Eur Food Res Technol (2001) 213:8–11
DOI 10.1007/s002170100322

ORIGINAL ARTICLE

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Identification of new diketopiperazines in roasted coffee

Received: 4 December 2000 / Published online: 20 June 2001

Abstract This paper reports on the identification of new diketopiperazines (DKPs) in roasted coffee. The roasted coffee was extracted with hot water and $CHCl_3$ to achieve both a cleanup and a concentration of the DKPs. To get rid of the huge caffeine peak in both HPLC and GC analysis, this extract was cleaned up again by ion-exchange chromatography on Dowex 50×8 followed by gel chromatography on Sephadex G10. The identification was accomplished by HPLC-ESI-MS, HPLC-ESI-MS/MS, GC-EI-MS and by comparison with synthesised reference compounds. In roasted coffee the following DKPs were identified: cyclo(pro-gly), cyclo(pro-ala), cyclo(phe-val), cyclo(phe-leu), and cyclo(phe-ile). Except for cyclo(pro-gly), where only one isomer can be formed, each DKP was present in both possible isomeric forms.

Keywords Roasted coffee · Diketopiperazines · Bitterness · LC-ESI-MS · GC-EI-MS

Introduction

Diketopiperazines (DKPs) have been detected in a variety of natural products, especially in thermally treated or fermented foods and beverages. Seven pro-based DKPs were identified in beer [1]. Pro- and phe-based DKPs are reported to give a bitter taste at concentrations ranging from 10 to 50 ppm when tasted in aqueous solutions. Recently, five pro-based DKPs were identified in roasted coffee proteins and roasted coffee itself [2]. These were cyclo(pro-ile), cyclo(pro-leu), cyclo(pro-phe), cyclo(pro-pro), and cyclo(pro-val). Due to the ring-structure, two isomers are possible, a *cis*- and a *trans*-isomer as shown in Fig. 1. Each DKP was present in both isomeric forms [2].

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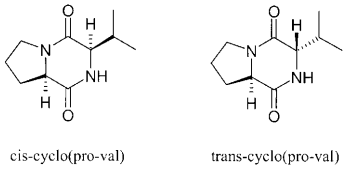

Fig. 1 Isomers of cyclo(pro-val)

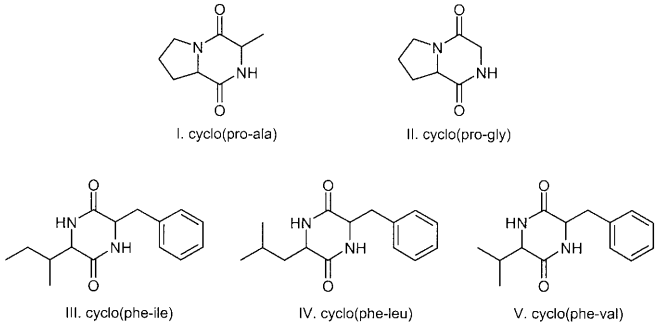

Fig. 2 Structures of identified diketopiperazines in roasted coffee

In this paper we report on the identification of five minor DKPs in roasted coffee: cyclo(pro-ala), cyclo(pro-gly), cyclo(phe-ile), cyclo(phe-leu), and cyclo(phe-val) (Fig. 2). As for the five pro-based DKPs, again both isomers of each DKP except for cyclo(pro-gly)were detected.

Materials and methods

Water. The water used in this study was HPLC grade throughout.

Coffee. The roasted coffee used was a commercial sample purchased from a local retail outlet.

Hot water extraction. Roasted and ground coffee (40 g) was extracted with hot water (1000 ml), filtered and freeze-dried.

$CHCl_3$ extraction. After cooling to room temperature the hot water extract was extracted three times, with 50 ml chloroform each

time. The $CHCl_3$ extracts were pooled, HCl (0.001 N, 200 ml) was added as a preservative and the $CHCl_3$ was removed in vacuo.

Anion exchange chromatography. A 1.5×20 cm column filled with Dowex 50×8 was used for separation. The column was treated with 500 ml of 0.1 N HCl and 500 ml of 0.001 N HCl. The $CHCl_3$-free extract was added and the column was washed with 500 ml of 0.001 N HCl. The eluate was again extracted with $CHCl_3$ (four times, each with 50 ml), the solvent was removed under reduced pressure and the residue dissolved in methanol/water.

Gel chromatography. The extract was added to a 1.5×60 mm Sephadex G10 column and eluted with water. Fractions of 25 ml were collected.

RP-HPLC. Analytical RP-HPLC was carried out on a Nucleosil 100–5 C18 HD 250×4 mm column with a pre-column of Nucleosil 100–5 C18 HD 11×4 mm (Macherey-Nagel, Dueren, FRG). The eluents were water (A) and ethanol (B), with isocratic elution (5% B) for 5 min, followed by a linear gradient to 50% B in 45 min and reset. The flow rate was 0.5 ml min^{-1}, the detection wavelength 220 nm, and the injection volume 20 µl. The sample concentration was approximately 1 mg ml^{-1} in 5% ethanol.

LC-ESI-MS. A Bruker Esquire mass spectrometer was used in the positive ESI-mode. Settings were as follows: scan range 50–1000*m/z*, nebuliser pressure 50 psi, dry gas (N_2) flow 10 l/min, dry temperature 365 °C, capillary 2500 V, endplate 2000 V, capillary exit 120 V, skimmer I 40 V; skimmer II 10 V, cut off (trap drive) 55*m/z*. For HPLC conditions, see above.

Mass spectroscopic data for compounds **I–V** (Fig. 2)**:**

I. Cyclo(pro-ala); ESI-MS, *m/z* 169. ESI-MS/MS of 169 (%): *m/z* 169 (1.1, $[M+H]^+$), 152 (13.7), 141 (21.1), 124 (4.2), 113 (4.2), 96 (10.5), 70 (100).
II. Cyclo(pro-gly); ESI-MS *m/z* 155. ESI-MS/MS of 155 (%): *m/z* 155 (2.1, $[M+H]^+$), 138 (6.6), 127 (100), 110 (6.5), 99 (39.1), 82 (34.8), 70 (50.0).
III. Cyclo(phe-ile); ESI-MS *m/z* 261. ESI-MS/MS of 261 (%): *m/z* 261 (0.2, $[M+H]^+$), 233 (58.3), 216 (25.4), 198 (1.1), 188 (18.6), 120 (100), 103 (4.3), 86 (20.5).
IV. Cyclo(phe-leu); ESI-MS *m/z* 261. ESI-MS/MS of 261 (%): *m/z* 261 (0.1, $[M+H]^+$), 233 (55.2), 216 (27.1), 198 (2.1), 188 (14.6), 120 (100), 103 (7.3), 86 (14.6).
V. Cyclo(phe-val); ESI-MS *m/z* 247. ESI-MS/MS of 247 (%): *m/z* 247 (0.2, $[M+H]^+$), 219 (81.3), 202 (17.8), 174 (26.1), 120 (100), 103 (5.2).

GC-EI-MS. The instrument was a Hewlett-Packard GC 5890 (Series II) with a MSD 5972. Splitless injection (200 °C), DB-5 MS (60 m x 0.25 mm i.d., 0.25 µm); the carrier was He (1.1 ml min^{-1}); the temperature program was 200 °C (25 min isothermal), increasing to 300 °C in 10 min. MS settings: source temperature 167 °C, 1.5 scans s^{-1}, 70 eV.

Mass spectroscopic data for compounds **I–V** (Fig. 2):

I. Cyclo(pro-ala); EI-MS *m/z* (%) 168 (68.1, M^+), 152 (5.5), 140 (12.5), 125 (37.5), 97 (33.3), 70 (100), 55 (9.7).
II. Cyclo(pro-gly); EI-MS *m/z* (%) 154 (100, M^+), 126 (22), 111 (100), 98 (26.4), 83 (75.0), 70 (52.8), 55 (25.1).
III. Cyclo(phe-ile); EI-MS *m/z* (%) 260 (33.3, M^+), 204 (47), 169 (17.3), 141 (54.8), 113 (69.2), 86 (42.2), 91 (100), 65 (9.1), 57 (15).
IV. Cyclo(phe-leu); EI-MS *m/z* (%) 260 (33.3, M^+), 204 (48.6), 169 (16.7), 141 (55.6), 113 (69.4), 86 (41.7), 91 (100), 65 (9.7), 57 (13.9).
V. Cyclo(phe-val); EI-MS *m/z* (%) 246 (47.2, M^+), 204 (9.7), 175 (4.2), 155 (18.1), 127 (47.2), 113 (18.1), 91 (100), 72 (11.1), 57 (8.3).

Synthesis of DKPs. Chemical synthesis was basically carried out as described by Pickenhagen et al. [3]. Alternatively, the corre-

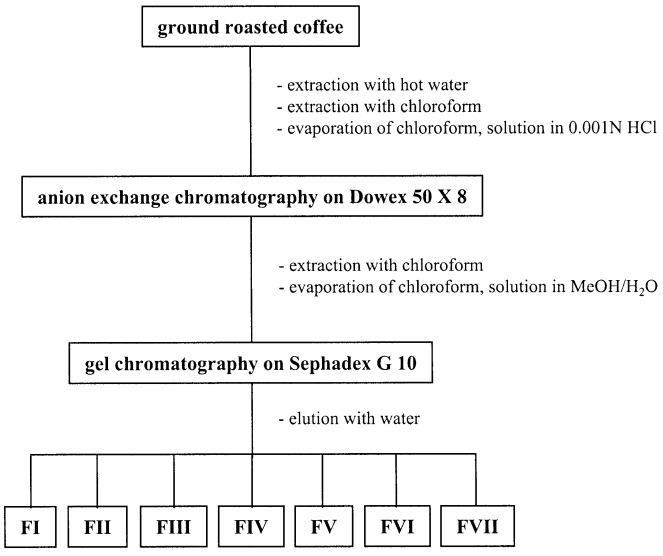

Fig. 3 Cleanup for roasted coffee

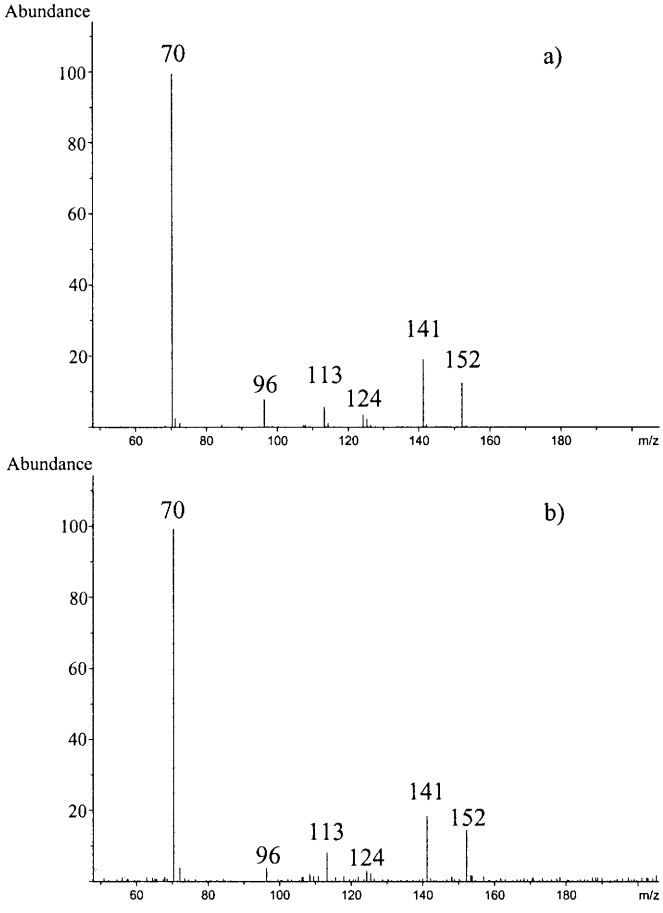

Fig. 4a,b LC-ESI-MS/MS of *cis*-cyclo(pro-ala): **a** reference compound; **b** fraction III of the Sephadex G10 fractionation at the same retention time

sponding amino acids were heated with sea sand at 290 °C for 2 min [4]. After cooling, the mixture was dissolved in water, extracted with $CHCl_3$ and purified by preparative RP-HPLC (Nucleosil 100–7 C-18, 250×21 mm; Macherey-Nagel, Dueren, FRG) with a water/ethanol gradient [isocratic elution (20% B) for 5 min, followed by a linear gradient to 80% B in 45 min and reset] at 5 ml/min and detection at 220 nm. In the case of chemical synthesis only one geometric isomer was observed (*cis*) while the thermal treatment yielded both forms. The isolated DKPs were chemically characterised by ESI-MS and ^{1}H-NMR (data not shown).

Results and discussion

In the search for the bitter principle of roasted coffee, a fractionation according to the procedure developed by Chen [5] recently led to the identification of five pro-based DKPs in roasted coffee [2]. The $CHCl_3$ extract in which both isomers of cyclo(pro-ile), cyclo(pro-leu), cyclo(pro-phe), cyclo(pro-pro) and cyclo(pro-val) were identified included a number of smaller unknown signals together with a dominating caffeine peak. For further enrichment and separation of the DKPs from caffeine an alternative fractionation including a decaffeination step was developed. Standard decaffeination techniques such as extraction with organic solvents are not suitable, as the DKPs are also extracted. Weidner [6] analysed the coffee alkaloids to characterise different coffee varieties and used anion exchange resin for decaffeination. Based on this method and a further cleanup by gel chromatography on Sephadex G10, the fractionation scheme given in Fig. 3 was used for DKP isolation from roasted coffee. The resulting Sephadex G10 fractions were analysed by GC-EI-MS and LC-ESI-MS, respectively. By LC-ESI-MS, the known five pro-based DKPs were observed as major compounds in fractions III and IV, present in both isomeric forms. Furthermore three minor signals with retention times of 6.8 min, 9.2 min, and 10.3 min were determined in fraction III as having an ESI-MS/MS fragmentation pattern including an *m/z* 70 fragment, typical for pro-based DKPs [2]. By comparison with synthesised DKPs these compounds were identified as cyclo(pro-gly) at 6.8 min, *cis*-cyclo(pro-ala) at 9.2 min, and *trans*-cyclo(pro-ala) at 10.3 min. In Fig. 4 the LC-ESI-MS/MS spectra of the *cis*-cyclo(pro-ala) reference (a) and of fraction III of the Sephadex G10 fractionation (b) is shown. As for the pro-based DKPs [2] the typical fragmentation pattern of losses of –28 *m/z* and of –17 *m/z* from the $[M+H]^+$ ion and the intensive *m/z* 70 fragment were observed for these three pro-based DKPs. The assignment of the signals to cyclo(pro-gly), *cis*-cyclo(pro-ala), and *trans*-cyclo(pro-ala) was confirmed by means of GC-EI-MS analysis (data not shown) with the same method used for the pro-based DKPs [2].

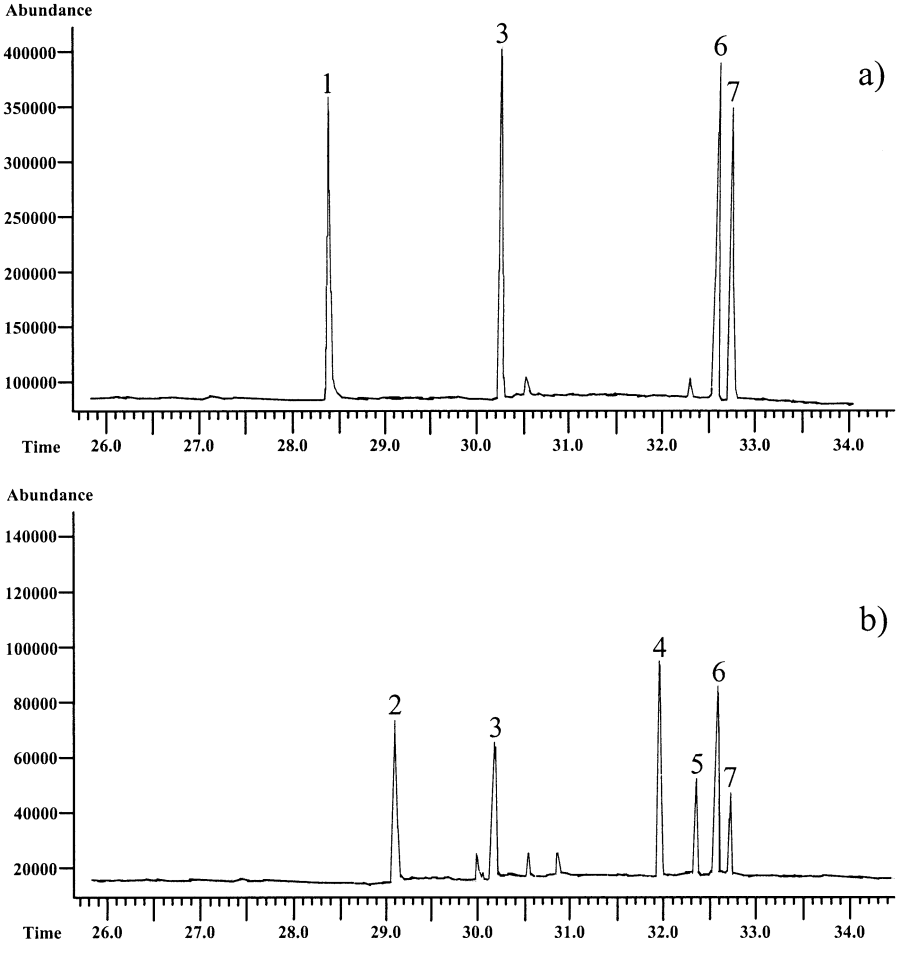

Fig. 5a,b GC-EI-MS separation of diketopiperazines (MS detection: TIC shown): **a** reference compounds; **b** fraction VI of the Sephadex G10 fractionation. **1** *cis*-cyclo(phe-ala), **2** *trans*-cyclo(phe-val), **3** *cis*-cyclo(phe-val), **4** *trans*-cyclo(phe-ile), **5** *trans*-cyclo(phe-leu), **6** *cis*-cyclo(phe-ile), **7** *cis*-cyclo(phe-leu)

All Sephadex G10 fractions were analysed by GC-EI-MS. In fraction VI a group of six signals was present with a major *m/z* 91 fragment. Under the GC-EI-MS conditions applied this fragment is typical for phe-based compounds, and had already been observed in the cyclo(pro-phe) isomers [2]. Close inspection led to the conclusion that these compounds are the phe-based DKPs: cyclo(phe-val), cyclo(phe-ile), and cyclo(phe-leu). Again, as observed for the pro-based DKPs both isomers of each of these three DKPs were detected. In Fig. 5a the GC-EI-MS chromatogram of a mixture of the synthesised reference compounds is given and compared to fraction VI of the Sephadex G10 fractionation in Fig. 5b. Looking at the retention times the GC-EI-MS spectra of the identified compounds in comparison to the synthesised reference compounds are similar (data not shown).

Furthermore, the identification of the phe-based DKPs was verified by the LC-ESI-MS analysis. By comparison with the synthesised DKPs again, the retention times, the MS-spectra and the ESI-MS/MS spectra were identical. As for the pro-based DKPs the fragmentation pattern included losses of –28 *m/z* and –17 *m/z*, indicating the fragmentation of the peptide bonds. Therefore the presence of phe-based DKPs in roasted coffee is evident.

In conclusion, the presence of phe-based DKPs was rather unexpected. In contrast to pro-based DKPs by the cyclodehydration of e.g. phe and leu no double-ring system is formed. This ring structure, in the case of the pro-based compounds, is likely to stabilise the DKP during thermal treatment. The phe content is about the same in green and roasted coffee [7]. This explains the formation of phe-based DKPs during the roasting process, but it might be speculated that these DKPs, in contrast to pro-based DKPs, are at least partly degraded in dark roasted coffee.

Acknowledgements The project was granted by the German Minister of Economy via the Arbeitskreis Industrieller Forschungsvereinigungen/Forschungskreis der Ernährungsindustrie (AIF-FV: 11793N). The support of German Coffee Trade Association is gratefully acknowledged. Thanks are due to Allan Bradbury and Hartmut Balzer (Kraft Foods, Munich) for advice and the permission to use the GC-MS equipment.

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