Chemical Characterization of Diketopiperazines in Beer

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Diketopiperazines (DKPs) corresponding to cyclic dipeptides have been detected in a variety of natural products as well as in processed foods, beverages, and food and beverage ingredients. A series of seven proline-based diketopiperazines, namely, cyclo(Ala-Pro), cyclo(Val-Pro), cyclo(Ile-Pro), cyclo(Leu-Pro), cyclo(Met-Pro), cyclo(Phe-Pro), and cyclo(Pro-Pro), has now been identified in beer. A marketplace cross-section of five commercial beers was studied, involving products manufactured in different countries using distinctly different raw materials and brewing styles; maximum concentrations of individual diketopiperazines in various beers ranged from below detection limit to approximately 24 ppm. The flavor characteristics of these compounds were described variously as bitter, mouth coating, drying, astringent, salty, metallic, and grainy when evaluated in water at concentrations ranging from 10 to 50 ppm. However, it is questionable whether or not the diketopiperazines reported here make a significant contribution to either the aroma or taste of most beers.

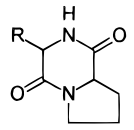
Keywords: *Amino acid; beer; diketopiperazine; gas chromatography−mass spectrometry; flavor; proline; volatile component*

INTRODUCTION

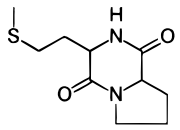
Relatively few food or beverage products have been as widely studied as beer and the raw materials of brewing, namely, barley malt (and adjuncts), hops (and hop-derived products), and yeast. Indeed, more than 800 individual beer flavor components have so far been identified in beer, including acetals, alcohols, aldehydes, esters, ethers, fatty acids, furans, ketones, phenols, pyrazines, terpenes, thiazoles, thioesters, thiols, and thiophenes (e.g. Engan, 1981; Meilgaard, 1981; Meilgaard and Peppard, 1986). Of these, the volatile nitrogen-containing constituents of beer are particularly important, because many of them are present at levels significantly above their very low flavor thresholds; their occurrence also frequently provides a point of distinction between different types of beer, such as ales and lagers (Pickett *et al*., 1976).

Volatile nitrogen-containing components of beer include biogenic and other amines, amino acid ethyl esters, pyrazines, pyridines, pyrroles, and thiazoles (e.g. Palamand *et al*., 1969; Kavanagh *et al*., 1974; Harding *et al*., 1977; Peppard and Halsey, 1980, 1981; Izquierdo-Pulido *et al*., 1996). While Sakamura *et al*. (1978) identified five bitter proline-based diketopiperazines in a roasted malt, at levels ranging from 6 to 56 mg/kg, they did not report whether these compounds could be detected in beer brewed from such malt. Though no specific details were given, Tressl *et al*. (1985) cited the formation of cyclo(Pro-Pro) in wort under conditions of high-temperature boiling; it was again unclear, however, whether or not this compound could also be detected in the resulting beer. It is interesting to note that Kitabatake *et al*. (1980) identified a number of cyclic tetrapeptides (produced by mold contamination) to be inducers of "gushing" in beer. This paper now reports the identification of seven proline-based diketopiperazines (structures **I**−**VII** in Chart 1) in a range of commercial beers obtained from the marketplace.

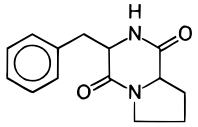
Chart 1. Structures of Diketopiperazines Found in Beer



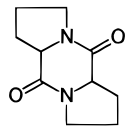
I. cyclo(Ala-Pro): R = methyl
II. cyclo(Val-Pro): R = *iso*-propyl
III. cyclo(Ile-Pro): R = *sec*-butyl
IV. cyclo(Leu-Pro): R = *iso*-butyl



V. cyclo(Met-Pro)



VI. cyclo(Phe-Pro)



VII. cyclo(Pro-Pro)

Diketopiperazines (DKPs) corresponding to cyclic dipeptides have been detected in a variety of natural products, as well as in processed foods, beverages and food and beverage ingredients. The former include marine sponges and/or associated endobionts (e.g. Schmitz *et al*., 1983; Adamczeski *et al*., 1995; Jayatilake *et al*., 1996) and fungal culture media (e.g. Stopsack *et al*., 1991; Ayer and Trifonov, 1994), while the latter include various cereal grains (Dansi *et al*., 1970), cocoa (Pickenhagen *et al*., 1975a; Ney, 1986; Rizzi, 1989), Comte cheese (Roudot-Algaron *et al*., 1993), hydrolyzed vegetable protein, HVP (Eriksen, 1977, 1980), aged sake (Takahashi *et al*., 1974), and dried squid (Kawai *et al*., 1991). In addition, Hilton *et al*. (1992) measured levels of cyclo(His-Pro) in the following foods and beverages: noodle, potted meat, nondairy creamer, hot dog, ham,

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S0021-8561(97)00099-X CCC: $14.00

egg, white bread, tuna, whole milk, chocolate milk, fish sauce, and dried shrimp. One of the principal degradation products of the high-intensity sweetener, aspartame, is 3-carboxymethyl-6-benzyl-2,5-diketopiperazine (e.g. Tateo *et al*., 1988; Prodolliet and Bruelhart, 1993); the latter constituent can often therefore be found in certain diet foods and beverages. However, in many of the instances where DKPs have been reported, one of the constituent amino acids is proline. Rizzi (1989) carried out model studies of DKP formation in cocoa and concluded that the mechanism involved intramolecular cyclization of linear peptide precursors rather than stepwise cyclic condensation involving individual amino acids.

DKPs have been shown by several researchers to be intensely bitter in taste and to contribute, for example, to the bitter taste of cocoa (e.g. Pickenhagen *et al*., 1975a) and aged sake (Takahashi *et al*., 1974). On the other hand, DKPs were *not* found to be a source of bitterness in the case of a commercial soybean HVP (Eriksen, 1977, 1980). Cocoa DKPs apparently act synergistically with theobromine (Pickenhagen *et al*., 1975a,b; Ney, 1986). Ney (1986) showed that the bitterness of DKPs followed the so-called "Q rule", developed previously for amino acids, peptides, and proteins, which relates perceived bitterness to amino acid composition. Ishibashi *et al*. (1988) also proposed a mechanism for the bitter taste sensation elicited by DKPs and certain other peptides. Gardner (1980) demonstrated good correlations ($R > 0.94$; $p < 0.01$) between bitterness thresholds of DKPs (and related compounds) and molecular connectivity. Cyclo(Asp-Phe) has been used to help simulate the taste and aroma of cheese (Smith *et al*., 1977). Esser and Essig (1980) employed DKPs in a patented method for preparing beverages with bitter taste. Pickenhagen *et al*. (1975b) also used cyclic (as well as open-chain) dipeptides, in combination with theobromine, to impart desirable bitter taste to food and beverage formulations.

MATERIALS AND METHODS

Materials. Beers were purchased from a local liquor store. Sodium chloride was ACS/Reagent grade (VWR Scientific, West Chester, PA). Dichloromethane was OmniSolv HR-GC grade (EM Science, Gibbstown, NJ). Methanol and water were both ACS/HPLC grade (Fisher Scientific, Pittsburgh, PA). GC retention index standards (straight-chain ethyl esters) were obtained from various suppliers and were used without further purification. All reagents used in synthesis of DKPs were purchased from Fluka (Buchs, Switzerland), except for the protected amino acids which were purchased from Bachem (Bubendorf, Switzerland). Small samples of DKPs **II** and **III** were generously provided by Hoffmann-La Roche, Nutley, NJ.

Synthesis and Chemical Characterization of DKPs. Diketopiperazines **I** and **IV**−**VII** were prepared using standard peptide chemistry (Bodanszky, 1984) in moderate to good overall yields. Equipment/methods used in their chemical characterization were as follows. Mp: Büchi apparatus (Dr. Tottoli), in open capillaries, not corrected. Optical rotation $[\alpha]_D$: Perkin-Elmer 241 polarimeter, in 10 cm cell. IR: Nicolet 510 FT-IR spectrometer, absorptions reported in cm^{-1}. ^1H NMR spectra: Bruker AVANCE DPX-400 (400 MHz), δ in ppm relative to TMS, J in Hz. ^{13}C NMR spectra: Bruker AVANCE DPX-400 (100 MHz), δ in ppm relative to TMS. MS: Finnigan MAT model 212, peak intensities given as percentage of base peak in parentheses. GC: Carlo Erba GC 6000 Vega Series 2, column DB-1701, 30 m × 0.32 mm i.d.

*(3S,6S)-3-(2-Methylpropyl)hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (**IV**).* To a precooled solution (−15 °C) of 20 g (93 mmol) of Boc-Pro-OH in 400 mL of tetrahydrofuran (THF) was added dropwise 10.2 mL (93 mmol) of *N*-methylmorpholine (NMM) followed by 13.2 mL (93 mmol) of $ClCO_2iBu$. After 2 min of stirring, a precooled solution (−15 °C) of 16.9 g (93 mmol) of H-Leu-OCH_3·HCl and 10.2 mL (93 mmol) of NMM in 160 mL of dimethylformamide (DMF) was added slowly. The mixture was stirred at −15 °C for 1 h, the cooling bath was removed, and stirring continued until the reaction was complete (monitoring by GC). The reaction mixture was diluted with EtOAc and washed with a 3% citric acid solution, saturated $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$) and concentrated *in vacuo* to give 31 g (97%) of a yellowish oil. This material was dissolved in 50 mL of CH_2Cl_2; 50 mL of trifluoroacetic acid was added dropwise, and the mixture was stirred at room temp. (*ca*. 22 °C) until complete conversion was observed by GC. The solvent was evaporated *in vacuo*, and the residue was taken up in EtOAc and washed with saturated $NaHCO_3$ and brine. The aqueous phase was adjusted to pH 10 with NaOH and extracted twice with EtOAc; the combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to give 21.1 g (96%) of an orange oil. This product was dissolved in 100 mL of EtOAc, and the solution was warmed to reflux temperature. After complete conversion (GC monitoring) the reaction mixture was cooled slowly to room temperature with slight stirring. The crystalline precipitate was separated by filtration, washed with cold EtOAc, and dried to give 7.8 g (40%) of pure diketopiperazine **IV**. Mp 162−164 °C; $[\alpha]_D = -144.0$ ($c = 1.0$, $CHCl_3$); ^1H NMR $\delta = 0.95$ (d, $J = 6.6$ Hz, CH_3), 1.00 (d, $J = 6.6$ Hz, CH_3), 1.49−1.57 (m, 1H, CH_2), 1.74−1.85 (m, 1H, CH), 1.86−1.94 (m, 1H, CH_2), 1.97−2.19 (m, 3H, CH_2), 2.30−2.39 (m, 1H, CH_2), 3.50−3.63 (m, 2H, NCH_2), 4.02 (dd, $J = 3.2$, 9.5 Hz, 1H, CH), 4.12 (dd, $J = 7.8$, 8.6 Hz, 1H, CH), 6.41 (br, NH); ^{13}C NMR $\delta = 21.18$ (CH_3), 22.71 (CH_2), 23.24 (CH_3), 24.57 (CH), 28.04 (CH_2), 38.49 (CH_2), 46.44 (CH_2N), 53.35 (CH), 58.93 (CH), 166.22 (CO), 170.34 (CO); IR (KBr) 3260 m, 2950 m, 2875 m, 1680 s, 1670 s, 1635 s, 1435 m; MS (EI) *m*/*z* (%) 154 (96), 125 (12), 86 (26), 70 (100), 55 (12), 43 (17), 41 (23), 30 (13).

*(3S,6S)-3-Methylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione (**I**).* According to the procedure described for the synthesis of diketopiperazine **IV**, 50 g (0.23 mol) of Boc-Pro-OH and 32.4 g (0.23 mol) of H-Ala-OCH_3·HCl were condensed to give 3.8 g (10%) of crystalline diketopiperazine **I**. Mp 172−174 °C; $[\alpha]_D = -126.5$ ($c = 0.2$, EtOH); ^1H NMR $\delta = 1.48$ (d, $J = 6.8$ Hz, CH_3), 1.89−1.96 (m, 1H, CH_2), 1.98−2.07 (m, 1H, CH_2), 2.09−2.15 (m, 1H, CH_2), 2.32−2.37 (m, 1H, CH_2), 3.52−3.65 (m, 2H, NCH_2), 4.09−4.16 (m, 2H, CH), 6.94 (br, NH); ^{13}C NMR $\delta = 15.83$ (CH_3), 22.70 (CH_2), 28.10 (CH_2), 45.38 (CH_2N), 51.12 (CH), 59.21 (CH), 166.36 (CO), 170.53 (CO); IR (KBr) 3265 m, 2980 w, 2880 w, 1715 s, 1690 s, 1665 s, 1645 s, 1455 m, 1410 m; MS (EI) *m*/*z* (%) 168 (35, M^+), 140 (10), 125 (34), 97 (33), 70 (100), 55 (17), 44 (90), 41 (49), 28 (51).

*(3S,6S)-3-(2-Methylsulfanylethyl)hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (**V**).* According to the procedure described for the synthesis of diketopiperazine **IV**, 26.8 g (0.125 mol) of Boc-Pro-OH and 25.0 g (0.125 mol) of H-Met-OCH_3·HCl were condensed to give 0.53 g (1.8%) of diketopiperazine **V** after recrystallization of the crude product. Mp 142−146 °C; $[\alpha]_D = -113.6$ ($c = 1.01$, $CHCl_3$); ^1H NMR $\delta = 1.85$−1.97 (m, 1H, CH_2), 1.98−2.12 (m, 3H, CH_2), 2.13 (s, 3H, CH_3), 2.30−2.43 (m, 2H, CH_2), 2.65−2.76 (m, 2H, SCH_2), 3.51−3.65 (m, 2H, NCH_2), 4.11 (dd, $J = 6.8$, 8.4 Hz, 1H, CH), 4.21 (t, $J = 5.6$ Hz, 1H, CH), 7.69 (s, 1H, NH); ^{13}C NMR $\delta = 15.06$ (CH_3), 22.55 (CH_2), 28.06 (CH_2), 28.78 (CH_2), 29.79 (CH_2), 45.31 (CH_2), 54.20 (CH), 58.91 (CH), 165.46 (CO), 170.74 (CO); IR (KBr) 3270 m, 2930 w, 2820 w, 1715 s, 1690 s, 1645 s, 1635 s, 1430 m, 1305 w; MS (EI) *m*/*z* (%) 228 (32, M^+), 167 (44), 154 (99), 139 (32), 70 (100), 61 (28), 56 (21), 41 (32), 28 (26).

*(3S,6S)-3-Benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione (**VI**).* According to the procedure described for the synthesis of diketopiperazine **IV**, 50 g (0.23 mol) of Boc-Pro-OH and 49.6 g (0.23 mol) of H-Phe-OCH_3·HCl were condensed to give 17.8 g (32%) of crystalline diketopiperazine **VI**. Mp 132−134 °C, $[\alpha]_D = -190.5$ ($c = 1.0$, $CHCl_3$); ^1H NMR $\delta = 1.85$−2.03 (m, 3H, CH_2), 2.26−2.36 (m, 1H, CH_2), 2.82 (dd, $J = 10.1$, 14.5 Hz, 1H, CH_2), 3.52−3.68 (m, 2H, NCH_2), 3.58 (dd, $J = 3.8$, 14.5 Hz, 1H, CH_2), 4.06 (dd, $J = 7.3$, 8.2 Hz, 1H, CH), 4.28 (dd, $J = 3.8$, 10.1 Hz, 1H, CH), 5.94 (br, NH), 7.22−7.36

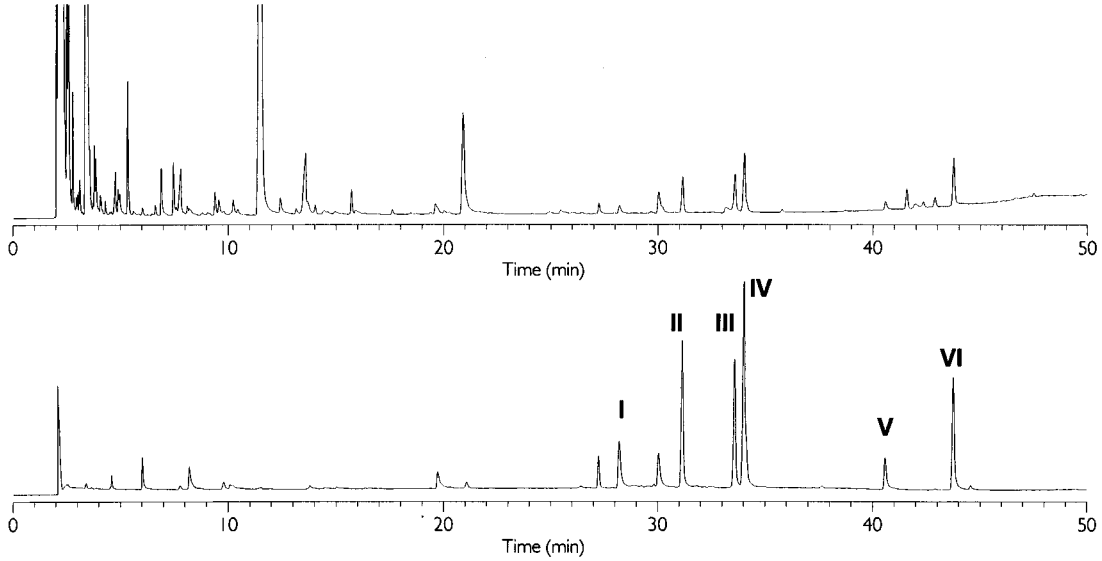

Figure 1. GC analysis of Diketopiperazines in beer extract. Upper trace, flame ionization detection (FID); lower trace, nitrogen/phosphorus detection (NPD). GC column: DB-1 stationary phase, 30 m × 0.53 mm i.d. × 1.5 μm film thickness. Temperature program: 80−260 °C at 4 °C/min. See Table 1 for identification of individual DKPs.

Table 1. GC and MS Measurements of Diketopiperazines in Beer

structure	DKP	GC retention index (DB-1)	NPD response	SCD response	principal MS features: *m/z* (% relative abundance)
I	cyclo(Ala-Pro)	1259	+		*a*
II	cyclo(Val-Pro)	1376	+		196 (M^+, 1), 154 (100), 125 (33), 72 (38), 70 (96), 41 (36)
III	cyclo(Ile-Pro)	1478	+		210 (M^+, 0.1), 154 (100), 125 (14), 86 (18), 70 (72), 41 (27)
IV	cyclo(Leu-Pro)	1496	+		*a*
V	cyclo(Met-Pro)	1780	+	+	*a*
VI	cyclo(Phe-Pro)	1935	+		*a*
VII	cyclo(Pro-Pro)	1503	+		*a*

[a] See Materials and Methods.

(m, 5H); ^{13}C NMR δ = 22.40 (CH_2), 28.22 (CH_2), 36.65 (CH_2), 45.29 (NCH_2), 56.11 (CH), 59.00 (CH), 127.36 (CH), 129.06 (CH), 129.08 (CH), 135.88 (C), 164.95 (CO), 169.33 (CO); IR (KBr) 3265 m, 2980 w, 2880 w, 1710 m, 1690 s, 1660 s, 1645 s, 1455 m, 1410 m; MS (EI) *m/z* (%) 244 (32, M^+), 153 (51), 125 (100), 91 (52), 70 (88), 43 (8), 41 (27), 28 (34).

*(3S,6S)-Octahydrodipyrrolo[1,2-a; 1′,2′-d]pyrazine-5,10-dione (**VII**).* To a precooled solution (−15 °C) of 22.4 g (89.8 mmol) of *Z*-Pro-OH in 170 mL of THF was added dropwise 9.9 mL (89.9 mmol) of NMM followed by 11.8 mL (89.8 mmol) of $ClCO_2$*i*Bu. After 2 min of stirring, a precooled solution (−15 °C) of 15.1 g (89.9 mmol) H-Pro-OCH_3·HCl and 9.9 mL (89.9 mmol) of NMM in 760 mL of DMF was added slowly. The mixture was stirred at −10 °C for 1 h, the cooling bath was removed, and stirring continued until the reaction was complete (monitoring by GC). The reaction mixture was diluted with EtOAc and washed with a 3% citric acid solution, saturated $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$) and concentrated *in vacuo* to give 25 g of a yellowish oil. Purification by flash chromatography (silica gel, methyl *tert*-butyl ether/hexane 1:4) gave 11.05 g of the coupling product that was dissolved in 250 mL of MeOH and hydrogenated over 1.0 g of Pd on charcoal (10%). After complete reaction (TLC monitoring) the catalyst was separated by filtration over Celite, the solvent was removed *in vacuo*, and the resulting solid purified by flash chromatography (silica gel, EtOAc/MeOH 2:1) to give 3.8 g (22%) of crystalline diketopiperazine **VII**. Mp 132−133 °C; $[\alpha]_D$ = −145.8 (c = 1.03, $CHCl_3$); ^{1}H NMR δ = 1.86−1.98 (m, 2H, CH_2), 1.99−2.08 (m, 2H, CH_2), 2.14−2.24 (m, 2H, CH_2), 2.29−2.36 (m, 2H, CH_2), 3.53 (d, J = 8.6 Hz, 2H, NCH_2), 3.55 (d, J = 8.6 Hz, 2H, NCH_2), 4.19 (t, J = 8.0 Hz, NCH); ^{13}C NMR δ = 23.20 (CH_2), 27.53 (CH_2), 45.04 (CH_2), 60.37 (CH), 166.22 (CO); IR (KBr) 3310 w, 2975 m, 2885 w, 2845 w, 1665 s, 1430 s, 1335 w, 1290 w, 1210 w, 1165 m; MS (EI) *m/z* (%) 194 (24, M^+), 166 (5), 138 (7), 124 (5), 110 (7), 96 (13), 70 (100), 55 (10), 41 (37), 28 (17).

Preparation of Beer Flavor Extracts. Beer (710 mL) was diluted to 900 mL with deionized water. Sodium chloride (10 g) was added to the sample, which was then extracted with dichloromethane (3 × 40 mL). Extracts were combined, and the volume reduced to *ca.* 20 mL by evaporation of solvent using a rotory evaporator; the volume was then reduced further to 0.5 mL by evaporation under a gentle stream of nitrogen at 40 °C in a fume hood. The extract finally obtained was stored at −10 °C prior to instrumental analysis.

Instrumental Analysis of Beer Flavor Extracts. These were analyzed by gas chromatography employing various methods of detection: (i) mass spectrometry (MS); (ii) flame ionization detection (FID), (iii) sulfur-specific chemiluminescence detection (SCD), and (iv) nitrogen/phosphorus detection (NPD) in nitrogen-specific mode.

Gas Chromatography−Mass Spectrometry (GC−MS). This was carried out on a Hewlett-Packard 5880A gas chromatograph interfaced with a Hewlett-Packard 5970 mass selective detector. Separations were mostly performed using a 30 m × 0.25 mm (i.d.) capillary column coated with a 1 μm film of DB-1 stationary phase (J&W Scientific, Folsom, CA). Helium was used as the carrier gas, with a column head pressure of 15 psi. The oven temperature program used was 60−270 °C at a rate of 5 °C/min. A final temperature hold for 18 min resulted in a total run time of 60 min. The injector and transfer line temperatures were held at 250 °C. Injection volume was 0.1 μL, with a split ratio of 50:1. The mass spectrometer was operated with an ionization voltage of 70 eV and electron multiplier voltage of 2200 V and was scanned from *m/z* 35 to 400 at 1 scan/s.

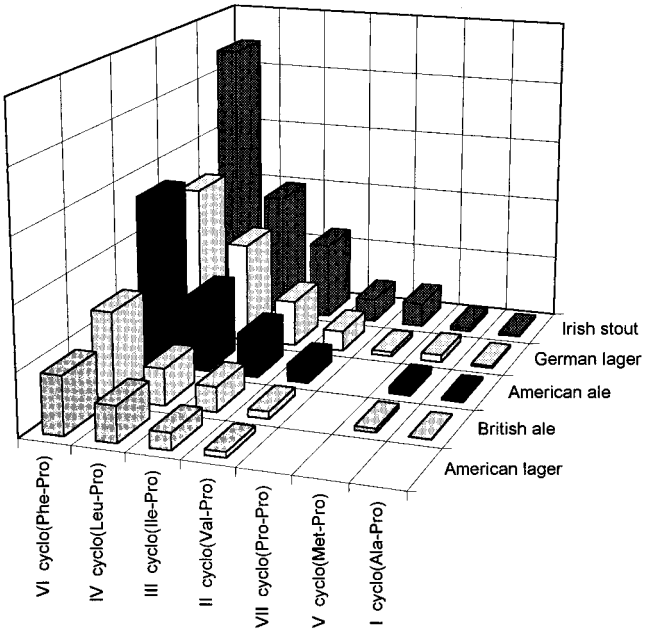

Figure 2. Relative levels of diketopiperazines found in commercial beers.

Gas Chromatography-FID. Quantitation was performed using GC with flame ionization detection, on a Perkin-Elmer Sigma 2000 gas chromatograph. Separations were performed using a 30 m × 0.32 mm (i.d.) capillary column coated with a 1 μm film of DB-1 stationary phase. Helium was used as the carrier gas, with a column head pressure of 12 psi. The oven temperature program used was 60−270 °C at a rate of 5 °C/min, with a final temperature hold of 18 min, for a total run time of 60 min. Injection volume was 0.1 μL, with a split ratio of 50:1. The injection port temperature was 240 °C, and the detector temperature was 280 °C.

High-Performance Liquid Chromatographic Analysis of Beer. The stout was analyzed for cyclo(Phe-Pro) by high-performance liquid chromatography (HPLC), using a Hewlett Packard 1100 system employing photodiode array (PDA) detection. Beer was degassed using a sonicator, filtered through a 0.45 μm Nylon filter (Phenomenex, Torrance, CA), and 20 μL was injected onto a 220 mm × 4.6 mm Brown Lee Spheri-5 column (Bodman, Aston, PA) containing 5 μm C_{18}-bonded silica reversed-phase packing. The mobile phase was pumped at 1 mL/min, according to the following gradient: 60% water/40% methanol for 6 min, adjusted to 50/50 over the next 4 min, then post-run to 100% methanol over 1 min and held as such for an additional 3 min, before finally returning to initial composition over 1 min (total cycle time, 15 min). UV spectra were acquired from 190 to 400 nm (peak width, 0.1 min; threshold, 1.0 mAU), with quantitation of cyclo(Phe-Pro) based on peak height measurements at 205 nm relative to a linear external standard calibration plot (0, 10, 20, 50, and 100 ppm in 5% methanol/95% water; $R = 0.999$).

Sensory Evaluation of Diketopiperazines. DKPs were evaluated for aroma and taste, in water, at concentrations ranging from 10 to 50 ppm depending in each case on an initial informal flavor assessment (concentrations suitable for assessors to be able to detect and describe flavor characteristics). These tests were each carried out by a panel of 5−7 experienced creative flavorists. No set list of flavor attributes was provided to the assessors, who were instead allowed a free choice of what descriptive terms to use.

RESULTS AND DISCUSSION

During the course of our continuing studies into the flavor of alcoholic beverages, we noted a series of unidentified late-eluting components which were evident in the gas chromatogram obtained on analysis of an extract of beer. Analysis of this extract by capillary GC, employing a column effluent splitter and simultaneous flame ionization and nitrogen-specific detection,

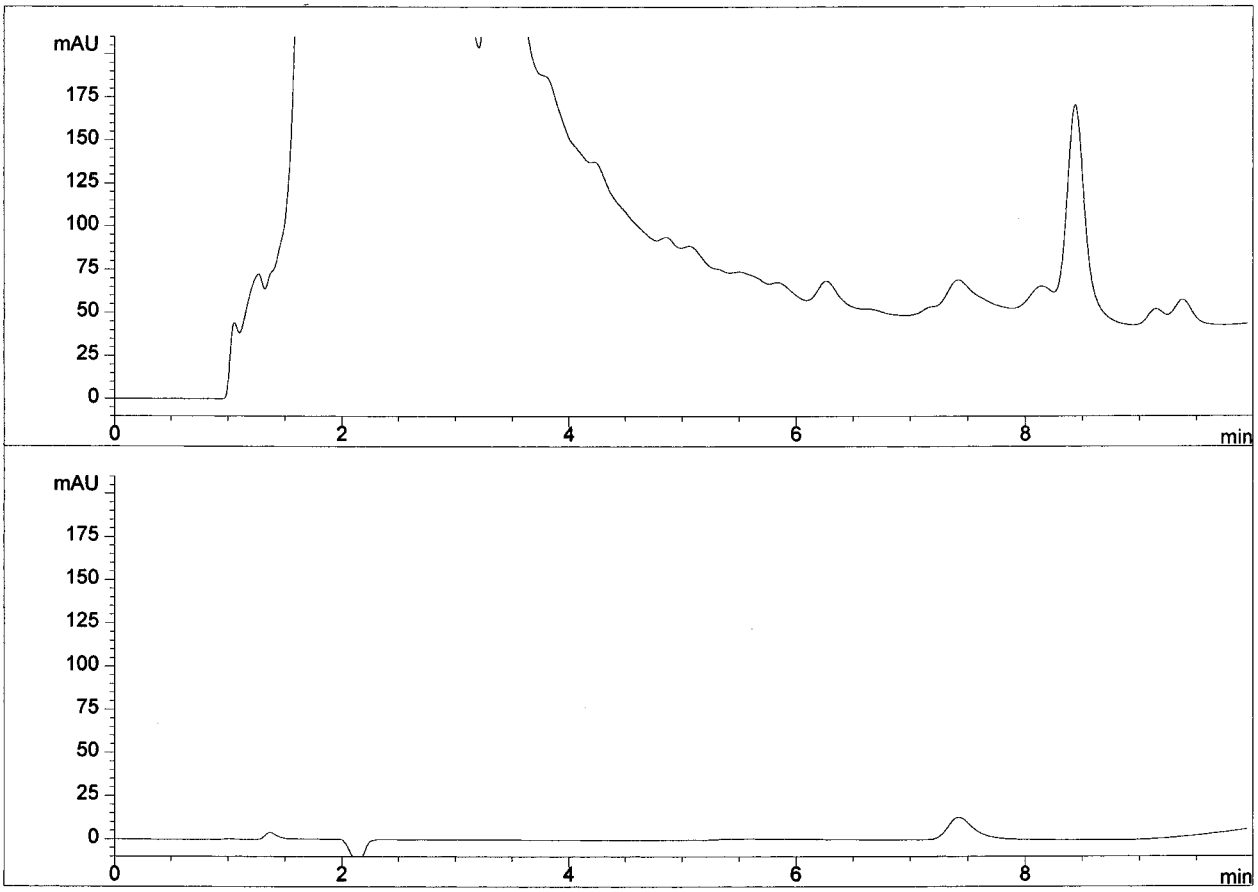

Figure 3. HPLC analysis of cyclo(Phe-Pro). Upper trace, direct injection of beer; lower trace, 20 ppm cyclo(Phe-Pro) standard.

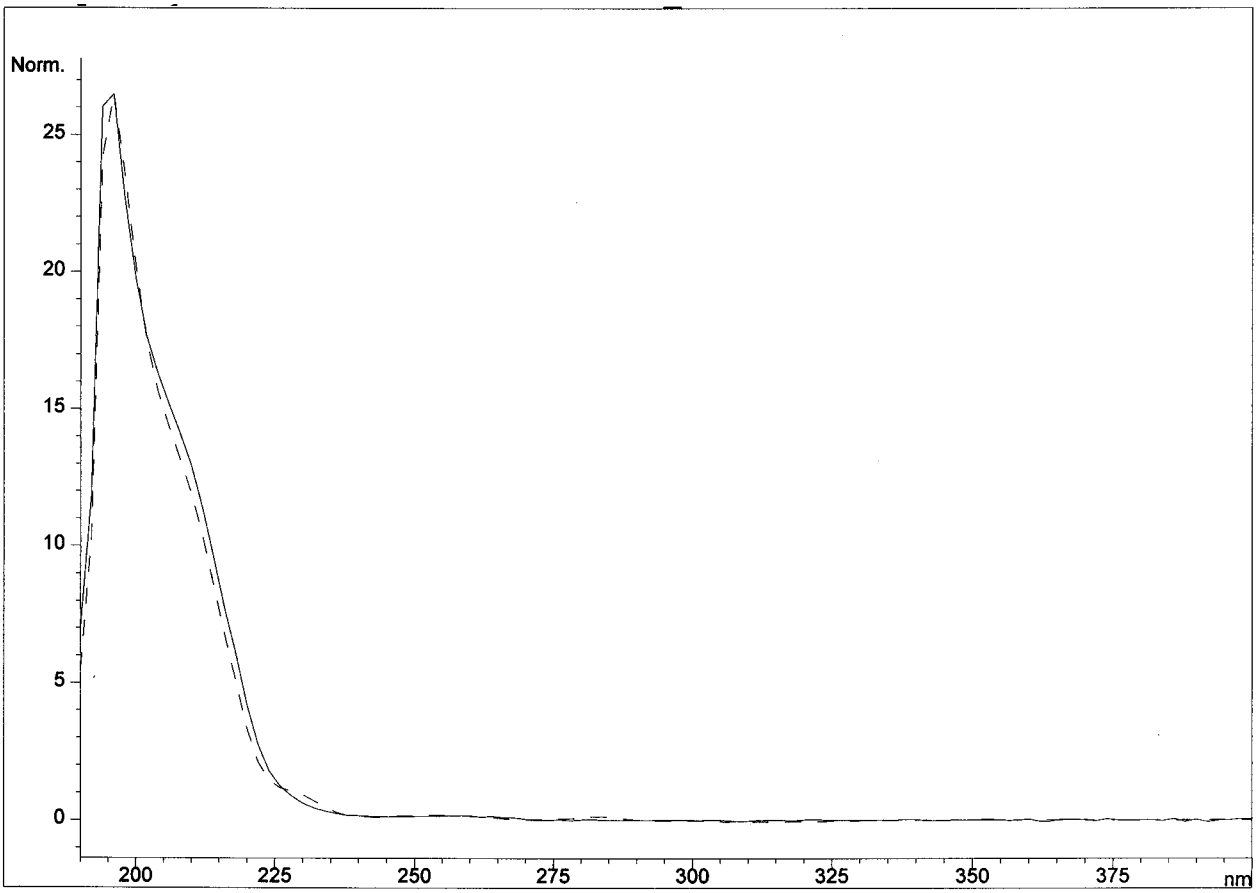

Figure 4. Background-corrected UV spectra of cyclo(Phe-Pro). Dashed line, direct injection of beer; solid line, 20 ppm cyclo-(Phe-Pro) standard.

Table 2. Sensory Evaluation of Diketopiperazines in Water

structure	DKP	description
I	cyclo(Ala-Pro)	weak, drying, very slightly astringent at 50 ppm
II	cyclo(Val-Pro)	lingering, metallic, salty taste at 10 ppm
III	cyclo(Ile-Pro)	lingering, salty taste at 30 ppm
IV	cyclo(Leu-Pro)	slightly bitter, salty character at 30 ppm
V	cyclo(Met-Pro)	mouth coating, drying character at 30 ppm
VI	cyclo(Phe-Pro)	drying, slightly astringent at 50 ppm
VII	cyclo(Pro-Pro)	weak, slightly bitter, grainy taste at 50 ppm

indicated that several peaks in the 25−45 min retention time region (see Figure 1) corresponded to nitrogen-containing components. Moreover, it was apparent that these were *major* nitrogen-containing volatiles of beer. Further analysis of the extract by capillary GC employing sulfur-specific chemiluminescence detection indicated that one of these peaks in addition contained sulfur. Thereafter, analysis of the extract by GC−MS, including interpretation of mass spectral fragmentation patterns as well as comparison of the mass spectral data obtained with several commercial and proprietary mass spectral libraries, subsequently indicated this series of peaks to be due to diketopiperazines. The individual compounds tentatively identified by GC-MS were cyclo-(Ala-Pro), cyclo(Val-Pro), cyclo(Ile-Pro), cyclo(Leu-Pro), cyclo(Met-Pro), and cyclo(Phe-Pro); cyclo(Pro−Pro) was later also identified. All seven of these DKPs (structures **I**−**VII**) were then obtained, either as gifts or by synthesis (see Materials and Methods), and were shown (on the basis of comparison of mass spectral and GC retention index data) to be the same compounds as those tentatively identified in beer. Table 1 summarizes the various GC and MS measurements made on the DKPs found in beer.

Some or all of the seven DKPs listed above were found in each of a series of five commercial beers, originating from a number of different countries and brewed using distinctly different raw materials and brewing styles. Figure 2 lists the country of origin and product type for these beers and shows *relative* levels of individual DKPs found in each. As can be seen from Figure 2, the stout generally contained the highest levels of DKPs, especially cyclo(Phe-Pro), with the American ale and German lager each containing similar but lower amounts; the American lager contained the lowest levels of all five beers. The relative profile of DKPs was generally similar for all five products, with cyclo(Phe-Pro) being most prominent, cyclo(Leu-Pro) and cyclo(Ile-Pro) being next most prominent, and cyclo(Ala-Pro), cyclo(Met-Pro), and cyclo(Pro-Pro) in several cases being present only at trace levels or not detectable at all. Study of brew-to-brew variation among DKP levels for any particular brand of beer, and possible changes in DKP levels which may occur during prolonged storage of beer, are topics which remain for future study. However, it was demonstrated that DKPs in beer did not arise through artifact formation, at least in the case of cyclo(Phe-Pro), by direct determination using HPLC with PDA detection

(see Materials and Methods). The presence of cyclo(Phe-Pro) in beer was confirmed by matching retention times (Figure 3; R.T. = 7.42 min) and background-corrected UV spectra (Figure 4), while the concentration of cyclo(Phe-Pro) in the stout was determined to be 23.9 ppm (average of 4 measurements; RSD = 5.6%).

DKPs are presumably formed during the normal manufacturing processes associated with beer production in which first malt, then wort, are exposed to elevated temperatures for prolonged periods of time. Dark malts, for example, typically are dried and kilned for 1−2 days, with temperatures maintained in excess of 100 °C for up to 4 h (see Briggs *et al*., 1981, Chapter 6), while it is common brewhouse practice to boil wort (prior to its fermentation to beer) for a period of 1−2 h (see Hough *et al*., 1982, Chapter 14). In general, the relative levels of DKPs found in this study were highest in the case of beers made with the darkest (i.e. most highly kilned) malts. From consideration of published lists of amino (and imino) acid concentrations found in malt, wort (e.g. Briggs *et al*., 1981, Chapter 9), and beer (Hough *et al*., 1982, Chapter 22), it appears that relative levels of these compounds (other than perhaps in the case of proline) do not correlate well with the types and concentrations of DKPs found in the various beers investigated in the present study. The latter is presumably also determined by the relative ease of formation and stability of the compounds in question.

The flavor characteristics of each DKP were determined in water by sensory evaluation, as described in Materials and Methods. Table 2 summarizes the results that were obtained for DKP concentrations in the range of 10−50 ppm; it was noticeable that none of the DKPs had appreciable odor. Since the levels of DKPs found in the various beers comprising the present study were in the majority of cases considerably below the maximum determined level of 24 ppm for cyclo(Phe-Pro) in the stout, it is questionable whether or not these compounds will have a significant effect on the flavor of most beers, even allowing for possible additive effects among DKPs. This statement is made in view of the highly complex nature of beer and beer flavor, which in any case tends already to be characterized by bitter, mouth coating, drying, and astringent notes (resulting from components such as the hop-derived, so-called *iso*-α acids, barley, and hop polyphenols, etc), similar to those elicited by the various DKPs. However, determining the true impact of DKPs on beer flavor awaits future, more detailed sensory evaluation work.

ACKNOWLEDGMENT

We thank C. Flynn for carrying out the sensory evaluations, M. Werner and G. Seferovič for carrying out the syntheses, J. Märki for recording the NMR spectra, A. Kuhn for performing FTIR and optical rotation analyses, and finally the officers of Givaudan-Roure Corp. for permission to publish this work.

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Received for review February 5, 1997. Revised manuscript received May 16, 1997. Accepted May 16, 1997.[⊗]

JF9700992

[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1997.