigma-Aldrich, Steinheim, Germany); and (*E*)-3,4-dihydroxycinnamic acid, potassium carbonate, L-aspartic acid, D-aspartic acid, L-dopa, L-glutamic acid, L-tyrosine, sodium sulfate, pyridine, and tetrahydrofuran (Merck, Darmstadt, Germany). Solvents were of HPLC

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10.1021/jf050458q CCC: $30.25
Published on Web 06/02/2005

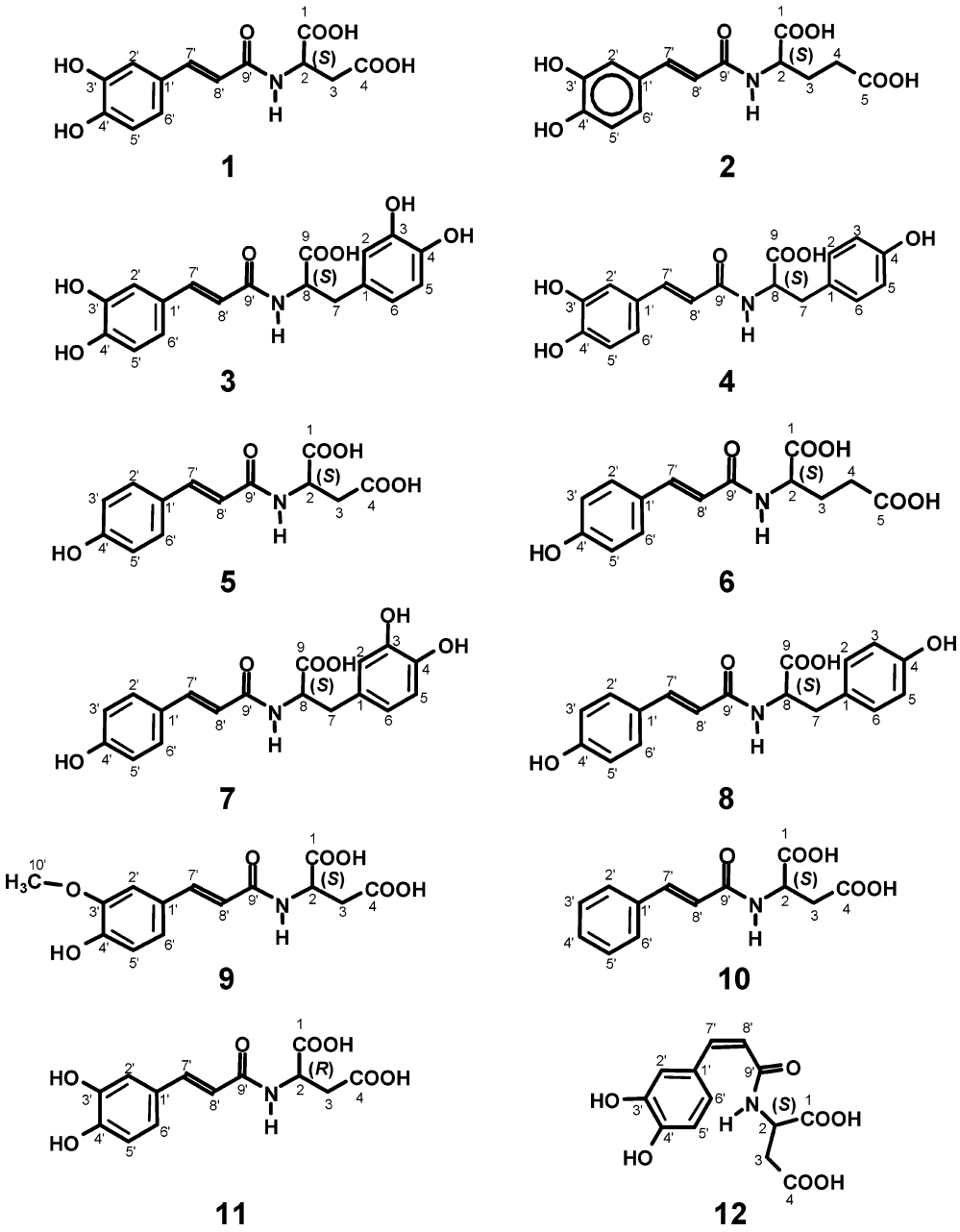

Figure 1. Structures of *N*-phenylpropenoyl amino acids **1**–**12**.

grade (Merck). Deuterated solvents were obtained from Euriso-Top (Gif-Sur-Yvette, France). Bottled water (Vittel; low mineralization, 405 mg/L) adjusted to pH 6.0 with aqueous hydrochloric acid (0.1 mol/L) was used for sensory evaluation. Nonfermented, washed cocoa beans were collected in Sulawesi.

Sensory Analyses. *Training of the Sensory Panel.* Twelve subjects (five women and seven men, ages 25−38 years) with no history of known taste disorders were trained to evaluate the taste of aqueous solutions (3 mL each) of the following standard taste compounds by using a triangle test as described in the literature (*3*): sucrose (50 mmol/L) for sweet taste; lactic acid (20 mmol/L) for sour taste; NaCl (20 mmol/L) for salty taste; caffeine (1 mmol/L) for bitter taste; sodium glutamate (3 mmol/L) for umami taste; and gallustannic acid (0.05%) for astringency. The assessors had participated earlier at regular intervals for at least two years in sensory experiments and were, therefore, familiar with the techniques applied. Sensory analyses were performed in a sensory panel room at 22−25 °C in three different sessions.

Pretreatment of Fractions. Prior to sensory analysis, the fractions or compounds isolated were suspended in water, and, after removal of the volatiles in high vacuum (<5 mPa), were freeze-dried twice. GC-MS and ion chromatographic analysis revealed that food fractions treated by that procedure are essentially free of the solvents and buffer compounds used.

Half-Tongue Test. Taste dilution factors as well as human astringency recognition thresholds were determined by means of the recently developed half-tongue test (*4*) using bottled water as the solvent. Serial 1:1 dilutions of the samples were presented in order of increasing concentrations to a trained panel of 12 persons in three different sessions using the sip-and-spit method. When the panelist selected correctly, the same concentration was presented again besides one blank as a proof for the correctness of the data. The geometric mean of the last and the second last concentration was calculated and taken as the individual recognition threshold. The values between individuals and between five separate sessions differed by not more than plus or minus one dilution step; that is, a threshold value of 26 μmol/L for (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine represents a range from 13 to 52 μmol/L.

Syntheses of *N*-Phenylpropenoyl-L-amino Acids, 1−11 (Figure 1). Acetic anhydride (25 mmol) was added dropwise to a solution of (*E*)-caffeic acid, (*E*)-*p*-coumaric acid, or (*E*)-ferulic acid (10 mmol each), respectively, in pyridine (5 mL) and (dimethylamino)pyridine (0.25 mmol) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C

and, then, poured onto crushed ice. The aqueous phase was acidified to pH 2.0 with aqueous hydrochloric acid (2 mol/L) and extracted with a mixture of ethyl acetate and tetrahydrofuran (3:1, v/v; 3 × 50 mL), and the combined organic extracts were dried over Na_2SO_4, filtered, and freed from solvent in a vacuum, affording a colorless, amorphous powder. An aliquot (1.0 mmol) of the acetylated phenylpropenoic acid derivative obtained was mixed with thionyl chloride (1.0 mmol) and was then heated under reflux until no further formation of hydrogen chloride was observable. After cooling to room temperature, the reaction mixture was dried under a stream of nitrogen, and either L-aspartic acid, D-aspartic acid, L-glutamic acid, L-dihydroxyphenylalanine, or L-tyrosine (each 1.0 mmol) dissolved in dry tetrahydrofuran (30 mL) was added. After this solution had been stirred for up to 72 h at room temperature, the solvent was evaporated in a vacuum, and the residue was dissolved in a solution of K_2CO_3 (0.25 mol/L; 20 mL) in water/methanol (1:1; v/v) and stirred at room temperature. After 20 min, Amberlyst 15 ion-exchange resin was added with stirring until a pH value of 3.5 was reached. The resin was then filtered off and washed with water (20 mL), and the filtrate was freed from methanol in a vacuum and, finally, freeze-dried. The residue was taken up in water (3 mL) and applied onto the top of a water-cooled glass column (40 × 140 mm) filled with a slurry of LiChroprep 25−40 μm RP-18 material (Merck) in aqueous formic acid (0.1% in water; pH 2.5). Chromatography was performed using aqueous formic acid (0.1% in water; pH 2.5) as the effluent, followed by aqueous formic acid (0.1% in water; pH 2.5) containing increasing amounts of methanol. The target compounds were detected by monitoring the effluent at 300 nm, and the fractions containing these compounds were confirmed by RP-HPLC-DAD. After the individual fractions had been freeze-dried three times, the corresponding *N*-phenylpropenoyl-L-amino acids were obtained as white, amorphous powders in high purities of >99%.

(+)-N-[3′,4′-Dihydroxy-(E)-cinnamoyl]-L-aspartic acid ***1*** *(0.33 mmol; 33% yield) (**Figure 1**):* $[\alpha]_D^{20}$ +9.3° (in H_2O); CD (in MeOH), $\lambda_{ext}(\Delta\epsilon)$ = 237 nm (−2.53); UV_{max}, 207, 285, 309 nm; LC-MS (ESI^+), *m/z* 613 (100, $[2M + Na]^+$), 296 (95, $[M + 1]^+$), 591 (55, $[2M + 1]^+$), 924 (52, $[3M + K]^+$), 908 (46, $[3M + Na]^+$), 629 (38, $[2M + K]^+$), 318 (25, [M + Na), 163 (21, $[M - 132]^+$); ^{1}H NMR (400 MHz, DMSO-d_6), δ 2.62 [dd, 1H, *J* = 7.0, 16.3 Hz, H−C(3a)], 2.73 [dd, 1H, *J* = 5.9, 16.3 Hz, H−C(3b)], 4.63 [m, 1H, H−C(2)], 6.44 [d, 1H, *J* = 15.7 Hz, H−C(8′)], 6.75 [d, 1H, *J* = 8.2 Hz, H−C(5′)], 6.85 [dd, 1H, *J* = 1.8, 8.2 Hz, H−C(6′)], 6.95 [d, 1H, *J* = 1.8 Hz, H−C(2′)], 7.25 [d, 1H, *J* = 15.7 Hz, H−C(7′)], 8.29 [d, 1H, *J* = 8.2 Hz, H−N], 9.16 [s, 1H, HO−C(3′)], 9.41 [s, 1H, HO−C(4′)], 12.55 [s, 2H, HOOC-(1, 4)]; ^{13}C NMR (100 MHz, DMSO-d_6), δ 36.7 [C-3], 48.9 [C-2], 114.1 [C-2′], 116.0 [C-5′], 118.2 [C-8′], 120.8 [C-6′], 126.5 [C-1′], 140.1 [C-7′], 145.7 [C-3′], 147.7 [C-4′], 165.4 [C-9′], 172.0 [C-4], 172.8 [C-1].

(−)-N-[3′,4′-Dihydroxy-(E)-cinnamoyl]-L-glutamic acid ***2*** *(0.50 mmol; 50% yield) (**Figure 1**):* $[\alpha]_D^{20}$ −5.9° (H_2O); UV_{max}, 207, 285, 309 nm; LC-MS (ESI^+), *m/z* 619 (100, $[2M + 1]^+$), 310 (35, $[M + 1]^+$), 163 (5, $[M - 146]^+$); ^{1}H NMR (400 MHz, DMSO-d_6), δ 1.82 [m, 1H, H−C(3a)], 2.01 [m, 1H, H−C(3b)], 2.30 [m, 2H, H−C(4)], 4.33 [m, 1H, H−C(2)], 6.41 [d, 1H, *J* = 15.7 Hz, H−C(8′)], 6.75 [d, 1H, *J* = 8.2 Hz, H−C(5′)], 6.85 [dd, 1H, *J* = 2.0, 8.2 Hz, H−C(6′)], 6.95 [d, 1H, *J* = 2.0 Hz, H−C(2′)], 7.25 [d, 1H, *J* = 15.7 Hz, H−C(7′)], 8.20 [d, 1H, *J* = 7.9 Hz, H−N], 9.10 [s, 1H, HO−C(3′)], 9.33 [s, 1H, HO−C(4′)], 12.37 [s, 2H, HOOC(1, 5)]; ^{13}C NMR (100 MHz, DMSO-d_6), δ 26.7 [C-3], 30.2 [C-4], 51.4 [C-2], 114.1 [C-2′], 115.9 [C-5′], 118.1 [C-8′], 120.6 [C-6′], 126.4 [C-1′], 140.0 [C-7′], 145.7 [C-3′], 147.6 [C-4′], 165.6 [C-9′], 173.2 [C-5], 173.5 [C-1].

(−)-N-[3′,4′-Dihydroxy-(E)-cinnamoyl]-3-hydroxy-L-tyrosine ***3*** *(0.32 mmol; 32% yield) (**Figure 1**):* $[\alpha]_D^{20}$ −13.0° (in MeOH); UV_{max}, 225, 255, 297 nm; LC-MS (ESI^+), *m/z* 719 (100, $[2M + 1]^+$), 360 (45, $[M + 1]^+$), 163 (25, $[M - 196]^+$); ^{1}H NMR (400 MHz, DMSO-d_6), δ 2.72 [dd, 1H, *J* = 8.9, 13.6 Hz, H−C(7a)], 2.91 [dd, 1H, *J* = 4.6, 13.6 Hz, H−C(7b)], 4.42 [m, 1H, H−C(8)], 6.42 [d, 1H, *J* = 15.7 Hz, H−C(8′)], 6.47 [dd, 1H, *J* = 1.8, 8.2 Hz, H−C(6)], 6.60 [d, 1H, *J* = 8.2 Hz, H−C(5)], 6.62 [d, 1H, *J* = 1.8 Hz, H−C(2)], 6.74 [d, 1H, *J* = 8.2 Hz, H−C(5′)], 6.82 [dd, 1H, *J* = 1.8, 8.2 Hz, H−C(6′)], 6.94 [d, 1H, *J* = 1.8 Hz, H−C(2′)], 7.19 [d, 1H, *J* = 15.7 Hz, H−C(7′)], 8.14 [d, 1H, *J* = 7.9 Hz, H−N], 8.63, 8.68, 9.09, 9.32 [4 × s, 4 × 1H, HO−C(3, 4,3′,4′)], 12.56 [s, 1H, HOOC(9)]; ^{13}C NMR (100 MHz, DMSO-d_6), δ 35.0 [C-7], 54.3 [C-8], 114.1 [C-2′], 115.5 [C-5], 116.0 [C-5′], 116.7 [C-2], 118.4 [C-8′], 120.0 [C-6], 120.6 [C-6′], 126.5 [C-1′], 128.7 [C-1], 139.7 [C-7′], 144.0 [C-4], 145.1 [C-3], 145.7 [C-3′], 147.6 [C-4′], 165.4 [C-9′], 173.7 [C-9].

(−)-N-[3′,4′-Dihydroxy-(E)-cinnamoyl]-L-tyrosine ***4*** *(0.40 mmol; 40% tield) (**Figure 1**):* $[\alpha]_D^{20}$ −35.6° (in MeOH); UV_{max}, 225, 255, 297 nm; LC-MS (ESI^+), *m/z* 687 (100, $[2M + 1]^+$), 344 (27, $[M + 1]^+$), 163 (10, $[M - 180]^+$); ^{1}H NMR (400 MHz, DMSO-d_6), δ 2.78 [dd, 1H, *J* = 9.2, 13.9 Hz, H−C(7a)], 2.98 [dd, 1H, *J* = 4.8, 13.9 Hz, H−C(7b)], 4.48 [m, 1H, H−C(8)], 6.41 [d, 1H, *J* = 15.7 Hz, H−C(8′)], 6.65 [d, 2H, *J* = 8.5 Hz, H−C(3, 5)], 6.74 [d, 1H, *J* = 8.1 Hz, H−C(5′)], 6.82 [dd, 1H, *J* = 2.0, 8.2 Hz, H−C(6′)], 6.94 [d, 1H, *J* = 2.0 Hz, H−C(2′)], 7.03 [d, 2H, *J* = 8.5 Hz, H−C(2, 6)], 7.20 [d, 1H, *J* = 15.7 Hz, H−C(7′)], 7.82 [d, 1H, *J* = 8.1 Hz, H−N], 9.10, 9.16, 9.32 [3s, 3 × 1H, HO], 12.60 [s, 1H, HOOC(9)]; ^{13}C NMR (100 MHz, DMSO-d_6), δ 36.4 [C-7], 54.2 [C-8], 114.1 [C-2′], 115.2 [C-3, 5], 115.9 [C-5′], 118.2 [C-8′], 120.7 [C-6′], 126.5 [C-1′], 127.9 [C-1], 130.2 [C-2, 6], 139.8 [C-7′], 145.7 [C-3′], 147.6 [C-4′], 156.1 [C-4], 165.5 [C-9′], 173.5 [C-9].

(+)-N-[4′-Hydroxy-(E)-cinnamoyl]-L-aspartic acid ***5*** *(0.45 mmol; 45% yield) (**Figure 1**):* $[\alpha]_D^{20}$ +1.2° (in MeOH); UV_{max}, 207, 285, 309 nm; LC-MS (ESI^+), *m/z* 280 (100, $[M + 1]^+$), 559 (70, $[2M + 1]^+$), 597 (50, $[2M + K]^+$), 147 (26, $[M - 132]^+$); ^{1}H NMR (400 MHz, DMSO-d_6), δ 2.65 [dd, 1H, *J* = 7.0, 16.4 Hz, H−C(3a)], 2.73 [dd, 1H, *J* = 5.7, 16.4 Hz, H−C(3b)], 4.64 [m, 1H, H−C(2)], 6.52 [d, 1H, *J* = 15.7 Hz, H−C(8′)], 6.79 [d, 2H, *J* = 8.6 Hz, H−C(3′, 5′)], 7.34 [d, 1H, *J* = 15.7 Hz, H−C(7′)], 7.40 [d, 2H, *J* = 8.6 Hz, H−C(2′, 6′)], 8.27 [d, 1H, *J* = 8.2 Hz, H−N], 9.85 [s, 1H, HO−C(4′)], 12.62 [s, 2H, HOO−C(1, 4)]; ^{13}C NMR (100 MHz, DMSO-d_6), δ 36.5 [C-3], 49.0 [C-2], 116.0 [C-3′, 5′], 118.3 [C-8′], 126.0 [C-1′], 129.5 [C-2′, 6′], 139.7 [C-7′], 159.2 [C-4′], 165.4 [C-9′], 171.9 [C-4], 172.7 [C-1].

(−)-N-[4′-Hydroxy-(E)-cinnamoyl]-L-glutamic acid ***6*** *(0.55 mmol; 55% yield) (**Figure 1**):* $[\alpha]_D^{20}$ −6.2° (in MeOH); UV_{max}, 207, 285, 309 nm; LC-MS (ESI^+), *m/z* 918 (100, $[3M + K]^+$), 903 (75, $[3M + Na]^+$), 294 (55, $[M + 1]^+$), 587 (35, $[2M + 1]^+$), 147 (19, $[M - 146]^+$); ^{1}H NMR (400 MHz, DMSO-d_6), δ 1.84 [m, 1H, H−C(3a)], 1.98 [m, 1H, H−C(3b)], 2.32 [m, 2H, H−C(4)], 4.34 [m, 1H, H−C(2)], 6.50 [d, 1H, *J* = 15.7 Hz, H−C(8′)], 6.80 [d, 2H, *J* = 8.6 Hz, H−C(3′,5′)], 7.34 [d, 1H, *J* = 15.7 Hz, H−C(7′)], 7.40 [d, 2H, *J* = 8.6 Hz, H−C(2′,6′)], 8.23 [s, 1H, H−N], 9.83 [s, 1H, HO−C(4′)], 12.64 [s, 2H, HOO−C(1, 5)]; ^{13}C NMR (100 MHz, DMSO-d_6), δ 26.5 [C-3], 30.0 [C-4], 51.2 [C-2], 115.7 [C-3′,5′], 118.0 [C-8′], 125.7 [C-1′], 129.2 [C-2′,6′], 139.3 [C-7′], 158.9 [C-4′], 165.4 [C-9′], 173.3 [C-5], 173.5 [C-1].

(−)-N-[4′-Hydroxy-(E)-cinnamoyl]-3-hydroxy-L-tyrosine ***7*** *(0.30 mmol; 30% yield) (**Figure 1**):* $[\alpha]_D^{20}$ −24.2° (in MeOH); UV_{max}, 225, 255, 297 nm; LC-MS (ESI^+), *m/z* 1395 (100, $[4M + Na]^+$), 1068 (85, $[3M + K]^+$), 725 (46, $[2M + K]^+$), 1411 (44, $[4M + K]^+$), 147 (23, $[M - 196]^+$), 344 (15, $[M + 1]^+$); ^{1}H NMR (400 MHz, DMSO-d_6), δ 2.73 [dd, 1H, *J* = 8.8, 13.8 Hz, H−C(7a)], 2.91 [dd, 1H, *J* = 4.8, 13.8 Hz, H−C(7b)], 4.40 [m, 1H, H−C(8)], 6.46 [dd, 1H, *J* = 2.0, 8.0 Hz, H−C(6)], 6.50 [d, 1H, *J* = 15.7 Hz, H−C(8′)], 6.59 [d, 1H, *J* = 8.0 Hz, H−C(5)], 6.61 [d, 1H, *J* = 2.0 Hz, H−C(2)], 6.78 [d, 2H, *J* = 8.6 Hz, H−C(3′,5′)], 7.27 [d, 1H, *J* = 15.7 Hz, H−C(7′)], 7.38 [d, 2H, *J* = 8.6 Hz, H−C(2′,6′)], 8.08 [d, 1H, *J* = 8.0 Hz, H−N]; ^{13}C NMR (100 MHz, DMSO-d_6), δ 36.4 [C-7], 54.1 [C-8], 115.2 [C-5], 115.6 [C-3′,5′], 116.4 [C-2], 119.0 [C-8′], 119.7 [C-6], 125.7 [C-1′], 128.5 [C-1], 129.1 [C-2′,6′], 138.9 [C-7′], 143.7 [C-4], 144.8 [C-3], 158.8 [C-4′], 165.0 [C-9′], 173.3 [C-9].

(−)-N-[4′-Hydroxy-(E)-cinnamoyl]-L-tyrosine ***8*** *(0.55 mmol; 55% yield) (**Figure 1**):* $[\alpha]_D^{20}$ −26.9° (in MeOH); UV_{max}, 225, 255, 297 nm; LC-MS (ESI^+), *m/z* 677 (100, $[2M + Na]^+$), 693 (51, $[2M + K]^+$), 1020 (38, $[3M + K]^+$), 1004 (32, $[3M + Na]^+$), 147 (30, $[M - 180]^+$), 328 (25, $[M + 1]^+$), 350 (24, $[M + Na]^+$); ^{1}H NMR (400 MHz, DMSO-d_6), δ 2.81 [dd, 1H, *J* = 9.3, 13.9 Hz, H−C(7a)], 3.00 [dd, 1H, *J* = 4.7, 13.9 Hz, H−C(7b)], 4.49 [m, 1H, H−C(8)], 6.50 [d, 1H, *J* = 15.7 Hz, H−C(8′)], 6.66 [d, 2H, *J* = 8.3 Hz, H−C(3,5)], 6.79 [d, 2H, *J* = 8.4 Hz, H−C(3′,5′)], 7.03 [d, 2H, *J* = 8.3 Hz, H−C(2, 6)], 7.30 [d, 1H, *J* = 15.7 Hz, H−C(7′)], 7.38 [d, 2H, *J* = 8.4 Hz, H−C(2′,6′), 8.16 [d, 1H, *J* = 8.0 Hz, H−N]; ^{13}C NMR (100 MHz, DMSO-d_6), δ

36.4 [C-7], 54.2 [C-8], 115.2 [C-3,5], 116.0 [C-3′,5′], 118.4 [C-8′], 126.0 [C-1′], 127.9 [C-1], 129.5 [C-2′,6′], 130.2 [C-2,6], 139.5 [C-7′], 156.1 [C-4], 159.1 [C-4′], 165.6 [C-9′], 173.5 [C-9].

(+)-N-[4′-Hydroxy-3′-methoxy-(E)-cinnamoyl]-L-aspartic acid ***9*** *(0.4 mmol; 40% yield) (**Figure 1**):* $[\alpha]_D^{20}$ +10.9° (in H_2O); UV_{max}, 219, 303 nm; LC-MS (ESI^+), *m/z* 310 (100, $[M + 1]^+$), 619 (75, $[2M + 1]^+$), 177 (62, $[M - 132]^+$); ^1H NMR (400 MHz, DMSO-d_6), δ 2.65 [dd, 1H, J = 7.0, 16.6 Hz, H−C(3a)], 2.74 [dd, 1H, J = 5.7, 16.6 Hz, H−C(3b)], 3.81 [s, 3H, H−C(10′)], 4.65 [m, 1H, H−C(2)], 6.58 [d, 1H, J = 15.7 Hz, H−C(8′)], 6.79 [d, 1H, J = 8.1 Hz, H−C(5′)], 7.00 [dd, 1H, J = 1.8, 8.1 Hz, H−C(6′)], 7.16 [d, 1H, J = 1.8 Hz, H−C(2′)], 7.34 [d, 1H, J = 15.7 Hz, H−C(7′)], 8.21 [d, 1H, J = 7.9 Hz, H−N], 9.44 [s, 1H, HO−C(4′)], 12.65 [s, 2H, HOO−C(1,4)]; ^{13}C NMR (100 MHz, DMSO-d_6), δ 36.5 [C-3], 48.9 [C-2], 55.7 [C-10′], 110.9 [C-2′], 115.8 [C-5′], 118.7 [C-8′], 122.0 [C-6′], 126.5 [C-1′], 139.9 [C-7′], 148.0 [C-3′], 148.6 [C-4′], 165.6 [C-9′], 172.0 [C-4], 172.7 [C-1].

(+)-N-(E)-Cinnamoyl-L-aspartic acid ***10*** *(0.55 mmol; 55% yield) (**Figure 1**):* $[\alpha]_D^{20}$ +1.7° (in MeOH); UV_{max}, 207, 261 nm; LC-MS (ESI^+), *m/z* 264 (100, $[M + 1]^+$), 527 (65, $[2M + 1]^+$), 549 (20, $[2M + 23]^+$), 131 (20, $[M - 132]^+$); ^1H NMR (400 MHz, DMSO-d_6), δ 2.67 [dd, 1H, J = 7.0, 16.5 Hz, H−C(3a)], 2.76 [dd, 1H, J = 5.5, 16.5 Hz, H−C(3b)], 4.67 [m, 1H, H−C(2)], 6.75 [d, 1H, J = 15.8 Hz, H−C(8′)], 7.38−7.44 [m, 3H, H−C(3′,5′,4′)], 7.44 [d, 1H, J = 15.8 Hz, H−C(7′)], 7.57 [d, 2H, J = 8.1 Hz, H−C(2′,6′)], 8.39 [d, 1H, H−N], 12.58 [s, 2H, HOO−C(1, 4)]; ^{13}C NMR (100 MHz, DMSO-d_6), δ 36.3 [C-3], 48.9 [C-2], 121.9 [C-8′], 127.8 [C-2′,6′], 129.1 [C-3′,5′], 129.8 [C-4′], 135.0 [C-1′], 139.5 [C-7′], 165.0 [C-9′], 171.9 [C-4], 172.6 [C-1].

(−)-N-[3′,4′-Dihydroxy-(E)-cinnamoyl]-D-aspartic acid ***11*** *(0.33 mmol; 33% yield) (**Figure 1**):* $[\alpha]_D^{20}$ −9.3° (in H_2O); $[\alpha]_D^{20}$ −6.0° (in MeOH); CD (in MeOH), $\lambda_{ext}(\Delta\epsilon)$ = 238 nm (+1.68); UV, LC-MS, and NMR data were identical to those obtained for the corresponding (+)-L-enantiomer **1**.

Solvent Extraction of Cocoa Beans. Washed beans (100 g) were peeled by hand, frozen in liquid nitrogen, crushed in a grinding mill, and then extracted with *n*-pentane (5 × 300 mL) at room temperature for 30 min. After centrifugation, the residual cocoa material was then extracted five times with a mixture (7:3, v/v; 300 mL each) of acetone and water for 45 min at room temperature with stirring. After filtration, the liquid layer was freed from acetone under reduced pressure at 30 °C, and the aqueous solution obtained was extracted with dichloromethane (5 × 150 mL) to remove theobromine and caffeine and then freeze-dried to give the acetone/water extract.

Fractionation of the Acetone/Water Extract by Solid-Phase Extraction. Aliquots (1 g each) of the acetone/water extract were dissolved in Millipore water (10 mL), filtered, and then applied onto the top of a C18 Sep-Pak cartridge (Waters, Millipore) preconditioned with methanol, followed by water. Fractionation was performed by flushing the column with water (50 mL, fraction S1), followed by methanol/water (20:80, v/v; 40 mL; fraction S2) and methanol (40 mL; fraction S3). The fractions S1−S3 collected were concentrated in a vacuum and, after freeze-drying twice, the yields and the taste quality of the individual fractions were determined. The intensely astringent fraction S1 was taken up in aqueous formic acid (0.1% in water; pH 2.5; 3 mL), the astringent fraction S2 was dissolved in a mixture (20:80, v/v; 5 mL) of methanol and aqueous formic acid (0.1% in water; pH 2.5), and both fractions were separately applied onto the top of a water-cooled glass column (40 × 140 mm) filled with a slurry of LiChroprep 25−40 μm RP-18 material (Merck) in the same solvent mixture. Chromatography was performed by using aqueous formic acid (0.1% in water; pH 2.5), followed by the same formic acid solution containing increasing amounts of methanol up to 60%. The effluent was collected in 13 subfractions, namely, S1-I−XIII and S2-I−XIII, respectively. These fractions were used for taste dilution analysis as well as for chemical structure determination.

Taste Dilution Analysis (TDA). Aliquots (250 mg) of fraction S1 were separated by semipreparative HPLC on RP-18, ODS-Hypersil, 5 μm (ThermoHypersil), and the effluent was collected to give seven subfractions, which were freeze-dried, taken up in water (2 mL), and then sequentially diluted 1:2 with bottled water. The serial dilutions of each of these fractions were then presented to the sensory panel in order of ascending concentrations, and each dilution was evaluated by means of the half-tongue test. The dilution at which a taste difference between the diluted extract and one blank (water) could just be detected was defined as the taste dilution (TD) factor (*1*). The TD factors evaluated by four different assessors in three different sessions were averaged. The TD factors between individuals and separate sessions did not differ by more than one dilution step.

Isolation of (+)-*N*-[3′,4′-Dihydroxy-(*E*)-cinnamoyl]-L-aspartic Acid (1) and (+)-*N*-[4′-Hydroxy-(*E*)-cinnamoyl]-L-aspartic Acid (5) from RP18 Fractions S1-VIII and S1-IX, Respectively. Aliquots of fractions S1-VIII and S1-IX were fractionated by semipreparative HPLC on RP-18, ODS-Hypersil, 5 μm (ThermoHypersil), to afford compounds **1** and **5**, respectively, by using methanol/water mixtures as the mobile phase (flow rate = 3 mL/min). For the isolation of compound **1**, chromatography was performed with a mixture (20:80, v/v) of methanol and aqueous formic acid (0.1% in water; pH 2.5) for 10 min, increasing the methanol content to 60% over 30 min and then to 100% over 0.5 min; thereafter, elution with 100% methanol for 10 min was performed. For the isolation of compound **5**, chromatography was performed with a mixture (25:75, v/v) of methanol and aqueous formic acid (0.1% in water; pH 2.5) for 10 min, increasing the methanol content to 60% over 30 min and then to 100% over 0.5 min; thereafter, elution with 100% methanol for 10 min was performed. The target compounds were collected, freed from solvent in a vacuum, and freeze-dried three times to give compounds **1** and **5** as white amorphous powders in high purity of >99%. Spectroscopic (^1H NMR, MS, UV, $[\alpha]_D^{20}$) and chromatographic data (retention time) as well as sensory activity (taste threshold) of the isolated materials were identical to the data obtained for the corresponding synthesized reference materials.

Identification of (−)-*N*-[3′,4′-Dihydroxy-(*E*)-cinnamoyl]-L-glutamic Acid (2), (−)-*N*-[4′-Hydroxy-(*E*)-cinnamoyl]-L-glutamic Acid (6), (+)-*N*-[4′-Hydroxy-3-methoxy-(*E*)-cinnamoyl]-L-aspartic Acid (9), and (+)-*N*-(*E*)-Cinnamoyl-L-aspartic Acid (10) in RP18 Fraction S1-X. Aliquots of fraction S1-X were separated by analytical RP-HPLC (flow rate = 0.8 mL/min) starting with a mixture (20:80, v/v) of methanol and aqueous formic acid (0.1% in water; pH 2.5) for 10 min, then increasing the methanol content to 60% over 30 min and, finally, to 100% over 0.5 min, thereafter eluting with 100% methanol for 10 min. Comparison of chromatographic data (retention times), spectroscopic data (LC-MS, UV), and sensory data with those of the synthesized reference compounds led to the identification of compounds **2**, **6**, **9**, and **10** (**Figure 1**) as (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid, (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid, (+)-*N*-[4′-hydroxy-3-methoxy-(*E*)-cinnamoyl]-L-aspartic acid, and (+)-*N*-(*E*)-cinnamoyl-L-aspartic acid, respectively.

Isolation of (−)-*N*-[3′,4′-Dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (3), (−)-*N*-[3′,4′-Dihydroxy-(*E*)-cinnamoyl]-L-tyrosine (4), (−)-*N*-[4′-Hydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (7), and (−)-*N*-[4′-Hydroxy-(*E*)-cinnamoyl]-L-tyrosine (8) from RP18 Fractions S2-VIII, S2-X, and S2-XII, Respectively. Aliquots of fractions S2-VIII, S2-X, and S2-XII were separated by semipreparative HPLC on RP-18, ODS-Hypersil, 5 μm (ThermoHypersil), using the following methanol/water gradients at a flow rate of 3 mL/min. For the isolation of compound **3** from fraction S2-VIII, chromatography was performed starting with a mixture (30:70, v/v) of methanol and aqueous formic acid (0.1% in water; pH 2.5) for 5 min, then increasing the methanol content to 50% over 30 min and, finally, to 100% over 0.5 min, thereafter eluting with 100% methanol for 10 min. For the isolation of compounds **4** and **7** from fraction S2-X, chromatography was performed starting with a mixture (25:75, v/v) of methanol and aqueous formic acid (0.1% in water; pH 2.5) for 10 min, then increasing the methanol content to 40% over 30 min and, finally, to 100% over 0.5 min, thereafter eluting with 100% methanol for 10 min. For the isolation of compound **8** from fraction S2-XII, chromatography was performed starting with a mixture (30:70, v/v) of methanol and aqueous formic acid (0.1% in water; pH 2.5) for 10 min, then increasing the methanol content to 50% over 30 min and, finally, to 100% over 0.5 min, thereafter eluting with 100% methanol for 10 min. The fractions containing the target compounds were collected, the solvents were removed in a vacuum, and the fractions were freeze-dried three times to give compounds **3**, **4**, **7**, and **8** as white, amorphous powders in

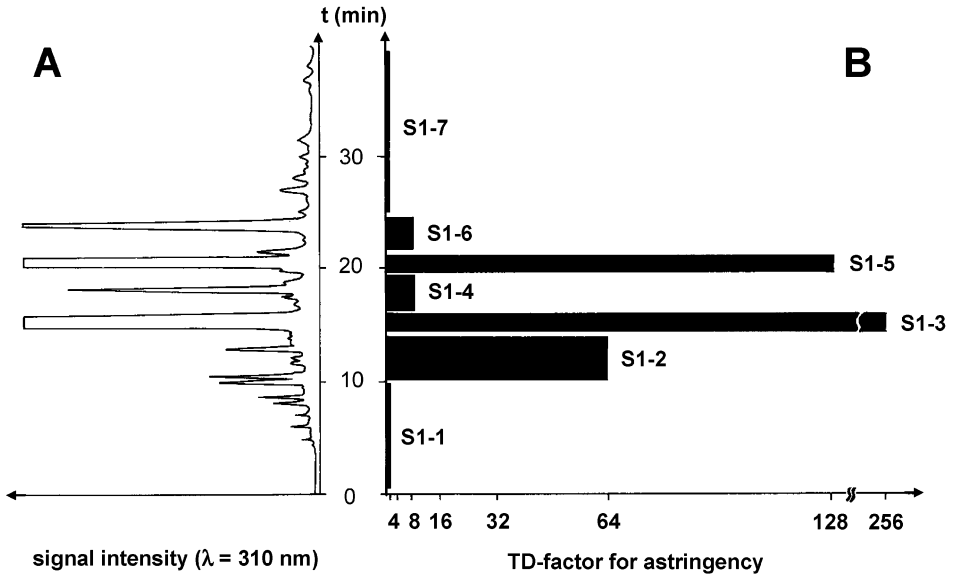

Figure 2. (**A**) RP-HPLC chromatogram and (**B**) TD chromatogram of fraction S1 isolated from cocoa beans by means of solid-phase extraction.

purities of >99%. Spectroscopic data (^{1}H NMR, MS, UV, $[\alpha]_D^{20}$) and chromatographic data (retention time) as well as sensory activity (threshold) of the isolated materials were identical to those obtained for the corresponding synthesized reference materials.

Isolation of (+)-*N*-[3′,4′-Dihydroxy-(*Z*)-cinnamoyl]-L-aspartic Acid (12). A solution of compound **1** (0.5 mmol) in methanol/water (20:80, v/v; pH 3.0; 2 mL) was exposed to UV light (λ = 365 nm) for 1 h by means of a Spectroline model CM-10 (Spectronics Corp., Westbury, NY). Aliquots of the light-exposed mixture were separated by semipreparative HPLC on RP-18, ODS-Hypersil, 5 μm (Thermo-Hypersil). Using a flow rate of 3 mL/min, chromatography was performed starting with a mixture (20:80, v/v) of methanol and aqueous formic acid (0.1% in water; pH 2.5) for 10 min, then increasing the methanol content to 60% over 30 min and, finally, to 100% over 0.5 min. The fraction containing the corresponding (*Z*)-isomer was collected, freed from solvent in a vacuum, and freeze-dried three times to give (+)-*N*-[3′,4′-dihydroxy-(*Z*)-cinnamoyl]-L-aspartic acid (**12**) as a white, amorphous powder (46% yield) with a purity of >99%: UV_{max}, 207, 285, 309 nm; LC-MS (ESI^+), *m*/*z* 613 (100, $[2M + Na]^+$), 296 (95, $[M + 1]^+$), 591 (55, $[2M + 1]^+$), 924 (52, $[3M + K]^+$), 908 (46, $[3M + Na]^+$), 629 (38, $[2M + K]^+$), 318 (25, $[M + Na]^+$), 163 (21, $[M - 132]^+$); ^{1}H NMR (400 MHz, CD_3OD), δ 2.79 [dd, 1H, J = 6.9, 16.8 Hz, H−C(3a)], 2.84 [dd, 1H, J = 5.5, 16.8 Hz, H−C(3b)], 4.80 [m, 1H, H−C(2)], 5.83 [d, 1H, J = 12.6 Hz, H−C(8′)], 6.59 [d, 1H, J = 12.6 Hz, H−C(7′)], 6.70 [d, 1H, J = 8.1 Hz, H−C(5′)], 6.90 [dd, 1H, J = 2.0, 8.1 Hz, H−C(6′)], 7.12 [d, 1H, J = 2.0 Hz, H−C(2′)].

Quantitative Analysis of Light-Induced (*E*)/(*Z*)-Isomerization. Aliquots (50 μL) of solutions of the *N*-phenylpropenoyl amino acids (5 μmol/L) in methanol/water (1:1, v/v) were mixed with Millipore water (2 mL) adjusted to pH 3, 5, or 7 with aqueous hydrochloric acid (0.1 mmol/L), placed into a crystallization dish (4.6 cm i.d.), and then radiated for 1 h with UV light at λ = 254 or 365 nm by means of a Spectroline model CM-10 at a distance of 13 cm. After light exposure, the samples were analyzed by means of HPLC-DAD and LC-MS/MS, and the relative amounts of (*E*)- and (*Z*)-isomers were calculated from the ratio of the corresponding peak areas.

Polarimetry. The optical rotation of solutions of *N*-phenylpropenoyl amino acids (20 mg) in Millipore water (2 mL) or methanol (2 mL) was performed on a 241 MC polarimeter (Perkin-Elmer, Rodgau-Jügesheim, Germany) equipped with a 100 mm cell and operating at 589 nm.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a HPLC pump system PU 1580 with an in-line degasser (DG-1580-53), a low-pressure gradient unit (LG-1580-02), and a diode array detector (DAD) type MD 1515. Chromatography was performed on stainless steel columns packed with ODS-Hypersil, 5 μm, RP-18 material (ThermoHypersil) in either an analytical (250 × 4.6 mm i.d.; flow rate = 0.8 mL/min) or a semipreparative scale (250 × 10 mm i.d.; flow rate = 3.0 mL/min).

Liquid Chromatography−Mass Spectrometry (LC-MS). An analytical ODS-Hypersil, 5 μm, HPLC column (Phenomenex, Aschaffenburg, Germany) was coupled to an LCQ mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) operating in the positive (ESI^+) and negative (ESI^-) electrospray ionization mode. After injection of the sample (20−100 μL), analyses were performed using the solvent gradient reported above.

Circular Dichroism (CD) Spectroscopy. For CD spectroscopy, methanolic solutions of the samples were analyzed by means of a Jasco J600 spectropolarimeter (Hachioji, Japan).

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, and DEPT-135 NMR experiments were performed on an AV-360 spectrometer (Bruker, Rheinstetten, Germany). ^{1}H, COSY, HMQC, and HMBC measurements were performed on an AMX 400-III spectrometer (Bruker). Evaluation of the experiments was carried out using 1D- and 2D-WIN-NMR (version 6.1) as well as XWin-NMR software (version 3.5; Bruker). Tetramethylsilane was used as the internal standard.

RESULTS AND DISCUSSION

With the aim of isolating the highly polar *N*-phenylpropenoyl amino acids, freshly ground, peeled cocoa beans were defatted by extraction with *n*-pentane, and the residual cocoa material was then extracted with acetone/water as reported recently (*2*). After removal of the acetone in a vacuum and extraction of the liquid layer with dichloromethane, the aqueous phase was freeze-dried to give an acetone/water extract that was further fractionated by means of solid-phase extraction using RP-18 cartridges. Elution with water, followed by methanol/water (20:80), and methanol, revealed fractions S1, S2, and S3, respectively, after freeze-drying. These three fractions, strongly differing in color and taste, were then analyzed by means of HPLC-DAD, HPLC-MS, and HPLC-degustation. In particular, fractions S1 and S2 exhibited a pronounced astringent and intense mouth-coating oral sensation.

Isolation and Identification of *N*-Phenylpropenoyl-L-Amino Acids (1, 2, 5, 6, 9, and 10) from Fraction S1. To locate the key taste compounds in the strongly astringent tasting fraction S1, this fraction was further separated by HPLC using RP18 material as the stationary phase (**Figure 2A**). With the chromatography monitored at 310 nm, the effluent was collected in seven fractions S1-1−7, which were individually freeze-dried, then dissolved in the same amount of water, and, finally, used

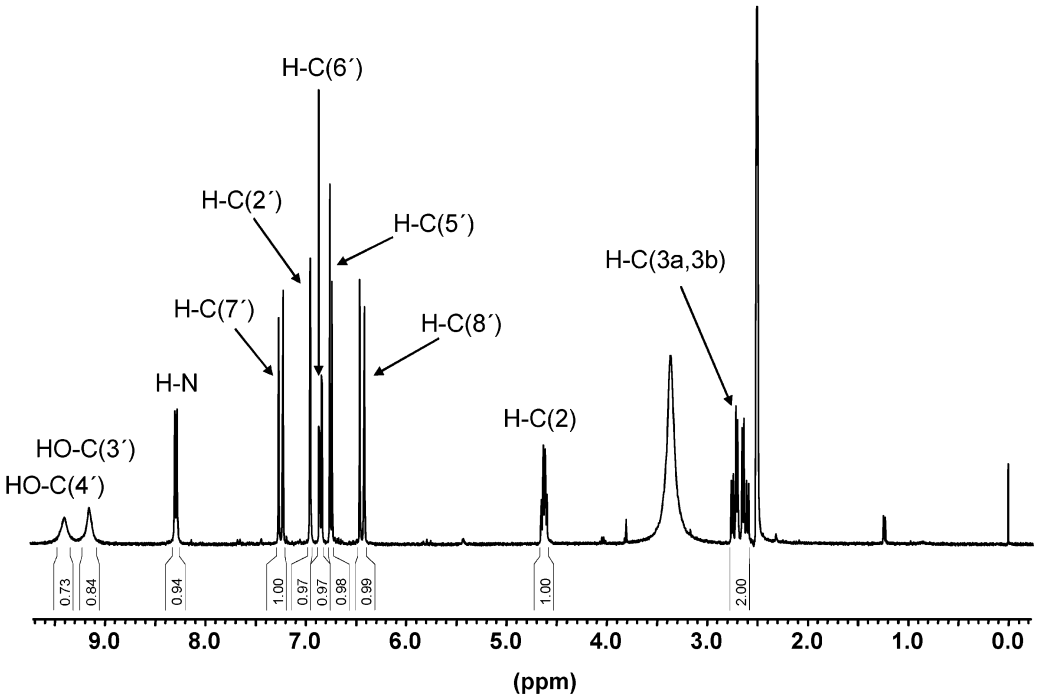

Figure 3. ^{1}H NMR (400 MHz; DMSO-d_6) of (+)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**1**) identified in HPLC fraction S1-3.

for the TDA. To achieve this, each solution was stepwise diluted 1:1 with water, and the dilutions were then presented in order of increasing concentrations to trained sensory panelists, who were asked to judge the astringent oral sensation by application of the recently developed half-tongue test (*4*) until the threshold was reached. As this so-called TD factor, obtained for each fraction, is related to its taste activity in water, the seven HPLC fractions were rated according to their relative astringency impact (**Figure 2B**). Due to their high TD factors of 256 and 128, fractions S1-3 and S1-5 were evaluated with by far the highest taste impacts for astringency, followed by HPLC fraction S1-2 judged with a TD factor of 64. In comparison, the other fractions were evaluated with significantly lower astringent taste impact and should therefore not contribute to cocoa taste.

With the aim of isolating the taste compounds exhibiting the astringent and mouth-coating oral sensation of HPLC fractions S1-3 and S1-5, fraction S1 was isolated from the acetone/water extract (31 g) obtained from 222 g of cocoa beans and then fractionated by preparative column chromatography on RP-18 material. With the effluent monitored at 300 nm, the effluents of the individual peaks detected were collected and freed from solvent in a vacuum to give 13 subfractions (S1-I−XIII). Final purification of the individual subfractions was performed by semipreparative RP-HPLC, thus giving rise to highly pure astringent taste compounds, which were analyzed by means of UV−vis, LC-MS, and NMR spectroscopy.

The astringent compounds detected in subfractions S1-VIII−X showed similar UV absorption spectra exhibiting maxima at 207, 285, and 309 nm. LC-MS analysis showed $[M + H]^+$ ions with *m*/*z* 296 and 280 for the compounds eluting in fractions S1-VIII and S1-IX, respectively, indicating that one nitrogen atom is incorporated in both molecules. MS/MS analysis of the ion *m*/*z* 296 or 280 further demonstrated a loss of 132 amu, most likely corresponding to an aspartate moiety, to generate the ions *m*/*z* 163 and 147 fitting well with the structure of a 3,4-dihydroxycinnamic acid or 4-hydroxycinnamic acid, respectively. To further confirm the structure of the suggested aspartate moiety, 1D- and 2D-NMR experiments were performed.

The ^{1}H NMR spectrum of compound **1**, displayed in **Figure 3**, showed 11 resonance signals each integrating for one proton. Upon the addition of trace amounts of D_2O, the proton signals of two hydroxy groups and one imino group resonating at 8.29, 9.16, and 9.41 ppm disappeared. When these observations were taken into account and the 13 carbon atoms detected in the ^{13}C NMR spectrum considered, the sum formula of $C_{13}H_{13}NO_7$ was proposed for compound **1**. The double doublet of H−C(3a,b) resonating at 2.68 ppm showed a geminal coupling with a coupling constant of 16.5 Hz and coupling constants of 7.0 and 5.9 Hz, respectively, assigned as the vicinal couplings with the proton H−C(2) detected at 4.63 ppm. In addition, a homonuclear, double-quantum filtered δ,δ-correlation experiment (DQF-COSY) revealed coupling between the proton H−C(2) and the N−H proton resonating at 8.29 ppm. Furthermore, the coupling constant of 15.7 Hz for the homonuclear coupling of H−C(8′) and H−C(7′), resonating at 6.44 and 7.25 ppm, led to their assignment as two (*E*)-configured olefinic protons as expected for cinnamic acid derivatives. Three aromatic protons showing ortho and meta couplings were detected between 6.74 and 6.95 ppm. Comparison of the 13 carbon signals observed in the ^{13}C NMR spectrum with the results of a DEPT-135 experiment, which showed 7 signals, identified six carbon signals as quaternary carbon atoms. Unequivocal assignment of these quaternary carbon atoms and the hydrogen-substituted carbon atoms, respectively, could be successfully achieved by means of heteronuclear multiple-bond correlation spectroscopy (HMBC) optimized for $^{2}J_{C,H}$ and $^{3}J_{C,H}$ coupling constants as well as heteronuclear single-quantum correlation spectroscopy (HSQC) optimized for $^{1}J_{C,H}$ coupling constants.

For an unequivocal confirmation of the proposed structure, we also measured heteronuclear δ,δ-correlations between the nitrogen atom and neighboring protons by means of ^{15}N/^{1}H heteronuclear single-bond (^{15}N-HMQC) and multiple-bond

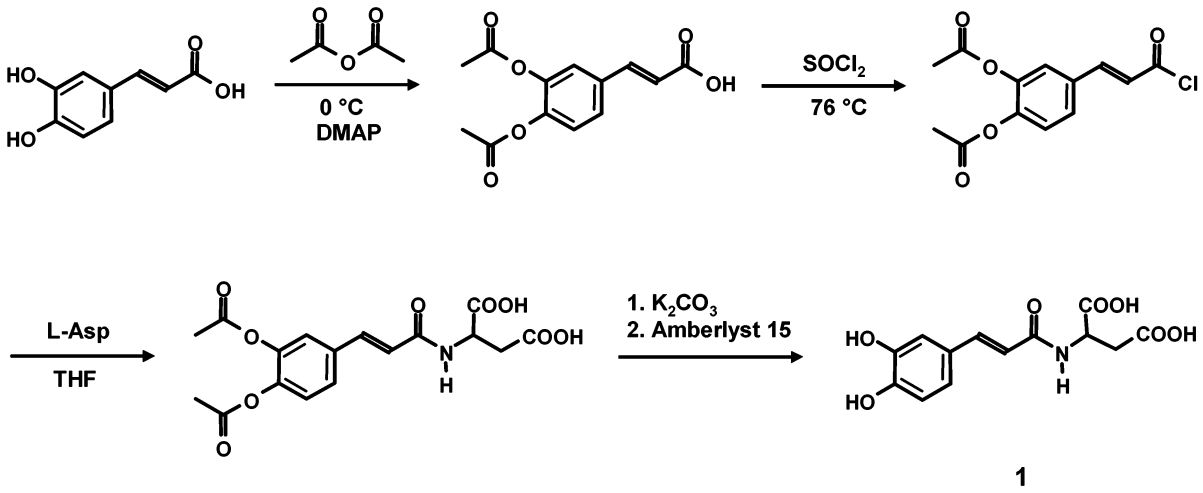

Figure 4. Synthetic sequence used for the preparation of (+)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**1**).

correlation spectroscopy (^{15}N-HMBC). In the ^{15}N-HMQC experiment, just one coupling was observed, thus allowing the conclusion that only one proton, resonating at 8.29 ppm, is bound to the nitrogen atom. In addition, the ^{15}N-HMBC experiment showed the expected heteronuclear coupling between the nitrogen atom and the protons H−C(3a) and H−C(3b) of *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid **1** (**Figure 1**) resonating at 2.62 and 2.73 ppm.

To further confirm the structure of compound **1**, *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid was synthesized following the reaction sequence outlined in **Figure 4**. (*E*)-Caffeic acid was acetylated with acetic anhydride in pyridine/(dimethylamino)pyridine, converted into the corresponding chloride using thionyl chloride, and, finally, reacted with L-aspartic acid in dry tetrahydrofuran. After K_2CO_3-catalyzed cleavage of the protecting groups and Amberlite treatment, the target compound **1** was isolated by column chromatography on RP-18 material, followed by a final HPLC purification yielding *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**Figure 1**) as a white, amorphous powder in a high purity of >99%. The mass spectroscopic and NMR, as well as the sensory data were well in line with those determined for the authentic compound isolated from cocoa beans.

To identify the enantiomer of compound **1** existing naturally in cocoa beans, the optical rotation of compound **1** isolated from cocoa beans, synthetic *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid, and *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-D-aspartic acid synthesized from D-aspartic acid following the procedure shown in **Figure 4** for the L-enantiomer was measured by means of polarimetry. The synthetic D-enantiomer showed an optical rotation of $[\alpha]_D^{20}$ −9.3° (in H_2O), whereas the L-enantiomer revealed a value of +9.3° (in H_2O), fitting well with the optical rotation found for the naturally occurring enantiomer isolated from cocoa beans. In addition, CD measurements were performed with these samples, showing that the isolated compound **1** and the synthesized (+)-L-enantiomer showed the same extinction value of $\lambda_{ext}(\Delta\epsilon) = 237$ nm (−2.5), whereas the (−)-D-enantiomer exhibited an extinction value of $\lambda_{ext}(\Delta\epsilon) = 238$ nm (+1.68), thus clearly demonstrating that cocoa beans contain exclusively the (+)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**1**) (**Figure 5**).

Taking all of these data into consideration, LC-MS/MS, 1D- and 2D-NMR experiments, and CD spectroscopy as well as enantiopure synthesis led to the unequivocal identification of the astringent compound **1** isolated from fraction S1-VIII as (+)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**Figure 1**). By means of HPLC-degustation, HPLC-MS, and HPLC-DAD, compound **1** was identified as the taste compound in HPLC fraction S1-3, which was evaluated with a high TD factor for its astringent and mouth-coating oral sensation (**Figure 2B**).

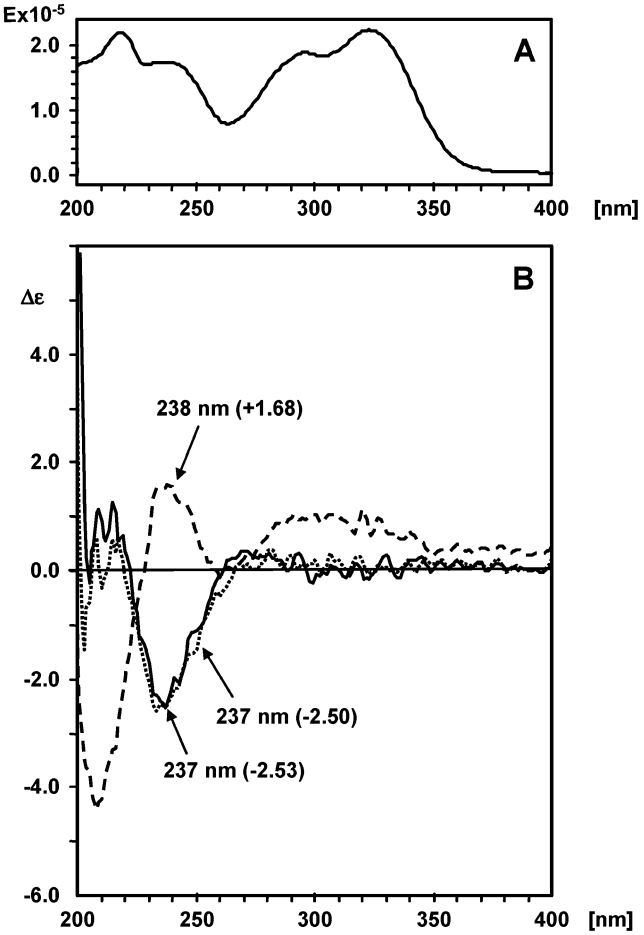

Figure 5. (**A**) UV spectrum, and (**B**) CD spectrum of the synthetic (+)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**1**; • • •), the synthetic (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-D-aspartic acid (**11**; - - -), and the sample of compound **1** isolated from cocoa (—).

Using the same analytical strategy, LC-MS/MS studies, 1D- und 2D-NMR experiments, and polarimetry led to the identification of compound **5** detected in fraction S1-IX and HPLC fraction S1-5 (**Figure 2B**) as (+)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**Figure 1**). The structure of that taste compound

was further confirmed by synthesis using the procedure reported above for compound **1**, but starting with 4′-hydroxy-(*E*)-cinnamic acid instead of (*E*)-caffeic acid. Although this compound has been earlier reported in cell suspension cultures of *Arabidopsis thaliana* (*5*), the taste activity of that amino acid has not been previously described.

In addition, four astringent compounds were detected in subfraction S1-X showing similar UV absorption spectra as shown for compounds **1** and **5** and showing $[M + H]^+$ ions with *m*/*z* 310 (compounds **2** and **9**), 294 (compound **6**), and 264 (compound **10**), thus suggesting that the structures of these tastants might be (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**2**), (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**6**), (+)-*N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-aspartic acid (**9**), and (+)-*N*-(*E*)-cinnamoyl-L-aspartic acid (**10**). To further strengthen this assumption, compound **2** was synthesized from 3′,4′-dihydroxy-(*E*)-cinnamic acid and L-glutamic acid, compound **6** from 4′-hydroxy-(*E*)-cinnamic acid and L-glutamic acid, compound **9** from 4′-hydroxy-3′-methoxy-(*E*)-cinnamic acid and L-aspartic acid, and, finally, compound **10** from (*E*)-cinnamic acid and L-aspartic acid using the synthetic procedure reported above for compound **1**. Comparison of retention times, UV, and LC-MS/MS as well as sensory data of the compounds isolated from subfraction S1-X with those of the synthesized reference compounds led to the unequivocal identification of these compounds as (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**2**), (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**6**), (+)-*N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-aspartic acid (**9**), and (+)-*N*-(*E*)-cinnamoyl-L-aspartic acid (**10**), respectively. Comparison of retention times and LC-MS/MS data as well as cochromatography with the synthetic compounds revealed these taste compounds are present in HPLC fractions S1-4 (compound **2**), S1-6 (compound **6**), and S1-7 (compounds **9** and **10**) evaluated with lower taste dilution factors for astringency (**Figure 2B**). Although compound **9** has been earlier reported in cell cultures of *Beta vulgaris* (*6*), the taste activity of that compound has yet not been reported. To the best of our knowledge, compounds **2**, **6**, and **10** have not been reported previously.

Isolation and Identification of *N*-Phenylpropenoyl-L-Amino Acids (3, 4, 7, and 9) from Fraction S2. Aimed at isolating the taste compounds exhibiting the astringent and mouth-coating oral sensation in fraction S2, an aliquot of this fraction obtained from 222 g cocoa beans was fractionated by preparative column chromatography on RP-18 material. Monitoring the effluent at 300 nm, the effluents of the individual peaks detected were collected and freed from solvent in a vacuum to give 13 subfractions (S2−I to S2-XIII) which were evaluated by the sensory panel. Final purification of the taste-active subfractions was performed by semipreparative RP-HPLC, thus giving rise to highly pure astringent taste compounds which were analyzed by means of UV/Vis, LC-MS, and NMR spectroscopy.

LC-MS analysis of the astringent compounds isolated from these subfractions, all of which exhibiting similar absorption maxima at 225, 255, and 297 nm, revealed $[M+H]^+$ ions with *m*/*z* 360 for the compound **3** eluting in fraction S2−VIII, *m*/*z* 344 for compounds **4** and **7** detected in fraction S2-X, and *m*/*z* 328 for compound **8** detected in fraction S2-XII. These data clearly indicated that one nitrogen atom is incorporated in the molecules under investigation. MS/MS analysis of the ions *m*/*z* 360 and 344, corresponding to the second peak in fraction S2-X, revealed the loss of 196 and 180 amu, respectively, to generate the ion *m*/*z* 163 fitting well with the structure of a 3,4-dihydroxycinnamic acid and corresponding to the cleavage of one molecule of 3-hydroxy-tyrosine and tyrosine, respectively. In addition, MS/MS analysis of the ion *m*/*z* 344, corresponding to the first peak in fraction S2-X, as well as the ion *m*/*z* 328 revealed the loss of 196 and 180 Da, respectively, to generate the ion *m*/*z* 147 fitting well with the structure of a hydroxycinnamic acid and corresponding to the cleavage of one molecule of 3-hydroxy-tyrosine and tyrosine. To further confirm the phenylpropenoic acid group in the molecules and to identify the amino acid moieties, 1D- and 2D-NMR experiments were performed.

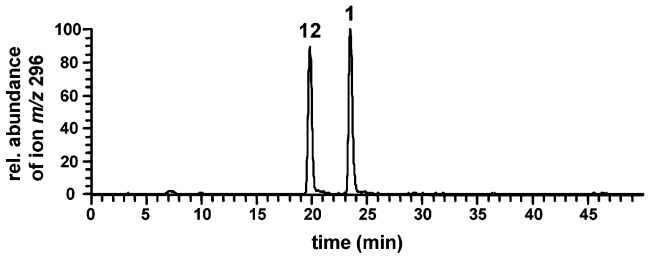

Figure 6. HPLC-MS analysis of an aqueous solution of (+)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**1**) exposed to UV light with a wavelength of 365 nm.

In brief, LC-MS/MS as well as DQF−COSY-, HMBC- and HSQC NMR experiments, revealing a complete picture of chemical shifts, homo- and heteronuclear single- and multiple-bond correlations, led to the unequivocal identification of the astringent compounds in fraction S2−VIII, S2-X, and S3-XII as (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**3**), (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine (**4**), (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**7**), and (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (**8**), respectively (**Figure 1**). Finally, the structures of all of these compounds were synthesized from 3′,4′-dihydroxy-(*E*)-cinnamic acid or 4′-hydroxy-(*E*)-cinnamic acid and L-tyrosine or 3-hydroxy-L-tyrosine, respectively, using the synthetic procedure reported above for compound **1**. Although compound **3**, named clovamide, was earlier identified in red clover (*Trifolium pratense*) (*7*−*9*), together with compound **8**, named deoxyclovamide, earlier reported as a constituent of the bark of African blackwood (*Dalbergia melanoxylon)* (*10*) and as cocoa antioxidants (*11*), the taste activity of these phenolic amino acid derivatives has not been previously published. In addition, compound **4** has been earlier identified in cocoa flowers (*12*) and raw robusta coffee beans (*13*), but its taste activity has not been investigated so far. To the best of our knowledge, compound **7** has not previously been identified in any food material.

(*E*)/(*Z*)-Isomerization of *N*-Phenylpropenoyl Amino Acids. During isolation of the amino acid amides from cocoa as well as during purification of the corresponding synthetic compounds, we observed that the (*E*)-configured olefinic double bond in these molecules is partially isomerized in the presence of light. To study the influence of light on this isomerization process, an aqueous solution of compound **1** was exposed to UV light with a wavelength of 365 nm, and, thereafter, the mixture was analyzed by RP-HPLC. As shown in **Figure 6**, besides compound **1**, a second compound was formed, which was isolated by HPLC and unequivocally identified as (+)-*N*-[3′,4′-dihydroxy-(*Z*)-cinnamoyl]-L-aspartic acid (**12**) by means of LC-MS/MS and NMR experiments.

To investigate the influence of light on this isomerization process more quantitatively, solutions of *N*-phenylpropenoyl amino acids differing in the pH value were exposed to UV light with the wavelength of 254 or 365 nm, respectively (**Table 1**).

Table 1. Isomerization of *N*-Phenylpropenoyl Amino Acids upon UV Light Exposure

		ratio of isomers (%) after radiation at			
		254 nm		365 nm	
	compd	(*Z*)-iso[a]	(*E*)-iso[a]	(*Z*)-iso[a]	(*E*)-iso[a]
pH 3.0	**1**	16	84	46	54
	2	12	88	43	57
	3	23	77	62	38
	4	16	84	51	49
	5	21	79	64	36
	9	42	58	70	30
	10	29	71	3	97
pH 5.0	**1**	17	83	43	57
	2	14	86	45	55
	3	25	75	69	31
	4	18	82	54	46
	5	22	78	64	36
	9	43	57	70	30
	10	27	73	2	98
pH 7.0	**1**	20	80	43	57
	2	16	84	45	55
	3	26	74	66	34
	4	20	80	55	45
	5	22	78	64	36
	9	44	56	70	30
	10	24	76	2	98

[a] The corresponding isomers have been detected by HPLC-DAD as well as HPLC-MS/MS.

After light exposure, the solutions were subsequently analyzed by HPLC coupled to a diode array detector or a mass spectrometer. Compounds **1**−**5** and **9**, containing either a 4′-hydroxy-(*E*)-cinnamoyl, a 3′,4′-dihydroxy-(*E*)-cinnamoyl, or a 4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl group, were most strongly isomerized into the corresponding (*Z*)-configured compound at pH 7 when exposed to light with a wavelength of 365 nm. In comparison, exposure to UV light with a wavelength of 254 nm was significantly less active in facilitating the isomerization process; for example, at pH 3 the (*Z*)- and (*E*)-configurations of **1** were detected in ratios of 16:84 and 46:54 when exposed to light with the wavelength of 254 or 365 nm, respectively. In contrast, compound **10**, bearing an (*E*)-cinnamoyl moiety, was not significantly isomerized upon light exposure at 365 nm, but was converted by 29% into the (*Z*)-isomer when exposed to light at 254 nm (pH 3.0). Independent from the pH value, compound **9** showed favorably isomerization at 254 nm as well as at 365 nm; for example, only 30% the (*E*)-isomer was recovered after exposure to light with the wavelength of 365 nm. In contrast, compounds **1** and **2** were least susceptible to isomerization under the conditions used. Due to different UV−vis absorption maxima, the *N*-phenylpropenoic acid moiety is driving the acceptability for (*E*)/(*Z*)-isomerization.

As the naturally occurring (*E*)-configured *N*-phenylpropenoyl amino acids were shown to be rapidly converted into the corresponding (*Z*)-isomers, quantitative analysis of these compounds in food and plant materials, such as cocoa, has to be done very carefully in the absence of light to prevent any artifact formation.

Sensory Activity of *N*-Phenylpropenoyl Amino Acids. Prior to sensory analysis, the purity of all compounds was checked by HPLC-MS as well as ^{1}H NMR spectroscopy. To study the sensory activity of these *N*-phenylpropenoyl amino acids, the human sensory recognition thresholds were determined in bottled water using the half-tongue test for the astringent and a triangle test for the sour impression (**Table 2**). The oral sensation imparted by the *N*-phenylpropenoyl amino acids was described as mouth-drying and puckering astringent, with threshold concentrations ranging from 26 to 190 μmol/L. Comparison of the taste thresholds obtained for the aspartic acid derivatives showed that the sensory activity was not strongly influenced by the hydroxyl groups at the cinnamoyl moiety; for example, the aspartic acid amides containing (*E*)-cinnamic acid, 4′-hydroxy-(*E*)-cinnamic acid, and 3′,4′-dihydroxy-(*E*)-cinnamic acid, respectively, showed very similar taste thresholds of 220, 180, and 170 μmol/L. In contrast, compound **9**, containing a 4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl moiety, showed a 3-fold lower taste threshold when compared to compound **1**.

Table 2. Human Recognition Taste Thresholds of *N*-Phenylpropenoyl Amino Acids

taste compd	taste threshold concn for astringency[a] (μmol/L)
(+)-*N*-[(*E*)-cinnamoyl]-L-aspartic acid (**10**)	220[b]
(−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**2**)	190[b]
(+)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**5**)	180[b]
(+)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**1**)	170[b]
(−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-D-aspartic acid (**11**)	170[b]
(+)-*N*-[3′,4′-dihydroxy-(*Z*)-cinnamoyl]-L-aspartic acid (**12**)	170[b]
(−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**6**)	170[b]
(−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (**8**)	100
(−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine (**4**)	83
(+)-*N*-[4′-hydroxy-3-methoxy-(*E*)-cinnamoyl]-L-aspartic acid (**9**)	57[b]
(−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**7**)	55
(−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**3**)	26

[a] Taste threshold concentrations were determined by means of the half-tongue test. [b] These compounds exhibit sour taste at the recognition taste threshold concentration of 1500 μmol/L, which was determined by means of a triangle test as reported recently (*3*).

Sensory analysis of the L- and D-configured compound **1** as well as the (*Z*)-configured isomer **12** revealed the same threshold concentration of 170 μmol/L for these isomers, thus demonstrating that the stereochemistry does not play any role for the sensory activity of these amino acid amides.

In contrast, the amino acid moiety seems to be of major importance for the sensory impact. For example, the taste threshold of the *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-amino acids decreased from 170 and 190 μmol/L for the aspartic acid and glutamic acid derivatives **1** and **2**, respectively, by a factor of 2 to 83 μmol/L for the tyrosine derivative **4** and by 7-fold to 26 μmol/L for the dopa derivative **3** (**Table 2**). Besides the astringent taste quality, the amides of the aliphatic amino acids asparatic acid (**1**, **5**, and **9**−**12**) and glutamic acid (**2** and **6**) exhibited significant sour taste at concentrations >1.5 mmol/L, whereas the amides of the aromatic amino acids tyrosine (**4** and **8**) and 3-hydroxytyrosine (**3** and **7**) did not induce any sour taste perception.

Aimed at demonstrating the contribution of the *N*-phenylpropenoyl amino acids to the typical astringent taste of roasted cocoa, quantitative studies, taste reconstitution, and taste omission experiments are currently in progress and will be published elsewhere.

ACKNOWLEDGMENT

We thank H.-U. Humpf, University of Muenster, for performing the CD measurements.

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Received for review March 1, 2005. Revised manuscript received April 28, 2005. Accepted April 28, 2005. This research project was supported by a grant from FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn, Germany), the AiF (Arbeitsgemeinschaft industrieller Forschung), and the German Ministry of Economics (Project 13235 N).

JF050458Q