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Sonochemical transformation of thymidine: A mass spectrometric study

Jisha Chandran [a], Usha K. Aravind [b], C.T. Aravindakumar [a,c,*]

[a] Inter University Instrumentation Centre, Mahatma Gandhi University, Kottayam 686560, Kerala, India
[b] Advanced Centre of Environmental Science and Sustainable Development, Mahatma Gandhi University, Kottayam 686560, Kerala, India
[c] School of Environmental Sciences, Mahatma Gandhi University, Kottayam 686560, Kerala, India

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ABSTRACT

Ultrasound is extensively used in medical field for a number of applications including targeted killing of cancer cells. DNA is one of the most susceptible entities in any kind of free radical induced reactions in living systems. In the present work, the transformation of thymidine (dT) induced by ultrasound (US) was investigated using high resolution mass spectrometry (LC–Q-ToF–MS). dT was subjected to sonolysis under four different frequencies (200, 350, 620 and 1000 kHz) and at three power densities (10.5, 24.5 and 42 W/mL) in aerated as well as argon saturated conditions. A total of twenty modified nucleosides including non-fully characterized dT dimeric compounds were detected by LC–Q-ToF–MS. Out of these products, seven were obtained only in the argon atmosphere and two only in the aerated conditions. Among the identified products, there were base modified products and sugar modified products. The products were formed by the reaction of hydroxyl radical and hydrogen atom. Under aerated conditions, the reactions proceed via the formation of hydroperoxides, while in argon atmosphere disproportionation and radical recombinations predominate. The study provides a complete picture of sonochemical transformation pathways of dT which has relevance in DNA damage under ultrasound exposure.

1. Introduction

The reactive oxygen species (ROS) induced modification of DNA is a much investigated topic of research because of its important physiological as well as pathological consequences. Hydroxyl radical (·OH) is one of the major ROS formed in the body by transition metal mediated Fenton reactions and on exposure to ionizing radiation, causing oxidative degradation of cellular DNA [1,2]. It is capable of producing double and single strand breaks, abasic sites and modified bases which if left unrepaired accumulate in cells and leads to mutations and cancer [3,4]. The analysis of the initially formed transient species and end products in the DNA constituent level helps to elucidate the otherwise complex mechanism of interaction of DNA with the ROS [5–7]. A wealth of knowledge is available in literature on the transformation of the various nitrogen bases, nucleosides and nucleotides under gamma radiation, UV radiation and photosensitized electron transfer and Fenton reaction [2,7–11].

It is known that exposure to low frequency high power ultra sound (US) causes oxidative stress on the cells due to the production of ·OH [12]. It is substantiated by the decrease in the glutathione content after US exposure. Ultrasound is extensively used in medical field as an imaging technique, for bone healing, disruption of kidney stone, and targeted killing of cancer cells [13]. Even the diagnostic US, which is pulsed and high frequency, is found to produce single strand breaks in human leukocytes *in vitro* [13]. Identification of these lesions is important as most of them have great biological implications due to their lethal, mutagenic and genotoxic effects.

The physical or chemical activity of US is due to a phenomenon called acoustic cavitation which is the formation, growth and collapse of gas filled bubbles when it is passing through liquids. High temperature and pressure are created by the final collapse of the bubble. Thermolysis of water produces the hydrogen atom (H·) and highly oxidizing ·OH [14]. There are studies showing evidence for the production of ·OH in medical US using terephthalate dosimeter and degradation of polymers [15]. All these evoke the interest on the effect of US on biomolecules especially DNA as it is an important target for these reactive species [16].

The effect of US on DNA was reported *in vitro* and by taking nitrogen bases as model compounds [17,18]. Among the bases, thymine was found to be the most reactive [17]. The end products of the three pyrimidine bases on sonolysis were documented by TLC and GC–MS analyses and it was found that the products obtained were identical to that obtained by radiolysis of their

* Corresponding author at: Inter University Instrumentation Centre, Mahatma Gandhi University, Kottayam 686560, Kerala, India. Fax: +91 481 2731009.
E-mail address: cta@mgu.ac.in (C.T. Aravindakumar).

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aqueous solutions [19–21]. Fuciarelli et al. reported several base lesions in DNA solution by the cavitational activity of ultrasound at a frequency of 2.17 MHz [18]. Studies have also been done on the sonochemical activity of thymine over a range of US intensities which also help to understand the kinetics of the reaction [22]. Even though there are many studies on the sonochemically modified nitrogen bases, sonochemical transformation studies on nucleosides would give a better understanding of the mechanistic aspects of effect of US on cellular DNA. In the present study, we investigated the effect of US on thymidine (dT), a relevant nucleoside used as a target of various ROS generating systems [1,10]. The variation of the sonochemical activity of dT with US frequency at three different power densities is explained along with the influence of argon on sonolysis. Moreover, the study is a reinvestigation of the free radical induced transformation products of dT using high resolution mass spectrometry. Argon atmosphere simulates the hypoxic conditions in cells and hence details of the products in its presence would be highly beneficial. The accurate mass measurement using high resolution mass spectrometer as well as the MS/MS facility helps to get an insight into the structure [23].

2. Materials and methods

2.1. Sonolysis of dT

dT was purchased from Pharma Waldhof and used as such. All solutions were prepared in water purified by a Cascada™ Lab Water Systems and of resistivity 18.2 MΩ cm. Solutions of dT (10^{-4} mol/L, 250 mL) was subjected to sonolysis at four different frequencies (200, 350, 620 and 1000 kHz) at three different power densities, 10.5, 24.5 and 42 W/mL for 2 h. Two frequencies (620 kHz and 1 MHz), among the four studied, fall under the range used for the surgical non disruptive applications. The sonolysis was carried out in a glass reactor with an L3 ELAC Nautik ultrasound generator powered by an Allied Signal R/F generator (T & C power conversion, Model AG 1006). For the experiments under argon saturated atmosphere, the solution was bubbled with argon gas for half an hour at a pressure of 5 psi prior to sonolysis and Ar was bubbled throughout the experiment. Sonolysis was carried out only at a power density of 42 W/mL under argon atmosphere, where there was maximum degradation in the case of aerated conditions. Two transducers were used with resonances 212 and 620 kHz and 350 and 1000 kHz respectively. The temperature was maintained at 25 ± 1 °C.

2.2. HPLC analysis

The percentage degradation of dT on sonolysis was monitored by HPLC (Shimadzu prominence UFLC, LC 20 AD) connected with a diode array detector (SPD-M20 A). An Enable C18 (25 cm × 4.5 mm × 5 μm) column was used at a flow rate of 1 mL/min. The sonolysed mixture was eluted isocratically with water and methanol (95:5) as mobile phases.

2.3. Analysis of end products by LC–Q-ToF–MS

The end products were analyzed by Waters Xevo G2 QToF with an electrospray ionization (ESI) source. It was coupled to Acquity H class UPLC with BEH C18 (50 mm × 2.1 mm × 1.7 μm) column and Acquity TUV detector. The instrument was tuned with the parent compound, dT. The ionization conditions are as follows: capillary voltage: 2.5 V, sampling cone voltage: 30 V and extraction cone voltage: 4 V. The source and desolvation temperatures were kept at 135 °C and 350 °C respectively. Nitrogen was used as the desolvation and nebulizing gas at a flow rate of 50 and 900 L/h, respectively. For the mass spectrometric analyses, the dT was sonolysed for 1 h under normal conditions and for 30 min. in the case of argon saturated conditions at a power density of 42 W/mL. The modified nucleosides were eluted isocratically with 0.1% formic acid in water and methanol (95:5) and detected in negative mode of ionization. For mass spectrometric analyses dT samples were sonolysed for 60 min. at frequency 620 kHz and power density 42 W/mL.

3. Results and discussion

3.1. Effect of frequency and power on the transformation

The dT solutions (10^{-4} mol/L) were subjected to four US frequencies (200, 350, 620 and 1000 kHz) at three different power densities (10.5, 24.5 and 42 W/mL) under aerated conditions. The degradation profile is given in Fig. 1. At a power density of 10.4 W/mL the degradation of dT was higher at a frequency of 350 kHz, but as power increases to 24.5 W/mL the degradation at 350 and 620 kHz became comparable. At 42 W/mL, the degradation was found to be almost equal for 350, 620 and 1000 kHz. As power increases, the rate of degradation increases at all frequencies. The reaction was found to follow pseudo first order kinetics as inferred from Fig. 1. The experiment was repeated in the presence of argon atmosphere at 620 kHz and power density of 42 W/mL. The solution was saturated with argon and an argon atmosphere was maintained throughout the sonolysis. A comparatively higher rate of decomposition of dT was observed in argon atmosphere as shown in Fig. 1(c).

The results clearly indicate that the rate of the sonochemical reaction depends on the power and frequency of ultrasound. The increased sonochemical activity observed at frequencies 350 and 620 kHz is expected as it was reported that the overall free radical production was greater in this range of frequency [24]. When power density increases, there is an enhancement in the energy of cavitation facilitating the production of higher number of free radicals [25] which explains the general increase in the rate of degradation with power.

The chemical transformations of molecules on sonolysis are caused by pyrolysis and reaction with the ·OH and H· produced by water pyrolysis [14,26,27]. Since dT is non volatile, the contribution from the pyrolytic reactions can be considered negligible [28] and the major transformation pathway could be the reaction with free radicals. The intermediate radicals formed from dT on sonolysis by the addition of ·OH and H· were detected earlier by spin trapping studies [28] which further support the free radical mediated mechanism. From the pulse radiolysis studies, it is reported that ·OH adds to the base with a reaction rate of the order of 3–10 × 10^9 L/mol s and abstracts hydrogen from the methyl group of thymine as well as the sugar ring with a rate constant of 2 × 10^9 L/mol s, while the rate constant of H· reaction is of the order ten times lesser than the former (1–5 × 10^8 L/mol s) [29]. The pseudo first order nature of the reaction observed in the present case proposes that the major reactive species is the highly reactive ·OH radical.

A slightly higher rate of decomposition of dT is observed under argon atmosphere. Compared to air, argon possesses higher polytropic index and lower thermal conductivity. Also, it has higher solubility which facilitates more cavitation events to take place compared to the aerated conditions [30]. This results in an efficient production of free radicals contributing to the increased rate of decomposition of dT in the presence of argon.

3.2. Mass spectrometric analysis of the transformed products

Sonolysis of dT yielded a mixture of products which were analyzed using LC–Q-ToF–MS. The total ion chromatogram obtained

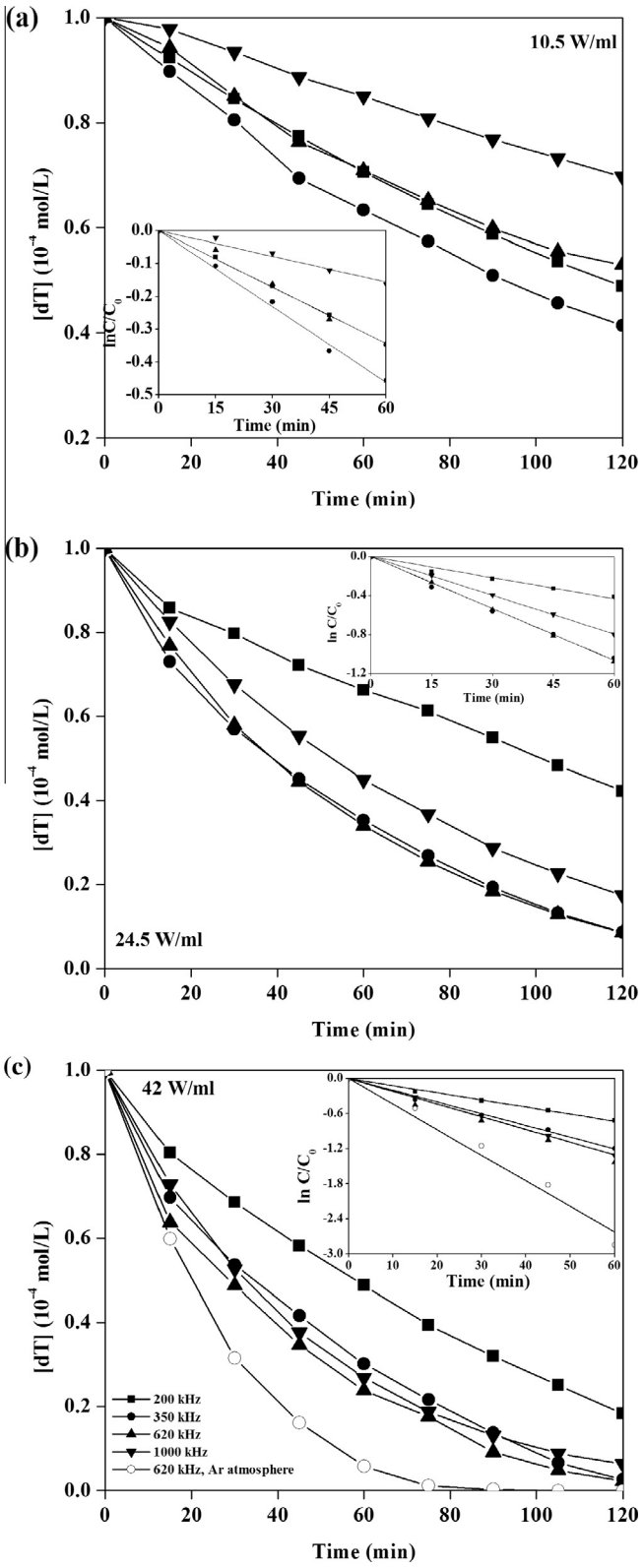


Fig. 1. Effect of frequency and power on the degradation of dT (a) 10.5 W/mL, (b) 24.5 W/mL and (c) 42 W/mL. Inset: the pseudo first order nature of the reaction.

under aerated and Ar saturated conditions are given in the Fig. 2. The measured mass accuracy for all the products was less than 5 ppm. The structures were assigned from the mass, elemental composition and fragmentation pattern of the corresponding ions. The twenty identified products are given in Table 1.

The major identified products include *cis* and *trans*-thymidine glycols (**1**, **6**), 1-(dR)-5-hydroxy-5-methylbarbituric acid (**12**), 1-(dR)-5-hydroxy-5-methylhydantoin (**13**), 5-(hydroxymethyl)-2′-deoxyuridine (**15**), 5-formyl-2′-deoxyuridine (**20**). These products were identified from the ESI fragmentation pattern. Interestingly, these were reported in the case of radiation induced chemical transformation of dT [7]. The product **8** was proposed to be formed from **12** by hydrolysis. Two products were detected which were assumed to be arising from the reaction of ·OH with the sugar moiety: thymine (**17**) and the product, **19**. The proposed structure of **19** is given in Fig. 5. The product **5** containing two additional hydrogen atoms than dT as inferred from the elemental composition, is proposed to be 5,6-dihydrothymidine. There were four additional base modified products formed only in the argon saturated conditions. Under Ar saturated conditions three dimeric products were also detected (**4**, **7** and **11**). The rest of the products are likely to be formed from these initially formed stable end products.

In order to find out the fate of the initially formed radicals in the absence of oxygen, the sonolysis was carried out in argon atmosphere. All the major oxidatively generated products obtained in the above experiments were also observed under argon atmosphere except two base modified products (**2** and **18**). In addition to that three radical recombination products (**4**, **7** and **11**) were also found to be formed under Argon atmosphere. The products **2** and **18** were found to be arising from the primary sonolysis products of dT by an oxygen mediated mechanism. In spite of the low intensity obtained, the formation of hydantoin derivative (**13**) was also confirmed from the fragmentation pattern (Supporting Information Fig. S1).

The online UV spectra acquired for the pure components eluted in the HPLC is given in Fig. 3. The initial peaks in the chromatogram were found to be due to co eluting polar base damage products and hence could not acquire the spectra of individual compounds. The polar base damage products were found to be having a maximum absorption around 260–270 nm. The 5-formyl-2′-deoxyuridine was identified by the absorption maximum at about 279 nm [2].

3.2.1. Formation of base modified products of dT

Cis and *trans*-5,6-dihydroxy-5,6-dihydrothymidine (dTg$_2$ and dTg$_1$), 1-(dR)-5-hydroxy-5-methylbarbituric acid (**12**), 1-(dR)-5-hydroxy-5-methylhydantoin (**13**), 5-(hydroxymethyl)-2′-deoxyuridine (**15**) and 5-formyl-2′-deoxyuridine (**20**) are the base modified products found in the present case.

Two products detected with m/z = 275.08 were identified as thymidine glycols as evident from the fragmentation pattern. *Cis*-thymidine glycols were reported to be the major oxidatively generated products from thymidine. It is well known that thymidine glycols exists as a pair of *cis* and *trans* diastereomers [31]. The fragmentation patterns were similar for both the glycols identified, differing only in the relative abundances of peaks. In order to distinguish the *cis* and *trans* forms from the mass spectrum, MS/MS spectra were acquired at different collision energies. The relative percentage of loss of water molecules from the $[M-H]^-$ ion at different collision energies is given in Fig. 4. The relative propensity of water loss from the second compound was found to be greater as compared to that from the first. The MS/MS spectrum was recorded at different collision energies ranging from 10 to 20 eV. At all collision energies, water loss was found to be a facile process in the case of dTg$_1$ than dTg$_2$. For the elimination of water molecule to be a facile process, both the eliminating H and the hydroxyl group should be antiperiplanar to each other which is possible in the case of *cis* isomer than the *trans* [32]. In the present case, dTg$_2$ showed higher possibility of water loss indicating that it is the *cis* isomer and dTg$_1$ the *trans* form.

The fragmentation of **15** showed major peaks at m/z 214.07, 166.05 and 124.04. The first two peaks correspond to the neutral

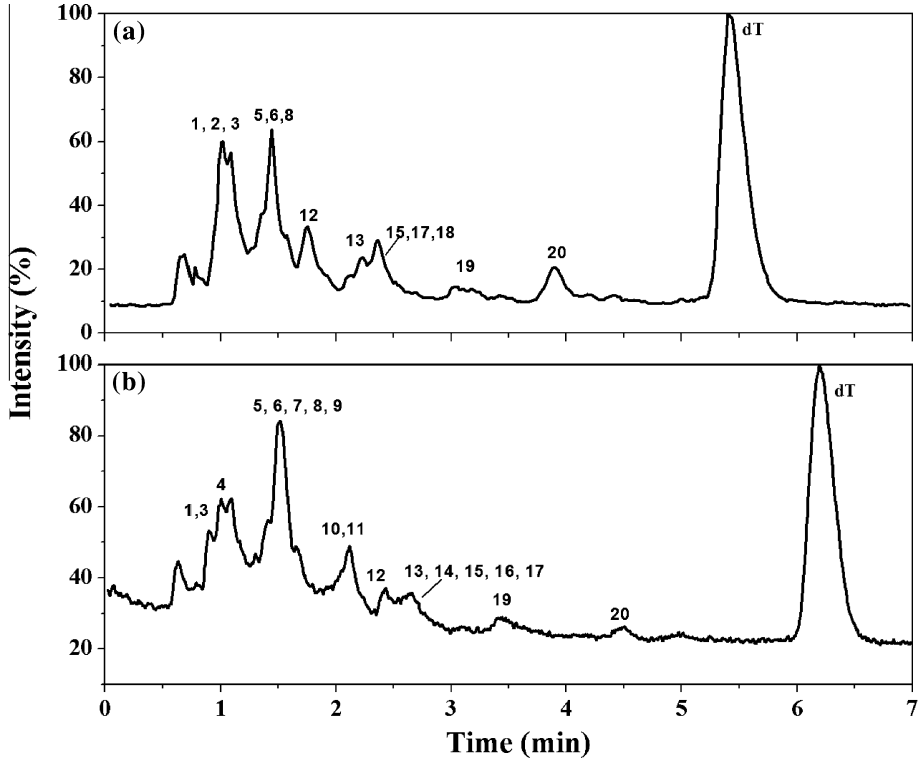


Fig. 2. The total ion chromatogram (TIC) of sonolysed dT under (a) aerated and (b) Ar saturated conditions. The numbers of the corresponding products, as in Table 1, are shown above most peaks.

loss of NHCO and an S_1 type fragmentation. The peak at 124.04 was found to be formed from the fragment with m/z = 214.07 by another S_1 fragmentation. Applying these fragmentation rules to the MS/MS spectra of the ion with m/z = 255.06 led to the structural assignment of **20** as 5-formyl-2′-deoxyuridine. In the case of **12**, the peaks at 230.06 and 183.04 correspond to the loss of NHCO, the S_1 type fragmentation of $[M-H]^-$ ion and the compound is identified as 1-(dR)-5-hydroxy-5-methylbarbituric acid. The base peak results from the cleavage of C2–N3 and C6–N1 bonds which leads to a loss of 159 mass units. The same loss of 159 mass units is observed in the MS/MS spectrum of **13** also, and this compound has been identified as 1-(dR)-5-hydroxy-5-methylhydantoin.

The predominant reaction of ·OH with dT is the addition to the C5–C6 double bond of the thymine moiety and abstraction of hydrogen from the exocyclic methyl group of thymine as well as from the sugar ring [5]. The reactions at the base part are more common and the most studied. In aerated solutions the reactions proceed through the formation of relatively stable hydroperoxides, which then decompose to other products [1,33]. The structures of the initially formed radicals and the formation of the hydroxyhydroperoxides under the aerated conditions are given in Scheme 1. The mechanism of formation of all these major base modified products is well described in the radiation studies of dT [1,7].

3.2.2. Formation of 5,6-dihydrothymidine

The product whose measured m/z in the negative mode was 243.06 (**5**), has been identified as 5,6-dihydrothymidine. It is the major product formed by γ-irradiation of DNA or dT under anoxic conditions. It was reported that its formation was initiated by the solvated electrons and the production was found to decrease when the amount of oxygen increases [9,34]. This fact makes its presence on sonolysis of dT under aerated conditions interesting. The formation of solvated electrons, even though reported on sonolysis, usually occurs at a pH above 9.1 [16]. In the present case, pH of the dT solution was in the range 3.7–5. This fact rules out the mechanism initiated by hydrated electron. Therefore, a H· initiated reaction mechanism is proposed for the formation of **5** on sonolysis. H· adds to the C5 and C6 positions and the resulting radicals can undergo a disproportionation by electron transfer (Scheme S1, Supporting Information). The anionic species produced by the disproportionation of the C5-yl and C6-yl radicals incorporate a proton from the medium to form **5**. An intermediate radical produced by the addition of H· to dT on sonolysis was detected earlier in the spin trapping studies [28] which further supports this assignment. Under oxygenated conditions, this product is not expected as the radicals formed by the addition of H· and it may react with the molecular oxygen. As a result of long term sonolysis, the oxygen molecules may be consumed by the reaction with the carbon centered radicals, providing a deaerated condition. Further, no air bubbling was maintained throughout the sonolysis which justifies the formation of 5,6-dihydrothymidine and the proposed mechanism.

3.2.3. Products resulting from ·OH attack on sugar moiety

Formation of thymine was detected from the HPLC analysis and was also confirmed from the mass spectrometric analysis. Base release from the nucleosides on sonolysis was reported earlier for all the four 2′-deoxyribonucleosides in DNA [35]. There are reports showing that ·OH reacts with the 2′-deoxyribonucleosides ring by the abstraction of H from various positions. The H· abstraction from C1′ and C4′ is of particular interest as it leads to abasic sites [36,37]. H· abstraction from C1′ and C4′ positions followed by the rearrangement of the sugar ring subsequently releases thymine. The product **19**, with a molecular mass 239.06 is only two hydrogens less than the dT. The MS/MS spectrum consists of a fragment 125.03 corresponding to the unmodified base, and from the close examination of the fragmentation pattern (Fig. S3, Supporting Information), it was possible to tentatively identify the product to be 3′-keto-dT (Fig. 5). Another possible product to

Table 1
The sonolytically modified nucleosides detected under aerated and argon saturated conditions at a frequency of 620 kHz and power density 42 W/mL, in the order of elution from the column.

Sl. No.	Compound (dR = 2-deoxy-β-D-*erythro*-pentofuranosyl)	*m/z* of $[M-H]^-$ ion detected in sonolysis	γ-Ray irradiation	Fenton reaction
1	*trans*-5,6-Dihydroxy-5,6-dihydrothymidine (dTg_1)	275.0878	Yes	Yes
2	5-Formyl-5,6-dihydroxy-5,6-dihydro-2′-deoxyuridine	289.0669[#]	nr	nr[§]
3	N-(2-deoxy-β-D-erythro-pentouranosyl)-([2-hydroxypropanoyl]carbamoyl) carbamic acid	291.0823	nr	nr[§]
4	Dimer **1**	517.1772[*]	nr	nr
5	5,6-Dihydrothymidine	243.0617	Yes[⊥]	nr
6	*cis*-5,6-Dihydroxy-5,6-dihydrothymidine (dTg_2)	275.0877	Yes	Yes
7	Dimer **2**	517.1782[*]	nr	nr
8	N^1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-N^3-tartronoylurea	291.0826	nr	nr[§]
9	(5R)-5-hydroxy-5,6-dihydrothymidine (dTOH1)	259.0923[*]	Yes	nr
10	(5S)-5-hydroxy-5,6-dihydrothymidine (dTOH2)	259.0921[*]	Yes	nr
11	Dimer **3**	517.1776[*]	nr	nr
12	1-(dR)-5-hydroxy-5-methylbarbituric acid	273.0722	Yes	nr
13	1-(dR)-5-hydroxy-5-methylhydantoin	245.0774	Yes	Yes
14	6-Hydroxy-5,6-dihydrothymidine (dTOH3)	259.0922[*]	Yes	nr
15	5-(Hydroxymethyl)-2′-deoxyuridine	257.0774	Yes	Yes
16	6-Hydroxy-5,6-dihydrothymidine (dTOH4)	259.0923[*]	Yes	nr
17	Thymine	125.0349	Yes	Yes
18	Unknown	289.0668[#]	nr	nr
19	3′-Ketothymidine	239.0662	nr	nr
20	5-Formyl-2′-deoxyuridine	255.0617	Yes	Yes

[*] Detected only in the sonolysis of dT under argon atmosphere.
[#] Detected only in the sonolysis of dT under aerated conditions.
[§] Not detected in the case of dT but the probable evidence of formation was given by the Fenton reaction of dTpdT.
[⊥] Detected but the mechanism of formation is different.

be considered here is the 5′-aldehyde derivative of dT. The assignment was based on the fragmentation pattern which supports the 3′-keto-dT (Fig. S3, Supporting Information). It could be formed by the abstraction of H atom from the C3′ position. Under aerated conditions, the carbon centered radical reacts with molecular oxygen to give the peroxyl radical which on elimination of an $HO_2^{\cdot}$ results in product **19** [16]. Formation of this sugar modified product is highly significant as it directly throws some light to the DNA strand break mechanism on sonolysis. Oxidation reactions on 2-deoxyribose moiety of DNA are significant as they are the prime proponents of strand breaks in DNA.

3.2.4. Products formed from the primary end products of sonolysis

The proposed structure of the product with *m/z* 289.08 observed only in aerated conditions are given in Fig. 6. The structural assignment is based on the major fragments found in the MS/MS spectrum (Figs. S4 and S5, Supporting Information). The product **2** showed a peak with *m/z* = 261.07 corresponding to a loss of CO. This can be from the exocyclic aldehyde group. There is also a peak at *m/z* = 172.02 which is obtained by the *N*-glycosidic bond cleavage of the proposed structure **2**. The formation of **2** is likely to be the result of H-abstraction by ·OH from the methyl group of thymine from dTg_1 or dTg_2. It follows the same pathway of formation of **20**. There is another possibility of its formation: by the addition of ·OH to the C5–C6 double bond of the product **20**. In the case of the compound **18**, the fragment formed as a result of *N*-glycosidic bond cleavage appeared at *m/z* = 175.03, corresponding to the elemental composition $C_5H_7N_2O_5$. This is also the base peak. There was no peak corresponding to the loss of CO as in the case of **18**. It is predicted to be formed from **8** by the abstraction H from 3′ position following a pathway similar to that of the base modified product discussed in Section 3.2.2. The structure

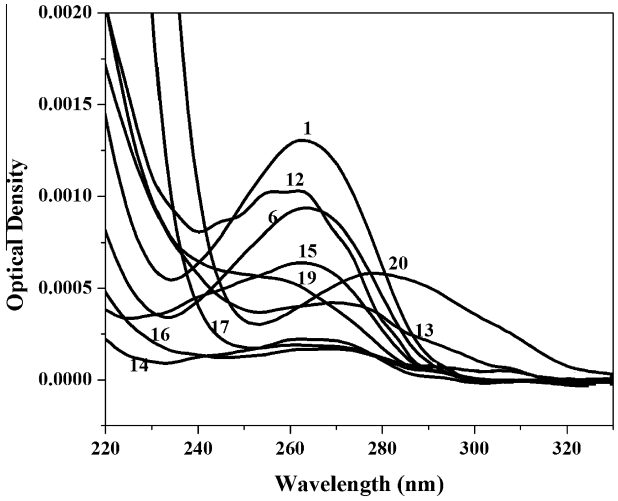


Fig. 3. The online UV spectra of the sonochemically transformed products of dT.

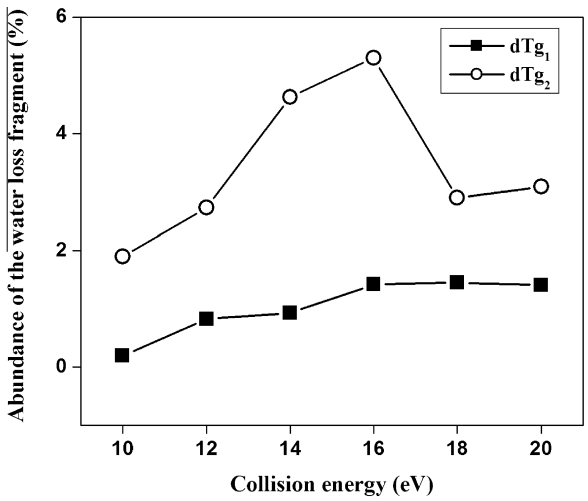
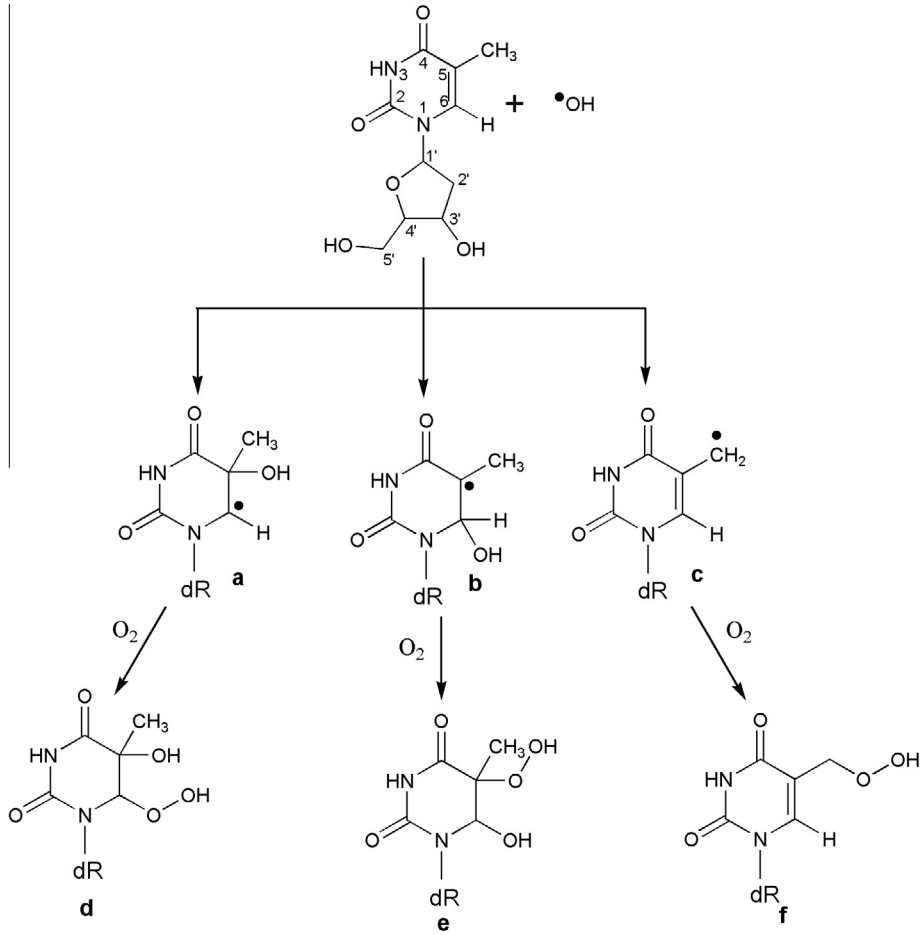


Fig. 4. Variation of relative propensity of water loss from the dTg's with collision energy. The percentage water loss is greater for the *trans*-isomer, **1**, relative to the *cis*-isomer, **6**.



Scheme 1. The formation and structures of the initially formed radicals and the hydroxyhydroperoxides from dT. Similar schemes for products **3**, **8**, **9**, **10**, **14** and **16** are provided in the Supplementary Information (Schemes S2 and S3).

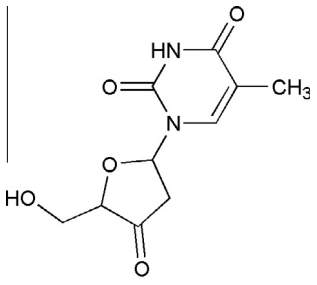


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Fig. 5. Structure of the sugar modified product.

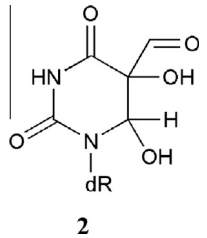

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Fig. 6. The proposed structure of the polar product **2** with $m/z = 289.06$ as determined from the fragmentation pattern (Supporting Information, Fig. S4).

could not be assigned with certainty but it is obvious that its formation requires aerated conditions. Their formation can be justified by the fact that dT after 1 h of sonolysis was nearly 25% in aerated and 10% in argon saturated conditions most probably less than the concentration of primary sonolysis products.

The products **3** and **8** were supposed to be the hydroperoxides with the m/z value of the $[M-H]^-$ ions (291.08); however, their presence in the Ar atmosphere ruled out this possibility. From the elemental composition and the fragmentation pattern, two possibilities were suggested as N-(2-deoxy-β-D-*erythro*-pentouranosyl)-([2-hydroxypropanoyl]carbamoyl) carbamic acid and N^1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-N^3-tartronoylurea. The latter is reported earlier as a thermal decomposition product of 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**d**), **12** being the intermediate compound [33] and we propose similar hydrolytic route for the formation of **3** (Supporting Information Scheme S2). From the information on the mass spectrometric fragmentation pattern, the assignment of **3** is only tentative. As the formation of **12** under the Ar atmosphere is confirmed from the LC–Q-ToF analysis, the formation of **3** and **8** under this condition can also be explained. The formation of these secondary oxidation products may not be significant in the damage to cellular DNA as secondary products can be formed only by continuation reaction of ·OH.

3.2.5. The products formed under argon atmosphere

All the major oxidation products obtained in the aerated conditions except **2** and **18** were detected in argon saturated atmosphere. The product profile shows that extensive recombination and

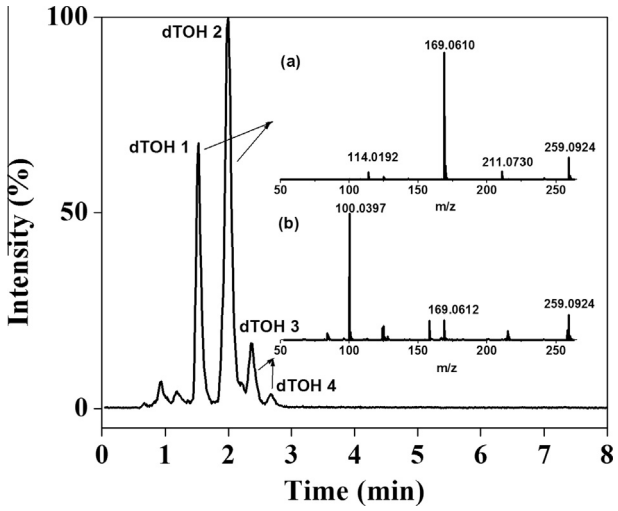

Fig. 7. The total ion chromatogram acquired in the MSMS mode for **9**, **10**, **14** and **16** or m/z = 259.09. The fragmentation pattern of the two the corresponding 5-hydroxy- and 6-hydroxy-5,6-dihydrothymidine are given in the inset (a) and (b) respectively.

dimerization reactions take place in the absence of oxygen. Four products (dTOH 1, dTOH 2, dTOH 3 and dTOH 4) with mass 259.09 were detected when dT was sonolysed in argon atmosphere which were not observed in aerated conditions under the selected time of sonication. dTOH 1 and dTOH 2 showed similar MS/MS spectrum. Similarly, dTOH 3 and dTOH 4 have also shown similar MS/MS spectrum (Fig. 7). The accurate mass measured and the fragmentation pattern helped in the proposal of the structure of dTOH 1 and dTOH 2 as 5-hydroxy-5,6-dihydrothymidine. The base peak was at 169.06, corresponding to the fragment formed by the S_1 type fragmentation commonly occurring in nucleosides [38]. In the case of dTOH3 and dTOH4, even though the peak corresponding to an S_1 type fragmentation is present, the base peak is the one with m/z 100.03, which could be formed by cleavage of C2–N3 and C6–N1 bonds. When the hydroxyl group is present at the C6 position, its electron withdrawing effect may weaken the C6–N1 bond favoring this fragmentation than the S_1 type as in the former case. The structure of dTOH 3 and dTOH 4 is assigned to be isomers of 6-hydroxy-5,6-dihydrothymidine. In the case of 6-hydroxy-5,6-dihydrothymidine, four *cis* and *trans* diastereomers are possible: 5*S*,6*S*, 5*R*,6*R*, 5*S*,6*R* and 5*R*,6*S* [31]. Assignment of the correct stereo chemical structure of dTOH 3 and dTOH 4 could not be done using the present set of data only. The presence of a peak at m/z = 114.01 in the MS/MS spectrum of dTOH 1 and dTOH 2 is crucial as it arises only from a structure with a hydroxyl group in the C5 position. It was present in the MS/MS spectrum of **12** also with a C5-hydroxyl group. Since the two compounds gave similar fragment ions in the MS/MS spectrum, they are likely diastereomers. There are reports on the separation of the 5S and 5R forms of 5-hydroxy-5,6-dihydrothymidine on reversed phase HPLC columns [39]. From the order of elution it was found that dTOH 1 is 5R isomer and dTOH 2 the 5S.

Three dimeric products were detected with same m/z and similar MS/MS spectrum. The proposed structures of the products are given in Fig. 8 (the spectrum is given in the Supporting Information, Fig. S7). Due to the close similarity of the spectra, the exact structure could not be elucidated, but from the m/z value, it was inferred that they are formed from the dimerization of the radical a and b. There can be an a–a type, b–b type and a–b type dimers as reported earlier in the case of radiolysis of thymine under N_2O flushed conditions [40]. Accordingly, the possible structures of the dimers in the case of dT are given in the Fig. 8. This suggests a predominant recombination and disproportionation mechanism in the absence of oxygen as reported earlier by Cadet et al. [41].

3.2.6. Mechanism of formation of the products in argon atmosphere

5-Hydroxy-5,6-dihydrothymidine and 6-hydroxy-5,6-dihydrothymidine are reported to be formed along with the thymidine glycols when aqueous solution of dT was subjected to either gamma radiolysis or photolysis under oxygen free atmosphere [42]. In the present case, the mass spectrometric analyses were carried out after 1 h of sonolysis at 620 kHz at a power density of 42 W/mL. In the presence of oxygen, the radicals **a** and **b** react with O_2 thus resulting in the production of glycols (dTg) predominantly. But in the argon atmosphere, the radicals may undergo disproportionation reactions by electron transfer giving the carbocations and carbanions. The positive ions on addition of a water molecule end up as glycols while the negative ions get add on a proton giving the dTOH's (Scheme S3, Supporting Information). The reaction between the reducing **a** and the oxidizing **b** may also take place

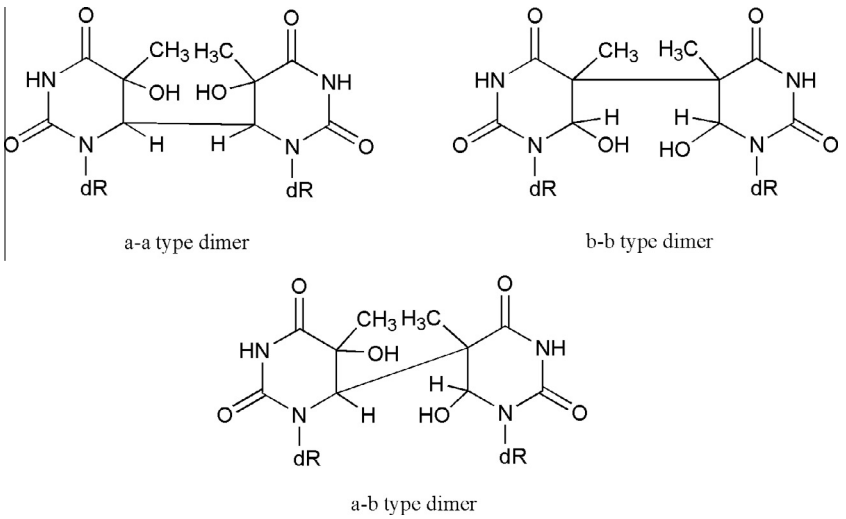

Fig. 8. The possible structures of radical recombination products, producing dimers **4**, **7** and **11**.

resulting in the production of *cis* and *trans* dTg's and the 6-hydroxy-5,6-dihydrothymidine (dTOH 3 and dTOH 4).

The reducing radical **c** may react with the oxidizing radical **b** [5], converting **c** to a carbocation. It may incorporate a water molecule subsequently resulting in **15** and **20**. The formation of 1-(dR)-5-hydroxy-5-methylbarbituric acid (**12**), 1-(dR)-5-hydroxy-5-methylhydantoin (**13**) and 3′-ketothymidine (**19**) in the absence of oxygen is not reported earlier. It is probable that the secondary oxidation reactions under the ultrasonic field lead to the formation of **12** and **13**. The product **19** may be formed by H· abstraction from C3′ position followed by redox or disproportionation reactions of the radical. The formation of **5** in the argon atmosphere might be following the same mechanism as in the case of aerated conditions as it is mediated by disproportionation reactions.

3.2.7. A comparison of product profile between sonolysis, radiolysis and Fe^{2+} mediated Fenton reactions of dT

The Table 1 shows a comparison of the transformation products formed in sonolysis, radiolysis [7] and Fenton reactions [2]. It is interesting to note that most of the low molecular weight and ring opened products as observed in radiolysis experiments and Fenton reaction were not detected in the case of sonolysis. For example, *N*-(2-deoxy-β-D-*erythro*-pento-furanosyl) formylamine, *N*-(2-deoxy-β-D-*erythro*-pento-furanosyl) urea, N-formyl-N-(2-deoxy-β-D-*erythro*-pento-furanosyl) urea and N^1-(2-deoxy-β-D-*erythro*-pento-furanosyl)-N^2-pyruvyl urea were absent in the present case. We identified three recombination products of 5-hydroxy- and 6-hydroxy-5,6-dihydrothymidyl radicals as in the case of thymine under radiolysis in N_2O flushed conditions. The products **2**, **18** (*m*/*z* 289.06), **3** and **8** (*m*/*z* = 291.08) are reported here which were not identified either in the case of radiation or in Fenton reaction. However, the presence of their *m*/*z* values can be seen in the report on the Fenton reaction of thymidine dinucleoside monophosphate (dTpdT) using FAB-MS [2]. 5,6-Dihydrothymidine (**5**), is a reported product under radiolysis, however with a different mechanism of formation as described in Section 3.2.1. A number of sugar modified products were reported in the radiolysis and Fenton reaction of dT, such as 2-deoxyribonolactone and 2-deoxytetradialdose [2]. In the present case we observed only 3′-ketothymidine as the sugar modified product. The reported eight new products in the present case including dimers were not detected either in radiolysis or in Fenton reaction. Among the thirteen identified products under aerated conditions, only six were reported in the case of Fenton reaction of dT. No data are available on the Fenton reaction of dT in the absence of oxygen.

4. Conclusions

There is an ever increasing use of US in medical field as it is considered as a non ionizing radiation. Out of the four frequencies selected for this study, two (620 kHz and 1 MHz) fall under the range used for the surgical non disruptive applications [43]. From the analysis of the products, it is concluded that US on long exposure causes the same oxidatively generated damage on dT, a DNA model compound. The formation of 5,6-dihydrothymidine under sonolysis is interesting since this product is reported only in a reductive environment where hydrated electron is the main reactive entity (Section 3.2.1). A mechanism of its formation involving H· is proposed. Four products were detected which are the result of a free radical reaction on the primary degradation products of dT. The sonochemical formation of 3′-ketothymidine, a sugar modified product, is highly significant as it may result in DNA strand breaks on sonolysis. The detection of three dimers clearly indicates the predominant disproportionation and recombination reactions of radicals under the argon atmosphere. The product profile provides a thorough understanding of the sonochemical transformation of dT in aqueous medium which could be very well connected with DNA damage mechanism in US assisted cancer therapy and the possible damaging effect on DNA in normal cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ultsonch.2015.05.016.

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