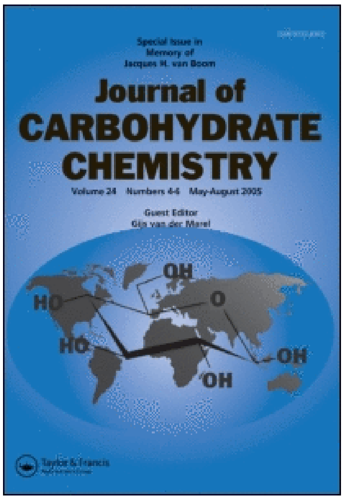

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3-(D-ERYTHRO-TRIHYDROXYPROPYL)-1-NEOPENTYL PYRROLE-2-CARBOXALDEHYDE, A NOVEL NONENZYMATIC BROWNING PRODUCT OF GLUCOSE

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ABSTRACT

The reaction of equimolar amounts of D-glucose and neopentylamine in phosphate buffer under physiological conditions of pH and temperature has been investigated. From the diethyl ether extract, we have identified a C-8 condensation product and have demonstrated that its formation does not proceed via a free-radical mechanism. Based on ^{1}H and ^{13}C NMR and other spectroscopic techniques, including UV, IR, EI-MS, CI-MS of the TMS derivative, the structure 3-(D-erythro-trihydroxypropyl)-1-neopentylpyrrole-2-carboxaldehyde, was assigned to this advanced Maillard reaction product.

INTRODUCTION

Nonenzymatic browning reactions between sugars and amino acids through the Maillard reaction are known to be responsible for deterioration of stored or heated foodstuffs.[1-4] In recent years it has become evident that the initial step of this reaction occurs *in vivo* in a variety of cellular, extracellular, and membrane proteins.[5-8] However, very little information on the chemistry of the late-stage Maillard process under physiological conditions is available, and, as a result of this, it has been difficult to develop a clear understanding of the role of this reaction in structural and functional changes in proteins in diabetes and aging.

In previous work,[9] we described the isolation and characterization of three glucose-derived pyrrole compounds and a gamma-dihydropyranone from petroleum ether and diethyl ether extracts - compounds that had been formed under physiological conditions of pH and temperature. However, we could not completely characterize one of the major compounds that was extractable in diethyl ether whose molecular weight was 237. An analogous compound has been detected by Kato's group[10] during GC-MS analysis of reaction products of butylamine and glucose at elevated temperatures, but its structure has remained unidentified.

In an extension of that work, we have now identified the unknown compound from the ether extract to be 3-(D-*erythro*-trihydroxypropyl)-1-neopentylpyrrole-2-carboxaldehyde (1). This compound has not been previously reported in the Maillard literature. A possible pathway for the formation of this compound will be discussed.

RESULTS AND DISCUSSION

Equimolar concentrations of D-glucose and neopentylamine were reacted under physiological conditions of pH and temperature. After

10 days of incubation, the reaction was stopped and the melanoidin-like precipitate was removed by filtration. The aqueous layer was exhaustively extracted with petroleum ether, followed by diethyl ether. Major compounds in petroleum ether, as evidenced by TLC and HPLC, were identified as 2-acetyl-1-neopentylpyrrole, 5-hydroxymethyl-1-neopentylpyrrole-2-carboxaldehyde 2, and 2-(2-hydroxyacetyl)-1-neopentylpyrrole 3, as previously described.[9] The diethyl ether extract contained four major compounds: in the less polar fraction (as evidenced by faster migration on TLC), 5-hydromethyl-1-neopentylpyrrole-2-carboxaldehyde (not completely extracted by petroleum ether) was isolated, while in the moderately polar fraction a 2,3-dihydro-3,5-dihydroxy-4H-pyran-4-one was identified.[9] Two compounds with molecular weights of 237 and 213, respectively, were contained in the more polar fraction of the ether extract. The latter could not be identified due its instability during purification. However, the former, compound 1, which is the subject of this paper, was obtained from this fraction in 0.07% overall yield (calculated from starting glucose). Its purification was effected by use of TLC and HPLC as described in experimental section. This compound had a UV absorption maximum at 291 nm comparable to that of other pyrrole aldehydes reported in literature;[9,11-14] however, it differed from the other pyrroles by the absence of a shoulder at 265 nm and a small shift in absorption towards longer wavelength. IR spectrum of 1 was also very similar to that of pyrrole aldehyde previously described[9,11,12] with characteristic aldehydic C-H stretch absorption frequencies at ca. 2825 and 2720 cm^{-1} and a strong carbonyl absorption at 1650 cm^{-1}.

The ^{13}C NMR spectrum of 1 (FIG. 1, below) showed eleven signals which included a distinct aldehyde carbonyl absorption at δ 179.46, four pyrrole ring carbon signals at δ 136.58, 131.29, 128.93 and 109.31, two methine carbon signals with -OH substituent at δ 74.56 and 70.09, two methylene carbons at δ 63.71 and 59.14, a tertiary carbon at δ 33.51, and three equivalent methyl carbons at δ 27.60. The structure of this compound was partially determined by comparison of its ^{13}C NMR signals with those of previously established pyrrole compounds shown below. ^{13}C NMR frequencies for the neopentyl moiety were practically identical for all three pyrroles and so were the aldehyde groups of compounds 1 and 2. The presence of

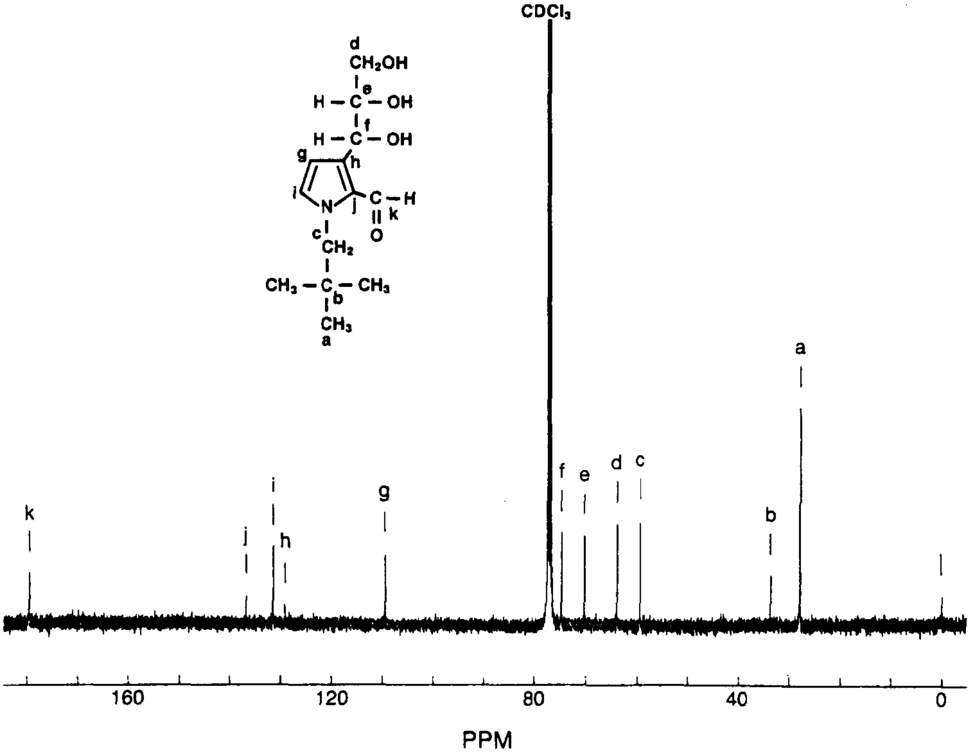
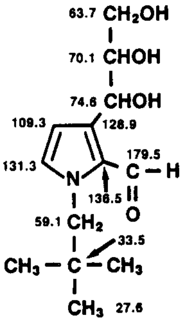


FIG. 1. 100-MHz ^{13}C NMR spectrum of 1.

two quaternary carbons on the pyrrole backbone of compound 1, as indicated by negative ^{13}C signals by Attached Proton Test (APT)[15] (at δ 128.9 and 136.5), was indicative of ring disubstitution. Absorption frequency of 1 at δ 109.3 could unequivocally be assigned to C-4 from comparison of compounds 2 and 3 signals at δ 109.9 and 108.2, respectively. The carbon atom at position 5 (C-5), a tertiary carbon, had a resonance at 131.3 ppm which was comparable to C-5 resonance of 3 which absorbed at 131.8 ppm and differed appreciably with the C-5 substituted resonance of 2 (142.9 ppm). Assignment of C-2 signal at (136.5) was made by comparison with that of 2 which was 133.8 ppm. The final resonance of the pyrrole ring of 1,



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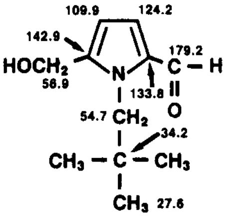

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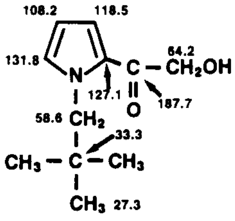

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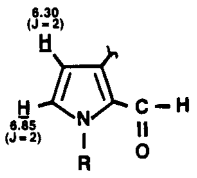
128.9 ppm, could only be assigned to C-3 since it was quaternary in nature and had a lower downfield shift than either 2 or 3, a shift that could be explained by presence of substitution at C-3 of 1.

The pattern of substitution on 1 was further clarified by comparison of its ^{1}H NMR spectra with those of 2, 3 and a few other substituted pyrrole compounds reported in literature.[16] Important information was especially obtained by observing the presence of a single signal at 6.85 ppm, a region where protons on C-3 and C-5 resonate.[11,16,17] Such a resonance was also observable for both compound 2 and 3 as shown below.



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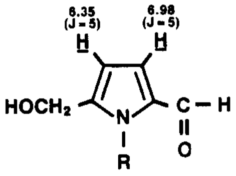

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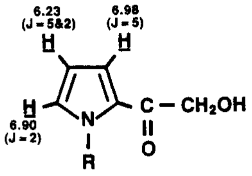

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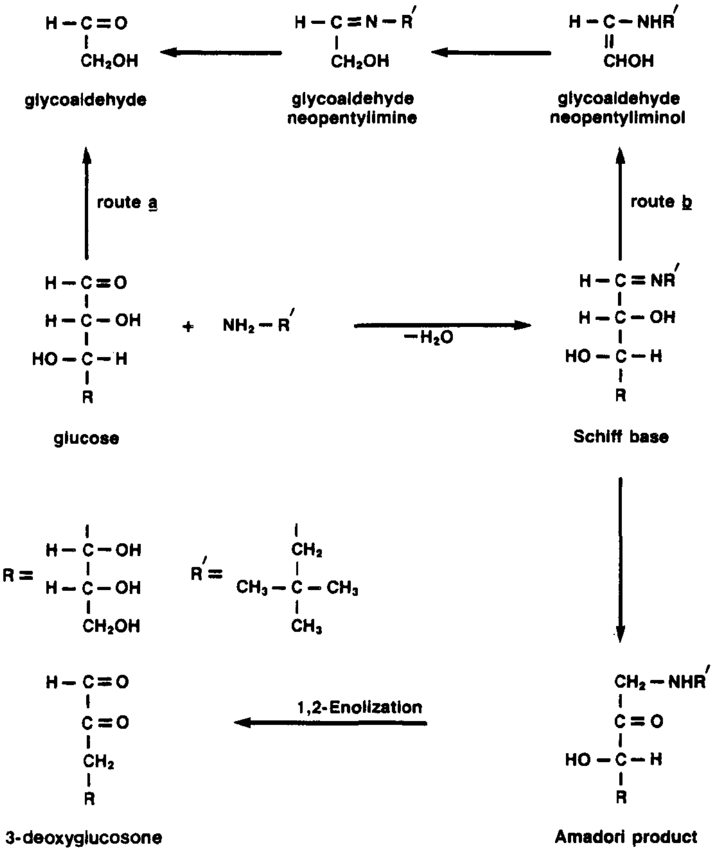
Additional information that supported the assignment of the single proton resonating at $\delta = 6.85$ to C-H at position 5 was the observation that the coupling constant for the vicinal protons of 1 was $J_{4,5} = 2$ Hz, unlike that for vicinal protons of 2 where $J_{3,4} = 5$. This observation was also in agreement with values reported in literature whereby the J-coupling constant values for C-H (4-5) have been reported to be 1-2 Hz, while C-H (3-4) were 3-4 Hz for pyrrole compounds.[16] Our observed coupling constant of 2 Hz clearly indicated that the two adjacent protons were in position 4 and 5 of the pyrrole ring.

Acetylation of 1, using conditions previously reported by Tsuchida and coworkers,[18] gave three distinguishable O-acetyl groups as indicated by three acetate methyl proton resonances at δ 2.00, 2.08 and 2.12. This result further confirmed that 1 was a trihydroxy compound.

GC-MS of 1 using the electron-impact (EI) mode of ionization exhibited a peak at m/z 237 with other major fragmentation patterns very similar to that of 2. This ion was attributed to molecular ion minus water molecule (M^+-H_2O). Although the acetylated derivative of 1 did not chromatograph well for GC-MS analysis, its trimethylsilyl derivative gave a single peak on GC and exhibited a peak of m/z 471, corresponding to the molecular ion of the silylated 1. Other fragments were observed at m/z 456 (M^+-CH_3), 415 [M - $C(CH_3)_3]^+$, 368 (M^+- CH_2OTMS), and a base peak at 266 arising from [M^+ - CH(OTMS)CH_2OTMS]. Based on the data described above, the structure of compound 1 was postulated.

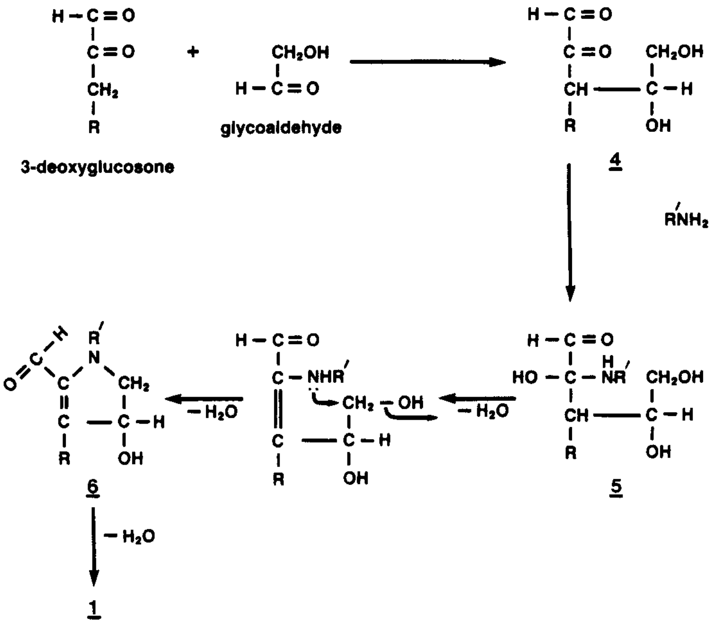
It is conceivable that compound 1 was formed via a base-catalyzed condensation reaction of 3-deoxyglucosone,[19,20] with glycoaldehyde, followed by subsequent reaction with neopentylamine. 3-Deoxyglucosone is a well-documented dicarbonyl compound formed by the reaction of glucose and amine through an Amadori product, followed by a 1,2-enolization step as shown in Scheme 1. Glycoaldehyde, the other reactant postulated in the formation of 1, is a C-2 fragmentation product of glucose. Its formation has been extensively studied by Namiki and coworkers,[21,22] and the latter have established that the two-carbon fragmentation occurs prior to Amadori rearrangement. They have also clearly shown that glycoaldehyde forms independently from 3-deoxyglucosone. One possible mechanism for the formation of glycolaldehyde from a glucose molecule is fission by reverse-aldol condensation occuring at the C2-C3 bond (route a, Scheme 1) that has been postulated to occur under basic conditions.[1,21] An alternative mechanism (route b) would involve cleavage of an aminated sugar rather than by cleavage of the sugar itself. The Schiff's base formed can undergo a base-catalysed reverse-aldol-type reaction to give rise to glycoaldehyde neopentyl-



Scheme 1

amine (enol form). The latter can then rearrange to glycoaldehyde imine, a compound that can be easily hydrolysed to glycoaldehyde.

Reaction of glycoaldehyde with 3-deoxyglucosone would give rise to a dicarbonyl 4 (Scheme 2). Intermediate 4 can then react with an amine to form the iminol 5 that can readily cyclize to give dihydropyrrole aldehyde 6. The latter intermediate can lose a molecule of water to give compound 1. Thus, in contrast to the other pyrroles previously described,[9] the proposed mechanism stipulates, that not all four ring carbon atoms are derived from the same glucose molecule.



Scheme 2

In an effort to establish whether 1 was formed through a mechanism involving free-radical intermediates of glucose, we incubated glucose and neopentylamine with and without an ion chelator (EDTA) in presence of N_2 and O_2, respectively. The rate of formation of 1 for the two experiments was followed using reverse-phase HPLC, monitoring absorbance at 285 nm. The time-course study on the formation of 1 under the two conditions is shown in FIG. 2 below. From this study, it is apparent that formation of compound 1 is not mediated by a mechanism involving fragmentation of glucose by free oxygen radicals. The proposed mechanism for the formation of 1 and its possible occurence in vivo remain to be demonstrated.

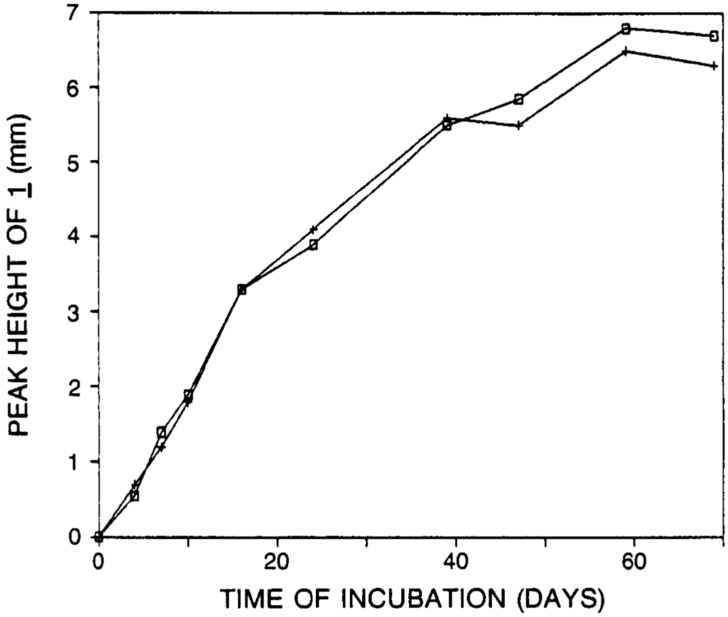


FIG. 2. Kinetics of formation of 1 under atmospheres of air (□) and nitrogen with EDTA (+). Reactions were conducted as described under 'Experimental' and quantitation was performed by HPLC.

EXPERIMENTAL

General Methods. ^1H NMR were recorded at 200 MHz on a Varian XL-200 Fourier-transform spectrophotometer. ^{13}C NMR spectra were obtained using Bruker WH-400 NMR spectrometer operating at 100.6 MHz, equipped with quadrature phase detection and interfaced with an ASPECT 3000 data system. The attached Proton Test (APT) experiment was done as described by Le Cocq.[15] For both ^1H and ^{13}C NMR analysis, compounds were dissolved in deuterated chloroform using tetramethylsilane as an internal standard. UV spectra were measured in chloroform with a Hewlett Packard 8450A UV/Vis spectrophotometer.

Combined gas chromatographic-mass spectrometry (GC-MS) analysis was performed using an HP 5985A mass spectrometer (Hewlett Packard).

A glass column (1.8 M x 2 mm i.d.) packed with 3% OV-17 on gaschrom-Q 100/120 mesh (Applied Science College Station, PA) was used for GC analysis. The column temperature was held at 180°C (isothermal) with an injection port temperature was 250°C. Helium was used as a carrier gas at a flow rate of 20 mL/min. The mass spectrum was determined using an ionizing energy of 70 eV and ion source temperature of 200°C.

Thin layer chromatography (TLC) was run on aluminum sheets precoated with Silica Gel-60 F_{254} (E. Merck). Flash chromatography was performed by the method of W.C. Still[23] using a 40-65 μm (400-230 mesh) Silica Gel-60 (E. Merck No. 9385).

HPLC chromatography employed a Waters 510 dual-pump system equipped with a Model 411 absorbance detector, automated gradient controller and a Waters 740 Data Module. Reverse-phase separations and analyses were made using a Vydac C-18 (RP) 10-μm column (3.2 x 250 mm) and water-acetonitrile gradient system.

Reaction of glucose with neopentylamine phosphate salt. The reaction was conducted as described previously.[9] Glucose (90.0 g, 0.5 mol) was dissolved in 200 mL of purified water, and 43.6 g (58 mL, 0.5 mol) neopentylamine was added. The pH of the reaction mixture was adjusted to pH 7.2 by a stepwise addition of 85% phosphoric acid. The reaction mixture was incubated at 37 °C for 10 days, during which time the solution turned from clear to dark-brown, and some melanoidin-like material precipitated out. The mixture was filtered, and the aqueous filtrate was saturated with sodium chloride and exhaustively extracted with petroleum ether to remove the more non-polar compounds previously described.[9] After petroleum ether extraction, the aqueous fraction was further extracted with diethyl ether (5 x 400 mL). The diethyl ether was removed by rotary evaporation to yield 0.20 g of crude product.

<u>Isolation of 3-(D-erythro-trihydroxypropyl)-1-neopentylpyrrole 2-carboxaldehyde (1) from ether extract</u>. The ether fraction obtained from above was charged on a silica gel column (20 x 150 mm) which was packed with hexanes-ethyl acetate (4:1). The column was then eluted with hexanes-ethyl acetate (3:2) and ethyl acetate-methanol (8:1), successively. After analysis by TLC [solvent system $CHCl_3$-MeOH (8:1)] and subsequent 2,4-dinitrophenylhydrazine (DNP) spray detection, most of compound 1 was shown to be eluted with ethyl acetate - methanol (8:1). However, after further analysis with HPLC and TLC using the same solvent indicated above, fractions containing compound 1 were found to be contaminated with some other minor carbonyl-containing compounds. Further purification of 1 was effected by preparative TLC using $CHCl_3$-MeOH (8:1) as the eluting solvent. Elution of the band containing 1 was performed in chloroform. The solvent was evaporated, and the concentrate was dissolved in CH_3CN-H_2O and purified further with preparative HPLC to give 25 mg (0.07%) of pure 1 as a yellow oil: ^1H NMR ($CDCl_3$) δ 1.00 (s, 9 H, CH_3), 2.00-2.50 (brs, 3 H, OH), 3.65-3.95 (m, 3 H, H-2' and 2 H-3') 4.10 (s, 2 H, CH_2), 5.05 (d, J = 5 Hz, 1 H, H-1'), 6.35 (d, $J_{4,5}$ = 2 Hz, 1 H, H-4), 6.85 (d, J = 2 Hz, 1 H, H-5), 9.75 (s, 1 H, CHO). ^{13}C NMR ($CDCl_3$) δ 27.61 ($(CH_3)_3$), 33.52 ($\underline{C}(CH3)_3$), 59.14 (CH_2N), 63.72 (CH_2OH), 70.09 ($\underline{CO}HCH_2OH$), 74.57 (ArCHOH), 109.32 (C-4) 128.94 (C-3), 131.30 (C-5), 136.58 (C-2), and 179.47 (CHO). ^{13}C APT NMR resulted in the following sign for the signals: 27.61 (-ve), 33.52 (+ve), 59.14 (+ve), 63.72 (+ve), 70.09 (-ve), 74.57 (-ve), 109.32 (-ve), 128.94 (+ve), 131.30 (-ve), 136.58 (+ve) and

179.47 (-ve). IR 3380, 2950, 2870, 1645, 1495, 1365, and 1030 cm^{-1}. GC-MS gave one major peak m/z 237 ($M^+ - H_2O$), 195 [M^+ - (-CH(OH)CH_2OH)], and others at m/z 190, 120, 124, 109, and 107. The TMS derivative of 1 also gave one major peak on GC with m/z 471 (M^+); other major fragments were observed at 414 [M^+ - (C$(CH_3)_3$)], 368 (M^+ - CH_2OTMS), 292, 266 (M^+ - CH(-CHOTMS)-CH_2OTMS), 180, and 165 (M^+- (CH(OTMS)CH(OTMS)CH_2OTMS). UV ($CHCl_3$) λ max 291 nm.

Acetylation of compound 1. Compound 1 (10 mg) was dissolved in 2 mL of an equimolar mixture of acetic anhydride and pyridine and allowed to stand for 2 days at room temperature. The mixture was then poured into 15 mL of ice water and extracted with chloroform (5 x 50 mL). The extract was washed with dilute sodium bicarbonate solution (2 x 20 mL), water (2 x 20 mL), and dried over anhydrous sodium sulfate. After evaporation of the chloroform, the acetylated compound was chromatographed on preparative TLC and then eluted with EtOAc/hexanes (1:6). Scrapings from preparative TLC were extracted several times with chloroform. After concentration of the chloroform extract, a light-yellow oil was obtained whose ^1H NMR in $CDCl_3$ showed signals at δ 1.05 (s, 9 H, CH_3), 2.00 (s, 3 H, -$COCH_3$) 2.08 (s, 3 H, $COCH_3$), 2.12 (s, 3H, $COCH_3$), 4.10-4.46 (m, 3 H, H-2' and 2 H-3') 4.15 (s, 2 H, CH_2), 5.50 (dd, J = 6.0 Hz, 1 H, H-1'), 6.30 (d, $J_{4,5}$ = 2 Hz, 1 H, H-4) 6.85 (d, $J_{4,5}$ = 2, 1 H, H-5), and 9.94 (s, 1 H, CHO).

Silyation of compound 1 for GC-MS analysis. The TMS derivative was obtained by treating 0.5 mg of 1 with of Tri-SIL'TBT' (1 mL) (Pierce Chemical Co., Rockford, IL) in a Reacti-Vial for 10 min at room temperature and then 20 min at 60 °C.

Time-course study of formation of 1 in oxygen and under oxygen metal-free conditions. In order to follow the progress of formation of compound 1 in both free-radical conducive and non-conducive conditions, two sets of reactions were prepared. Reaction 1: 21.7 g (29 mL, 0.25 mol) of neopentylamine was diluted with 200 mL of distilled water, and the pH was adjusted to pH 7.2 by addition of 87% phosphoric acid. Ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA) 0.9 g (2.5 mmol) was added, followed by 45.0 g (0.25 mol) of D-glucose. The solution was degassed, perfused with nitrogen, and stored at 37 °C. Aliquots (3 mL) of the incubated sample were taken for a period of 60 days. Each aliquot was diluted with 20 mL of H_2O. The diluted solution was then saturated with NaCl and extracted with (2 x 40 mL) of diethyl ether, dried over Na_2SO_4, and concentrated. The concentrate was then dissolved in 10 mL of *n*-butanol. For HPLC analysis of compound 1, 100 μL of the butanol solution was diluted ten times with the same solvent. From the diluted solution, 10 μL were injected into HPLC and analyzed using a C-18 (RP-18) 10 μm system. Compound 1 eluted at retention time 23.4 min using water-acetonitrile in a gradient system. Reaction 2: This was conducted in a similar manner to reaction 1 described above, with exception that no addition of EDTA and no degassing or introduction of nitrogen was performed. Results for the progress of formation of compound 1 in both incubation mixtures 1 and 2 are shown in FIG. 2.

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