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Detailed Investigation of the Production of the Bread Flavor Component 6-Acetyl-1,2,3,4-tetrahydropyridine in Proline/1,3-Dihydroxyacetone Model Systems

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The production of 6-acetyl-1,2,3,4-tetrahydropyridine (ATHP), an important Maillard flavor component, in the reaction of L-(−)-proline and 1,3-dihydroxyacetone was investigated as a function of different reaction conditions. The two major side products from the reaction were identified as 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine and 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine, the last one being a new compound described here for the first time. A maximum yield of ATHP of 2.7 mol % from L-(−)-proline and 1,3-dihydroxyacetone was noted at 130 °C in the presence of 2 equiv of sodium bisulfite. The role of sodium bisulfite as a reducing species, and as a stabilizing agent for 6-acetyl-1,2,3,4-tetrahydropyridine, was clarified. In view of the new data obtained, the hypothesized mechanism of formation of 6-acetyl-1,2,3,4-tetrahydropyridine was confirmed, and the reaction mechanisms leading to 2,3-dihydro-1*H*-pyrrolizines were reconsidered.

KEYWORDS: 6-Acetyl-1,2,3,4-tetrahydropyridine; bread flavor; 2,3-dihydro-1*H*-pyrrolizine; model reaction; Maillard flavor

INTRODUCTION

Already in 1963, Wiseblatt and Zoumut isolated a substance with a bread-like flavor after boiling of fermented liquid brews, containing only glucose and yeast in an inorganic aqueous buffer solution (*1*). The authors described the importance of 1,3-dihydroxyacetone in Maillard browning and synthesized the bread flavor compound from a reaction of proline with 1,3-dihydroxyacetone. A structure identification was, however, only published in 1969, when Hunter et al. (*2*) isolated the bread flavor compound from a modified Wiseblatt reaction of proline with 1,3-dihydroxyacetone in the presence of sodium bisulfite and identified it as 6-acetyl-1,2,3,4-tetrahydropyridine. Ever since then, many publications have focused on the occurrence, mechanism of formation, and synthesis of this flavor compound (*3*, *4*). 6-Acetyl-1,2,3,4-tetrahydropyridine (which occurs in tautomeric equilibrium with 6-acetyl-2,3,4,5-tetrahydropyridine) is regarded nowadays as a very important Maillard flavor compound. It has a typical roasty odor, resembling the flavor of crackers and popcorn, and displays a very low odor threshold of only 1 ppb in water (*5*). 6-Acetyl-1,2,3,4-tetrahydropyridine contributes to the aroma of several bakery products: potato chips (*6*), bread crust (*7*), toast (*8*), popcorn (*9*), corn tortillas (*10*), and rice cakes (*11*). This compound, as such or as its bisulfite complex or its salts, was claimed to be useful for flavoring bread and other bakery products (*12*, *13*), and different synthetic strategies were developed (*14*−*18*).

The so-called "Hunter reaction", for the production of 6-acetyl-1,2,3,4-tetrahydropyridine from proline and 1,3-dihydroxyacetone, has been referred to many times, but it remained always more or less obscure. Yields were unknown, and side products, described by Hunter and co-workers, have never been identified. The crucial role of bisulfite in the development of the flavor component has never been unraveled. In this publication, the model reaction of proline and 1,3-dihydroxyacetone was studied in detail. The influence of the reaction conditions and of the addition of different reagents was investigated, optimal reaction conditions were determined, and reaction products were identified. All data were collected to shed more light on the exact mechanism of formation of 6-acetyl-1,2,3,4-tetrahydropyridine and side products of the Hunter reaction.

MATERIALS AND METHODS

Chemicals. L-(−)-Proline (Pro, 99+%), 1,3-dihydroxyacetone (DHA, dimer 98%), sodium bisulfite ($NaHSO_3$, powder p.a.), 1,3-dibromoacetone (tech, 70%), chloroacetone (96%), 1,3-dichloroacetone (99%), silica gel (0.035−0.070 mm, pore diameter ca. 6 nm), chloroform-*d* (0.03 *v*/*v* % TMS, 99.8+ atom % D), ethyl acetate, hexane, and diethyl ether (c.p., stabilized with BHT) were from Acros Organics (Geel, Belgium). Internal standard collidine (99%), hydroxy-2-propanone

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(acetol, tech. 90%), sodium hydrosulfite (dithionite $Na_2S_2O_4$, techn. 85%), and sodium thiosulfate were from Sigma-Aldrich (Bornem, Belgium).

Model Reactions. L-(−)-Proline (0.05 mol), 1,3-dihydroxyacetone, and $NaHSO_3$ (both in varying amounts) were mixed and grounded in a mortar. The powder was put in a two-necked 250-mL round-bottom flask equipped with a condenser and a mechanical stirrer (Heidolph) that was then placed in a preheated oil bath for 20 min at constant temperature (±5 °C). During the heating period, the mixture fused, gradually rose as a foamy mass to fill the flask, collapsed, and finally dried up. An intense browning occurred during the course of the reaction. After 20 min, the oil bath was replaced by an ice bath, and the mixture was allowed to cool. The reaction products were dissolved in 100 mL of 2 N NaOH and extracted three times with 50 mL of diethyl ether. The yellow-colored extract was boiled for 10 min with decolorizing charcoal and was dried over $MgSO_4$. After filtration, the extract was concentrated by evaporation and analyzed by GC-MS. Quantification was accomplished with collidine as an internal standard. In these quantifications, a response factor of 1 was assumed, because pure and stable standards of the evaluated compounds are difficult to obtain.

For the different variations of the reaction described, all reagents were carefully mixed before the reaction was started, or were dissolved in water or a buffer solution, and the same reaction procedure was applied.

Mass Spectrometry. For the analysis of the extracts, a Hewlett-Packard 6890 GC Plus coupled with a HP 5973 MSD (Mass Selective Detector-Quadrupole type), equipped with a CIS-4 PTV (Programmed Temperature Vaporization) injector (Gerstel), and a HP5-MS capillary column (30 × 0.25 mm i.d.; coating thickness 0.25 μm) was used. Working conditions were as follows: injector 250 °C; transfer line to MSD 250 °C; oven temperature start 35 °C, hold 5 min, programmed from 35 to 60 °C at 2 °C min^{-1} and from 60 to 220 °C at 20 °C min^{-1}, hold 5 min; carrier gas (He) 1 mL min^{-1}; split 1/10; ionization EI 70 eV. When the MS was operated in SIM mode for the detection of 2-acetyl-1-pyrroline, ions 111, 83, and 69 were monitored.

Column Chromatography. The different compounds in the extracts were separated by chromatography over a short silica column (15 cm, i.d. 3 cm) using a solvent mixture of hexane and ethyl acetate (7/3). Spots were visualized on TLC by iodine vapors.

NMR Spectroscopy. High-resolution ^{1}H NMR (270 MHz) and ^{13}C NMR (68 MHz) spectra were taken in $CDCl_3$ as solvent (tetramethylsilane as internal standard) with a JEOL EX 270 NMR spectrometer.

5-Acetyl-6-methyl-2,3-dihydro-1H-pyrrolizine **5** (later referred to as "pyrrolizine") (data in agreement with ref *21*). ^{1}H NMR (270 MHz, $CDCl_3$): δ(ppm) 2.38 (3H, s, CH_3), 2.39 (3H, s, CH_3CO), 2.43 (2H, quintet, J = 7.4 Hz, CH_2CH_2N), 2.77 (2H, t, J = 7.6 Hz, CH_2CN), 4.29 (2H, t, J = 7.2 Hz, CH_2N), 5.74 (1H, s, =CH). ^{13}C NMR (67 MHz, $CDCl_3$): δ(ppm) 15.9 (CH_3), 24.1 (*C*H_2CN), 26.8 (*C*H_2CH_2N), 29.2 (*C*H_3CO), 49.2 (CH_2N), 104.5 (=CH), 125.5 (HC=*C*N), 132.9 (*C*CH$_3$), 143.7 (*C*COCH$_3$), 187.2 (C=O). IR (KBr, cm^{-1}) $\nu_{C=O}$ = 1630; mp 47°C; MS (70 eV) *m*/*z* (%) 148 (100), 163 (81), 120 (27), 149 (26), 65 (13), 91 (13), 77 (11), 93 (9), 92 (8), 43 (7).

5-Acetyl-6-hydroxymethyl-2,3-dihydro-1H-pyrrolizine **6** ("pyrrolizine-OH"). ^{1}H NMR (270 MHz, $CDCl_3$): δ(ppm) 2.47 (3H, s, CH_3CO), 2.50 (2H, quintet, J = 7.4 Hz, CH_2CH_2N), 2.83 (2H, t, J = 7.6 Hz, CH_2CN), 4.27 (2H, t, J = 7.2 Hz, CH_2N), 4.63 (2H, s, CH_2OH), 5.94 (1H, s, =CH). ^{13}C NMR (67 MHz, $CDCl_3$): δ(ppm) 24.2 (*C*H_2CN), 26.8 (*C*H_2CH_2N), 28.4 (*C*H_3CO), 49.2 (CH_2N), 59.2 (CH_2OH), 103.4 (=CH), 126.0 (HC=*C*N), 138.5 (*C*CH$_2$OH), 148.8 (*C*COCH$_3$), 187.1 (C=O). IR (KBr, cm^{-1}) ν_{OH} = 3450, $\nu_{C=O}$ = 1610; mp 86 °C; MS (70 eV) *m*/*z* (%) 179 (100), 164 (48), 148 (38), 133 (36), 132 (32), 134 (32), 108 (32), 163 (19), 43 (17), 106 (14).

1-(1-Pyrrolidinyl)-2-propanone **7** (data in agreement with ref *19*). MS (70 eV) *m*/*z* (%) 84 (100), 42 (26), 55 (16), 85 (7), 43 (4), 41(4), 127 (3), 83 (2), 81 (2).

Sensory Evaluation. Odor evaluation and determination of the odor threshold of 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine were performed by 18 untrained panelists. Determination of the odor threshold was accomplished according to the method of "triangular-forced-choice" (ASTM E679-91 odor standard). Solutions of the pure compound in odorous-free distilled water were presented to the panelists in colorless and odorless glass Erlenmeyers. Five ascending concentration steps, ranging from about 0.1 to 10 ppm, were used, with a concentration factor of 3 in each step. Each step comprised two blanks and one solution of the 2,3-dihydro-1*H*-pyrrolizine, and the panelists were asked to identify the differing sample.

RESULTS AND DISCUSSION

For the production of the bread flavor compound 6-acetyl-1,2,3,4-tetrahydropyridine **3** (ATHP), Hunter and co-workers reacted L-(−)-proline and 1,3-dihydroxyacetone in the presence of excess sodium bisulfite at 92 °C for 30 min, without solvent (*2*). This condensation reaction gave rise to low yields (not quantified) of relatively pure ATHP, in the presence of some unidentified side products. In this work, the importance of the different reagents, the influence of the reaction conditions, and the evolution of the reaction products were systematically studied to realize an optimal and reproducible bread flavor formation. Reaction products were identified, and the reaction mechanisms involved were reconsidered.

Identification of Reaction Products. Model reactions of L-(−)-proline (**1**) and 1,3-dihydroxyacetone (**2**) were performed applying different reaction conditions. Upon heating, a foamy mixture was formed that rose to fill the flask and then collapsed to a brown sticky mass. The resulting reaction mixture was dissolved in an aqueous sodium hydroxide solution and extracted with diethyl ether. Gas chromatographic analysis of most extracts revealed the presence of the two stable tautomeric forms of the bread flavor compound ATHP (**3** and **4**), and of two other main compounds. A typical gas chromatogram is shown in **Figure 1**. Two side products occur in relatively high amounts, but were previously not identified in the so-called Hunter reaction. After separation by column chromatography, their structure was determined by a combination of mass spectrometry, IR spectroscopy, and NMR spectroscopy. A first side product was identified as 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine (**5**) by comparison with literature data. Different 2,3-dihydro-1*H*-pyrrolizines have been identified in model reactions of proline with monosaccharides, that is, glucose, rhamnose, arabinose, erythrose, and glyceraldehyde (*20*, *21*), in a heated xylose-lysine model system (*22*) and in a threonine-sucrose model reaction (*23*). Concerning their presence in food, only the identification of 5-acetyl-2,3-dihydro-1*H*-pyrrolizine in malt and beer has been described. L-Proline is the major free amino acid in malt, and flavors are formed during kilning, wort boiling, pasteurization, and elevated storage conditions (*24*). The second side product had similar spectra, but did not exactly match any of the previously described 2,3-dihydro-1*H*-pyrrolizines (*20*, *21*). By detailed NMR spectroscopy, it could be tentatively identified as 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (**6**), a compound which is described here for the first time. Although the overall odor of the extract remains roasty cracker-like in the presence of low amounts of these 2,3-dihydro-1*H*-pyrrolizine side products, especially 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (**6**) has an acrid and unpleasant odor. The aroma of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine (**5**) has been described in the literature as smoky, bitter, and medicine-like (*20*). An odor panel evaluated the flavor of both 2,3-dihydro-1*H*-pyrrolizine side products and described the odor of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine as slightly roasted, but stale. The odor of 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine gave no roasted attributes, but was mainly described as stale and unpleasant. The odor threshold of the new compound 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (**6**) was determined as 0.2 ppm by sensorial evaluation.

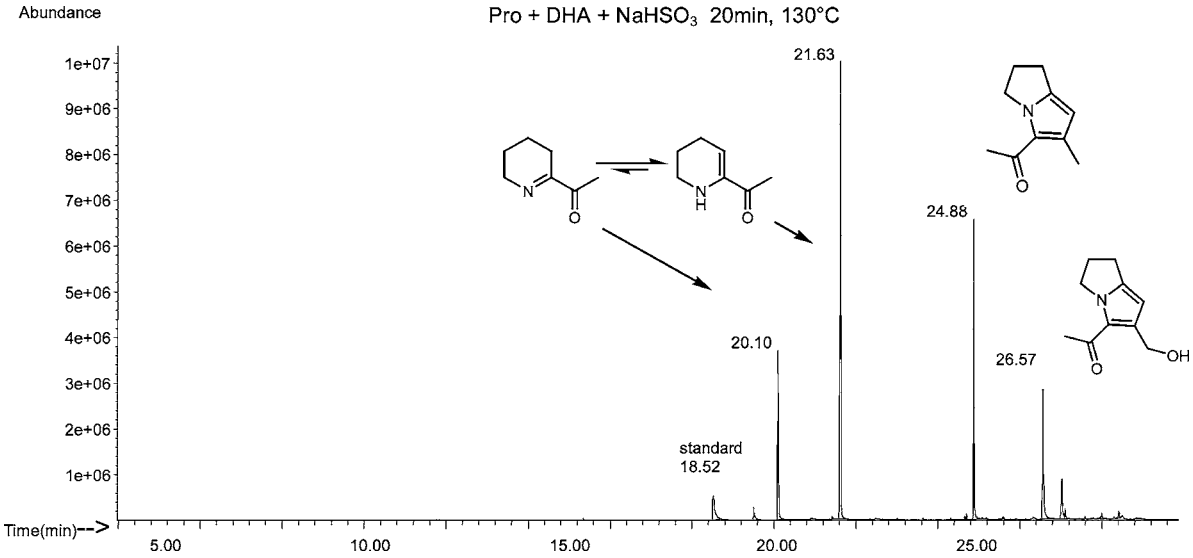


Figure 1. Gas chromatogram of reaction products from the reaction of L-(–)-proline with equimolar amounts of 1,3-dihydroxyacetone and sodium bisulfite (20 min, 130 °C).

Scheme 1. Reaction Products of the Condensation of L-(–)-Proline and 1,3-Dihydroxyacetone

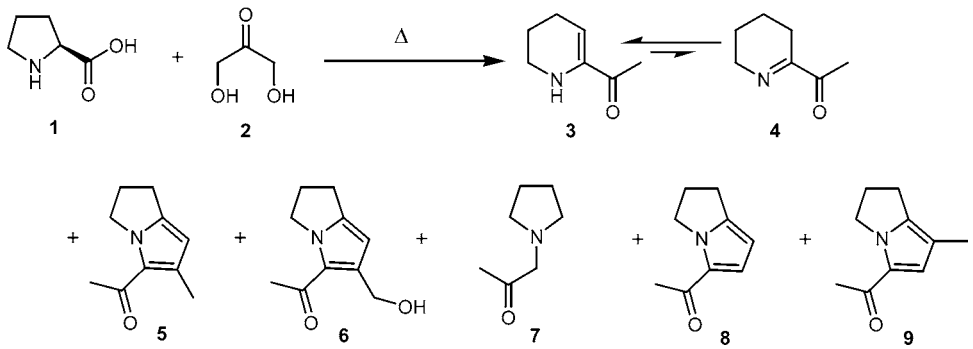


Table 1. Identified Reaction Products of the Condensation of L-(–)-Proline and 1,3-Dihydroxyacetone with Their Retention Index (on HP5 Column)

compound	Kovats index	literature data
1-(1-pyrrolidinyl)-2-propanone (**7**)	1019	proline/sugar model experiments (*19*)
5-acetyl-2,3-dihydro-1*H*-pyrrolizine (**8**)	1391	proline/sugar model experiments (*21*)
5-acetyl-7-methyl-2,3-dihydro-1*H*-pyrrolizine (**9**)	1496	proline/sugar model experiments (*21*)
5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine (**5**)	1530	proline/sugar model experiments (*21*)
5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (**6**)	1754	

Other minor compounds could be identified in the reaction mixture and are displayed in **Table 1**. The condensation reaction of L-(−)-proline (**1**) and 1,3-dihydroxyacetone (**2**) is shown in **Scheme 1**.

Nitrogen-containing heterocycles in food have been correlated, not only with flavor development, but also in some cases with toxicity. Therefore, fundamental knowledge on their formation is essential in the search for food quality control.

Influence of Reaction Conditions. The reaction of L-(−)-proline and 1,3-dihydroxyacetone in equimolar amounts was performed at different temperatures varying from 100 to 200 °C (shown in **Figure 2**). The amounts of ATHP formed were very low (0.01−0.03 mol %) and remained more or less constant with increasing temperatures. The production of the 2,3-dihydro-1*H*-pyrrolizines, that is, 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine (**5**) ("pyrrolizine") and 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (**6**) ("pyrrolizine-OH"), increased with increasing temperature. To obtain an optimal bread flavor, implying a maximal formation of the bread crust flavor ATHP and a limited formation of the unpleasantly smelling 2,3-dihydro-1*H*-pyrrolizines, heating at moderate temperatures appears to serve best.

In the reaction described by Hunter and co-workers (*12*), the addition of bisulfite seems critical: the formation of the bread flavor compound ATHP is promoted, and the isolation thereof from the reaction mixture is facilitated. Therefore, the influence of the addition of sodium bisulfite to the reaction of proline and 1,3-dihydroxyacetone was investigated at a moderate temperature of 90 °C (**Figure 3**). From this graph can be concluded that the addition of 1 equiv of sodium bisulfite leads to a substantial increase (more than 100 fold) of the amount of ATHP recovered. When an excess of bisulfite was used (more than 2 equiv), the yield of ATHP was not improved significantly.

Sodium bisulfite is a reducing species, able to release 2 electrons for 1 equiv of reagent. Other reducing agents tested in the reaction, such as $FeSO_4$, KI, and sodium thiosulfate $Na_2S_2O_3$, resulted in a black mass of reaction products and a very difficult extraction afterward. Traces of 2,3-dihydro-1*H*-pyrrolizines (**5** and **6**) and of ATHP could be detected, but quantification was not relevant, due to the low recovery. Good yields resulted from the reaction of proline and 1,3-dihydroxyacetone in the presence of the reducing agent sodium dithionite ($Na_2S_2O_4$), as is shown in **Figure 4**. In this graph, a comparison

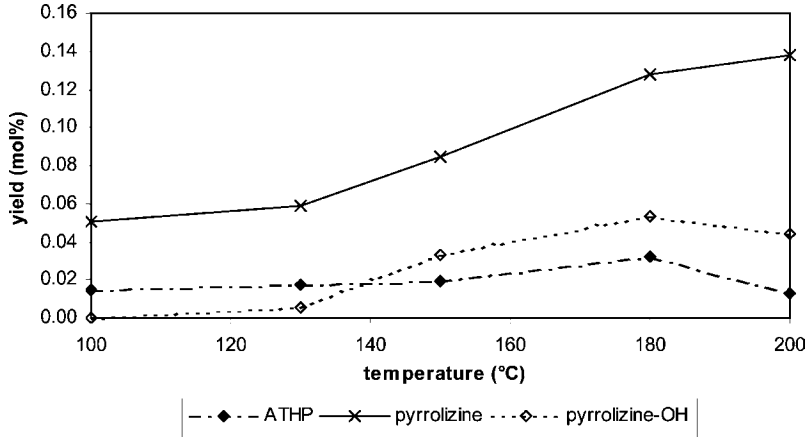


Figure 2. Yield (mol %) of ATHP and 2,3-dihydro-1*H*-pyrrolizine side products from the reaction of L-(–)-proline and 1,3-dihydroxyacetone in equimolar amounts, as a function of reaction temperature.

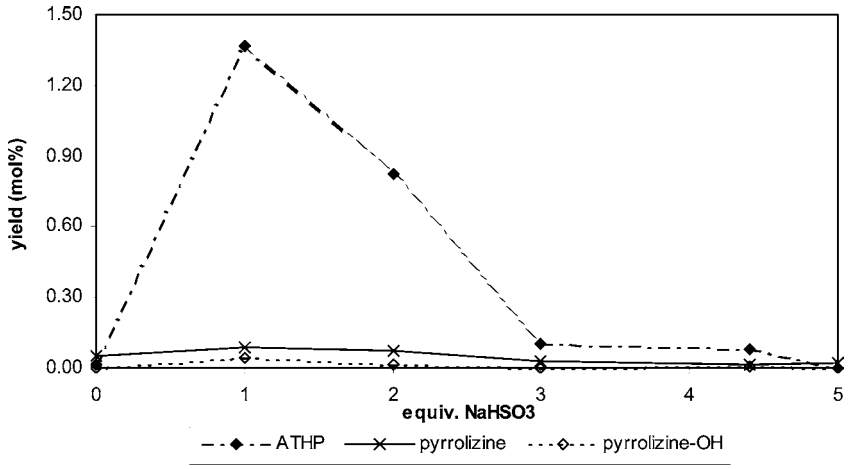


Figure 3. Influence of the addition of different amounts of sodium bisulfite in the reaction of L-(–)-proline and 1,3-dihydroxyacetone (90 °C, 20 min) on the yield (mol %) of ATHP and 2,3-dihydro-1*H*-pyrrolizine side products.

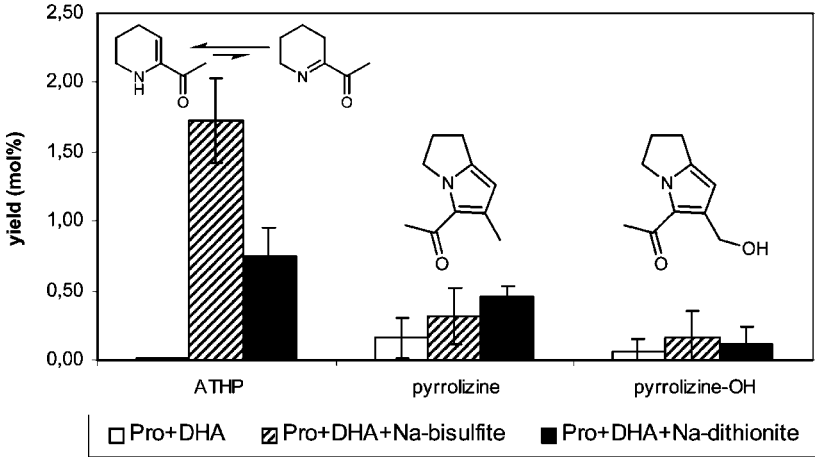


Figure 4. Influence of the addition of 1 equiv of $NaHSO_3$ and of 1 equiv of $Na_2S_2O_4$ (sodium dithionite) in the reaction of L-(–)-proline and 1,3-dihydroxyacetone (130 °C, 20 min) on the yield (mol %) of ATHP and 2,3-dihydro-1*H*-pyrrolizine side products.

is made between the yields of the reaction of equimolar amounts of proline and 1,3-dihydroxyacetone performed at 130 °C without additives, with 1 equiv of bisulfite $NaHSO_3$ and with 1 equiv of dithionite $Na_2S_2O_4$. In comparison with the addition of $NaHSO_3$, less ATHP was formed when $Na_2S_2O_4$ was applied, but similar amounts of 2,3-dihydro-1*H*-pyrrolizines (**5** and **6**) were recovered.

In addition to its reducing activity, bisulfite is known to stabilize imines by nucleophilic addition, thus preventing hydrolytic cleavage (*25*, *26*). This fact might explain the facilitated isolation and improved recovery of ATHP in the presence of bisulfite. To release ATHP from its stable bisulfite addition product, alkali was added to the reaction mixture prior to extraction. When dithionite is applied as a reducing species,

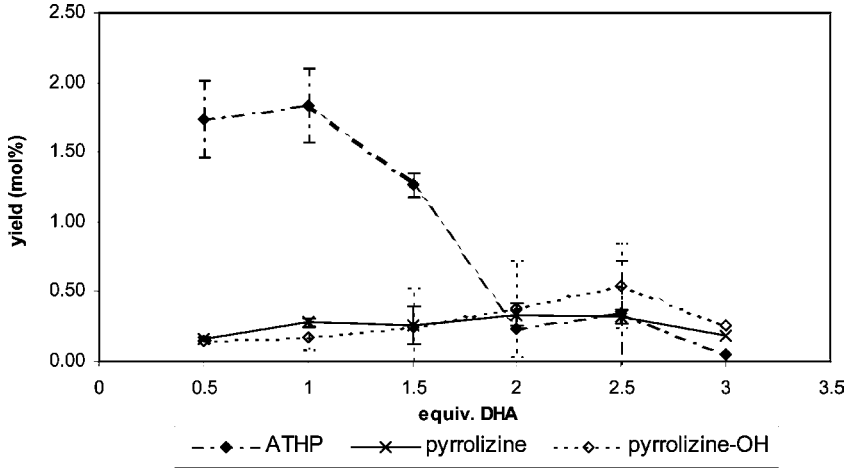


Figure 5. Influence of the amount of DHA used in the reaction of L-(–)-proline and 1,3-dihydroxyacetone (1 equiv of $NaHSO_3$, 120 °C, 20 min) on the yield (mol %) of ATHP and 2,3-dihydro-1*H*-pyrrolizine side products.

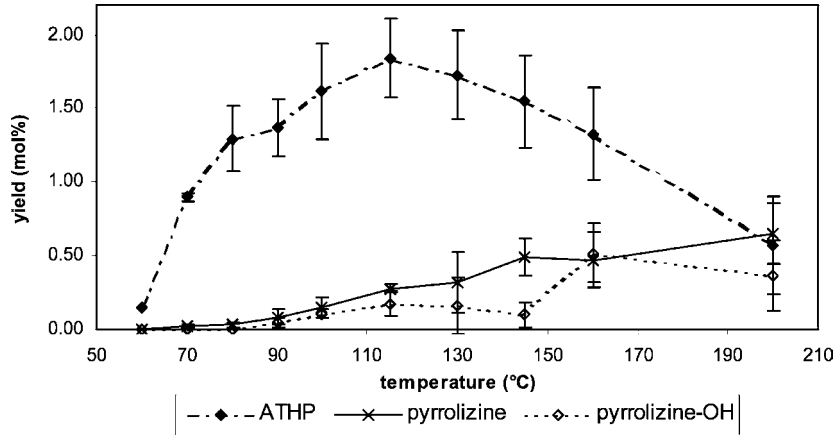


Figure 6. Influence of temperature on the yield (mol %) of ATHP and 2,3-dihydro-1*H*-pyrrolizine side products in the reaction of L-(–)-proline and 1,3-dihydroxyacetone (1 equiv of $NaHSO_3$, 20 min).

bisulfite is formed by oxidation and can thus stabilize the ATHP formed. This might explain the positive effect of $Na_2S_2O_4$ in contrast with the other reducing species tested.

The influence of the use of a different number of equivalents of 1,3-dihydroxyacetone in the reaction with proline was investigated. In **Figure 5** is shown that, in the presence of bisulfite, maximum yields were accomplished when 1 equiv of 1,3-dihydroxyacetone was used, but 0.5 equiv of 1,3-dihydroxyacetone provided high yields as well. This indicates that 1,3-dihydroxyacetone is not a limiting reagent. However, too much 1,3-dihydroxyacetone reduced the yield of ATHP substantially, because side reactions increasingly occurred.

The reaction of equimolar amounts of L-(−)-proline and 1,3-dihydroxyacetone with 1 equiv of $NaHSO_3$ was performed at temperatures ranging from 60 to 200 °C. The graph shown in **Figure 6** displays the average of two or three replications with the standard deviation as the error bar. It is clear that for the ATHP production, a maximum is reached between 110 and 130 °C. At higher temperatures, the unstable ATHP formed is probably degraded again, because less of the principal bread flavor compound was recovered from the reaction. The amount of 2,3-dihydro-1*H*-pyrrolizines (**5** and **6**) produced, however, increased with increasing temperatures between 60 and 200 °C. These results are in agreement with the influence of temperature on the reaction of proline and 1,3-dihydroxyacetone without the addition of $NaHSO_3$ (**Figure 2**). This allows the conclusion that an optimal heating temperature can be found, where the formation of the bread flavor compound ATHP is maximal, but where the production of undesirable side products remains limited. Although extrapolation from this model system to a real food system is not straightforward, this should be taken into account, because baking at higher temperatures can have negative consequences for the flavor of the food product.

In the reaction of L-(−)-proline and 1,3-dihydroxyacetone in the presence of 1 equiv of $NaHSO_3$, optimal yields of 1.8 ± 0.3 mol % ATHP were found at 115−130 °C. The addition of 2 equiv of sodium bisulfite also improved the formation of ATHP, and at 130 °C the yields of ATHP were 1.7 ± 1.0 mol %. The maximum yield of ATHP was accomplished from the reaction of L-(−)-proline and 1,3-dihydroxyacetone in the presence of 2 equiv of $NaHSO_3$ at 130 °C and amounts to 2.7 mol % of ATHP (3.3 mg ATHP per mmol of L-(−)-proline). It must be noted that the reproducibility of the reaction decreased when more equivalents of sodium bisulfite were used. This is probably due to a concentration effect. The yields reported here are moderate, but establish, as compared to literature data (*30*), where 45 μg of ATHP per mmol of proline was obtained in the reaction with glucose, a very significant increase. Due to the very low odor threshold of ATHP (1 ppb in water), the flavor developed in the reaction is very strong, despite the low yields.

A reaction time of 20 min was chosen because it could be visually observed that in most cases the rising and most significant browning of the reaction mixture were terminated after 15−20 min. To investigate the influence of the reaction

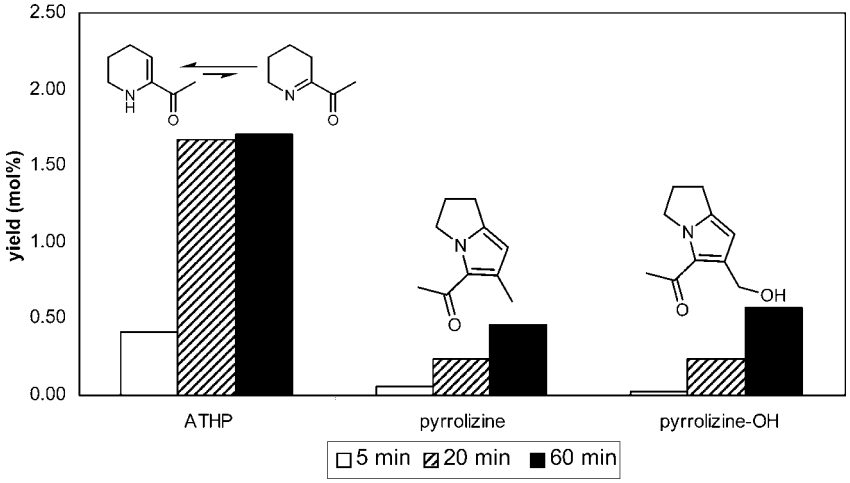
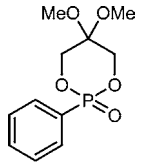


Figure 7. Influence of reaction time on the yield (mol %) of ATHP and 2,3-dihydro-1*H*-pyrrolizine side products in the reaction of L-(–)-proline and 1,3-dihydroxyacetone (2 equiv of $NaHSO_3$, 130 °C).

Scheme 2. 5,5-Dimethoxy-2-phenyl-1,3,2-dioxaphosphinane 2-Oxide, Used in the Reaction with L-(–)-Proline



10

time, the reaction of proline and 1,3-dihydroxyacetone (2 equiv of $NaHSO_3$, 130 °C) was stopped after 5 min, on one hand, and the reagents were allowed to react for 1 h, on the other hand. The results are shown in **Figure 7**. As is the case for the influence of the reaction temperature, an optimal reaction time (around 20 min) can be found, where the yield of ATHP is maximal, and where the production of 2,3-dihydro-1*H*-pyrrolizines is limited. After this time, 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine (**5**) and 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (**6**) are increasingly formed, while the recovery of the unstable ATHP does not increase. Because almost no ATHP (0.03%) was recovered after heating proline and 1,3-dihydroxyacetone without bisulfite for 1 h, this confirms the formation of a stabilized salt of ATHP with bisulfite.

The reaction of proline and 1,3-dihydroxyacetone was also performed in the presence of phosphates. The aim of the addition of Na_3PO_4 and NaH_2PO_4 was to stimulate in situ the formation of 1,3-dihydroxyacetone monophosphate and hence increase bread flavor formation. The catalytic role of phosphates and their influence on the course of the Maillard reaction has been shown (*27*, *28*). When reactions are performed in phosphate buffer, the presence of phosphate may have a more pronounced effect than the pH itself. Under the tested conditions of phosphate additions to the dry model system, however, extraction became very difficult, and little flavor compounds were recovered. A positive effect could not be demonstrated. Also, the reaction of the dihydroxyacetone phosphate precursor **10** (*29*), shown in **Scheme 2**, with proline, did not generate any flavor compounds.

Blank et al. (*30*) studied in detail Maillard model reactions of glucose and proline in phosphate buffer solutions. The highest yields of Maillard reaction products were obtained at pH 7 and 8. Acetic acid was the most important product of the reaction; up to 0.04 mol % ATHP and very low yields (up to 0.004 mol %) of 2-acetyl-1-pyrroline were obtained.

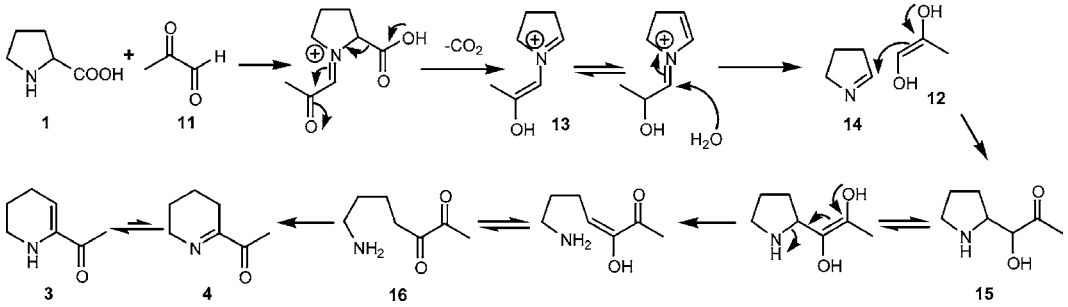
Performing the Hunter reaction in phosphate buffer solutions (pH 7) requires a dilute reaction system. The extracts obtained from the reaction of proline, 1,3-dihydroxyacetone, and sodium bisulfite in phosphate buffer showed a high impurity, and little ATHP was formed. When the reaction was performed by refluxing in a small amount of water (5 mL) without pH adjustment, the yield of ATHP was only one-fourth of the reaction yield under dry conditions. The presence of water seems to inhibit formation of ATHP and of 2,3-dihydro-1*H*-pyrrolizines. This is in agreement with the results of an investigation of the effect of moisture content on flavor formation in a microwave-heated propylene glycol-based proline-xylose model system (10 min, 130 °C). The content of 2,3-dihydro-1*H*-pyrrolizines decreased strongly with increasing moisture content (from 0% to 5% H_2O) (*31*).

The negative influence of water in the reaction makes it difficult to evaluate the influence of pH. Proline was reacted with 1,3-dihydroxyacetone in the presence of concentrated HCl (1 equiv) as an acid, on one hand, and in the presence of $NaHCO_3$ (1 equiv) as a base, on the other hand. The yields of ATHP diminished slightly (with 22%, in the case of addition of $NaHSO_3$ and $NaHCO_3$) to drastically (with 90%, in the case of addition of $NaHSO_3$ and HCl). Sodium bisulfite can react with HCl with the formation of sulfur dioxide gas. Therefore, the positive effect of bisulfite is probably lost in the presence of acid, and yields cannot be compared. In addition, the negative influence of the water added when concentrated HCl was used lowered the yield. Addition of $NaHCO_3$ alone gave very low yields, comparable with the yields without additives.

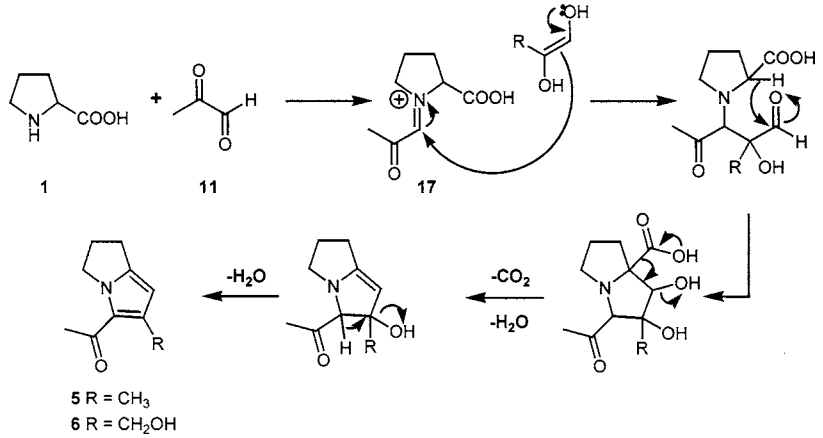
As is shown in the different experiments described, many factors influence the formation of these Maillard flavor compounds. The monitoring of some isolated factors in this model reaction illustrates the extremely complex pattern of these reactions in real food systems.

Mechanism of Formation. For the formation of 6-acetyl-1,2,3,4-tetrahydropyridine, the Hodge mechanism has long served as the standard mechanism (*32*). It was observed, however, that the proposed intermediate *N*-2-oxopropyl-4-aminobutanal did not generate 6-acetyl-1,2,3,4-tetrahydropyridine upon heating (*33*). Accordingly, the so-called Hodge mechanism is most probably not operative. A different mechanism was proposed (*34*, *35*) for the formation of 6-acetyl-1,2,3,4-tetrahydropyridine, starting from 1-pyrroline and hydroxy-2-propanone. These two compounds are formed by Strecker

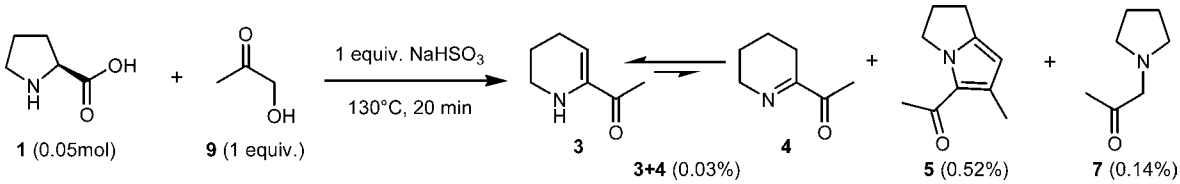
Scheme 3. Mechanism of Formation of ATHP (According to Hofmann and Schieberle (*34*))



Scheme 4. Proposed Mechanism of Formation of 5-Acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine and 5-Acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (Alternative to Tressl et al. (*21*))



Scheme 5. Yields of Reaction Products of the Reaction of L-(–)-Proline and Hydroxy-2-propanone



degradation of proline (**1**), initiated by an α-dicarbonyl compound, in this case 2-oxopropanal (**11**) (**Scheme 3**). The intermediate 2-(1-hydroxy-2-oxopropyl)pyrrolidine (**15**) thus formed was synthesized by Hofmann and Schieberle (*34*) and was shown to yield 6-acetyl-1,2,3,4-tetrahydropyridine (**4**) upon heating.

In the Hunter reaction described here, 2-oxopropanal (**11**) is formed by dehydration of 1,3-dihydroxyacetone (**2**). The negative influence of water as a solvent in the reaction can be explained by its inhibiting effect on this dehydration. Sodium bisulfite (or dithionite as a substitute) serves as a reductant to reduce 2-oxopropanal (**11**) to hydroxy-2-propanone (**12**), which is a reagent in the reaction as well. When 1,3-dihydroxyacetone (**2**) was heated (130 °C, 10 min) in the presence of $NaHSO_3$ (1 and 2 equiv), traces of hydroxy-2-propanone (**12**) are detected. Still, hydroxy-2-propanone can be present in the reaction mixture as a result of the Strecker degradation of proline. Use of an excess of $NaHSO_3$ reduces the yield, because too much reductive activity eliminates essential reagents and the presence of several carbonyl groups remains essential in the reaction sequence. The presence of 1-(1-pyrrolidinyl)-2-propanone (**7**) in the extracts of the experiments performed confirms the pathway presented in **Scheme 3**, because it can be formed by reduction of intermediate **13**. This is another indication that too much reductive activity eliminates important intermediates in the reaction sequence, and thus reduces the yield.

Under the given reaction conditions and with the analysis method described, 2-acetyl-1-pyrroline was never detected, not even when the extracts were analyzed by GC−MS−SIM (selecting only those ions specific for 2-acetyl-1-pyrroline). However, both important flavor compounds, 6-acetyl-1,2,3,4-tetrahydropyridine and 2-acetyl-1-pyrroline, are essentially formed from the same intermediates. Hofmann and Schieberle (*34*) found that 2-acetyl-1-pyrroline is formed when 2-oxopropanal is present in high amounts, whereas in the presence of the reduction product hydroxy-2-propanone, the formation of ATHP is favored, as is confirmed here. Furthermore, the formation of 2-acetyl-1-pyrroline was suggested to result from 1-pyrroline and 2-oxopropanal hydrate, because the amounts of 2-acetyl-1-pyrroline formed were much lower under dry-heating conditions. In the reaction described here, both the presence of bisulfite and the absence of water lead to the exclusive formation of ATHP.

For the formation of 2,3-dihydro-1*H*-pyrrolizines, a general mechanism was proposed by Tressl et al. (*21*). This mechanism involves the formation of an iminium ion by the reaction of proline with 2-oxopropanal. This iminium ion undergoes either an aldol condensation reaction with an α-hydroxy carbonyl compound or a nucleophilic attack by an α-hydroxy carbonyl compound followed by either a Michael addition or an aldol condensation. A specific proposal of this mechanism, applied to the 2,3-dihydro-1*H*-pyrrolizines identified here, is displayed

in **Scheme 4**. The iminium ion **17** undergoes a nucleophilic attack of an enolized hydroxy-2-propanone (or 1,3-dihydroxy-2-propanone) (Mannich reaction). Intramolecular aldol condensation forms the bicyclic transient compound, after which decarboxylation and dehydration yield 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine (**5**) when hydroxy-2-propanone (**12**) is a reagent, while 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (**6**) is formed from 1,3-dihydroxyacetone (**2**) as the reagent. 5-Acetyl-7-methyl-2,3-dihydro-1*H*-pyrrolizine (**9**) is formed when the nucleophilic attack of hydroxy-2-propanone (**12**) in **Scheme 4** originates from carbon-1 instead of carbon-2.

Because 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (**6**) is formed from 1,3-dihydroxyacetone, the presence of bisulfite has no influence on its production, as can be deducted from **Figure 3**, but the more 1,3-dihydroxyacetone is added, the more 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (**6**) is formed (**Figure 5**). The information obtained from the investigation of the reaction conditions confirms the reaction mechanism proposed.

The "Hunter" reaction was also performed with hydroxy-2-propanone (**12**) as a reagent instead of 1,3-dihydroxyacetone (**2**) (**Scheme 5**). At 130 °C, with 1 equiv of $NaHSO_3$, hydroxy-2-propanone and proline reacted with the formation of 0.03% of ATHP (cf. 1.72% with 1,3-dihydroxyacetone) and 0.5% of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine (cf. 0.32% with 1,3-dihydroxyacetone). No 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine was detected, in contrast with the relatively large amounts of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine. This is in agreement with the mechanism of formation proposed, because the formation of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine requires hydroxy-2-propanone, while 1,3-dihydroxyacetone is needed for the formation of 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine.

To collect some more mechanistic information, instead of 1,3-dihydroxyacetone, halogenated acetone derivatives, that is, chloroacetone, 1,3-dichloroacetone, and 1,3-dibromoacetone, were reacted with proline, in water. Because halogens are much better leaving groups than the hydroxyl group, these reactions were expected to yield in situ 2-oxopropanal and produce ATHP, although possible side effects of the resulting HCl or HBr must be taken into account. Different reaction conditions were applied: with and without $NaHSO_3$, in water and in basic solution. However, no ATHP could be detected, nor any 2,3-dihydro-1*H*-pyrrolizines. Only condensation products of the carbonyl derivatives, containing no nitrogen, were detected in the extracts.

In conclusion, the reaction of L-(−)-proline and 1,3-dihydroxyacetone gave rise to a maximal yield of 2.7% 6-acetyl-1,2,3,4-tetrahydropyridine, when heated at 130 °C in the presence of 2 equiv of sodium bisulfite. The positive influence of sodium bisulfite is due to a combination of its reductive activity, providing necessary reagents for the reaction, and of its stabilizing effect on 6-acetyl-1,2,3,4-tetrahydropyridine, improving the recovery of this unstable compound from the reaction mixture. Side reactions yield 2,3-dihydro-1*H*-pyrrolizines, among which 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine was quantitatively the most important. 5-Acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine was described as a new compound, tentatively identified here for the first time.

ABBREVIATIONS USED

Pro, L-(−)-proline; DHA, 1,3-dihydroxyacetone; ATHP, 6-acetyl-1,2,3,4-tetrahydropyridine; pyrrolizine, 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine; pyrrolizine-OH, 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine.

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