Degradation of Tetrahydro-β-carbolines in the Presence of Nitrite: HPLC−MS Analysis of the Reaction Products

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Motivated by the identification of numerous novel tetrahydro-β-carboline-carboxylic acids in food samples, we studied the reactions of tetrahydro-β-carbolines in the presence of nitrosating agents. The anticipated formation of nitroso derivatives from unsubstituted tetrahydro-β-carbolines, and from tetrahydro-β-carboline-3-carboxylic acids was indicated by HPLC−MS/MS analysis and validated by the characteristic product ion spectra of the respective nitroso compounds. In addition, oxidative decarboxylation resulted in formation of the corresponding dihydro-β-carbolines, and in the generation of the β-carbolines harman or norharman. Subsequently, we studied the reactivity of tetrahydro-β-carboline-1-carboxylic acids derived from the Pictet−Spengler condensation of indole amines with α-oxo acids. Again, in the presence of nitrosating agents the rapid disappearance of the starting material was obvious, but no nitroso derivatives could be observed. Instead, further HPLC−MS/MS studies demonstrated that dihydro-β-carbolines were the major products of tetrahydro-β-carboline-1-carboxylic acids. Finally, we demonstrated that freshly isolated nitroso-precursors spontaneously decomposed to yield harman alkaloids. In conclusion, we revealed that nitroso-tetrahydro-β-carbolines can represent intermediates involved in the generation of β-carbolines, and we established a novel pathway for the formation of harman alkaloids from nutritional tetrahydro-β-carbolines.

Keywords: *Tetrahydro-β-carbolines; nitroso compounds; harman alkaloids; HPLC−MS; tandem mass spectrometry*

INTRODUCTION

In the presence of dietary nitrite sources, *N*-nitrosation of amines, amides, and nitrogen-containing heterocycles is a common reaction, and most of the resulting nitroso derivatives have been found to be mutagenic substances (*3*). The DNA alkylating intermediates causing these genotoxic effects are formed either by spontaneous decomposition or after metabolic activation of the respective nitroso compounds. In addition, *N*-nitroso indoles can exert genotoxicity mediated by an alternative mechanism that relies on the transfer of the nitroso group to DNA bases (*4*). Besides numerous indole amines, tetrahydro-β-carbolines have been demonstrated to exert mutagenic effects after nitrosation (*5*−*9*). Yet, the identity of the mutagenic products derived from reaction of tetrahydro-β-carbolines with nitrite remains to be established (*9*−*11*).

Nitrosation of tetrahydro-β-carbolines under acidic conditions with aqueous sodium nitrite is likely to yield N^2-nitroso-tetrahydro-β-carbolines. In addition, the reversible reaction at indole nitrogen *N*-9 has been described (*12*−*13*). According to model reactions (*14*−*16*), one can expect the presence of N^2-nitroso-tetrahydro-β-carbolines in nitrite-treated food samples as well as the endogenous formation of such metabolites in the digestive system. Rather surprisingly, tetrahydro-β-carbolines are common food constituents (*17* and references therein), but only the occurrence of 2-nitroso-tetrahydro-β-carboline-3-carboxylic acid **9** in cured meat products and of 1-methyl-2-nitroso-tetrahydro-β-carboline-3-carboxylic acid **10a/b** in soy sauce has been reported (*18*−*20*).

Motivated by the identification of novel tetrahydro-β-carbolines in food samples (*1, 2*), we examined the reaction of various tetrahydro-β-carbolines **1**−**8** (Figure 1) with nitrosating agents. The initial focus of this study was to confirm the predicted formation of nitroso-tetrahydro-β-carbolines from the ubiquitously occurring, but rather reactive, tetrahydro-β-carbolines **5**−**8**. Consequently, we planned to develop an analytical method for the structure-specific detection of labile nitroso-compounds by means of HPLC coupled with tandem mass spectrometry (HPLC−MS/MS) analysis. In addition, we aimed to characterize the major products obtained by the nitrosation of tetrahydro-β-carbolines and intended to study the mechanistic aspects of the nitrite-induced degradation reaction.

MATERIALS AND METHODS

Caution. Nitroso-tetrahydro-β-carbolines and related nitrosation products are potential mutagens and should be handled with extreme caution.

Apparatus. HPLC analysis was performed with a binary high-pressure gradient HPLC system (Knauer, Berlin, Germany) equipped with analytical pumpheads, a dynamic mixing chamber (200 μL), and a Rheodyne 7125 injector with a 20-μL sample loop. For chromatographic separation a Eurospher 100-C_{18} column (250 mm × 4 mm i.d., 5 μm particle size; Knauer, Berlin, Germany) was applied. UV detection was achieved with an HP photodiode array detector 1040A (Hewlett-

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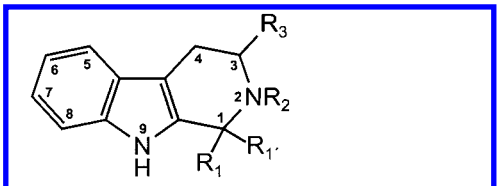
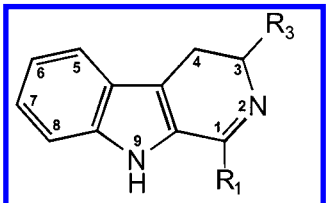


1:	$R_1 = R_{1'} = R_2 = H$; $R_3 = COOH$
2a/b:	$R_1 = R_2 = H$; $R_{1'} = CH_3$; $R_3 = COOH$
3:	$R_1 = R_{1'} = R_2 = R_3 = H$
4:	$R_1 = R_2 = R_3 = H$; $R_{1'} = CH_3$
5a/b:	$R_1 = R_3 = COOH$; $R_{1'} = R_2 = H$
6a/b:	$R_1 = R_3 = COOH$; $R_{1'} = CH_3$; $R_2 = H$
7:	$R_1 = COOH$; $R_{1'} = R_2 = R_3 = H$
8:	$R_1 = COOH$; $R_{1'} = CH_3$; $R_2 = R_3 = H$
9:	$R_1 = R_{1'} = H$; $R_2 = NO$; $R_3 = COOH$
10a/b:	$R_1 = H$; $R_{1'} = CH_3$; $R_2 = NO$; $R_3 = COOH$
11:	$R_1 = R_{1'} = H$; $R_2 = NO$; $R_3 = H$
12:	$R_1 = H$; $R_{1'} = CH_3$; $R_2 = NO$; $R_3 = H$

Figure 1. Tetrahydro-β-carbolines under study.



13:	$R_1 = H$; $R_3 = COOH$
14:	$R_1 = CH_3$; $R_3 = COOH$
15:	$R_1 = R_3 = H$
16:	$R_1 = CH_3$; $R_3 = H$

Figure 2. Dihydro-β-carbolines under study.

Packard, Waldbronn, Germany). Sampling interval was 640 ms for 1 scan from 190 to 400 nm. For data evaluation chromatograms were obtained at 220 and 280 nm for compounds **1**−**12** (Figure 1), at 300 nm for norharman **17** and harman **18**, and at 354 nm for dihydro-β-carbolines **13**−**16** (Figure 2). Solvent A was 0.05% trifluoroacetic acid (TFA) in water (v/v), and solvent B was acetonitrile. For linear gradient elution HPLC was programmed as follows: 0 min, 10% B; 20 min, 30% B; 22 min, 100% B; 27 min, 100% B. The flow rate was 1 mL/min. Quantification was achieved with the help of external calibration using reference substances dissolved in water at concentrations from 5 μg/mL to 80 μg/mL.

HPLC−electrospray ionization−MS/MS analysis was performed utilizing a TSQ 7000 tandem mass spectrometer system equipped with an electrospray ionization (ESI) interface (Finnigan MAT, Bremen, Germany) as has been described previously (*1*, *2*, *21*). Chromatographic separation for ESI-MS/MS experiments was performed on a Eurospher 100-C_{18} column (100 × 2.0 mm i.d., 5 μm particle size; Knauer, Berlin, Germany) using a binary gradient. Solvent A was 0.05% TFA in water (v/v), and solvent B was acetonitrile. The HPLC was programmed as follows: pressurizing with 50% B, equilibration time 10 min at 10% solvent B, and linear gradient elution: 0 min, 10% B; 20 min, 30% B; 30 min, 100% B; and 35 min, 100% B. The flow rate was 200 μL/min and the injection volume was 5 μL. For the pneumatically assisted electrospray ionization spray the capillary voltage was set to 3.5 kV and the temperature of the heated capillary was 180−240 °C. Positive ions were detected with a total scan duration of 1.0 s for a single spectrum (mass range *m*/*z* 150 to *m*/*z* 500). MS/MS experiments such as product ion scans (mass range *m*/*z* 50 to *m*/*z* 500) were performed at a collision gas pressure of 2.0 mTorr Ar (0.26 Pa Ar) with a total scan duration of 3.0 s for a single spectrum.

For HPLC−ESI-MS with postcolumn splitting a 20-μL sample was injected. After chromatographic separation and passage through an UV detector (270 nm, Knauer, Berlin, Germany), a Peek micro-splitter valve (Upchurch Scientific, Oak Harbor, WA) was applied to divide the eluate. Identity of nitroso-tetrahydro-β-carboline was confirmed by ESI-MS/MS (flow rate 20 μL/min, 40 psi N_2 as sheath gas), and the remaining 90% of the eluate containing the purified nitroso-tetrahydro-β-carbolines was collected, concentrated to dryness in a gentle stream of N_2, redissolved in 40 μL of water, and immediately reanalyzed by HPLC−MS 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 10 min at 10% solvent B, and linear gradient elution: 0 min, 10% B; 20 min, 50% B; 21 min, 100% B; and 27 min, 100% B.

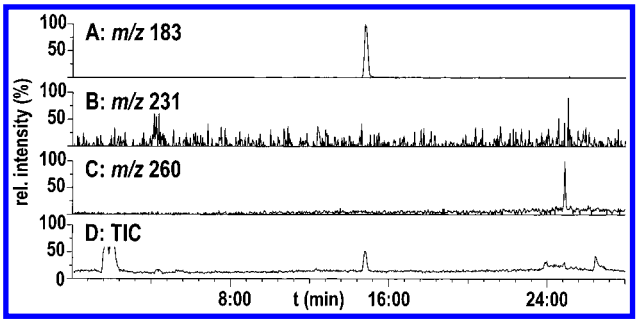


Figure 3. HPLC−MS analysis of products obtained by the nitrosation of 0.1 mg/mL tetrahydro-β-carboline **2a**. A−C, Mass chromatograms of the precursor **2a** (*m*/*z* 231, 3B) and the products harman **18** (*m*/*z* 183, 3A) and nitroso-tetrahydro-β-carboline **10a** (*m*/*z* 260, 3C); D, total ion chromatogram.

Reagents. Water and acetonitrile (both of HPLC gradient grade), and trifluoroacetic acid (spectroscopic grade), were from Merck (Darmstadt, Germany). Norharman **17** and harman **18** (purity >99%) were supplied by Aldrich and Fluka (Deisenhofen, Germany), respectively. Synthesis, isolation, and spectroscopic characterization of tetrahydro-β-carbolines **1**−**8** in Figure 1 have been described previously (*1*, *2*).

Nitrosation of Tetrahydro-β-carbolines. A 0.3-mg aliquot of each of the tetrahydro-β-carbolines **1**−**8** was dissolved in 1 mL of water; we added 0.1 mL of nitrosation reagent (1 M aqueous solution of $NaNO_2$, KNO_2, or nitrite pickling salt) plus 0.2 mL of ethanol and adjusted the mixture to pH 3 with 0.1 M HCl (*11*). For isolation of nitroso-tetrahydro-β-carbolines **10a** and **12** we applied 3 mg of **2a** or **4**; for the initial nitrosation experiment (Figure 3) we utilized 0.1 mg of tetrahydro-β-carboline **2a**. The reaction vials were incubated in the dark at 37 °C for up to 1 h. Subsequently, the nitrosation products were analyzed immediately without any further cleanup. As control samples, 0.3 mg of tetrahydro-β-carbolines **1**−**8** in 1.1 mL of water and 0.2 mL of ethanol were treated accordingly.

Characterization of Nitrosation Products: Nitroso-tetrahydro-β-carbolines 9−**12.** 2-Nitroso-tetrahydro-β-carboline-3-carboxylic acid **9** was detectable only at the very beginning of the nitrosation reaction (incubation time less than 5 min); it decomposed during extended incubation times as well as during isolation. ESI-MS (heated capillary 180 °C): *m*/*z* 246 $[M + H]^+$, 287 $[M + MeCN + H]^+$, 216, 257; ESI-MS/MS product ions (precursor ion *m*/*z* 287, collision energy 10 eV): *m*/*z* 246, 216 $[M - NO + H]^+$, 200.

1-Methyl-2-nitroso-tetrahydro-β-carboline-3-carboxylic acid **10a/b** decomposed during isolation. ESI-MS (heated capillary 240 °C): *m*/*z* 260 $[M + H]^+$, 301 $[M + MeCN + H]^+$, 214, 230, 271. ESI-MS/MS product ions (precursor ion *m*/*z* 260, 12 eV): *m*/*z* 230 $[M - NO + H]^+$, 214, 186, 168, 156, 142, 130.

2-Nitroso-tetrahydro-β-carboline **11** was detectable only at the very beginning of the nitrosation reaction (incubation time less than 5 min); it decomposed during extended incubation times as well as during isolation. ESI-MS (heated capillary 200 °C): *m*/*z* 202, 243, 284 ($[M + n \times MeCN + H]^+$, $n = 0-2$), 172, 213, 254; ESI-MS/MS product ions (precursor ion *m*/*z* 202, 10 eV): *m*/*z* 172 $[M - NO + H]^+$, 143.

1-Methyl-2-nitroso-tetrahydro-β-carboline **12** decomposed during isolation. ESI-MS (heated capillary 220 °C): *m/z* 216 $[M + H]^+$, 257 $[M + MeCN + H]^+$, 186, 227; ESI-MS/MS product ions (precursor ion *m/z* 216, collision energy 16 eV): *m/z* 186 $[M - NO + H]^+$, 170, 156, 142.

Characterization of Nitrosation Products: 3,4-Dihydro-β-carbolines 13−16 (Figure 2). 3,4-Dihydro-β-carboline-3-carboxylic acid **13** decomposed during isolation. ESI-MS: *m/z* 215 $[M + H]^+$; ESI-MS/MS product ions (precursor ion *m/z* 215, 17 eV): *m/z* 197 $[M - H_2O + H]^+$, 169 $[M - HCOOH + H]^+$.

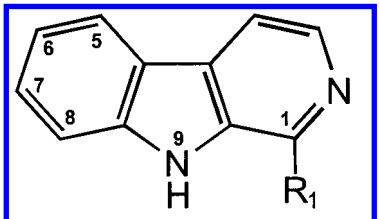
1-Methyl-3,4-dihydro-β-carboline-3-carboxylic acid **14** could be isolated and purified as follows. Nitrosation products obtained from 9 mg of **2a/b** were concentrated to dryness by lyophilization. The residue was extracted with 4 mL of MeOH, diluted with water, filtered, and concentrated again. Final purification by HPLC yielded 2 mg (22%) of **14**. ESI-MS: *m/z* 229 $[M + H]^+$. ESI-MS/MS product ions (precursor ion *m/z* 229, 21 eV): *m/z* 211 $[M - H_2O + H]^+$, 188 $[M - CH_3CN + H]^+$, 183, 146. ^{1}H NMR (400 MHz, CD_3OD): δ 1.84 (s, 3H, CH_3), 3.26 (dd, 1H, $H4_{ax}$), 3.42 (dd, 1H, $H4_{eq}$), 4.24 (dd, 1H, H3), 6.99 (dd, 1H, H6), 7.08 (dd, 1H, H7), 7.26 (d, 1H, H8), 7.53 (d, 1H, H5), $J_{3,4ax} = 9$ Hz, $J_{3,4eq} = 5$ Hz, $J_{4ax,4eq} = 14$ Hz, $J_{5,6} = 8$ Hz, $J_{6,7} = 7$ Hz, $J_{7,8} = 8$ Hz.

3,4-Dihydro-β-carboline **15** decomposed during isolation. ESI-MS: *m/z* 171 $[M + H]^+$. ESI-MS/MS product ions (precursor ion *m/z* 171, 24 eV): *m/z* 154 $[M - NH_3 + H]^+$, 144 $[M - HCN + H]^+$, 118 [indole + H]$^+$.

1-Methyl-3,4-dihydro-β-carboline **16** could be isolated and purified as follows. Nitrosation products obtained from 9 mg of **4** were concentrated to dryness by lyophilization. The residue was extracted with 4 mL of MeOH, diluted with water, filtered , and concentrated again. Final purification by HPLC yielded 6 mg (66%) of **16**. ESI-MS: *m/z* 185 $[M + H]^+$. ESI-MS/MS product ions (precursor ion *m/z* 185, 21 eV): *m/z* 168 $[M - NH_3 + H]^+$, 144 $[M - CH_3CN + H]^+$. ^{1}H NMR (400 MHz, CD_3OD): δ 2.03 (s, 3H, CH_3), 2.74 (m, 2H, H4), 3.97 (m, 2H, H3), 7.20 (dd, 1H, H6), 7.46 (dd, 1H, H7), 7.53 (d, 1H, H8), 7.73 (d, 1H, H5), $J_{5,6} = 8$ Hz, $J_{6,7} = 7$ Hz, $J_{7,8} = 9$ Hz.

Identification of Nitrosation Products: β-Carbolines 17 and 18. β-Carboline (norharman) **17** was available as an authentic reference compound and could be identified as follows. ESI-MS: *m/z* 169 $[M + H]^+$. ESI-MS/MS product ions (precursor ion *m/z* 169, 31 eV): *m/z* 142 $[M - HCN + H]^+$, 115 $[M - C_3H_4N + H]^+$.

1-Methyl-β-carboline (harman) **18** was available as an authentic reference compound and could be identified as follows. ESI-MS: *m/z* 183 $[M + H]^+$. ESI-MS/MS product ions (precursor ion *m/z* 183, 31 eV): *m/z* 168, 115 $[M - C_4H_6N + H]^+$.



17: $R_1 = H$; norharman
18: $R_1 = CH_3$; harman

RESULTS AND DISCUSSION

Nitrosation of Tetrahydro-β-carbolines: Analysis of the Products by HPLC−ESI-MS/MS. Previous studies on the analysis of β-carbolines in food samples had revealed that alkaloids such as **1**−**8** were effectively ionized by the electrospray process yielding exclusively protonated molecule ions in the positive mode. Consequently, all tetrahydro-β-carbolines could be identified unambiguously by means of HPLC−MS/MS with the help of their characteristic product ion spectra showing the specific RDA fragmentation of the tetrahydropyrido moiety (*1*, *2*). To establish an analytical method for the detection of nitroso-tetrahydro-β-carbolines we initially studied the products obtained by the nitrosation of 1-methyl-tetrahydro-β-carboline-3-carboxylic acid **2a/b** (*18*, *19*). As demonstrated by the mass chromatograms derived from the HPLC−MS analysis after 1 h of incubation time (Figure 3), the reaction of the major diastereomer *cis*-**2a** with $NaNO_2$ resulted in the complete degradation of the tetrahydro-β-carboline precursor (Figure 3B; *m/z* 231). As expected, formation of a product showing the molecular ion of the corresponding nitroso derivate could be detected by HPLC−MS (Figure 3C; *m/z* 260). In addition, a compound with the molecular ion $[M + H]^+$ at *m/z* 183 was generated from **2a** (Figure 3A). Subsequently, identity of this major degradation product as harman **18** was established with the help of an authentic reference compound that showed identical retention time, UV spectra, and product ions. Comparable results were obtained when we studied the nitrosation of diastereomer **2b** or varied the nitrite source for the model experiments.

The ESI mass spectra of **10a** shown in Figure 4 demonstrated the extraordinarily labile character of this nitroso-tetrahydro-β-carboline. Together with the molecular ion *m/z* 260, additional cluster ions $[M + MeCN + H]^+$ at *m/z* 301 could be detected and demonstrated that the ionization conditions were obviously rather gentle. Still, decomposition of the nitroso compound during the electrospray process resulted in the neutral loss of NO and yielded ions *m/z* 230 and *m/z* 271 (Figure 4A). By reducing the temperature of the heated inlet capillary to 220 °C we enhanced the intensity of the molecular ion *m/z* 260, thus demonstrating that the degradation of the nitroso-tetrahydro-β-carboline happened in the interface and not during the chromatographic separation. By taking advantage of additional skimmer CID at 10 eV we successfully suppressed the formation of cluster ions $[M + MeCN + H]^+$ (Figure 4B). Nitroso-tetrahydro-β-carboline **11** was even more unstable than **10a** and could be detected only within the first minutes of the nitrosation reaction. To record molecular ions $[M + H]^+$ at *m/z* 202 of **11**, the temperature of the heated inlet capillary was further reduced to 200 °C. Yet, it was obvious that besides extensive formation of cluster ions $[M + n \times MeCN + H]^+$ *m/z* 243 and 284, significant loss of NO yielded ions *m/z* 172, 213, and 254 (Figure 4C). Comparable decomposition reactions of labile nitroso compounds have already been observed during ESI-MS analysis of *N*-nitroso-peptides and *S*-nitroso-conjugates (*23*−*25*).

The extraordinarily labile character of the nitroso-tetrahydro-β-carbolines was obvious from the ESI mass spectra. Accordingly, all attempts to isolate and concentrate the compounds for further identification by NMR spectroscopy resulted in the degradation of the nitroso derivatives. Yet, the product ion spectra of all compounds under study could be reproduced without observation of significant alterations. Consequently, the structural characterization of the nitrosation products relied on the thorough interpretation of the MS/MS experiments which are discussed now in detail.

Besides characteristic retention times and molecular ions, all nitroso-tetrahydro-β-carbolines showed the indicative loss of NO $[M + H - 30]^+$ in the ESI mass spectra and in the product ion spectra (Figure 5). As was established before, tetrahydro-β-carboline-carbox-

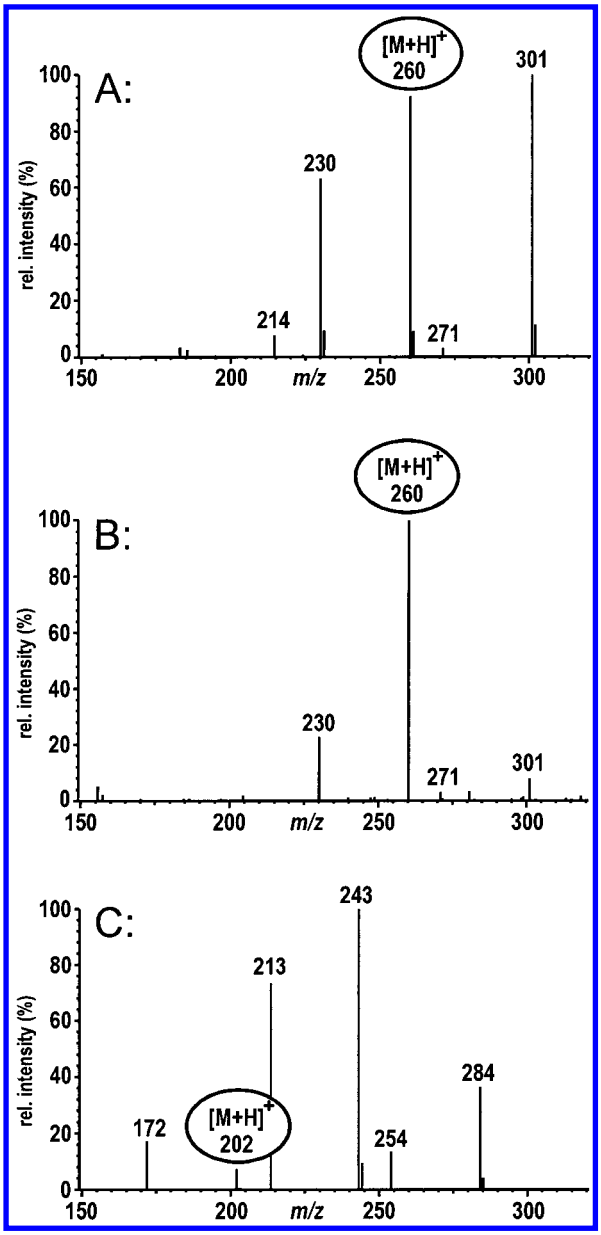


Figure 4. ESI mass spectra of nitroso-tetrahydro-β-carbolines **10a** (4A, heated cap. 240 °C; 4B, heated cap. 220 °C plus skimmer CID 10 eV) and **11** (4C, heated cap. 200 °C).

ylic acids can be identified unambiguously by the specific RDA cleavage of the tetrahydropyrido moiety (*1*, *2*). Consequently, the absence of these prominent ions $[M + H - 73]^+$ at *m/z* 173 (**9**) and 187 (**10a/b**) demonstrated the modification of the tetrahydropyrido nitrogen of tetrahydro-β-carboline-carboxylic acids **1** and **2a/b** which was indicative for N^2-nitroso derivatives **9** and **10a/b**. In contrast, 1-methyl-2-nitroso-tetrahydro-β-carboline-carboxylic acids **10a/b** yielded fragments at *m/z* 186 from neutral loss of CO_2 and NO. Product ions *m/z* 214 and 168 in Figure 5 were formed by consecutive loss of formic acid (or H_2O and CO) from the carboxy moiety and H_2NNO (or H_2O and N_2). This demonstrated that **10a/b** most likely were *N*-nitroso-carboxylic acids and did not represent nitrite anhydrides such as 1-methyl-tetrahydro-β-carboline-CO_2NO. In addition, performing our nitrosation reactions in the presence of ethanol further reduced the risk of obtaining nitrite esters. Product ion spectra of both nitroso-tetrahydro-β-carbolines **11** and **12** revealed base peaks formed as a result

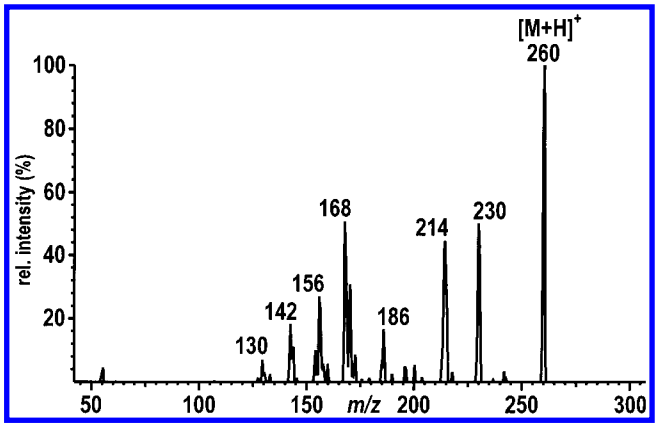


Figure 5. Product ion spectrum of nitroso-tetrahydro-β-carboline **10a** (collision energy 12 eV).

of the typical loss of NO. In addition, **12** was also characterized by elimination of H_2NNO, whereas **11** showed the RDA product ion *m/z* 143 ($[M - NO - HNCH_2 + H]^+$, intensity <10%). Taken together, the MS/MS experiments suggested the formation of nitroso-tetrahydro-β-carbolines **9**−**12** which were most likely modified at the tetrahydropyrido nitrogen. It should be noted that *syn*- and *anti*-isomers of these nitroso compounds coeluted (*26*) and could not be differentiated by HPLC−MS.

Analyzing the nitrosation reactions with tryptophan-derived tetrahydro-β-carboline-1,3-dicarboxylic acids **5**−**6** or tryptamine-related tetrahydro-β-carboline-1-carboxylic acids **7**−**8**, we could not detect any of the respective nitroso-tetrahydro-β-carbolines by means of HPLC−MS/MS. Instead, the dihydro-β-carbolines **13**−**16** in Figure 2 represented the major degradation products of all tetrahydro-β-carbolines having a carboxy group at C1. Pyruvate-derived 1-methyl-dihydro-β-carbolines **14** and **16** were more stable than the Pictet−Spengler products of glyoxylic acid **13** and **15**. Consequently, we could isolate **14** and **16** and collect further evidence by NMR spectroscopy to support the proposed structures. The proton NMR data were consistent with previously published results (*22*, *27*). According to the missing H1, combined with the signals and typical coupling constants of H3 and the methylene group at C4, the double bonds in **14** and **16** could be unambiguously located at C1−N2. Besides elimination of NH_3, the product ion spectra of both 1-methyl-3,4-dihydro-β-carbolines **14** and **16** showed fragments which were formed by the neutral loss of acetonitrile and proved to be characteristic for the methyl substituent. In analogy, diagnostic ions such as the loss of HCN implied the structure of labile 3,4-dihydro-β-carboline **15**. Accordingly, the unstable 3,4-dihydro-β-carboline-3-carboxylic acid **13** was tentatively identified with the help of its UV absorbance, molecular ion $[M + H]^+$ at *m/z* 215, and product ion *m/z* 169 ($[M - HCOOH + H]^+$), which confirmed the presence of the carboxyl group. Hence, we established that oxidative decarboxylation of tetrahydro-β-carboline-1-carboxylic acids **5**−**8** yielded the corresponding 3,4-dihydro-β-carbolines **13**−**16**. In addition, HPLC−MS/MS analysis demonstrated formation of **13**−**16** as minor products following the reaction of tetrahydro-β-carbolines **2**−**4** in the presence of nitrite.

Nitrosation of Tetrahydro-β-carbolines: Mechanistic Considerations. Although thermal or electrochemical breakdown of tetrahydro-β-carbolines has been reported before (*28*, *29*), our experiments (which are

Table 1. Nitrosation of Tetrahydro-β-carbolines

precursor[a]	products
1 (55%)	**9** (1%); **13** (n.d.); **17** (44%)
2 (8%)	**10** (28%); **14** (10%); **18** (54%)
3 (61%)	**11** (6%); **15** (25%); **17** (8%)
4 (86%)	**12** (3%); **16** (10%); **18** (1%)
5 (10%)	**13** (88%); **17** (2%)
6 (16%)	**14** (78%); **18** (6%)
7 (18%)	**15** (82%); **17** (n.d.)
8 (4%)	**16** (96%); **18** (n.d.)

[a] **2**, **5**, **6**, and **10** as mixture of diastereomers.

summarized in Table 1) demonstrated for the first time the facile degradation of tetrahydro-β-carbolines in the presence of commonly used nitrosation agents. More important, these reactions established a novel chemical pathway for the formation of bioactive harman alkaloids from indole amines via their respective nitroso derivatives. Obviously, tetrahydro-β-carboline-3-carboxylic acids **1** and **2a/b** represented the most effective progenitors of norharman **17** or harman **18**, and yielded nitroso-tetrahydro-β-carbolines and dihydro-β-carbolines as side products. In contrast, isomeric tetrahydro-β-carboline-1-carboxylic acids **7** and **8** were as reactive, but yielded exclusively dihydro-β-carbolines **15** and **16** by oxidative decarboxylation. As important, neither the corresponding nitroso-tetrahydro-β-carbolines nor formation of harmanes could be observed following the reaction of tetrahydro-β-carboline-1-carboxylic acids **7** and **8** with nitrite. Obviously, direct oxidation of dihydro-β-carbolines **15** and **16** was not involved in formation of **17** or **18**. As consequence, one pathway to the harman alkaloids did proceed via two consecutive oxidative decarboxylation reactions of tetrahydro-β-carboline-1,3-dicarboxylic acids **5** and **6** with dihydro-β-carboline-3-carboxylic acids as likely intermediates (*29*).

Comparing the influence of substituents on the reactivity of tetrahydro-β-carbolines, the data in Table 1 demonstrate that 1-carboxylic acids were more reactive than the 3-carboxylic acids, whereas tetrahydro-β-carbolines **3** and **4** were the most inert educts. Likewise, the methyl group at position 1 stabilized both nitroso-tetrahydro-β-carbolines **10** and **12** as well as the dihydro-carbolines **14** and **16**. Analyzing the reactivity of tetrahydro-β-carbolines under study, compounds **1**−**4** yielded product patterns which were clearly different from results obtained for 1-carboxylic acid derivatives **5**−**8**. Particularly, the formation of nitroso-tetrahydro-β-carbolines **9**−**12** from tetrahydro-β-carbolines **1**−**4** was obvious, which was paralleled with higher yields of harman alkaloids. In addition, reactions of tetrahydro-β-carbolines **3** and **4** demonstrated that, independent from the oxidative decarboxylation mechanism we discussed above, a second pathway significantly contributed to the generation of harmanes. Consequently, we adressed the question whether nitroso-tetrahydro-β-carbolines **9**−**12** were involved in the formation of harman **18** and norharman **17**.

Potential of Nitroso-tetrahydro-β-carbolines To Act as Precursors of Harman Alkaloids. To confirm that labile nitroso-precursors were intermediates of harman alkaloid synthesis, we isolated nitroso-tetrahydro-β-carbolines **10a** and **12** from the reaction mixtures by HPLC−MS analysis with postcolumn splitting. A minor part of the eluate was directly analyzed by online ESI-MS/MS and confirmed the identity of the individual nitroso-tetrahydro-β-carbolines while most of the fractionated material was gently concentrated with a stream of nitrogen. Instantaneous HPLC−MS analysis of the purified nitroso-compound **10a** revealed almost complete degradation of this precursor together with formation of harman **18** as the major product (Figure 6). The same results were obtained following the isolation of nitroso-tetrahydro-β-carboline **12**. The presence of the educt **2a** (or **4**, respectively), as demonstrated by Figure 6B, indicates that the degradation of nitroso-tetrahydro-β-carbolines **10a** and **12** can be initiated by the loss of the NO group and may proceed via consecutive radical chain reactions.

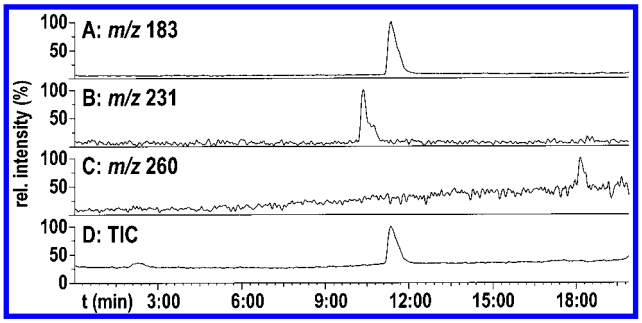


Figure 6. HPLC−MS analysis of products obtained by spontaneous decomposition of nitroso-tetrahydro-β-carboline **10a**. A−C, Mass chromatograms of the educt **10a** (*m*/*z* 260, 6C) and the products harman **18** (*m*/*z* 183, 6A) and tetrahydro-β-carboline **2a** (*m*/*z* 231, 6B); D, total ion chromatogram.

In conclusion, we revealed that the nitroso-tetrahydro-β-carbolines can represent intermediates involved in the generation of β-carbolines and established a novel pathway for the formation of harman from nutritional tetrahydro-β-carbolines in the presence of nitrite. Yet, it has to be acknowledged that analysis of food samples has revealed rather small concentrations of harman alkaloids while tetrahydro-β-carbolines were almost ubiquitously present in significantly higher amounts (*17* and references therein). Consequently, further research should address the identification of technological factors or chemical compounds, such as antioxidative treatments or naturally occurring radical traps, that could provide extended protection from the undesirable formation of harman alkaloids in the presence of nitrite.

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