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Bioorganic & Medicinal Chemistry 13 (2005) 2741–2747
Bioorganic & Medicinal Chemistry

Isolation of brominated quinones showing chemiluminescence activity from luminous acorn worm, *Ptychodera flava*

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Received 31 January 2005; revised 16 February 2005; accepted 16 February 2005

Abstract—Luminous acorn worm, *Ptychodera flava* emits green light by stimulating with diluted hydrogen peroxide. We have recently reported isolation and structure determination of 2,3,5,6-tetrabromohydroquinone as a luminous substance and riboflavin as a possible light emitter. There are three other luminous substances in the extracts from *P. flava*, so here we report the isolation and structure determination of other luminous substances as 2,3,5-tribromohydroquinone, tetrabromo-1,4-benzoquinone, and 2,3,5-tribromo-6-(2,3,5-tribromo-4-hydroxy-phenoxy)-benzene-1,4-diol. Besides, this is the first report of isolation of tetrabromo-1,4-benzoquinone from acorn worm. Structure–activity relationship of chemiluminescence activity of halogenated quinone derivatives reveals that a highly halogen substitution and 1,4-quinone skeleton are important for high chemiluminescence activity.

1. Introduction

Acorn worms live in a shallow sea sand and some of them are well known as luminous organisms. In 1961, Dure and Cormier reported that *Balanoglossus biminiensis* (*B. biminiensis*; a kind of acorn worm found in Sapelo Island, Georgia) showed bioluminescence, and that *B. biminiensis* showed a luciferin–luciferase reaction.[1] They reported the first example of requiring hydrogen peroxide (H_2O_2) for a bioluminescence. In the *B. biminiensis* bioluminescence, an unidentified luciferin was peroxidized with H_2O_2 catalyzed by a luciferase or a horseradish peroxidase.[2] Though so many efforts have been paid for elucidating the structure of both a luciferin and a luciferase, the structures still remain unclear.[2–5]

Ptychodera flava (*P. flava*), a smaller luminous acorn worm than *B. biminiensis*, was found in Kattore bay, Kohama Island, Okinawa by Higa and co-workers.[6,7] They isolated some halogenated compounds in order to elucidate the environmental significance of excretion from *P. flava*, but little was known about the bioluminescence of *P. flava* except the fact that they emit light with H_2O_2. *P. flava* emits green light from the whole body when applied to a diluted aqueous hydrogen peroxide, and the resultant bioluminescence intermittently continued for about a minute and the bioluminescence spectrum was recorded to show an emission spectrum centered at 528 nm by using a live specimen (Fig. 1).

In order to understand the bioluminescence system of *P. flava*, we have reported the isolation and structure determination of a luminous substance and a light emitter.[8] Luminous substance was determined to be 2,3,5,6-tetrabromohydroquionone (TetraBHQ) and a green fluorescent compound was determined to be a riboflavin as a possible light emitter.

In the presence of both TetraBHQ and riboflavin, chemiluminescence emission centering at 521 nm, close to the bioluminescence emission at 528 nm, was observed. In the absence of TetraBHQ or riboflavin, no luminescence was observed. This result strongly suggested that TetraBHQ and riboflavin related to the bioluminescence of *P. flava*.

We also found three other luminous substances in extract of *P. flava*. In the current studies, isolation and structure determination of other luminous components from *P. flava* and structure–activity relationship of chemiluminescence are described.

Keywords: *Ptychodera flava*; Bioluminescence; 2,3,5-Tribromohydroquinone; Tetrabromo-1,4-benzoquinone.
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doi:10.1016/j.bmc.2005.02.028

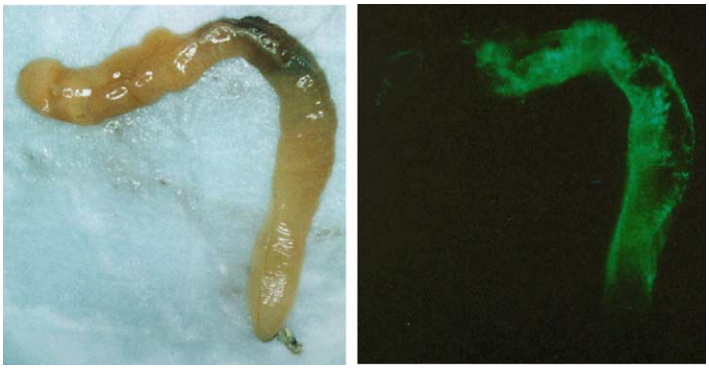


Figure 1. *Ptychodera flava* and its bioluminescence.

2. Results and discussion

Luminous substances were successfully extracted with ethyl acetate. HPLC analysis of ethyl acetate extract gave four chemiluminescence peaks and these peaks were named **1**, **2**, **3**, and **4** (Fig. 2).

An ODS column chromatographic separation of 1.48 g of ethyl acetate extract gave each peaks as a single constituent. Luminous compounds (**1**, **2**, **3**, and **4**) were obtained 379.0, 706.6, 8.0, and 47.3 mg, respectively. Structure of **2** exhibiting the highest intensity on UV and chemiluminescence chromatogram was determined to be TetraBHQ as a major luminous substance by NMR, MS, and X-ray analysis and was previously reported.[8] ^{1}H NMR measurement of **1** in DMSO-d_6 gave one hydroxyl proton and one aromatic proton and ^{13}C NMR gave six peaks in aromatic region. An ESI-Q-TOF-MS analysis of **1** showed molecular ion peak at *m*/*z* 342.8, 344.8, 346.8, and 348.8 $[M-H]^-$. It was suggested that **1** had 3 bromine atoms and the structure of **1** was determined to be 2,3,5-tribromohydroquinone, which was a known compound (Fig. 3).[7]

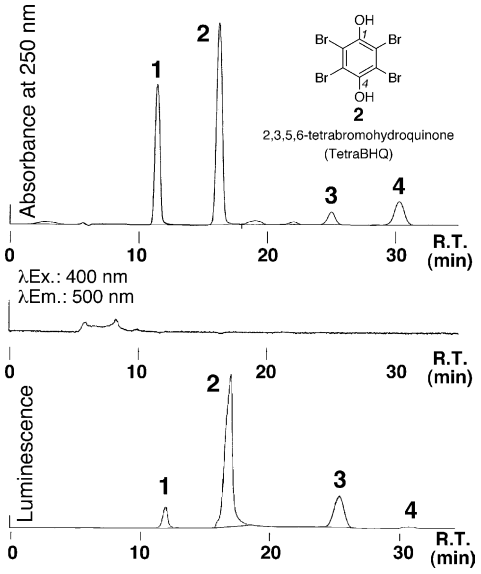


Figure 2. HPLC chromatogram of ethyl acetate extract from *Ptychodera flava* and structure of **2**.

Trace amount of **3** relative to other fractions did not give any peak in ^{1}H NMR spectrum and gave only two peaks at 137.7 and 169.2 ppm in ^{13}C NMR. In the studies on structural relationships of chemiluminescence activity of quinone analogs described below, **3** was found to be the same retention time of HPLC analysis as an authentic tetrabromo-1,4-benzoquinone (Fig. 4). ^{13}C NMR spectrum of **3** was in agreement with those of the authentic tetrabromo-1,4-benzoquinone.

An ESI-Q-TOF-MS analysis of **4** gave molecular ion peaks at *m*/*z* 684.5, 686.5, 688.5, 690.5, 692.5, 694.5, and 696.5 $[M-H]^-$ (Fig. 5). Seven isotopic peaks indicated the existence of six bromine atoms and MS/MS measurements gave two informative fragments, ***a*** and ***b***, which was formed by a cleavage of ether linkage.

^{1}H spectrum of **4** gave three hydroxy protons and one aromatic proton. ^{13}C NMR spectrum of **4** gave 12 peaks in aromatic region. The structure of **4** was determined to be 2,3,5-tribromo-6-(2,3,5-tribromo-4-hydroxy-phenoxy)-benzene-1,4-diol on the basis of HMBC and MS/MS experiments and the structure of **4** was reported by Higa and Sakemi (Fig. 6).[7]

3. Structure–activity relationships of chemiluminescence activity of quinone analogs

Study for the relationships between structure and chemiluminescence activity, some authentic and isolated quinone derivatives were subjected to a chemiluminescence assay (Fig. 7).

Figure 8 shows chemiluminescence activity of each quinone derivatives. All compounds showed higher chemiluminescence activity at pH 12.0 than pH 8.5. Hydroquinone has been known to show a weak luminescence, but in these conditions, chemiluminescence activity of hydroquinone and *p*-benzoquinone was less than detection limit of chemiluminescence detector. TetraBHQ showed higher luminescence activity than 2,3,5-tribromohydroquinone. Catechols and resorcinol

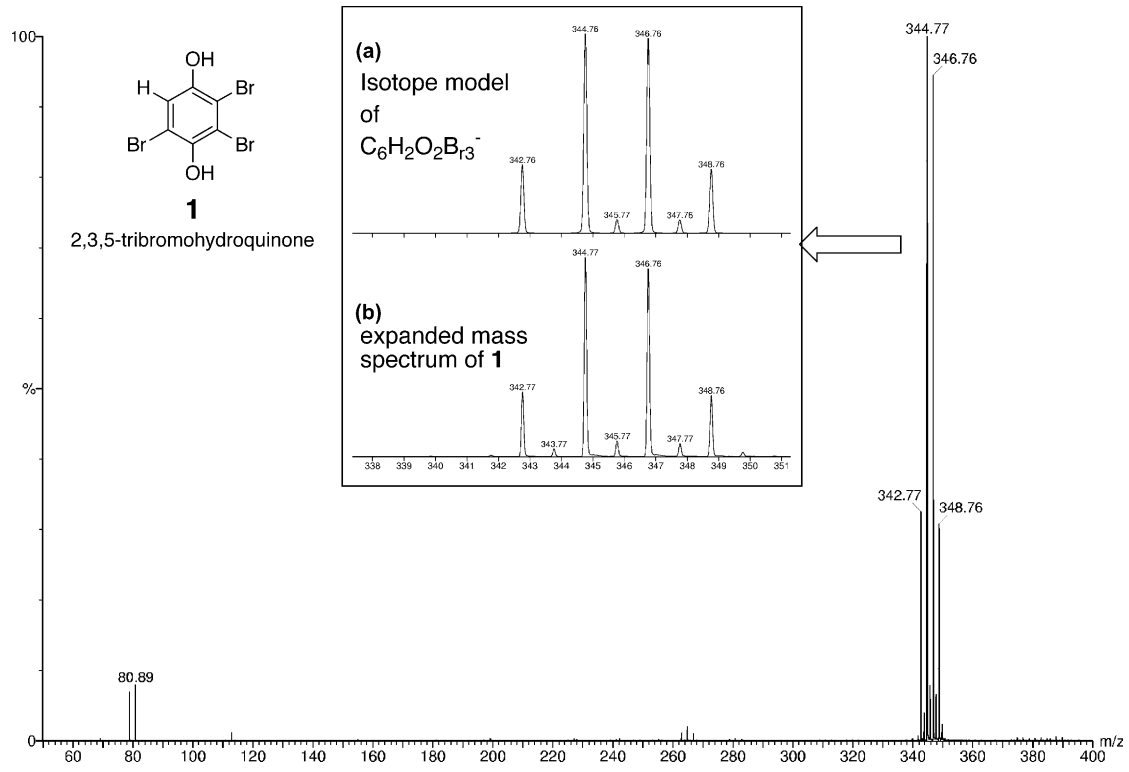


Figure 3. Structure and ESI-Q-TOF mass spectra of **1**: (a) isotope model calculated as $C_6H_2O_2Br_3^-$ in the negative mode, (b) expanded spectrum of molecular ion.

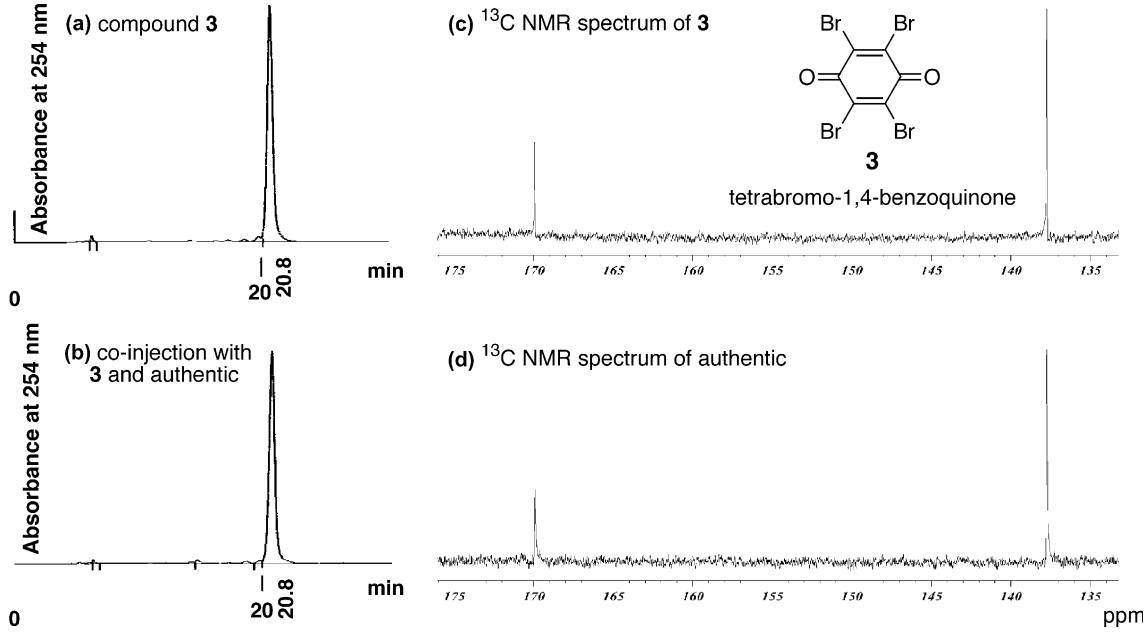


Figure 4. HPLC chromatogram and ^{13}C NMR spectra: (a) HPLC chromatogram of **3**, (b) co-injection with authentic tetrabromo-1,4-benzoquinone, (c) ^{13}C NMR of **3**, (d) ^{13}C NMR of authentic tetrabromo-1,4-benzoquinone.

showed a trace activities. To our surprise, halogenated *p*-benzoquinone, especially tetrachloro-1,4-benzoquinone showed the highest activities of all hydroquinone derivatives. From these results, highly brominated or chlorinated structure and 1,4-quinone structure play important roles for high chemiluminescence activity.

4. Conclusion

2,3,5-Tribromohydroquinone, tetrabromo-1,4-benzoquinone, and 2,3,5-tribromo-6-(2,3,5-tribromo-4-hydroxyphenoxy)-benzene-1,4-diol were isolated and determined as minor luminous substances by means of

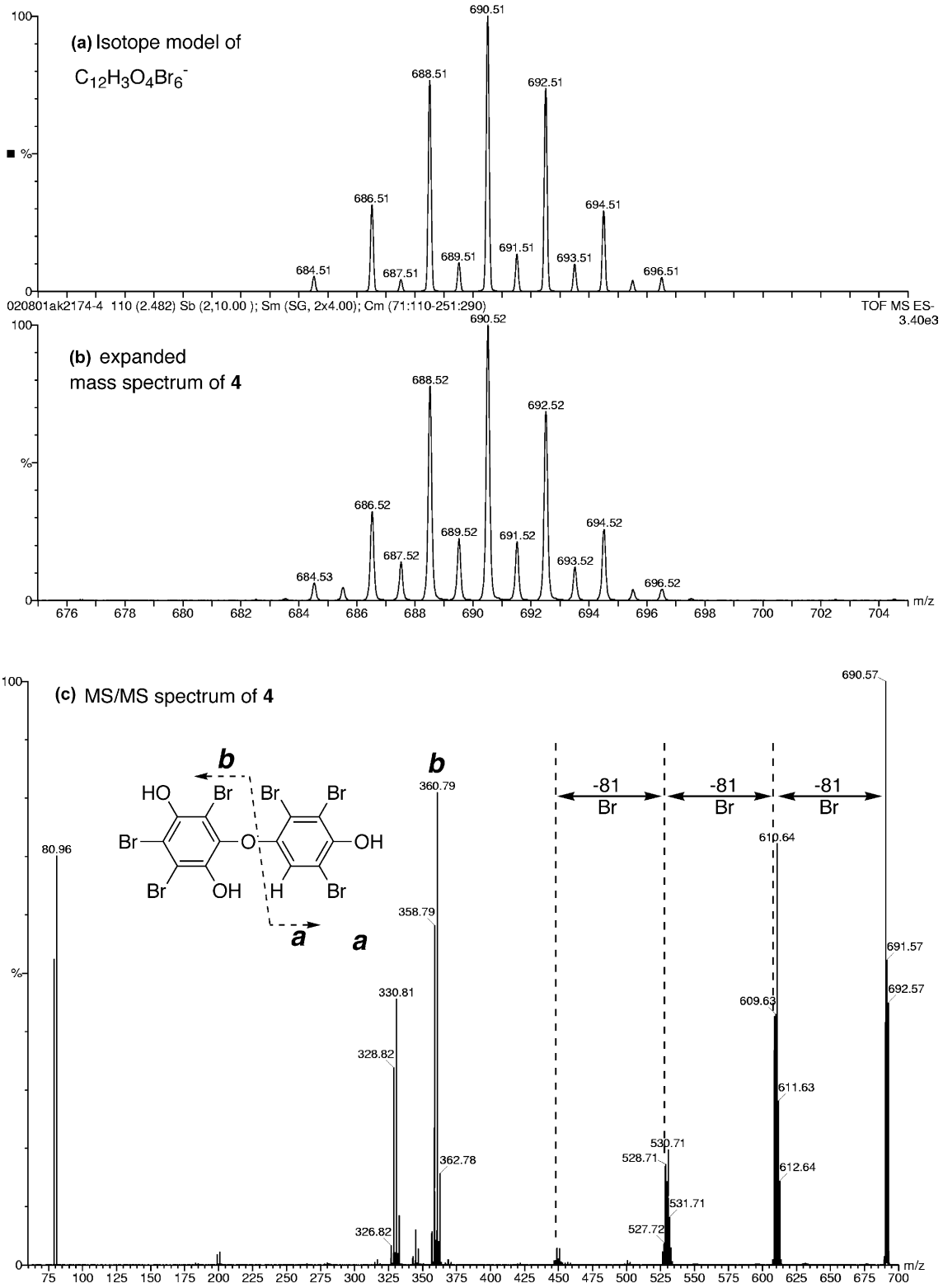
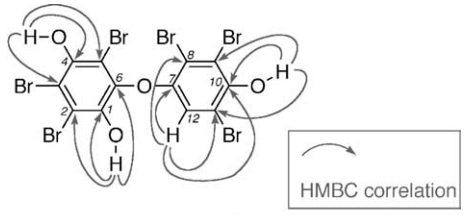


Figure 5. ESI-Q-TOF-MS and MS/MS spectra of **4**: (a) isotope model calculated as $C_{12}H_3O_4Br_6^-$ in the negative mode, (b) mass spectrum of **4**, (c) MS/MS spectrum of **4**.

NMR and MS analyses. Although tribromohydroquinone and 2,3,5-tribromo-6-(2,3,5-tribromo-4-hydroxyphenoxy)-benzene-1,4-diol have been isolated by Higa and co-workers, this report is the first isolation of tetrabromo-1,4-benzoquinone from acorn worm. Structure–activity relationship of chemiluminescence activity found that highly brominated or chlorinated structure as well as 1,4-quinone structure was found to be important for high chemiluminescence activity. Further study will reveal the bioluminescence system of *P. flava*.



4
2,3,5-tribromo-6-(2,3,5-tribromo-4-hydroxy-phenoxy)-benzene-1,4-diol

Figure 6. Structure of **4**.

5. Experimental

5.1. Reagents

The phenols such as 2,3,5,6-tetrabromohydroquinone, tetrachlorocatechol, and tetrabromocatechol were purchased from Lancaster Co. Ltd. (Morecambe, UK). Riboflavin, hydroquinone and *p*-benzoquinone were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Tetrachloro-1,4-benzoquinone, 2,4,6-tribromoresorcinol, and tetrabromo-1,4-benzoquinone were purchased from Aldrich Chem Co. (Milw., WI).

5.2. Instrumentation

HPLC analyses were carried out using JASCO PU-980 HPLC pump systems equipped with a JASCO UV-970 UV/VIS detector, a JASCO FP-920 Fluorescence Detector, a JASCO CL-925 CL Detector and a JASCO 807-IT integrator. UV spectra were recorded on a JASCO Ubest-50 UV/VIS Spectrophotometer. Fluorescence and luminescence spectra were measured with a JASCO FP-770 Spectrofluorometer. IR spectra were taken on a JASCO FT/IR-8300 and were reported in wave number (cm^{-1}). Luminescence activities were measured with a Jasco 825-CL CL Detector, which was specially equipped with a dark box in front of photomultiplier tube recording photons with a SIC Chromatocorder 12. Proton NMR spectra were recorded on a Bruker AMX-600 at 600 MHz. Chemical shifts (δ) are given in parts per million relative to DMSO-d_6 (δ 2.49) as an internal standard. Carbon NMR spectra were recorded on a Bruker AMX-600 at 150.9 MHz. Chemical shifts are (δ) given in parts per million relative to DMSO-d_6 (δ 39.7) as an internal standard. Elemental analysis was performed by Analytical Laboratory of Graduate School of Bioagricultural Sciences, Nagoya University.

A Q-TOF Mass Spectrometer instrument equipped with a Z-spray ESI source (Micromass Ltd., Manchester, UK) was used. MS measurements were performed in the negative ion mode. The sample cone voltage was set at 25 eV and the sample was dissolved in a mixture of water and acetonitrile 1:1 (v/v). The source block temperature was set at 100 °C and the desolvation temperature was also set at 100 °C. The scan time was 1.0 s for MS and 2.0 s for MS/MS, respectively. All the samples were sprayed at 5 μL/min by syringe pump unit.

5.3. Extraction and purification of luminous substances, 1, 2, 3, and 4

Protocol of extraction was followed by a previous report.[8] Twenty grams of lyophilized *P. flava*, which contained sea sand was homogenized in 100 mL of ethyl acetate and centrifuged at 10,000*g* for 10 min at 4 °C. Resultant precipitate was re-extracted in 100 mL of ethyl acetate twice and combined. The given supernatant gave 1.48 g of brown solid by concentration under a reduced pressure. Brown solid was dissolved in methanol and applied to ODS (Cosmosil 75C_{18}-OPN, nacalai tesque, Inc., Kyoto Japan) and the gel was evaporated

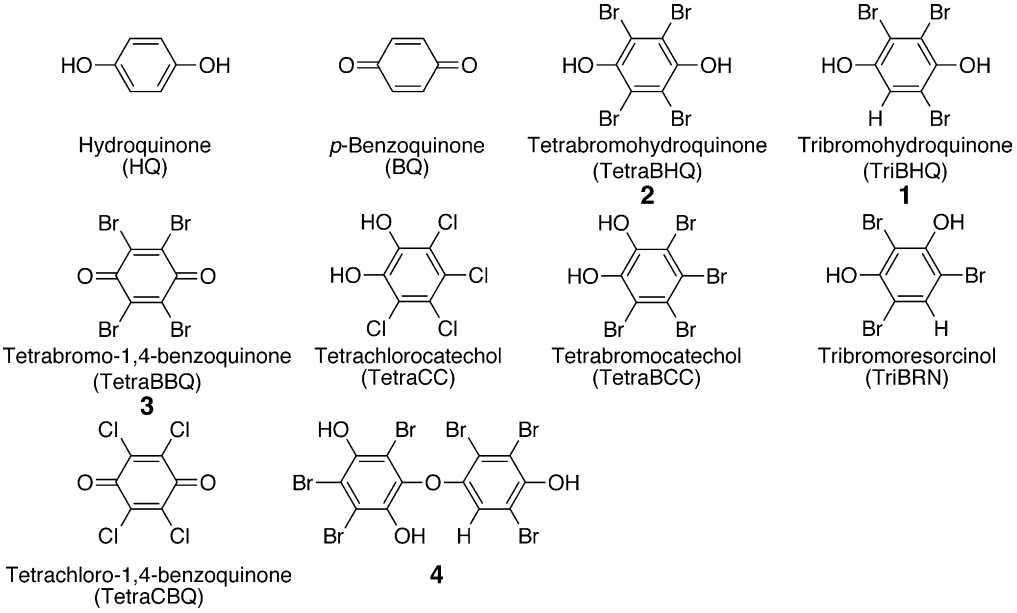


Figure 7. Structures of quinone derivatives.

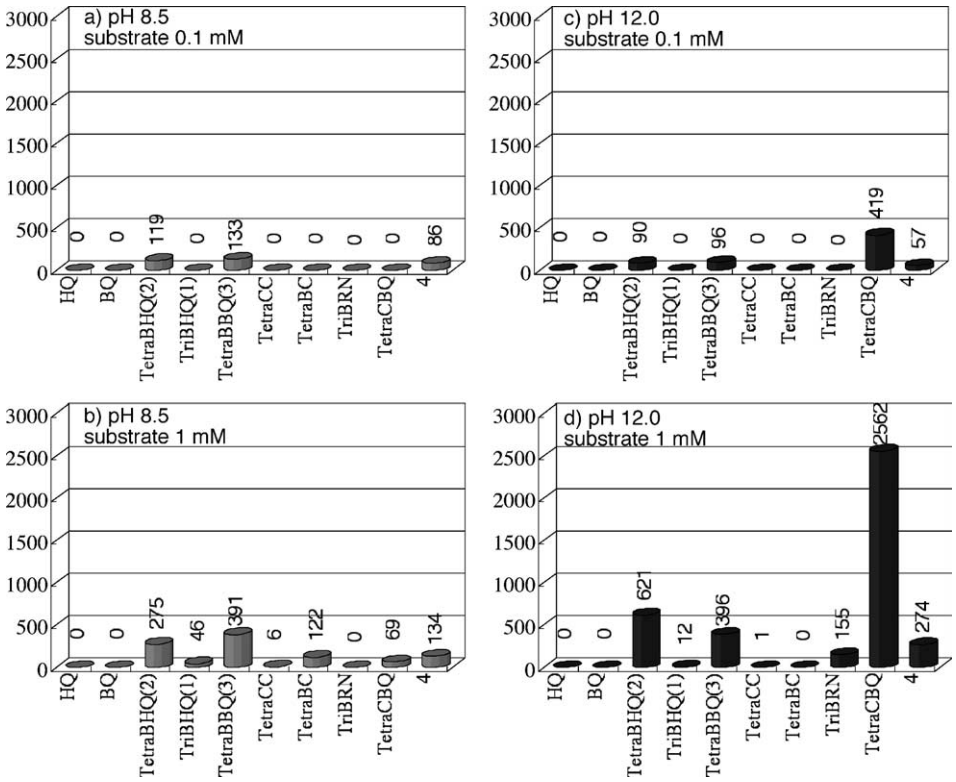


Figure 8. Chemiluminescence activities of halogenated quinone analogs.

to dryness. The resultant powder was packed into a glass column and roughly separated with methanol/H_2O containing 0.1% trifluoroaceticacid (TFA) as a mobile phase and fractionated. The fraction which contained **1**, **2**, **3**, and **4** were further purified by HPLC using an ODS column (10×250 mm, Cosmosil $75C_{18}$-OPN, Nacalai Tesque, Inc., Kyoto, Japan) twice. In the latter chromatogram, it was eluted by 70% methanol/H_2O containing 0.1% TFA at a flow rate 2.0 mL/min. Finally, **1**, **2**, **3**, and **4** were obtained 379.0, 706.6, 8.0, and 47.3 mg, respectively.

5.3.1. 2,3,5-Tribromohydroquinone (1). UV/VIS (MeOH) λ_{max} (ε) 309 nm (6.61×10^3). IR (KBr) λ_{max} cm^{-1} 3483, 1448, 1207. ^1H NMR (600 MHz, DMSO) δ 7.14 (s, H-6), 9.44 (s, H-4), 10.33 (s, H-1). ^{13}C NMR (150 MHz, DMSO) 111.53 (C-3), 112.37 (C-2), 117.0 (C-5), 118.09 (C-6), 144.62 (C-4), 149.42 (C-1).

Anal. Calcd for $C_6H_3O_2Br_3$: C, 20.78; H, 0.87. Found: C, 20.78; H, 0.82.

5.3.2. 2,3,5,6-Tetrabromohydroquinone (2). Data were reported in a previous report.[8]

5.3.3. Tetrabromo-1,4-benzoquinone (3). UV/VIS (AcOEt) λ_{max} (ε) 308 nm (8.41×10^3). IR (KBr) λ_{max} cm^{-1} 1682, 1214, 1055. ^{13}C NMR (150 MHz, DMSO) 137.7, 169.2.

5.3.4. 2,3,5-Tribromo-6-(2,3,5-tribromo-4-hydroxy-phenoxy)-benzene-1,4-diol (4). UV/VIS (MeOH) λ_{max} (ε) 308 nm (4.60×10^3). IR (KBr) λ_{max} cm^{-1}. 3394, 1413, 1293, 1184, 829. ^1H NMR (600 MHz, DMSO) δ 6.62 (s, H-12), 9.83 (s, H-4), 9.98 (s, H-10), 9.99 (s, H-1). ^{13}C NMR (150 MHz, DMSO) 107.79 (C-5), 111.14 (C-11), 113.73 (C-3), 114.47 (C-8), 115.20 (C-2), 116.46 (C-12), 117.65 (C-9), 140.09 (C-1), 142.95 (C-6), 146.03 (C-4), 147.52 (C-10), 148.01 (C-7).

5.4. Chemiluminescence activity

Measurement of chemiluminescence was done by the addition of luminescence buffer (1100 μL, 1.7% H_2O_2 in 70% 1,4-dioxane) to a mixture of sample solution (320 μL, ethyl acetate solution) and an alkaline buffer (pH 8.5, 100 mM Tris–HCl or pH 12.0, 100 mM Gly-NaOH).

Acknowledgements

We thank Professor T. Higa (Ryukyus Univ.) for his kind advice and help with collecting *P. flava*. This work was financially supported by a MEXT Grant-in-Aid for Specially Promoted Research (16002007) and a JSPS Research Fellowship for Young Scientists to A.K.

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