Reaction of Tryptophan with Carbohydrates: Identification and Quantitative Determination of Novel β-Carboline Alkaloids in Food

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The formation of various carbohydrate-derived β-carbolines was observed when model reactions of tryptophan with glucose were studied by means of HPLC with diode array detection, as well as by means of HPLC-MS. Isolation of these compounds and subsequent characterization by tandem mass spectrometry and NMR spectroscopy led to the identification of diastereomeric 1-(1,3,4,5-tetrahydroxypent-1-yl)-9*H*-pyrido[3,4-*b*]indoles (**1a/b**), 1-(1,4,5-trihydroxypent-1−yl)-9*H*-pyrido[3,4-*b*]indoles (**2a/b**), and *E*/*Z* isomers of 1-(1,5-dihydroxypent-3-en-1-yl)-9*H*-pyrido[3,4-*b*]indole (**3a/b**). HPLC-MS was used to prove the presence of these novel β-carboline alkaloids in various food samples. In addition, quantitative determination of β-carbolines **1a**, **1b**, and **2a/b** in numerous products was achieved by means of HPLC with fluorometric detection. Concentrations ranged from 12 to 1922 μg/L for **1a** and **1b** and from 3 to 644 μg/L for **2a/b**. The highest concentrations of all carbohydrate-derived β-carbolines under study were found in ketchup, soy sauce, and fish sauce.

Keywords: *L-Tryptophan; Maillard reaction; β-carbolines; electrospray ionization; HPLC-MS/MS*

INTRODUCTION

The Pictet−Spengler condensation of tryptophan with carbonyl compounds is a well-known reaction that occurs readily under mild conditions (*1*). Oxidative decarboxylation of the primarily formed 1,2,3,4-tetrahydro-β-carbolinecarboxylic acids (THCCs) yields β-carboline alkaloids (*2*). Several THCCs and β-carbolines such as norharman or harman have been detected in food samples including smoked meat, soy sauce, cheese, and yogurt and in alcoholic fermentation products such as beer, wine, and sake (*3*-*8*). THCCs as well as the respective β-carbolines exhibit a variety of pharmacologically relevant effects including interactions with benzodiazepine receptors (*9*, *10*) and inhibition of the human monoamine oxidase A (*11*). Harman has been demonstrated to have particular comutagenic and genotoxic properties (*12*). Furthermore, β-carbolines have been related to the outbreak of the eosinophilia−myalgia syndrome (*13*, *14*) and represent a chemical model to study the induction of Parkinson's disease (*15*, *16*).

The Pictet−Spengler condensation of indole amines with aldehydes has attracted reasonable attention. We studied the reaction of tryptophan with α-keto acids responsible for the formation of polar THCCs, which can serve as progenitors for harman alkaloids in food samples (*7*). In addition, we and others recently demonstrated the formation of polyhydroxylated THCCs and β-carbolines by condensation of tryptophan with reducing carbohydrates (*17*−*19*). Continuing our studies on the reaction of tryptophan with aldohexoses, we now demonstrate the isolation and identification of various β-carbolines by tandem mass spectrometry (MS/MS) and NMR spectroscopy. For the first time we report on the occurrence of these carbohydrate-derived harman alkaloids in food samples and present the results of their quantitative determination by means of HPLC with fluorometric detection.

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MATERIALS AND METHODS

Apparatus. For HPLC-MS we used an Applied Biosystems (BAI, Bensheim, Germany) 140b pump. MS and MS/MS analyses were performed utilizing a TSQ 7000 tandem mass spectrometer system equipped with an electrospray ionization (ESI) interface (Finnigan MAT, Bremen, Germany). Data acquisition and evaluation were conducted on a DEC 5000/33 (Digital Equipment, Unterföhring, Germany). High-resolution mass spectrometry (HRMS) data were acquired on a MAT 90 EI mass spectrometer (Finnigan MAT). For HPLC analysis with diode array detection (DAD) a Hewlett-Packard (Waldbronn, Germany) 1100 HPLC gradient pump and a Hewlett-Packard 1100 photodiode array detector were used, including Hewlett-Packard Chemstation software for data acquisition and evaluation. HPLC with fluorometric detection was performed on a Knauer (Berlin, Germany) HPLC system consisting of two model 64 HPLC pumps equipped with micro pump heads and a Jasco (Groβ-Umstadt, Germany) FP-1520 fluorescence detector. Data processing was performed with a Knauer HPLC data system. For identification and quantitative determination in food samples a Sunchrom Triathlon autosampler (BAI, Bensheim, Germany) was used for sample injection. UV−vis and fluorescence spectra were recorded on a Shimadzu (Duisburg, Germany) UV-2101PC spectrophotometer and a Shimadzu RF-540 spectrofluorophotometer, respectively. NMR spectra were acquired with a Bruker (Rheinstetten, Germany) DMX 600 spectrometer, calibrating the chemical shifts with the help of the solvent signal (CD_3OD: 3.31 ppm for ^1H NMR and 49.0 ppm for ^{13}C NMR) as reference.

Reagents. Water of HPLC gradient grade, L-tryptophan, D-glucose, TLC plates (SiO_2 60), and Lichroprep C18 (particle size = 43−60 μm) were from Merck (Darmstadt, Germany). Silica gel (particle size = 32−63 μm) was from ICN (Eschwege,

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Germany). Trifluoroacetic acid (TFA, spectroscopic grade) was from Fluka (Buchs, Switzerland), and acetonitrile of HPLC gradient grade was from Fisher (Loughborough, U.K.). All other chemicals were of analytical purity. Membrane filters with pore size = 0.2 μm were from Ziemer (Mannheim, Germany). Food samples were purchased at local markets.

Isolation of β-Carbolines from Model Reactions. For model reactions, 500 mg of L-tryptophan and 900 mg of glucose were dissolved in 8 mL of water and adjusted to pH 1 with 2 M HCl. Reaction mixtures were kept at 80 °C for 12 days. Model reactions were filtered, adjusted to pH 8 with 4 M NaOH, and extracted with ethyl acetate. Completeness of extraction was established by TLC on silica gel plates using acetone/chloroform/25% aqueous NH_4OH (6:2:1, v/v/v, use of organic phase) as eluent and detection of fluorescent spots with excitation at 366 nm. The combined ethyl acetate extracts were dried with anhydrous sodium sulfate, and the solvent was removed under vacuum at 40 °C. The extract was applied to a silica gel column (77 cm × 2.1 cm) and eluted with acetone/chloroform/25% aqueous NH_4OH (6:2:1, v/v/v, use of organic phase). Fractions (10 mL) were collected and checked by TLC as described above. Fractions showing only one fluorescent spot on TLC were pooled and concentrated by evaporation of the solvent under vacuum at 40 °C. The concentrated fractions were adjusted to pH 7 and further purified by column chromatography with Lichroprep C18 material. For the isolation of **1a/b** and **3a/b** column 1 (33 cm × 3.3 cm) was equilibrated and eluted with water/acetonitrile/TFA (90:10:0.05, v/v/v). For the isolation of **2a/b** column 2 (14 cm × 1.6 cm) was equilibrated with water/TFA (100:0.05, v/v) and then eluted with 100 mL of water/TFA (100:0.05, v/v) and 300 mL of water/acetonitrile/TFA (95:5:0.05, v/v/v). Fluorescent fractions were analyzed by HPLC-DAD (200−600 nm) with an Eurospher 100 C18 column (250 mm × 4 mm i.d., particle size = 5 μm; Knauer) using a linear binary gradient. Solvent A was 0.05% TFA in water (v/v), and solvent B was acetonitrile. HPLC was programmed as follows: 0 min, 5% B; 30 min, 30% B; 31 min, 100% B; 35 min, 100% B. The flow rate was 1 mL/min, and the injection volume was 20 μL for all analyses. Fractions containing only one β-carboline were pooled and freeze-dried. Fractions containing **3a** were further purified by preparative HPLC on an Eurospher 100 C18 column (250 mm × 16 mm i.d., particle size = 5 μm; Knauer) with water/methanol/TFA (70:30:0.05, v/v/v) as solvent and UV detection at 254 nm. The flow rate was 7.5 mL/min. The described separation procedures yielded the following compounds as pale yellow amorphous powders.

1a: ESI-MS, $[M + H]^+$ *m/z* 303; ESI-MS/MS (precursor ion *m/z* 303, 20 eV, 267 mPa Ar), *m/z* 303, 285, 267, 225, 207, 195; EI-HRMS, calcd for $C_{16}H_{18}N_2O_4$ (M^+) 302.1267, found 302.1267; UV λ_{max} (H_2O), 349 nm (3000 M^{-1} cm^{-1}), 288 nm (10000 M^{-1} cm^{-1}), 236 nm (24000 M^{-1} cm^{-1}), 211 nm (18000 M^{-1} cm^{-1}); fluorescence (H_2O/acetonitrile/TFA 75:25:0.05, v/v/v) excitation λ_{max} 256, 302, or 370 nm, emission λ_{max} 454 nm; R_f (acetone/chloroform/25% aqueous NH_4OH 6:2:1, v/v/v, use of organic phase) 0.18; ^1H NMR (600 MHz, CD_3OD) δ 8.55 (d, 1H, H-4), 8.39 (d, 1H, H-5), 8.34 (d, 1H, H-3), 7.81−7.77 (m, 2H, H-7 and H-8), 7.46 (dd, 1H, H-6), 5.80 (dd, 1H, H-1′), 3.86 (ddd, 1H, H-3′), 3.66 (dd, 1H, H-5′a), 3.57 (dd, 1H, H-5′b), 3.52 (ddd, 1H, H-4′), 2.48 (ddd, 1H, H-2′a), 2.23 (ddd, 1H, H-2′b); $J_{5,6}$ = 8.1 Hz, $J_{6,7}$ = 5.5 Hz, $J_{3,4}$ = 6.3 Hz, $J_{1',2'a}$ = 5.4 Hz, $J_{1',2'b}$ = 6.3 Hz, $J_{2'a,2'b}$ = 14.7 Hz, $J_{2'a,3'}$ = 2.8 Hz, $J_{2'b,3'}$ = 9.3 Hz, $J_{3',4'}$ = 6.3 Hz, $J_{4',5'a}$ = 4.3 Hz, $J_{4',5'b}$ = 6.0 Hz, $J_{5'a,5'b}$ = 11.3 Hz; ^{13}C NMR (150 MHz, CD_3OD) δ 145.4 (C-8a), 144.8 (C-1), 135.4 (C-4a), 133.0 (C-7 and C-9a), 129.3 (C-3), 124.0 (C-5), 123.0 (C-6), 121.3 (C-4b), 117.0 (C-4), 113.9 (C-8), 76.2 (C-4'), 70.3 (C-3'), 68.1 (C-1'), 64.3 (C-5'), 40.2 (C-2').

1b: ESI-MS, $[M + H]^+$ *m/z* 303; ESI-MS/MS (precursor ion *m/z* 303, 20 eV, 267 mPa Ar), *m/z* 303, 285, 267, 225, 207, 195; EI-HRMS, calcd for $C_{16}H_{18}N_2O_4$ (M^+) 302.1267, found 302.1264; UV λ_{max} (H_2O), 349 nm (3000 M^{-1} cm^{-1}), 288 nm (10000 M^{-1} cm^{-1}), 235 nm (23000 M^{-1} cm^{-1}), 211 nm (17000 M^{-1} cm^{-1}); fluorescence (H_2O/acetonitrile/TFA 75:25:0.05, v/v/v), excitation λ_{max} 258, 298, or 370 nm, emission λ_{max} 446 nm; R_f (acetone/chloroform/25% aqueous NH_4OH 6:2:1, v/v/v, use of the organic phase) 0.18; ^1H NMR (600 MHz, CD_3OD) δ 8.58 (d, 1H, H-4), 8.41 (d, 1H, H-5), 8.35 (d, 1H, H-3), 7.83−7.77 (m, 2H, H-7 and H-8), 7.47 (dd, 1H, H-6), 5.76 (dd, 1H, H-1′), 4.09 (ddd, 1H, H-3′), 3.71 (m, 1H, H-5′a), 3.63−3.57 (m, 2H, H-5′b and H-4′), 2.21 (ddd, 1H, H-2′a), 2.08 (ddd, 1H, H-2′b); $J_{5,6}$ = 8.0 Hz, $J_{6,7}$ = 6.5 Hz, $J_{3,4}$ = 6.2 Hz, $J_{1',2'a}$ = 10.0 Hz, $J_{1',2'b}$ = 3.1 Hz, $J_{2'a,2'b}$ = 14.4 Hz, $J_{2'a,3'}$ = 2.3 Hz, $J_{2'b,3'}$ = 10.3 Hz, $J_{3',4'}$ = 5.9 Hz; ^{13}C NMR (150 MHz, CD_3OD) δ 145.5 (C-8a and C-1), 135.6 (C-4a), 133.2 (C-7), 132.6 (C-9a), 129.4 (C-3), 124.1 (C-5), 123.1 (C-6), 121.3 (C-4b), 117.1 (C-4), 113.9 (C-8), 76.4 (C-4′), 69.7 (C-3′), 66.8 (C-1′), 64.4 (C-5′), 40.4 (C-2′).

2a/b: ESI-MS, $[M + H]^+$ *m/z* 287; ESI-MS/MS (precursor ion *m/z* 287, 20 eV, 267 mPa Ar), *m/z* 287, 269, 251, 233, 209; EI-HRMS, calcd for $C_{16}H_{18}N_2O_3$ (M^+) 286.1317, found 286.1313; UV λ_{max} (H_2O), 348 nm (3000 M^{-1} cm^{-1}), 288 nm (10000 M^{-1} cm^{-1}), 235 nm (24000 M^{-1} cm^{-1}), 211 nm (17000 M^{-1} cm^{-1}); fluorescence (H_2O/acetonitrile/TFA 75:25:0.05, v/v/v), excitation λ_{max} 256, 302, or 372 nm, emission λ_{max} 448 nm; R_f (acetone/chloroform/25% aqueous NH_4OH 6:2:1, v/v/v, use of organic phase), 0.23; compounds **2a/b** could not be separated chromatographically and were isolated as a mixture. However, two-dimensional NMR experiments allowed unequivocal identification of the signal sets for the individual diastereomers, as follows.

2a: ^1H NMR (600 MHz, CD_3OD) δ 8.56 (d, 1H, H-4), 8.39 (d, 1H, H-5), 8.35 (d, 1H, H-3), 7.81−7.76 (m, 2H, H-7 and H-8), 7.46 (dd, 1H, H-6), 5.61 (dd, 1H, H-1′), 3.67 (m, 1H, H-4′), 3.47 (m, 2H, H-5′a and H-5′b), 2.15 (m, 2H, H-2′a and H-2′b), 1.84 (m, 1H, H-3′a), 1.52 (m, 1H, H-3′b); $J_{5,6}$ = 8.0 Hz, $J_{6,7}$ = 6.4 Hz, $J_{3,4}$ = 6.2 Hz; ^{13}C NMR (150 MHz, CD_3OD) δ 145.5 (C-8a), 144.9 (C-1), 135.5 (C-4a), 133.1 (C-7), 132.8 (C-9a), 129.4 (C-3), 124.0 (C-5), 123.0 (C-6), 121.2 (C-4b), 117.1 (C-4), 113.9 (C-8), 72.9 (C-4′), 69.3 (C-1′), 67.2 (C-5′), 34.2 (C-2′), 29.7 (C-3′).

2b: ^1H NMR (600 MHz, CD_3OD) δ 8.56 (d, 1H, H-4), 8.39 (d, 1H, H-5), 8.35 (d, 1H, H-3), 7.81−7.76 (m, 2H, H-7 and H-8), 7.46 (dd, 1H, H-6), 5.56 (dd, 1H, H-1′), 3.62 (m, 1H, H-4′), 3.47 (m, 2H, H-5′a and H-5′b), 2.28 (m, 1H, H-2′a), 1.99 (m, 1H, H-2′b), 1.78 (m, 1H, H-3′a), 1.61 (m, 1H, H-3′b); $J_{5,6}$ = 8.0 Hz, $J_{6,7}$ = 6.4 Hz, $J_{3,4}$ = 6.2 Hz; ^{13}C NMR (150 MHz, CD_3OD) δ 145.5 (C-8a), 144.9 (C-1), 135.5 (C-4a), 133.1 (C-7), 132.8 (C-9a), 129.4 (C-3), 124.0 (C-5), 123.0 (C-6), 121.2 (C-4b), 117.1 (C-4), 113.9 (C-8), 72.9 (C-4′), 69.7 (C-1′), 67.2 (C-5′), 34.1 (C-2′), 30.0 (C-3′).

3a: ESI-MS, $[M + H]^+$ *m/z* 269; ESI-MS/MS (precursor ion *m/z* 269, 20 eV, 267 mPa Ar), *m/z* 269, 251, 233, 197, 182; EI-HRMS, calcd for $C_{16}H_{16}N_2O_2$ (M^+) 268.1212, found 268.1219; UV λ_{max} (H_2O), 350 nm, 289 nm, 235 nm, 210 nm; fluorescence (H_2O/acetonitrile/TFA 75:25:0.05, v/v/v), excitation λ_{max} 258, 304, or 374 nm, emission λ_{max} 446 nm; R_f (acetone/chloroform/25% aqueous NH_4OH 6:2:1, v/v/v, use of organic phase) 0.51; ^1H NMR (600 MHz, CD_3OD) δ 8.59 (d, 1H, H-4), 8.42 (d, 1H, H-5), 8.35 (d, 1H, H-3), 7.84−7.78 (m, 2H, H-7 and H-8), 7.48 (dd, 1H, H-6), 5.71−5.61 (m, 3H, H-1′, H-3′, and H-4′), 3.88 (dd, 1H, H-5′a), 3.80 (dd, 1H, H-5′b), 2.88 (m, 2H, H-2′a und H-2′b); $J_{5,6}$ = 8.1 Hz, $J_{6,7}$ = 6.4 Hz, $J_{3,4}$ = 6.2 Hz, $J_{4',5'a}$ = $J_{4',5'b}$ = 5.7 Hz, $J_{5'a,5'b}$ = 12.8 Hz; ^{13}C NMR data could not be acquired due to insufficient material.

3b: ESI-MS, $[M + H]^+$ *m/z* 269; ESI-MS/MS (precursor ion *m/z* 269, 20 eV, 267 mPa Ar), *m/z* 269, 251, 233, 197, 182; EI-HRMS, calcd for $C_{16}H_{16}N_2O_2$ (M^+) 268.1212, found 268.1207; UV λ_{max} (H_2O), 351 nm (3000 M^{-1} cm^{-1}), 288 nm (9000 M^{-1} cm^{-1}), 236 nm (21000 M^{-1} cm^{-1}), 211 nm (15000 M^{-1} cm^{-1}); fluorescence (H_2O/acetonitrile/TFA 75:25:0.05, v/v/v), excitation λ_{max} 257, 302, or 372 nm, emission λ_{max} 450 nm; R_f (acetone/chloroform/25% aqueous NH_4OH 6:2:1, v/v/v, use of organic phase) 0.51; ^1H NMR (600 MHz, CD_3OD) δ 8.57 (d, 1H, H-4), 8.40 (d, 1H, H-5), 8.35 (d, 1H, H-3), 7.82−7.77 (m, 2H, H-7 and H-8), 7.47 (dd, 1H, H-6), 5.77 (m, 1H, H-3′), 5.63 (dd, 1H, H-1′), 5.55 (dt, 1H, H-4′), 3.91 (d, 2H, H-5′), 2.86 (m, 1H, H-2′a), 2.76 (m, 1H, H-2′b); $J_{5,6}$ = 8.1 Hz, $J_{6,7}$ = 5.8 Hz, $J_{3,4}$ = 6.1 Hz, $J_{1',2'a}$ = 5.2 Hz, $J_{1',2'b}$ = 6.3 Hz, $J_{3',4'}$ = 15.4 Hz, $J_{4',5'}$ = 5.4 Hz; ^{13}C NMR (150 MHz, CD_3OD) δ 145.5 (C-8a), 144.1 (C-1), 135.5 (C-4a), 135.3 (C-4′), 133.1 (C-7), 132.9 (C-9a), 129.5 (C-3), 125.8 (C-3′), 124.0 (C-5), 123.0 (C-6), 121.3 (C-4b), 117.1 (C-4), 113.9 (C-8), 69.4 (C-1′), 63.1 (C-5′), 40.4 (C-2′).

Identification of β-Carbolines in Food Samples by HPLC-MS/MS. Food samples were filtered through membrane filters of pore size 0.2 μm. The ketchup samples were centrifuged at 6000*g* for 20 min, and the supernatant was membrane-filtered. The resulting solutions were directly subjected to HPLC-ESI-MS/MS analysis. Chromatographic separation was performed on a Symmetry C18 column (150 mm × 2.1 mm i.d., particle size = 5 μm; Waters, Milford, MA) using a binary gradient. Solvent A was 0.05% TFA in water (v/v), and solvent B was acetonitrile. HPLC was programmed as follows: pressurizing with 50% B, equilibration time of 10 min at 5% B, and linear gradient elution (0 min, 5% B; 30 min, 30% B; 31 min, 100% B; 35 min, 100% B). The flow rate was 200 μL/min and the injection volume was 5 μL for all analyses. For pneumatically assisted ESI, the spray voltage was set to 3.5 kV; the temperature of the heated capillary was 220 °C. Nitrogen served both as sheath (60 psi) and auxiliary gas (10 units). Product ion scanning was performed at a collision gas pressure of 267 mPa Ar and a collision energy of 20 eV with a total scan duration of 1.0 s for a single spectrum. The most abundant product ions were chosen for the selected reaction monitoring (SRM) analysis with a scan duration of 0.5 s for each experiment. Selected ion pairs were as follows: SRM 1, *m/z* 303/285 and *m/z* 303/195 for **1a/b** (0−17 min of the chromatographic run), *m/z* 269/251 and *m/z* 269/197 for **3a/b** (17−35 min of the chromatographic run); SRM 2, *m/z* 367/229 and *m/z* 367/188 for established tryptophan glycoconjugates (cf. ref *17*; 0−14 min of the chromatographic run), *m/z* 287/269 and *m/z* 287/251 for **2a/b** (14−35 min of the chromatographic run).

Quantification of β-Carbolines 1a, 1b, and 2a/b in Food by HPLC with Fluorometric Detection. Food samples were filtered through membrane filters of pore size 0.2 μm. The ketchup samples were centrifuged at 6000*g* for 20 min, and the supernatant was membrane-filtered. The resulting solutions were diluted with water and 4 M KOH (ketchup 1, ketchup 2, soy sauce 1, fish sauce, 1:10 v/v; soy sauce 2, 1:5 v/v; Worcestershire sauce 1, Worcestershire sauce 2, plum juice 1, plum juice 2, pineapple juice 1, 1:3 v/v; vinegar 1, vinegar 2, 1:2 v/v; pineapple juice 2, 1:1.1 v/v) to adjust the pH to 6−7. Solid phase extraction (SPE) was performed with HLB cartridges (OASIS, 30 mg; Waters), which were conditioned with 1 mL of MeOH and 1 mL of water. After application of 1 mL of the neutralized sample (for analysis of pineapple juice 2 we applied 4 mL of the neutralized sample), each column was washed with 1 mL of MeOH/water (5:95, v/v) and 1 mL of MeOH/water (3:7, v/v) containing 2% aqueous NH_4OH (pH 11). The columns were eluted with 1 mL of MeOH/water (1:1, v/v) containing 2% acetic acid (pH 3). The eluate was diluted with water (1:10, v/v) and directly subjected to HPLC analysis. Chromatographic separation was performed on an XTerra MS C18 column (150 mm × 2.1 mm i.d., particle size = 3.5 μm; Waters) using a linear binary gradient. Solvent A was 0.05% TFA in water (v/v), and solvent B was acetonitrile. HPLC was programmed as follows: 0 min, 10% B; 26 min, 23% B; 28 min, 100% B; 31 min, 100% B. The flow rate was 200 μL/min and the injection volume was 5 μL for all analyses. The fluorescence detector was set to 300 nm for excitation and to 450 nm for emission. Quantitative determinations were performed with standard solutions (0, 1, 2, 5, 10, 20, 50, and 100 nmol/L) of reference compounds **1a** (0, 0.30, 0.60, 1.51, 3.02, 6.04, 15.10, and 30.20 μg/L) and **2a/b** (0, 0.29, 0.57, 1.43, 2.86, 5.72, 14.30, and 28.60 μg/L) in water for external calibration [calibration function for **1a**, $y = 0.4017x - 0.3708$ (correlation coefficient, $r = 0.9988$); calibration function for **2a/b**, $y = 0.448x - 0.3852$ (correlation coefficient, $r = 0.9995$)]. Concentrations were calculated from peak areas of **1a**, **1b**, and **2a/b**. Recoveries of **1a**, **1b**, and **2a/b** were estimated by comparing the respective peak areas of standard solutions after SPE cleanup with untreated standard solutions. The detection limit for the purified reference compounds was estimated (signal/noise = 3/1) with the help of the standard solutions. The limit of quantification (LOQ) of the method was calculated with the help of the calibration functions as $LOQ = y + 3.3s$ (with y = axis intercept of the calibration function and s = standard deviation of the measured values for the standard samples with 1 nmol/L).

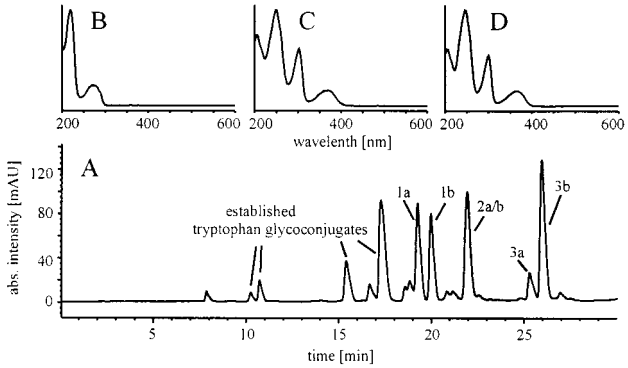


Figure 1. HPLC-DAD analysis of a tryptophan−glucose model reaction: (A) chromatogram detected at 254 nm; (B−D) UV spectra of (B) *gluco*-tetrahydro-β-carboline, (C) compound **1a**, and (D) harman.

RESULTS AND DISCUSSION

Detection and Characterization of Carbohydrate-Derived β-Carbolines by HPLC-MS/MS. Our recent investigations on the reaction of tryptophan with carbohydrates had revealed the formation of *N*-glycosides, *C*-glycosyl conjugates, and *glyco*-tetrahydro-β-carbolines in addition to established Amadori rearrangement products (*17*). Subsequent studies on alkaloids generated in model reactions of tryptophan with glucose demonstrated the occurrence of additional compounds, which showed characteristic UV spectra completely different from those of known tryptophan glycoconjugates (Figure 1). By comparison of the UV spectra obtained during these HPLC-DAD analyses with data from the reference alkaloid harman, the unknown substances **1**−**3** were tentatively identified as β-carboline derivatives. With the help of HPLC-ESI-MS analysis we determined the molecular ions *m/z* 303 $[M + H]^+$ for **1a** and **1b**, *m/z* 287 $[M + H]^+$ for **2a/b**, and *m/z* 269 $[M + H]^+$ for **3a** and **3b**. The product ion spectra (Figure 2; Table 1) as obtained by low-energy collision-induced dissociation showed fragmentation patterns dominated by the loss of H_2O. In addition, retro-aldol fragmentation, a reaction that already had been observed for polyol-β-carbolines (*17*), caused the characteristic neutral loss of CH_2O units. Clearly, product ions of **1a** and **1b** demonstrated the presence of three neighboring CH_2O moieties plus the presence of one additional hydroxyl group. Correspondingly, the product ion spectrum of **2a/b** confirmed the presence of two neighboring CH_2O moieties plus the presence of one additional hydroxyl group, whereas the product ion spectra of **3a** and **3b** confirmed the presence of one terminal CH_2O moiety plus the presence of one additional hydroxyl group. Furthermore, **3a** and **3b** showed a prominent product ion *m/z* 197, which indicated the loss of the olefinic alcohol C_4H_8O; consequently, **3a** and **3b** most likely were substituted β-carbolines featuring a $-CH_2-CH{=}CHCH_2OH$ moiety. Thus, the combined UV, MS, and MS/MS data demonstrated the presence of β-carboline alkaloids with glucose-derived side chains.

Identification of Carbohydrate-Derived β-Carbolines by NMR Spectroscopy. For structure elucidation of compounds **1**−**3** (Figure 3) we isolated these carbohydrate-derived alkaloids from model reactions of tryptophan and glucose. Identification was determined

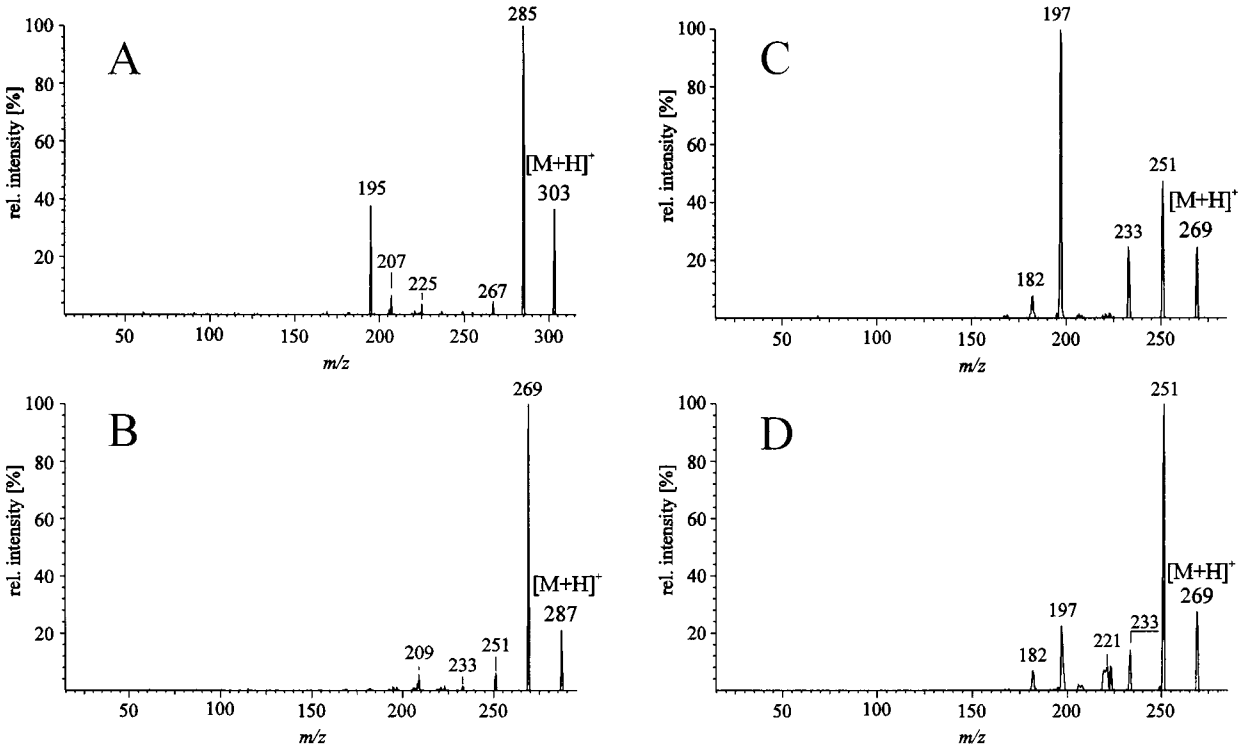
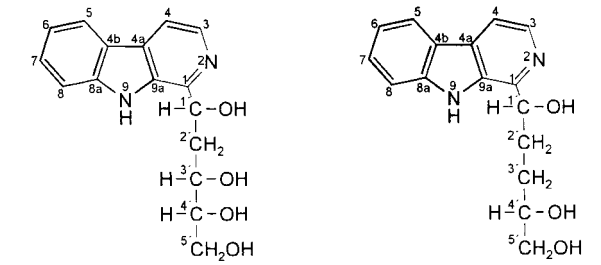


Figure 2. Product ion spectra of the β-carbolines **1a** (A), **2a/b** (B), **3a** (C), and **3b** (D) (20 eV, 267 mPa Ar).

Table 1. Product Ions of β-Carbolines 1a, 2a/b, and 3b

m/z	1a	2a/b	3b
303	$[M + H]^{+a}$		
287		$[M + H]^{+a}$	
285	$[303 - H_2O]^+$		
269		$[287 - H_2O]^+$	$[M + H]^{+a}$
267	$[285 - H_2O]^+$		
251		$[269 - H_2O]^+$	$[269 - H_2O]^+$
233		$[251 - H_2O]^+$	$[251 - H_2O]^+$
225	$[285 - C_2H_4O_2]^+$		
221			$[251 - CH_2O]^+$
209		$[269 - C_2H_4O_2]^+$	
207	$[267 - C_2H_4O_2]^+$		
197			$[269 - C_4H_8O]^+$
195	$[285 - C_3H_6O_3]^+$		

[a] Precursor ion.

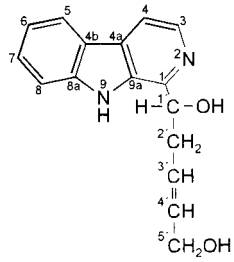


1-(1,3,4,5-Tetrahydroxypent-1-yl)-9*H*-pyrido[3,4-b]indol

1a/b

1-(1,4,5-Trihydroxypent-1-yl)-9*H*-pyrido[3,4-b]indol

2a/b



1-(1,5-Dihydroxypent-3-en-1-yl)-9*H*-pyrido[3,4-b]indol

3a/b

Figure 3. Carbohydrate-derived β-carbolines.

by NMR spectroscopy including DEPT, HH-COSY, HMBC, and HMQC experiments. All substances under study showed comparable signals in the ^{1}H NMR spectra arising from the protons H-3, H-4, H-5, H-6, H-7, and H-8 of the β-carboline skeleton. In contrast, **1**−**3** showed different ^{1}H and ^{13}C signals for the carbohydrate-derived substituents at C-1. For **1a** and **1b**, the NMR spectra confirmed the presence of a 1,3,4,5-tetrahydroxypentyl moiety, as we had postulated on the basis of the MS/MS data. The position of the methylene group at C-2′ was proved by the negative DEPT signal and by the HMQC correlations between C-2′ and the two protons H-2′a and H-2′b. **1a** and **1b** not only yielded identical molecular ions *m/z* 303 $[M + H]^+$ and product ion spectra, but also their NMR data were almost identical. Significant variations could be observed exclusively for the coupling constants $^3J_{H-1',H-2'a}$ (5.4 Hz for **1a**, 10.0 Hz for **1b**) and $^3J_{H-1',H-2'b}$ (6.3 Hz for **1a**, 3.1 Hz for **1b**) and for the chemical shifts of C-1′ (δ_C = 68.1 for **1a**, δ_c = 66.8 for **1b**). Therefore, **1a** and **1b** were evidently diastereomers with different configurations of the hydroxyl group at C-1′. Compounds **2a**/**b** could not be separated chromatographically and were isolated as a mixture. However, by two-dimensional NMR experiments we unequivocally identified them as diastereomeric 1-(1,4,5-trihydroxypent-1-yl)-β-carbolines. Substances **3a** and **3b** had the molecular composition $C_{16}H_{16}N_2O_2$ as determined by HRMS. From this molecular formula, as well as from the product ion spectra, it became evident that **3a** and **3b** had an additional double bond when compared with **1a**, **1b**, and **2a/b**. NMR spectra of compounds **3a/b** showed that protons

Table 2. Carbohydrate-Derived β-Carbolines in Food[a]

sample	**1a** (μg/L)	**1b** (μg/L)	**2a/b** (μg/L)	**3a**	**3b**
ketchup 1	785.7 (0.3%)	628.9 (0.6%)	99.9 (1.2%)	+[b]	+
ketchup 2	1921.6 (2.7%)	1577.0 (3.5%)	222.2 (1.1%)	+	+
soy sauce 1	1819.1 (2.5%)	1751.8 (1.7%)	643.5 (2.0%)	nd[c]	+
soy sauce 2	980.2 (2.1%)	769.1 (0.4%)	187.4 (1.2%)	nd	+
fish sauce	1536.4 (0.1%)	1375.9 (0.1%)	240.1 (1.9%)	nd	+
plum juice 1 (from concentrate)	252.7 (0.2%)	204.2 (0.1%)	67.6 (0.7%)	+	+
plum juice 2 (from concentrate)	395.4 (3.2%)	347.1 (2.9%)	89.1 (2.5%)	+	+
Worcestershire sauce 1	151.2 (1.0%)	121.4 (0.6%)	46.1 (2.2%)	nd	+
Worcestershire sauce 2	320.7 (0.8%)	255.8 (1.7%)	53.8 (2.6%)	+	+
balsamic vinegar 1	122.7 (0.7%)	111.2 (0.3%)	35.6 (1.4%)	+	+
balsamic vinegar 2	249.5 (1.5%)	204.2 (1.1%)	30.4 (0.3%)	+	+
wine vinegar	nd	nd	nd	nd	nd
pineapple juice 1 (from concentrate)	314.5 (2.1%)	225.0 (1.7%)	39.1 (0.5%)	+	+
pineapple juice 2 (not from concentrate)	14.8 (5.4%)	11.6 (5.2%)	2.8 (3.6%)	nd	nd
red wine	nd	nd	nd	nd	nd

[a] Quantitative values were determined as the mean of two separate SPE extractions; precision of the method is indicated by the coefficients of variation in parentheses. [b] +, detected. [c] nd, not detected.

H-3′ and H-4′ (δ_H = 5.8−5.5), as well as carbons C-3′ ($\delta_{C-3'}$ = 125.8 for **3b**) and C-4′ ($\delta_{C-4'}$ = 135.3 for **3b**) occurred with significant low-field shifts compared with the values for **2a/b** ($\delta_{H-3'a/b}$ = 1.84 and 1.52, $\delta_{H-4'}$ = 3.67, $\delta_{C-3'}$ = 29.7, and $\delta_{C-4'}$ = 72.9 for **2a**), thus confirming the existence of the carbon−carbon double bond (*20*). The large coupling constant $^3J_{H-3',H-4'}$ (15.4 Hz) of **3b** verified the *trans*-configuration of the double bond in the prominent isomer **3b**, which was identified as 1-(1,5-dihydroxypent-3-en-1-yl)-β-carboline. Accordingly, we identified the side product **3a** as isomeric 1-(1,5-dihydroxypent-3-en-1-yl)-β-carboline on the basis of its ^{1}H NMR spectrum, which was largely identical with data of **3b**. However, owing to strong overlap of the proton signals H-1′, H-3′, and H-4′ we could not determine the $^3J_{H-3',H-4'}$ coupling constant for **3a**. Therefore, **3a** most likely represented the *Z*-isomer of **3b** with unknown configuration at C-1′. Occurrence of carbohydrate-derived β-carbolines in reactions of sugars with tryptophan has been reported in the literature. Bräutigam and Severin (*21*) detected several 1-substituted β-carbolines, namely, 1-(2−furyl)-, 1-hydroxymethyl-, and 1-(1-hydroxy-3-butenyl)-β-carboline, after heating of tryptophan with xylose at 160 °C. Nakatsuka and co-workers (*22*) isolated the hydroxymethylfuryl-β-carboline derivatives flazin and perlolidin from soy sauce. Wang and co-workers (*18*) identified 1-(1,3,4-trihydroxybutyl)-β-carboline and 1-(1,4-dihydroxybutyl)-β-carboline in model reactions of tryptophan and xylose. Rönner and co-workers (*19*) investigated the oxidative decarboxylation of 1-(D-*gluco*-1,2,3,4,5-pentahydroxypentyl)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, which resulted in the formation of 1-(D-*gluco*-1,2,3,4,5-pentahydroxypentyl)-β-carboline. However, there are no reports available to date in which compounds **1**−**3** have been detected or identified.

Analysis of Carbohydrate-Derived β-Carbolines in Food Samples. To clarify the relevance of the newly identified β-carbolines, several food samples were analyzed by means of HPLC-ESI-MS/MS using the SRM mode. On the basis of the product ion spectra the following ions were selected: *m*/*z* 303/285 and *m*/*z* 303/195 for **1a** and **1b**, *m*/*z* 287/269 and *m*/*z* 287/251 for **2a/b**, and *m*/*z* 269/251 and *m*/*z* 269/197 for **3a** and **3b**. As a typical example, Figure 4 shows the corresponding mass chromatograms obtained by the analysis of a balsamic vinegar sample using HPLC-MS/MS with SRM experiments. In addition to the established tryptophan glycoconjugates (Figure 4D; *17*) we detected the novel

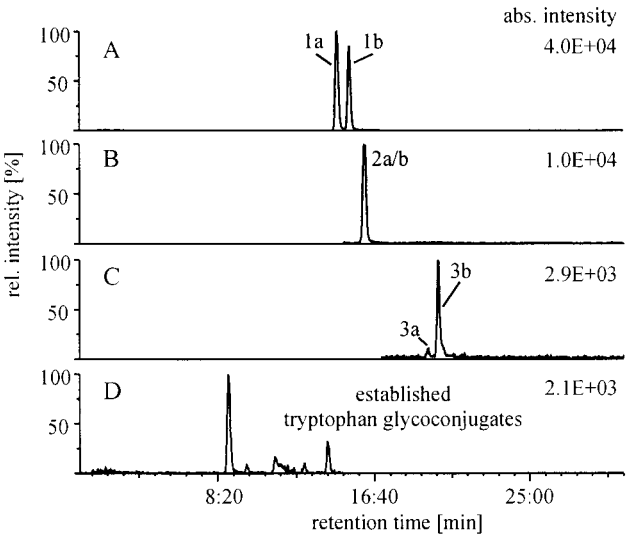


Figure 4. HPLC-MS/MS analysis of balsamic vinegar 2: (A) SRM *m*/*z* 303/285, *m*/*z* 303/195 for **1a/b** (B) SRM *m*/*z* 287/269, *m*/*z* 287/251 for **2a/b**; (C) SRM *m*/*z* 269/251, *m*/*z* 269/197 for **3a/b**; (D) SRM *m*/*z* 367/229, *m*/*z* 367/188 for established tryptophan glycoconjugates (*17*).

carbohydrate-derived β-carbolines **1**−**3** in significant amounts (Figure 4A−C). Subsequently, we identified the novel compounds **1**−**3** in most food samples studied (Table 2) by HPLC-MS/MS analyses. Only wine vinegar as well as the red wine sample contained neither the established tryptophan glycoconjugates nor the novel β-carbolines. In all other samples we detected both the glycoconjugates and the β-carbolines. For quantitative determination of the carbohydrate-derived β-carbolines **1a**, **1b**, and **2a/b**, we took advantage of the strong fluorescence of these compounds and used purified reference compounds as standards for external calibration by means of HPLC with fluorometric detection. Due to their small concentrations, **3a/b** could not be included within the quantitative analysis. The very first analysis of food samples by HPLC with fluorometric detection demonstrated that, in contrast to HPLC-MS/MS studies, SPE had to be included as a cleanup procedure prior to the quantitative evaluation of **1a**, **1b**, and **2a/b**. Subsequently, we developed an efficient extraction method based on the application of a mixed-mode macroporous [poly(divinylbenzene-*co*-*N*-vinylpyrrolidone)] copolymer (Oasis HLB cartridges) for SPE. By variation of the pH of the eluent and the concentration of the organic modifier we achieved selective isolation of compounds

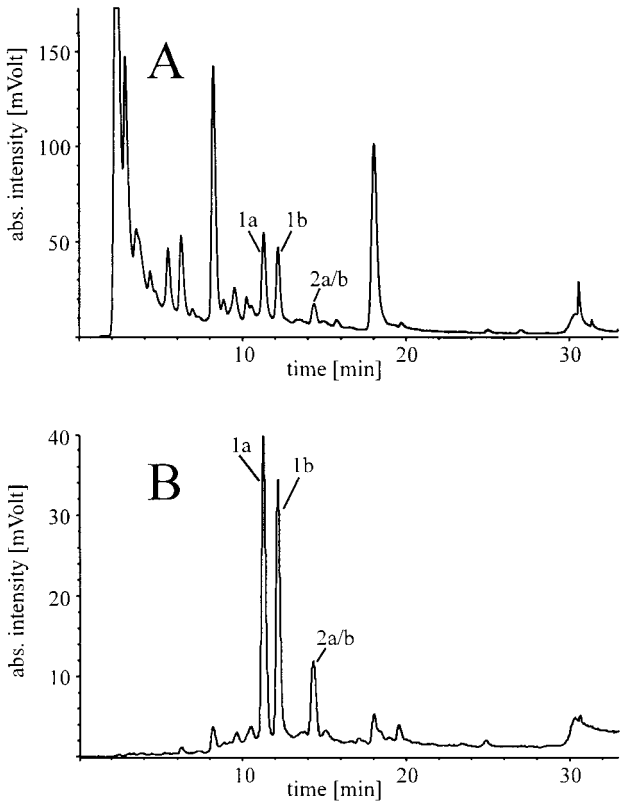


Figure 5. HPLC chromatograms of plum juice concentrate 1 detected at 300 nm for excitation and 450 nm for emission: (A) before SPE; (B) after SPE.

1−**3** (Figure 5). The β-carbolines **1**−**3** are basic analytes that were strongly retained at a high pH value and could be eluted at a more acidic pH. Thus, after application of the samples, the wash step used aqueous methanol with 2% aqueous NH_4OH to remove acidic and neutral substances. Then, elution with acidic aqueous methanol selectively extracted the basic compounds, such as the β-carbolines **1**−**3**. The recovery of the SPE procedure for **1a**, **1b**, and **2a/b** was determined with purified reference compounds and was >95%. As demonstrated by the coefficients of variation in Table 2, the precision of the method was also quite good. The detection limit for the purified reference material without matrix was 1 nmol/L (corresponding to 1.5 pg on column for **1a** and 1.4 pg on column for **2a/b**) at the signal/noise ratio of 3:1. The LOQs were 2.7 nmol/L (corresponding to 4.1 pg on column) for **1a** and 2.0 nmol/L (corresponding to 2.9 pg on column) for **2a/b**.

The results of the quantitative evaluations are summarized in Table 2. It became clear that the novel β-carbolines **1a**, **1b,** and **2a/b** were present in a variety of food samples. Concentrations exceeding 1 mg/L were detected in ketchup, soy sauce, and fish sauce. Our results indicated that formation of these β-carbolines depended on the reaction conditions during food production. This was evident from the analyses results of the vinegar samples: The balsamic vinegars 1 and 2 contained >100 μg/L of **1a/b** and >30 μg/L of **2a/b**, whereas we could not detect any of the β-carbolines in the wine vinegar examined. Wine vinegar is produced by fermentation of wine, whereas balsamic vinegar is made from heat-concentrated grape must. Accordingly, β-carbolines **1a/b** and **2a/b** may be suitable markers for the heat-processed balsamic vinegar. Varying β-carboline concentrations due to different processing conditions were also observed for the pineapple juice samples. Whereas pineapple juice 1 was made from concentrate, pineapple juice 2 was pasteurized fruit juice not made from concentrate. Accordingly, the concentrations of all carbohydrate-derived β-carbolines in pineapple juice 1 were ∼20 times higher compared to pineapple juice 2. Likewise, significant differences could be observed among individual samples of ketchup and soy sauce as well.

Taken together, we identified the carbohydrate-derived 1-(1,3,4,5-tetrahydroxypent-1-yl)-, 1-(1,4,5-trihydroxypent-1-yl)-, and 1-(1,5-dihydroxypent-3-en-1-yl)-β-carbolines (**1**−**3**) for the first time in model reactions of tryptophan with glucose, as well as in several food samples. In the future, detailed mechanistic examinations will answer the question of whether the established tryptophan C-glycoconjugates, *glyco*-tetrahydro-β-carbolines, or Amadori rearrangement products serve as direct progenitors of the carbohydrate-derived β-carbolines (**1**−**3**). Finally, studies concerning the conditions that favor formation of these novel compounds will establish the potential of these chemicals to serve as suitable marker substances for monitoring the application or progress of distinct food-processing procedures.

ABBREVIATIONS USED

DAD, diode array detection; DEPT, distortionless enhancement by polarization transfer; ESI, electrospray ionization; HH-COSY, homonuclear correlation spectroscopy; HMBC, heteronuclear multiple-bond connectivity; HMQC, ^{1}H-detected heteronuclear multiple-quantum coherence; HRMS, high-resolution mass spectrometry; LOQ, limit of quantification; MS/MS, tandem mass spectrometry; SPE, solid phase extraction; SRM, selected reaction monitoring; TFA, trifluoroacetic acid; THCCs, 1,2,3,4-tetrahydro-β-carbolinecarboxylic acids.

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