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New hydrolytic products detected in aqueous solutions of bromodeoxyuridine

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Abstract

The aqueous solution stability of 5-bromo-2′-deoxyuridine (BUdR) has been extensively investigated. Only a few hydrolysis products have been identified: 5-bromouracil (BU), 5-hydroxy-2′-deoxyuridine (HUdR) and 5-methyl-3(2H)-furanone. The last is an acidic conversion product of 2-deoxyribose. Another major non-UV-absorbing product, resulting from basic degradation of the uracil ring, has been mentioned but not identified. This paper reports the detection and identification of several more hydrolysis products, including the non-UV-absorbing chemical N-ureido-2′-deoxyriboside (ureadR). In the acid hydrolyzate, a major amount of BU and minor amounts of uracil and ureadR were detected. In base, ureadR was the major product. In addition, minor amounts of BU, HUdR, uracil, deoxyuridine, 6-hydroxyuracil and 6-hydroxydeoxyuridine were observed. Based on these products, a new hydrolytic pathway of BUdR in aqueous solutions is proposed. © 1998 Elsevier Science B.V.

Keywords: Bromodeoxyuridine; Hydroxydeoxyuridine; Ureidodeoxyriboside; Nucleosides

1. Introduction

5-Bromo-2′-deoxyuridine (BUdR) has a wide range of biological activities. It enhances the radiosensitivity of cells [1] and has been employed in studies of DNA replication [2] and electron transfer during radiolysis of nucleosides and nucleotides [3]. Clinically, it has been used as a radiation sensitizer in the treatment of cancer [4–6]. Its utility as a diagnostic tool in biology and medicine has been documented in a recent review [7]. Stability of BUdR in aqueous solutions has been studied extensively [8–12]. Most of the studies were monitored by UV [9–12] with the detection and identification of the final products achieved by thin-layer chromatography (TLC) [10,11]. They yielded little information on the identities of the hydrolysis products except 5-bromouracil (BU). van Schepdael et al. [8] followed the hydrolysis with the more specific and sensitive high-performance liquid chromatography (HPLC). In acidic and neutral aqueous solutions, BU was the reported product. In acid, a UV-absorbing product from 2-deoxyribose was also detected [13]. In alkali solutions, nearly complete decomposition of BUdR to non-UV-absorbing unknown(s) was observed [8,10]. Because of the chemical similarity of BUdR to the natural nucleoside thymidine and the importance of BUdR as a biological tool and chemotherapeutic agent, it is desirable to identify all key hydrolysis products of BUdR in order to more fully understand the hydrolysis pathway of BUdR and, perhaps, of thymidine.

This paper presents several new BUdR hydrolysis products, which have not been previously identified, including the major non-UV-absorbing product resulting from alkaline decomposition. The hydrolysis

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products were detected by HPLC with UV detection at 205 and 254 nm. They were identified by UV and mass spectral data analysis, and HPLC co-elution with authentic chemicals. Based on the appearance of these products, a new hydrolysis pathway of BUdR is presented.

2. Experimental

2.1. Reagents

5-Bromo-2′-deoxyuridine, 2-deoxyribose, urea and barbituric acid were obtained from Aldrich (Milwaukee, MI, USA), 5-bromouracil was from Krishell (Portland, OR, USA), and uracil was from Eastman (Rochester, NY, USA). Reagent-grade NaH_2PO_4 and HPLC-grade MeOH were purchased from Mallinckrodt (Paris, KY, USA). Solutions of 0.1 *M* HCl and NaOH were prepared from Dilute-it Analytical Concentrate from J.T. Baker (Phillipsburg, NJ, USA) with deionized water.

2.2. Hydrolysis

Stock solutions of BUdR (10 mg ml^{-1}) in water, 0.1 *M* HCl and 0.1 *M* NaOH were prepared in deionized water. From each stock ten 1-ml aliquots were individually transferred to separate 1-dram vials (VWR Scientific, San Francisco, CA, USA) and capped tightly. One vial from each stock was immediately put in a freezer. Three vials each were stored on the bench top (25°C), in a 50°C and in a 80°C heating block (H2025 Temp-Blok, Scientific Products, McGaw Park, IL, USA). At the end of the second, sixth and ninth days of storage, one vial of each stock was removed from each storage condition and transferred to the freezer. When ready for HPLC analysis, the vials were removed from the freezer and thawed. The content of each vial was mixed with 1.0 ml of MeOH, from which a 0.1-ml aliquot was individually diluted with 0.5 ml water to form the test solutions before HPLC analysis.

2.3. High-performance liquid chromatography

The HPLC system consisted of a Varian (Walnut Creek, CA, USA) 9010 pump, Varian 9050 UV–Vis detector, a Varian 9065 photodiode array detector (PAD), a Varian 9095 autosampler equipped with a 10 μl loop. Data were collected and analyzed with a Varian LC STAR workstation. Liquid chromatography–mass spectrometry (LC–MS) was performed with a Vestec 201 XL mass spectrometer equipped with an LC thermospray interface (Houston, TX, USA).

Test solutions (10 μl) were automatically injected onto a Phenomenex (Torrance, CA, USA) Spherisorb ODS (1), 250×4.6 mm I.D., 5 μm stainless-steel column. Chromatographic analysis was carried out by isocratic elution, with a 0.01 *M* KH_2PO_4 buffer (pH 4.8) containing 10% MeOH, at 1.0 ml min^{-1}. Detection was by UV at 205 nm, 254 nm, photodiode array detection (PAD) or LC–MS.

3. Results

3.1. Hydrolysis

Figs. 1–3 present the HPLC profiles of, respectively, the water, 0.1 *M* HCl and 0.1 *M* NaOH solutions of BUdR stored at different temperatures for up to nine days. The results are similar to reported findings [8–12]. BUdR is fairly stable in water and acidic solutions, except at elevated temperature. When the water solution was heated at 50°C (Fig. 1c and d), slight decrease in BUdR (1) was accompanied by a gradual and small increase of 2 and 3 plus the tiny peaks 4 and 6. At 80°C heating (Fig. 1e and f), 1 was completely converted to 2 and small peaks 3–7. The hydrolysis of 1 in 0.1 *M* HCl followed a similar but slightly faster pattern than in water (Fig. 2). The very minor decomposition peaks 4, 6 and 7 were replaced by small peaks 8–10. Peak 10 disappeared on extended heating. In 0.1 *M* NaOH, a noticeable amount of 3 was generated, even at room temperature (Fig. 3a and b). Heating the solution resulted in a drastic decrease of 1 with concomitant increase of 3 and several minor to trace peaks (2, 6, 9, 11 and 12, Fig. 3c–f).

3.2. Identity of hydrolysis products

Identities of the hydrolysis products 2–13 were elucidated with the aids of their UV spectra, mass

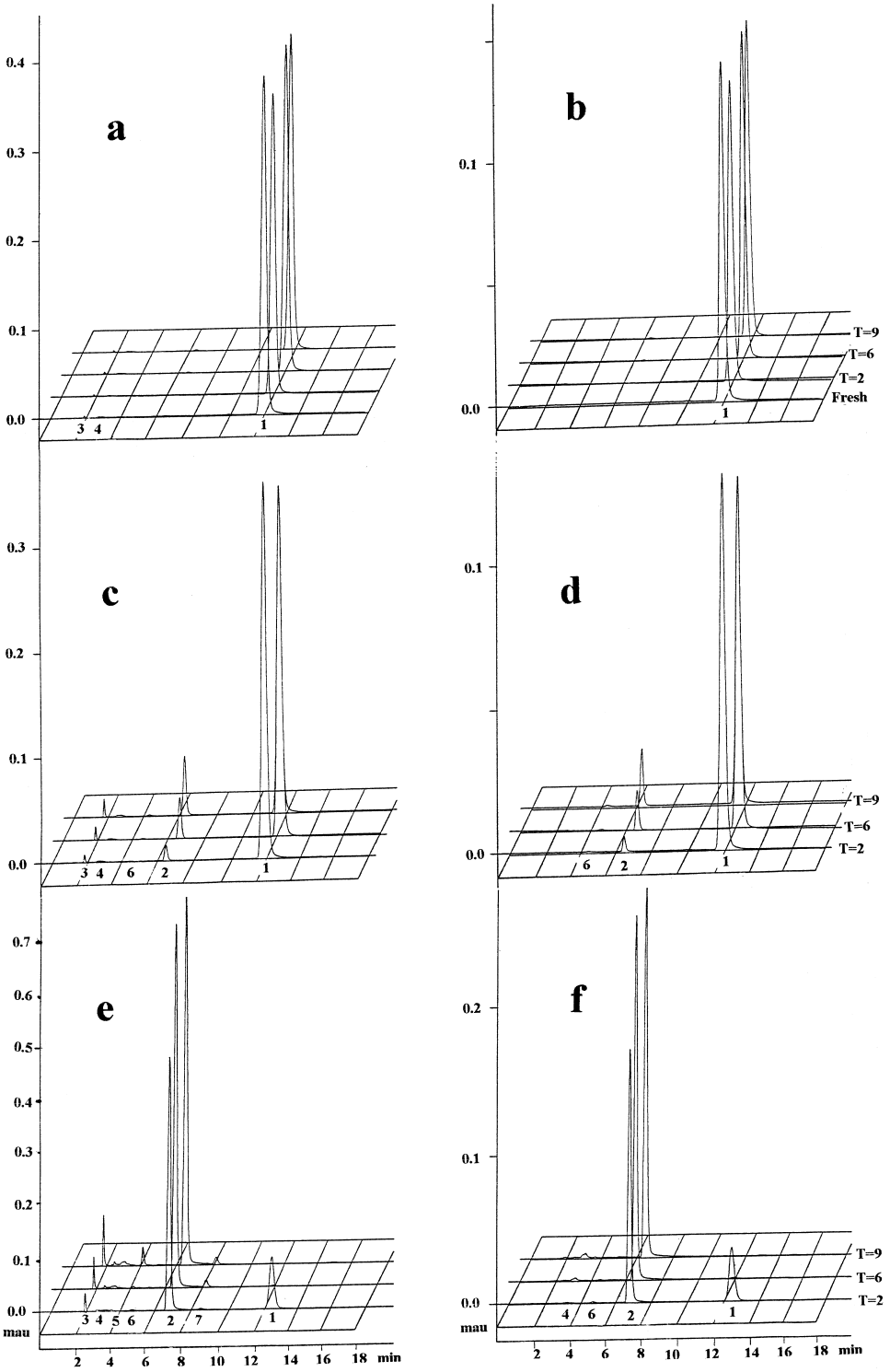

Fig. 1. HPLC of BUdR in water (10 mg ml^{-1}) stored at 25°C (a, b), 50°C (c, d) and 80°C (e, f). Left figures (a, c, e) are from 205 nm detection and right figures (b, d, f) are from 254 nm detection. *T* is the number of days in storage. See Section 2 for details.

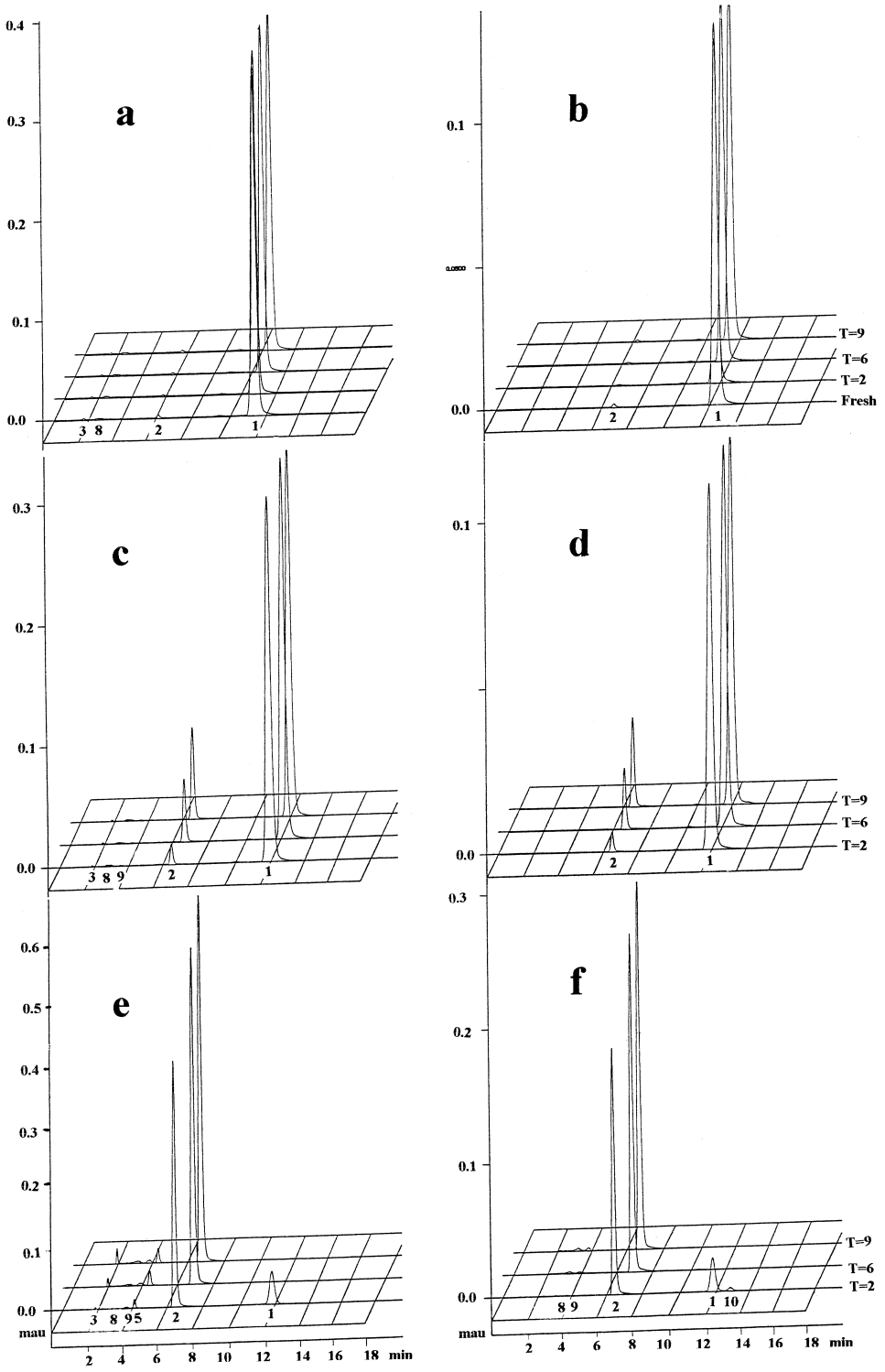

Fig. 2. HPLC of BUdR in 0.1 *M* HCl (10 mg ml^{-1}) stored at 25°C (a, b), 50°C (c, d) and 80°C (e, f). Left figures (a, c, e) are from 205 nm detection and right figures (b, d, f) are from 254 nm detection. *T* is the number of days in storage. See Section 2 for details.

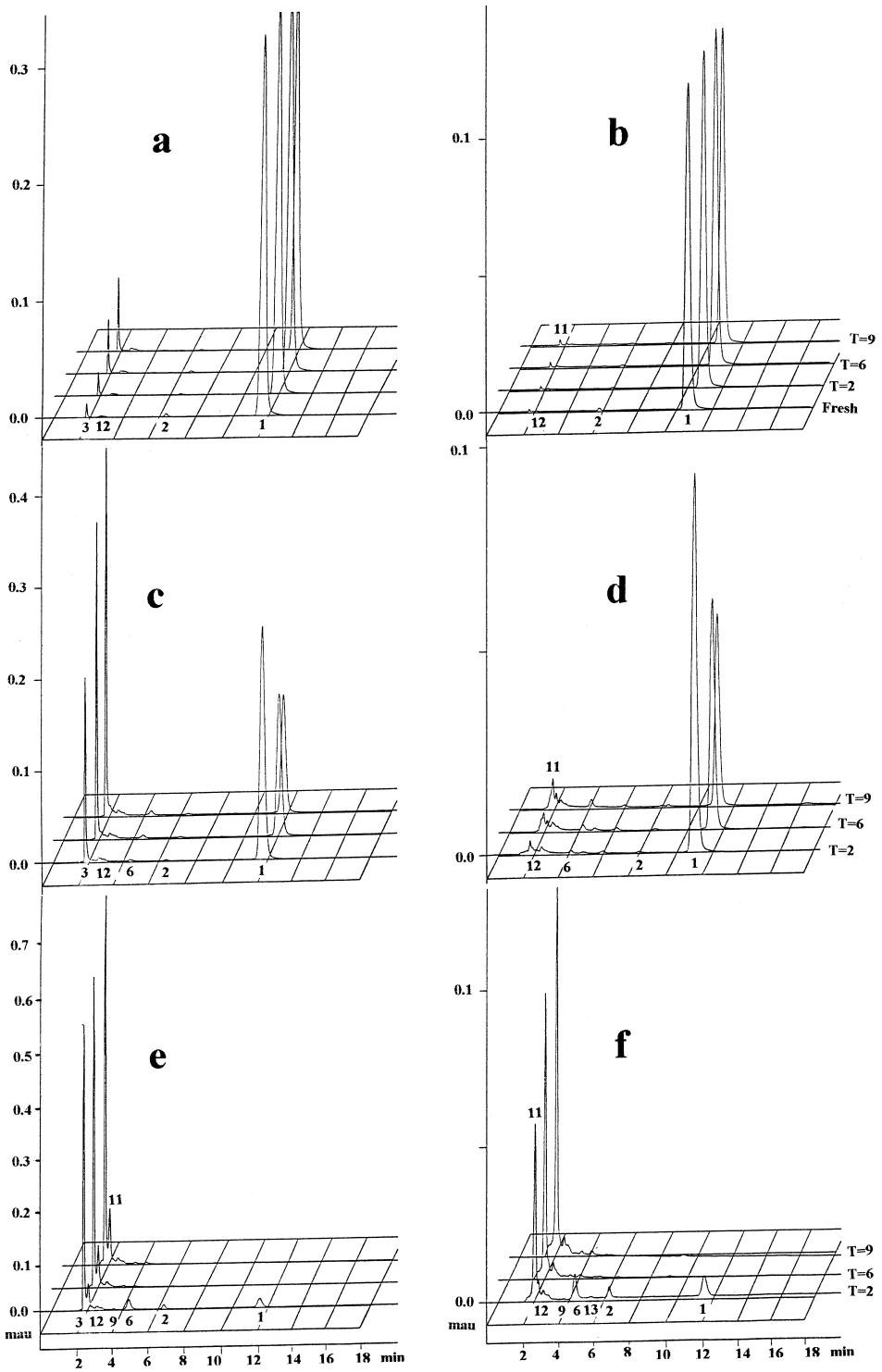

Fig. 3. HPLC of BUdR in 0.1 *M* NaOH (10 mg ml^{-1}) stored at 25°C (a, b), 50°C (c, d) and 80°C (e, f). Left figures (a, c, e) are from 205 nm detection and right figures (b, d, f) are from 254 nm detection. *T* is the number of days in solution. See Section 2 for details.

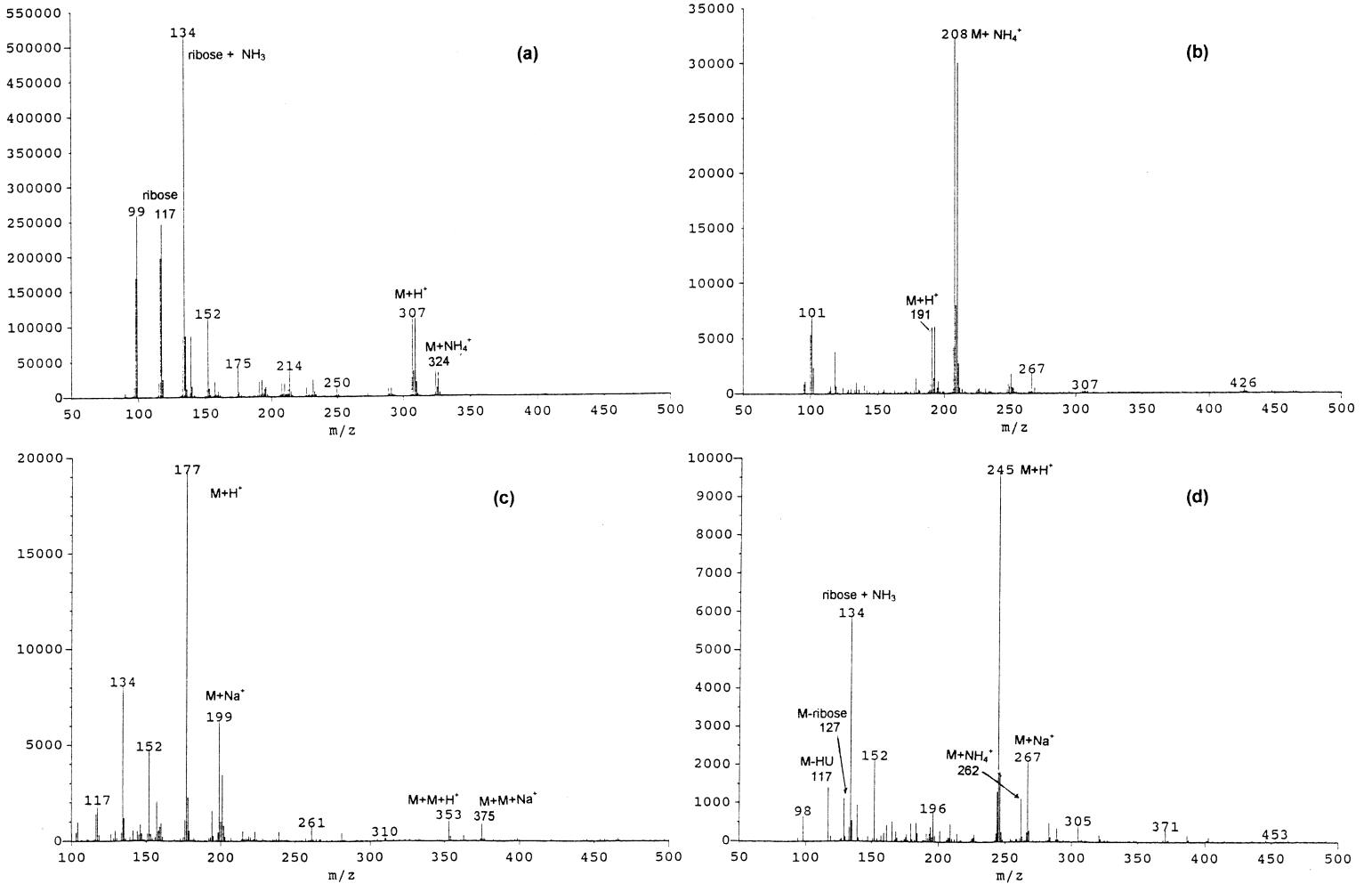

Fig. 4. Mass spectra of BUdR and its hydrolytic products. (a) 1 BUdR, (b) 2 BU, (c) 3 ureadR and (d) 6 HUdR. Mass spectra were obtained with LC–MS.

spectral data and enhancement of HPLC peaks with authentic chemicals. Many of the minor products were either not sensitive or not well separated enough to yield useful MS. As a consequence, meaningful mass spectral data were obtainable only for 1, 2, 3 and 6 which are presented in Fig. 4. Table 1 lists the UV properties and identities of 1–13 detected in Figs. 1–3. The UV spectra, obtained with PAD during HPLC, are presented in Fig. 5. Spectra for 7, 12 and 13 are very weak and could have been distorted, particularly in the short-wavelength region. Based on their UV profiles, 2–13 can be grouped into four types. Group I products (2, 4, 6, 7), with UV spectra similar to that of BUdR (1) having absorption maxima/minimum (max/min) at about 280/242 nm, retain the intact mono-substituted uracil structure. Group II members (9, 13) have the UV profile of uracil (U) with max/min at 258/228 nm and are probably the debrominated products. UV spectra of group III products (11, 12), with max/min at around 252/226 nm, suggest they are associated with barbituric acid (6-hydroxyuracil). Group IV (3, 5, 8, 10), which have either end absorption (<200 nm, 3 and 8) or single absorption maximum (5, 10), bear no resemblance to the uracil structure.

Based on their mass spectral data, the group I products 2 and 6 are, respectively, 5-bromouracil (m/z: 208/210=M·NH_4, 191/193=M·H) and hydroxydeoxyuridine (m/z: 245=M·H, 134=ribose·NH_3, 127=M−ribose, 117=M−hydroxyuracil). The absorption maximum of 6 is consistent with the reported value for 5-hydroxy-2′-deoxyuridine (279 nm [11,14]) but different from that of 6-hydroxyuracil or barbituric acid (255 nm [15]). Therefore, 6 is 5-hydroxy-2′-deoxyuridine (HUdR). Since 4 has UV profile identical to that of 6 but is less retained than 6 during HPLC, analogous to the relative retention of 2 and 1, 4 is probably 5-hydroxyuracil (HU). Though the max/min wavelengths of 7 are similar to the rest of this group, the absorption ratio of 214 nm/280 nm, which is about eight, is much larger than those of the rest in this group. The relatively strong HPLC retention of 7 suggests that the lipophilic BU moiety is still present. The strong absorption at 214 nm may have resulted from decomposition in the deoxyribose moiety. However, the identity of 7 remains to be elucidated.

In the group II products, 9 has been identified as uracil (U) by its UV data and by peak enhancement. The similarity of the UV spectra and the relative HPLC retention between U and 13 suggest that the latter is 2′-deoxyuridine (UdR). Similarly, the structures of 11 and 12 in group III are assigned 6-hydroxyuracil (barbituric acid, Ba) and 6-hydroxy-2′-deoxyuridine (BadR), respectively.

UV of the group IV products, which bear no resemblance to those of uracil or substituted derivatives, suggest that the products do not contain intact uracil moiety. 2-Deoxyribose is not detectable by UV at wavelength>195 nm, even for a large quanti-

Table 1
HPLC retention, UV maximum and minimun and proposed identities for BUdR and hydrolysis products shown in Figs. 1–3

Peak	t_R (min)	λ_{max} (min) (nm)	Identity	Detected in
1	12.5	209 (242) 278	BUdR	Figs. 1–3
2	7.0	208 (238) 275	BU	Figs. 1–3
3	2.4	196 sh	ureadR	Figs. 1–3
4	3.6	208 (240) 278	HU	Fig. 1
5	4.7	210	unknown	Figs. 1 and 2
6	5.2	207 (244) 279	HUdR	Figs. 1 and 3
7	8.7	214 (242) 280	unknown	Fig. 1
8	3.4	<190	unknown	Fig. 2
9	4.1	200 (226) 259	U	Figs. 2 and 3
10	13.3	261	ddR	Fig. 2
11	2.6	195 sh (226) 252	Ba	Fig. 3
12	3.1	200 (224) 255	BadR	Fig. 3
13	5.9	200 (228) 260	UdR	Fig. 3

t_R (retention time) is representative from Figs. 1–3. UV data were obtained by PAD. See Section 3.2 for identity. sh=Shoulder.

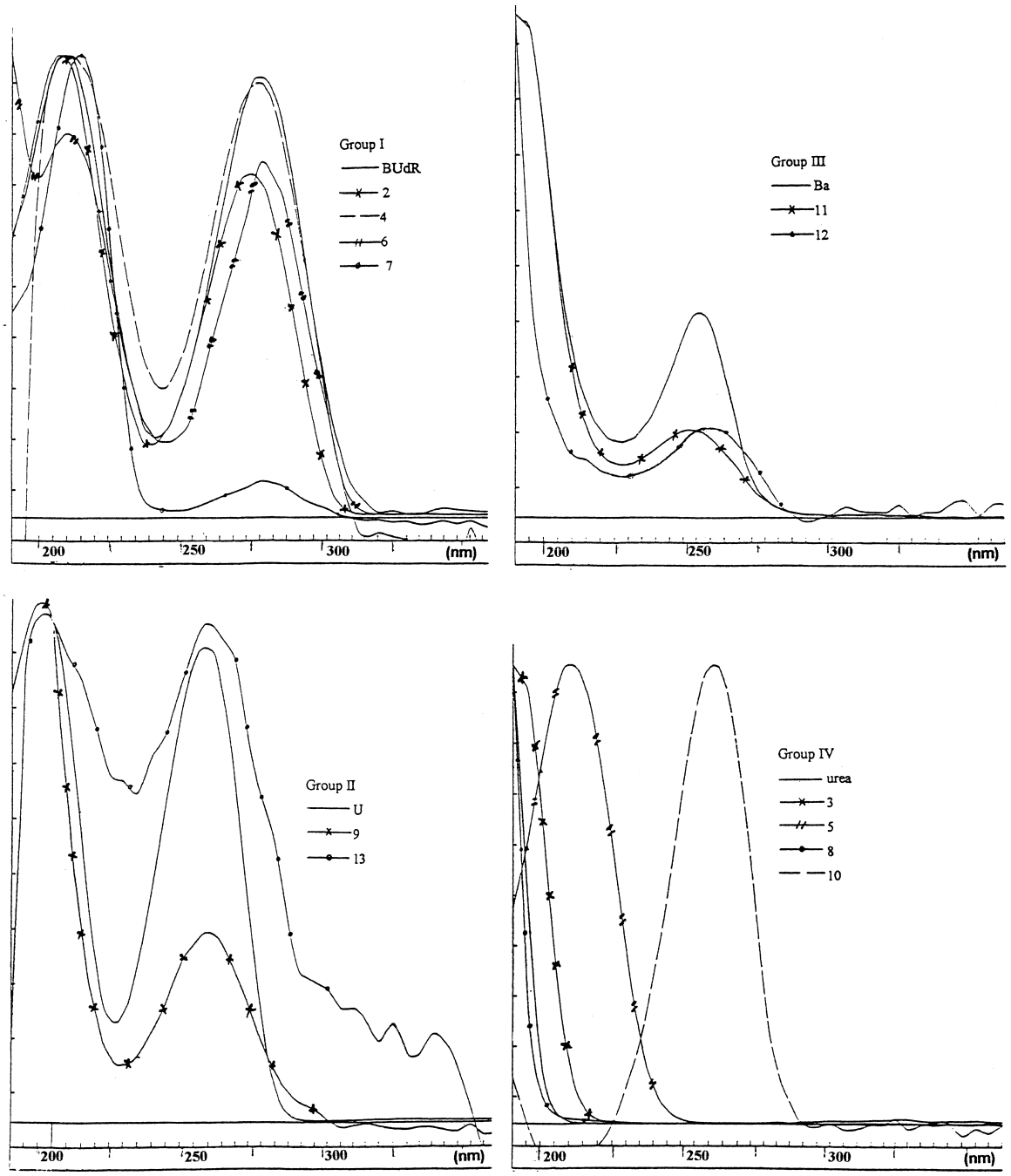

Fig. 5. UV spectra of BUdR and its hydrolysis products. (a) Group I and BUdR, (b) group II and uracil, (c) group III and barbituric acid co-injected with hydrolyzate and (d) group IV and urea. UV obtained and normalized with PAD.

ty, and therefore cannot account for any of the observed hydrolysis products. The major base product 3 is easily detected at 205 nm but not at 254 nm. Though the retention time of 3, slightly ahead of the unretained, bears resemblance to that of urea, their UV are different (Fig. 4, group IV). Product 3 is identified as N-ureido-2′-deoxyriboside, ureadR, by its MS data (m/z: 177=M·H, 160=M−NH_2, 134=M−$CONH_2$+2H or ribose·NH_3, 117=M−$NHCONH_2$). The UV spectrum and HPLC retention of 10 are indistinguishable from an UV-absorbing product generated by heating a 1 *M* HCl solution of 2-deoxyribose. This product has been identified as 5-methyl-3(2H)-furanone (ddR) by Seydel et al. [13]. The identities of 5 and 8 remain unknown.

4. Discussion

The stability of BUdR in aqueous solutions has been well documented [8–12]. Most of the studies were monitored with UV spectroscopy [9,11,12] and yielded little information on the identity of the hydrolysis products. Sano et al. [10], using TLC, detected and identified BU and 2-deoxyribose (dR) as the products when the hydrolysis was carried out at pH<7. At pH 11, only unknown non-UV-absorbing products were observed. Using the more selective and sensitive HPLC, van Schepdael et al. [8] showed that BU was the hydrolytic product of BUdR under acidic pH, though generation of ddR was implied. In alkaline, only small amounts of BU, HU and HUdR were identified as products. Mass balance calculation indicated that the major decomposition in base was non-UV-absorbing unknown(s). Using UV detection at 254 and 205 nm, we detected several additional decomposition products. Most of the products were detectable at both wavelengths. A few were detected by either 205 or 254 nm only. In 0.1 *M* HCl (Fig. 2), BUdR (1) was hydrolyzed primarily to the glycolysis product BU (2) and small amounts of ureadR (3), U (9), ddR (10) and unknowns 5 and 8. At neutral pH (Fig. 1), the hydrolysis took a path similar to that in acid with 2 being the major product. Increased amounts of ureadR and 5 were detected. Instead of 8, U and ddR, small amounts of 5-hydroxylated products HU (4) and HUdR (6) were observed. In 0.1 *M* NaOH (Fig. 3), the major product was the non-UV-absorbing ureadR and minor to trace amounts of hydroxylated or debrominated products—Ba (11), BadR (12), HUdR and UdR (13), though small amounts of glycolysis product BU were also present. The insignificant generation of BU indicates that the glycosidic bond of BUdR is fairly stable in basic solutions. Glycosidic bonds of 5- or 6-hydroxydeoxyuridines (6, 12) appear less stable resulting in the formation of hydroxyuracils (4, 11).

The above suggests that hydrolysis of BUdR proceeds in three pathways, debromination (A), glycolysis (B), and addition of water across the 5,6 double bond (C), as depicted in Fig. 6. Path A is insignificant in the hydrolysis of BUdR. At pH below the pK_a of BUdR (8.3 [10]), B is the dominant pathway which results in the generation of BU followed by a tiny amount of debromination to give rise to U. At pH higher than 8.3, ionization of the BU moiety promotes the addition of water across the C_5=C_6 bond (path C). Removal of HBr from the resulted intermediate 14 generates HUdR and BadR which in turn are hydrated to intermediate 15, deoxyuridine glycol (5,6-dihydroxy-2′-deoxyuridine). It has been well documented that the uracil ring is unstable when its 5,6 double bond is saturated. It opens and degrades to urea derivatives as indicated by the instability of dihydrouridine [16], thymine glycol [17] and thymidine glycol [17,18]. Thymidine glycol has been shown to convert to ureadR (3) quantitatively when incubated in alkali [17]. Similarly, the deoxyuridine glycol 15 and the intermediate 14 in the BUdR solutions decompose to ureadR (3). This probably accounts for the reported instability of HUdR in alkali [14,18].

Thymidine glycol and urea residue have been generated from thymidine residues in DNA by ionization radiation and oxidative stress [17–21]. They are inhibitive to DNA synthesis by blocking DNA polymerase activity. Though thymidine was very stable to neutral hydrolysis [9], the result may be unreliable since it was obtained by UV spectrophotometry where degradation of the uracil ring was not fully considered. Therefore, with the new information and the more selective and sensitive HPLC method, solution stability study of thymidine and other DNA nucleosides at physiological pH should be revisited.

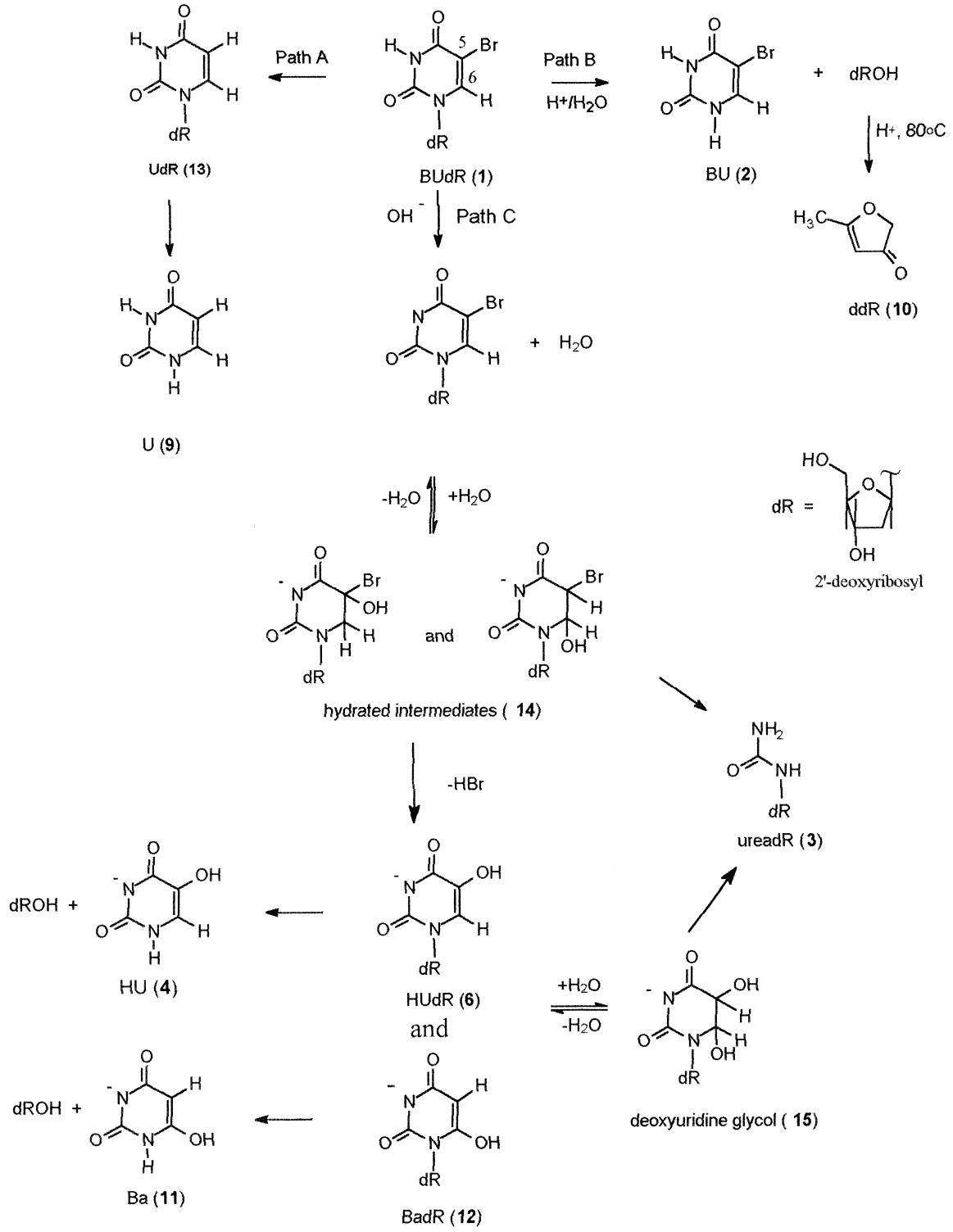

Fig. 6. Proposed hydrolysis pathway of BUdR.

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