Pergamon

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Tetrahedron Letters 44 (2003) 5583–5587

TETRAHEDRON LETTERS

Chemical studies of the antioxidant mechanism of theaflavins: radical reaction products of theaflavin 3,3′-digallate with hydrogen peroxide

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Received 17 April 2003; revised 2 June 2003; accepted 3 June 2003

Abstract—The objective of the current study is to characterize the reaction products of theaflavin 3,3′-digallate, one of the major characteristic polyphenols of black tea, with hydroxyl radicals generated by hydrogen peroxide, with the aim of gaining insights into specific mechanisms of antioxidant reactions in physiological systems. Two major reaction products were isolated and identified using high-field 1D and 2D NMR spectral analysis. Both of them are A-ring fission products. The observation of these compounds indicates that the A ring rather than the benzotropolone moiety is the initial site for formation of reaction products in the hydrogen peroxide oxidant system.

Theaflavins are a group of polyphenol pigments formed at the fermentation stage of black tea manufacturing.[1] They are orange or orange-red in color and possess a benzotropolone skeleton that is formed from co-oxidation of appropriate pairs of catechins, one with a *vic*-trihydroxy moiety, and the other with an *ortho*-dihydroxy structure.[2,3] It is known that theaflavins, which account for 2–6% of the dry weight of solids in brewed black tea,[4] contribute importantly to properties including its color[5], 'mouthfeel'[6] and extent of tea cream formation.[7] Their structures are well studied.[8–11] Recently, theaflavins have attracted considerable interest because of their beneficial health properties, including antimutagenicity in the rat liver S9 fraction,[12] suppression of cytochrome p450 1A1 in cell culture,[13] anticlastogenic effects in bone marrow cells of mice,[14] suppression of extracellular signals and cell proliferation,[15] and anti-inflammatory and cancer chemopreventive action (by suppressing the activation of NFκB through inhibition of IKK activity).[16] Theaflavins have also shown strong antioxidant activity against lipid oxidation detected in the rabbit erythrocyte ghost system[17] and rat liver homogenates,[18] against LDL oxidation in mouse macrophage cells,[19] as preventives for DNA oxidative damage in cell-free systems,[17] in the inhibition of xanthine oxidase and suppression of intracellular reactive oxygen species in HL-60 cells,[20] and through H_2O_2 scavenging ability.[20] As an important oxidant, hydrogen peroxide (H_2O_2) can normally be produced from many physiological sources in the aerobic environment of mammalian cells and tissues.[21] However, the specific mechanism of theaflavin oxidation by H_2O_2 remains unclear. In this study, we report the oxidation products formed by the reaction of H_2O_2 with theaflavin 3,3′-digallate (**1**), one of the major theaflavins.

Two major oxidation products (**2**–**3**) from the reaction of theaflavin 3,3′-digallate (**1**) with hydrogen peroxide were isolated and identified on the basis of their spectral data.[22]

Compound **2**, a reddish amorphous solid, was assigned the molecular formula $C_{42}H_{32}O_{22}$ based on positive-ion APCI-MS ($[M+H]^+$ at m/z 889) and ^{13}C NMR data. In

Keywords: theaflavin 3,3′-digallate; black tea; H_2O_2; hydroxyl radical; antioxidant mechanism.

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0040-4039/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0040-4039(03)01382-0

comparison with the ^{1}H NMR spectrum of **1**, the ^{1}H NMR of **2** also displayed three characteristic downfield signals for the benzotropolone group (δ 7.42 brs, H-c; 7.64 brs, H-e; 7.66 s, H-g); two sets of C-ring signals (δ 5.21 m, H-2; 5.74 m, H-3; 3.10 dd, $J=4.8$, 17.4 Hz, 2.90 brd $J=17.4$ Hz, H-4; 5.90 m, H-2′; 5.74 m, H-3′; 2.97 dd, $J=4.8$, 18.0 Hz, 2.68 brd, $J=18.0$ Hz, H-4′), and two singlets for the gallate groups (δ 6.88 s, H-13 and H-17; 6.86 s, H-12′ and H-16′). However, it only showed two signals for the A ring at δ 6.00 d, $J=2.4$ Hz and 6.09 d, $J=2.4$ Hz, instead of the expected four. In addition, two additional signals were observed at δ 3.97 d, $J=16.2$ Hz and 3.88 d, $J=16.2$ Hz. These two signals were assigned to one methylene group (δ_C 40.8) based on the HMQC spectrum. The ^{13}C NMR spectrum of **2** displayed 42 carbon signals, 14 of which were assigned to the gallate groups, 11 to the benzotropolone group, 6 to the C-ring of flavan-3-ols, 6 to one set of A rings and 5 additional signals (δ 175.5 s; 172.2 s; 161.9 s; 104.3 s; 40.8 t). These observations indicated that the

Figure 1. Structures of compounds **1**–**3**.

Figure 2. Significant HMBC (H→C) correlations for compounds **2** and **3**.

C rings, gallate groups and benzotropolone moiety of **2** did not undergo any change during oxidation. Rather, the changes were localized to a single A ring. As mentioned above, this modified A ring showed one methylene group (δ 40.8), two carbonyl groups (δ 175.5 and 172.2), and two quaternary carbons (δ 161.9 and 104.3). The HMBC spectral analysis (Fig. 2) yielded correlation peaks between H-7′ (δ 3.97 and 3.88) and C-5′ (δ 104.3), C-6′ (δ 161.9), C-8′ (δ 175.5); H-4′ (δ 2.68 and 2.97) and C-5′ (δ 104.3), C-6′ (δ 161.9), C-9′ (δ 172.2). Thus, the structure of the modified A ring was confirmed as shown in Figure 1. The remaining outstanding issue was whether this modified A ring belonged to the flavan-3-ol connected to the benzene or the tropolone part of the benzotropolone group. To resolve this question, a careful analysis of cross peaks in the HMBC spectrum (Fig. 2) was required. The HMBC spectrum showed correlations between H-g (δ 7.66) and C-2′ (δ 77.4); H-2′ (δ 5.90) and C-g (δ 122.6), C-k (δ 129.3), C-3′ (δ 67.9), and C-4′ (δ 30.8); H-c (δ 7.42) and C-2 (δ 80.8); H-e (δ 7.64) and C-2 (δ 80.8); H-2 (δ 5.21) and C-c (δ 118.2), C-d (δ 135.0), C-e (δ 126.3), C-3 (δ 70.2), and C-4 (δ 27.7). These data indicated that this modified A ring belongs to a flavan-3-ol connected to the benzene part of the benzotropolone group. Therefore, the structure of **2** was deduced as shown (Fig. 1). The complete interpretation of the NMR data was based on the results of HMQC and HMBC experiments (Table 1).

Compound **3** was isolated as a reddish amorphous solid. The positive-ion APCI-MS of **3** displayed a molecular ion peak at m/z $[M+H]^+$ 889, supporting a molecular formula of $C_{42}H_{32}O_{22}$, as noted above for **2**. The NMR spectra of **3** displayed signal patterns similar to those of **2**. The ^{1}H NMR spectrum of **3** also showed the three characteristic signals for the benzotropolone group (δ 7.37 brs, H-c; 7.64 brs, H-e; 7.67 s, H-g); two sets of C-ring signals (δ 5.26 m, H-2; 5.68 m, H-3; 2.92 brd, $J=17.4$ Hz, 2.68 brd, $J=17.4$ Hz, H-4; 5.79 m, H-2′; 5.63 m, H-3′; 3.19 brd, $J=16.8$ Hz, 2.91 brd, $J=16.8$ Hz, H-4′), two singlets for the gallate groups (δ 6.80 s, H-12 and H-16; 6.94 s, H-13′ and H-17′); two signals for the A ring (δ 6.01 s and 6.04 s); two additional signals for the methylene group (δ 4.01 d, $J=15.6$ Hz and 3.78 d, $J=15.6$ Hz). The ^{13}C NMR spectrum of **3** displayed modified A-ring signals at δ 174.8 s; 171.4 s; 162.0 s; 103.7 s; 40.0 t. Clearly, all of these features suggested that **3** was also an A-ring cleaved diacid derivative of **1**. In the structure of **3**, the modified A ring belonged to a flavan-3-ol connected to the tropolone part of the benzotropolone group rather than the benzene moiety found in the structure of **2**. This connectivity was demonstrated by the HMBC data (Fig. 2). In this latter case, the cross peaks involved H-7 (δ 4.01 and 3.78) with C-5 (δ 103.7), C-6 (δ 162.0), C-8 (δ 174.8); H-4 (δ 2.68 and 2.92) and C-5 (δ 103.7), C-6 (δ 162.0), C-9 (δ 171.4); H-g (δ 7.67) with C-2′ (δ 76.3); H-2′ (δ 5.79) with C-g (δ 122.5), C-k (δ 129.4), C-3′ (δ 68.0), and C-4′ (δ 28.3); H-c (δ 7.37) with C-2 (δ 82.2); H-e (δ 7.64) with C-2 (δ 82.2); H-2 (δ 5.26) with C-c (δ 117.8), C-d (δ 133.6), C-e (δ 126.1), C-3 (δ 69.2), and C-4 (δ 29.7). Therefore, the structure of **3** was determined as shown (Fig. 1). The complete interpretation of the NMR data was based on the results of HMQC and HMBC experiments (Table 1).

The purpose of this investigation was to isolate and characterize the reaction products of theaflavin 3,3′-digallate, one of the major theaflavins in black tea, in a hydrogen peroxide oxidant system. Numerous mechanistic studies have been conducted on tea catechins, the precursor compounds of theaflavins, with different oxidants such as the peroxyl radical system, the peroxidase/hydrogen peroxide oxidant system, and the DPPH oxidant system.[23–27] These reports indicate that

Table 1. δ_H (600 MHz) and δ_C (150 MHz) NMR spectral data of compounds **2** and **3** (CD_3OD) (δ in ppm, J in Hz)

	2		**3**	
	δ_H	δ_C	δ_H	δ_C
2	5.21 m	80.8 d	5.26 m	82.2 d
3	5.74 m	70.2 d	5.68 m	69.2 d
4	3.10 dd 4.8, 17.4 2.90 brd 17.4	27.7 t	2.92 brd 17.4 2.68 btd 17.4	29.7 t
5		158.7 s		103.7 s
6	6.00 d 2.4	96.6 d		162.0 s
7		158.7 s	4.01 d 15.6 3.78 d 15.6	40.0 t
8	6.09 d 2.4	95.3 d		174.8 s
9		157.1 s		171.4 s
10		99.8 s		167.5 s
11		168.0 s		121.6 s
12		121.5 s	6.80 s	109.7 d
13	6.88 s	110.8 d		146.7 s
14		146.9 s		140.5 s
15		140.6 s		146.7 s
16		146.9 s	6.80 s	109.7 d
17	6.88 s	110.8 d		
2′	5.90 m	77.4 d	5.79 m	76.3 d
3′	5.74 m	67.9 d	5.63 m	68.0 d
4′	2.97 dd 18.0, 4.8 2.68 brd 18.0	30.8 t	3.19 brd 16.8 2.91 brd 16.8	28.3 t
5′		104.3 s		158.5* s
6′		161.9 s	6.04 s	95.8 d
7′	3.97 d 16.2 3.88 d 16.2	40.8 t		158.7* s
8′		175.5 s	6.01 s	96.5 d
9′		172.2 s		158.1 s
10′		167.8 s		100.5 s
11′		121.5 s		168.0 s
12′	6.86 s	110.0 d		121.6 s
13′		146.9 s	6.94 s	110.0 d
14′		140.6 s		146.7 s
15′		146.9 s		140.5 s
16′	6.86 s	110.0 d		146.7 s
17′			6.94 s	110.0 d
a		186.4 s		186.4 s
b		156.1 s		156.1 s
c	7.42 s	118.2 d	7.37 s	117.8 d
d		135.0 s		133.6 s
e	7.64 s	126.3 d	7.64 s	126.1 d
f		130.0 s		130.3 s
g	7.66 s	122.6 d	7.67 s	122.5 d
h		147.2 s		147.2 s
I		152.2 s		151.8 s
J		122.8 s		122.8 s
K		129.3 s		129.4 s

* Assignments may be interchanged in each column.

the use of different oxidants can result in the formation of different oxidation products from catechins; moreover, the main site of catechins antioxidant action appear to be the *o*-dihydroxy B-ring, or *vic*-trihydroxy B-ring or gallate group through the one electron transfer or H-atom abstraction mechanism. These hypotheses are supported by several theoretical studies of tea catechins by measuring their bond dissociation enthalpy (BDE) and ionization potential (IP) value, or pulse radiolysis and laser photolysis study.[28,29] Despite knowing the chemical structures of these two major oxidant products by this study, it is difficult to hypothesize a mechanism initiated on the B ring or the gallate group that lead to bond cleavage in the A ring. Halliwell and Gutteridge reported that the reaction of a hydroxyl radical with aromatic ring structures could be proceeded by the addition mechanism.[30] For the formation of these two major products, the possible initial step is the attack on the A ring by the hydroxyl radical. The A ring radical then undergoes a series of further reactions, including cleavage of the A ring. If the hydroxyl radical attacks the A ring of the flavan-3-ol connected to the benzene part of the benzotropolone group, **2** will be formed; conversely, **3** should be formed if the reaction is initiated at the A ring of the flavan-3-ol connected to the tropolone part of the benzotropolone group. It is noteworthy that the initial site for the formation of these two major reaction products is the A ring, not the benzotropolone group or the gallate group, in the hydrogen peroxide oxidant system. The identification of oxidant-specific products may provide an analytical framework for evaluating the antioxidant actions of theaflavins in biological systems. This study represents a first approach to determination of the antioxidant mechanism in theaflavins. Further study will be necessary to indicate if the mechanism is indeed the hydroxyl radical addition mechanism. Using glutathione to trap the intermediate radical will be helpful to understand the mechanism. These reaction products can be used as standards in metabolic studies of theaflavins in *vivo*.

Acknowledgements

This work was supported by NIH Grant PO1 CA88961. Funds to purchase the 600 MHz NMR spectrometer at CUNY College of Staten Island were provided by the New York State Dormitory Authority and the New York State Higher Education Applied Technology Program.

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22. 500 mg theaflavin 3,3′-digallate in 10 mL 10% acetone/water were treated with 1 mL 30% H_2O_2. The mixtures were reacted at room temperature for 48 h until most of the theaflavin 3,3′-digallate disappeared. After evaporation of the solvent *in vacuo*, the residue was first applied to a Sephadex LH-20 column and eluted with 45% acetone to obtain a mixture of the two major reaction products. This mixture was injected into a RP C-18 column and eluted with 35% methanol/water to give 16 mg of compound **2** (3.13%) and 10 mg compound **3** (1.95%).
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