

## Molecular Definition of the Taste of Roasted Cocoa Nibs (*Theobroma cacao*) by Means of Quantitative Studies and Sensory Experiments

TIMO STARK,<sup>†</sup> SABINE BAREUTHER,<sup>†</sup> AND THOMAS HOFMANN<sup>\*,‡</sup>

Deutsche Forschungsanstalt für Lebensmittelchemie, Lichtenbergstrasse 4, D-85748 Garching, Germany, and Institut für Lebensmittelchemie, Universität Münster, Corrensstrasse 45, D-48149 Münster, Germany

Sensory-guided decomposition of roasted cocoa nibs revealed that, besides theobromine and caffeine, a series of bitter-tasting 2,5-diketopiperazines and flavan-3-ols were the key inducers of the bitter taste as well as the astringent mouthfeel imparted upon consumption of roasted cocoa. In addition, a number of polyphenol glycopyranosides as well as a series of *N*-phenylpropenoyl-L-amino acids have been identified as key astringent compounds of roasted cocoa. In the present investigation, a total of 84 putative taste compounds were quantified in roasted cocoa beans and then rated for the taste contribution on the basis of dose-over-threshold (DoT) factors to bridge the gap between pure structural chemistry and human taste perception. To verify these quantitative results, an aqueous taste reconstitute was prepared by blending aqueous solutions of the individual taste compounds in their "natural" concentrations. Sensory analyses revealed that the taste profile of this artificial cocktail was very close to the taste profile of an aqueous suspension of roasted cocoa nibs. To further narrow down the number of key taste compounds, finally, taste omission experiments and human dose/response functions were performed, demonstrating that the bitter-tasting alkaloids theobromine and caffeine, seven bitter-tasting diketopiperazines, seven bitter- and astringent-tasting flavan-3-ols, six puckering astringent *N*-phenylpropenoyl-L-amino acids, four velvety astringent flavonol glycosides,  $\gamma$ -aminobutyric acid,  $\beta$ -aminoisobutyric acid, and six organic acids are the key organoleptics of the roasted cocoa nibs.

**KEYWORDS:** Cocoa; taste; taste reconstruction; taste omission; dose/response functions

### INTRODUCTION

Because of its pleasant aroma and its attractive taste, the fermented and roasted seeds of the cocoa tree *Theobroma cacao* are widely enjoyed by consumers as the desirable key ingredient in cocoa-based beverages and chocolate confectionary. Some of the sensory criteria used to describe the taste quality of roasted cocoa is its balanced bitterness, its typical astringent mouthfeel perceived as a long-lasting puckering and shrinking sensation in the oral cavity, as well as the slight sour taste, all together imparting rich mouthfeel, complexity, and palatability to cocoa-containing products.

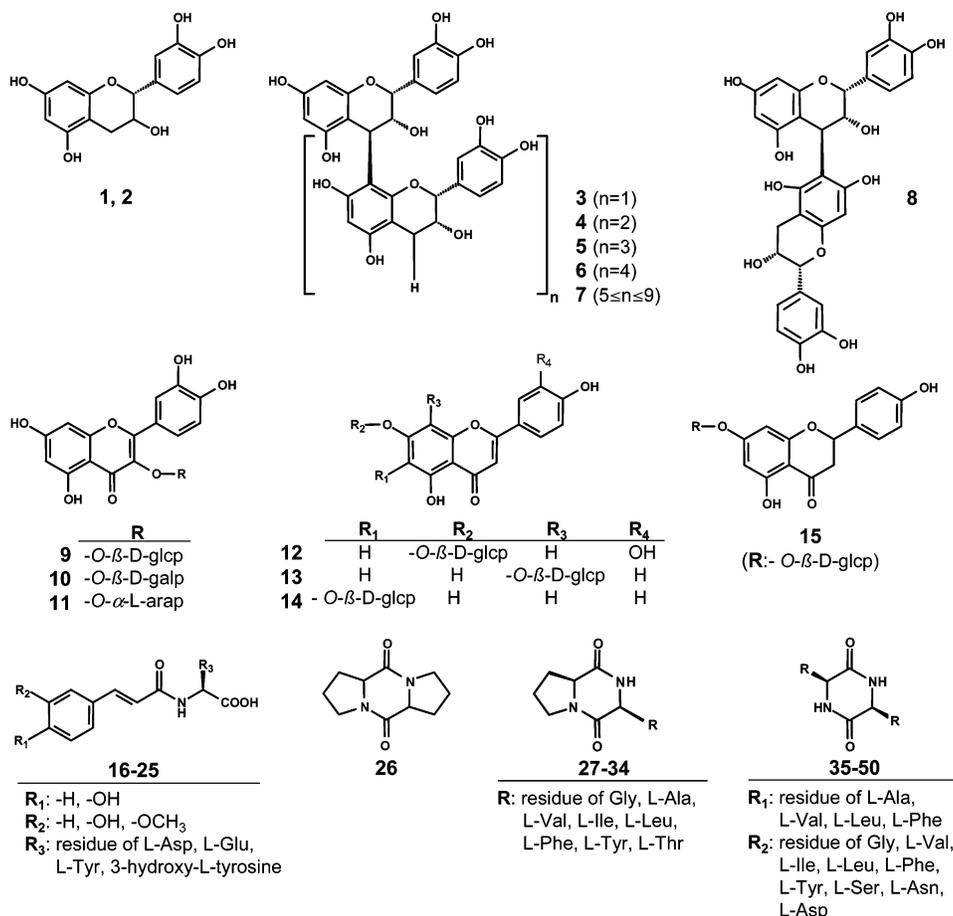
Numerous attempts have been made to find a correlation between the results obtained from sensory panelists and the molecules reflecting the typical taste profile of roasted cocoa (1–8). Although theobromine (1, 2) and 2,5-diketopiperazines (3, 4) are believed to be responsible for the cocoa bitterness and flavan-3-ols (5–8) are proposed as key contributors of

astringency of cocoa, it is agreed that further systematic studies are necessary to fully understand the typical taste of roasted cocoa on a molecular level (4). Aimed at identifying the nonvolatile, key taste compounds responsible for the typical taste of roasted cocoa nibs, we applied the recently developed taste dilution analysis (9) on fractions isolated from ground roasted cocoa nibs. This bioresponse-directed fractionation led to the detection of numerous taste-active compounds in cocoa nibs (10–12), the structures of which had been unequivocally determined as the following (Figure 1): the flavan-3-ols, epicatechin (1) and catechin (2), the (4 $\beta$   $\rightarrow$  8)-linked flavan-3-ol oligomers, procyanidin B2 (3), procyanidin C1 (4), [epicatechin-(4 $\beta$   $\rightarrow$  8)]<sub>3</sub>-epicatechin (5), [epicatechin-(4 $\beta$   $\rightarrow$  8)]<sub>4</sub>-epicatechin (6), and [epicatechin-(4 $\beta$   $\rightarrow$  8)]<sub>5-9</sub>-epicatechin (7), as well as the (4 $\beta$   $\rightarrow$  6)-linked procyanidin B5 (8) have been identified as puckering astringent and bitter-tasting compounds; the polyphenol glycosides, quercetin-3-*O*- $\beta$ -D-glucopyranoside (9), quercetin-3-*O*- $\beta$ -D-galactopyranoside (10), quercetin-3-*O*- $\alpha$ -L-arabinopyranoside (11), luteolin-7-*O*- $\beta$ -D-glucopyranoside (12), apigenin-8-*C*- $\beta$ -D-glucopyranoside (13), apigenin-6-*C*- $\beta$ -D-glucopyranoside (14), and naringenin-7-*O*- $\beta$ -D-glucopyranoside (15), were found to induce a velvety astringent mouth-

\* To whom correspondence should be addressed. Telephone: +49-251-83-33-391. Fax: +49-251-83-33-396. E-mail: thomas.hofmann@uni.muenster.de.

<sup>†</sup> Deutsche Forschungsanstalt für Lebensmittelchemie.

<sup>‡</sup> Institut für Lebensmittelchemie.



**Figure 1.** Chemical structures of taste-active compounds identified in roasted cocoa nibs by means of the taste dilution analysis.

coating; the polyphenol amino acid conjugates, (+)-*N*-[(*E*)-cinnamoyl]-L-aspartic acid (**16**), (+)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**17**), (+)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**18**), (+)-*N*-[4'-hydroxy-3'-methoxy-(*E*)-cinnamoyl]-L-aspartic acid (**19**), (–)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**20**), (–)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**21**), (–)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**22**), (–)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**23**), (–)-*N*-[4'-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (**24**), and (–)-*N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine (**25**), were identified to impart a puckering astringent sensation to the oral cavity without exhibiting any bitter taste; and furthermore, a number of 25 different 2,5-diketopiperazines were identified as metallic-like bitter-tasting compounds, namely, cyclo(L-Pro-L-Pro) (**26**), cyclo(L-Pro-Gly) (**27**), cyclo(L-Pro-L-Ala) (**28**), cyclo(L-Pro-L-Val) (**29**), cyclo(L-Pro-L-Ile) (**30**), cyclo(L-Pro-L-Leu) (**31**), cyclo(L-Pro-L-Phe) (**32**), cyclo(L-Pro-L-Tyr) (**33**), cyclo(L-Pro-L-Thr) (**34**), cyclo(L-Ala-L-Val) (**35**), cyclo(L-Ala-L-Ile) (**36**), cyclo(L-Ala-L-Leu) (**37**), cyclo(L-Ala-L-Phe) (**38**), cyclo(L-Ala-L-Tyr) (**39**), cyclo(L-Val-L-Val) (**40**), cyclo(L-Val-L-Leu) (**41**), cyclo(L-Val-L-Phe) (**42**), cyclo(L-Val-L-Tyr) (**43**), cyclo(L-Leu-Gly) (**44**), cyclo(L-Phe-Gly) (**45**), cyclo(L-Phe-L-Leu) (**46**), cyclo(L-Phe-L-Ile) (**47**), cyclo(L-Phe-L-Ser) (**48**), cyclo(L-Phe-L-Asp) (**49**), and cyclo(L-Phe-L-Asn) (**50**).

To evaluate the taste contribution of these individual compounds more precisely, the objectives of the present investigation were to quantify putative taste compounds in roasted cocoa nibs, to rate them on the basis of a dose/activity relationship, and, finally, to confirm the taste contribution of the key compounds by means of taste reconstitution and taste omission experiments, as well as human dose/response functions.

## MATERIALS AND METHODS

**Chemicals.** The following compounds were obtained commercially: caffeine, theobromine (Fluka, Neu-Ulm, Germany), (+)-catechin, (–)-epicatechin,  $\gamma$ -aminobutyric acid (Sigma, Steinheim, Germany), sucrose, glucose, fructose (Merck, Darmstadt, Germany), naringenin-7-*O*- $\beta$ -D-glucopyranoside, luteolin-7-*O*- $\beta$ -D-glucopyranoside, quercetin-3-*O*- $\beta$ -D-glucopyranoside, quercetin-3-*O*- $\beta$ -D-galactopyranoside, apigenin-8-*C*- $\beta$ -D-glucopyranoside, apigenin-6-*C*- $\beta$ -D-glucopyranoside (Roth, Karlsruhe, Germany), cyclo(L-Asp-L-Phe), cyclo(Gly-L-Phe), cyclo(L-Ala-L-Leu), cyclo(L-Leu-L-Phe), cyclo(L-Phe-L-Pro), cyclo(L-Pro-L-Thr), cyclo(L-Pro-L-Tyr), *cis*-cyclo(L-Pro-L-Val), cyclo(L-Val-L-Val), cyclo(L-Phe-L-Ser), and *trans*-cyclo(D-Ala-L-Val) (Bachem, Bubendorf, Switzerland). Solvents were high-performance liquid chromatography (HPLC)-grade (Merck, Darmstadt, Germany). The *N*-phenylpropenoyl-L-amino acids **16–25** as well as the diketopiperazines, cyclo(L-Ala-L-Leu), cyclo(L-Ala-L-Ile), cyclo(L-Ala-L-Tyr), cyclo(L-Val-L-Leu), cyclo(L-Val-L-Tyr), cyclo(L-Ala-L-Phe), cyclo(L-Ala-L-Pro), cyclo(L-Ala-L-Val), cyclo(L-Asn-L-Phe), cyclo(L-Ile-L-Phe), cyclo(L-Ile-L-Pro), cyclo(L-Pro-L-Pro), cyclo(L-Val-L-Phe), and *trans*-(L-Val-D-Phe), were synthesized as reported elsewhere (*11, 12*). Procyanidins B2, B5, and C1, [epicatechin-(4 $\beta$   $\rightarrow$  8)]<sub>3</sub>-epicatechin, [epicatechin-(4 $\beta$   $\rightarrow$  8)]<sub>4</sub>-epicatechin, and quercetin-3-*O*- $\alpha$ -L-arabinopyranoside were isolated from nonfermented and nonroasted cocoa as detailed recently (*10*). Cocoa beans, fermented in Ghana for 5 days, were roasted by the food industry.

**Preparation of an Aqueous Extract from Roasted Cocoa Nibs (RCN Extract).** Cocoa nibs were frozen with liquid nitrogen and ground in a lab mill. Aliquots of the cocoa powder (1.0 g) were suspended in water (25 mL) and stirred for 15 min at room temperature prior to filtration using a cellulose filter. For sensory experiments, the aqueous RCN extract was used directly.

**Quantitative Analyses.** For quantitative analysis of taste compounds, powdered roasted cocoa nibs (5.0 g) were spiked with solutions of the internal standards of diketopiperazine (*12*) and *N*-phenylpropenoyl

amino acids (13) (100  $\mu$ L, each) in methanol (1.0 mg/mL) and the mixture was homogenized in a laboratory shaker for 30 min. After equilibration, the cocoa powder was extracted with *n*-pentane (5  $\times$  30 mL) at room temperature for 30 min. The residual cocoa material was then extracted 5 times with acetone/water (70:30, v/v; 30 mL each) for 45 min at room temperature with stirring. After centrifugation, the liquid layer was freed from acetone under reduced pressure at 30 °C and then freeze-dried to give the acetone/water extract as reported recently (10). After membrane filtration, aliquots (5  $\mu$ L) were analyzed by means of liquid chromatography/mass spectrometry (LC–MS/MS) using gradients and mass transitions described below.

**Flavonol Glycosides 9–15.** Following the procedure reported above, an aliquot of the acetone extract (120 mg) isolated from cocoa nibs was dissolved in a mixture (50:50, v/v; 10 mL) of acetonitrile and water adjusted to pH 2.5 with formic acid. After membrane filtration, aliquots (5  $\mu$ L) were analyzed by means of HPLC–MS/MS on Zorbax Eclipse XDB-C8 150  $\times$  2 mm i.d., 5  $\mu$ m column (Agilent) using the following transition reactions given in parentheses: quercetin-3-*O*- $\alpha$ -L-arabinopyranoside (*m/z* 433.1  $\rightarrow$  299.9), quercetin-3-*O*- $\beta$ -D-glucopyranoside (*m/z* 463.1  $\rightarrow$  299.9), quercetin-3-*O*- $\beta$ -D-galactopyranoside (*m/z* 463.1  $\rightarrow$  299.9), naringenin-7-*O*- $\beta$ -D-glucopyranoside (*m/z* 433.1  $\rightarrow$  270.9), luteolin-7-*O*- $\beta$ -D-glucopyranoside (*m/z* 447.1  $\rightarrow$  284.9), apigenin-8-*C*- $\beta$ -D-glucopyranoside (*m/z* 431.1  $\rightarrow$  310.9), apigenin-6-*C*- $\beta$ -D-glucopyranoside (*m/z* 431.1  $\rightarrow$  310.9) and using the following solvent gradient at a flow rate of 0.25 mL/min. Starting with a mixture (17:83, v/v) of acetonitrile (0.1% formic acid) and aqueous formic acid (0.1%, pH 2.5), held at 17% for 15 min, the acetonitrile content was increased to 30% in 30 min, increased to 100% within 5 min, and, finally, held at 100% for 10 min. Quantitative analysis was performed by comparing the peak areas obtained for the corresponding mass traces with those of defined standard solutions of each reference compound in methanol.

***N*-Phenylpropenoyl Amino Acids 16–25.** Quantitative analysis was performed using the stable isotope dilution assays with LC–MS/MS detection reported recently (13).

**2,5-Diketopiperazines 26–50.** Quantification was performed using the diastereomer dilution analysis with LC–MS/MS detection published elsewhere (12). By means of the multiple-reaction-monitoring (MRM<sup>+</sup>) mode, the individual diketopiperazines were analyzed on Luna phenyl hexyl 150  $\times$  2 mm i.d., 5  $\mu$ m (Phenomenex) using the following transition reactions given in parentheses: *cis*-cyclo(L-Leu-L-Pro) (*m/z* 211.0  $\rightarrow$  70.0), *cis*-cyclo(L-Ile-L-Pro) (*m/z* 211.0  $\rightarrow$  70.0), *cis*-cyclo(L-Leu-L-Phe) (*m/z* 261.0  $\rightarrow$  120.0), *cis*-cyclo(L-Ile-L-Phe) (*m/z* 261.0  $\rightarrow$  120.0), *cis*-cyclo(L-Val-L-Pro) (*m/z* 197.0  $\rightarrow$  70.0), *cis*-cyclo(L-Pro-L-Thr) (*m/z* 199.0  $\rightarrow$  70.0), *cis*-cyclo(L-Phe-L-Ser) (*m/z* 235.0  $\rightarrow$  120.0), *cis*-cyclo(L-Ala-L-Ile) (*m/z* 185.0  $\rightarrow$  86.0), *cis*-cyclo(L-Ala-L-Leu) (*m/z* 185.0  $\rightarrow$  86.0), *cis*-cyclo(L-Val-L-Phe) (*m/z* 247.0  $\rightarrow$  120.0), *cis*-cyclo(L-Val-L-Val) (*m/z* 199.0  $\rightarrow$  72.0), cyclo(L-Leu-Gly) (*m/z* 171.0  $\rightarrow$  86.0), *cis*-cyclo(L-Phe-L-Pro) (*m/z* 245.0  $\rightarrow$  120.0), *cis*-cyclo(L-Ala-L-Phe) (*m/z* 219.0  $\rightarrow$  120.0), *cis*-cyclo(L-Pro-L-Pro) (*m/z* 195.0  $\rightarrow$  70.0), *cis*-cyclo(L-Asp-L-Phe) (*m/z* 263.0  $\rightarrow$  91.0), *cis*-cyclo(L-Ala-L-Tyr) (*m/z* 235.0  $\rightarrow$  107.0), cyclo(Gly-L-Pro) (*m/z* 155.0  $\rightarrow$  70.0), cyclo(Gly-L-Phe) (*m/z* 205.0  $\rightarrow$  120.0), *cis*-cyclo(L-Ala-L-Pro) (*m/z* 169.0  $\rightarrow$  70.0), *cis*-cyclo(L-Pro-L-Tyr) (*m/z* 261.0  $\rightarrow$  136.0), *cis*-cyclo(L-Ala-L-Val) (*m/z* 171.0  $\rightarrow$  72.0), *cis*-cyclo(L-Val-L-Tyr) (*m/z* 263.0  $\rightarrow$  136.0), *cis*-cyclo(L-Asn-L-Phe) (*m/z* 262.0  $\rightarrow$  120.0), *cis*-cyclo(L-Val-L-Leu) (*m/z* 213.0  $\rightarrow$  72.0), and *cis*-cyclo(L-Val-L-Ile) (*m/z* 213.0  $\rightarrow$  72.0).

**Flavan-3-ols 1–7 and 8.** For the quantification of catechin, epicatechin, and oligomeric procyanidins, an aliquot of the acetone extract (10 mg) isolated from cocoa nibs as reported recently (10) was dissolved in a mixture (50:50, v/v; 10 mL) of methanol and water adjusted to pH 2.5 with formic acid and, after membrane filtration, aliquots (5  $\mu$ L) were analyzed by means of LC–MS/MS on a RP-18 Synergi Fusion, 150  $\times$  2 mm i.d., 5  $\mu$ m column (Phenomenex Aschaffenburg, Germany) using mixtures of methanol containing 0.1% formic acid and aqueous formic acid (0.1% in water, pH 2.5) as follows: Operating at a flow rate of 0.2 mL/min, starting with a mixture (5:95, v/v) of methanol (0.1% formic acid) and aqueous formic acid (0.1%, pH 2.5), held at 5% for 5 min, the methanol content was increased to 40% in 45 min, increased to 100% within 5 min, and, finally, held at 100% for 10 min. After identification of the individual flavan-3-ol, on the basis of identical LC–MS/MS and UV/vis data as

well as the retention time with the corresponding reference, quantification was performed by comparing the peak area obtained for the corresponding mass transition with those of defined standard solutions of each reference compound in methanol. By means of the multiple-reaction-monitoring (MRM<sup>+</sup>) mode, the individual flavan-3-ols and procyanidins were analyzed using the following transition reactions given in parentheses: (–)-epicatechin (*m/z* 289.1  $\rightarrow$  245.0), (+)-catechin (*m/z* 289.1  $\rightarrow$  245.0), procyanidin B-2 epicatechin-(4 $\beta$   $\rightarrow$  8)-epicatechin (*m/z* 577.2  $\rightarrow$  289.0), procyanidin C-1 epicatechin-(4 $\beta$   $\rightarrow$  8)<sub>2</sub>-epicatechin (*m/z* 865.3  $\rightarrow$  125.0), [epicatechin-(4 $\beta$   $\rightarrow$  8)]<sub>3</sub>-epicatechin (*m/z* 1153.4  $\rightarrow$  125.0), and [epicatechin-(4 $\beta$   $\rightarrow$  8)]<sub>4</sub>-epicatechin (*m/z* 1441.5  $\rightarrow$  125.2).

**Theobromine and Caffeine.** For the quantification of purine alkaloids, ground roasted cocoa beans (1.0 g) were suspended in boiling water (70 mL). After the suspension was maintained at boiling temperature for 20 min and cooled to room temperature, Carrez solution I and II (1 mL each) were added, the suspension was made up to 100 mL with water, and after filtration, the filtrate obtained was analyzed by means of analytical RP-HPLC using a ODS-Hypersil, 250  $\times$  4.6 mm i.d., 5  $\mu$ m column (Shandon Frankfurt, Germany). Chromatography was performed isocratically with a mixture (15/85; v/v) of acetonitrile and aqueous sodium acetate (0.4% in water). Monitoring the effluent at 254 nm, quantification of caffeine and theobromine was performed by comparing the peak area obtained with those of defined standard solutions of caffeine and theobromine in water.

**Soluble Carbohydrates and Organic Acids.** Sugars and organic acids were quantitatively determined in the acetone extract obtained from cocoa nibs (10) using enzymatic test kits (R-Biopharm GmbH, Darmstadt, Germany) closely following the experimental protocols given by the manufacturers. Oxalic acid was quantitatively determined by means of an enzymatic test purchased from Sigma–Aldrich (Deisenhofen, Germany). The volatile acetic acid was determined in an aqueous suspension of roasted cocoa nibs.

**Free Amino Acids.** For amino acid analysis, the acetone extract (15.0 mg) isolated from cocoa nibs (10) was dissolved in aqueous buffer solution (1 mL, 0.1 mol/L) containing sodium acetate (8.2 g/L), methanol (7.5%), formic acid (0.3%), acetic acid (1.5%), and octanoic acid (0.001%), membrane filtered, and analyzed either directly or after 1:9 dilution with aqueous buffer solution by means of an LC 3000 amino acid analyzer (Biotronic, Maintal, Germany) equipped with a 75  $\times$  6.0 mm i.d. BTC F guard column and a 145  $\times$  3.2 mm i.d. BTC 2410 main column (Eppendorf-Netheler-Hinz, Maintal, Germany). For amino acid analysis, the column temperature was maintained at 47 °C for 26.5 min, then adjusted to 49 °C from 26.5 to 41.8 min, to 50 °C from 41.8 to 55.8 min, to 52 °C from 55.8 to 70.8 min, to 56 °C from 70.8 to 80.8 min, and, finally, to 60 °C from 80.8 to 98.8 min. After postcolumn derivatization with ninhydrine, the amino acids were quantified by monitoring the effluent at 440 nm for primary amino acids and at 570 nm for proline.

**Sensory Analyses. Panel Training.** To familiarize the subjects with the taste language used by our sensory group and to get them trained in recognizing and distinguishing different qualities of oral sensations, 12 assessors with no history of known taste disorders (5 women and 7 men, age 25–38 years) participated for at least 2 years in weekly training sessions. For example, the subjects were trained to evaluate the taste of aqueous solutions (5 mL each) of the following standard taste compounds in bottled water (Vittel, low mineralization of 405 mg/L) adjusted to pH 6.0 with aqueous hydrochloric acid (0.1 mol/L), sucrose (12.5 mmol/L) for sweet taste, lactic acid (20 mmol/L) for sour taste, NaCl (12 mmol/L) for salty taste, caffeine (1 mmol/L) for bitter taste, sodium glutamate (3 mmol/L) for umami taste, and iron(II) gluconate (0.03 mmol/L) for metallic taste. For the puckering astringency and the velvety astringent, mouth-drying oral sensation, the panel was trained by using gallotannic acid (0.05%) and quercetin-3-*O*- $\beta$ -D-glucopyranoside (0.01 mmol/L), respectively, using the half-tongue test (14). Sensory analyses were performed in a sensory panel room at 22–25 °C in three independent sessions.

**Taste Recognition Threshold Concentrations.** Using triangle tests as reported recently (15), threshold concentrations for sour-, sweet-, bitter-, salty-, metallic-, and umami-tasting compounds were determined in bottled water adjusted to pH 5.5 with hydrochloric acid (0.1 mol/

L). To overcome memory effects of astringent compounds, threshold concentrations of astringent compounds were determined by means of the recently developed half-tongue test (12, 14).

**Taste Recombinants.** To prepare an artificial taste imitate of the aqueous cocoa bean extract, the "natural" amounts of the 84 taste compounds, summarized in **Table 1**, were suspended in bottled water and the pH value of the solution was then adjusted to 5.5 by the addition of sodium hydroxide (0.1 mmol/L). The taste reconstitute was equilibrated for 10 min in an ultrasonic bath, and the overall taste quality was then evaluated by means of the taste profile analysis using nose clips. To avoid degradation of labile phenols, freshly prepared preparations were used exclusively.

**Taste Omission Experiments.** To investigate the taste contribution of the individual taste compounds, 14 partial taste recombinants were prepared one by one by omitting either individual tastant groups or single taste compounds from the complete taste reconstitute. Each of the partial recombinants was presented to the panelists in comparison with the complete taste reconstitute, using a triangle test. Panelists were asked to evaluate whether the solutions were identical in the overall taste or not. Those panelists, who detected the taste difference correctly, were asked to rate the intensity of the given taste descriptors on a scale from 0 (not detectable) to 5 (strongly detectable).

**Taste Profile Analysis.** Freshly prepared aqueous extracts of cocoa beans, the taste recombinants, as well as partial taste recombinants were presented to the sensory panel, who was asked to score the taste qualities astringent, bitter, sour, sweet, salty, and umami on a scale from 0 (not detectable) to 5 (strongly detectable). While wearing nose clips, the samples were briefly swirled around in the mouth and then expectorated.

**Recording of Human Dose/Response Functions.** The acquisition of dose/response functions was performed closely following the procedure reported earlier (12). Serial 1:1 dilutions of the samples in water were prepared starting at the level of 128- or 256-fold above the recognition threshold concentration of the bitter or astringent test compounds and ending at the concentration level two steps below the individual recognition threshold concentration. To fit the dose/response functions into a five-point intensity scale, first, the taste intensity of the individual compounds was compared at the highest concentration level by means of the recently reported half-tongue tasting method. To achieve this, the solutions of the individual compounds were applied in binary combinations to one side of the tongue and the assessors were asked to determine which side showed the stronger sensation (14). On a five-point scale with 0.25 scale subunits, a 10 mmol/L solution of epigallocatechin-3-gallate, used as the reference compound for astringency, or a 30 mmol/L solution of salicin, used as the reference compound for bitter taste, was evaluated with the highest sensory intensity and set to the maximum score of 5.0. After the sensory intensity of each test compound at its maximum concentration had been rated, the sensory intensities of the other dilutions were determined by using the half-tongue tasting method. To achieve this, first, one dilution of an individual compound was rated against the intensity of the next lower as well as the next higher concentration of the same compound and the intensity of this solution was approximated by a comparison to the taste intensity of aqueous solutions containing increasing concentrations of the reference compound epigallocatechin 3-gallate and salicin, respectively. Human response functions with dose-over-threshold (DoT) factors on the x axis and taste intensities on the y axis were recorded for each individual subject in triplicates. The intensity values between trained individuals and separate sessions did not differ more than  $\pm 0.4$  units.

**HPLC.** The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a PU 1580 HPLC-pump system with a DG-1580-53 in-line degasser, a LG-1580-02 low-pressure gradient unit, and a MD 1515 diode array detector (DAD). Chromatography was performed on a stainless-steel column packed with ODS-Hypersil, 250  $\times$  4.6 mm i.d., 5  $\mu$ m material (ThermoHypersil), operating with a flow rate of 0.8 mL/min.

**LC-MS/MS.** LC-MS/MS analysis was performed using an Agilent 1100 HPLC system connected to the API 3200 LC-MS/MS (Applied Biosystems, Darmstadt, Germany) running in the positive or negative electrospray ionization mode. By means of the MRM mode, the

individual taste compounds were analyzed using the mass transitions described above or in the literature (13).

## RESULTS AND DISCUSSION

A freshly prepared aqueous suspension of powdered roasted cocoa nibs imparted the typical complex and attractive cocoa taste and was used for taste profile analysis. To achieve this, a trained sensory panel was asked to rate the intensity of the taste qualities bitter, sour, sweet, salty, umami, and astringency on a scale from 0 (not detectable) to 5 (intensely detectable). High scores were found for the intensity of the bitter taste (4.1), followed by the astringent, mouth-coating taste sensation (3.0) as well as the sour taste sensation (2.8) (**Figure 2**). In comparison, saltiness, sweetness, and umami taste was not detectable.

As reported recently, sequential application of solvent extraction, gel permeation chromatography, and RP-HPLC in combination with the taste dilution analysis revealed that, besides the alkaloids theobromine and caffeine, the monomeric and oligomeric flavan-3-ols **1–8** as well as the metallic, blood-like bitter-tasting 2,5-diketopiperazines **26–50** were among the key compounds contributing to the bitter taste of roasted cocoa (10, 12). Besides the flavan-3-ols **1–8**, the velvety astringent polyphenol glycosides **9–15** as well as the puckering astringent *N*-propenoyl amino acids **16–25** have been identified as key mouthfeel compounds of roasted cocoa nibs (10, 11). To confirm the importance of these molecules as key tastants of roasted cocoa nibs, to evaluate the taste contribution of additional putative taste compounds, e.g., amino acids, organic acids, and soluble carbohydrates, and to demonstrate a correlation between single taste compounds and individual taste qualities, we aimed at preparing an taste imitate containing these taste compounds in their "natural" concentrations and to compare the taste profile of this biomimetic taste reconstitute to that of the authentic aqueous suspension of roasted cocoa nibs. To achieve this, first, all of the individual taste compounds needed to be quantified in roasted cocoa nibs and the taste recognition thresholds needed to be determined.

**Concentrations and DoT Factors of Taste Compounds.** To evaluate the taste contribution of the individual taste compounds, the flavan-3-ols **1–8**, polyphenol glycosides **9–15**, *N*-phenylpropenoyl-L-amino acids **16–25**, diketopiperazines **26–50**, 2 alkaloids theobromine and caffeine, and, in addition, 20 amino acids, 6 organic acids, and 6 soluble carbohydrates were quantitatively determined in roasted cocoa beans and the taste quality as well as the taste recognition threshold of each substance was evaluated by the trained sensory panel. As we aimed to elucidate the key contributors for each individual taste quality, the single taste compounds were grouped into six classes differing in their taste qualities (**Table 1**).

The 25 diketopiperazines **26–50**, the alkaloids theobromine and caffeine, as well as the amino acids L-isoleucine, L-leucine, L-phenylalanine, L-tyrosine, L-valine, L-lysine, L-arginine, and L-histidine were classified into taste group I representing compounds imparting a bitter taste (**Table 1**). By means of the triangle test, the human sensory recognition thresholds of these compounds were determined (10, 12). Fitting well with our previous data, the taste imparted by the diketopiperazines was described as metallic-type bitter, with threshold concentrations spanning from 190 to 3910  $\mu$ mol/kg for the bitter taste. Quantitative analysis revealed theobromine as the quantitatively predominating bitter taste compound, followed by cyclo(L-Pro-L-Val), L-leucine, and caffeine, e.g., 63.565 mmol/kg of theobromine, 8.878 mmol/L of *cis*-cyclo(L-Pro-L-Val), 6.990 mmol/kg of L-leucine, and 5.218 mmol/kg of caffeine have been found

**Table 1.** Taste Qualities, Taste Thresholds, Concentrations, and DoT Factors of Selected Taste Compounds in Roasted Cocoa Nibs

taste compound	threshold concentration <sup>a</sup> ( $\mu\text{mol/kg}$ )	concentration ( $\mu\text{mol/kg}$ )	DoT <sup>b</sup> factor
group I: bitter-tasting compounds			
theobromine	800.0	63 564.4	79.4
caffeine	750.0	5218.3	7.0
cyclo(L-Pro-L-Val) (29)	1280.0	8877.8	6.9
cyclo(L-Val-L-Leu) (41)	470.0	817.1	1.7
cyclo(L-Ala-L-Ile) (36)	540.0	639.5	1.2
cyclo(L-Ala-L-Leu) (37)	680.0	734.0	1.1
cyclo(L-Pro-L-Ile) (30)	480.0	537.0	1.1
cyclo(L-Pro-L-Ala) (28)	1490.0	1357.0	0.9
cyclo(L-Pro-L-Leu) (31)	1190.0	711.2	0.6
cyclo(L-Ala-L-Val) (35)	1470.0	633.5	0.4
cyclo(L-Phe-L-Leu) (46)	190.0	47.9	0.3
cyclo(L-Phe-L-Ile) (47)	190.0	61.5	0.3
cyclo(L-Val-L-Val) (40)	1260.0	237.6	0.2
cyclo(L-Ala-L-Phe) (38)	570.0	72.0	0.1
cyclo(L-Val-L-Phe) (42)	1000.0	58.0	<0.1
cyclo(L-Pro-L-Phe) (32)	1020.0	64.3	<0.1
cyclo(L-Phe-Gly) (45)	610.0	11.3	<0.1
cyclo(L-Phe-L-Asp) (49)	3810.0	0.7	<0.1
cyclo(L-Pro-L-Thr) (34)	630.0	22.9	<0.1
cyclo(L-Pro-L-Pro) (26)	2580.0	32.5	<0.1
cyclo(L-Pro-L-Tyr) (33)	480.0	4.0	<0.1
cyclo(L-Val-L-Tyr) (43)	190.0	8.8	<0.1
cyclo(L-Phe-L-Asn) (50)	960.0	14.6	<0.1
cyclo(L-Ala-L-Tyr) (39)	530.0	1.6	<0.1
cyclo(L-Phe-L-Ser) (48)	210.0	3.0	<0.1
cyclo(L-Pro-Gly) (27)	3250.0	2.1	<0.1
cyclo(L-Leu-Gly) (44)	590.0	<1.2	<1.2
L-leucine	12 000.0	6990.4	0.6
L-tyrosine	5000.0	2719.6	0.5
L-isoleucine	11 000.0	2716.4	0.2
L-valine	21 000.0	4049.8	0.2
L-lysine	85 000.0	578.0	<0.1
L-arginine	75 000.0	723.4	<0.1
L-histidine	48 000.0	568.4	<0.1
L-phenylalanine	58 000.0	4761.5	<0.1
group II: bitter and puckering astringent compounds			
epicatechin (1)	800 <sup>g</sup> /800 <sup>d</sup>	8613.1	10.8 <sup>g</sup> /10.8 <sup>d</sup>
procyanidin B2 (3)	500 <sup>g</sup> /200 <sup>d</sup>	2082.8	4.2 <sup>g</sup> /10.4 <sup>d</sup>
procyanidin C1 (4)	400 <sup>g</sup> /300 <sup>d</sup>	1628.0	4.1 <sup>g</sup> /5.4 <sup>d</sup>
[epicatechin-(4 $\beta$ $\rightarrow$ 8)] <sub>3</sub> -epicatechin (5)	290 <sup>g</sup> /150 <sup>d</sup>	1158.9	4.0 <sup>g</sup> /7.7 <sup>d</sup>
[epicatechin-(4 $\beta$ $\rightarrow$ 8)] <sub>4</sub> -epicatechin (6)	200 <sup>g</sup> /70 <sup>d</sup>	802.1	4.0 <sup>g</sup> /11.5 <sup>d</sup>
catechin (2)	1000 <sup>g</sup> /600 <sup>d</sup>	2363.9	2.4 <sup>g</sup> /3.9 <sup>d</sup>
procyanidin B5 (8)	900 <sup>g</sup> /400 <sup>d</sup>	791.5	0.9 <sup>g</sup> /2.0 <sup>d</sup>
[epicatechin-(4 $\beta$ $\rightarrow$ 8)] <sub>5-9</sub> -epicatechin (7) <sup>e</sup>	>601 <sup>g</sup> /30 <sup>d</sup>	623.3	<1.0 <sup>g</sup> /20.7 <sup>d</sup>
group III: purely astringent compounds			
$\gamma$ -aminobutyric acid	20.0 <sup>f</sup>	5011.4	250.6
$\beta$ -aminoisobutyric acid	120.0 <sup>f</sup>	1349.3	11.2
quercetin-3-O- $\beta$ -D-glucopyranoside (9)	0.65	101.4	155.7
quercetin-3-O- $\beta$ -D-galactopyranoside (10)	0.43	33.8	78.6
quercetin-3-O- $\alpha$ -L-arabinopyranoside (11)	21.6	497.5	22.6
luteolin-7-O- $\beta$ -D-glucopyranoside (12)	5.2	3.6	0.7
apigenin-8-C- $\beta$ -D-glucopyranoside (13)	8.7	<1.0	<0.1
apigenin-6-C- $\beta$ -D-glucopyranoside (14)	10.8	<1.0	<0.1
naringenin-7-O- $\beta$ -D-glucopyranoside (15)	13.0	<1.0	<0.1
N-[3',4'-dihydroxy-(E)-cinnamoyl]-3-hydroxy-L-tyrosine (23)	26.0	851.5	32.8
N-[3',4'-dihydroxy-(E)-cinnamoyl]-L-aspartic acid (18)	170.0	1615.6	9.5
N-[4'-hydroxy-(E)-cinnamoyl]-L-aspartic acid (17)	180.0	551.6	3.1
N-[4'-hydroxy-(E)-cinnamoyl]-L-tyrosine (24)	100.0	132.6	1.3
N-[3',4'-dihydroxy-(E)-cinnamoyl]-L-tyrosine (25)	83.0	39.6	0.5
N-[4'-hydroxy-(E)-cinnamoyl]-3-hydroxy-L-tyrosine (22)	55.0	27.0	0.5
N-[4'-hydroxy-3'-methoxy-(E)-cinnamoyl]-L-aspartic acid (19)	57.0	<20.0	<0.3
N-[4'-hydroxy-(E)-cinnamoyl]-L-glutamic acid (20)	170.0	<15.0	<0.1
N-[3',4'-dihydroxy-(E)-cinnamoyl]-L-glutamic acid (21)	190.0	<15.0	<0.1
N-[(E)-cinnamoyl]-L-aspartic acid (16)	220.0	<10.0	<0.1
group IV: sour compounds			
citric acid	2600.0	30 974.9	11.9
acetic acid	2000.0	16 717.7	8.3
succinic acid <sup>g</sup>	900.0	1725.0	1.9
malic acid	3700.0	3581.2	1.0
lactic acid	15 400.0	9260.7	0.6
oxalic acid	5600.0	2810.5	0.5
phosphoserine	3125.0	744.7	0.2
phosphoethanolamine	3000.0	298.5	<0.1

Table 1. (Continued)

taste compound	threshold concentration <sup>a</sup> ( $\mu\text{mol/kg}$ )	concentration ( $\mu\text{mol/kg}$ )	DoT <sup>b</sup> factor
group V: sweet-tasting compounds			
L-alanine	8000.0	6115.5	0.8
$\beta$ -alanine	1200.0	339.0	0.3
L-serine	30 000.0	1823.4	<0.1
glycine	30 000.0	873.0	<0.1
L-proline	26 000.0	2475.0	<0.1
L-threonine	40 000.0	1899.0	<0.1
sucrose	12 500.0	8827.3	0.7
glucose	50 000.0	1669.1	<0.1
fructose	52 000.0	4834.1	<0.1
raffinose	25 000.0	1068.3	<0.1
stachyose	25 000.0	533.6	<0.1
galactose	25 000.0	1110.1	<0.1
group VI: umami-like taste compounds			
glutamic acid	3000.0	1781.8	0.6
aspartic acid	4000.0	1357.9	0.3

<sup>a</sup> Taste threshold concentrations were determined in bottled water by means of a triangle test for bitter, sweet, umami, and sour compounds and by means of the half-tongue test for astringent compounds. <sup>b</sup> DoT factor is calculated as the ratio of the concentration and taste threshold. <sup>c</sup> Taste threshold and DoT factor for bitter taste.

<sup>d</sup> Taste threshold and DoT factor for astringency. <sup>e</sup> A mixture of procyanidins containing the oligomers from the hexamer to the decamer in their natural ratio was used for the sensory experiments. Yields were determined by weight. <sup>f</sup> Taste threshold concentration was taken from the literature (17). <sup>g</sup> This compound exhibits umami-like taste at the threshold level of 700  $\mu\text{mol/L}$  corresponding to a taste activity value of 2.4 for umami-like taste quality.

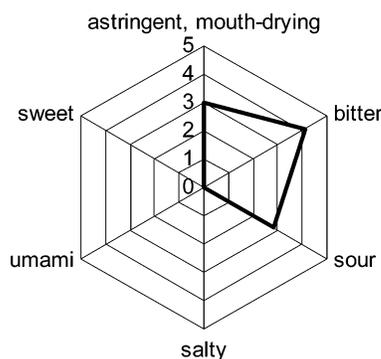


Figure 2. Taste profiles of a freshly prepared cocoa bean extract.

in roasted cocoa nibs. In comparison to the concentration of cyclo(L-Pro-L-Val), the concentrations of the other diketopiperazines were significantly lower, e.g., 633.5 and 3.0  $\mu\text{mol/kg}$  have been found for cyclo(L-Ala-L-Val) and cyclo(L-Phe-L-Ser). A comparison between the two alkaloids revealed that the concentration detected for theobromine was 12 times higher than for caffeine and that the concentrations for bitter-tasting amino acids, except for L-lysine, L-arginine, and L-histidine (568–723  $\mu\text{mol/kg}$ ), were in the concentration range between 2716  $\mu\text{mol/L}$  found for L-isoleucine and 6990  $\mu\text{mol/kg}$  found for L-leucine (Table 1). To gain first insights into the taste impact of these compounds, these were rated on the basis of their DoT factors, defined as the ratio of the concentration and the taste recognition threshold of a compound (15). The calculation of the DoT factors for bitter taste revealed an extraordinary high value of 79.4 for theobromine. With somewhat lower DoT factors of 7.0 and 6.9, caffeine and cyclo(L-Pro-L-Val) were evaluated with high taste impacts, whereas cyclo(L-Val-L-Leu), cyclo(L-Ala-L-Ile), cyclo(L-Ala-L-Leu), and cyclo(L-Pro-L-Ile) just slightly exceeded their bitter thresholds (Table 1). In contrast, the concentration of all of the other bitter-tasting compounds in tastant group I was found to be below their taste threshold concentration. Among these, the DoT factors of the cyclo(L-Pro-L-Ala), cyclo(L-Pro-L-Leu), cyclo(L-Ala-L-Val), cyclo(L-Phe-L-Leu), cyclo(L-Phe-L-Ile), cyclo(L-Val-L-Val), and cyclo(L-Ala-L-Phe), as well as the amino acids L-leucine and L-isoleucine,

were between 0.1 and 1.0, whereas for all further amino acids and diketopiperazines, DoT factors <0.1 were calculated (Table 1).

Tastant group II, summarizing compounds imparting bitterness and a puckering astringent oral sensation, contained the individual flavan-3-ols 1–6 and 8, as well as a mixture of oligomeric procyanidins consisting of hexamers to decamers on their “natural” ratios and concentrations (Table 1). Fitting well with our previous data (10), the oral sensation imparted by the flavan-3-ols was described as astringent, with threshold concentrations spanning from 30 to 800  $\mu\text{mol/kg}$ , and as bitter, with threshold concentrations spanning from 200 to 1000  $\mu\text{mol/kg}$ . Among this group of taste compounds, the flavan-3-ol monomers were found in highest concentrations, e.g., 8613 or 2364  $\mu\text{mol/kg}$  of (–)-epicatechin (1) and (+)-catechin (2). The calculation of the DoT factors for the astringent sensation of these compounds revealed high values for all of the flavan-3-ols. Among these, the mixture of hexameric to decameric procyanidins (7) was evaluated to exceed its taste threshold by a factor of more than 20. With somewhat lower DoT factors, also the flavan-3-ols 1–6 were evaluated with high taste impacts for the astringent taste sensation. Relating the concentrations of these compounds to their bitterness recognition thresholds revealed the highest DoT factor of 10.8 for epicatechin, followed by the procyanidins B2 and C1 as well as the tetrameric and pentameric procyanidins 5 and 6 just exceeding their taste thresholds by a factor of 4. In comparison, (+)-catechin (2) was evaluated with just a DoT factor of 2.4, and procyanidin B5 as well as the polymeric fraction 7 did not reach their bitter taste thresholds (Table 1).

Taste group III, summarizing the compounds imparting an astringent and mouth-drying oral sensation without any bitter taste, contained the polyphenol glycosides 9–15, the *N*-phenylpropenoyl amino acids 16–25, as well as the amino acids  $\gamma$ -aminobutyric acid and  $\beta$ -aminoisobutyric acid (Table 1). In comparison to the flavan-3-ols in group II, the glycosides 9–15 were found to induce a mouth-drying and mouth-coating sensation at very low threshold concentrations spanning from 0.43  $\mu\text{mol/kg}$  for quercetin-3-*O*- $\beta$ -D-galactopyranoside to 21.6  $\mu\text{mol/kg}$  found for quercetin-3-*O*- $\alpha$ -L-arabinopyranoside. Also, the *N*-phenylpropenoyl amino acids induced an astringent sensation at significantly lower concentrations when compared

to the flavan-3-ols; astringent threshold concentrations were found to range from 26.0  $\mu\text{mol/L}$  for *N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-*L*-3-hydroxy-tyrosine to 220  $\mu\text{mol/kg}$  for *N*-(*E*)-cinnamoyl-*L*-aspartic acid (Table 1). In contrast to these polyphenols, the amino acids  $\gamma$ -aminobutyric acid and  $\beta$ -aminoisobutyric acid were found to induce a mouth-drying sensation at low threshold concentrations of 20 and 120  $\mu\text{mol/kg}$  (water), respectively (16). Quantitative analysis of the individual compounds of this taste group revealed by far the highest concentration of more than 5 mmol/kg for  $\gamma$ -aminobutyric acid in roasted cocoa beans. In comparison, all of the other taste compounds summarized in group III were present in lower concentrations ranging between 1616  $\mu\text{mol/kg}$  found for *N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-*L*-aspartic acid (18) and less than 1.0  $\mu\text{mol/kg}$  found for apigenin-6-*C*- $\beta$ -*D*-glucopyranoside, apigenin-8-*C*- $\beta$ -*D*-glucopyranoside, and naringenin-7-*O*- $\beta$ -*D*-glucopyranoside (13–15) (Table 1). The calculation of the DoT factors of these compounds revealed by far the highest values of 251, 156, and 79, respectively, for  $\gamma$ -aminobutyric acid, quercetin-3-*O*- $\beta$ -*D*-glucopyranoside, and quercetin-3-*O*- $\beta$ -*D*-galactopyranoside, followed by quercetin-3-*O*- $\alpha$ -*L*-arabinopyranoside and  $\beta$ -aminoisobutyric acid evaluated with somewhat lower DoT factors. In the group of the puckering astringent *N*-phenylpropenoyl amino acids, the highest DoT factor of 33 was found for *N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-*L*-3-hydroxy-tyrosine, followed by *N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-*L*-aspartic acid, *N*-[4'-hydroxy-(*E*)-cinnamoyl]-*L*-aspartic acid, and *N*-[4'-hydroxy-(*E*)-cinnamoyl]-*L*-tyrosine. The concentration of all of the other taste compounds in group III did not exceed their taste threshold concentration and, therefore, should not have a major contribution to cocoa taste.

Taste group IV contained the sour-tasting organic acids succinic acid, oxalic acid, malic acid, citric acid, acetic acid, and lactic acid, as well as phosphoserine and phosphoethanolamine (Table 1). The highest concentration of more than 30 mmol/kg was found for citric acid, whereas acetic acid, lactic acid, malic acid, oxalic acid, and succinic acid were present in lower concentrations between 16.7 and 1.7 mmol/kg. On the basis of quantitative data and taste threshold concentrations, DoT factors were calculated, demonstrating the highest values of 11.9 and 8.3 for citric acid and acetic acid, respectively. Significantly lower DoT factors were determined for succinic acid and malic acid, whereas all of the other sour-tasting compounds of group IV did not exceed their taste threshold concentrations.

Finally, soluble carbohydrates and sweet-tasting amino acids were grouped into the sweet-tasting group V, and *L*-aspartic acid and *L*-glutamic acid were grouped into the umami-like-tasting group VI (Table 1). Quantitative analysis and sensory studies revealed that the "natural" concentration of each of these compounds in roasted cocoa beans was below the corresponding recognition threshold concentration. When these data are taken into consideration, the sweet- as well as the umami-like-tasting compounds might not be of major importance for the typical taste of roasted cocoa beans.

**Taste Reconstitution Experiments.** To confirm the results of the instrumental analysis and to check whether the compounds already identified can create the typical taste of an aqueous suspension of roasted nibs, we prepared an aqueous taste reconstitute containing the 84 compounds in "natural" concentrations as given in Table 1 and compared the taste profile of that complete taste reconstitute with that of a freshly prepared aqueous suspension of roasted nibs. To achieve this, all of the compounds summarized in the taste groups I–VI were dissolved in bottled water in their "natural" concentrations and the pH value as well as the color tone was adjusted to those of an authentic aqueous cocoa extract by adding some trace amounts

**Table 2.** Taste Profile Analysis of the Authentic Cocoa Bean Extract, the Complete Taste Recombinant Containing 84 Compounds, and a Reduced Taste Recombinant Containing 34 Compounds

taste quality	intensities of individual taste qualities in <sup>a</sup>		
	authentic cocoa bean extract	total taste recombinant	reduced taste recombinant <sup>b</sup>
bitter	4.1	3.7	3.7
astringent, mouth-drying	3.0	3.0	3.0
sour	2.8	2.6	2.6
sweet	0	0	0
salty	0	0	0
umami	0	0	0

<sup>a</sup> Intensities were judged on a scale from 0 (not detectable) to 5 (strongly detectable). <sup>b</sup> The selection of these 31 compounds was done based on the results of taste omission experiments.

of sodium hydroxide as well as sugar couleur. The trained sensory panel was then asked to evaluate the taste profile of these samples by scoring the taste descriptors, given in Table 2, on a scale from 0 (not detectable) to 5 (strongly detectable). Sensory evaluation of this complete taste recombinant revealed a high intensity for bitterness evaluated with a score of 3.7, thus being very close to the bitter taste intensity (4.1) of the authentic aqueous roasted cocoa extract. In addition, the astringent sensation was judged with a score of 3.0 in both samples (Table 2). Also sourness of the recombinant judged with an intensity of 2.6 was rather close to that of the authentic cocoa nibs evaluated with a score of 2.8. Sweetness, saltiness, and umami taste were not detected for both solutions. As demonstrated in Figure 2, the taste profile of the recombinant was very close to that of the original cocoa, and the trained panelists concluded that the typical taste of the freshly prepared aqueous cocoa extract could be completely reconstituted by the blend of the 84 compounds given in Table 1.

**Taste Omission Experiments.** After the taste impact of 84 nonvolatiles was evaluated in the aqueous suspension of roasted cocoa nibs on the basis of DoT values as well as taste reconstitution experiments, the following experiments aimed at confirming the taste contribution of the taste groups I–VI as well as the individual compounds by means of taste omission experiments. To achieve this, individual taste recombinants either lacking in a taste group or in one or more individual taste compounds were evaluated by means of triangle tests using two samples of the complete taste reconstitute as the control. Those panelists, who detected the taste difference correctly, were asked to rate the intensity of the key taste descriptors astringent, bitter, and sour on a scale from 0 to 5.

In a first set of experiments, the complete taste group I, containing the bitter-tasting theobromine, caffeine, diketopiperazines 26–50, as well as amino acids, was omitted from the taste recombinant. As given in Table 3, all eight panelists were able to detect this sample in a triangle test with two samples of the complete recombinant as the reference. These panelists rated the partial recombinant as being significantly less intense in bitterness (–3.2) when compared to the total recombinant and detected the loss of the balanced, cocoa-typical bitter quality. To investigate the taste contribution of the individual taste classes in tastant group I, additional partial recombinants were prepared lacking in either alkaloids, diketopiperazines, or amino acids. Seven of eight panelists successfully detected the omission of the alkaloids theobromine and caffeine from the complete taste recombinant and described this partial recombinant as being significantly less bitter (–2.7) (Table 3). In addition, the sensory panel reported that the partial recombinant exhibited a delayed

**Table 3.** Influence of Tastant Groups or Individual Taste Compounds on the Taste Profile of the Taste Recombinant

tastants omitted	number of panelists <sup>a</sup> that detect a difference	description of taste difference <sup>b</sup> (change in intensity)
taste group I	8	less bitter (3.7 → 0.5), loss of typical cocoa-type bitter quality
theobromine, caffeine	7	less bitter (3.7 → 1.0)
diketopiperazines 26–50	7	late bitterness, long on-set less bitter (3.7 → 2.9) caffeine-like bitterness, short on-set
bitter amino acids	0	nd
taste group II	8	less bitter (3.7 → 3.2) and astringent (3.0 → 1.8)
monomers 1 and 2	6	less bitter (3.7 → 3.3) and astringent (3.0 → 2.0)
oligomers 3–6 and 8	5	less bitter (3.7 → 3.5) and astringent (3.0 → 2.2)
polymers 7	0	nd
taste group III	8	less astringent (3.0 → 1.8)
amino acid amides 16–25	6	less astringent (3.0 → 2.2)
$\gamma$ -aminobutyric acid and glycosides 9–15	5	less astringent (3.0 → 2.5)
taste group IV	8	loss of sourness (2.6 → 0.2)
taste group V	0	nd
taste group VI	0	nd

<sup>a</sup> Eight panelists were asked to detect the recombinant lacking certain tastants by means of a triangle test. <sup>b</sup> If the sample was detected correctly (cf. footnote a in **Table 3**), the changes in taste intensities should be evaluated on a scale from 0 (not detectable) to 5 (strongly detectable). nd = no difference describable.

bitter perception with a rather late on-set. These findings are well in line with the high DoT factors calculated for the alkaloids (cf. **Table 1**). Also, seven of eight panelists successfully detected the omission of the diketopiperazines from the complete recombinant with significantly less intense bitterness (−0.8) and reported that the omission of diketopiperazines induced a change of the balanced, cocoa-like bitter taste to a caffeine-like bitterness with rapid on-set (**Table 3**). Although just one diketopiperazine, namely, the cyclo(L-Pro-L-Val), was evaluated with a relatively high DoT factor above 1, it is obvious that diketopiperazines are essential for a balanced cocoa bitter quality. In contrast, omission of bitter-tasting amino acids from the taste recombinant was not detectable by any of the sensory panelist, thus excluding the amino acids as contributors to the taste of roasted cocoa nibs. Consequently, theobromine, caffeine, and diketopiperazines have been elucidated as key contributors to the bitter taste of roasted cocoa nibs, thus confirming earlier suggestions in the literature (1, 2).

In a second experiment, taste group II, containing the puckering astringent and bitter-tasting monomeric and oligomeric flavan-3-ols 1–8, was omitted from the complete taste recombinant. As shown in **Table 3**, all eight panelists were able to pick up the taste difference by means of a triangle test using two samples of the complete recombinant as the control. The panelist rated this partial recombinant as being significantly less astringent (−1.2) and less bitter (−0.5) than the total recombinant, thus fitting well with the high DoT factors calculated for the flavan-3-ols (**Table 1**). To further clarify the taste contribution of the individual compounds in taste group II, partial recombinants lacking either the monomers, catechin and epicatechin, the dimeric to the pentameric procyanidins, or the polymeric procyanidins from hexamers to decamers were compared to the complete recombinant by means of triangle tests. Six of eight panelists detected the omission of the monomers and evaluated the taste difference with a modest reduction in bitterness (−0.4) and astringency (−1.0). Five of eight panelists detected the lack of dimeric to pentameric procyanidins and described the taste difference with a slight reduction in bitter taste intensity (−0.2) as well as astringency (−0.8) (**Table 3**). It is interesting to note that omission of the procyanidin polymer fraction, containing hexamers to decamers, was not detectable by any of the sensory panelists, thus excluding these high-molecular-weight polyphenols as key

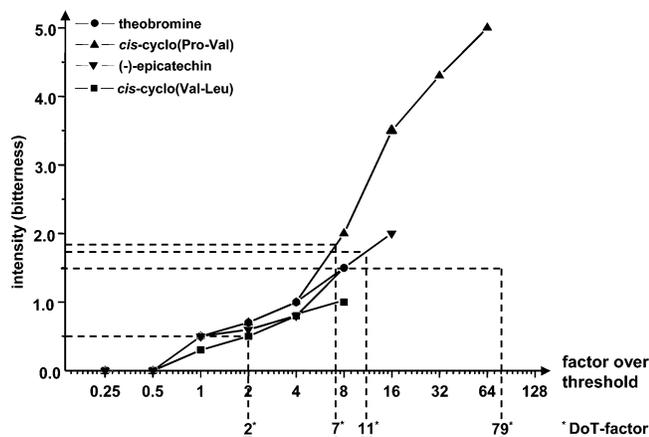
contributors to the taste of roasted cocoa beans. Consequently, these findings confirm catechin and epicatechin as well as dimeric to pentameric procyanidins as contributors to the astringency and bitter taste of roasted cocoa nibs, whereas the procyanidin oligomers above the pentamer do not show major taste contributions to astringency or to the bitter taste of cocoa.

To investigate the importance of molecules exhibiting just astringency without bitterness, either the taste group III, the puckering astringent *N*-phenylpropenoyl-L-amino acids 16–25, or the velvety astringent polyphenol glycosides 9–15 and mouth-drying  $\gamma$ -aminobutyric acid and  $\beta$ -aminoisobutyric acid were omitted from the complete taste recombinant (**Table 3**). All eight panelists were able to detect the lack of the whole group III, and these panelists rated the partial recombinant as being less astringent (−1.2) compared to the total recombinant. Six or five of eight panelists were able to detect a taste difference when the *N*-phenylpropenoyl acid amides or the glycosides,  $\gamma$ -aminobutyric acid, and  $\beta$ -aminoisobutyric acid were omitted from the complete taste recombinant. The panelists described both partial recombinants as being less astringent; the intensity for astringency dropped by 0.8 and 0.5 scores when the *N*-phenylpropenoyl acid amides and the glycosides were omitted, thus indicating that both classes of astringent compounds influence the astringency of roasted cocoa nibs.

In a fourth experiment, the sour-tasting group IV, containing organic acids, phosphoserine, and phosphoethanolamine, were omitted from the complete taste recombinant. As shown in **Table 3**, all eight panelists were able to detect this sample by means of a triangle test using two samples of the complete recombinant as the control. The panelist did not detect any sourness in this partial recombinant, thus fitting well with the high DoT factor calculated for the organic acids.

Finally, partial recombinates have been prepared lacking in either the sweet-tasting group V or the umami-like-tasting group VI. As given in **Table 3**, none of the eight panelists was able to detect any taste difference between these partial recombinates and the complete taste recombinant. Consequently, the sweet-tasting amino acids and soluble carbohydrates as well as the umami-like-tasting amino acids do not contribute to the taste of the aqueous suspension of roasted cocoa beans.

When all of these findings are taken into account, it can be concluded that alkaloids, diketopiperazines, monomeric to pentameric flavan-3-ols, *N*-phenylpropenoyl-L-amino acids,



**Figure 3.** Human dose/response functions of selected key bitter taste compounds of roasted cocoa nibs. The end of each curve represents the solubility maximum of each compound.

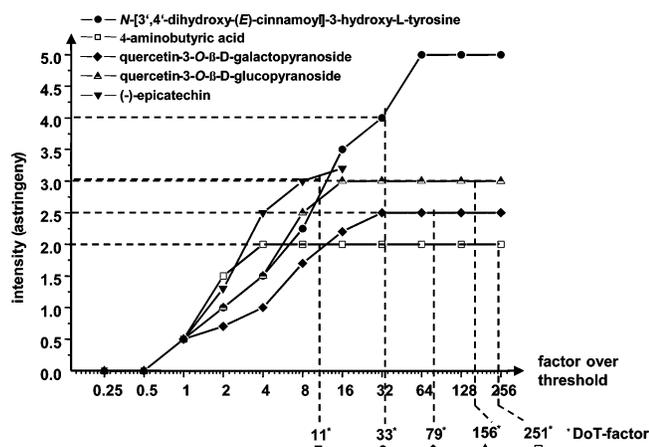
polyphenol glycosides, organic acids,  $\gamma$ -aminobutyric acid, and  $\beta$ -aminoisobutyric acid are the key taste compounds of roasted cocoa nibs, whereas soluble carbohydrates and all of the other amino acids do not have any significant taste impact. To further narrow down the number of key taste compounds, finally, a reduced recombinant was prepared containing the taste compounds evaluated with DoT factors  $\geq 0.5$ , with the exception of the procyanidin polymers (7), which already have shown to be not important for cocoa taste. The reduced recombinant contained the bitter-tasting alkaloids, theobromine and caffeine, the bitter-tasting diketopiperazines, 28–31, 36, 37, and 41, the puckering-astringent and bitter-tasting flavan-3-ols, 1–6 and 8, the polyphenol glycosides, 9–12, as well as the puckering astringent *N*-phenylpropenoyl amino acid amides, 17, 18, and 22–25, the sour-tasting citric acid, acetic acid, succinic acid, malic acid, lactic acid, oxalic acid,  $\gamma$ -aminobutyric acid, and  $\beta$ -aminoisobutyric acid. The taste profile of the partial recombinant containing these 34 taste compounds was then evaluated by the sensory panel. As given in Table 2, the taste profile of this partial recombinant did not differ from that of the complete taste recombinant containing all 84 taste compounds. Consequently, these 34 compounds were successfully identified as the key taste compounds contributing to the bitterness, sourness, and astringency of roasted cocoa nibs.

Although the calculated DoT factors for the astringent compounds in group III, in particular, the high values of 251 and 156 for  $\gamma$ -aminobutyric acid and quercetin-3-*O*- $\beta$ -D-glucopyranoside, were by far higher than those determined for the flavan-3-ols in group II, the omission of taste groups II and III, respectively, induced the same decrease in astringency intensity by 1.2 scores when compared to the total taste recombinant (Tables 1 and 3). Furthermore, a comparison of the DoT factors of the *N*-phenylpropenoyl amino acids showing DoT factors between 0.5 and 33 with those of 251 and 156 for  $\gamma$ -aminobutyric acid and quercetin-3-*O*- $\beta$ -D-glucopyranoside suggested the predominant taste contribution of the later compounds. However, the omission experiments clearly demonstrated that the omission of *N*-phenylpropenoyl amino acids induced a more pronounced reduction in astringency ( $-0.8$ ) when compared to the omission of  $\gamma$ -aminobutyric acid and polyphenol glycosides ( $-0.5$ ). To obtain more detailed insight into the human perception of the cocoa taste compounds, human dose/response functions were recorded in the following.

**Human Dose/Response Functions.** Aimed at recording human dose/response functions for selected cocoa bitter and astringent taste compounds (Figure 3, 4), serial aqueous 1:1

dilutions of the samples were prepared by starting at the solution of maximum solubility of the individual compound and ending at the concentration level two steps below the individual recognition threshold concentration. Independent of their sensory training status, panelists have difficulties in memorizing the intensity of a taste compound for a longer period of time; they are known to give different ratings for the same solution of the test compound tasted at different time intervals. Consequently, recording dose/response functions with standard sensory methodologies usually leads to unreliable curves with very high error margins. To overcome the carry-over problem mentioned above, we therefore applied the recently reported half-tongue testing (14), thus offering the possibility of a direct comparison of the sensory impact of two samples. On a five-point numerical scale with 0.25 scale subunits, human dose/response functions were determined for each individual subject for the bitter taste compounds theobromine, cyclo(L-Pro-L-Val), cyclo(L-Val-L-Leu), and (-)-epicatechin, evaluated with the highest DoT factors, using standard solutions of salicin as the reference to define the bitter intensity represented by the individual scores (Figure 3). After the taste intensity of each compound at its maximum solubility had been rated, the taste intensities of the other dilutions were determined by using the half-tongue tasting method so that one dilution of an individual compound was rated against the intensity of another dilution of the same compound and the intensity of this solution was approximated by a comparison to the taste intensity of aqueous solutions containing the reference compound salicin in defined concentrations. Human response functions with DoT factors on the *x* axis and taste intensities on the *y* axis were recorded for each individual subject in triplicates. The intensity values between trained individuals and separate sessions did not differ more than  $\pm 0.4$  units (Figure 3).

The results, outlined in Figure 3, clearly demonstrated that the gustatory response for different bitter key taste compounds identified in roasted cocoa nibs is following rather different dose/response functions. On the five-point scale, cyclo(L-Pro-L-Val) was evaluated with the highest taste intensity of 5.0 at its maximum solubility of 81.9 mmol/L, exceeding the threshold concentration by 64-fold. In comparison, cyclo(L-Val-L-Leu) and theobromine or (-)-epicatechin reached their solubility level already 8- or 16-fold above their individual taste threshold concentrations. Because the gustatory response at the factor over threshold, displayed on the *x* axis in Figure 3, is reflecting the taste intensity of a compound at its calculated DoT factor (Table 1), the taste activity of each individual compound can be reliably elucidated at its "natural" concentration level in roasted cocoa. For example, at its DoT factor of 6.9, cyclo(L-Pro-L-Val) was evaluated with an intensity score of 1.8. Although (-)-epicatechin was rated with a higher DoT factor of 10.8, the taste intensity of that flavan-3-ol was evaluated with a somewhat lower intensity score of 1.7. Even more interestingly, theobromine reached its maximum solubility already at a concentration level of 8-fold above its threshold concentration. Therefore, on the basis of its high DoT factor of 79.4, calculated on the basis of quantitative data and threshold values, the taste contribution of theobromine is strongly overrated, because this compound reached maximum solubility at 8-fold threshold concentrations, imparting a bitter taste intensity of 1.5 only. In conclusion, the DoT factors alone do not allow a reliable evaluation of the taste impact of taste compounds despite the higher DoT factors determined for theobromine and (-)-epicatechin; the cyclo(L-Pro-L-Val) was demonstrated to induce a stronger human response of bitterness. Contrary to literature data (2, 3), it has to be concluded that, of the group of bitter compounds, the 2,5-diketopiperazine cyclo(L-Val-L-Pro) is the single most important



**Figure 4.** Human dose/response functions of selected key astringent compounds of roasted cocoa nibs. The end of each curve represents the solubility maximum of each compound.

bitter compound contributing to the bitter taste of an aqueous suspension of roasted cocoa.

Using the same approach, human dose/response functions were recorded for the astringent sensation of (–)-epicatechin,  $\gamma$ -aminobutyric acid, quercetin-3-*O*- $\beta$ -D-glucopyranoside, quercetin-3-*O*- $\beta$ -D-galactopyranoside, and *N*-(*E*)-caffeoyl-L-3-hydroxy-tyrosine, for which the highest DoT factors were calculated on the basis of quantitative data and threshold concentrations. As outlined in **Figure 4**, also for the individual astringent compounds, the gustatory response is following rather different dose/response functions. Apart from (–)-epicatechin, which reached its maximum solubility at 16-fold of the threshold concentration, all of the other astringent compounds were soluble up to concentrations of at least 256 times above their individual taste thresholds. The highest taste intensity rated with a score of 5.0 was observed for an aqueous solution of *N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-3-hydroxy-tyrosine, exceeding the threshold concentration by 64-fold. In the 16-fold threshold concentration, *N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-3-hydroxy-tyrosine was evaluated with a score of 3.5 for astringency, followed by (–)-epicatechin of 3.2, quercetin-3-*O*- $\beta$ -D-glucopyranoside of 3.0, quercetin-3-*O*- $\beta$ -D-galactopyranoside of 2.2, and  $\gamma$ -aminobutyric acid of 2.0 (**Figure 4**). Surprisingly,  $\gamma$ -aminobutyric acid already approximated its maximum gustatory response after exceeding its threshold concentration by a factor of 4, thus demonstrating that the taste impact expected from the extraordinarily high DoT factor of 251 calculated for that amino acid in cocoa nibs is strongly overrated. The same was observed for quercetin-3-*O*- $\beta$ -D-glucopyranoside, the bio-response of which approximated a maximum intensity of 3.0 already at a 16-fold threshold concentration. Although a high DoT factor of 156 was calculated for that compound, it can just reach a maximum taste intensity of 3.0. Also for the *N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-3-hydroxy-tyrosine, we found saturation but not before exceeding the threshold concentration by a factor of 64. Consequently, *N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-3-hydroxy-tyrosine, which was evaluated with a DoT factor of 33, induced a human bioresponse with an intensity of 4.0. In contrast to the literature (5, 6), it can be concluded that, of the group of key astringent compounds, *N*-[3',4'-dihydroxy-(*E*)-cinnamoyl]-L-3-hydroxy-tyrosine is the single most important compound contributing to the astringent taste sensation of an aqueous suspension of roasted cocoa.

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