Studies on the 1,1-Diphenyl-2-picrylhydrazyl Radical Scavenging Mechanism for a 2-Pyrone Compound

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The radical scavenging mechanisms for the 2-pyrone compound, 4-hydroxy-3,6-dimethyl-2H-pyran-3-yl (1), and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (4) in several solvent systems were evaluated by the quantitative change in compounds detected at 270 nm and subsequent HPLC analyses. The HPLC profile for each condition suggested that the reaction proceeded by a different mechanism in each solvent system. In organic solvents (CHCl₃, i-Proan, and EtOH), 1-[4-(3,4-dihydro-3,6-dimethyl-2,4-dioxo-2H-pyran-3-yl) phenyl]-1-phenyl-2-picrylhydrazine (2) was produced as an adduct of the DPPH radical and 1. On the other hand, the reaction in a buffer solution (an acetate buffer at pH 5.5) gave several degradation products with 1-[4-(2,3-dihydro-2,5-dimethyl-3-oxo-fur-2-y1) phenyl]-1-phenyl-2-picrylhydrazine (5), this being structurally elucidated by spectroscopic analyses. The decrease of the DPPH radical in each reaction system suggests that compound 1 could scavenge about 1.5-1.8 equivalents of the radical in organic solvents and about 3.5-3.9 in the buffer solution.

Key words: 1,1-diphenyl-2-picrylhydrazyl; radical scavenger; radical scavenging mechanism; 2-pyrone

In the course of our screening program for radical scavengers from the fermentation broth of microorganisms, we have previously reported the isolation and structural determination of four ansamycin-type,1′ two oxazolyl-type and one α-pyrone-type compounds3 as free radical scavengers. We applied our screening method that uses the bactericidal action of the hydroxyl radical. Another screening program for free radical scavengers3 gave us eight sorbicillin-related compounds as DPPH radical scavengers,4,5,6 USF2550A (1, 4-hydroxy-3,6-dimethyl-2H-pyran-2-one) as an indophenol-reducing compound was isolated from unidentified fungal strain USF-2550. In addition, compound 1 also reacted with the DPPH radical in EtOH to yield 1-[4-(3,4-dihydro-3,6-dimethyl-2,4-dioxo-2H-pyran-3-yl) phenyl]-1-phenyl-2-picrylhydrazine (2) as an adduct of the DPPH radical and 1.7 In this present study, we elucidate the radical scavenging mechanisms for compound 1 and the DPPH radical on the basis of the behavior of compounds in the reaction mixture.

Materials and Methods

Chemicals. DPPH, α-tocopherol and the other reagents were analytical-grade products from Wako Pure Chemical Industries, Japan. USF2550A (1) was isolated from the fermentation broth of fungal strain USF-2550, and compound 2 was prepared by using the previously reported method.7

Instruments. Spectroscopic measurements were taken with the following instruments: a JEOL Alpha-400 spectrometer (NMR), a Hitachi 270-50 infrared spectrometer (IR), a Shimadzu UV-160A spectrometer (UV and visible spectra), a JEOL DX-303 or JEOL JMS-SX102 spectrometer (MS and HR-MS), and a Horiba SEPA-200 high-sensitivity polarimeter (optical rotation). HPLC was carried out with Jasco PU-980 pump connected to a Jasco UV-970 spectrometer (270 nm).

Measurement of the DPPH-radical scavenging activity. An ethanol solution of a sample (2 ml) was mixed with a 0.5 mM DPPH ethanol solution (1 ml) and 0.1 M acetate buffer (pH 5.5; 2 ml). After standing for 0.5 hr and monitoring at 0.5, 3, 6 and 24 hr, the absorbance of the mixture at 517 nm was measured. The DPPH-radical scavenging activity of each sample was determined as the percentage decrease in the absorbance shown by a blank test.

Analysis of the reaction products between 1 and the DPPH radical (4) from time-course studies. One

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Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; HMBC, 1H-detected heteronuclear multiple-bond connectivity; HPLC, high-performance liquid chromatography
ml of a 3.5 mM USF2550A (I) ethanol solution was slowly added to 4 ml of a 1.0 mM DPPH ethanol solution, and the mixed solution was stirred at room temperature for 8 hr. At 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 hr after starting the reaction, a 15-μl aliquot of the solution was withdrawn and injected into an analytical HPLC system under the following conditions: column, Wakosil-II 5C18 HG (θ7.5×250 mm, Wako Pure Chemical Industries, Japan); solvent system, 0.15% KH2PO4 (pH 3.5) solution (solvent A) and CH3CN (solvent B), a gradient program made up of linear segments with 15% of solvent B (from 0 to 5 min), from 15% of solvent B to 60% of solvent B (from 5 to 10 min), from 60% of solvent B to 80% of solvent B (from 10 to 25 min), and from 80% of solvent B to 15% of solvent B (from 25 to 27 min); flow rate, 2.6 ml/min; detection, 270 nm. The peak area was integrated by a Chromatocorder 21 (SIC, Japan).

One ml of a 3.0 mM I iso-propanol solution was slowly added to 4 ml of a 0.9 mM DPPH iso-propanol solution, and the reaction mixture was stirred at room temperature for 8 hr. The reaction products were analyzed in the same manner as that already described.

One ml of a 0.75, 2.2 or 10 mM I chloroform solution was slowly added to 4 ml of a 1.1, 1.1 or 1.9 mM DPPH chloroform solution, respectively, and each mixed solution was stirred at room temperature for 8 hr. The reaction products were analyzed in the same manner as that already described.

One ml of a 3.1 or 0.13 mM I ethanol solution was slowly added to a mixture of 2 ml of a 1.4 or 0.93 mM DPPH ethanol solution and 2 ml of a 0.1 M acetic acid buffer (pH 5.5) solution, and the reaction mixture was stirred at room temperature for 8 hr. The reaction products were analyzed in the same manner.

Analysis of the reaction products between 2 and the DPPH radical (4) from time-course studies. One ml of a 0.95 or 0.12 mM compound 2 ethanol solution was slowly added to a mixture of 2 ml of a 0.25 or 0.93 mM DPPH ethanol solution and 2 ml of a 0.1 M acetic acid buffer (pH 5.5) solution, and the reaction mixture was stirred at room temperature for 8 hr. The reaction products were analyzed in the same manner.

Isolation of reaction product 5. Compound 2 (21.2 mg) was dissolved in ethanol (8 ml), and the solution stirred at ambient temperature. Four ml of a 0.1 M acetic acid buffer (pH 5.5) solution was then added. The reaction mixture was stirred for 24 hr at room temperature. Ethanol was removed in vacuo from the reaction mixture, and then 10 ml of distilled water was added to the residual solution. The reaction products were extracted with the same volume of ethyl acetate. The resulting ethyl acetate extract was dried over Na2SO4 and concentrated in vacuo to give an oily residue. This residue was chromatographed on Sephadex LH-20, eluting with ethanol, to give the desired fraction. This fraction was concentrated in vacuo and subsequently applied to preparative HPLC under the following conditions: column, Capcell pak C18 SG120 (φ15×250 mm, Shiseido, Japan); solvent system, acetonitrile-H2O (55:45, 0.2% trifluoroacetic acid); flow rate, 8.9 ml/min; detection, 270 nm. Pure compound 5 (6.4 mg) was given as reddish-brown powder.

1-[4-(2,3-dihydro-2,5-dimethyl-3-oxo-fur-2-yl) phenyl]-1-phenyl-2-picyrylhydrazine (5). Reddish-brown powder; IR

\[ \nu_{\text{max}} \text{ (KBr) cm}^{-1}: 3300, 1705, 1625, 1600, 1545, 1515, 1345, 1305; \text{HREAB-MS} \text{ } m/z: 506.1272 \left( M^+ \right), 506.1312 \text{ for } C_{21}H_{19}N_{10}O_{6}; \text{UV } \nu_{\text{max}} \text{ nm (c, CH}_{2}OH): 256.5 (39, 200), 301 (sh 21,800). \] 1H-NMR (400 MHz, CDCl3) \( \delta \): 10.12 (1H, s, NH), 9.19 (1H, br, s, H-5"H or 3'-H), 8.50 (1H, br, s, 3'-H or 5"-H), 7.45 (2H, d, \( J = 8.8 \text{ Hz}, 3'-'H \) and 5"-H), 7.35 (2H, d, \( J = 8.8 \text{ Hz}, 3'-'H \) and 5"-H), 7.21 (1H, dd, \( J = 7.4 \text{ and } 7.4 \text{ Hz}, 4^\prime-',H \)), 7.09 (2H, d, \( J = 8.8 \text{ Hz}, 2'-'H and 6'-H)), 7.07 (2H, d, \( J = 8.8 \text{ Hz}, 2'-'H and 6'-H)), 5.40 (1H, s, 4.2, 3.31 (3H, s, 5-CH3), 1.71 (3H, s, 2-CH3).\) 13C-NMR (100 MHz, CDCl3) \( \delta \): 204.5 (s, C-3), 188.6 (s, C-5), 145.8 (s, C-1"'), 145.8 (s, C-1''), 142.0 (s, C-1'-'), 136.7 (s, C-4"'), 135.6 (s, C-4'), 129.7 (d, C-3-" and C-5"), 126.3 (d, C-4"'), 126.0 (d, C-3'-' and C-5''), 125.1 (d, C-3" or C-5"), 125.0 (d, C-5" or C-3''), 121.0 (d, C-2'-' and C-6"), 120.1 (d, C-2'-' and C-6''), 102.2 (d, C-4), 90.0 (s, C-2), 24.4 (q, 2-CH3), 16.9 (q, 5-CH3). C-2"- and C-4"- did not appear.

Methyl 5-hydroxy-2-methyl-3-oxo-2-[4-(1-phenyl-2-picyrylhydrazino)phenylene]-4-hexenoate (6). Compound 2 was applied to a Sephadex LH-20 column, eluting with methanol, to give a new compound yielded by methanolysis as reddish-brown powder.

IR \( \nu_{\text{max}} \text{ (KBr) cm}^{-1}: 3300, 1740, 1620, 1600, 1540, 1300, 1260; \text{HREAB-MS} \text{ } m/z: 565.1454 \left( M^+ \right), 565.1454 \text{ for } C_{26}H_{23}N_{10}O_{6}; \text{EI-MS} \text{ } m/z: 565, 480, 393, 254, 240, 194; \text{UV } \nu_{\text{max}} \text{ nm (c, CH}_{2}OH): 269 (29,300). \] 1H-NMR (400 MHz, CDCl3) \( \delta \): 15.01 (1H, br, s, 5-CH3), 10.15 (1H, br, s, NH), 9.18 (1H, br, s, 5-"H or 3-"H), 8.48 (1H, br, s, 3-"H or 5-"H), 7.35 (2H, d, \( J = 8.0 \text{ and } 8.0 \text{ Hz}, 3'-'H \) and 5"-H), 7.29 (2H, d, \( J = 8.8 \text{ Hz}, 3'-'H \) and 5"-H), 7.21 (1H, brt, \( J = 8.0 \text{ Hz}, 4^\prime-,H \) and 4"-H), 7.06 (2H, d, \( J = 8.8 \text{ Hz}, 2'-'H \) and 6'-'H)), 5.38 (1H, s, 4-CH3), 3.74 (3H, s, OCH3), 2.00 (3H, s, 6-CH3), 1.80 (3H, s, 2-CH3). 13C-NMR (100 MHz, CDCl3) \( \delta \): 196.7 (s, C-3), 187.1 (s, C-5), 172.2 (s, C-1), 145.4 (s, C-1"'), 145.3 (s, C-1''), 141.8 (s, C-1'-'), 139.9 (s, C-2'-' or C-6"'), 136.7 (s, C-4"'), 135.7 (s, C-4"'), 133.7 (s, C-6" or C-2"'), 129.7 (d, C-3-" and
Results and Discussion

**DPPH-radical scavenging activity**

USF2550A (1) and $\alpha$-tocopherol were submitted to the assay system with DPPH. The DPPH-radical scavenging activity of USF2550A (1) was evaluated by periodically measuring the absorbance at 517 nm and as expressed as the percentage decrease in the absorbance shown by a blank test. The dose-dependent manner at concentrations ranging from 25 to 800 $\mu$M of 1 at each measurement time is shown in Fig. 2. The results of this experiment suggest that the DPPH-radical scavenging activity of 1 depended on the reaction period. In other words, this radical scavenging reaction between 1 and the DPPH radical proceeded gradually. The scavenging activity of $\alpha$-tocopherol, as a positive control, did not show such a time-dependent manner.

**Analysis of the reaction products between USF2550A (1) and the DPPH radical (4) from time-course studies**

HPLC profiles of the reaction mixture between 1 and the DPPH radical (4) in organic solvents and in a 0.1 M acetate buffer (pH 5.5) solution are shown in Fig. 3. In organic solvents (EtOH, isopropanol and CHCl$_3$), four peaks were detected with retention times of 3.9, 16.2, 17.5, and 18.4 min in the chromatograms (Fig. 3A). The respective peaks were assigned to 2550A (1, 3.9 min), compound 2 (16.2 min), 1,1-diphenyl-2-picyrhydrazine (DPPH + H, 3, 17.5 min) produced by reduction of the DPPH radical, and the DPPH radical (4, 18.4 min) on the basis of the isolation study and structural elucidation of the reaction products. On the other hand, in a 0.1 M acetate buffer (pH 5.5) solution, three main peaks were observed of 3.9 (1), 17.5 (3) and 18.4 min (4). The peak of the adduct (2) at 16.2 min was not detected under this condition (Fig. 3B). The time-course characteristics for the accumulation of each compound, as shown in Fig. 4, were analyzed by HPLC. A, B and C in Fig. 4 and Fig. 5 indicate...
that, independent of the relative proportions of USF2550A (1) and the DPPH radical (4), 1 USF2550A (1) that disappeared from the reaction mixture was utilized for adduct 2, 2 all the removed DPPH radical (4) was changed to 2 or 3, and 3 only about 25-45% of hydrogen atoms were obscured in the organic solvent systems. Furthermore, the DPPH-radical (4) scavenging reaction proceeded faster in CHCl₃ than in EtOH or in iso-propanol. In contrast, Fig. 4D shows that consumed 1, which was detected as adduct 2 in Fig. 4A-C, was completely missing, whereas the DPPH radical (4) was gradually scavenged in the buffer solution to yield DPPH + H (3).

**Analysis of the reaction products between the adduct (2) and the DPPH radical (4) from time-course studies**

Observation of the reaction between 2550A (1) and the DPPH radical (4) in the buffer solution suggested that adduct 2 was degraded to give several products under the prevailing conditions and that some of the degradation products could scavenge the DPPH radical (4). The time-course characteristic for the accumulation of each compound in the reaction between adduct 2 and the DPPH radical (4) are shown in Fig. 5. Adduct 2 disappeared rapidly in the reaction mixture, while DPPH + H (3) increased slowly. It seems that the result of the experiment supports some of the degradation products from 2 being DPPH-radical scavengers. An attempt to induce the degradation products from 2 in the buffer solution provided one major product (5) and many minor ones. Product 5 was inactive against the DPPH radical; therefore, 5 was one of the terminal compounds in the reaction between 1 and the DPPH radical (4) in the buffer solution. The minor degradation products which had DPPH radical scavenging activity could not be identified.

**Structural determination of the degradation product (5)**

Adduct 2 was dissolved in ethanol and then a 0.1 M acetate buffer (pH 5.5) solution was added to the solution. The reaction mixture was stirred for 24 hr
Fig. 5. Time-Course Studies on the Accumulation of Compounds 1, 2, 3 and 4 with Different Proportions of 1 and 4 in CHCl₃.
A USF2550A (1):DPPH (4) = 1:2; B USF2550A (1):DPPH (4) = 1:6; • USF2550A (1); ◆ Adduct 2; ▲ DPPH + H (3); ■ DPPH (4).

Fig. 6. Time-Course Studies on the Accumulation of Each Compound in the Reaction between Adduct 2 and the DPPH Radical (4).
◆ Adduct (2); ■ DPPH (4); ◆ DPPH + H (3); ◆ Compound 5.

at room temperature. Sephadex LH-20 column chromatography with MeOH converted residual adduct 2 into methanolysis product 6; hence, purification of degradation product 5 was performed by using ethanolic LH-20 chromatography and subsequently preparative HPLC. Major degradation product 5 derived from the adduct (2) was obtained as reddish-brown powder and formulated as C₉₄H₅ₐN₂O₃ from the HREI-MS data. The UV and visible spectra of 5 were similar to those of DPPH + H (3) and the adduct (2). The ¹H- and ¹³C-NMR spectra in CDCl₃ indicate that the structure of 5 was merely changed in the 3,4-dihydro-3,6-dimethyl-2,4-dioxo-2H-pyran-3-yl moiety of adduct 2. Assignments of the ¹H- and ¹³C-NMR spectral data of 5 were obtained from the HMBC experiments, a summary of the HMBC experiment for 5 being shown in Fig. 7. The cross peaks between two methyl groups and three methines and the carbons, which were given by the HMBC spectrum, enabled a planar 4-(2,3-dihydro-2,5-dimethyl-3-oxo-fur-2-yl)phenyl moiety to be constructed; cross peaks were between 2-CH₃ (δH 1.71) and C-2 (δC 90.0), C-3 (δC 204.5) and C-4' (δC 135.6), between 5-CH₃ (δH 2.32) and C-4 (δC 102.2) and C-5 (δC 188.6), between 4-H (δH 5.40) and C-2 and C-5, between 5'-H (δH 7.45) and C-2, and between 3'-H (δH 7.45) and C-2. As a result, we determined the structure of degradation product 5 to be that shown in Fig. 1.

Proposed mechanism for the DPPH radical scavenging activity of USF2550A (1)

Our proposed mechanisms for the DPPH radical scavenging activity of 1 are illustrated in Fig. 8. In organic solvents, USF2550A (1) donated a hydrogen atom to the DPPH radical to yield a DPPH-H radical pair, and a newly generated USF2550A radical connected with another DPPH radical at the position without steric hindrance to give a USF2550A-DPPH radical pair. As the result of an “in-cage reaction”, adduct 2 and DPPH + H (3) were produced and detected in the reaction mixture. Time-course studies for the accumulation of each compound in the reaction revealed some interesting information: 1) All consumed USF2550A (1) was changed into adduct 2. 2) The quantity of the DPPH radical (4) missing from the reaction mixture is consistent with the sum of adduct 2 and DPPH + H (3). 3) If the reaction proceeded as an “in-cage reaction” system, the concentration of consumed USF2550A (1), produced DPPH + H (3) and produced adduct 2, respectively, gave the same value; however, that of
produced 3 was 25–45% less than that of produced 2 [i.e. consumed 1]. It seems that 25–45% of the DPPH-H radical pair may have been resolved into a DPPH radical molecule and a hydrogen atom, which might have been absorbed by the organic solvents through an “out-of-cage reaction”. The observation of the experiments shows that one molecule of USF2550A (1) could consequently scavenge about 1.5–1.8 molecules of the DPPH radical.

On the other hand, in the reaction between 1 and the DPPH radical (4) with the 0.1 M acetate buffer (pH 5.5) solution system, it seems that DPPH-H (3) and adduct 2 were produced in the same manner that as just described; however, adduct 2 was rapidly degraded in the system. We expect, therefore, that adduct 2, whose quantity was the same as that of consumed 1, and DPPH + H (3), whose quantity was about 40% less than that of consumed 1, were formed in the first step, and in the next step, adduct 2 was completely degraded and the degradation products of 2 scavenged additional molecules of the DPPH radical (4). For example, at 8 hr in Fig. 4D, the production of 2 (0.28 mM) and 3 (0.17 mM) was expected to consume 0.28 mM of 1, and 0.45 mM of 4 was scavenged. After the adduct 2 had been completely degraded, the products derived from 2 could
scavenge the remaining molecules of 4 (0.09 mM) to finally give detectable 0.26 mM of 3. This premise is supported by the observation that degradation of adduct 2 also occurred while stirring 2 alone in the buffer solution. Furthermore, the increase of DPPH + H (3) in a time-dependent manner in the buffer reaction system between 2 and the DPPH radical (4) indicates that the degradation products of 2 could gradually scavenge the DPPH radical (4) (Fig. 6). The results reveal that the degradation products from one molecule of 2 could quench over one molecule of the DPPH radical (4) and, simultaneously, the degradation products of 2 were finally changed into several terminal compounds, including compound 5.

In addition, we performed experiments to estimate the ability of USF2550A (1) and adduct 2 to each scavenge the DPPH radical (4) in a 0.1 M acetate buffer solution. The results of the experiments are shown in Fig. 9. Figure 9A indicates that USF2550A (1) could scavenge gradually the DPPH radical (4), and 3.5–3.9 moles of the DPPH radical (4) were scavened with one mole of consumed USF2550A (1). In this experiment, 2.5–2.9 moles of DPPH + H (3) were yielded. On the other hand, Fig. 9B shows that the number of molecules of yielded DPPH + H (3) was equal to that of the missing DPPH radical (4) for the reaction between adduct 2 and the DPPH radical (4). These results mean that one molecule each of USF2550A (1) and the DPPH radical (4) could produce one molecule of adduct 2 in the buffer solution, and then adduct 2 was rapidly degraded. The degradation products arising from adduct 2 may be able to scavenge two additional molecules of the DPPH radical (4) to gradually yield the same number of molecules of DPPH + H (3) in the buffer solution; hence, one molecule of the consumed DPPH radical (4) was lost in the reaction between USF2550A (1) and the DPPH radical (4). Adduct 2, expected to be produced in the buffer solution, could scavenge 2 molecules of the DPPH radical (4). On the other hand, the isolated adduct 2 could scavenge 4 molecules of 4 (Fig. 9B). It seems that the gradual supply of adduct 2 in the buffer solution might decrease its ability to scavenge the DPPH radical (4). In conclusion, the ability that one molecule of USF2550A (1) could scavenge about 3.5–3.9 molecules of the DPPH radical (4) in the buffer solution is inferred from calculation of the accumulation of DPPH + H (3) and from the results of the experiments in organic solvents.

Studies on the antioxidant mechanism that was elucidated from the structures of reaction products have recently been reported. Masuda et al. have clarified the stable radical termination products from curcumin and examined the antioxidation process of curcumin on the basis of these products, and they have indicated that a dimer of curcumin was a radical-terminated product in the initial radical-scavenging stage. Yoshida et al. have also reported that gallic acid produced a dimer of gallic acid in the reaction with the DPPH radical. Sakata et al. have clarified that the reaction between (+)-catechin and the DPPH radical changed the two hydroxy groups of the B-ring in (+)-catechin into an ortho-quinone structure on the basis of their NMR analysis.

However, clarification of the radical scavenging mechanisms for antioxidants is not yet sufficient to explain their implication. Various types of antioxidant are required to prevent and provide therapy for diseases; hence, it is important to evaluate their characteristics. DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one) saponins are known as pyrone-type antioxidants. We have also reported that a 2-pyrene compound gave two oxidized products in the reaction between the 2-pyrene and the Fenton reagent. We described in the previous paper that the reaction between USF2550A (1) and the DPPH radical gave an adduct (2), this being the first adduct formed between a radical scavenger and the DPPH radical. In this report, on an analytical-HPLC basis, we have revealed a new type of an-
Radical Scavenging Mechanism for a 2-Pyrone Compound

A oxidative mechanism between USF2550A (1) and the DPPH radical in organic solvents and in a buffer solution.

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