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DETECTION OF D-GLUCOSE-DERIVED PYRROLE COMPOUNDS DURING MAILLARD REACTION UNDER PHYSIOLOGICAL CONDITIONS*

F. GEORGE NJOROGE[†,‡], LAWRENCE M. SAYRE[§], AND VINCENT M. MONNIER[‡]

Institute of Pathology, School of Medicine, and Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106 (U.S.A.)

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ABSTRACT

5-Hydroxyl-1-neopentylpyrrole-2-carbaldehyde, 2-(2-hydroxyacetyl)-1-neopentylpyrrole, and 2-acetyl-1-neopentylpyrrole, in decreasing order of abundance, have been isolated and the structures characterized. These compounds were obtained from the reaction of a mixture of D-glucose and neopentylamine under physiological conditions of pH and temperature. In addition, 4*H*-dihydropyran-4-one, a known intermediate product of the Maillard reaction, was detected. The neopentylamine adducts were already detectable after one week of incubation, but rapid acid and alkaline degradation explains the lack of detection in body proteins.

INTRODUCTION

During the past ten years, it has become evident that the initial step of the Maillard reaction, nonenzymatic glycosylation, occurs *in vivo*. Reducing sugars react with, *e.g.*, protein-based primary amino groups to form a Schiff base which slowly undergoes an Amadori rearrangement resulting in a more stable ketoamine adduct. This posttranslational modification has been encountered in a wide variety of tissues and may be responsible for certain long-term complications of diabetes mellitus[1]. A number of proteins, for example, hemoglobin[2], albumin[3], collagen[4], lens crystallins[5], erythrocyte membrane proteins[6], and peripheral nerve proteins[7] have been found to undergo nonenzymatic glycosylation with D-glucose. In the advanced stage of the Maillard reaction, a series of dehydration and rearrangement reactions of the glycosylated adducts occur which lead to brown-colored, highly fluorescent compounds[8]. Monnier and Cerami[9] hypothesized that the advanced stage of the Maillard reaction could occur spontaneously *in vivo* and could explain

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†To whom correspondence should be addressed.
‡Institute of Pathology, Case Western Reserve University.
§Department of Chemistry, Case Western Reserve University.

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the age-related increase in fluorescence and brown pigment, as well as crosslinks in long-lived proteins such as lens crystallins and collagen. When incubated with D-glucose for several days, these proteins showed striking similarities in u.v., fluorescence, and crosslinking properties with those from aging or diabetic tissue[10–12]. These findings have raised the possibility that the Maillard reaction may account for some of the changes observed during aging *in vivo* and, at an accelerated rate, in diabetes.

The late stages of the Maillard reaction under physiological conditions, as well as its role in the structural and functional changes occurring in tissue, are presently poorly understood. Food chemists working on the late-stage products of the Maillard reaction have isolated several pyridine, pyrazine, imidazole, and pyrrole derivatives. However, the reaction mixtures were subjected to very high temperatures and extreme pH's. Pongor *et al.*[13] reported the formation of a furan–imidazole derivative of glucose and protein (FFI) that was detected by radioimmunoassay in serum albumin and hemoglobin[14]. Because acid hydrolysis was used to isolate the original compound, uncertainty persists as to the original structure of this compound. Therefore, it was of interest to investigate the reaction between D-glucose and a primary amine under physiological conditions of pH and temperature, in order to facilitate the elucidation of the structures and roles of the late-stage Maillard compounds *in vivo*. We have investigated the reaction between neopentylamine and D-glucose and we report herein the first chemical evidence for the formation of pyrrole compounds under physiological conditions of pH and temperature.

EXPERIMENTAL

Methods. — U.v. spectra were recorded for solutions in chloroform with a Hewlett–Packard 8450 UV/Vis spectrophotometer, and i.r. spectra recorded with a Beckman IR-8 or IR-10 spectrophotometer. ^{1}H-N.m.r. spectra were recorded at 200 MHz with a Varian XL-200 Fourier-transform spectrophotometer, and ^{13}C-n.m.r. spectra with a Bruker WH-400 NMR spectrometer operating at 100.6 MHz and equipped with quadrature-phase detection and interfaced with an ASPECT 3000 data system; for both ^{1}H- and ^{13}C-n.m.r. spectrometry, compounds were dissolved in (^{2}H)chloroform containing tetramethylsilane as an internal standard. T.l.c. was run on aluminum sheets precoated with Silica gel 60 F_{254}. L.c. chromatography was performed with a Waters model 510, dual-pump system, at 7239 Pa (1000 Psi), accompanied by a model 450 variable-wavelength detector. Flash chromatography was performed by the method of Still[15] using 40–63 μm (400–230 Mesh) Silica gel 60 (E. Merck No. 9385). Combined gas–liquid chromatography–mass spectrometry (GC-MS) analysis was performed with an HP 5985A mass spectrometer (Hewlett–Packard), equipped with a glass column (1.8 m × 2 mm i.d.), packed with 3% OV-17 on Gas Chrom Q, 100–120 mesh (Applied Science College Station, PA); the column temperature was held at 180° (isothermal) and the injection-port

temperature was 250°; He was the carrier gas, at a flow rate of 20 mL/min. The mass spectra were recorded at an ionizing energy of 70 eV and an ion-source temperature of 200°.

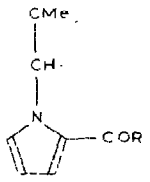
Reaction of neopentylamine with D-*glucose.* — In a 500-mL bottle, D-glucose (90.0 g, 0.5 mol) was dissolved in double distilled, de-ionized water (200 mL) and neopentylamine (43.6 g, 58 mL, 0.5 mol) was added. The pH was adjusted to 7.2 by a stepwise addition of 87% H_3PO_4. The mixture was incubated at 37° for 10 days. During this period, the solution turned from clear to dark brown and some water-insoluble material precipitated out. The precipitate was filtered off, washed with distilled water (3 × 100 mL), and air dried for 6 h to yield a dark-brown solid (7.3 g, 5.5% from combined weight of reactants). The aqueous filtrate was saturated with NaCl and exhaustively extracted with petroleum ether (30–75°) and then diethyl ether, and the solvents were removed with a rotary evaporator at a temperature <40°. The resulting amorphous solids from both extractions were subjected to flash chromatography in 9:1 and 4:1 hexane–ethyl acetate. The compounds isolated by flash chromatography were analyzed by l.c. using an acetonitrile–water gradient system and t.l.c. in hexane–ethyl acetate. Major compounds isolated were further characterized by ^1H- and ^{13}C-n.m.r. spectroscopy, mass spectrometry, and u.v. and i.r. spectroscopy.

Incubation of 5-hydroxymethyl-2-furaldehyde with neopentylamine. — To a solution of 5-hydroxymethyl-2-furaldehyde (0.2 g, 1.6 mmol) in water (0.2 mL) was added neopentylamine (0.13 g, 0.18 mL, 1.6 mmol). The pH of the mixture was adjusted to 7.2 with 87% H_3PO_4. The reaction progress was monitored by t.l.c. in 1:1 ethyl acetate–toluene. T.l.c. plates were first viewed under u.v. light and then sprayed with 2,4-dinitrophenylhydrazine followed by ninhydrin reagent.

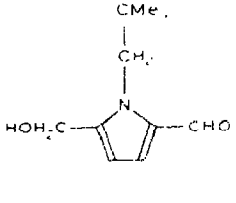
Acid hydrolysis of pyrrole compounds. — To the pyrrole compound (0.1 mg) dissolved in chloroform was added 6M HCl (2 mL). The mixture was purged with N_2 and incubated for 10 h at 110°. The effect of 6M HCl on the pyrrole compounds was assessed by comparison of the chromatographic properties of acid-treated samples to those of untreated ones.

RESULTS

Equimolar amounts of D-glucose and neopentylamine reacted under near-physiological conditions of pH and temperature. After 10 days of incubation, the reaction was stopped and the solid that had precipitated during the course of the reaction was filtered off to yield a dark-brown material (5.5%, w/w). The aqueous layer was extracted with petroleum ether, followed by diethyl ether, and the removal of solvents from each extract yielded 0.14 g (0.1%) and 1.5 g (1.1%) of brown material, respectively. T.l.c. (3:2 hexane–ethyl acetate) of the petroleum ether extract indicated the presence of three major compounds, **P1, P2,** and **P3** (R_F 0.70, 0.53, and 0.28, respectively). T.l.c. (same solvent system) of the diethyl ether extract indicated the presence of four major compounds, **E1, E2, E3,** and **E4,** (R_F 0.28, 0.11, 0.06, and 0.02, respectively).



1 R = CH_3
2 R = CH_2OH



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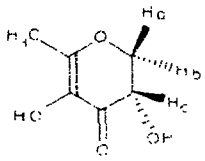

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Compound **P1** was obtained in 0.2% yield and was found to react readily with 2,4-dinitrophenylhydrazine to give a brown spot. G.l.c.–m.s. and ^{1}H-n.m.r. analysis indicated that it was 2-acetyl-1-neopentylpyrrole (**1**): e.i.m.s.; *m/z* 179 (M^+) 164 ($M^+ - CH_3$), 136 ($M^+ - COCH_3$), and 122 [$M^+ - C(CH_3)_3$].

The fragmentation pattern was similar to that reported by Gianturco *et al.*[16] for similar compounds. The ^{1}H-n.m.r. spectrum of **1** was similar to that reported in the Varian high-resolution n.m.r. spectra catalog[17], exhibiting signals at δ 0.95 (s, 9 H), 2.47 (s, 3 H), 4.37 (s, 2 H), 6.15 (dd, 1 H, *J* 4 Hz), 6.83 (d, 1 H, *J* 4 Hz), and 6.98 (m, 1 H, *J* 2 Hz).

Compound **P2**, isolated in 11% yield, showed a positive Pauly's test but was unexpectedly unreactive with 2,4-dinitrophenylhydrazine. However, the i.r. spectrum showed a strong peak at ν_{max} 1660 cm^{-1} that indicated the presence of a carbonyl group as reported previously[18]; other ν_{max} were at 3480, 1380, and 1360 cm^{-1}: ^{1}H-n.m.r. of **P2**; δ 0.95 (s, 9 H), 3.58 (br. s, 1 H), 4.38 (s, 2 H), 4.68 (s, 2 H), 6.23 (dd, 1 H, *J* 5.0, 2 Hz), and 6.98 (m, 2 H, *J* 5 Hz); ^{13}C-n.m.r.: δ 27.23 [$C(CH_3)_3$], 33.27 [$C(CH_3)_3$], 58.1 (CH_2OH), 64.15 (CH_2N), 108.18 (C-4), 118.49 (C-3), 127.09 (C-2), 131.85 (C-5), and 187.87 (C=O); e.i.m.s.: *m/z* 195 (M^+), 164 ($M^+ - CH_2OH$), and 136 ($M^+ - COCH_2OH$); λ_{max} 291 and 251 nm (shoulder). From the aforementioned spectroscopic data, the structure of **P2** was established to be 2-(2-hydroxyacetyl)-1-neopentylpyrrole (**2**). Comparison of this ketone with an analogous ketone isolated by Ledl[19] * from the reaction of propylamine with D-glucose at elevated temperatures revealed close similarities in u.v., n.m.r., and chromatographic properties. Treatment of **P2** with 6M HCl for 10 h at 110° led to complete decomposition of this compound.

*Sample donation from his laboratory.

The third major compound (**P3**), isolated from the petroleum ether extract by flash chromatography, was 5-hydroxymethyl-1-neopentylpyrrole-2-carbaldehyde (**3**). It was isolated in 20% yield from combined petroleum ether and diethyl ether concentrates, and was found to react with 2,4-dinitrophenylhydrazine, giving a dark-brown spot on t.l.c. It also appeared as a pink spot after spraying with the Ehrlich's reagent. The chromatographic properties of **P3** on silica gel were similar to those of analogous pyrrolecarbaldehyde isolated by Ledl[19,*]. The u.v. spectrum was very similar to that reported by Kato and Fujimaki[18] for similar pyrrole compounds obtained by heating reducing sugars with amines; it displayed a strong absorption at λ_{max} 293 nm and a shoulder at ~254 nm. Compound **P3** was also found to be extractable by diethyl ether (compound **E1**). Compound **E1** and **P3** had similar physical as well as chemical properties and, therefore, were considered to be the same compound: ν_{max} 3390, 1650, 1400, 1370, and 1048 cm^{-1}; ^{1}H-n.m.r. δ 0.98 (s, 9 H), 1.05 (br.s, 1 H), 4.75 (s, 4 H), 6.35 (d, 1 H, *J* 5 Hz), 6.93 (d, 1 H, *J* 5 Hz), and 10.48 (s, 1 H); ^{13}C-n.m.r. δ 27.66 [$(CH_3)_3$], 34.26 [$C(CH_3)_3$], 58.61 (CH_2N), 56.92 (CH_2OH), 109.97 (C-3), 124.24 (C-4), 133.83 (C-5), 142.87 (C-2), and 179.14 (CHO): e.i.m.s.: *m/z* 195 (M^+), 180 ($M^+ - CH_3$), 178 ($M^+ - OH$), 166 ($M^+ - CHO$), and 139 [$M^+ - (CH_3)_3C + H$]. An attempt to obtain **P3** by incubating 5-hydroxymethyl-2-furfural with neopentylamine at physiological conditions of temperature and pH were unsuccessful; even after 12 days of incubation, no formation of **P3** could be detected in the petroleum ether extract of the reaction mixture by t.l.c. analysis. Incubation of **P3** with 6M HCl for 10 h at 110° led to complete decomposition of this compound.

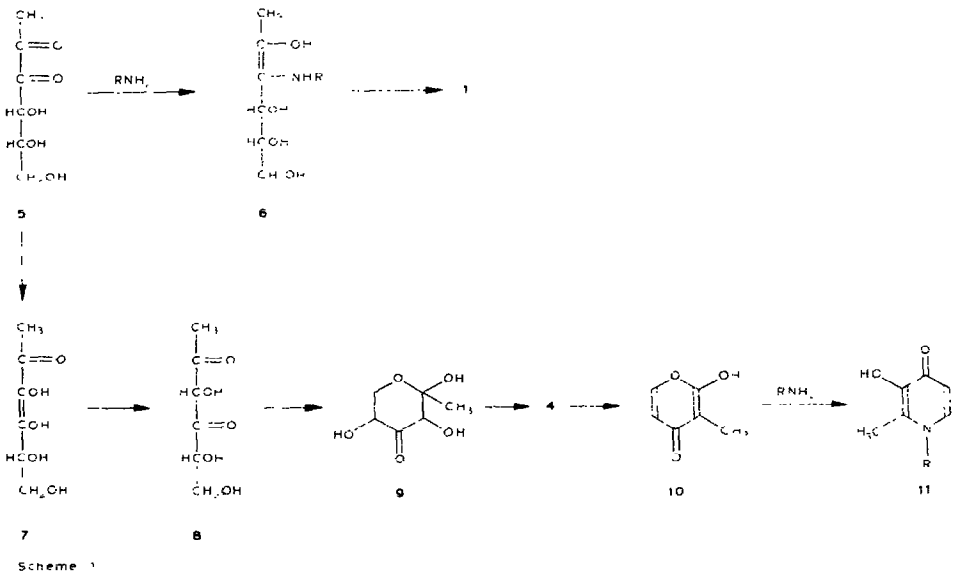
Compound **E2**, obtained by flash chromatography of the ether extract in 4.3% yield, was found to be 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (**4**); it showed a yellow spot after spraying the t.l.c. plate with 2,4-dinitrophenylhydrazine and keeping it overnight, ν_{max} 3510 and 3400 (br.) (H-bonded OH), 1735 (very weak), 1668, and 1618 cm^{-1} (α-substituted α,β-enone): ^{1}H-n.m.r.: δ 2.18 (s, 3 H), 4.05 (m, H-3), 4.30 (ABq, 1 H, *J* 6, 12 Hz, H-2b), 4.45 (ABq, 1 H, 6, *J* 12 Hz, H-2b), and 4.45–4.70 (br., 2 H): e.i.m.s.: *m/z* 144 (M^+), 101, 73, 72, 55, 45, and 43 (same as those reported by Fisher *et al.*[20]).

The structures of compounds **E3** and **E4** from the diethyl ether extract remain unidentified. Both gave a positive reaction with 2,4-dinitrophenylhydrazine and have molecular masses of 237 and 213, respectively. ^{1}H-N.m.r. spectroscopy and mass spectroscopy of **E3** strongly indicated that it is a disubstituted *N*-neopentylpyrrolecarbaldehyde.

DISCUSSION

The reaction of D-glucose with neopentylamine was studied as a model for understanding the chemical nature of the changes occurring when proteins are exposed to D-glucose. The use of a simple aliphatic amine as a model for protein-based amino groups excludes some of the complications that would result from the

*Sample donation from his laboratory.



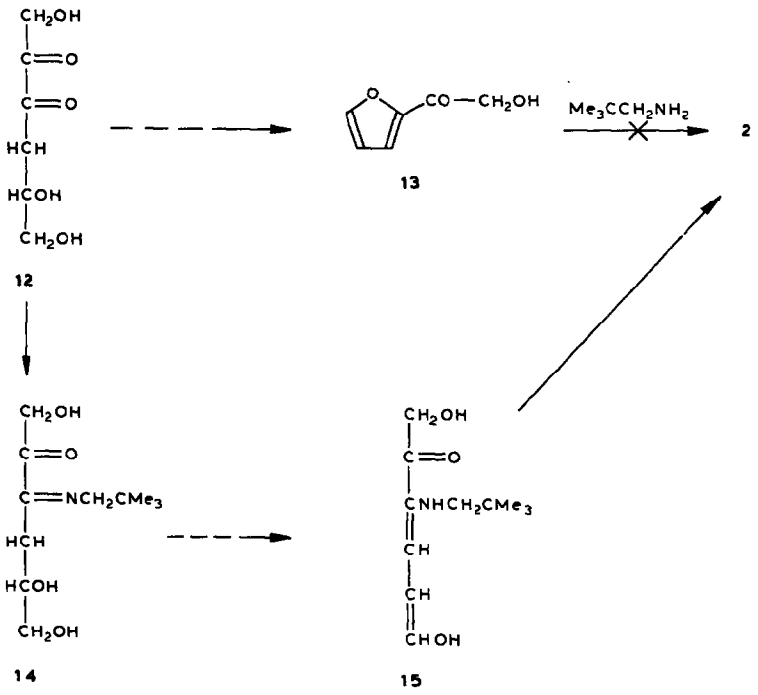
Scheme 1

use of amino acids as model compounds, such as the formation of Strecker degradation products[21,22], and isolation problems due to the presence of charged groups (*e.g.*, where lysine is used). The three pyrrole compounds isolated from the reaction between neopentylamine and D-glucose at physiological conditions of pH and temperature are late products of the Maillard reaction. Although pyrrole compounds similar to those reported in this study have been isolated[23], this is the first report of their formation under very mild conditions.

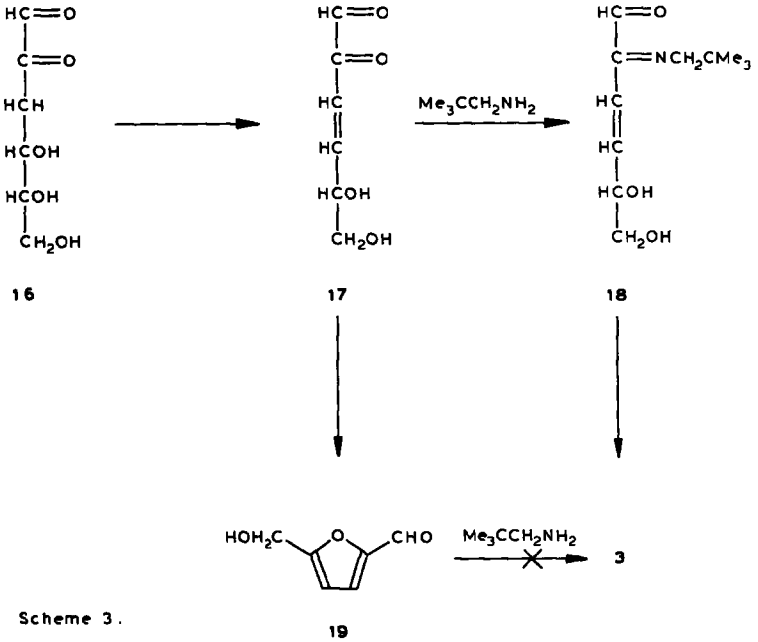
2-Acetylpyrrole (**1**) is possibly formed from the reaction of 1-deoxyglucosone[24] (**5**) with neopentylamine to form an iminol intermediate **6** that undergoes cyclization, followed by a series of dehydrations (see Scheme 1). The keto group of **1** could potentially act as a crosslink with amino groups *via* Schiff base formation.

2-(2-Hydroxyacetyl)pyrrole (**2**) has been postulated to be formed *via* 4-deoxy-glucosone[25] (**12**). Through a series of dehydrations and eventual cyclization. **12** can be transformed into 2-hydroxyacetylfuran (**13**). This would be expected to react with neopentylamine at elevated temperatures to give **2** (Scheme 2). However, attempts by Jurch and Tatum[26] to synthesize an analog of **2**, where the neopentyl group is replaced by a methyl group, from **13** and methylamine failed. An alternative sequence involving cyclization–dehydration through a Schiff base **14** and subsequent conversion to an enaminol **15** could lead to **2** as shown in Scheme 2. Unlike **1, 2** cannot react with an amine in forming a Schiff base as it was found to be unreactive towards 2,4-dinitrophenylhydrazine.

5-Hydroxymethylpyrrole-2-carbaldehyde (**3**) formation has been documented



Scheme 2.



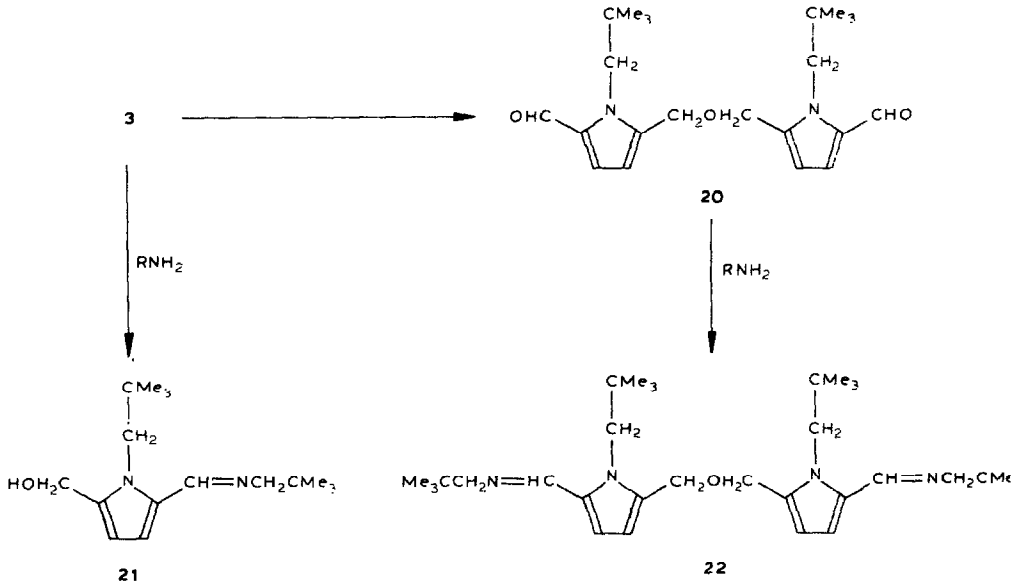
Scheme 3.

by several researchers[18,26]. It could conceivably be formed from the reaction of 3-deoxyglucosone (**16**) with neopentylamine *via* intermediate 3,4-dideoxy-hexosulos-3-ene **17** and Schiff base **18** (Scheme 3). The last-named compound could undergo cyclization and subsequent dehydration to form the pyrrolecarbaldehyde[24] **3**. At higher temperatures, pyrrolecarbaldehyde **3** can be formed from the reaction of 5-hydroxymethyl-2-furfural (**19**) with an amine, as reported by Jurch and Tatum[26]; however, under physiological conditions of pH and temperature, we could not detect any formation of **3** from **19** incubated with neopentylamine. This suggests that, at physiological conditions, **3** is probably formed directly *via* the Schiff base (**18**) route rather than through the **19** intermediate.

The hydroxymethyl group of **3** can serve as a site of further reaction resulting in two ether-bond-linked pyrrolecarbaldehydes (**20**), as demonstrated by Olsson *et al.*[24]. This could react with amino groups to form either mono- or di-aldimines (**22**) (Scheme 4). Alternatively, **3** could form a Schiff base (**21**) with an amine without condensing with a second molecule of **3**. If these reactions were to occur in proteins, they could result in the formation of inter- or intra-molecular crosslinks.

Compound **4**, obtained from the ether extract, possibly was formed from 1-deoxyglucosone (**5**) (see Scheme 1) by tautomerization to a 2,4-diketone (**7, 8**) which then cyclized to form a pyranone intermediate[9], the latter subsequently being dehydrated into **4**. As Mills *et al.*[27] have shown, **4** may undergo a further dehydration step to give maltol (**10**), a compound that can in turn react with an amino group to give a pyridone[28] **11**.

The observation that the pyrrole compounds **2** and **3** were completely destroyed after incubation with 6M hydrochloric acid for 10 h at 110° explains the lack



Scheme 4

of detection of these compounds *in vivo*, as acid hydrolysis is commonly used to break down proteins prior to analysis. However, it is of interest that Scott and assoc.[29,30] found proteolytic digest of connective tissues to contain material that reacted rapidly at room temperature with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent). This Ehrlich chromogen of pyrrolic nature was found to be associated with a three-chain peptide chain from human-type III and IV collagen. From their results, these authors proposed that collagen crosslinking could result from such an Ehrlich chromogen. The identification of carbaldehyde and ketopyrrole compounds as products of the late-stage Maillard reaction under physiological conditions provides a starting point from which further studies of the chemistry of this process can proceed.

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