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Structures, Sensory Activity, and Dose/Response Functions of 2,5-Diketopiperazines in Roasted Cocoa Nibs (*Theobroma cacao*)

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The taste compounds inducing the blood-like, metallic bitter taste sensation reported recently for a dichloromethane extract prepared from roasted cocoa nibs were identified as a series of 25 diketopiperazines by means of HPLC degustation, LC−MS/MS, and independent synthesis. Among these 25 compounds, 13 cis-configured diketopiperazines, namely, cyclo(L-Ile-L-Phe), cyclo(L-Val-L-Leu), cyclo(L-Pro-L-Pro), cyclo(L-Ile-L-Pro), cyclo(L-Val-L-Tyr), cyclo(L-Ala-L-Tyr), cyclo(L-Phe-L-Ser), cyclo(L-Ala-L-Ile), cyclo(L-Leu-L-Phe), cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Thr), cyclo(L-Pro-L-Tyr), and cyclo(L-Val-L-Val) were identified for the first time in cocoa. In addition, the taste recognition thresholds for the metallic as well as the bitter taste of the diketopiperazines were determined, and after quantitative analysis by using two diastereomeric diketopiperazines as the internal standards, the sensory impact of the diketopiperazines was evaluated on the basis of their dose-over-threshold (DoT) factors calculated as the ratio of the concentration and the threshold concentration of a compound. These data revealed DoT factors above 1.0 exclusively for *cis*-cyclo(L-Pro-L-Val), *cis*-cyclo(L-Val-L-Leu), *cis*-cyclo(L-Ala-L-Ile), *cis*-cyclo(L-Ala-L-Leu), and *cis*-cyclo(L-Ile-L-Pro), whereas all of the other diketopiperazines were present below their individual bitter taste threshold concentrations and should therefore not contribute to the cocoa taste. Because the DoT factors do not consider the nonlinear relationship between the concentration and gustatory response of an individual compound, we, for the first time, report on the recording of dose/response functions describing the human bitter taste perception of diketopiperazines more precisely.

KEYWORDS: Cocoa; bitter taste; metallic taste; *cis*-cyclo(L-Pro-L-Val); diketopiperazines; threshold concentrations; dose/response functions

INTRODUCTION

Besides the pleasant aroma, the sensory quality of the fermented and roasted seeds of *Theobroma cacao* is mainly driven by its alluring gustatory profile centering around the typical bitter taste, a slight sour note, as well as its long-lasting astringent and mouth-drying sensation. These enhance the complexity and palate length of cocoa beverages and chocolate confectionary.

Because of its low detection threshold of 10 mg/L in water (*1*), the methylxanthine theobromine, which is present in cocoa beans in concentrations between 1.8 and 3.8 g/100 g of dry weight (*2*), is believed to contribute to the typical bitter taste. In addition, some diketopiperazines, generated during the roasting of the fermented cocoa beans from hydrophobic amino acids, are believed to contribute to the bitter taste of roasted cocoa (*3*). Among these dilactams, *cis*-cyclo(Ala-Gly) and *cis*-cyclo(Ala-Val) have been found as the predominating diketopiperazines in roasted cocoa samples (*3*). In contradiction to these findings, recent studies identified *cis*-cyclo(Pro-Gly) as the major diketopiperazine in cocoa (*4*). Although the diketopiperazines *cis*-cyclo(Val-Phe) and *cis*-cyclo(Ala-Phe) have been detected in roasted cocoa only in trace amounts, model experiments demonstrated that these diketopiperazines, when mixed with theobromine, induce a bitter taste sensation similar to that perceived from an aqueous suspension of cocoa powder (*3*). High-performance liquid chromatography in combination with mass spectrometry using a moving belt interface was used to monitor diketopiperazines in cocoa powders (*5*), but a comparison of quantitative data and the sensorially perceived bitter intensity of the products led to the conclusion that further systematic studies are necessary to understand the typical taste of roasted cocoa on a molecular level (*4*).

With the aim at investigating the key molecules imparting the typical bitterness and astringent taste sensation of roasted cocoa, very recently, an aqueous acetone extract prepared from

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powdered roasted cocoa nibs and reflecting the typical taste profile of roasted cocoa was sequentially extracted with dichloromethane and ethyl acetate and, after removing the solvents in high vacuum, the dichloromethane solubles, the ethyl acetate extractables, as well as the water solubles were evaluated sensorially in aqueous solution by means of a comparative taste profile analysis (*6*). The highest scores for bitter taste, astringency, and sour taste were found for the water solubles as well as the ethyl acetate extractables, whereas the dichloromethane solubles exhibited a metallic-like bitter taste perceived with high taste intensity (*6*). Activity-guided analysis of the key taste contributors in the water as well as the ethyl acetate fraction revealed that, besides the theobromine and caffeine, the flavan-3-ols epicatechin, catechin, procyanidin B-2, procyanidin B-5, procyanidin C-1, [epicatechin-(4β → 8)]$_3$-epicatechin, and [epicatechin-(4β → 8)]$_4$-epicatechin were among the key compounds contributing to the bitter taste as well as the astringent mouth feel. In addition, a series of quercetin, naringenin, luteolin, and apigenin glycopyranosides imparting a smooth, velvety type of astringent sensation as well as a family of *N*-phenylpropenoyl amino acids such as (+)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid and (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine, inducing a puckering type of astringency, have been identified as contributors to the astringent taste of cocoa (*6*, *7*). In contrast, the molecules inducing the bloody, metallic bitter taste of the dichloromethane fraction still need to be investigated.

With the aim at completing the knowledge on the taste active nonvolatiles in roasted cocoa, the objectives of the present investigation were, therefore, to identify and quantify the metallic- and bitter-tasting compounds in the dichloromethane solubles isolated from fermented and roasted cocoa, to determine their human threshold concentrations and to evaluate their taste contribution based on dose/response considerations.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: caffeine, theobromine, triethylamine (Fluka, Neu-Ulm, Germany), 2,2-dimethoxypropane, palladium 10% on charcoal (Sigma−Aldrich, Steinheim, Germany), hydrochloric acid (Merck, Darmstadt, Germany), *Z*-IIe-Pro-OH, *Z*-Ala-Val-OH, *Z*-Ala-Pro-OH, *Z*-Pro-Pro-OH, *Z*-Val-Leu-OH, *Z*-Ala-Leu-OH, *Z*-Val-Tyr-OH, *Z*-Ala-IIe-OH, *Z*-Val-Phe-O-CH_3, *Z*-IIe-Phe-O-CH_3, *Z*-Ala-Phe-O-CH_3, *Z*-Asn-Phe-O-CH_3, *Z*-Ala-Tyr-O-CH_3, *cis*-cyclo(Gly-Pro) (**1**), *cis*-cyclo(Phe-Pro) (**2**), *cis*-cyclo(Leu-Pro) (**3**), *cis*-cyclo(Gly-Phe) (**4**), *cis*-cyclo(Asp-Phe) (**5**), *cis*-cyclo(Leu-Gly) (**6**), *cis*-cyclo(Pro-Thr) (**7**), *cis*-cyclo(Pro-Tyr) (**8**), *cis*-cyclo(Leu-Phe) (**9**), *cis*-cyclo(Pro-Val) (**10**), *cis*-cyclo(Phe-Ser) (**11**), *cis*-cyclo(Val-Val) (**12**), *cis*-cyclo(Ala-Ala) (**13**), *cis*-cyclo(Gly-Gly) (**14**), *cis*-cyclo(Ala-Gly) (**15**), *trans*-cyclo(D-Ala-L-Pro) (**16**), and *trans*-cyclo(D-Ala-L-Val) (**17**) (Bachem, Bubendorf, Switzerland). Solvents were of HPLC grade (Merck, Darmstadt, Germany). Deuterated solvents were obtained from Euroiso-Top (Gif-Sur-Yvette, France). Cocoa beans, fermented in Ghana for 5 days, were roasted by the food industry. 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.

Sensory Analyses. *Training of the Sensory Panel.* A total of 12 subjects (5 women and 7 men, ages 25−38 years) with no history of known taste disorders were trained to evaluate the taste of aqueous solutions (2 mL each) of the following standard taste compounds in bottled water adjusted to pH 6.0 with aqueous hydrochloric acid (0.1 mol/L) or aqueous sodium hydroxide solution (0.1 mmol/L) by using a triangle test as described below: sucrose (25 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, gallustannic acid (0.05%) and quercetin-3-*O*-β-D-glucopyranoside (0.002 mmol/L) for the puckering astringency and the velvety astringent, mouth-drying oral sensation, respectively, and iron-(II) gluconate (0.03 mmol/L) for metallic taste. The assessors had participated earlier at regular intervals for at least 2 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.

Determination of Taste Threshold Concentrations. Taste recognition thresholds, which means the concentrations at which the typical taste qualities of the compounds were just detectable, were determined in bottled water by means of a triangle test as reported recently (*6*). The values between individuals and between three separate sessions differed by not more than plus or minus one dilution step; that is, a threshold value of 40 μmol/L for the metallic taste of *cis*-cyclo(L-Leu-L-Phe) represents a range from 20 to 80 μmol/L.

Recording of Dose/Response Functions. Serial 1:1 dilutions of the samples in water were prepared starting at the solution of maximum solubility of each individual taste compound 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 were compared at the highest concentration level by means of the recently reported half-tongue tasting method (*6*, *8*), thus offering a direct comparison of the taste impact and a reliable evaluation of the gustatory response of different compounds. To achieve this, the solutions of the individual compounds were applied in binary combinations to one side of the tongue and the panelist were asked to determine which side showed the stronger sensation. On a five-point scale, the compound evaluated with the highest taste intensity at its maximum concentration, that is, *cis*-cyclo(L-Val-L-Pro) at its maximum solubility of 81.92 mmol/L (water) in the present study, was set to the maximum score of 5.0. 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 by cross-checking the taste intensity between the different compounds at the same dilution. The dose/response functions of three individual sessions were averaged. The intensity values between trained individuals and separate sessions did not differ more than ±0.3 units.

Synthesis of 2,5-Diketopiperazines. After a synthetic strategy, reported in the literature (*3*, *9*) with some modifications, 2,2-dimethoxypropane (0.6 mmol) and hydrochloric acid (125 μL, 32%) were added to a solution of the corresponding dipeptide (1.2 mmol) *Z*-IIe-Pro-OH, *Z*-Ala-Val-OH, *Z*-Ala-Pro-OH, *Z*-Pro-Pro-OH, *Z*-Val-Leu-OH, *Z*-Ala-Leu-OH, *Z*-Val-Tyr-OH, or *Z*-Ala-IIe-OH in methanol (2.0 mL). After stirring the mixture for 24 h at room temperature, the solvent was removed in a vacuum and the residue was dissolved in a minimum amount of absolute methanol. Addition of diethyl ether (50 mL) resulted in crystallization of the product; the supernatant diethyl ether was discarded; the procedure was repeated twice; and then the sample was freed from solvent in a vacuum. The dipeptide methyl ester obtained was dissolved in methanol (80 mL), and the N-protecting group was removed upon hydrogenation with hydrogen (5 bar) using palladium (10% on charcoal; 100 mg) as the catalyst. After 30 min, the catalyst was filtered off, triethylamine (2 mmol) was added, and the solution was stirred for 48 h. The solvent was then removed in a vacuum, and the residue obtained was taken up in a mixture (20:80, v/v; 5 mL) of methanol and aqueous formic acid (0.1%, pH 2.5) and was applied onto the top of a water-cooled glass column (140 × 40 mm i.d.) filled with a slurry of LiChroprep 25−40 μm RP-18 material (Merck, Darmstadt, Germany) in the same solvent mixture. When the effluent was monitored at 220 nm, chromatography was performed starting with a mixture (20:80, v/v) of methanol and aqueous formic acid (0.1%, pH 2.5), thereafter increasing the methanol content to 70%. The fractions containing the title compounds were freed from solvent in a vacuum, freeze-dried, and finally purified by means of automated HPLC (Jasco, Groβ-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) using a semipreparative RP-18 column, ODS-Hypersil, 250 × 10 mm, 5 μm (ThermoHypersil, Kleinostheim, Germany) as the stationary phase and

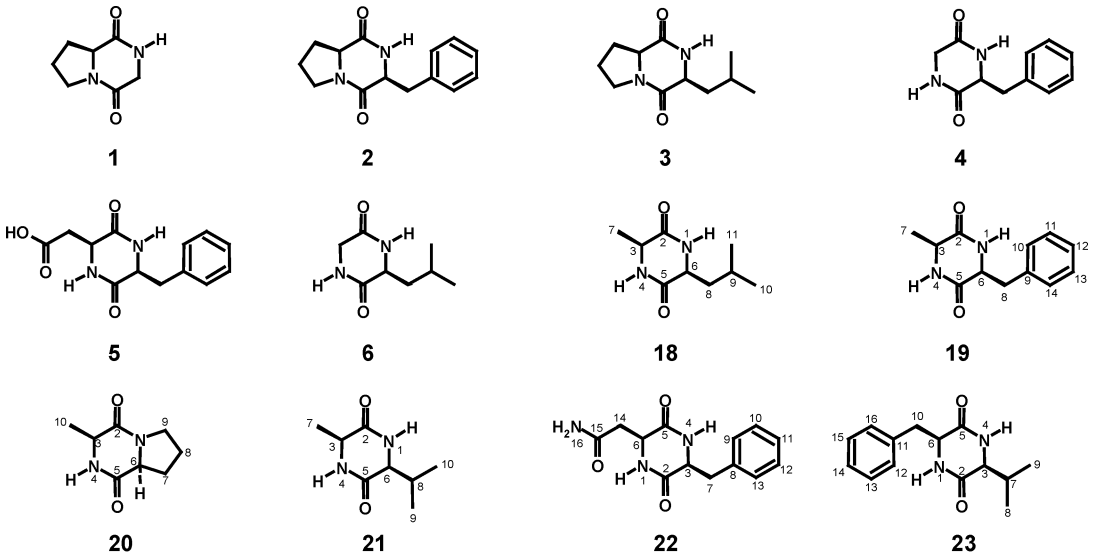

Figure 1. Chemical structures of cis-configured 2,5-diketopiperazines reported earlier in roasted cocoa.

Table 1. Mass Spectrometric Data of 2,5-Diketopiperazines[a]

2,5-diketopiperazine	*m/z* of $[M + 1]^+$	*m/z* (%) of product ion
cis-cyclo(Gly-L-Pro) (**1**)	155	70 (100), 155 (90), 127 (53), 82 (28), 113 (10), 58 (6)
cis-cyclo(L-Phe-L-Pro) (**2**)	245	120 (100), 70 (75), 245 (53), 154 (51), 217 (17), 172 (11), 98 (7)
cis-cyclo(L-Leu-L-Pro) (**3**)	211	70 (100), 183 (37), 138 (29), 86 (28), 26 (211), 26 (114), 155 (15), 98 (12)
cis-cyclo(Gly-L-Phe) (**4**)	205	120 (100), 132 (28), 114 (25), 149 (21), 177 (20), 205 (20), 91 (18)
cis-cyclo(L-Asp-L-Phe) (**5**)	263	175 (100), 203 (84), 91 (44), 245 (28), 130 (24), 263 (21), 158 (20), 172 (12), 120 (8)
cis-cyclo(L-Leu-Gly) (**6**)	171	86 (100), 72 (19), 69 (15), 114 (14), 126 (14), 171 (13), 143 (5)
cis-cyclo(L-Pro-L-Thr) (**7**)	199	153 (100), 70 (27), 125 (20), 181 (17), 199 (11), 97 (7), 74 (6)
cis-cyclo(L-Pro-L-Tyr) (**8**)	261	136 (100), 216 (46), 70 (28), 107 (19), 155 (18), 233 (6)
cis-cyclo(L-Leu-L-Phe) (**9**)	261	120 (100), 86 (55), 188 (40), 261 (39), 114 (32), 216 (29), 233 (24), 170 (5)
cis-cyclo(L-Val-L-Pro) (**10**)	197	70 (100), 124 (72), 72 (50), 100 (31), 197 (31), 169 (26), 98 (20), 154 (15), 141 (13)
cis-cyclo(L-Phe-L-Ser) (**11**)	235	120 (100), 162 (26), 144 (15), 235 (14), 207 (11), 147 (6)
cis-cyclo(L-Val-L-Val) (**12**)	199	72 (100), 126 (54), 199 (35), 171 (30), 100 (24), 154 (23)
cis-cyclo(L-Ala-L-Ala) (**13**)	143	72 (100), 98 (73), 70 (56), 143 (27), 55 (11), 115 (10)
cis-cyclo(Gly-Gly) (**14**)	115	58 (100), 59 (66), 115 (65), 87 (50)
cis-cyclo(L-Ala-Gly) (**15**)	129	72 (100), 84 (80), 55 (60), 101 (39), 129 (38)
trans-cyclo(D-Ala-L-Pro) (**16**)	169	70 (100), 169 (24), 141 (12), 96 (11), 72 (9), 113 (5), 98 (5)
trans-cyclo(D-Ala-L-Val) (**17**)	171	72 (100), 98 (81), 126 (41), 100 (37), 171 (17), 143 (12)
cis-cyclo(L-Ala-L-Leu) (**18**)	185	86 (100), 140 (50), 114 (37), 112 (26), 72 (21), 185 (20), 157 (7)
cis-cyclo(L-Ala-L-Phe) (**19**)	219	120 (100), 146 (9), 191 (6), 174 (6), 219 (5), 128 (4), 72 (3)
cis-cyclo(L-Ala-L-Pro) (**20**)	169	70 (100), 169 (24), 141 (12), 96 (11), 72 (9), 113 (5), 98 (5)
cis-cyclo(L-Ala-L-Val) (**21**)	171	72 (100), 98 (81), 126 (41), 100 (37), 171 (17), 143 (12)
cis-cyclo(L-Asn-L-Phe) (**22**)	262	203 (100), 245 (65), 175 (53), 262 (31), 130 (20), 120 (8), 91 (7)
cis-cyclo(L-Val-L-Phe) (**23**)	247	120 (100), 174 (63), 219 (46), 72 (23), 247 (21), 202 (17), 156 (14), 100 (10), 148 (7)
cis-cyclo(L-Ala-L-Ile) (**24**)	185	86 (100), 140 (50), 114 (37), 112 (26), 72 (21), 185 (20), 157 (7)
cis-cyclo(L-Ala-L-Tyr) (**25**)	235	107 (100), 135 (78), 235 (30), 129 (20), 162 (16), 207 (9)
cis-cyclo(L-Val-L-Leu) (**26**)	213	72 (100), 86 (86), 140 (60), 213 (50), 100 (35), 168 (35), 185 (35), 84 (18), 113 (17)
cis-cyclo(L-Val-L-Tyr) (**27**)	263	136 (100), 235 (85), 263 (57), 72 (28), 107 (15)
cis-cyclo(L-Ile-L-Phe) (**28**)	261	120 (100), 86 (55), 188 (40), 261 (39), 114 (32), 216 (29), 233 (24), 170 (5)
cis-cyclo(L-Ile-L-Pro) (**29**)	211	70 (100), 183 (37), 138 (29), 86 (28), 26 (211), 26 (114), 155 (15), 98 (12)
cis-cyclo(L-Pro-L-Pro) (**30**)	195	195 (100), 70 (19), 98 (18)
trans-cyclo(L-Val-D-Phe) (**31**)	247	120 (100), 174 (63), 219 (46), 72 (23), 247 (21), 202 (17), 156 (14), 100 (10), 148 (7)

[a] MS data were obtained by ESI^+ ionization.

a mixture of methanol and aqueous formic acid (0.1% in water, pH 2.5) as the mobile phase. After the solvents were removed in high vacuum (10^{-6} Pa), the title compounds were suspended in water (10 mL) and freeze-dried 3 times to afford the corresponding 2,5-diketopiperazines as white, amorphous powders in high purities of more than 99%.

(3*S*,6*S*)-3-Methyl-6-(2-methylpropyl)-piperazine-2,5-dione, *cis*-cyclo(L-Ala-L-Leu), **18** (**Figure 1**). Yield: 0.82 mmol (68%). LC−MS (ESI^+) *m/z*: 185 (100, $[M + 1]^+$). LC−MS/MS (ESI^+): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 0.86 [d, 3H, J = 6.6 Hz, H-C(10/11)], 0.88 [d, 3H, J = 6.6 Hz, H-C(10/11)], 1.27 [d, 3H, J = 7.0 Hz, H-C(7)], 1.47 [m, 1H, H-C(8a)], 1.62 [m, 1H, H-C(8b)], 1.83 [m, 1H, H-C(9)], 3.77 [m, 1H, H-C(6)], 3.86 [m, 1H, H-C(3)], 8.06 [s, 1H, H-N(4)], 8.07 [s, 1H, H-N(1)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 19.7 [C(7)], 22.0 [C(10/11)], 23.1 [C(10/11)], 23.8 [C(9)], 42.7 [C(8)], 50.1 [C(3)], 52.8 [C(6)], 168.5 [C(5)], 169.0 [C(2)].

(3*S*,6*S*)-3-Methyl-6-(phenylmethyl)-piperazine-2,5-dione, *cis*-cyclo(L-Ala-L-Phe), **19** (**Figure 2**). Yield: 1.04 mmol (87%). LC−MS (ESI^+) *m/z*: 219 (100, $[M + 1]^+$). LC−MS/MS (ESI^+): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 0.49 [d, 3H, J = 7.0 Hz, H-C(7)], 2.87 [dd, 1H, J = 5.0, 13.4 Hz, H-C(8a)], 3.12 [dd, 1H, J = 3.8, 13.4 Hz, H-C(8b)], 3.63 [m, 1H, H-C(6)], 4.17 [m, 1H, H-C(5)], 7.22 [m, 5H, H-C(10−14)], 7.98 [s, 1H, H-N(1)], 8.06 [s, 1H, H-N(4)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 19.6 [C(7)], 38.2 [C(8)], 49.6 [C(6)], 55.3 [C(3)], 126.5 [C(12)], 127.9 [C(11, 13)], 130.3 [C(10, 14)], 136.0 [C(9)], 165.7 [C(2)], 167.6 [C(5)].

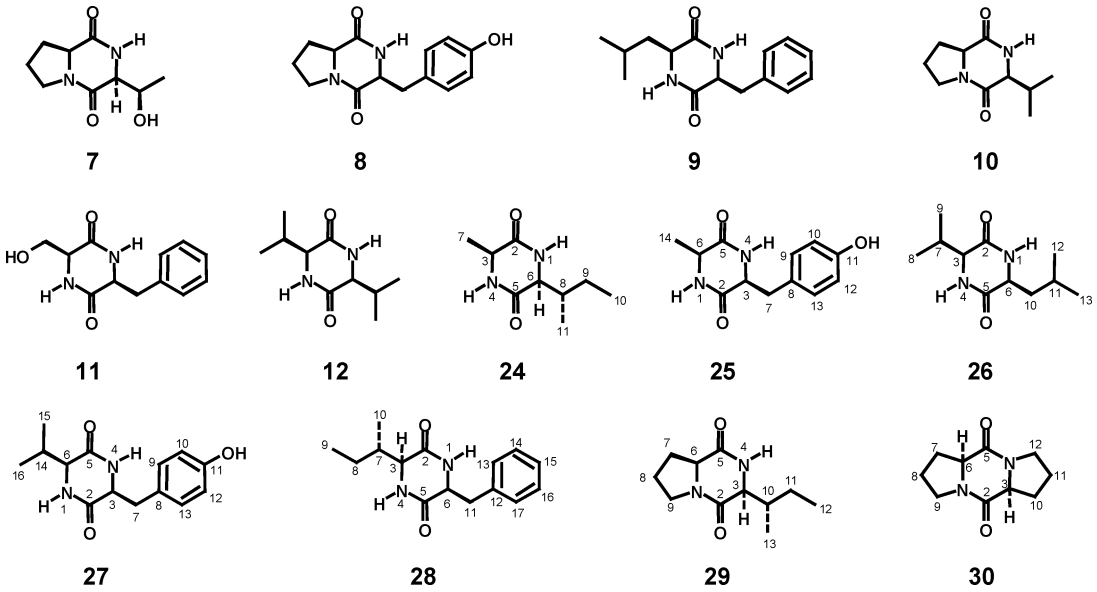

Figure 2. Chemical structures of cis-configured 2,5-diketopiperazines previously not reported in roasted cocoa.

(3*S*,6*S*)-3-Methyl-hexahydropyrrolo-[1,2-*a*]-pyrazine-1,4-dione, *cis*-cyclo(L-Ala-L-Pro), **20** (**Figure 1**). Yield: 0.72 mmol (60%). LC−MS (ESI^+) *m*/*z*: 169 (100, $[M + 1]^+$); LC−MS/MS (ESI^+): cf. **Table 1**. ^{1}H NMR (400 MHz, MeOH-d_4, COSY) δ: 1.38 [d, 3H, J = 6.8 Hz, H-C(10)], 1.90−2.01 [m, 3H, H-C(7a, 8)], 2.31 [m, 1H, H-C(7b)], 3.52 [m, 2H, H-C(9)], 4.18 [m, 1H, H-C(3)], 4.25 [m, 1H, H-C(6)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 15.7 [C(10)], 23.6 [C(8)], 29.2 [C(7)], 46.4 [C(9)], 52.1 [C(3)], 60.5 [C(6)], 169.0 [C(2)], 172.6 [C(5)].

(3*S*,6*S*)-3-Methyl-6-(1-methylethyl)-piperazine-2,5-dione, *cis*-cyclo-(L-Ala-L-Val), **21** (**Figure 1**). Yield: 0.42 mmol (35%). LC−MS (ESI^+) *m*/*z*: 171 (100, $[M + 1]^+$). LC−MS/MS (ESI^+): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 0.85 [d, 3H, J = 6.6 Hz, H-C(9/10)], 0.95 [d, 3H, J = 7.2 Hz, H-C(9/10)], 1.28 [d, 3H, J = 6.9 Hz, H-C(7)], 2.15 [m, 1H, J = 3.3, 6.9 Hz, H-C(8)], 3.68 [m, 1H, H-C(6)], 3.88 [q, 1H, J = 6.9 Hz, H-C(3)], 7.95 [s, 1H, H-N(1)], 8.01 [s, 1H, H-N(4)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 16.4 [C(9/10)], 18.0 [C(9/10)], 19.6 [C(7)], 30.6 [C(8)], 49.2 [C(3)], 58.9 [C(6)], 166.1 [C(5)], 168.2 [C(2)].

(3*S*,6*S*)-3-Phenylmethyl-6-amidocarboxymethyl-piperazine-2,5-dione, *cis*-cyclo(L-Asn-L-Phe), **22** (**Figure 1**). Yield: 1.08 mmol (90%). LC−MS (ESI^+) *m*/*z*: 262 (100, $[M + 1]^+$); LC−MS/MS (ESI^+): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 1.42 [dd, 1H, J = 8.6, 15.9 Hz, H-C(14a)], 2.29 [dd, 1H, J = 4.1, 15.9 Hz, H-C(14b)], 2.96 [dd, 1H, J = 4.9, 13.7 Hz, H-C(7a)], 3.10 [dd, 1H, J = 4.9, 13.7 Hz, H-C(7b)], 4.02 [m, 1H, H-C(6)], 4.18 [m, 1H, H-C(3)], 6.89 [s, 2H, H-N(16)], 7.25 [m, 5H, H-C(9−13)], 7.58 [s, 1H, H-N(1)], 8.08 [s, 1H, H-N(4)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 38.0 [C(7)], 38.1 [C(14)], 51.1 [C(6)], 55.1 [C(3)], 126.6 [C(11)], 128.0 [C(10, 12)], 130.0 [C(9, 13)], 136.3 [C(8)], 166.3 [C(2)], 166.8 [C(5)], 171.5 [C(15)].

(3*S*,6*S*)-3-(1-Methylethyl)-6-(phenylmethyl)-piperazine-2,5-dione, *cis*-cyclo(L-Val-L-Phe), **23** (**Figure 1**). Yield: 0.72 mmol (60%). LC−MS (ESI^+) *m*/*z*: 247 (100, $[M + 1]^+$). LC−MS/MS (ESI^+): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 0.31 [d, 3H, J = 6.9 Hz, H-C(8/9)], 0.67 [d, 3H, J = 6.9 Hz, H-C(8/9)], 1.70 [m, 1H, H-C(7)], 2.88 [dd, 1H, J = 5.0, 13.4 Hz, H-C(10a)], 3.14 [dd, 1H, J = 4.3, 13.4 Hz, H-C(10b)], 3.53 [m, 1H, H-C(6)], 4.20 [m, 1H, H-C(3)], 7.21 [m, 5H, H-C(12−16)], 7.88 [s, 1H, H-N(1)], 8.06 [s, 1H, H-N(4)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 17.1 [C(8/9)], 19.1 [C(8/9)], 31.9 [C(7)], 38.7 [C(10)], 55.9 [C(3)], 60.1 [C(6)], 127.3 [C(14)], 128.8 [C(13, 15)], 131.2 [C(12, 16)], 137.2 [C(11)], 167.3 [C(2)], 167.4 [C(5)].

(3*S*,6*S*)-3-Methyl-6-[(1*S*)-1-methylpropyl)]-piperazine-2,5-dione, *cis*-cyclo(L-Ala-L-IIe), **24** (**Figure 2**). Yield: 0.42 mmol (35%). LC−MS (ESI^+) *m*/*z*: 185 (100, $[M + 1]^+$). LC−MS/MS (ESI^+): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 0.85 [t, 3H, J = 7.4 Hz, H-C(10)], 0.91 [d, 3H, J = 7.2 Hz, H-C(11)], 1.18 [m, 1H, H-C(9a)], 1.27 [d, 3H, J = 7.0 Hz, H-C(7)], 1.41 [m, 1H, H-C(9b)], 1.85 [m, 1H, H-C(8)], 3.74 [m, 1H, H-C(6)], 3.88 [m, 1H, H-C(3)], 7.93 [s, 1H, H-N(1)], 8.07 [s, 1H, H-N(4)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 12.9 [C(10)], 15.1 [C(11)], 20.0 [C(7)], 24.3 [C(9)], 38.1 [C(8)], 49.8 [C(3)], 58.9 [C(6)], 166.7 [C(5)], 168.7 [C(2)].

(3*S*,6*S*)-3-[(4-hydroxyphenyl)methyl]-6-methyl-piperazine-2,5-dione, *cis*-cyclo(L-Ala-L-Tyr), **25** (**Figure 2**). Yield: 0.60 mmol (50%). LC−MS (ESI^+) *m*/*z*: 235 (100, $[M + 1]^+$). LC−MS/MS (ESI^+): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 0.54 [d, 3H, J = 7.1 Hz, H-C(14)], 2.73 [dd, 1H, J = 4.8, 13.6 Hz, H-C(7a)], 3.01 [dd, 1H, J = 3.6, 13.6 Hz, H-C(7b)], 3.60 [m, 1H, H-C(6)], 4.07 [m, 1H, H-C(3)], 6.65 [d, 2H, J = 8.5 Hz, H-C(10, 12)], 6.93 [d, 2H, J = 8.5 Hz, H-C(9, 13)], 7.97 [s, 1H, H-N(1)], 8.02 [s, 1H, H-N(4)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 20.0 [C(14)], 37.8 [C(7)], 49.9 [C(6)], 55.8 [C(3)], 115.0 [C(10, 12)], 126.1 [C(8)], 131.5 [C(9)], 156.3 [C(11, 13)], 166.1 [C(2)], 167.9 [C(5)].

(3*S*,6*S*)-3-(1-Methylethyl)-6-(2-methylpropyl)-piperazine-2,5-dione, *cis*-cyclo(L-Val-L-Leu), **26** (**Figure 2**). Yield: 0.67 mmol (56%). LC−MS (ESI^+) *m*/*z*: 213 (100, $[M + 1]^+$). LC−MS/MS (ESI^+): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 0.85 [d, 3H, J = 7.1 Hz, H-C(8/9)], 0.86 [d, 3H, J = 6.6 Hz, H-C(12/13)], 0.88 [d, 3H, J = 6.6 Hz, H-C(12/13)], 0.95 [d, 3H, J = 7.1 Hz, H-C(8/9)], 1.45 [m, 1H, H-C(10a)], 1.63 [m, 1H, H-C(10b)], 1.85 [m, 1H, H-C(11)], 2.11 [m, 1H, H-C(7)], 3.62 [m, 1H, H-C(3)], 3.76 [m, 1H, H-C(6)], 8.01 [s, 1H, H-N(4)], 8.13 [s, 1H, H-N(1)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 17.5 [C(8/9)], 18.9 [C(8/9)], 21.9 [C(12/13)], 23.2 [C(12/13)], 23.7 [C(11)], 31.6 [C(7)], 44.1 [C(10)], 52.6 [C(6)], 59.7 [C(3)], 167.0 [C(2)], 168.6 [C(5)].

(3*S*,6*S*)-3-[(4-Hydroxyphenyl)methyl]-6-(1-methylethyl)-piperazine-2,5-dione, *cis*-cyclo(L-Val-L-Tyr), **27** (**Figure 2**). Yield: 0.29 mmol (24%). LC−MS (ESI^+) *m*/*z*: 263 (100, $[M + 1]^+$). LC−MS/MS (ESI^+): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 0.37 [d, 3H, J = 7.0 Hz, H-C(15/16)], 0.69 [d, 3H, J = 7.0 Hz, H-C(15/16)], 1.74 [m, 1H, H-C(14)], 2.77 [dd, 1H, J = 4.8, 13.8 Hz, H-C(7a)], 3.01 [dd, 1H, J = 4.5, 13.8 Hz, H-C(7b)], 3.51 [m, 1H, H-C(6)], 4.10 [m, 1H, H-C(3)], 6.62 [d, 2H, J = 8.4 Hz, H-C(10, 12)], 6.96 [d, 2H, J = 8.4 Hz, H-C(9, 13)], 7.85 [s, 1H, H-N(1)], 7.95 [s, 1H, H-N(4)], 9.10 [s, 1H, HO-C(11)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 16.5 [C(15/16)], 18.5 [C(15/16)], 31.3 [C(14)], 37.4 [C(7)], 55.5 [C(3)], 59.4 [C(6)], 114.9 [C(10, 12)], 126.5 [C(8)], 131.3 [C(9, 13)], 156.3 [C(11)], 166.6 [C(5)], 166.9 [C(2)].

(3*S*,6*S*)-3-[(1*S*)-1-Methylpropyl]-6-(phenylmethyl)-piperazine-2,5-dione, *cis*-cyclo(L-IIe-L-Phe), **28** (**Figure 2**). Yield: 1.02 mmol (85%). LC−MS (ESI^+) *m*/*z*: 261 (100, $[M + 1]^+$). LC−MS/MS (ESI^+): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 0.54 [d, 3H, J = 7.0 Hz, H-C(10)], 0.57 [m, 3H, H-C(9)], 0.66 [m, 2H, H-C(8)], 1.38

[m, 1H, H-C(7)], 2.86 [dd, 1H, J = 5.0, 13.5 Hz, H-C(11a)], 3.15 [dd, 1H, J = 4.1, 13.5 Hz, H-C(11b)], 3.57 [m, 1H, H-C(3)], 4.20 [m, 1H, H-C(6)], 7.21 [m, 5H, H-C(13−17)], 7.84 [s, 1H, H-N(4)], 8.05 [s, 1H, H-N(1)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 12.0 [C(9/10)], 14.7 [C(9/10)], 23.4 [C(8)], 37.8 [C(11)], 38.0 [C(7)], 55.3 [C(6)], 59.0 [C(3)], 126.7 [C(15)], 128.1 [C(14, 16)], 130.7 [C(13, 17)], 136.5 [C(12)], 166.6 [C(5)], 166.7 [C(2)].

(3*S*,6*S*)-3-[(1*S*)-1-Methylpropyl]-hexahydropyrrolo-[1,2-*a*]-pyrazine-1,4-dione, *cis*-cyclo(L-IIe-L-Pro), **29** (**Figure 2**). Yield: 1.06 mmol (88%). LC−MS (ESI$^+$) *m*/*z*: 211 (100, [M + 1]$^+$). LC−MS/MS (ESI$^+$): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 0.82 [t, 3H, J = 7.6 Hz, H-C(12)], 0.98 [d, 3H, J = 7.1 Hz, H-C(13)], 1.31 [m, 2H, H-C(11)], 1.81 [m, 3H, H-C(7a, 8)], 2.03 [m, 1H, H-C(10)], 2.14 [m, 1H, H-C(7b)], 3.36 [m, 2H, H-C(9)], 3.95 [s, 1H, H-C(3)], 4.11 [t, 1H, J = 7.7 Hz, H-C(6)], 7.91 [s, 1H, H-N(4)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 12.5 [C(12)], 15.2 [C(13)], 22.2 [C(8)], 24.1 [C(11)], 28.1 [C(7)], 35.1 [C(10)], 44.8 [C(9)], 58.4 [C(6)], 59.4 [C(3)], 165.5 [C(2)], 170.3 [C(5)].

(3*S*,6*S*)-Octahydrodipyrrolo-[1,2-*a*; 1′,2′-*d*]-pyrazine-5,10-dione, *cis*-cyclo(L-Pro-L-Pro), **30** (**Figure 2**). Yield: 0.89 mmol (74%). LC−MS (ESI$^+$) *m*/*z*: 195 (100, [M + 1]$^+$). LC−MS/MS (ESI$^+$): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 1.70−1.90 [m, 6H, H-C(7a, 10a, 8, 11)], 2.13 [m, 2H, H-C(7b, 10b)], 3.36 [m, 4H, H-C(9, 12)], 4.27 [t, 2H, J = 8.0 Hz, H-C(3, 6)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 23.0 [C(8, 11)], 27.3 [C(7, 10)], 44.7 [C(9, 12)], 59.9 [C(3, 6)], 166.1 [C(2, 5)].

(3*S*,6*R*)-3-(1-Methylethyl)-6-(phenylmethyl)-piperazine-2,5-dione, *trans*-cyclo(L-Val-D-Phe), **31**. Yield: 0.38 mmol (32%). LC−MS (ESI$^+$) *m*/*z*: 247 (100, [M + 1]$^+$). LC−MS/MS (ESI$^+$): cf. **Table 1**. ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ: 0.76 [d, 3H, J = 6.9, H-C(8/9)], 0.83 [d, 3H, J = 6.9 Hz, H-C(8/9)], 2.04 [m, 1H, H-C(7)], 2.88 [dd, 1H, J = 4.9, 13.5 Hz, H-C(10a)], 2.97 [m, 1H, H-C(3)], 3.14 [dd, 1H, J = 4.1, 13.5 Hz, H-C(10b)], 4.17 [m, 1H, H-C(6)], 7.21 [m, 5H, H-C(12−16)], 7.93 [s, 1H, H-N(4)], 8.21 [s, 1H, H-N(1)]. ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ: 16.8 [C(8/9)], 18.3 [C(8/9)], 31.7 [C(7)], 38.1 [C(10)], 54.9 [C(6)], 59.1 [C(3)], 126.7 [C(14)], 128.1 [C(13, 15)], 130.3 [C(12, 16)], 136.3 [C(11)], 167.2 [C(5)], 167.5 [C(2)].

Isolation and Identification of 2,5-Diketopiperazines in Roasted Cocoa. Roasted cocoa nibs (100 g) were 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 5 times with acetone/water (70:30, v/v; 300 mL each) for 45 min at room temperature while 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) and then freeze-dried to give the dichloromethane extractables. An aliquot (100 mg) of the dichloromethane extract was suspended in aqueous hydrochloric acid (0.001 N HCl in water; 5 mL) and then applied on top of a glass column (170 × 15 mm i.d.) filled with a slurry of Dowex 50WX 8, 50−100 mesh (Merck, Darmstadt, Germany), which was preconditioned with 0.1 mol/L aqueous hydrochloric acid (500 mL), followed by 0.001 mol/L aqueous hydrochloric acid (500 mL). Using 0.001 mol/L aqueous hydrochloric acid (500 mL) as the eluent, five fractions (100 mL each) were collected, freeze-dried, and analyzed by means of LC−MS/MS on a 150 × 2 mm i.d., 4 μm, Synergi Fusion-RP column (Phenomenex, Aschaffenburg, Germany). Starting with aqueous formic acid (0.1% in water, pH 2.5) at a flow rate of 0.2 mL/min, after 10 min, the methanol content was increased to 60% within 40 min, increased to 100% within 10 min, and finally, held at 100% for 10 min. A comparison of retention time and spectroscopic data with those obtained for the corresponding reference compounds led to the unequivocal identification of the 2,5-diketopiperazines **1**−**12** and **18**−**30** (**Figures 1** and **2**) in the roasted cocoa nibs.

Quantitative Analysis of 2,5-Diketopiperazines (DKPs). Ground, roasted cocoa nibs (50.0 g) were spiked with the internal standards *trans*-cyclo(D-Ala-L-Val) (1.4 mg) and *trans*-cyclo(L-Val-D-Phe) (1.9 mg) dissolved in methanol (2 mL). After homogenization and equilibration, the dichloromethane extractables were isolated as reported above, and an aliquot (100 mg) of this isolate was fractionated by cation-exchange chromatography following the procedure described above. The residue was taken up in a mixture (50:50, v/v; 3 mL) of methanol and aqueous formic acid (0.1% in water, pH 2.5), and after membrane filtration, aliquots (5−20 μL) were analyzed by means of LC−MS on a 150 × 2 mm i.d., 4 μm, Synergi Fusion-RP column (Phenomenex) using the same gradient described above. All of the 2,5-diketopiperazines containing the aromatic amino acids L-phenylalanine or L-tyrosine, respectively, were quantified by using *trans*-cyclo(L-Val-D-Phe) (**31**) as the internal standard. The remaining 2,5-diketopiperazines were quantified by using *trans*-cyclo(D-Ala-L-Val) (**17**) as the internal standard. The amounts of the individual 2,5-diketopiperazines were calculated using response curves that had been determined by analysis of solutions containing defined amounts of the internal standards and the target compounds in different concentrations.

Liquid Chromatography/Mass Spectrometry (LC−MS/MS). Aliquots (5−20 μL) were analyzed by means of LC−MS on a 150 × 2 mm i.d., 4 μm, Synergi Fusion-RP column (Phenomenex) coupled to a TSQ-Discovery-MS (Thermo Finnigan MAT GmbH, Bremen, Germany) using positive (ESI$^+$) electronspray ionization. Starting with aqueous formic acid (0.1% in water, pH 2.5) at a flow rate of 0.2 mL/min, after 10 min, the methanol content was increased to 60% within 40 min, increased to 100% within 10 min, and finally, held at 100% for 10 min. Using the selected reaction monitoring (SRM) mode and the multiple reaction monitoring (MRM) mode, the 2,5-diketopiperazines were detected by measuring the corresponding mass traces as given in **Table 1**.

Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{1}H, ^{13}C, and DEPT-135 NMR experiments were performed on Bruker AV-360 spectrometers. ^{1}H, COSY, HMQC, and HMBC measurements were performed on a Bruker AMX 400-III spectrometer (Bruker, Rheinstetten, Germany). Evaluation of the experiments was carried out using 1D- and 2D-WIN NMR as well as XWin-NMR software (version 3.5; Bruker, Rheinstetten, Germany). DMSO-d_6 and MeOH-d_4 were used as solvents, and tetramethylsilane (TMS) was used as the internal standard.

RESULTS AND DISCUSSION

With the aim at identifying the key molecules imparting the metallic and bitter taste sensation observed recently for a dichloromethane isolate of roasted cocoa (*6*), roasted cocoa nibs were fully extracted with acetone/water and the aqueous solution obtained was extracted with dichloromethane closely following the procedure reported recently (*6*). Analysis of this dichloromethane extract by means of HPLC/DAD and HPLC−MS revealed theobromine as the major constituent, followed by a large series of compounds each in comparatively low concentrations. HPLC/degustation revealed that these compounds imparted a significant bitter taste accompanied by a blood-like metallic taste sensation.

Identification of Compounds Imparting Metallic and Bitter Taste. To further characterize the chemical structure of these bitter compounds, the major amounts of theobromine needed to be removed first. To achieve this, the dichloromethane solubles were placed on the top of a cation-exchange column preconditioned with 0.001 mol/L aqueous hydrochloric acid. Using 0.001 mol/L aqueous hydrochloric acid as the eluent, the effluent was collected in five fractions, freeze-dried, and analyzed by means of LC−MS/MS. Fractions 1−4 containing the compounds under investigation were combined, and the structure of the individual compounds were evaluated by means of LC−MS/MS experiments.

Each of the compounds detected showed a characteristic mass spectrum that was well in line with the mass spectrometric data reported for 2,5-diketopiperazines (*10*). For example, the MS/MS spectrum of the pseudomolecular ion *m*/*z* 247 detected for the diketopiperazine *cis*-cyclo(L-Val-L-Phe) (**23**) revealed the characteristic ions with *m*/*z* 72 and 120, which are well in line with the expected immonium ions formed from the valine or

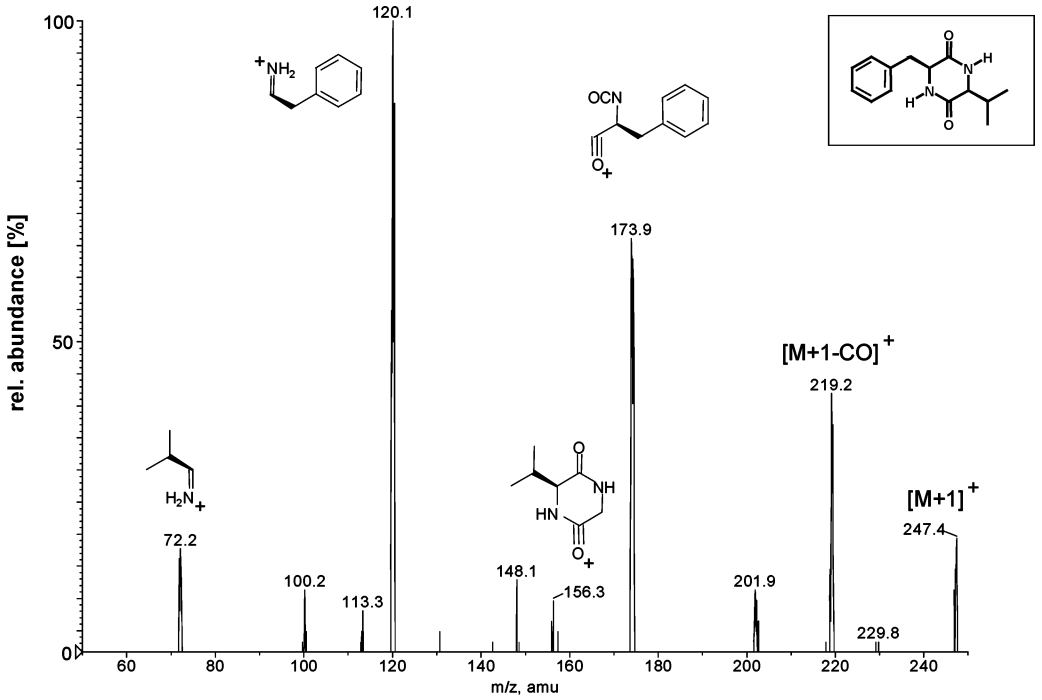

Figure 3. MS/MS (ESI+) spectrum of *cis*-cyclo(L-Val-L-Phe).

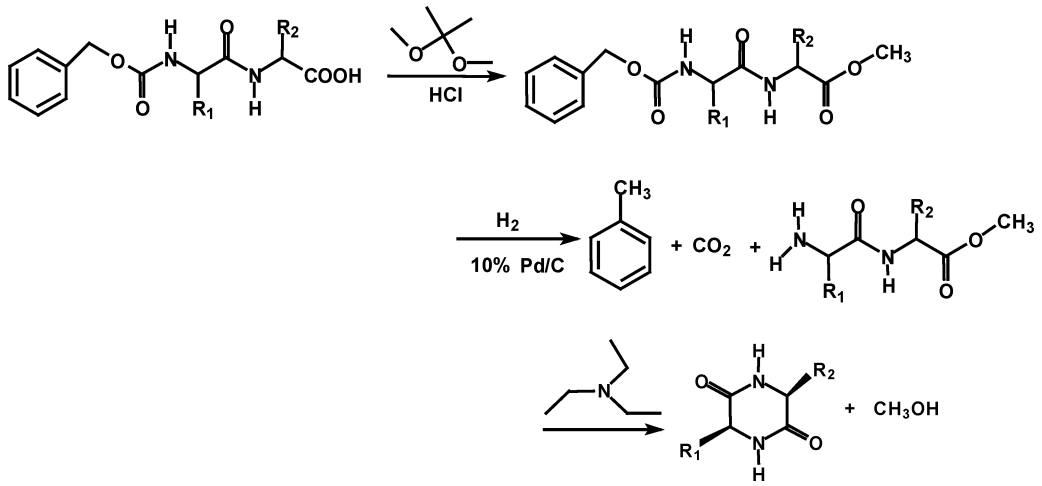

Figure 4. Reaction sequence used for the synthesis of 2,5-diketopiperazines.

the phenylalanine moiety, respectively (**Figure 3**). Using the characteristic immonium ions expected for the individual diketopiperazines containing proline (*m*/*z* 70), alanine (*m*/*z* 44), valine (*m*/*z* 72), phenylalanine (*m*/*z* 120), tyrosine (*m*/*z* 136), leucine/isoleucine (*m*/*z* 86), threonine (*m*/*z* 74), and serine (*m*/*z* 60), the cocoa extract has been screened for the following 14 cis-configured 2,5-diketopiperazines, which were reported earlier as cocoa ingredients (*3*−*5*, *11*): *cis*-cyclo(Gly-L-Pro) (**1**), *cis*-cyclo(L-Phe-L-Pro) (**2**), *cis*-cyclo(L-Leu-L-Pro) (**3**), *cis*-cyclo(Gly-L-Phe) (**4**), *cis*-cyclo(L-Asp-L-Phe) (**5**), *cis*-cyclo(L-Leu-Gly) (**6**), *cis*-cyclo(L-Ala-Gly) (**15**), *cis*-cyclo(L-Ala-L-Leu) (**18**), *cis*-cyclo(L-Ala-L-Phe) (**19**), *cis*-cyclo(L-Ala-L-Pro) (**20**), *cis*-cyclo(L-Ala-L-Val) (**21**), *cis*-cyclo(L-Asn-L-Phe) (**22**), *cis*-cyclo(L-Val-L-Phe) (**23**), and *cis*-cyclo(L-Pro-L-Asn). With the exception of *cis*-cyclo(L-Ala-Gly) and *cis*-cyclo(L-Pro-L-Asn), all of these cis-configured diketopiperazines could be detected in the dichloromethane fraction isolated from roasted cocoa nibs (**Figure 1**). In addition, the presence of 13 additional cis-configured diketopiperazines, namely, *cis*-cyclo(L-Pro-L-Thr) (**7**), *cis*-cyclo(L-Pro-L-Tyr) (**8**), *cis*-cyclo(L-Leu-L-Phe) (**9**), *cis*-cyclo(L-Pro-L-Val) (**10**), *cis*-cyclo(L-Phe-L-Ser) (**11**), *cis*-cyclo(L-Val-L-Val) (**12**), *cis*-cyclo(L-Ala-L-Ile) (**24**), *cis*-cyclo(L-Ala-L-Tyr) (**25**), *cis*-cyclo(L-Val-L-Leu) (**26**), *cis*-cyclo(L-Val-L-Tyr) (**27**), *cis*-cyclo(L-Ile-L-Phe) (**28**), *cis*-cyclo(L-Ile-L-Pro) (**29**), and *cis*-cyclo(L-Pro-L-Pro) (**30**), were suggested on the basis of their characteristic immonium ions detected by MS/MS experiments (**Figure 2**).

Finally, the identity of the diketopiperazines **1**−**12** was confirmed by comparison of LC−MS/MS data, retention times, and sensory activity with those obtained from commercially available reference compounds. To confirm the chemical structures of the compounds **18**−**30**, these diketopiperazines were synthesized following the reaction sequence given in **Figure 4**. The corresponding N-protected dipeptide was esterified with 2,2-dimethoxypropane and hydrochloric acid, and then the N-protecting group was removed by hydrogenation using palladium on charcoal as the catalyst. After triethylamine-calalyzed cyclization, the target compounds **18**−**30** (**Figures 1** and **2**) were isolated by column chromatography on RP-18 material, followed by a final HPLC purification, yielding the corresponding cis-configured diketopiperazines as white, amorphous powders in a high purity of more than 99%.

Table 2. Human Taste Recognition Thresholds of 2,5-Diketopiperazines

compound (number)	taste threshold concentration (μmol/L)[a] for metallic sensation	bitter taste
cis-cyclo(Gly-Gly) (**14**)	>8000	>8000
cis-cyclo(Gly-L-Phe) (**4**)	50	610
cis-cyclo(Gly-L-Pro) (**1**)	384	3250
cis-cyclo(L-Ala-Gly) (**15**)	1950	3910
cis-cyclo(L-Ala-L-Ala) (**13**)	>7000	>7000
cis-cyclo(L-Ala-L-Val) (**21**)	406	1470
cis-cyclo(L-Ala-L-Ile) (**24**)	168	540
cis-cyclo(L-Ala-L-Leu) (**18**)	30	680
cis-cyclo(L-Ala-L-Phe) (**19**)	144	570
cis-cyclo(L-Ala-L-Pro) (**20**)	387	1490
cis-cyclo(L-Ala-L-Tyr) (**25**)	430	530
cis-cyclo(L-Val-L-Val) (**12**)	510	1260
cis-cyclo(L-Val-L-Leu) (**26**)	120	470
cis-cyclo(L-Val-L-Tyr) (**27**)	100	190
cis-cyclo(L-Val-L-Phe) (**23**)	40	1000
cis-cyclo(L-Val-L-Pro) (**10**)	320	1280
cis-cyclo(L-Leu-Gly) (**6**)	529	590
cis-cyclo(L-Leu-L-Phe) (**9**)	40	190
cis-cyclo(L-Leu-L-Pro) (**3**)	120	1190
cis-cyclo(L-Phe-L-Pro) (**2**)	131	1020
cis-cyclo(L-Phe-L-Ser) (**11**)	40	210
cis-cyclo(L-Phe-L-Asp) (**5**)	3810	3810
cis-cyclo(L-Ile-L-Phe) (**28**)	40	190
cis-cyclo(L-Asn-L-Phe) (**22**)	380	960
cis-cyclo(L-Pro-L-Thr) (**7**)	631	1261
cis-cyclo(L-Pro-L-Pro) (**30**)	760	2580
cis-cyclo(L-Pro-L-Tyr) (**8**)	190	480
cis-cyclo(L-Ile-L-Pro) (**29**)	120	480
trans-cyclo(D-Ala-L-Pro) (**16**)	387	830
trans-cyclo(D-Ala-L-Val) (**17**)	188	818
trans-cyclo(L-Val-D-Phe) (**31**)	40	500

[a] Taste recognition threshold concentrations were determined by means of a triangle test in bottled water.

Among all of these diketopiperazines identified in the present study in cocoa, compounds **2**, **3**, **10**, **29**, and **30** were previously reported in roasted malt (*12*), compound **2** in aged sake (*13*), compounds **2**, **3**, **10**, **20**, **29**, and **30** in beer (*14*), compounds **1**−**3**, **7**, **9**, **10**, **18**, **20**, **21**, **23**, **24**, **26**, and **28**−**30** in chicken essence (*10*), compounds **1**−**3**, **9**, **10**, **22**, **23**, and **28**−**30** in roasted coffee (*15*, *16*), compounds **2**, **3**, **10**, **20**, and **30** in comte cheese (*17*), and compounds **9**, **12**, and **23** in hydrolyzed vegetable protein (*18*). Although the structures are known in synthetic chemistry (*19*), to the best of our knowledge, the compounds *cis*-cyclo(L-Phe-L-Ser) (**11**), *cis*-cyclo(L-Val-L-Tyr) (**27**), and *cis*-cyclo(L-Ala-L-Tyr) (**25**) identified in the present study in cocoa have never been reported before in any food product.

Sensory Evaluation of 2,5-Diketopiperazines. Prior to sensory analysis, trace amounts of solvents were removed in high vacuum, and after triple freeze drying, the purity of all of the compounds was checked by LC−MS as well as ^{1}H NMR spectroscopy. To study the sensory activity of the diketopiperazines, the human sensory recognition thresholds were determined in water by means of the triangle test. The oral sensation imparted by the diketopiperazines was described as metallic and bitter, with threshold concentrations ranging from 30 to 1950 μmol/L for the blood-like metallic sensation and from 190 to 3910 μmol/L for the bitter taste (**Table 2**). For all of these compounds, the taste threshold for the metallic sensation was found to be equal or below the threshold concentration determined for the bitter taste. For the metallic taste sensation, the diketopiperazines *cis*-cyclo(L-Ala-L-Leu) (**18**) showed the lowest threshold concentration of 30 μmol/L, whereas *cis*-cyclo(L-Phe-L-Asp) (**5**) and *cis*-cyclo(L-Ala-Gly) (**15**) showed the highest threshold concentrations of 3810 and 1950 μmol/L. *Cis*-cyclo(Gly-Gly) (**14**) and *cis*-cyclo(L-Ala-L-Ala) (**13**) did not show any metallic taste sensation in concentrations below 7000 μmol/L. For the bitter taste threshold, *cis*-cyclo(L-Leu-L-Phe) (**9**), *cis*-cyclo(L-IIe-L-Phe) (**28**), and *cis*-cyclo(L-Val-L-Tyr) (**27**) were evaluated with the lowest threshold concentration of 190 μmol/L for bitter taste, whereas *cis*-cyclo(L-Ala-Gly) (**15**) exhibited with 3910 μmol/L the highest threshold concentration.

A comparison of the thresholds of the tastant pairs *cis*-cyclo(L-Ala-L-Phe) (**19**) and *cis*-cyclo(L-Ala-L-Tyr) (**25**) or *cis*-cyclo(L-Val-L-Phe) (**23**) and *cis*-cyclo(L-Val-L-Tyr) (**27**) revealed that the threshold concentration of the metallic sensation of phenylalanine-containing diketopiperazines (40 or 140 μmol/L for **23** or **19**) is below the threshold concentration of a diketopiperazine containing a tyrosine residue (100 or 430 μmol/L for **27** or **25**). Vice versa, tyrosine-containing diketopiperazines were found to show lower taste thresholds for bitterness when compared to the corresponding diketopiperazines bearing a phenylalanine moiety; for example, *cis*-cyclo(L-Val-L-Phe) (**23**) and *cis*-cyclo(L-Ala-L-Phe) (**19**) did not show any bitter taste below 1000 and 570 μmol/L, respectively, whereas *cis*-cyclo(L-Val-L-Tyr) (**27**) and *cis*-cyclo(L-Ala-L-Tyr) (**25**) already showed bitter taste at the threshold concentrations of 190 and 530 μmol/L (**Table 2**).

To study the influence of the second amino acid residue on the bitter taste threshold, we compared the threshold concentrations of the alanine-containing 2,5-diketopiperazines **13**, **15**, **18**−**21**, **24**, and **25** differing in the structure of the second amino acid moiety. The data showed that the taste threshold concentration for bitter taste increases with increasing polarity of the second amino acid residue; e.g., *cis*-cyclo(L-Ala-L-Tyr), *cis*-cyclo(L-Ala-L-IIe), *cis*-cyclo(L-Ala-L-Phe), and *cis*-cyclo(L-Ala-L-Leu) showed threshold concentrations between 530 and 680 μmol/L, and *cis*-cyclo(L-Ala-L-Val) and *cis*-cyclo(L-Ala-L-Pro) showed thresholds of 1470 and 1490 μmol/L, whereas *cis*-cyclo(L-Ala-Gly) and *cis*-cyclo(L-Ala-L-Ala) exhibited threshold concentrations of 3910 or higher. This observation is well in line with literature data pinpointing hydrophobic amino acids such as phenylalanine, tyrosine, and leucine as the molecular key responsible for the bitter taste of peptides (*20*−*24*).

To finally investigate the influence of the stereochemistry on the taste threshold concentrations of diketopiperazines, we compared the threshold concentrations of *cis*-cyclo(L-Val-L-Phe) (**23**), *cis*-cyclo(L-Ala-L-Val) (**21**), and *cis*-cyclo(L-Ala-L-Pro) (**20**) with those determined for *trans*-cyclo(L-Val-D-Phe) (**31**), *trans*-cyclo(D-Ala-L-Val) (**17**), and *trans*-cyclo(D-Ala-L-Pro) (**16**), respectively. As summarized in **Table 2**, the taste thresholds determined for bitter taste as well as the metallic taste sensation were not significantly influenced by the stereochemistry, thus confirming earlier literature reports (*3*); for example, compounds **23** and **31** both showed a threshold concentration of 40 μmol/L and very close thresholds for bitterness at 1000 and 500 μmol/L (**Table 2**).

Quantitative Analysis of Diketopiperazines and Calculation of Dose-over-Threshold (DoT) Factors. With the aim at evaluating the taste contribution of the individual diketopiperazines, 25 cis-configured diketopiperazines were quantitatively determined in the roasted cocoa nibs by means of a diastereomer dilution assay. For the quantitative analysis, the cocoa nibs were spiked with synthetic *trans*-cyclo(L-Val-D-Phe) as the internal standard for the quantification of the 2,5-diketopiperazines containing the aromatic amino acids L-phenylalanine or L-tyrosine, respectively, and with synthetic *trans*-cyclo(D-Ala-L-Val) as a second internal standard for the quantification of the remaining 2,5-diketopiperazines bearing aliphatic side chains. After isolation of the dichloromethane fraction from the

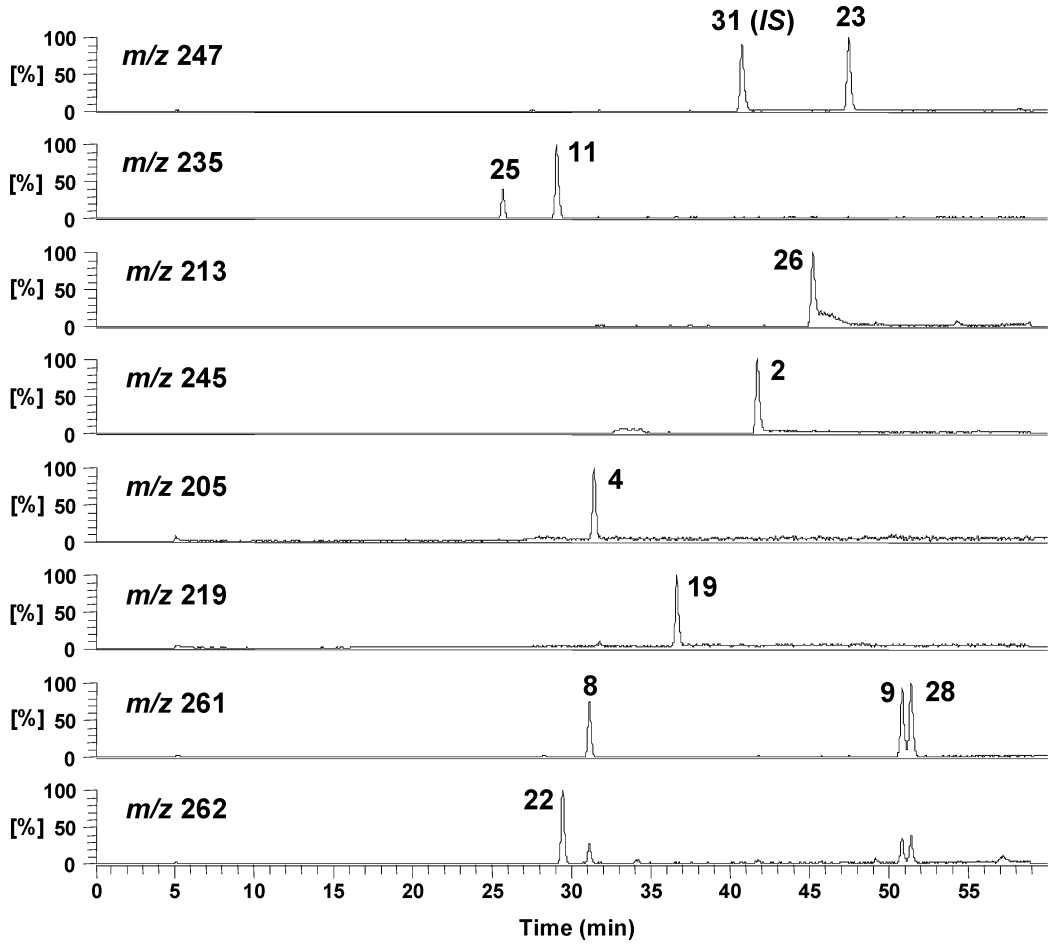

Figure 5. Quantification of selected diketopiperazines in roasted cocoa by means of LC–MS using *trans*-cyclo(L-Val-D-Phe) (**31**) as the internal standard (IS).

powdered cocoa nibs and removal of theobromine by means of cation-exchange chromatography as detailed above, quantification was performed by analytical RP-HPLC−MS as shown for example in **Figure 5** for analysis of the phenylalanine- and tyrosine-containing diketopiperazines. By far, the highest concentrations of 8878.0 and 1357.0 μmol/kg were found for *cis*-cyclo(L-Val-L-Pro) and *cis*-cyclo(L-Ala-L-Pro), followed by *cis*-cyclo(L-Val-L-Leu), *cis*-cyclo(L-Leu-L-Pro), *cis*-cyclo(L-Ala-L-Leu), *cis*-cyclo(L-Ala-L-Ile), *cis*-cyclo(L-Ala-L-Val), and *cis*-cyclo(L-Ile-L-Pro) with concentrations between 537.0 and 817.1 μmol/kg. In contrast, just very low concentrations of 0.7 and 1.6 μmol/kg were determined for *cis*-cyclo(L-Asp-L-Phe) and *cis*-cyclo(L-Ala-L-Tyr) (**Table 3**).

To gain first insights into the taste contribution of these compounds, these were rated in their sensory impact based on the ratio of the concentration and the taste recognition threshold of a compound. Calculation of these DoT factors (*25*) revealed that exclusively the concentrations of *cis*-cyclo(L-Pro-L-Val) (**10**), *cis*-cyclo(L-Val-L-Leu) (**26**), *cis*-cyclo(L-Ala-L-Ile) (**24**), *cis*-cyclo(L-Ala-L-Leu) (**18**), and *cis*-cyclo(L-Ile-L-Pro) (**29**) in the roasted cocoa nibs exceeded their taste threshold concentrations by a factor of 6.9, 1.7, 1.2, and 1.1, respectively (**Table 3**). All of the other diketopiperazines were present below their individual bitter taste threshold concentrations. Because the DoT factors do not consider the nonlinear relationship between the concentration and gustatory response of an individual compound as well the maximum solubility of such a compound, in the following, dose/response functions describing the human bitter taste perception of diketopiperazines were recorded for some selected compounds.

Human Dose/Response Functions. As independent from their sensory training status, panellists 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 (*26*). Consequently, recording dose/response functions with standard sensory methodologies usually leads to unreliable curves with very high error margins. In contrast, the recently reported half-tongue testing (*6, 8*) offers the possibility of a direct comparison of the taste impact of two samples, thus overcoming the memory problem mentioned above. In this test, each compound is applied onto one side of the tongue in random order and the panellist is asked to determine which side shows the stronger sensation. To adopt this technique to the recording of dose/response functions, serial 1:1 dilutions of the samples in water were prepared 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. 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 half-tongue tasting method, thus offering a direct comparison of the taste impact and a reliable evaluation of the gustatory response of different compounds.

On this five-point scale, the compound evaluated with the highest taste intensity at its maximum concentration, that is *cis*-cyclo(L-Val-L-Pro) (**10**) at its maximum solubility of 81.92 mmol/L (water), was set to the maximum score of 5.0. After the taste intensity of each compound at its maximum solubility had been rated, the taste intensities of the other dilutions were

Table 3. Concentrations and DoT Factors of 2,5-Diketopiperazines Identified in Roasted Cocoa Nibs

compound (number)	concentration[a] in (mg/kg)	concentration[a] in (μmol/kg)	DoT factors[b] for bitter taste
cis-cyclo(L-Val-L-Pro) (**10**)	1742.3	8877.8	6.9
cis-cyclo(L-Val-L-Leu) (**26**)	173.5	817.1	1.7
cis-cyclo(L-Ala-L-Ile) (**24**)	117.8	639.5	1.2
cis-cyclo(L-Ala-L-Leu) (**18**)	135.8	734.0	1.1
cis-cyclo(L-Ile-L-Pro) (**29**)	112.9	537.0	1.1
cis-cyclo(L-Ala-L-Pro) (**20**)	228.2	1357.0	0.9
cis-cyclo(L-Leu-L-Pro) (**3**)	149.6	711.2	0.6
cis-cyclo(L-Ala-L-Val) (**21**)	107.8	633.5	0.4
cis-cyclo(L-Leu-L-Phe) (**9**)	12.5	47.9	0.3
cis-cyclo(L-Ile-L-Phe) (**28**)	16.0	61.5	0.3
cis-cyclo(L-Val-L-Val) (**12**)	47.1	237.6	0.2
cis-cyclo(L-Val-L-Phe) (**23**)	14.3	58.0	0.1
cis-cyclo(L-Ala-L-Phe) (**19**)	15.7	72.0	0.1
cis-cyclo(L-Phe-L-Pro) (**2**)	15.7	64.3	0.1
cis-cyclo(L-Val-L-Tyr) (**27**)	2.3	8.8	<0.1
cis-cyclo(Gly-L-Phe) (**4**)	2.3	11.3	<0.1
cis-cyclo(L-Asn-L-Phe) (**22**)	3.8	14.6	<0.1
cis-cyclo(L-Pro-L-Thr) (**7**)	4.5	22.9	<0.1
cis-cyclo(L-Pro-L-Pro) (**30**)	6.3	32.5	<0.1
cis-cyclo(L-Pro-L-Tyr) (**8**)	1.0	4.0	<0.1
cis-cyclo(L-Asp-L-Phe) (**5**)	0.2	0.7	<0.1
cis-cyclo(L-Ala-L-Tyr) (**25**)	0.4	1.6	<0.1
cis-cyclo(L-Phe-L-Ser) (**11**)	0.7	3.0	<0.1
cis-cyclo(Gly-L-Pro) (**1**)	0.3	2.1	<0.1
cis-cyclo(L-Leu-Gly) (**6**)	<0.2	<1.2	<0.1

[a] The quantitative data are given as the mean of triplicates. The standard deviation of the quantitative data for the individual diketopiperazines was less than 10%. [b] DoT factors are calculated as the ratio of the concentration and the taste recognition threshold of a compound.

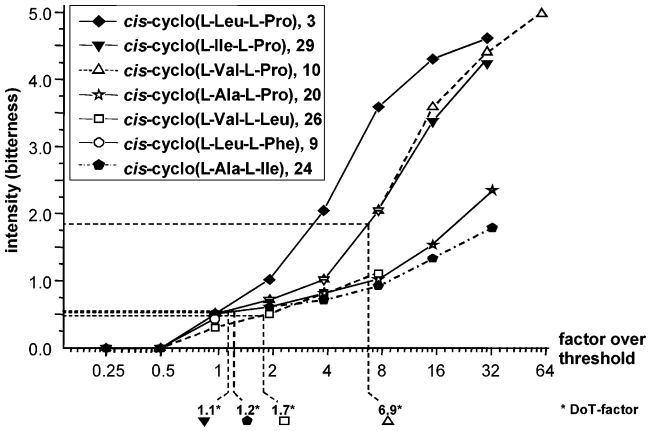

Figure 6. Human dose/response functions of selected diketopiperazines.

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 by cross-checking the taste intensity between the different compounds at the same dilution. Although this methodology is laborious, it gives much more reliable results with much smaller error margins than classical sensory tools used to measure dose/response curves. For example, the intensity values between trained individuals and separate sessions did not differ more than ± 0.3 units.

Using this methodology, dose/response functions were recorded for *cis*-cyclo(L-Val-L-Pro) (**10**), *cis*-cyclo(L-Val-L-Leu) (**26**), *cis*-cyclo(L-Leu-L-Phe) (**9**), *cis*-cyclo(L-Ala-L-Ile) (**24**), *cis*-cyclo(L-Ala-L-Pro) (**20**), and *cis*-cyclo(L-Ile-L-Pro) (**29**), and *cis*-cyclo(L-Leu-L-Pro) (**3**). The results, outlined in **Figure 6**, clearly demonstrated that the gustatory response for different diketopiperazines follows rather different dose/response functions. In particular, the perception of diketopiperazines *cis*-cyclo(L-Val-L-Pro), *cis*-cyclo(L-Leu-L-Pro), and *cis*-cyclo(L-Ile-L-Pro), consisting of L-proline and a second hydrophobic amino acid moiety, is reflected in rather high slopes and high taste intensities at higher concentration levels. The highest taste intensity of 5.0 was found for an aqueous solution of *cis*-cyclo(L-Val-L-Pro), exceeding the threshold concentration by 64-fold. Also, *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Ile-L-Pro) reached a high maximum bioresponse with scores of 4.6 and 4.2; a further increase of taste activity was prevented by the limited solubility of these compounds. In comparison, the *cis*-cyclo(L-Ala-L-Pro) containing the less hydrophobic amino acid L-alanine did not reach the same taste intensity as found for *cis*-cyclo(L-Leu-L-Pro) or *cis*-cyclo(L-Ile-L-Pro) and was just perceived with an intensity score of 2.3 in 32-fold threshold concentration. It is interesting to note that all of the tested diketopiperazines lacking proline in the molecule were very limited in solubility; for example, *cis*-cyclo(L-Leu-L-Phe) or *cis*-cyclo(L-Leu-L-Val) bearing a phenylalanine or a valine moiety instead of the proline showed maximum solubility already at the taste threshold concentration of 190 μmol/L (**9**) or after exceeding the threshold concentrations just by a factor of 8.

It appears that the detection threshold concentration of a taste compound alone does not enable a reliable evaluation of the taste impact of a compound. This value just considers the very beginning of the dose/response function, but there seems to be no correlation between the threshold and the maximal gustatory activation of a taste compound.

Another advantage of recording these dose/response functions is that the gustatory response at the factor-over-threshold given on the *x* axis in the dose/response functions reflects the taste intensity of a compound at the DoT factor calculated on the basis of the threshold concentration and the concentration of an individual compound in the cocoa. This opens the possibility to reliably elucidate the taste impact of each individual compound at the "natural" concentration level in cocoa. For example, the *cis*-cyclo(L-Val-L-Pro) calculated with a DoT factor of 6.9 exhibits a taste intensity of 1.8, whereas at the DoT factors of 1.7, 1.2, or 1.1, the diketopiperazines *cis*-cyclo(L-Val-L-Leu), *cis*-cyclo(L-Ala-L-Ile), and *cis*-cyclo(L-Ile-L-Pro) just reach taste intensity scores of about 0.5. On the basis of these results, it can be concluded that, out of the group of diketopiperazines, the *cis*-cyclo(L-Val-L-Pro) derivative is the most important bitter compound contributing to the bitter taste of roasted cocoa.

With the aim at demonstrating the contribution of individual compounds to the overall taste of roasted cocoa nibs, taste reconstruction and omission experiments using these compounds in their "natural" concentrations as well as the recording of additional dose/response functions for other cocoa taste compounds are currently in progress and will be published elsewhere.

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