European Journal of Chemistry

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Spectroscopic characterization of thiol adducts formed in the reaction of 4-methylcatechol with DPPH in the presence of *N*-acetylcysteine

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RESEARCH ARTICLE

10.5155/eurjchem.9.4.386-393.1794

Received: 04 October 2018
Received in revised form: 29 October 2018
Accepted: 30 October 2018
Published online: 31 December 2018
Printed: 31 December 2018

KEYWORDS

Quinones
Oxidation
Thiol adduct
4-Methylcatechol
N-Acetylcysteine
2,2-Diphenyl-1-picrylhydrazyl (DPPH)

ABSTRACT

Nucleophiles such as thiol compounds have enhancing effects on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of polyphenols. Several authors have suggested that regeneration of the catechol structure from *o*-quinone plays a key role in enhanced radical scavenging activity. We therefore explored the reaction of 4-methyl catechol (MC) with DPPH in the presence of *N*-acetylcysteine (NACys) to clarify the mechanism underlying activity enhancement. Four types of NACys adducts were isolated and purified by preparative HPLC after the reactions reached equilibrium and their structures were characterized spectroscopically using UV-Vis absorption, NMR, and LC-MS. Oxidation of MC using a periodate resin and subsequent reaction with NACys were also studied. LC-MS analyses revealed that a mono-NACys adduct is produced as the major product in the reaction of MC quinone with NACys, and direct reduction by NACys occurs in reactions with NACys MC quinones.

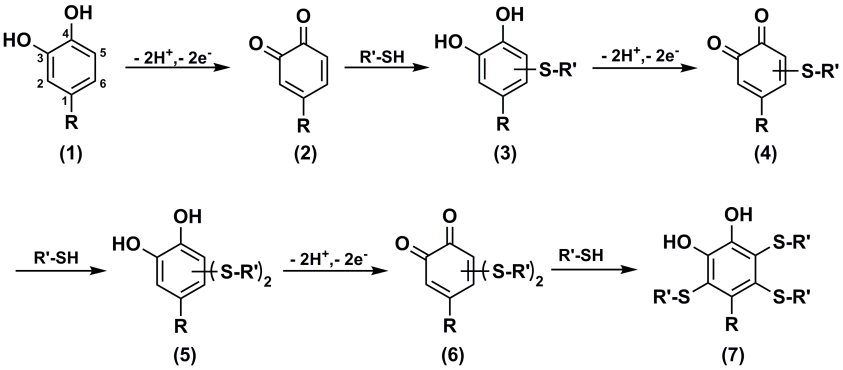
Cite this: *Eur. J. Chem.* **2018**, *9(4)*, 386-393 **Journal website**: www.eurjchem.com

1. Introduction

Polyphenols are a ubiquitous class of compounds in plants and are important sources of natural antioxidants. Flavonoids and phenolic acids are the principal types of plant polyphenols. Catechins are the most predominant flavonoids in plants, of which flavan-3-ols are predominant in green tea leaves, and chlorogenic acids (CGAs), and especially hydroxylcinnamates, are the major phenolic acids found in coffee beans [1-4]. Flavonoids and phenolic acids with an ortho-diphenolic (catecholic) structure act as strong antioxidants [5] and exert their antioxidant action through the radical-scavenging activity linked to their hydrogen- or electron-donating ability. Thus, the catechol moiety in a molecule likely plays a key role in antioxidant activity [6-9].

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method is the most commonly used method for evaluating the antioxidant activities of polyphenols [10,11]. A number of papers have reported the DPPH radical scavenging activities of plant polyphenols and these findings have been summarized in excellent review papers [12-14].

Several studies have shown that the antioxidant activities of phenolic compounds depend on the number and arrangement of hydroxyl groups in the molecule [12,15-17]. In addition to the structural characteristics of polyphenols, experimental factors also influence their DPPH radical scavenging activities [14] and include the type of solvent, temperature, and the presence of nucleophiles. Some authors have reported enhancing effects of nucleophiles, such as amine and thiol compounds, on the DPPH radical scavenging activities of polyphenols. For example, Fujimoto *et al.* [18] found a synergistic effect of the cysteinyl thiol on the antioxidant activities of caffeates, and Saito and Kawabata reported that the radical scavenging activity of protocatechuic acid increases



Scheme 1. Postulated radical scavenging mechanism of catechol compounds in the presence of a thiol nucleophile.

in the presence of nucleophiles such as thiols and amine compounds [19].

The enhancement of DPPH radical scavenging activity was attributed to regeneration of the catechol structure [18,19] resulting from the addition reaction of nucleophiles to the *o*-quinone intermediate. Thiol nucleophiles are reported to be far more reactive compared to amine nucleophiles [20].

The radical scavenging reaction of catechol compounds in the presence of a thiol nucleophile is postulated to proceed as shown in Scheme 1. In the first step, catechols (**1**) are oxidized by DPPH to form *o*-quinones (**2**). *o*-Quinones are highly electrophilic species that undergo attack by nucleophilic reagents such as thiols or amines [21]. In the case of thiol nucleophiles, this reaction affords catechol-thiol adducts (**3**), which lead to regeneration of the catechol structure. In the presence of excess DPPH and thiols, the initial mono-thiol adducts (**3**) are further oxidized to give their corresponding quinones (**4**) and subsequent di-thiol adducts (**5**). When starting from mono-substituted catechols (**1**), up to a tri-thiol adduct (**7**) can theoretically be formed. Since a single thiol addition to a quinone species represents a formal two-electron reduction, sequential thiol adducts formation leads to enhanced radical scavenging activity. The reduction of *o*-quinones by thiols should also be taken into account for regeneration of the catechol structure, since the thiol (−SH) group behaves both as a nucleophile and as a reducing agent [22].

Despite extensive studies, there is insufficient information to understand the detailed radical scavenging reactions of polyphenols in the presence of thiols. Spectroscopic characterization of the reaction products is indispensable for gaining further insight into the reaction mechanism. In this work, we focused on the reaction of 4-methylcatechol (MC) with DPPH in the presence and absence of the thiol nucleophile *N*-acetylcysteine (NACys). MC was used as a model polyphenol because it has the *o*-diphenol motif of catechins and CGAs. NACys is a typical *N*-substituted cysteine which provides a simple cysteinyl thiol model. We first examined the enhancing effects of thiols on the DPPH radical scavenging activity of MC. The oxidation products of MC in the DPPH radical scavenging reaction were analyzed by LC-MS in the absence and presence of NACys. The major reaction products, MC-NACys adducts, were isolated by preparative HPLC and their structures characterized from their 1D and 2D NMR spectra.

We also studied the periodate oxidation of MC and subsequent reaction of *o*-quinone with NACys. The periodate oxidation of catechol compounds proceeds via the formation of *o*-quinones [23-26]. The use of periodate resins allowed separation of the oxidant from the reaction mixture by simply filtering off the periodate resin. Thus, we could follow the *o*-quinone formation and NACys addition steps separately using UV-Vis absorption spectroscopy and LC-MS.

2. Experimental

2.1. DPPH radical-scavenging activity

The radical scavenging activities of samples were assayed according to the method proposed by Mensor *et al.* [27] with slight modifications. DPPH (19.7 mg) was dissolved in ethanol (100 mL) (500 μM) in a volumetric flask. A fresh DPPH solution was prepared daily and kept at room temperature in the dark. Stock solutions of MC and thiol compounds were prepared by dissolving each standard in 50% ethanol at a concentration of 12.5 μM for MC and 25 μM for thiol compounds, to adjust the radical scavenging equivalence. Typically, to a mixed solution of MC (2 mL) and a thiol compound (2 mL) in a test tube was added 1 mL of DPPH solution. The solution was mixed vigorously with a vortex mixer and incubated for 30 min at room temperature, then the absorbance change at 517 nm of the mixed solution was measured using a Shimadzu UV-2500 spectrophotometer at room temperature. The percent scavenging by the DPPH radical was calculated using the equation:

$$\text{Scavenging activity (SA, \%)} = [(A_{control}-A_{sample})/A_{control}] \times 100 \quad (1)$$

where A_{sample} and $A_{control}$ are the absorbance of the DPPH solution mixed with the sample solution or the same amount of solvent, respectively. All samples were analyzed in triplicate.

2.2. Reaction using periodate resin

Periodate resin was prepared according to the method of Harrison and Hodge [28] with slight modifications. A 25.0 g aliquot of a highly porous anion exchange resin (Diaion HPA25, Mitsubishi Chemical Corp., Japan) in 200 mL of Milli-Q water was mixed with 20 g of sodium periodate (Wako Pure Chemical Industries, Ltd., Japan). The solution was stirred at room temperature for 2 h and then the water was drained off using an inert filter. The periodate resin thus obtained was rinsed with 200 mL of water four times, then washed twice with 100 mL of THF (tetrahydrofuran) and twice with 100 mL of ether. The resin was transferred to 50 mL test tubes and dried in a vacuum oven at 35 °C overnight.

Solutions of MC and MC-NACys adducts were prepared by dissolving the designated amount of each sample in acetonitrile. An aliquot of periodate resin was added to the solution and left stirring for the indicated reaction time. After the reaction appeared to reach equilibrium, the resin was filtered off from the solution and NACys was added. Solutions with and without NACys were analyzed by UV-Vis and LC-MS.

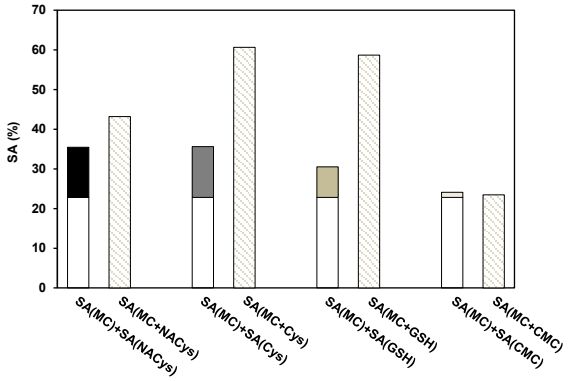


Figure 1. Comparison between the theoretical and practical scavenging activities of combinations of MC and various thiol compounds. Abbreviations: SA, Radical Scavenging Activity; NACys, *N*-acetyl-L-cysteine; Cys, L-cysteine; GSH, glutathione; CMC, *S*-carboxymethyl-L-cysteine.

2.3. Preparative HPLC

The major MC-NACys adducts were isolated by reversed-phase preparative HPLC using a Waters 2545 Binary Gradient Module equipped with a Waters 2487 Dual Absorbance Detector (Waters, USA), a YMC Triart C18 column (250 × 20 mm ID, 5 µm particle size, YMC, Japan).

2.4. UV-Vis spectra

UV-Visible absorption spectra were recorded on a Shimadzu UV-2500 spectrophotometer (Shimadzu Corp., Japan) with a band path width of 2 nm, using a quartz cuvette (path length, 1 cm). All measurements were carried out in the wavelength range 200-700 nm at room temperature.

2.5. LC-MS analysis

LC-MS was performed using an ultra-performance liquid chromatograph (Acquity, Waters, USA) coupled to a time-of-flight mass spectrometer (Synapt HDMS, Waters, USA). Analytes were separated using a YMC Triart C18 column (100 × 2.1 mm ID, 1.9 µm particle size, YMC, Japan). Data were collected in centroid mode over the mass range *m/z* 50-1000 with an acquisition time of 0.2 s and an inter-scan delay of 0.1 ms. The software package MassLynx 4.1 (Waters, Manchester, UK) was used for data acquisition and processing.

2.6. NMR spectra

The ^{1}H- and ^{13}C-NMR measurements were obtained with a JNM-ECA 500 spectrometer (JEOL Ltd., Japan) operating at 500 MHz (for ^{1}H) and at 125 MHz (for ^{13}C) in CD_3OD solution, and chemical shifts were expressed in δ (ppm) with reference to TMS. The coupling constant (*J*) is given in Hertz. The 2D-NMR (^{1}H-^{1}H COSY, heteronuclear multiple quantum coherence (HMQC) and ^{1}H-^{13}C heteronuclear multiple bond coherence (HMBC)) spectra were measured using the same instrument.

3. Results and discussion

3.1. Effect of thiols on the radical scavenging activity of MC

Synergistic effects were evaluated by determining the DPPH radical scavenging activity (SA) of binary SA(MC+thiol), and for each SA(MC) and SA(thiol) individually under the same test conditions. The four thiol antioxidants *N*-acetyl-L-cysteine (NACys), L-cysteine (Cys), glutathione (GSH) and *S*-carboxymethyl-L-cysteine (CMC) were chosen as thiol nucleophiles. NACys, Cys and GSH have a free thiol group whereas in CMC the hydrogen atom of the thiol group is replaced by a carboxymethyl group. Figure 1 compares the practical and theoretical scavenging activities of the SA(MC+thiol) combination, where the theore-tical value is the arithmetic sum of the value for each antioxidant, SA(MC)+SA(thiol). As defined by Hamdo *et al.* [29], the synergistic effect is evaluated by the difference between the practical and the theoretical SA values, ΔSA = SA(MC+thiol)-{SA(MC)+SA(thiol). When ΔSA > 0, there are synergies between the antioxidants, and when SA ≤ 0, there is an absence of synergy between the antioxidants [30]. The radical scavenging activity of MC can be enhanced by the presence of other thiol compounds. Binary systems containing NACys, Cys or GSH give rise to positive ΔSA values of +7.7, + 25.0 and +28.2%, respectively. On the other hand, the binary system with CMC has a negative value of -0.7%. These results suggest that a free thiol group is required to obtain positive synergistic effects.

3.2. Identification of reaction products by LC-MS in the DPPH radical scavenging reaction

MC and DPPH were reacted in the presence or absence of NACys, followed by LC-MS. Figure 2a shows HPLC profiles of reaction mixtures of MC and DPPH at various concentration ratios. HPLC analysis was conducted 120 min after the start of the reaction. In Figure 2b, HPLC analysis was conducted 8 h after adding NACys (4 equiv.) to the reaction mixture ([MC]:[DPPH], 1:1). The final concentrations were 1.25, 2.5 and 5.0 mM for MC, DPPH and NACys, respectively. MS and UV data for respective HPLC peaks are summarized in Table 1.

The DPPH radical scavenging reaction proceeds via the formation of *o*-quinone [31,32]. As shown in Figure 2a, only two peaks are observed, at retention times of around 4.2 and 6.6 min, for reaction mixtures of MC and DPPH. Peak 2 is assigned to unreacted MC, since it shows UV absorption at 282 nm and $[M-H]^-$ ions at *m/z* 123, both of which are characteristic of MC. Peak 1 can be assigned to MC quinone based on the characteristic UV absorption at 390 nm. The peak area of MC quinone (peak 1) increases as that of MC decreases (peak 2) when the relative DPPH concentration is increased. However, $[M-H]^-$ ions for peak 1 are unexpectedly observed at *m/z* 123, which is the same mass number as MC. This rather unusual MS feature was similarly reported for 1,2-benzoquinone by Albarran *et al.* [33], who suggested the formation of hydride adduct during the ionization process. It is interesting to note that MC quinone has a much shorter retention time (4.15 min) than does MC (6.58 min). *o*-Benzoquinone was reported to have a very strong dipole moment (5.1 D) in benzene [34] compared to catechol (2.62 D) [35].

Table 1. MS and UV data for HPLC peaks following MC/DPPH and MC/DPPH/NACys reactions.

Peak no	t_R (min)	λ_{max} (nm)	MW	[M-H]$^-$ (m/z)	Identification [a]
1	4.23	397.9	122.04	123.00	MC quinone
2	6.57	281.9	124.05	123.00	MC
3	5.07	307.1	446.08	445.05	Di-NACys adduct-1
4	5.73	316.9	607.10	606.16	Tri-NACys adduct
5	6.46	293.9, 253.9	285.07	284.02	Mono-NACys adduct
6	6.63	284.02	446.08	445.10	Di-NACys adduct-2

[a] Di-NACys adduct-1 and -2 denote a different regioisomer.

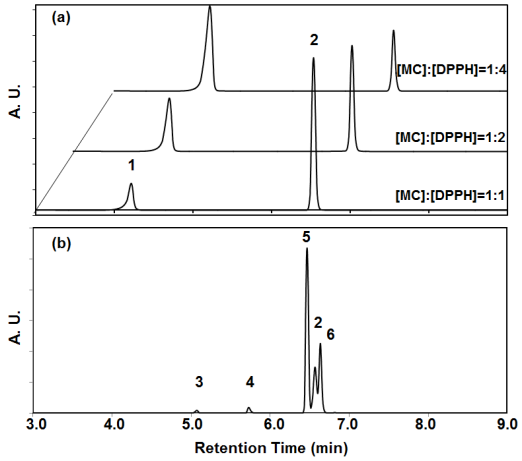


Figure 2. LC analysis of reaction mixtures of MC with DPPH (a) in the absence and (b) in the presence of NACys. Peak 2 = MC.

The larger dipole moment value of *o*-quinone may be one reason for the shorter retention time of *o*-benzoquinone.

In the presence of NACys, the reaction mixture shows the formation of several products, as presented in Figure 2b. Based on the MS spectra, peak 5 is assigned to mono-NACys adduct and peaks 3 and 6 are assigned to di-NACys adducts with different regioselectivities, with di-NACys adduct-2 (peak 6) being the major product based on the peak areas. A tri-NACys adduct is also observed at 5.73 min. The present observations suggest the sequential formation of thiol adducts in the reaction of MC and DPPH in the presence of NACys.

3.3. HPLC isolation and NMR characterization of major NACys adducts

We used preparative HPLC and NMR to respectively isolate and characterize the molecular and regioisomeric structures of NACys adducts corresponding to the mono-NACys adduct (peak 5) and the two di-NAC adducts (peaks 3 and 6). The tri-NACys adduct (peak 4) was also isolated. Several attempts to isolate MC quinone at 4.23 min (peak 1) were unsuccessful due to the high reactivity and instability of the compound. For ease of comparison with other publications, we adopted the atom numbering system for the catechol ring shown in Figure 3. The obtained NMR data are summarized as follows:

5-NACys MC (Peak 5): ^{1}H NMR (500 MHz, CD_3OD, δ, ppm): 6.76 (s, 1H, H-6), 6.69 (s, 1H, H-2), 4.39 (dd, 1H, *J* = 4.5, 8.0 Hz, NACys -CH-), 3.36-3.32 (m, 1H, NACys -CH_2-), 3.15-3.10 (m, 1H, NACys -CH_2-), 2.12 (s, 3H, H-7), 1.84 (s, 3H, NACys CH_3-CO). ^{13}C NMR (125 MHz, CD_3OD, δ, ppm): 174.0 (NACys CH_3-CO-), 173.4 (NACys -COOH), 146.5 (C-3), 144.7 (C-4), 130.7 (C-1), 126.4 (C-6), 120.7 (C-5), 117.6 (C-2), 54.2 (NACys -CH-), 37.1 (NACys -CH_2-), 22.5 (NACys -CH_3), 20.9 (C-7).

5,6-NACys MC (Peak 3): ^{1}H NMR (500 MHz, CD_3OD, δ, ppm): 6.75 (s, 1H, H-2), 4.52 (dd, 1H, *J* = 4.5, 7.3 Hz, NACys -CH-), 4.42 (dd, 1H, *J* = 4.0, 8.0 Hz, NACys -CH-), 3.37-3.27 (m, 3H, NACys -CH_2-), 3.11-3.07 (m, 1H, NACys -CH_2-), 2.40 (s, 3H, H-7), 1.98 (s, 3H, NACys CH_3-CO), 1.96 (s, 3H, NACys CH_3-CO). ^{13}C NMR (125 MHz, CD_3OD, δ, ppm): 173.5 (NACys CH_3-CO-), 173.3 (NACys CH_3-CO-), 173.0 (NACys -COOH), 172.9 (NACys -COOH), 146.9 (C-3), 146.5 (C-4), 136.8 (C-1), 127.5 (C-6), 125.5 (C-5), 118.7 (C-2), 53.6 (NACys -CH-), 53.5 (NACys -CH-), 38.9 (NACys -CH_2-), 38.5 (NACys -CH_2-), 22.5 (NACys -CH_3), 22.2 (NACys -CH_3), 22.0 (C-7).

2,5-NACys MC (Peak 6): ^{1}H NMR (500 MHz, CD_3OD, δ, ppm): 6.84 (s, 1H, H-6), 4.53 (dd, 1H, *J* = 4.5, 8.5 Hz, NACys -CH-), 4.43 (dd, 1H, *J* = 4.0, 8.0 Hz, NACys -CH-), 3.40-3.36 (m, 1H, NACys -CH_2-), 3.30-3.26 (m, 1H, NACys -CH_2-), 3.15-3.11 (m, 1H, NACys -CH_2-), 3.07-3.03 (m, 1H, NACys -CH_2-), 2.36 (s, 3H, H-7), 1.94 (s, 3H, NACys CH_3-CO), 1.94 (s, 3H, NACys CH_3-CO). ^{13}C NMR (125 MHz, CD_3OD, δ, ppm): 175.1 (NACys CH_3-CO-), 173.4 (NACys CH_3-CO-), 173.2 (NACys -COOH), 173.1 (NACys -COOH), 147.8 (C-3), 144.1 (C-4), 134.4 (C-1), 125.6 (C-6), 122.3 (C-5), 119.7 (C-2), 53.9 (NACys -CH-), 53.6 (NACys -CH-), 36.8(NACys -CH_2-), 36.0 (NACys -CH_2-), 22.2 (NACys -CH_3), 20.6 (NACys -CH_3), 20.6 (C-7).

2,5,6-NACys MC (Peak 4): ^{1}H NMR (500 MHz, CD_3OD, δ, ppm): 4.50 (dd, 1H, *J* = 4.5, 8.0 Hz, NACys -CH-), 4.45 (dd, 1H, *J* = 5.0, 8.0 Hz, NACys -CH-), 4.40 (dd, 1H, *J* = 4.0, 8.5 Hz, NACys -CH-), 3.41-3.35 (m, 2H, NACys -CH_2-), 3.34-3.27 (m, 2H, NACys -CH_2-), 3.13-3.07 (m, 2H, NACys -CH_2-), 2.71 (s, 3H, H-7), 1.98 (s, 3H, NACys CH_3-CO), 1.96 (s, 3H, NACys CH_3-CO), 1.95 (s, 3H, NACys CH_3-CO). ^{13}C NMR (125 MHz, CD_3OD, δ, ppm): 173.6 (NACys CH_3-CO-), 173.5 (NACys CH_3-CO-), 173.5 (NACys CH_3-CO-), 173.3 (NACys -COOH), 173.3 (NACys -COOH), 173.2 (NACys -COOH), 149.0 (C-3), 146.3 (C-4), 140.5 (C-1), 128.7 (C-6), 127.2 (C-5), 121.9 (C-2), 53.9 (NACys -CH-), 53.8 (NACys -CH-), 53.7 (NACys -CH-), 39.1 (NACys -CH_2-), 38.5 (NACys -CH_2-), 36.8 (NACys -CH_2-), 22.5 (NACys -CH_3), 22.5 (NACys -CH_3), 22.4 (NACys -CH_3), 21.3 (C-7).

In the ^{1}H NMR spectrum of the mono-NACys adduct (peak 5), two proton signals are observed in the aromatic region, indicating that the addition of NACys occurs on the benzene ring. Due to the lack of an AB coupling pattern in the same region, this addition likely occurs on either the 5-position or the 6-position.

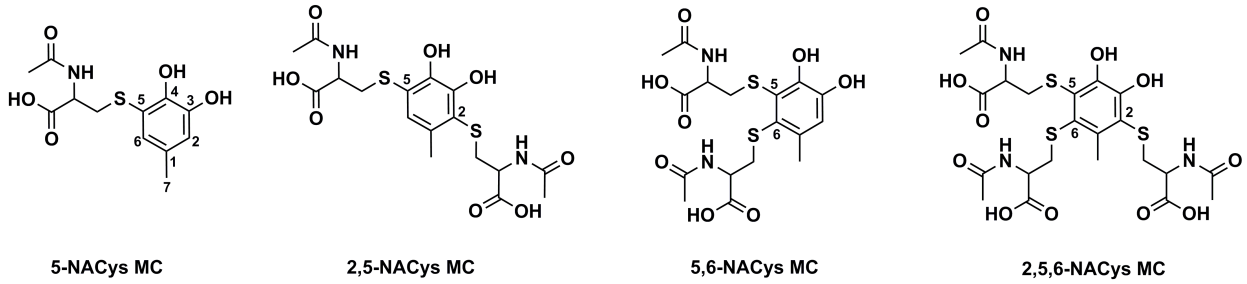


Figure 3. Structures of isolated products in the reaction of MC and DPPH in the presence of NACys.

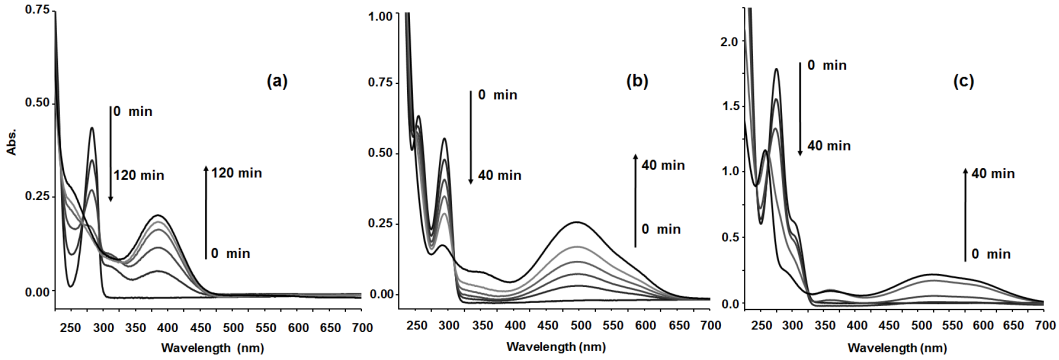


Figure 4. UV-Vis absorption spectral change during the reaction of MC and its NACys adducts with periodate resin in CH_3CN. (a) MC (b) 5-NACys MC (c) 2,5-NACys MC.

Moreover, in the HMBC experiment, the CH_3 carbon (C-7) at 22.5 ppm was correlated with the two singlet aromatic protons at δ 6.76 (H-6) and δ 6.69 ppm (H-2). These findings indicate that the attachment position is not adjacent to the methyl group, but rather at position 5. Thus, the mono-NACys adduct was identified as 5-NACys MC.

The ^{1}H NMR spectrum of the di-NACys adduct-1 (peak 3) shows only a single aromatic proton, at δ 6.75 ppm. In the HMBC spectrum, this proton signal was correlated with C-4 at δ 146.5 and C-6 at δ 127.5 ppm. From these NMR data, the di-NACys adduct-1 was identified as 5,6-NACys MC.

The di-NAC adduct-2 (peak 6) also shows only a single aromatic proton, at δ 6.84 ppm in the ^{1}H NMR spectrum. In the HMBC spectrum, C-2 at δ 119.7, C-4 at δ 144.1, and C-7 at δ 20.6 ppm were correlated with this proton signal. From these NMR data, the di-NACys adduct-2 was identified as 2,5-NACys MC.

No proton signal was observed for the tri-NACys adduct (peak 4) in the aromatic region of the ^{1}H NMR spectrum. Protons originating from the three NACys groups at the C-2, C-5 and C-6 positions were observed as a separate set of signals. These findings conclusively confirm the structure of 2,5-6-tri-NACys MC.

To date, we have succeeded in isolating 5-NACys MC, 2,5-NACys MC, 5,6-NACys MC and 2,5,6-NACys MC as reaction products for the DPPH oxidation of MC in presence of NACys. The addition reactions of thiol nucleophiles to *o*-quinones have been extensively studied and comprehensively reviewed by Yang *et al.* [36]. Several authors have focused their studies on the regioselectivity of this reaction. Quantitative product analysis for the reaction of enzymatically generated dopaquinone with cysteine revealed that 5-cysteinyldopa is the primary product, with 2-cysteinyldopa being produced as a regioisomer [37,38]. Huang et al. have studied the electrochemical oxidation of *N*-acetyldopamine in the presence of *N*-acetylcysteine and demonstrated that the major adducts are 5-adducts [39]. The reaction of *N*-acetylcysteine with *N*-acetyldopamine quinone also gives similar products [40]. Thus, the reaction products obtained in the present work show regioselectivities similar to that observed for cysteinyldopa.

3.4. Oxidation of MC derivatives using periodate resin and subsequent NACys addition

We also used polymer-supported periodate resin as an oxidant for the oxidation of MC. The oxidant resin was removed from the reaction solution by filtration following a designated reaction time, then aliquots of this solution were removed and reacted with NAC. The use of periodate resins enabled us to follow the *o*-quinone formation and NACys addition steps separately using UV absorption and LC-MS spectral measurements.

3.4.1. Oxidation of MC and NACys adducts by periodate resin

As shown in Figure 4a, MC in CH_3CN solution shows an absorption band at 284 nm (ε 2,600), characteristic of the π-π* electron transitions of phenolic compounds. When periodate resin was added to the MC solution, the colorless MC solution turned pale yellow and the yellow absorption band gradually decreased in intensity as the intensity of the 391 nm absorption band increased. The reaction reached equilibrium after a 120 min incubation period and no further spectral changes were observed. A similar spectral change was reported for the electrolytic oxidation of MC [26,41] and the absorption at 391 nm was assigned to the π-π* transition of *o*-quinone [42]. No clear isosbestic point was observed for this UV-Vis spectral change, suggesting that minor oxidation products in addition to *o*-quinone may be involved in this reaction. By assuming that MC quinone is the only oxidation product, we estimated that ~95% of MC is converted to MC quinone, based on HPLC analysis. In accordance with the UV results, LC-MS analysis of the solution at 120 min showed two peaks, assignable to MC quinone and MC, which correspond to peak 1 and peak 2 at 4.22 min ($[M-H]^-$ at *m/z* 123) and 6.57 min ($[M-H]^-$ at *m/z* 123), respectively.

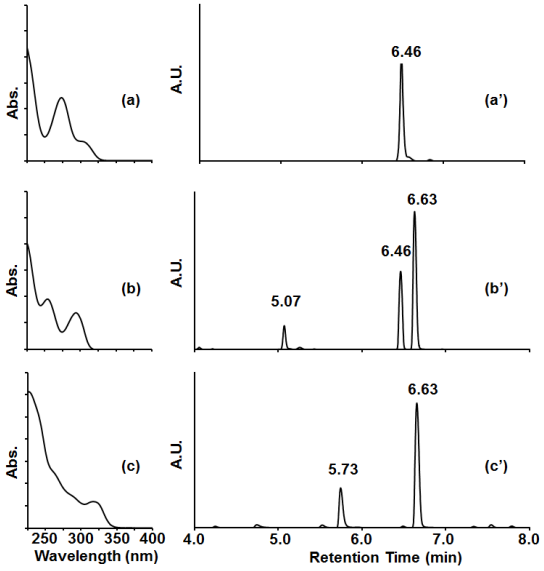


Figure 5. UV-Vis absorption spectra and LC-MS chromatograms of reaction mixtures of NACys and quinone. (a) (a') NACys (4 equiv.) was added to a solution of MC quinone at a reaction time of 120 min. (b) (b') NACys (4 equiv.) was added to a solution of 5-NACys MC quinone at a reaction time of 40 min. (c) (c') NACys (4 equiv.) was added to a solution of 2,5-NACys MC quinone at a reaction time of 40 min.

Upon adding periodate resin to the 5-NACys MC solution, the colorless 5-NACys MC solution turned orange. As shown in Figure 4b, 5-NACys MC showed two absorptions, at 255 (ε 3,200) and 295 nm (ε 2,800), and the intensities of these absorption bands gradually decreased and a new broad band appeared, centered at 502 nm, which can be assigned to the π-π* transition of 5-NACys MC quinone. The reaction appears to reach equilibrium after 40 min. LC-MS analysis of the solution at 40 min showed two LC peaks, at 5.30 ($[M-H]^-$ at *m/z* 282) and 6.46 min ($[M-H]^-$ at *m/z* 284), which can be reasonably assigned to 5-NACys MC quinone and 5-NACys MC, respectively. The conversion ratio of 5-NACys MC to 5-NACys MC quinone was estimated to be ~98%, based on the HPLC analysis.

When periodate resin was added to the 2,5-NACys MC solution, the colorless 2,5-NACys MC solution turned pale violet. As shown in Figure 4c, 2,5-NACys MC showed absorption at 277 nm (ε 9,150) with a shoulder at 305 nm (ε 3,240). Upon adding periodate resin, these absorption bands gradually decreased in intensity and a new broad band appeared, centered at 525 nm with a sharp absorption at 260 nm, and can be assigned to the π-π* transition of 2,5-NACys MC quinone. The reaction appeared to reach equilibrium after 40 min, and LC-MS analysis showed two LC peaks, at 5.69 min ($[M-H]^-$ at *m/z* 443) and 6.64 min ($[M-H]^-$ at *m/z* 445), which can be reasonably assigned to 2,5-NACys MC quinone and 2,5-NACys MC, respectively. The conversion ratio of 2,5-NACys MC to 2,5-NACys MC quinone was estimated to be ~96%, based on HPLC analysis.

The absorption maxima of the *o*-quinone species moved from 391 nm for MC quinone to longer wavelengths: 502 nm for 5-NACys MC quinone and 525 nm for 2,5-NACys MC quinone. These systematic absorption shifts may be explained in terms of the substituent effect, since an electron-donating substituent generally shifts the π-π* absorption band of the conjugate system to longer wavelengths [42].

3.4.2. Reaction of MC and NACys MC quinones with NACys

Immediately after the oxidation reactions, shown in Figures 4a-c, reached equilibrium, the periodate resin was removed by filtration and NACys was added to the solution. The addition of NACys to the quinone solutions resulted in UV spectral changes, including rapid disappearance of quinone absorptions in the visible region. In this section, we discuss the UV and LC-MS spectra of these reaction samples.

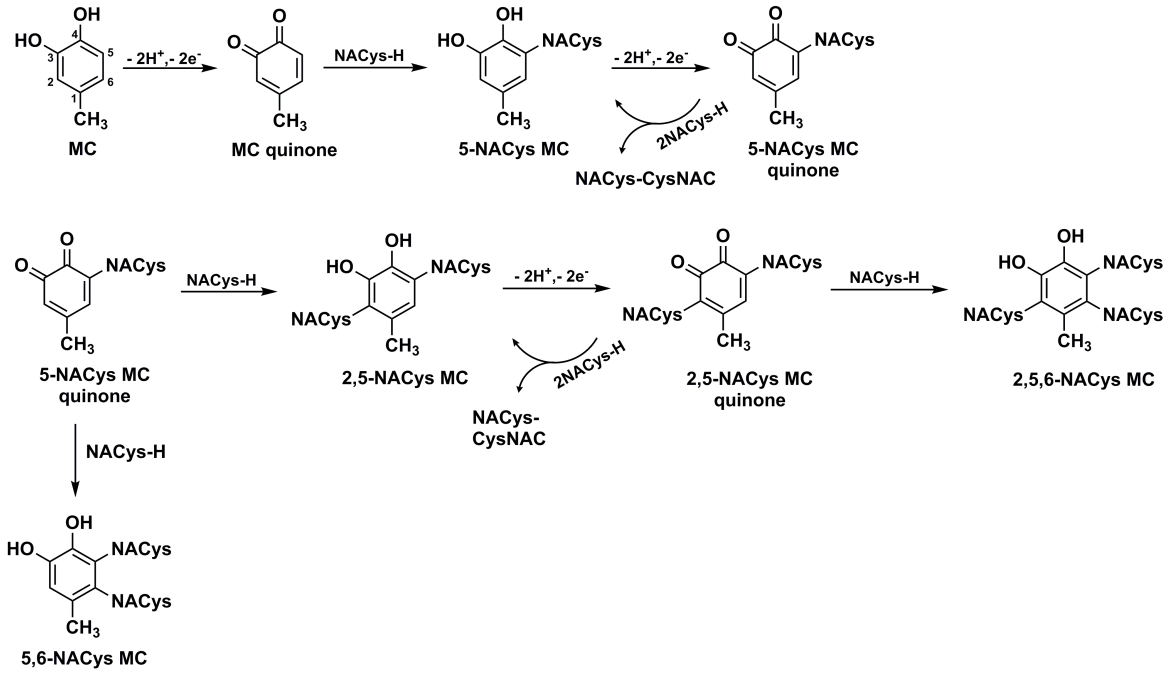
Figures 5a and 5a' show the UV absorption spectra and HPLC chromatogram obtained 120 min after NACys was added to an MC quinone solution. The UV absorption spectra show two absorptions, at 255 and 295 nm, which are characteristic of 5-NACys MC. The HPLC chromatogram shows a single peak, at 6.46 min, with an $[M-H]^-$ at *m/z* 284. These observations indicate that MC quinone formed in the periodate oxidation reaction is exclusively transformed into 5-NACys MC.

The reaction of NACys with 5-NACys MC quinone results in a rather obscure UV spectral pattern, with an absorption at 295 nm, as shown in Figure 5b. HPLC peaks are observed at 6.46 ($[M-H]^-$ at *m/z* 284) and 6.63 min ($[M-H]^-$ at *m/z* 445), with a minor peak at 5.07 min ($[M-H]^-$ at *m/z* 445). The retention times of these peaks correspond to 5-NACys MC, 2,5-NACys MC and 5,6-NACys MC, respectively. These results indicate that 5-NACys MC quinone formed in the periodate oxidation reaction is transformed into 2,5-NACys MC and 5,6-NACys MC as the major and minor reaction products, respectively. Since a considerable amount of 5-NACys MC, the starting material, is also detected, 5-NACys MC quinone produced is likely partly reduced by NACys back to 5-NACys MC.

The reaction of 2,5-NACys MC quinone with NACys gives rise to a rather unresolved spectral pattern, as shown in Figure 5c. HPLC peaks are observed at 6.63 min ($[M-H]^-$ at *m/z* 445) as the major component and at 5.73 min ($[M-H]^-$ at *m/z* 606) as a minor component, with the former being assigned to 2,5-NACys MC and the latter to 2,5,6-NACys MC. These findings indicate that 2,5-NACys MC quinone formed in the periodate oxidation reaction is mainly reduced back to 2,5-NACys MC and some is transformed into 2,5,6-NACys MC by the addition of NACys.

4. Conclusion

In summary, we propose the reaction mechanism for the oxidation of MC and subsequent reaction with NACys shown in Scheme 2. Under the reaction conditions used in this work, MC undergoes oxidation to form *o*-quinone (MC quinone), then subsequent nucleophilic addition of NACys results in the formation of 5-NACys MC as the major mono-NACys adduct.



Scheme 2. Plausible reaction mechanism for the DPPH radical scavenging reaction of MC in the presence of NACys.

In the next cycle of oxidation, 5-NACys MC is transformed into 5-NACys MC quinone. By reacting with excess NACys, this quinone is converted through nucleophilic addition mainly to 2,5-NACys MC, with 5,6-NACys MC as a minor product. This reaction process is completed with direct reduction by NACys to form 5-NACys MC, producing the cystine compound NACys-NACys. Oxidation of 2,5-NACys MC gives rise to 2,5-NACys MC quinone, then subsequent reaction with NACys results in direct reduction primarily to give 2,5-NACys MC and a small amount of 2,5,6-NACys MC through nucleophilic addition of NACys.

The production of NACys adducts in the nucleophilic addition step leads to regeneration of the catechol structure. One step of NACys adduct formation corresponds to a formal two-electron reduction. Thus, sequential NACys adduct formation leads to enhanced DPPH radical scavenging activity.

Acknowledgements

This work was supported by Kanazawa University SAKIGAKE project. The authors thank Dr. M. Suda of Technical Support Division, Institute of Science and Engineering, Kanazawa University for assistance with the NMR measurements.

Disclosure statement

Conflict of interests: The authors declare that they have no conflict of interest.
Author contributions: All authors contributed equally to this work.
Ethical approval: All ethical guidelines have been adhered.
Sample availability: Samples of the compounds are available from the author.

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