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SPECIFIC DEPROTONATION REACTIONS OF THE PYRIMIDINE RADICAL CATION RESULTING FROM THE MENADIONE MEDIATED PHOTOSENSITIZATION OF 2′-DEOXYCYTIDINE

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Menadione(2-methyl-1,4-naphthoquinone) was shown to sensitize 2′-deoxycytidine to near ultraviolet light according to two main mechanisms. Reaction of a water molecule with the initially photo-induced pyrimidine radical cation and subsequent addition of molecular oxygen leads to the preponderant formation of the four *cis* and *trans* diastereoisomers of 5,6-dihydroxy-5,6-dihydro-2′-deoxyuridine. Pyrimidine ring opening and rearrangement products are also generated through the intermediate 6-hydroxy-5,6-dihydro-2′-deoxyurid-5-yl radical. The competitive deprotonation reaction of the radical cation is likely to involve two sites. Loss of an amino group proton is the likely initial event to explain the formation of 2′-deoxyuridine which is resistant to further photooxidation. The second deprotonation reaction involves the osidic carbon C(1′). The resulting radical will further react with oxygen leading to the release of free cytosine with concomitant formation of 2-deoxy-D-ribono-1,4-lactone. This reaction which is not prevented by hydroxyl radical scavengers constitutes to our knowledge the first example of a pyrimidine radical which is able to initiate selective intramolecular reaction at position 1 within the sugar moiety.

KEY WORDS: Photooxidation, pyrimidine radical cation, photosensitization, 2-methyl-1,4-naphthoquinone, 2′-deoxycytidine, photoproducts.

INTRODUCTION

2-methyl-1,4-naphthoquinone or menadione which is a component of vitamin k_3 is able to photooxidize several pyrimidine nucleobases and nucleosides including thymine, cytosine and thymidine when exposed to near ultraviolet light.[1,2] A likely intermediate of these photoreactions is the pyrimidine radical cation which results from an electron transfer reaction between the nucleic acid component and the menadione in a triplet excited state.[3] It is worth noting that similar radicals are expected to be generated through the direct effects of ionizing radiation on related DNA components.[4,5] However, the use of frozen aqueous solutions which is required to produce these radical cations upon gamma radiolysis has been shown to affect their conversion to diamagnetic degradation products after thawing.[6] Therefore, the menadione mediated photosensitization reactions provide an alternative model system for investigating the chemical reactions of pyrimidine radical cations in aqueous solutions. One of the main conversion pathways of thymine and thymidine radical cations

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has been shown to be the hydration reaction which leads to the specific formation of the corresponding 6-hydroxy-5,6-dihydropyrimid-5-yl radical.[7] The second main competitive reaction is a deprotonation process which occurs at the N−1 position of thymine and within the methyl group of thymidine respectively.[1,2] As an extension to the work on thymine nucleobase and nucleoside we report results of investigations dealing with the two deprotonation reactions of the menadione mediated 2′-deoxycytidine radical cation.

MATERIALS AND METHODS

A. Chemicals

2′-deoxycytidine was purchased from Pharma-Waldhorf GmbH, Düsseldorf, GFR, and used without further purification. 2-methyl-1,4-naphthoquinone was obtained from Merck, Darmstadt, GFR.

[2-^{14}C]-2′-deoxycytidine was obtained from NEN, Boston, Massachussetts. The radioactive 2′-deoxyribonucleoside was purified prior to use on a Nucleosil octadecylsilyl silicagel column with water (pH 6.5) as the mobile phase to remove self-radiolysis decomposition products. [^{3}H] sodium borohydride was obtained from the Service des Molécules Marquées, Commissariat à l'Energie Atomique, Saclay, France.

B. Synthesis of [5′-^{3}H]-2′-deoxycytidine

The preparation of 5′-aldehyde-2′-deoxycytidine, a key intermediate, was made following an extension of the Moffat procedure.[8] 100 mg of 3′-0-acetyl-2′-deoxycytidine obtained by acetylation and subsequent detritylation of 5′-dimethoxytrityl-2′-deoxycytidine was dissolved in 5 ml of dimethylsulfoxide. To the resulting solution containing phosphorous pentoxide (Prolabo, Paris, France), 1 g of anhydrous N,N′-dicyclohexylcyclodiimide (Fluka, Bachs, Switzerland) was added with stirring. After 15 hours, 100 ml of 50% aqueous methanol were added to the reaction mixture. The resulting light white precipitate was removed by filtration and the solution evaporated to dryness under vacuum. The residue was dissolved in 6 ml of methanol saturated with ammonia and the resulting solution stirred overnight. The 5′-aldehyde-2′-deoxycytidine was purified by reversed phase HPLC on a Nucleosil C-18 column (0.74 × 25 cm) using water as the solvent at a flow-rate of 3 ml/min (k' = 10.4).

Reduction of the 5′-aldehyde-2′-deoxycytidine was carried out with [^{3}H] $NaBH_4$. A solution of 25 mCi of [^{3}H] $NaBH_4$ in 0.5 ml of methanol was added under stirring to a 2 ml methanol solution of the nucleoside. The reaction was complete after 1 hr and the excess of $NaBH_4$ was destroyed by addition of acetic acid. Evaporation to dryness gave an oily residue which after purification by RP-HPLC yielded 15% of [5′-^{3}H]-2′-deoxycytidine.

C. Spectroscopic measurements

Fast atom bombardment (FAB) mass spectrometry was carried out on a model MS50 spectrometer. Desorption of the molecules dissolved in a glycerol mull was effected by exposure to a beam of 8 keV xenon atoms. 250 MHz ^{1}H nuclear magnetic resonance spectra were recorded on a Bruker WM250 apparatus. Deuterium oxide was used as the solvent with 3-(trimethysilyl)proprionate-2,2,3,3-d_4 as the internal standard.

D. Irradiation procedure

Photolysis experiments were carried out with a Rayonet photochemical reactor (Southern New England Ultraviolet Co., Hamden, Connecticut, USA) using 16 RPR-3500 Å lamps, each lamp emitting about 1.5 to 5.10^6 photons $cm^{-2} s^{-1}$ of near UV light. The solution which consisted of 0.5 mM menadione and 5 mM 2'-deoxycytidine was irradiated in RQV quartz reaction vessels under oxygen bubbling. The temperature of the solution was maintained below 40°C by fan cooling.

E. Chromatographic analyses

Two dimensional thin-layer chromatographic separation of 2'-deoxycytidine photooxidation products was carried out on pre-coated silica gel 60 F_{254} plates with the two following solvent systems: 1/ lower phase of chloroform–methanol–water [4:2:1] to which was added 5% of methanol; 2/ ethyl acetate-2-propanol-water [75:16:9].

The high performance liquid chromatographic system consisted of a Waters M6000A dual piston pump (Waters Assoc., Mildford, Massachussetts, USA) and a 7125 Rheodyne loop injector. The two columns (30 × 0.75 cm) generally used were home packed with 10 μm octadecyl silica gel C-18 (Macherey-Nagel, Düren, GFR) and 10 μm Partisil silicagel phases (Whatman, Clifton, New Jersey, USA). A model R-401 differential refractometer (Waters Assoc., Mildford, Massachussetts, USA) was used as the detector.

F. Quantitative analysis of [5-^{3}H]-2-deoxy-D-ribonolactone and [2-^{14}C]-cytosine

A solution 5 mM of 2'-deoxycytidine (5.6 mg) and 0.5 mM menadione (0.4 mg) in 5 ml of methanol-water [1:1] to which was added [6-^{3}H]-2'-deoxycytidine (2 670 000 c.p.m.) and [2-^{14}C]-2'-deoxycytidine (2 250 000 c.p.m.) was irradiated for 3 hrs in the Rayonet reactor (λ_{max} = 360 nm) under oxygen saturation. After evaporation to dryness the resulting solid residue was dissolved in 1 ml of water and fractionated on the C-18 Nucleosil HPLC column using water (3 ml/min) as the solvent. The fractions eluting before 2'-deoxycytidine (k' = 16.5) and which contain [^{3}H]-2-deoxy-D-ribono-1,4-lactone and [^{14}C]-cytosine were collected and evaporated to dryness. The resulting residue to which was added cold 2-deoxy-D-ribonolactone and cytosine was further analyzed by HPLC on the Partisil silicagel column using ethyl acetate-2-propanol-water [75:16:9] as the eluent system with a flow rate of 3 ml/min. The collected [^{3}H] radioactivity for the 2-deoxy-D-ribonolactone peak (k' = 8.1) was 2250 c.p.m., representing 0.08% of the overall radioactivity. The fraction (k' = 0.79) which contains cytosine was contaminated by a relatively minor photoproduct. Purification of this fraction was achieved on the C-18 Nucleosil column using water as the solvent (*vide supra*). The [^{14}C] radioactivity (1760 c.p.m.) represents 0.08% of the overall ^{14}C activity. It should be noted that the yield of cytosine and 2-deoxy-D-ribonolactone is 4.5% of the total amount of the photoproducts. 2'-deoxyuridine and the photoproducts arising from the hydration reaction of the transient pyrimidine radical cation were generated in 12% and 83% yield respectively.

RESULTS

The near ultraviolet photolysis of an aerated aqueous solution of 5 mM 2'-deoxycytidine containing 0.5 mM menadione generated a complex mixture of degradation

products. These photoproducts which are predominantly nucleosidic (*vide infra*) have been separated either by two dimensional thin-layer chromatography on silicagel plates[9] or by high performance liquid chromatography using both normal and reversed phase columns. The characterization of the products based upon on ^{1}H NMR and FAB-MS analyses allowed us to group them into two classes according to their mechanism of formation.

Hydration of the cytosyl radical cation

Four main nucleosides which have lost the characteristic chromophore of 5,6-unsaturated 2,4-dioxopyrimidines have been identified as the four *cis* and *trans* diastereoisomers of 5,6-dihydroxy-5,6-dihydro-2′-deoxyuridine. The structural assignment made by 250 ^{1}H n.m.r. analysis was further supported by the chemical synthesis of the authentic samples through the intermediary of the two *trans* 2′-deoxyuridine bromohydrins.[10] Other important photoproducts are the nucleoside derivatives of carboxamide-α-imidazolidone and biurea. A last decomposition product whose structure is under investigation involves opening of the pyrimidine bond between positions 1 and 6. A common intermediate in the formation of these photoproducts is the 6-hydroxy-5,6-dihydro-2′-deoxycytid-5-yl radical which has been unambiguously characterized by ESR analyses of the paramagnetic adduct by spin-trapping with MNP.[7] Fast reaction of the 5-yl radical with molecular oxygen leads to the corresponding 6-hydroxy-5-hydroperoxyl radical which may undergo further reactions: reduction and subsequent protonation or dismutation followed by β cleavage of the resulting oxyl radicals.[11]

Deprotonation reaction

Emphasis has been placed on the isolation and the identification of the photoproducts which arise from the competitive deprotonation reaction of the initially produced pyrimidine radical cation. The main far ultraviolet absorbing photoproduct has been identified as 2′-deoxyuridine by comparison of its chromatographic properties and ^{1}H n.m.r. and FAB-MS spectroscopic features with those of the authentic sample. It is interesting to note that this nucleoside which is likely to arise from initial deprotonation of the pyrimidine moiety within the amino group is resistant to menadione mediated photooxidation. A second relatively minor photoproduct which also exhibits the characteristic ultraviolet absorption of 5,6-unsaturated 2,4-dioxo or 2-oxo-4-amino pyrimidines has been identified as free cytosine. In addition it should be noted that 2-deoxy-D-ribono-1,4-lactone which has been shown to be a radiation-induced degradation product of thymidine[12] and 2′-deoxyguanosine[13,14] has been also isolated and characterized on the basis of its characteristic ^{1}H n.m.r. features.[13] It has also been shown that the menadione mediated photooxidation of 2′-deoxycytidine labelled with [^{3}H] within the osidic moiety at the 5′ position and with [^{14}C] in the pyrimidine ring at the carbon C-2 gave rise to the stoichiometric formation of both [5-^{3}H]-2-deoxy-D-ribonolactone and [2-^{14}C]-cytosine. The presence of 50% methanol in the photolyzed solution rules out the possible involvement of the hydroxyl radical in the hydrogen abstraction at the carbon C(1′) of 2′-deoxycytidine which leads to the oxidation of the sugar moiety with concomitant release of the attendant pyrimidine base. It is also interesting to note that the release of free thymine and the formation of 2-deoxy-D-ribono-1,4-lactone were not observed when thymidine which is an

excellent substrate for the menadione photosensitization reaction (2) was used as the substrate. Altogether these various observations suggest that the release of free cytosine and the oxidation of the residue carbon C-1′ of 2′-deoxycytidine result from a common process which involves the deprotonation at position 1′ of the photoinduced cytosyl radical cation.

DISCUSSION

Menadione appears to be efficient in inducing photooxidation of 2′-deoxycytidine when exposed to near ultraviolet light in aerated aqueous methanol solution. It is likely, on the basis of the results of earlier flash photolysis experiments, that efficient electron transfer reaction occurs between various pyrimidine nucleic acid components and menadione in a triplet excited state. Further support for the involvement of a transient pyrimidine radical cation as a key intermediate in these photoreactions was provided by the characterization of the final photoproducts of thymine,[1] thymidine[2] and 2′-deoxycytidine (*vide supra*). A common process for the hydration reaction of the above radical pyrimidine radical cations is the formation of the corresponding 6-hydroxy-5-yl radicals (Figure 1). Similar reactions have been observed when pyrimidine nucleobases or nucleosides were exposed to the direct effects of ionizing radiations in frozen aqueous solutions and subsequent annealing.[4] On the other hand, the competitive deprotonation reaction of the initially generated radical cation is different for each of the three pyrimidines already studied. The deprotonation of the thymine radical cation was shown to occur at position 1 giving rise to the formation of addition products.[1] This reaction is prevented in thymidine due to the presence of a deoxyribose substituent at N − 1 position, the loss of the proton taking place at the methyl group.[2] The yield of the deprotonation reaction of the 2′-deoxycytidine radical cation ($\simeq 20\%$) is lower than those of thymine and thymidine ($\simeq 40\%$). One reaction concerns the amino group with subsequent thawing whereas the minor process

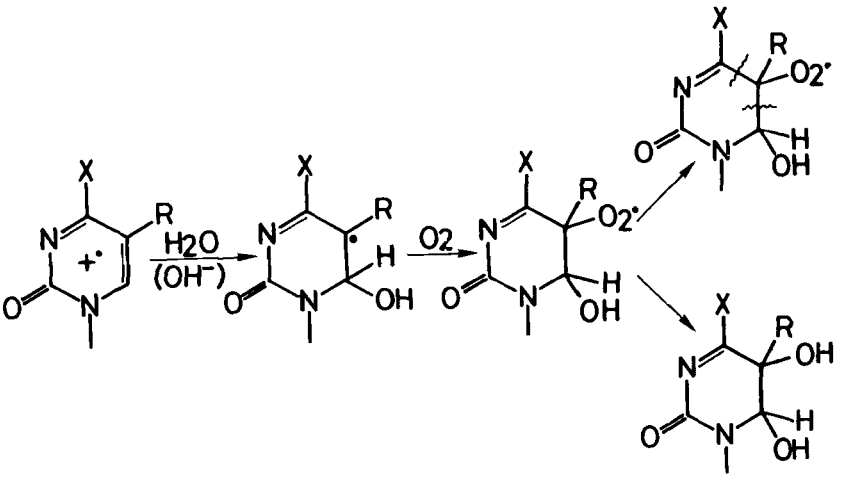

FIGURE 1 Specific hydration reaction of various pyrimidine radical cations with subsequent oxygen fixation and reaction of the resulting peroxyl radicals ($X = NH_2$, $R = H$: cytosine; $X = OH$, $R = CH_3$: thymine, thymidine).

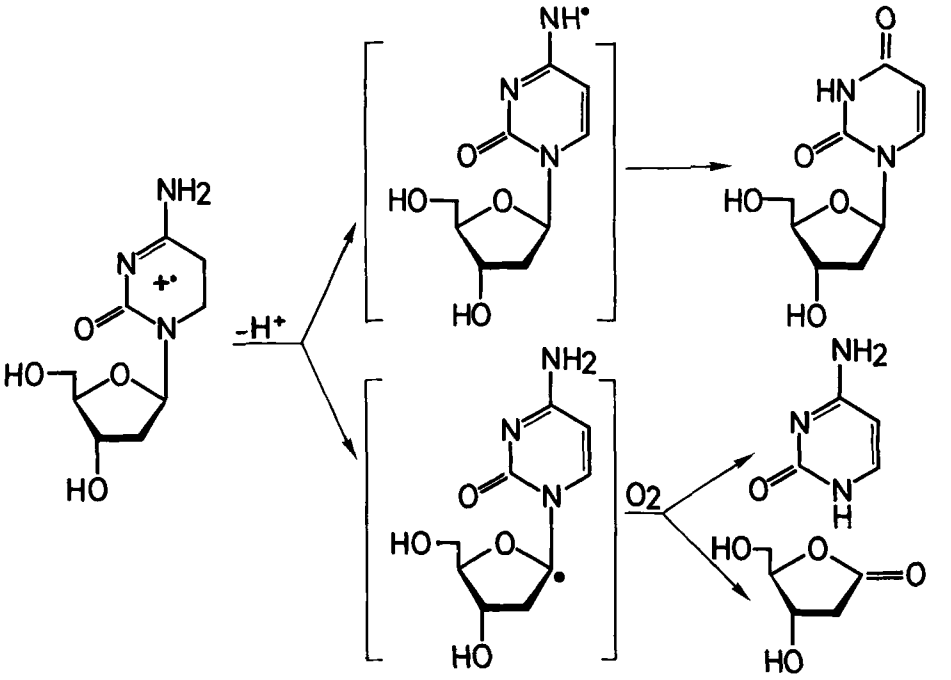

FIGURE 2 Deprotonation reactions of the menadione photoinduced 2′-deoxycytidine radical cation.

involves the deprotonation of carbon C(1′) (Figure 2). It is interesting to note that it has been proposed that the π-cation derived from N – 1 substituted cytosine derivatives following exposure to the direct effects of ionizing radiation[4] or as the result of photoionization[15] is expected to deprotonate within the exocyclic amino group and at the position 1′. The rupture of the N-glycosidic which accompanies the deprotonation at carbon C-1′ of 2′-deoxycytidine constitutes to our knowledge the first example of an intramolecular reaction within a nucleoside initiated by a pyrimidine radical. Work is in progress to determine whether the menadione photosensitization reaction may lead to a specific release of the cytosine base with concomitant formation of an alkali-labile site on the corresponding sugar moiety when DNA is used as the substrate.

The menadione photooxidation of thymidine and 2′-deoxycytidine may also be used as a reaction model for investigating the direct effects of ionizing radiation[4] and high powerful laser pulses[16–18] on these pyrimidine nucleosides. Recent experiments have shown that picosecond laser photolysis (λ = 266 nm) of thymidine generated similar degradation photoproducts to those formed by photosensitization as the result of a biphotonic process resulting in photoionization of the pyrimidine moiety.[18]

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