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Oxidation products of quercetin catalyzed by mushroom tyrosinase

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In honor of Professor Tadao Kamikawa's seventieth birthday

Abstract—Quercetin was oxidized as a substrate catalyzed by mushroom tyrosinase to the corresponding *o*-quinone and subsequent isomerization to *p*-quinone methide type intermediate; followed by the addition of water on C-2 yielding a relatively stable intermediate, 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone. In the presence of a catalytic amount of L-DOPA as a cofactor, the rate of this oxidation was enhanced. Fisetin, which lacks the C-5 hydroxyl group, was also oxidized but the rate of oxidation was faster than that of quercetin, indicating that the C-5 hydroxyl group is not essential but is associated with the activity.

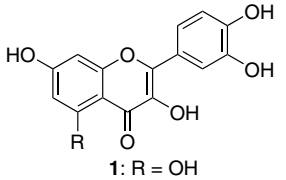
1. Introduction

In previous papers, a common flavonol, quercetin (**1**) was reported to inhibit the oxidation of L-DOPA catalyzed by mushroom tyrosinase.[1–3] This inhibitory activity was described to come from its ability to chelate copper in the enzyme since γ-pyrone moiety preferentially chelates copper.[4] As expected, the inhibition kinetics of quercetin was reported to be a competitive inhibitor analyzed by Lineweaver–Burk plots.[5] The chelation mechanism seems to be specific to flavonols as long as the 3-hydroxyl group is free. On the other hand, quercetin can be classified as a catechol derivative because of its B-ring structure. The catechols generally act as substrates on the tyrosinase reaction. Quercetin has recently been reported to be oxidized by polyphenol oxidase (PPO) from the chloroplast membranes of broad bean as well as mushroom tyrosinase through a complex mechanism caused by its γ-pyrone moiety.[6,7]

Although tyrosinase (EC 1.14.18.1) is known to catalyze a reaction between two substrates, a phenolic compound and oxygen, the discussion previously described was based on the experiment using mainly L-DOPA as a substrate. As the need arises, L-tyrosine was also assayed to see if the inhibitors characterized were effective on monophenolase activity. In both cases, the enzyme activity was monitored by dopachrome formation at 475 nm. The assay was carried out in air-saturated aqueous solution and as a result, oxygen, which was the other substrate, was disregarded. Hence, we examined tyrosinase action on flavonols with catechol moiety on the B-ring, particularly quercetin and fisetin (**2**), by combination techniques with spectrophotometry (dopachrome formation), polarography (oxygen consumption) and HPLC analysis.

2. Results and discussion

The oxygen consumption of quercetin (**1**) (see Fig. 1 for structures) was measured in the presence of tyrosinase (1.0 μg/mL). The reaction mixture consisting 50 or 100 μM of quercetin was incubated and oxygen consumption was measured up to 20 min. The results obtained are illustrated in Figure 2. The rate of oxygen consumption was dependent on quercetin concentrations. For



1: R = OH
2: R = H

Figure 1. Structures of quercetin (**1**) and fisetin (**2**).

Keywords: Mushroom tyrosinase; Oxidation; Flavonoid; Quercetin; Fisetin; *p*-Quinone methide.
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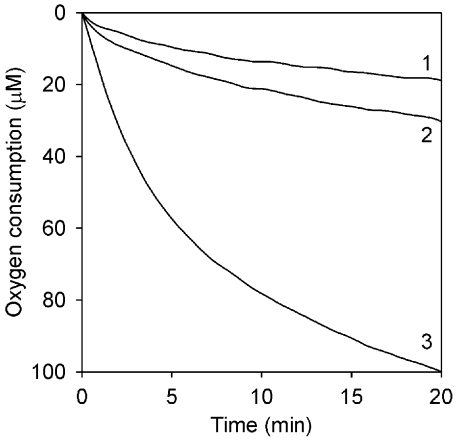


Figure 2. Oxygen consumption of quercetin (**1**) in the presence of tyrosinase. The concentration was selected at 50 μM (line 1) and 100 μM (line 2). Line 3 represents oxygen consumption by 100 μM L-DOPA as a control.

example, when 50 or 100 μM of quercetin was used, 14 or 25 μM of oxygen was absorbed, respectively. Supposedly, quercetin has a catechol moiety in its B-ring, which may be affected by tyrosinase. Figure 3 represents the consecutive spectra of quercetin (50 μM) under the same conditions of the oxygen uptake experiment. Interestingly, the original yellow color was noted to fade within 10 min. This oxidation was evidently characterized by a new peak with the maxima at 342 nm. More specifically, the UV/vis absorption spectrum changed rapidly and the characteristic shift to short wave lengths (378 → 342 nm) was observed.[6] These observations were not contradicted by the previous report[7] and the hypsochromic shift likely indicates that quercetin was oxidized. Absorbance at 378 nm mainly comes from $n \rightarrow \pi^*$ transition, and hence, the shift may be caused by structural changes including ketone moiety, aside from the change of catechol on the B-ring to corresponding *o*-quinone. The analysis of tyrosinase oxidation products was performed by HPLC as illustrated in Figure 4. Quercetin [retention time (t_R) 10.1 min] started being decreased and a new peak ***b*** (t_R 4.7 min) was gradually increased. The peak ***b*** was relatively stable but it seemed to be a small amount for determining the structure. On the other hand, quercetin was previously reported to be oxidized by Cu^{2+} to 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone (**3**) in aqueous conditions.[8] The UV spectrum of **3** previously reported was similar to Figure 3 and hence, the peak ***b*** was expected to come from **3**.[8] In order to identify the peak ***b***, the synthesis of **3** was first attempted to oxidize quercetin directly using $NaIO_4$. By the HPLC experiment, the peak ***b*** was identical to that of the enzymatic oxidation product of quercetin in the reaction mixture. However, **3** was hardly purified enough for structural characterization because of the coexistence of many by-products. On the other hand, it was found that $CuCl_2$ oxidation of quercetin was run effectively and allowed to produce **3**.[8,9] Accordingly, the peak ***b*** was consistent with **3** by chromatographic comparison using HPLC.

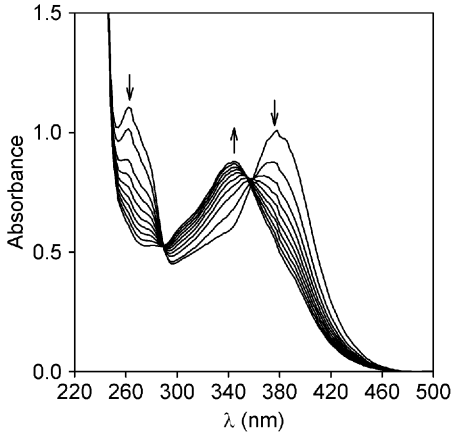


Figure 3. Consecutive spectra obtained in the oxidation of 50 μM of quercetin (**1**) by mushroom tyrosinase for 20 min. Scan speed was at 2 min intervals for 30 s. The arrows (↑) designate the evolution of the peak.

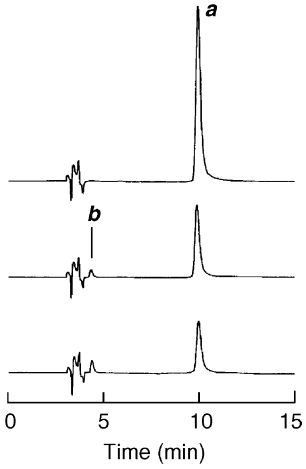
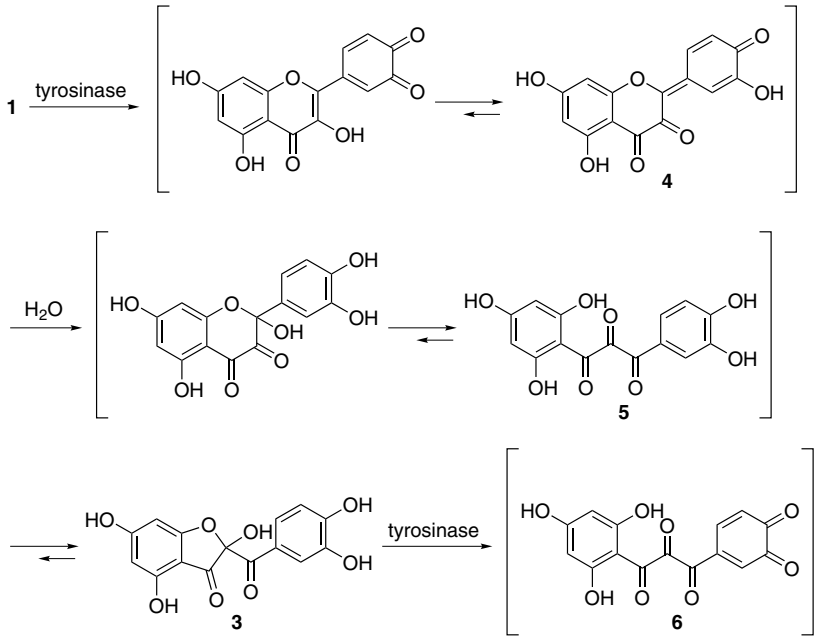


Figure 4. HPLC analysis of the reaction medium with 100 μM of quercetin (**1**) and tyrosinase. Sampling time was chosen at 0 min (upper), 20 min (middle), and 40 min (lower), respectively. The HPLC operating conditions were as follows: column; Capcell Pak C-18, solvent; 30% MeCN/H_2O containing 0.1% TFA, flow rate; 0.8 mL/min, detection; UV at 254 nm, injected amount; 20 μL from 3-mL assay system.

Proposed mechanism for the oxidative degradation of quercetin catalyzed by mushroom tyrosinase was illustrated in Scheme 1. Quercetin was rapidly oxidized to the corresponding *o*-quinone after the abstraction of $2e^-$ and $2H^+$ from the hydroxyl groups at C-3′ and -4′ and subsequent isomerization to *p*-quinone methide type intermediate (**4**),[10,11] followed by the addition of water on C-2 yielding the dione intermediate. This dione was tautomerized to the relatively stable protocatechuate intermediate (**3**) in aqueous solution via trione intermediate (**5**). In continuing spectrophotometrical experiment for 1 h, the changes in its UV/vis absorption spectrum started decreasing in absorbance at 342 nm but increasing at 270 and 400–550 nm (data not illustrated). Although the protocatechuate intermediate (**3**) was relatively stable, it was further oxidized, presumably to the corresponding *o*-quinone (**6**) at a slow rate.

Fisetin has a catechol moiety in its B-ring and also consumed oxygen as quercetin in the presence of tyrosinase,



Scheme 1. Proposed mechanism for the oxidative degradation of quercetin (**1**) catalyzed by mushroom tyrosinase.

but the uptake was faster than that of quercetin (Fig. 5). When 50 or 100 μM of fisetin was used, 17 or 31 μM of oxygen was consumed for 20 min, respectively. By spectroscopic experiments, hypochromic effect was observed at 375 nm that indicates the specific absorption by fisetin and this maximum absorption was shifted to 358 nm like quercetin (Fig. 6). Hence, fisetin can also be oxidized by mushroom tyrosinase. HPLC analysis was performed using reaction mixture with fisetin and tyrosinase under the same conditions of spectroscopic experiments (Fig. 7). It shows that peak ***a*** (6.2 min) which came from fisetin, was rapidly decreased and peak ***b*** (5.1 min) was mainly increased. It should be noted that by comparison with Figure 4, the decomposition of fisetin occurred intensively. The γ-pyrone is essential to show potent inhibitory activity on tyrosinase oxidation because it acts as a copper chelater. Also, as seen in the structure

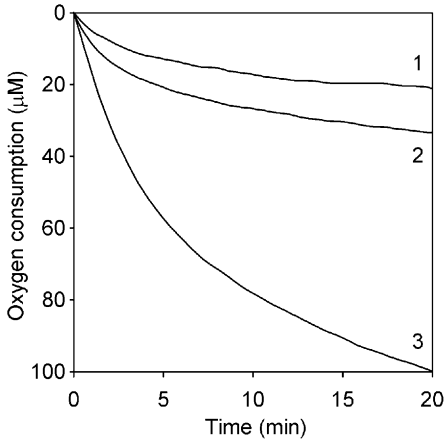


Figure 5. Oxygen consumption of fisetin (**2**) in the presence of tyrosinase. The concentration was selected at 50 μM (line 1) and 100 μM (line 2). Line 3 represents oxygen consumption by 100 μM L-DOPA as a control.

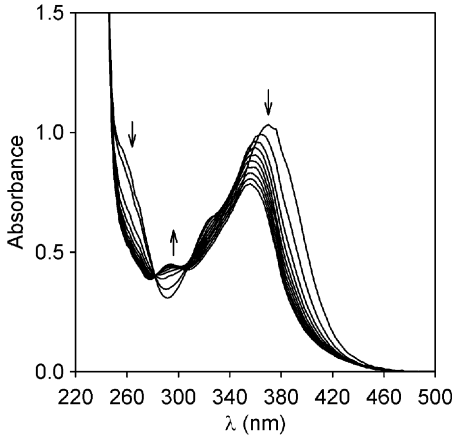


Figure 6. Consecutive spectra obtained in the oxidation of 50 μM of fisetin (**2**) by mushroom tyrosinase for 20 min. Scan speed was at 2 min intervals for 30 s. The arrows (↑) designate the evolution of the peak.

of hexylresorcinol and cardol, the resorcinol moiety is important for a tyrosinase inhibitor. Although quercetin has both γ-pyrone and resorcinol moieties in its structure, it is still easily oxidized. In contrast, fisetin is categorized in flavonols, which possesses γ-pyrone moiety on A-ring as only one significant function as copper chelater. It has also been reported that polyphenol oxidase from the chloroplast membranes of broad bean catalyzed the oxidation of fisetin.[12] The quick oxidation of fisetin could be explained in part by the absence of resorcinol moiety on its A-ring, although a diminished hydrogen bond between H-5 and ketone possibly affected the γ-pyrone coordination with copper on the tyrosinase active site.

Interestingly, the oxidation of quercetin catalyzed by tyrosinase was accelerated by the addition of the catalytic amount (10 μM) of L-DOPA. It has also been reported

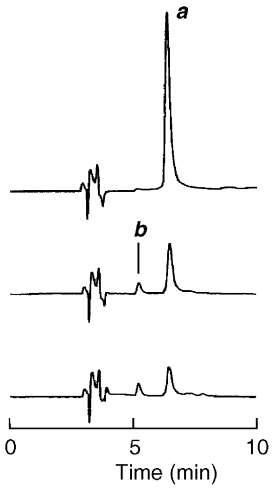


Figure 7. HPLC analysis of the reaction medium with 100 μM of fisetin (**2**) and tyrosinase. Sampling time was chosen at 0 min (upper), 10 min (middle), and 20 min (lower), respectively. The HPLC operating conditions were same as Figure 4.

that quercetin acts as cofactor.[4] Hence, L-DOPA enhanced the oxidation of quercetin except by the accumulation of *oxy*-form tyrosinase in the reaction mixture. To clarify how L-DOPA affected oxidation, HPLC analysis was performed in the presence of 0.5 mM of L-DOPA (Fig. 8). Remarkably, the decomposition rate of quercetin with L-DOPA was faster than the experiment without L-DOPA (Fig. 4). Moreover, some polar peaks around t_R 5.0 min emerged beside the peak of **3** whereas these peaks were not observed on L-DOPA oxidation. Recently, glutathionyl adducts were identified in the incubation experiment with quercetin and glutathione in the presence of tyrosinase by LC/MS analysis.[13] Although the detail is still largely unknown, it could be explained that quercetin was oxidized and subsequent formation of adducts with L-DOPA or its oxidized products.

As far as the cell-free experiment using mushroom tyrosinase is concerned, the inhibition kinetics reported did not exceed 30 s.[5] In the current experiment, quercetin was found to be rapidly oxidized to **3**, followed by further oxidation presumably to the corresponding *o*-quinone. As long as the enzyme activity was monitored by dopachrome formation at 475 nm, this peak was not detectable up to 20 min. Even if L-DOPA was oxidized, the oxidized intermediates form adducts with quercetin. The amount of L-DOPA oxidized within 30 s is negligible. Hence, the inhibition kinetics previously reported with respect to the oxidation of L-DOPA is still seemingly accurate, but it is not. Apparently, quercetin acts as a tyrosinase substrate and forms adducts in part with L-DOPA or its oxidation products. Hence, the inhibition mechanism could not follow simple Michaelis–Menten kinetics.

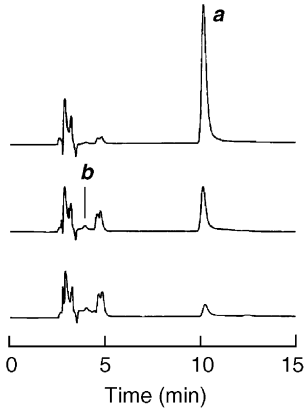


Figure 8. HPLC analysis of the reaction medium with 100 μM of quercetin (**1**) and tyrosinase in presence of 0.5 mM L-DOPA. Sampling time was chosen at 0 min (upper), 5 min (middle), and 20 min (lower), respectively. The HPLC operating conditions were same as Figure 4.

3. Experimental

3.1. General

Quercetin·$2H_2O$ and mushroom tyrosinase were purchased from Sigma (St. Louis, MO). $CuCl_2 \cdot 2H_2O$ was obtained from Mallinckrodt Chemicals (Phillipsburg, NJ). Acetonitrile (MeCN) was purchased from Alfa Aesar (Ward Hill, MA). Trifluoroacetic acid, dimethylsulfoxide (DMSO), and $NaIO_4$ were purchased from Aldrich (Milwaukee, WI). Fisetin was obtained from INDOFINE Chemical Co. (Hillsborough, NJ). Distilled water was purified by NANOpure (Barnstead International, Dubuque, IO).

3.2. Enzyme assay

The assay was performed as previously reported with some modifications.[14,15] Tyrosinase was purified by a method as previously described.[16] Oxygen consumption was measured on a Clark style electrode and an oxygraph equipped (YSI-53) with a water-jacketed chamber (Yellow Springs Instrument Co., Yellow Springs, OH) maintained at 30 °C. Calibration of an oxygen electrode was performed by using 4-*tert*-butylcatechol and excess tyrosinase according to the previous report.[17] Absorption measurements were recorded on a Spectra MAX plus spectrophotometer (Molecular device, Sunnyvale, CA) at 30 °C. For obtaining data of absorbance increment at 475 nm and oxygen consumption, 0.1 mL of a DMSO solution of flavonoid was mixed with 0.6 mL of 0.25 M sodium phosphate buffer (pH 6.8) and 2.2 mL of water. Then, 0.1 mL of the 0.05 M sodium phosphate buffer solution (pH 6.8) of tyrosinase (1.0 μg/mL) was added. When L-tyrosine was used as monophenol substrate, instead of 2.2 mL of water, 0.3 mL of 1.25 mM L-tyrosine solution and 1.9 mL of water were added. In the experiment by using cofactor, 6 μL of 5 mM L-DOPA solution was added at 5 min after enzymatic reaction started. Scan speed was selected for 2 min with 30 s intervals for obtaining consecutive spectra (220–500 nm).

3.3. HPLC analysis

HPLC analysis was performed on a LPG-1000 with an UV-7000 detector (Tokyo Rikakikai, Tokyo, Japan) and a 4.6 mm × 250 mm i.d., 5 μm, Capcell Pak C-18 column (Shiseido, Tokyo, Japan) as previously reported

with some modifications.[18] The operating conditions were as follows: solvent; 30% MeCN/H_2O containing 0.1% TFA, flow rate; 0.8 mL/min, detection; UV at 254 nm, injected amount; 20 μL from 3-mL assay system containing 100 μM flavonoid. The retention time (t_R) of **1** and **3** was identified as 10.1 and 4.7 min, respectively, by the coinjection with standard compounds.

3.4. Preparation of 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone (3)

The preparation of **3** was performed as previously reported with slight modification.[8,9] Quercetin·$2H_2O$ (25.0 mg, 73.8 μmol) was dissolved with 22.5 mL of aqueous 0.24 M HCl–MeCN (1:3) at 40 °C. Then, $CuCl_2$·$2H_2O$ (38.0 mg, 0.22 mmol) was added and the resultant mixture was stirred overnight at room temperature. After the most MeCN was removed in vacuo, the residue was partitioned between aqueous 1 M HCl solution and EtOAc. EtOAc layer was washed with brine and dried over Na_2SO_4. The filtrate was concentrated invacuo and the residue was subjected to Maxi-Clean C-18 Cartridge (Alltech, Deerfield, IL) eluted with 20% MeCN/H_2O containing 0.1% TFA. The eluate was directly subjected to preparative HPLC (solvent: 15% MeCN/H_2O containing 0.1% TFA, flow rate: 5 mL/min) equipped with a 10 mm × 250 mm, 10 μm, Econosil C-18 column (Alltech) to give **3** (16 mg, 68%) as a white powder. Data for **3**; ^{1}H NMR (400 MHz, CD_3CN) δ 6.01 (s, 1H), 6.08 (s, 1H), 6.82 (d, J = 8.8 Hz, 1H), 7.38 (d, J = 8.8 Hz, 1H), 7.39 (s, 1H); ^{13}C NMR (100 MHz, CD_3CN) δ 92.8, 98.7, 101.7, 103.6, 116.0, 117.1, 125.0, 125.6, 145.2, 152.1, 159.2, 169.9, 172.8, 190.1, 191.9; FAB-MS ($M+H^+$) 319. All spectra are consisted with those as previously reported.[8,9]

References and notes

1. Matsuda, H.; Higashino, M.; Chen, W. Z.; Tosa, H.; Imura, M.; Kubo, M. *Biol. Pharm. Bull.* **1995**, *18*, 1148.
2. Nagatsu, A.; Zhang, H. L.; Mizukami, H.; Sakakibara, J.; Tokuda, H.; Nishino, H. *Nat. Prod. Lett.* **2000**, *14*, 153.
3. Xie, L. P.; Chen, Q. Y.; Huang, H.; Wang, H. Z.; Zhang, R. Q. *Biochemistry—Moscow* **2003**, *68*, 487.
4. Kubo, I.; Kinst-Hori, I.; Chaudhuri, S. K.; Kubo, Y.; Sánchez, Y.; Ogura, T. *Bioorg. Med. Chem.* **2000**, *8*, 1749.
5. Chen, Q. X.; Kubo, I. *J. Agric. Food Chem.* **2002**, *50*, 4108.
6. Jiménez, M.; García-Carmona, F. *J. Agric. Food Chem.* **1999**, *47*, 56.
7. Fenoll, L. G.; García-Ruiz, P. A.; Varón, R.; García-Cánovas, F. *J. Agric. Food Chem.* **2003**, *51*, 7781.
8. Jungbluth, G.; Rühling, I.; Ternes, W. *J. Chem. Soc., Perkin Trans. 2* **2000**, 1946.
9. Utaka, M.; Takeda, A. *J. Chem. Soc., Chem. Commun.* **1985**, 1824.
10. Awad, H. M.; Boersma, M. G.; Vervoort, J.; Rietjens, I. M. C. M. *Arch. Biochem. Biophys.* **2000**, *378*, 224.
11. Blois, M. S. *Nature* **1959**, *181*, 1199.
12. Jiménez, M.; Escribano-Cebrián, J.; García-Carmona, F. *Biochim. Biophys. Acta* **1998**, *1425*, 534.
13. Awad, H. M.; Boersma, M. G.; Boeren, S.; van der Woude, H.; van Zanden, J.; van Bladeren, P. J.; Vervoort, J.; Rietjens, I. M. C. M. *FEBS Lett.* **2002**, *520*, 30.
14. Kubo, I.; Kinst-Hori, I. *Planta Med.* **1998**, *46*, 5338.
15. Nihei, K.; Yamagiwa, Y.; Kamikawa, T.; Kubo, I. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 681.
16. Espín, J. C.; Wichers, H. J. *J. Agric. Food Chem.* **1999**, *47*, 2638.
17. Rodríguez-López, J. N.; Ros-Martínez, J. R.; Varón, R.; García-Cánovas, F. *Anal. Biochem.* **1992**, *202*, 356.
18. Nihei, K.; Kubo, I. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2409.