Formation of Proline- and Hydroxyproline-Specific Maillard Products from [1-^{13}C]Glucose

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The Maillard reactions of [1-^{13}C]glucose with proline and hydroxyproline, respectively, were investigated to gain more insight into the reaction pathways involved. Among others, maltoxazine, cyclotene, cyclopenta[*b*]azepinones and pyridinones, 2-acetyltetrahydropyridine, dihydropyrrolizines, and several hydroxyproline-derived pyrrolyl and tetrahydroindolizinone compounds were analyzed by capillary GC/MS, and the extent and position of isotopic labeling were determined by interpretation of the MS data. The results were used to evaluate the so far postulated routes to these compounds. Caused by the observed distribution of the isotopic label, the structure of one of the products was reinvestigated and identified as 5-acetyl-7-methyl-1,2,3,4-tetrahydrofuro[3,4-*b*]pyridine (**15**), the first derivative of this type of bicyclic heterocycle isolated in Maillard model reactions.

INTRODUCTION

In a series of former publications concerning the Maillard reaction (Tressl et al., 1985a–e; Helak et al., 1989a,b) we demonstrated the outstanding role of proline hydroxyproline/glucose model experiments. The common attribute of both secondary amino acids is their capability to transform reactive sugar intermediates into stable, well detectable end products as a consequence of the blocked transamination during Strecker degradation. With this quality they take an intermediate position between the usual primary amino acids with their property to undergo complete Strecker degradation and 4-aminobutyric acid with its almost entirely suppressed Strecker degradation (Kersten, 1991). Therefore, most of the components so far characterized from proline or hydroxyproline/glucose systems contain significant structural elements which allow to a certain extent a reconstruction of their formation pathways. Nevertheless, the postulated generation routes of important proline-specific products, which are potent odorants and bitter-tasting components in boiled, baked, and roasted foods (Hunter et al., 1969; Shigematsu et al., 1975; Mills and Hodge, 1976; Doornbos et al., 1981; Pabst et al., 1984, 1985; Shaw and Ho, 1989), required to be proved unequivocally. For this purpose isotopic labeling experiments are most suitable (Koehler et al., 1969; Nyhammar et al., 1983). In this paper we demonstrate [1-^{13}C]glucose/proline and hydroxyproline model reactions which were carried out under elevated temperatures and analyzed by capillary GC/MS. The isotopic distribution among the formed products is calculated on the ratio of molecular mass ions M, M + 1, and M + 2, or characteristic fragment ions; labeling positions are estimated by MS data interpretation. By means of these deduction techniques most of the important proline- and hydroxyproline-derived products are confirmed due to their hitherto proposed formation pathways. The labeling experiments support the expected 1-deoxydiketose route leading to cyclotene via hexose reductone from the intact carbon chain of glucose and prove true an α-dicarbonyl cleavage (Simon and Heubach, 1965; Hayase and Kato, 1986) to form unlabeled cyclopenta[*b*]pyridinone and 2-propionylpyridine derivatives. Maltoxazine, the important guide component of malt, wort, and beer (Tressl et al., 1982), is proved to be generated from the intact glucose carbon chain via 3-deoxyaldoketose, whereas the so far supposed closely related formation of tentatively characterized 5-(1-hydroxyethylidene)-1,2,3,4,5,6-hexahydro-7*H*-cyclopenta[*b*]-pyridin-7-one (Helak et al., 1989b) is disqualified by this method. On the basis of this perception a new molecular structure and formation pathway are proposed for the latter component.

EXPERIMENTAL PROCEDURES

Sample Preparation. *Reaction of* L-*Proline and* L-*Hydroxyproline with [1-^{13}C]-*D-*Glucose.* Equimolar amounts (1 mmol) of anhydrous [1-^{13}C]glucose and L-proline and L-hydroxyproline, respectively, dissolved in water were autoclaved for 1.5 h at 150–160 °C in a stainless steel laboratory autoclave (Roth, I series) equipped with a 100-mL duran glass tube and heated by an electric heater with magnetic stirrer. After the mixture had cooled to room temperature, the pH was adjusted to 10 with 0.1 N NaOH and the compounds were extracted three times with freshly distilled diethyl ether. The combined ether extracts were dried over anhydrous sodium sulfate and concentrated to a volume of about 500 μL on a 20-cm Vigreux column. Aliquot amounts of the extracts were investigated by capillary GC/MS.

*Reaction of Pyrrolidine with [1-^{13}C]-*D-*Glucose.* After 90 mg of anhydrous [1-^{13}C]glucose was dissolved in ethanol, which was mixed with some drops of water, 100 μL of pyrrolidine was added. The mixture was refluxed for 5 h; 100 μL of glacial acetic acid was added, and it was heated for an additional 5 h at 80 °C. The dark brown reaction mixture was evaporated under reduced pressure and redissolved in dichlormethane, and bis(pyrrolidinohexose reductone) (**4**) was investigated by GC/MS.

Gas Chromatography (GC)/Mass Spectrometry (MS). The ether extracts prepared according to the described techniques were analyzed by GC/MS using a 30 m × 0.32 mm i.d. fused silica capillary column coated with Carbowax CAM and coupled with a Finnigan MAT 4500 quadrupole instrument. Temperature was programmed from 70 to 180 °C at 4 °C/min; ionization voltage was 70 eV and resolution 1000. Bis(pyrrolidinohexose reductone) (**4**) was analyzed on a 50 m × 0.32 mm i.d. DB-1 fused silica capillary column, programmed to 180 °C at 4 °C/min, coupled with a double-focusing mass spectrometer CH 5-DF (Varian MAT), ionization voltage 70 eV, resolution 2000 (10% valley).

MS Data Interpretation of Labeled Compounds. Isotopic labeling distributions were determined by calculating the ratio

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of molecular mass ion intensities M^+, $(M + 1)^+$, and $(M + 2)^+$ of the analyzed products, which were corrected to their natural content of ^{13}C isotopes and if necessary to any M − 1 fragmentation. Labeling positions were estimated by interpretation of characteristic mass fragmentation [supported by (direct analysis of daughter ions (DADI) in the case of maltoxazine].

^1H and ^{13}C NMR Spectroscopy. ^1H and ^{13}C spectra were recorded at 270 and 63 MHz, respectively, on a Bruker WM 270 NMR spectrometer in $CDCl_3$ solution. Chemical shifts are reported as parts per million relative to tetramethylsilane (Me_4Si); coupling constants are in hertz.

RESULTS AND DISCUSSION

As has been demonstrated in several former publications, model reactions of reducing sugars with proline and hydroxyproline, respectively, result in complex mixtures of amino acid specific components which were characterized by means of IR, MS, and NMR spectroscopy. The hindered transamination during Strecker degradation of proline prevents the formation of pyrazines and pyrroles, which are significant for primary amino acid reactions with sugars. From proline the reactive Strecker products pyrrolidine and 1-pyrroline are formed, generating *N*-pyrrolidines (**2**, **4**, **7**, **13**, **20**), cyclopent[*b*]azepinones (**5**, **6**, **21**, **22**), maltoxazine (**10**), tetrahydropyridines, cyclopenta-[*b*]pyridinones, and dihydropyrrolizines (**14**, **16**, **17**, **23**, **24**), while hydroxyproline leads to N-substituted pyrroles (**3**, **8**, **9**, **19**) and tetrahydroindolizinones (e.g., **11**) and others not mentioned in this paper. The chosen components are representatives resulting from different sugar degradation pathways which needed to be proved by definite isotopic labeling experiments. In our opinion ^{13}C-labeled precursors are the most promising tools to gain insight into the involved reaction sequences. Therefore, [1-^{13}C]glucose was heated under elevated temperatures with proline and hydroxyproline, respectively, and the reaction products were investigated by capillary GC/MS. In Table I the results of these studies are summarized. The presented integer intensities of the selected significant mass ions only give preliminary hints to labeling distributions and positions of the mentioned products, while detailed information is obtained from more exact intensity data.

Formation of Reductones from 1-Deoxydiketose. The mass spectra of cyclotene (**1a** and **1b**) are seen to be 100% singly labeled, which unmistakably demonstrates an exclusive formation from the intact hexose carbon chain, since recondensation from smaller fragments to give **1a**/**1b** would have led to a broader isotopic distribution. Cyclotene is formed as a major volatile product in the proline and hydroxyproline experiments, respectively, but the labeling position is found to be different in both reaction systems. In the proline-derived cyclotene 1a the fragmentation pattern definitely points out the labeling in the C-4 atom of the cyclopentenolone ring, while the hydroxyproline product **1b** obviously is labeled in the methyl group, which is indicated by the $(M-16)^+$ fragment ion at *m/z* 97. These tendencies are supported by other cyclopentenolone derivatives such as pyrrolidinyl compound **2** from proline, which is labeled in the C-4 ring position, and pyrrolyl compound **3** from hydroxyproline, labeled in the methyl group (M − 16; *m/z* 162). Bis-(pyrrolidinohexose reductone) (**4**), which has been synthesized from [1-^{13}C]glucose and pyrrolidine, is found to be 100% singly labeled but distributed in a 70:30 to 90:10 mixture of different isotopomers, depending on the reaction conditions. The major portion is provided by the C-4 ring-labeled version, whereas the minor one is labeled in the methyl group. The ratio was calculated by comparing the fragment ion intensities of $(CCH_3OH)^+$ at *m/z* 44 and of $(C[^{13}CH_3]OH)^+$ at *m/z* 45 (corrected to their natural ^{13}C content), which are formed by cleavages of the C-1/C-5 and C-4/C-5 bonds. The found isotopic distribution is comparable to the results of Simon (1962), who investigated piperidinohexose reductone prepared from [1-^{14}C]- and [6-^{14}C]glucose with piperidine, respectively. They examined the isotopic distribution by definite degradation reactions and found that 72% of the C-6 atoms and 28% of the C-1 atoms of the glucose were incorporated into the methyl group of the reductone. The remaining 72% of atoms from C-1 and 28% of those from C-6 of glucose were found in the C-4 ring position. As can be seen from these few examples, the formation of aminohexose reductones may strongly depend on the amine substituent, probably on its basicity. According to a scheme of Paulsen and Pflughaupt (1980), a general mechanism of aminohexose reductone generation involves the formation of Amadori products from correspondent glycosylamines, 2,3-enolization, elimination to 1-deoxydiketose, several keto–enol isomerizations, and subsequent elimination to "diacetylformoin" which can cyclize "with the participation of an amine" to the reductone on two complementary routes either by C-1/C-5 or by C-2/C-6 connection of the hexose skeleton. The isotopic distributions observed in the different amine and amino acid systems, respectively, indicate that the first route is favored by proline, pyrrolidine, and piperidine, whereas the second dominates in the hydroxyproline experiments. The most interesting aspect of these results is the correlation of the isotopic distributions with those of cyclotene from the corresponding amino acid systems. This observation suggests a common reaction pathway from glucose via hexose reductone to cyclotene.

Cyclopent[*b*]azepinones **5** and **6**, which are typical ring enlargement products of proline/cyclotene reactions (Tressl et al., 1985b; Pabst et al., 1985), also support the beforementioned tendency. They are 100% singly labeled in the C-6 ring position, which is in coincidence with the C-4 position in the proline-derived cyclotene. The labeled positions were estimated by comparing the mass spectra of labeled and unlabeled components. Fragment ions *m/z* 151 (M − 15) in the MS spectrum of **5** and *m/z* 149 (M − 15) in the MS spectrum of 6 indicate that no [1-^{13}C]atoms of the labeled glucose are incorporated into the methyl groups of the azepinones. Up to mass ions *m/z* 122 (compound **5**) and 120 (compound **6**), respectively, the spectra of labeled and unlabeled components are identical, which points out a $^{13}CH_2CHCH_3$ fragment split off from the cyclopentenolone moieties of the azepinones. In Figure 1 the isotopic distributions of cyclopentenone derivatives from proline and hydroxyproline model reactions with [1-^{13}C]glucose are summarized, giving a survey of the connection of hexose reductone, cyclotene, and cyclopent-[*b*]azepinone formation.

Formation of 3-Deoxyaldoketose Products. In acidic solution glucose is degraded to 5-(hydroxymethyl)-2-furfural. The reaction is initiated by an acid-catalyzed elimination of water from the C-3 position, yielding 3-deoxyaldoketose, which is rapidly dehydrated and cyclized to the furan aldehyde. In amine-catalyzed sugar degradation reactions the corresponding 2-(aminomethyl)-substituted furans represent the typical 3-deoxyaldoketose product, as can be seen from pyrrolidinyl compound **7** and pyrrolyl compound **8** from the proline and hydroxyproline systems, respectively. **7** and **8** are 100% singly labeled in the exocyclic *N*-methylene group, which can be deduced by the characteristic $M - CH_2OH$ fragment ions at *m/z* 151 (compound **7**) and 147 (compound **8**). In Figure 2 a

Table I. MS Data and $^1H/^{13}C$ NMR Data of Selected Products, Characterized in Proline and Hydroxyproline Model Experiments with [1-^{13}C]Glucose[a]

1a	from proline, 2-hydroxy-3-methyl-2-[4-^{13}C]cyclopenten-1-one ([4-^{13}C]cyclotene): 113 (100), 98 (4), 85 (19), 84 (21), 70 (35), 69 (16), 56 (46), 55 (27)
1b	from hydroxyproline, 2-hydroxy-3-[^{13}C]methyl-2-cyclopenten-1-one: 113 (100), 97 (10), 85 (23), 84 (31), 70 (50), 69 (25), 56 (46), 55 (33)
2	5-methyl-2-(1-pyrrolidinyl)-2-[4-^{13}C]cyclopenten-1-one: 166 (100), 165 (29), 151 (18), 138 (15), 137 (16), 122 (85), 108 (23), 95 (33), 94 (22), 81 (24), 80 (24), 70 (28)
3	2-hydroxy-5-[^{13}C]methyl-3-(1-pyrrolyl)-2-cyclopenten-1-one: 178 (100), 162 (7), 149 (18), 121 (37), 107 (13), 106 (11), 93 (22), 81 (51), 79 (20), 67 (26)
4	5-hydroxy-5-methyl-2,3-bis(1-pyrrolidinyl)-2-[4-^{13}C]cyclopenten-1-one and 5-hydroxy-5- [^{13}C]methyl-2,3-bis(1-pyrrolidinyl)-2-cyclopenten-1-one (mixture of two isotopomers): 251 (73), 236 (2), 235 (1), 234 (4), 208 (10), 207 (2), 182 (14), 181 (15), 180 (69), 165 (13), 152 (35), 139 (100), 124 (15), 110 (12), 109 (35), 97 (12), 96 (9), 94 (8), 70 (82), 55 (50), 44 (10), 43 (76)
5	7-methyl-2,3,4,5,6,7-hexahydro[6-^{13}C]cyclopent[*b*]azepin-8(1*H*)one: 166 (M^+, 100), 165 (36), 151 (18), 138 (29), 137 (30), 123 (25), 122 (45), 110 (12), 109 (40), 108 (25), 96 (13), 95 (58), 94 (22), 82 (17), 81 (18), 80 (18), 68 (18), 39 (6)
6	7-methyl-2,3,6,7-tetrahydro[6-^{13}C]cyclopent[*b*]azepin-8(1*H*)-one: 164 (71), 163 (100), 149 (12), 135 (5), 121 (5), 120 (10), 107 (6), 106 (12), 93 (13), 92 (10), 91 (8), 80 (10), 79 (11), 78 (9), 77 (11), 66 (11), 65 (12), 53 (9), 52 (11), 51 (7)
7	1-[5-(hydroxymethyl)-2-furyl[^{13}C]methyl]pyrrolidine: 182 (40), 181 (22), 151 (41), 112 (100), 85 (15), 84 (48), 82 (12), 70 (33), 43 (23)
8	1-[5-(hydroxymethyl)-2-furyl[^{13}C]methyl]pyrrol: 178 (49), 147 (13), 112 (100), 84 (33), 81 (6), 66 (19), 56 (33), 54 (15)
9	6-hydroxy-1-(1-pyrrolyl)-2,5-[1-^{13}C]hexandione: 196 (18), 165 (5), 137 (7), 115 (14), 81 (100), 53 (18)
10	8-oxo-1,2,3,3a,5,6,7,8-octahydro[8a-^{13}C]cyclopenta[*d*]pyrrolo[2,1-*b*][1,3]oxazine: 180 (60), 179 (15), 165 (1), 162 (2), 151 (100), 137 (13), 124 (11), 123 (17), 119 (7), 111 (7), 109 (15), 96 (21), 95 (17), 81 (15), 71 (7), 69 (15), 68 (21), 67 (12), 55 (21), 54 (20), 53 (29), 43 (32), 41 (38)
11	8-acetyl-5,6,7,8-tetrahydroindolizin-6-one (unlabeled): 177 (25), 134 (100), 122 (12), 108 (30), 106 (96), 80 (28), 67 (17), 52 (47), 43 (46) 8-acetyl-5,6,7,8-tetrahydro[5-^{13}C]indolizin-6-one: 178 (65), 135 (100), 121 (30), 107 (53), 105 (15), 80 (19), 43 (58)
12	1-hydroxy-2-[3-^{13}C]propanone (mixture with unlabeled compound): 75 (4), 74 (9), 44 (38), 43 (100)
13	1-(1-pyrrolidinyl)-2-[3-^{13}C]propanone (mixture with unlabeled compound): 128 (1), 127 (1), 85 (23), 84 (100), 56 (7), 55 (23), 43 (14), 42 (49)
14	2-[2-^{13}C]acetyl-3,4,5,6-tetrahydropyridine (mixture with unlabeled compound): 126 (42), 125 (59), 124 (11), 111 (2), 110 (4), 97 (2), 96 (2), 83 (26), 82 (100), 81 (5), 80 (11), 68 (8), 67 (5), 55 (46), 54 (75), 53 (14), 44 (22), 43 (56), 42 (11), 41 (16)
15	5-acetyl-7-methyl(or 5-[2-^{13}C]acetyl-7-methyl/or 5-acetyl-7-[^{13}C]methyl/or 5-[2-^{13}C]acetyl-7- [^{13}C]methyl)-1,2,3,4-tetrahydrofuro[3,4-*b*]pyridine (mixture of four isotopomers): 181 (25), 180 (68), 179 (71), 178 (29), 165 (8), 164 (7), 151 (7), 150 (5), 138 (7), 137 (47), 136 (46), 109 (12), 108 (13), 95 (5), 94 (14), 44 (66), 43 (100)
16	5-acetyl-6-methyl(or 5-[2-^{13}C]acetyl-6-methyl/or 5-acetyl-6-[^{13}C]methyl/or 5- [2-^{13}C]acetyl-6-[^{13}C]methyl-2,3-dihydro-1*H*-pyrrolizine (mixture of four isotopomers): 165 (11), 164 (32), 163 (28), 150 (12), 149 (66), 148 (100), 121 (9), 120 (14), 93 (9), 92 (10), 91 (9), 79 (5), 78 (6), 77 (11), 66 (11), 65 (17), 44 (7), 43 (17)
17	5-[2-^{13}C]acetyl-7-methyl-2,3-dihydro-1*H*-pyrrolizine (mixture with unlabeled compound): 164 (43), 163 (9), 149 (17), 148 (100), 120 (16), 118 (4), 104 (3), 93 (5), 92 (4), 91 (7), 79 (5), 77 (11), 66 (6), 65 (13), 44 (19), 43 (3)
18	3-hydroxy-2-[1-^{13}C]butanone (mixture with unlabeled compound): 89 (5), 88 (6), 46 (38), 45 (100), 44 (25), 43 (62)
19	3-(1-pyrrolyl)-2-[1-^{13}C]butanone (mixture with unlabeled compound): 138 (12), 137 (10), 95 (37), 94 (84), 44 (72), 43 (20)
20	1-(2-furyl-[^{13}C]methyl)pyrrolidine (mixture with unlabeled compound): 152 (34), 151 (48), 150 (16), 83 (20), 82 (100), 81 (73), 70 (24), 55 (11), 54 (23), 53 (21), 43 (12), 42 (50), 41 (17)
21	2,3,4,5,6,7-hexahydro[6-^{13}C]cyclopent[*b*]azepin-8(1*H*)-one (mixture with unlabeled compound): 152 (93), 151 (99), 150 (26), 137 (4), 136 (4), 135 (3), 124 (36), 123 (56), 122 (28), 109 (17), 108 (20), 96 (67), 95 (100), 94 (33), 81 (23), 80 (20), 68 (31), 67 (47), 55 (33), 54 (24), 53 (36)
22	2,3,6,7-tetrahydro[6-^{13}C]cyclopent[*b*]azepin-8(1*H*)-one (mixture with unlabeled compound): 150 (63), 149 (100), 148 (32), 136 (3), 135 (11), 134 (3), 122 (3), 121 (10), 120 (6), 107 (15), 106 (8), 94 (10), 93 (10), 92 (9), 80 (10), 79 (11), 66 (19), 65 (10), 53 (11), 52 (8)
23	1,2,3,4,5,6-hexahydro-7*H*-cyclopenta[*b*]pyridin-7-one (unlabeled compound): 137 (100), 136 (34), 109 (33), 108 (57), 94 (49), 81 (66), 80 (27), 79 (10), 68 (17), 67 (25), 54 (19), 53 (31), 52 (17), 41 (30)
24	5-methyl-1,2,3,4,5,6-hexahydro-7*H*-cyclopenta[*b*]pyridin-7-one (mixture of unlabeled, singly and doubly labeled isotopomers): 153 (11), 152 (42), 151 (27), 138 (29), 137 (98), 136 (100), 124 (3), 123 (6), 122 (3), 110 (5), 109 (27), 108 (33), 96 (3), 95 (6), 94 (5), 82 (10), 81 (15), 80 (12), 68 (4), 67 (8), 66 (6), 53 (21)

^1H and ^{13}NMR Data

15	^1H NMR (270 MHz, $CDCl_3$) δ 1.86 (m, 2 H, J = 6.5 Hz, H-3), 2.24 (s, 3 H, CH_3), 2.38 (s, 3 H, $COCH_3$), 2.98 (2 H, J = 6.5 Hz, H-4), 3.17 (dt, 2 H, H-2), possibly also a broad signal at δ 2.8 (NH) ^{13}C NMR (63 MHz, $CDCl_3$) δ 11.3 (q, CH_3), 21.2 (t, C-3), 22.5 (t, C-4), 26.1 (q, $COCH_3$), 42.6 (t, C-2), 124.1 (s, C-4a), 130.8 (s, C-7a), 137.2 (s, C-7), 145.0 (s, C-5), 187.1 (s, $COCH_3$)

[a] MS data: *m/z* (relative intensity). ^1H and ^{13}C NMR data: s, singlet; d, doublet; t, triplet; q, quartet; dt, doublet of triplets; m, multiplet.

scheme outlines the formation of 7 and 8 from corresponding proline- and hydroxyproline-derived 1-pyrrolinium intermediates which are also expected to be the precursors of maltoxazine (10) and tetrahydroindolizinone 11, respectively. The immonium intermediates are proposed to undergo dehydration and keto–enol tautomerization to form 6-hydroxy-1-(1-pyrrolyl)-2,5-hexanedione (9) in the hydroxyproline experiments (Tressl et al., 1985a), which is found to be 100% singly labeled in the C-1 position, indicated by the characteristic 1-pyrrolyl-[^{13}C]-methyl fragment ion *m/z* 81. In the proline experiments the correspondent 6-hydroxy-1-(1-pyrrolinium)-2,5-hexanedione may be formed which can cyclize to a cyclopentanone system with the hydroxymethyl residue in a sterically favored position to undergo ring closure to a 1,3-oxazine structure. A subsequent dehydration yields maltoxazine (10) as the dominating 3-deoxyaldoketose product (Tressl et al., 1982). The proposed reaction pathway is supported by different aspects. 10 is found to be nearly 100% singly labeled, which clearly shows a formation from the intact carbon chain of [1-^{13}C]glucose. This offers two probable isotopic distributions in compound 10: in the C-5 methylene group of the oxazine moiety or in the C-8a position adjacent to the nitrogen atom. The labeled position was calculated by interpretation of the MS data supported by the DADI method of the unlabeled component. The latter technique enables a concrete attachment to characteristic fragment ions. Transformed to the labeled component these data indicate a certain location at the C-8a ring position, which is due to the former C-1 atom of the postulated precursor.

From hydroxyproline a correspondent tricyclic ketone

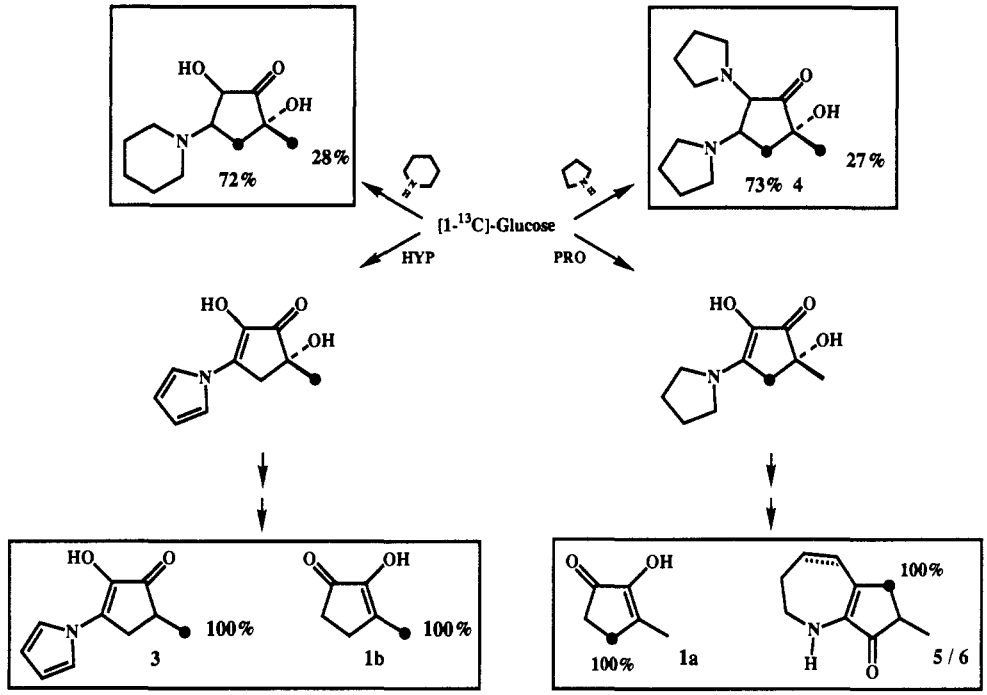

Figure 1. Isotopic distributions of cyclopentenone derivatives from proline and hydroxyproline model reactions with [1-^{13}C]glucose.

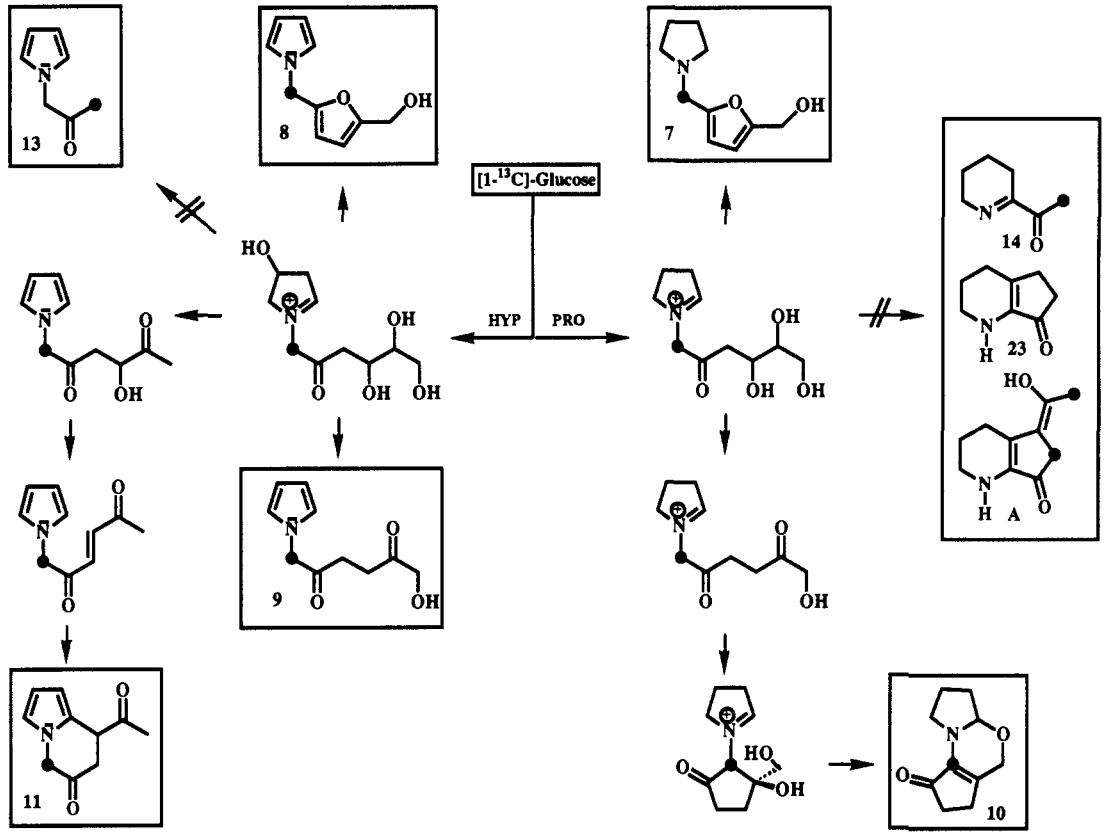

Figure 2. Formation pathways of 100% labeled hydroxyproline-specific (8, 9, 11) and proline-specific (7, 10) Maillard compounds in [1-^{13}C]glucose/amino acid model experiments.

containing a pyrrole moiety was not generated. Obviously, the strong tendency of the immonium intermediate to dehydrate to the aromatic pyrrole system in the early stages of the reaction prevents an analogous course. Instead, the tetrahydroindolizinone 11 is formed. 11 was characterized by means of its MS fragmentation, which matches those of other tetrahydroindolizinones, isolated from the hydroxyproline experiments and identified by ^{1}H NMR (Tressl et al., 1985a). The 100% singly labeled 11 must be derived from the intact carbon chain of [1-^{13}C]glucose. The MS data of both the unlabeled and labeled component 11 in Table I show base peaks at m/z 134 and 135, respectively, created by splitting off unlabeled acetyl fragments (M − 43). These results implicate a labeling of 11 in the C-7 position, i.e., at the *N*-methylene group which corresponds to C-1 of glucose. Thus, 11 is to be formed by a Michael-type intramolecular ring closure as outlined in Figure 2. Aromatization and a subsequent elimination in the aliphatic side chain lead to an α,β-unsaturated ketone, just suitable for cyclization to a six-membered

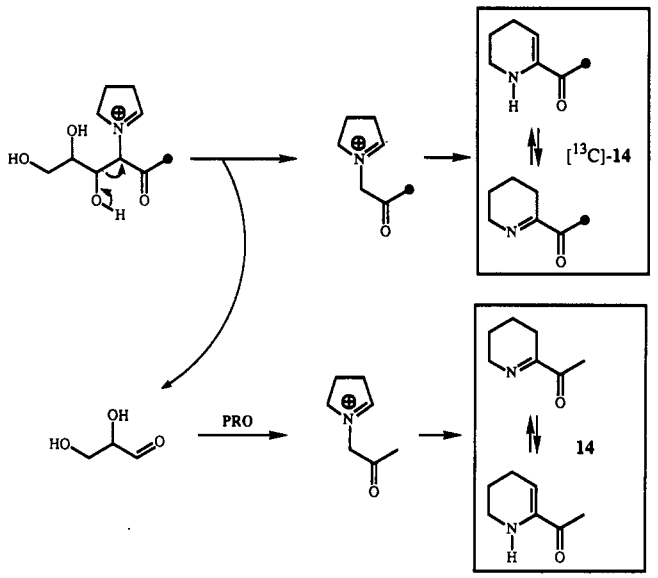

Figure 3. Formation of 2-acetyl-3,4,5,6-tetrahydropyridine (14) (mixture of unlabeled and singly labeled isotopomers) in proline/[1-^{13}C]glucose model experiments.

ring by intramolecular nucleophilic addition. As is also seen in Figure 2, the formation of components 13 and 14 as well of 23, 24, and the 5-(1-hydroxy-1-ethylidene) derivative of 23 (formula A in Figure 2) by this route is disqualified, because of the strong contrast between actually observed and expected labeled positions.

Formation of C_3, C_4, and C_5 Sugar Fragmentation Products. Along with the so far mentioned proline and hydroxyproline components formed from the intact carbon chain of glucose, several sugar fragmentation products are generated by retro aldol or α-dicarbonyl cleavages (Ledl et al., 1990). The formation of C_3 components, such as pyruvate, pyruvaldehyde, and acetol (12), from [1-^{13}C]-glucose plays an important role in the proline system with respect to providing the precursors of potent flavor compounds. 12 is found to be generated in a 70:30 mixture of unlabeled and singly labeled isotopomers, which points out that it is formed from C-1 to C-3 as well as from other triads of the hexose skeleton. The label is located in the methyl group, indicated by m/z 44. The correspondent pyrrolidinyl component 13 is also labeled at the methyl group but distributed in a 60:40 mixture of unlabeled and singly labeled isotopomers. In spite of this slight deviation 13 may result from the same reaction sequence as discussed for 12 (Tressl et al., 1992).

A corresponding 1-(1-pyrrolinium)-2-propanone precursor is postulated to undergo a typical proline-specific ring enlargement reaction to form 2-acetyl-3,4,5,6-tetrahydropyridine (14), which is detected to have the same isotopic distribution (60:40, unlabeled to singly labeled) and labeling position (methyl group) as found for 13. 14 is representative for its tautomeric 1,4,5,6-tetrahydro form and 2-acetylpyridine, all known to be potent flavor components of proline/sugar reaction systems and of several foodstuffs (Hunter et al., 1969; Schieberle, 1990). The isotopic distributions are estimated from characteristic acetyl fragment ions at m/z 43 and 44. 14 may be derived from glucose via appropriate 1-deoxydiketose intermediates as proposed in Figure 3, resulting in the observed isotopic distributions.

The highly reactive 1-piperidine 14, on the other hand, was proved to be a potential precursor of cyclopenta[*b*]-pyridinones, another type of proline-specific Maillard product. The most abundant representatives in proline/glucose systems are 1,2,3,4,5,6-hexahydro-7*H*-cyclopenta-[*b*]pyridin-7-one (23), its 5-methyl (24) derivative, and its only tentatively characterized 5-(1-hydroxy-1-ethylidene) derivative (A in Figure 2). The derivatives 24 and A were synthesized from 14 and acetaldehyde or pyruvaldehyde, respectively, by an aldol-type condensation and subsequent intramolecular Michael addition (Helak et al., 1989b). But from this observation the actual route of formation in the more complex proline/glucose system cannot be seriously deduced, and thus the hitherto postulated pathways to the pyridinones are preliminary to a certain degree.

The MS data of 23, 24, and the supported hydroxyethylidene derivative A generated in a proline/[1-^{13}C]-glucose system indicate very different labeling patterns. Whereas 23 is mostly unlabeled, the compounds 24 and A (15 in Table I) are found to be mixtures of unlabeled and singly and doubly labeled isotopomers. Obviously, there are at least two different pathways to the pyridinones in proline/sugar systems. 23 is generated from proline and an unlabeled precursor, e.g., a C_4 sugar degradation product (see below), but 24 and A must have at least two partially labeled precursors. The found ratios of isotopomers (24 36:55:9, A 36:47:17 with respect to unlabeled and singly and doubly labeled compounds) insinuate both correspondent structures and generation routes. In this case the observed ratios of isotopomers as well as the distinct positions of labeling are to be interpreted by the selected generation route.

At first sight route 1 outlined in Figure 4 complies with this condition. From the experimentally found 60:40 ratio of unlabeled and labeled precursors, a 36:48:16 statistical ratio of isotopomers is calculated for A, which is in coincidence with the detected ratio. In the case of 24 the deviations may result from different amounts of labeled C_2, C_3, and C_5 precursors involved in its formation. On the other hand, with respect to the labeling positions, route 1 is not consistent with the generation of compound A. From the characteristic fragment ions the labeling positions in A are located in a methyl group (m/z 164, M − 16), calculated to be labeled to 40%, and in an acetyl group (m/z 44, $^{13}COCH_3$), also found to be labeled to 40%. The values are estimated from the intensities of m/z 43 and 44 signals (acetyl group) and m/z 165 and 164 signals (methyl group) and represent the sum of isotopic labeling in these positions from both singly and doubly labeled products. This result is incompatible with the proposed structure of A, first with respect to the expected labeling positions (at least one ring position C-6 by route 1 as fulfilled with 24) and second with respect to missing a doubly labeled acetyl fragment ion which demonstrates both a methyl and an acetyl group in A. Consequently, a new structural proposal as well as another formation pathway is to be established.

The so far proposed structure does not entirely conform with the NMR data, which indicate two methyl groups at δ 2.24 and 2.38 in the ^{1}H NMR and δ 11.2 and 26.1 in the ^{13}C NMR spectrum. The data are in coincidence with chemical shift values for methyl groups of 5-methyl-2-acetylfurans (Kiewiet et al., 1974; Kim et al., 1974). Starting from the above-mentioned precursors, an aldol-type condensation followed by ketalization and subsequent dehydration, as outlined in route 2 of Figure 4, may lead to a 2-acetyl-5-methylfuran moiety, which is [3,4-*b*]-annellated to tetrahydropyridine. The new structure (15) is consistent with all spectroscopic demands and is the first furo[3,4-*b*]pyridine derivative so far described in the Maillard reaction.

In addition to compound 15, the 5,6-disubstituted 2,3-dihydro-1*H*-pyrrolizine 16 is found to have nearly the same distribution to unlabeled and singly and doubly labeled

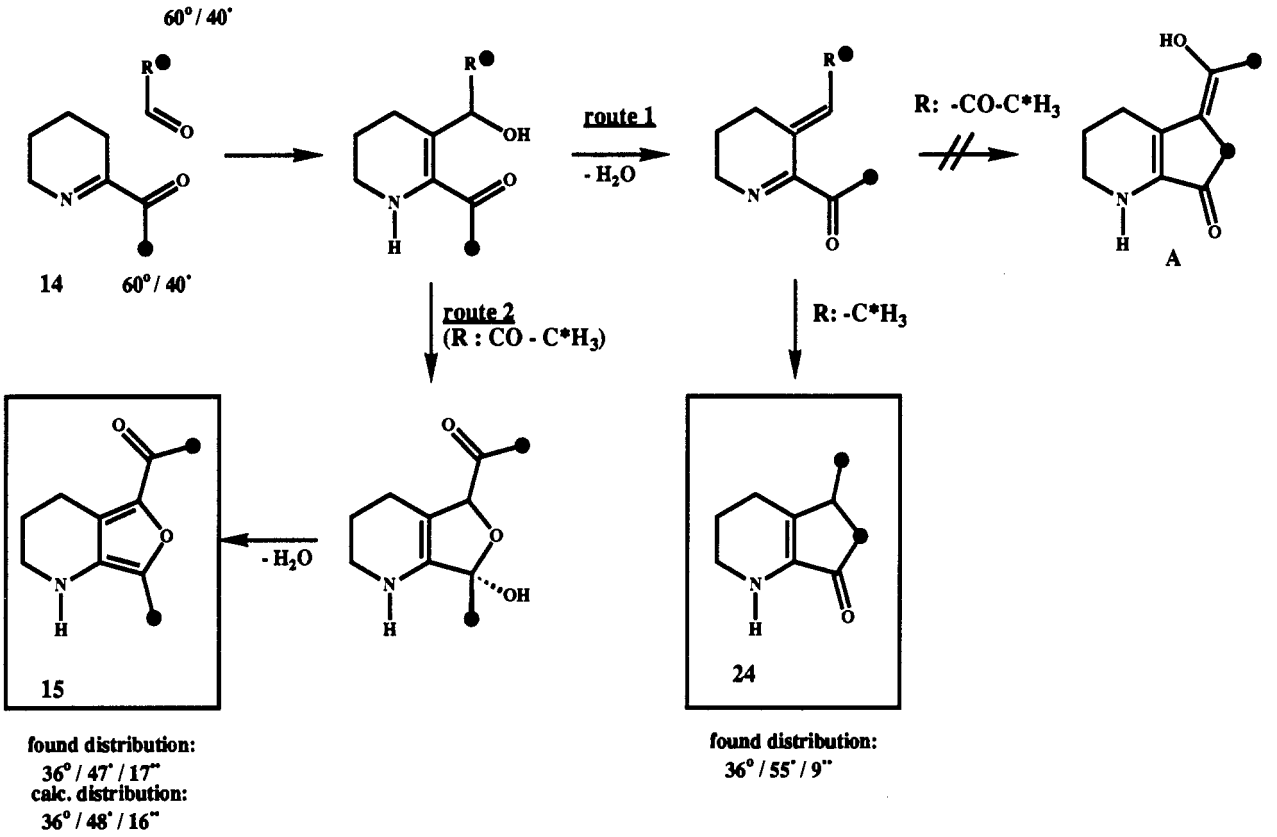

Figure 4. Formation of mixtures of unlabeled, singly and doubly labeled isotopomers of 5-methyl-1,2,3,4,5,6-hexahydro-7*H*-cyclopenta-[*b*]pyridin-7-one (24) and 5-acetyl-7-methyl-1,2,3,4-tetrahydrofuro[3,4-*b*]pyridine (15) in proline/[1-^{13}C]glucose model experiments.

product (34:48:17), which also indicates a generation from two C_3 sugar fragments with 60:40 ratios of unlabeled to singly labeled isotopomers. In contrast to 16, the 5,7-disubstituted pyrrolizine 17 is found to be 100% singly labeled in the methyl group of the acetyl moiety, indicating a formation from the intact carbon chain of glucose. The latter result demonstrates that 2,3-dihydro-1*H*-pyrrolizines (Tressl et al., 1985e) are formed from both retro aldol sugar fragments and intermediates derived from the intact sugar chain.

The four-carbon components in proline and hydroxyproline experiments are found to be mostly unlabeled, which indicates their formation from cleavage of the C-2/C-3 bond of [1-^{13}C]glucose or [1-^{13}C]-1-deoxydiketose, respectively. This route is supported by detection of labeled acetic acid which results from tetrose reductone formation according to Simon and Heubach (1965). Unlabeled products derived from unlabeled tetrose or tetrose reductone are 2-propionyltetrahydropyridines, known to be potent aroma components. 23 is a main constituent in malt, wort, and beer (Helak et al., 1989b). The homologous 5- and 6-alkyl-substituted cyclopenta-[*b*]pyridinones are found to be mixtures of unlabeled and singly and doubly labeled products, demonstrating their formation from retro aldol sugar fragments. Nevertheless, partly singly labeled C_4 components are also formed, which indicates a generation from different sugar cleavages as discussed above. Acetoin (18) is found to be singly labeled to an extent of 45% and the correspondent pyrrolyl compound 19 even to 60%, as can be estimated from the MS intensity data. These results suggest that labeled products such as acetoin or diacetyl may be formed in an advanced stage of the Maillard reaction from common retro aldol fragmentation, while others, unlabeled, are rapidly yielded in an early stage from unlabeled tetrose precursors.

One of the most surprising results of the labeling experiments was the detection of 100% unlabeled furfuryl alcohol, which must be yielded from [1-^{13}C]glucose by a α-cleavage and by splitting off labeled formic acid. This fragmentation must be suspected to happen in an early stage of the Maillard reaction, because all other five-carbon-derived products are detected to be partly singly labeled. This can be seen from furfurylpyrrolidinyl compound 20, which is found to be distributed 50:50 with regard to unlabeled and singly labeled product. A comparable isotopic distribution is detected for furfuraldehyde, suggesting that pyrrolidinyl component 20 is derived from the corresponding aldehyde by Strecker reaction. In addition, hexa- and tetrahydrocyclopent[*b*]-azepinones 21 and 22 are distributed to unlabeled and singly labeled products in ratios of 50:50 and 30:70, respectively. The expected precursors of the latter components are cyclopentenolones, which may be formed by retro aldol cleavage of suitable intermediates in an advanced reaction stage.

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