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Unique Proline–Benzoquinone Pigment from the Colored Nectar of "Bird's Coca Cola Tree" Functions in Bird Attractions

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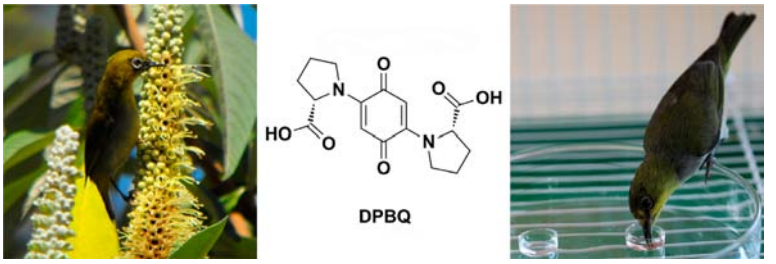
ABSTRACT



The major pigment responsible for the dark brown nectar of the "bird's Coca cola tree", *Leucosceptrum canum* (Labiatae), was isolated and identified as a unique symmetric proline-quinone conjugate, 2,5-di-(*N*-(−)-prolyl)-*para*-benzoquinone (DPBQ). Behavioral experiments with both isolated and synthetic authentic samples indicated that DPBQ functions mainly as a color attractant to bird pollinators.

The chemistry and ecological function of floral colored nectar have recently attracted much research interest.[1] In nature, flower nectar is usually a colorless liquid because its contents including nutrients and secondary metabolites are mostly colorless. However, at least 68 taxa from 20 genera belonging to 15 families have been documented to possess colored nectar, covering different broad categories of colors including yellow, amber-orange, red, brown, green, blue, and black,[2] and probably many other colored nectar plants are still to be discovered. Although a few such plants have been phytochemically and ecologically investigated, pigmented compounds in the colored nectar of most plants and their ecological functions are still largely unknown.

Leucosceptrum canum Smith, a large tree (up to 10 m high[3]) belonging to the family Labiatae (= Lamiaceae), has an unusual dark brown nectar and is the only colored nectar plant so far discovered in the Labiatae family. It is a favorite species of birds, and field observations have reported that it can attract over 40 kinds of birds for feeding within 40 min.[4] For this reason, *L. canum* is also called "bird's Coca cola tree" by ornithologists.[4] Most recently, Zhang et al.[5] reported that a purple anthocyanidin, 5-hydroxyflavylium, was the cause of the nectar coloration of *L. canum* and functioned as a foraging signal in bird pollination. Unfortunately, this compound was only characterized by UV and mass spectral methods,

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(1) (a) Olesen, J. M.; Ronsted, N.; Tolderlund, U.; Cornett, C.; Molgaard, P.; Madsen, J.; Jones, C. G.; Olsen, C. E. *Nature* **1998**, *393*, 529. (b) Hansen, D. M.; Beer, K.; Mueller, C. B. *Biol. Lett.* **2006**, *2*, 165–168.
(2) Hansen, D. M.; Olesen, J. M.; Mione, T.; Johnson, S. D.; Mueller, C. B. *Biol. Rev.* **2007**, *82*, 83–111.
(3) Peng, H.; Wu, Z. Y. *Acta Bot. Yunnan.* **2001**, *23*, 278–286.
(4) Han, L. X. *Nat. Watch* **2009**, *1*, 17–18.
(5) Zhang, F. P.; Cai, X. H.; Ren, Z. X.; Zachary, L. R.; Li, D. Z. *New Phytol.* **2012**, *193*, 188–195.

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and therefore its identification must be considered tentative. We have been interested in the secondary metabolites of *L. canum* and their ecological functions because of its uniqueness as a large woody Labiatae with colored nectar and have found that the glandular trichomes of *L. canum* harbor defensive sesterterpenoids with a novel C_{25} carbon framework.[6] To discover the pigmented compounds responsible for the colored nectar of *L. canum* and their ecological significance, we initiated an investigation three years ago and recently purified the major pigment and unambiguously determined its chemical structure with comprehensive spectroscopic analyses, especially 1D and 2D NMR and HRMS, and chemical synthesis. With the purified and synthetic authentic samples, we were also able to conduct behavioral experiments on a bird pollinator of *L. canum*.

L. canum blooms from early December to late March of the next year, and each plant can produce hundreds of inflorescences and thousands of white tubular flowers (Figure 1a–d). The tubular flowers are filled with dark brown colored nectar (Figure 1d) in the morning. The most common visitors are Japanese White-eye (*Zosterops japonicus*) (Figure 1a), Blue Winged Minla (*Minla cyanouroptera*) (Figure 1b), and Black-headed Sibia (*Heterophasia melanoleuca*) (Figure 1c) based on our own field observations. Large scale HPLC analysis of 109 nectar samples collected from the Botanical Garden of Kunming Institute of Botany and Dehong with a detection wavelength of 400 nm indicated that all samples contained a predominant peak with maximum UV absorptions at 215, 369, and 525 nm (Figure S1), which was accordingly targeted as the responsible pigmented compound of the dark brown nectar of *L. canum*. However, when we started to isolate this compound for spectroscopic identification and subsequent behavioral experiments, it was found to be so unstable that isolation and identification were very difficult. After two years of effort, we were finally able to obtain a 27.5 mg sample of this compound with sufficient purity for identification as a dark brown solid from 645 mL of *L. canum* nectar.

The pigmented compound was shown to have a molecular formula of $C_{16}H_{18}N_2O_6$ by positive and negative ESI mass spectrometry and high-resolution ESI-MS.[7] Eight carbon resonances were found in its ^{13}C NMR spectrum

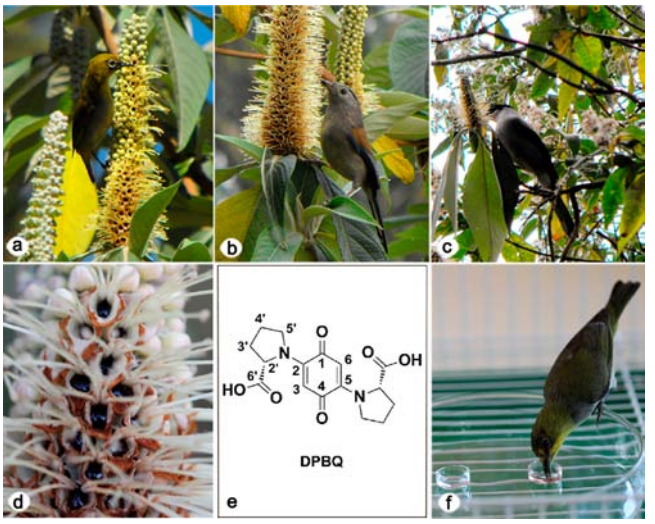

Figure 1. *Leucosceptrum canum* and its flower visitors. (a) Japanese White-eye (*Zosterops japonicus*); (b) *Minla cyanouroptera*; (c) *Heterophasia melanoleuca*; (d) Dark brown nectar of *L. canum*; (e) Chemical structure of 2,5-di-(*N*-prolyl)-*para*-benzoquinone (DPBQ); (f) Japanese White-eye probes DPBQ (500 μg/mL) in the behavioral experiments.

(Figure S4), which were further classified by DEPT experiments as three methylenes, two methines including an olefinic one (δ_C 100.1), and three quaternary carbons including an olefinic one (δ_C 148.6) and two carbonyl carbons (δ_C 179.9, and 173.4). Its ^1H NMR spectrum (Figure S3) displayed seven groups of signals, including an olefinic singlet at δ_H 5.23, a broad doublet at δ_H 4.99, a two-proton multiplet at δ_H 3.35, and four multiplets at δ_H 1.78–2.21, which were readily assigned to the corresponding methylene and methine carbons through a heteronuclear single-quantum correlation (HSQC) experiment (Figure S6). The $^1H-^1H$ COSY correlations (Figure S5) established a fragment of $-CHCH_2CH_2CH_2-$ with the terminal protons and carbons occurring relatively downfield, suggesting the existence of a tetrahydropyrrole moiety, which was substituted by a carboxylic group at C-2′ owing to the $^1H-^{13}C$ long-range correlations from H-2′ and H_2-3′ to the carbonyl carbon at δ_C 173.4 in the heteronuclear multiple bond coherence (HMBC) spectrum (Figure S7). Thus a proline unit was evident in the structure. The remaining signals in the NMR spectra were ascribable to an α,β-unsaturated keto residue, which was attached to the nitrogen of proline because of the HMBC correlations from H-2′ and H_2-5′ to the olefinic quaternary carbon and ROESY correlation between H-3/H-6 and H-2′ (Figure S8). In the HMBC spectrum (Figure S7), an unusual ^4J coupling from the proton at δ_H 5.23 to the carbon at δ_C 100.1 (from H-3 to C-6 and from H-6 to C-3) was also observed, indicating a symmetric structure for the compound. Considering its above molecular formula, the compound was straightforwardly established to be either 2,5-di-(*N*-prolyl)-*para*-benzoquinone or 2,5-di-(*N*-prolyl)-*ortho*-benzoquinone. However, it is difficult to distinguish

(6) Luo, S. H.; Luo, Q.; Niu, X. M.; Xie, M. J.; Zhao, X.; Schneider, B.; Gershenzon, J.; Li, S. H. *Angew. Chem., Int. Ed.* **2010**, *49*, 4471–4475.

(7) Natural DPBQ: Dark brown solid; $[\alpha]_D^{25} = -46.4$ ($c = 0.1$, water); UV/vis (water): λ_{max} (log ε) 215 (3.55), 369 (3.37), 525 (2.10) nm; IR (KBr): ν_{max} 3426, 2978, 1624, 1540, 1411, 1385, 1290, 1187, 1009 cm^{-1}; Positive ESI-MS: m/z (%) 357 (7) $[M+Na]^+$, 335 (5) $[M+H]^+$, 317 (9), 301 (40), 219 (31), 203 (100); Negative ESI-MS: m/z (%) 355 (42) $[M+Na-2H]^-$, 289 (100) $[M-COOH]^-$, 243 (41) $[M-2COOH-H]^-$, 217 (29), 215 (77); HR-ESI-MS: m/z 335.1169 $[M+H]^+$ (m/z_{calcd} $[C_{16}H_{19}N_2O_6]^+$ = 335.1238); 357.1060 $[M+Na]^+$ (m/z_{calcd} $[C_{16}H_{18}N_2O_6Na]^+$ = 357.1057); ^1H NMR (600 MHz, DMSO-d_6): δ 5.23 (s, 2H, H-3 and H-6), 4.99 (brd, J = 5.4 Hz, 2H, H_2-2′), 2.20 (m, 2H, H_2-3′a), 2.00 (m, 2H, H_2-3′b), 1.89 (m, 2H, H_2-4′a), 1.78 (m, 2H, H_2-4′b), 3.35 (m, 4H, H_4-5′); ^{13}C NMR (150 MHz, DMSO-d_6): δ 179.9 (s, 2C, C-1 and C-4), 173.4 (s, 2C, C_2-6′), 148.6 (s, 2C, C-2 and C-5), 100.1 (d, 2C, C-3 and C-6), 62.6 (d, 2C, C_2-2′), 51.1 (t, 2C, C_2-5′), 31.1 (t, 2C, C_2-3′), 21.6 (t, 2C, C_2-4′). Synthetic (−)-DPBQ: Dark brown solid; $[\alpha]_D^{19} = -41.1$ ($c = 0.1$, water). Synthetic (+)-DPBQ: Dark brown solid; $[\alpha]_D^{19} = +58.3$ ($c = 0.1$, water).

para-benzoquinone and *ortho*-benzoquinone and determine the absolute configuration of the proline in the structure by an NMR method, and an attempt to crystallize the compound for X-ray analysis also failed; therefore synthesis of this compound was necessary. By condensation of *para*-benzoquinone under alkaline conditions with L-(−)-proline and D-(+)-proline respectively, 2,5-di-(*N*-(−)-prolyl)-*para*-benzoquinone ((−)-DPBQ) and 2,5-di-(*N*-(+)-prolyl)-*para*-benzoquinone ((+)-DPBQ) were synthesized (Figure 2). Both products were found to have identical retention times and ^{1}H and ^{13}C NMR spectra to the isolated pigmented compound (Figures S9−S11). Therefore, the planar structure of the nectar pigment should be 2,5-di-(*N*-prolyl)-*para*-benzoquinone (DPBQ hereafter). Its CD curve was consistent with that of (−)-DPBQ but opposite to that of (+)-DPBQ (Figure 3), indicating that the proline in its structure was the L-(−)-form (Figure 1e), which was also supported by their optical rotations (DPBQ: $[\alpha]_D^{25} = -46.4$; (−)-DPBQ: $[\alpha]_D^{19} = -41.1$; (+)-DPBQ: $[\alpha]_D^{19} = +58.3$). Consequently, the nectar pigment was determined as 2,5-di-(*N*-(−)-prolyl)-*para*-benzoquinone.

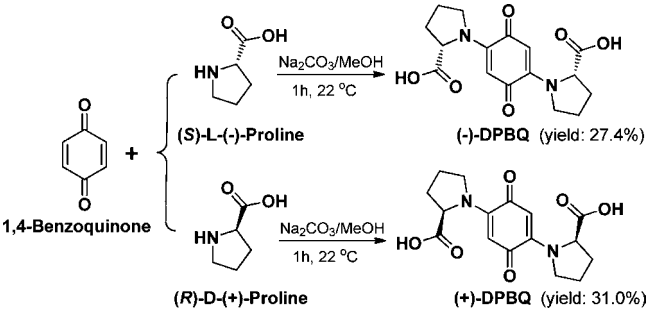

Figure 2. Synthetic routes of (−)-DPBQ and (+)-DPBQ.

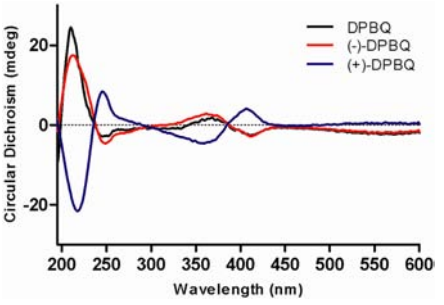

Figure 3. CD spectra of DPBQ, (−)-DPBQ, and (+)-DPBQ.

(8) (a) Shigemori, H.; Madono, T.; Sasaki, T.; Mikami, Y.; Kobayashi, J. *Tetrahedron* **1994**, *50*, 8347–8354. (b) Kobayashi, J.; Madono, T.; Shigemori, H. *Tetrahedron* **1995**, *51*, 10867–10874. (c) Takahashi, Y.; Kubota, T.; Ito, J.; Mikami, Y.; Fromont, J.; Kobayashi, J. *Bioorg. Med. Chem.* **2008**, *16*, 7561–7564. (d) Takahashi, Y.; Kubota, T.; Kobayashi, J. *Bioorg. Med. Chem.* **2009**, *17*, 2185–2188. (e) Takahashi, Y.; Ushio, M.; Kubota, T.; Yamamoto, S.; Fromont, J.; Kobayashi, J. *J. Nat. Prod.* **2010**, *73*, 467–471.

Figure 4. Color comparison of DPBQ in water at different concentrations (a−d: 100, 500, 1000, and 1500 μg/mL of DPBQ respectively) with *L. canum* nectar in deepest color (e).

As Figure 4 shows, DPBQ is the major cause of the dark brown color of *L. canum* nectar. Although a number of aminoquinones such as nakijiquinones have been reported from marine sponges,[8] quinone−animo acid conjugates are rarely found in the plant kingdom. DPBQ represents a novel type of natural pigment in floral nectar. DPBQ is generally unstable in organic solvents such as DMSO (degradation and presumably polymerization may occur) but is relatively stable in water especially under alkaline conditions such as in *L. canum* nectar (pH = 8.40 ± 0.17). We have found that the pH of *L. canum* nectar could fluctuate at least between 7.93 and 8.57. It can be therefore postulated that the color difference of *L. canum* nectar in different flower stages as pointed out by Zhang et al[5] could have been caused by different concentrations of DPBQ and pH changes in the nectar. Alternatively, it is possible that other amino acids besides proline could also conjugate with *para*-benzoquinone to result in other different colors. Such compounds were actually obtained as minor constituents of the nectar of *L. canum* but unfortunately were not fully identified due to their instability and paucity of sample amounts.

Before we could test DPBQ on the behavior of bird visitors, it was important to know the concentration of this pigment in the nectar of *L. canum*. However, when nectar was removed on the morning of the first day that the flower was open, we found that new nectar was secreted on the next day but the brown color became lighter. After nectar removal on successive days, the same thing occurred until the nectar finally had a purple-like color on the fifth sampling day when the flower senesced. These observations implied that DPBQ was regularly synthesized and secreted but that its concentration changed with flower stage and feeding by visitors. Therefore, HPLC was employed to quantify DPBQ in the nectar sampled from five inflorescences (each 10 flowers) over five consecutive days. It was found that the concentration of DPBQ was extremely high (up to 1.39 mg/mL) in nectar on the first two days of blooming, but the level gradually decreased with the flowering time and removal of old nectar. On the fifth day of blooming, as the flowers became senesced, the concentration of DPBQ dropped to a minimum

(Figure S2). The result was in good agreement with our above observations of changes in nectar color.

Naïve Japanese White-eyes (*Z. japonicus*) were used for testing their behavioral responses to nectar, nectar sugar, and DPBQ, with a method described in the literature[9] with suitable modifications (Methods in the Supporting Information). As shown in Figure 5, Japanese White-eyes probed the colored nectar of *L. canum* in an incredibly higher proportion (%) (85.40 ± 5.94) than the clear nectar sugar ($p < 0.001$) and water ($p < 0.001$) (Figure 5a). Each time the nectar was drunk up by birds in just one probe. After the exteriors of the glass dishes of nectar sugar and water were painted dark brown, this proportion dramatically decreased (56.67 ± 4.08) and proportions for nectar sugar and water obviously increased (Figure 5b). Under this circumstance, birds consumed much higher amounts (μL) of *L. canum* nectar (95.34 ± 2.44) and nectar sugar (85.63 ± 4.12) than water (34.02 ± 5.32) (Figure 5c). In parallel experiments, natural DPBQ and the synthetic (−)-DPBQ and (+)-DPBQ were also tested at a medium natural concentration (500 μg/mL). It was found that, in the first 20 trials, Japanese White-eyes absolutely preferred the pigmented compounds (DPBQ, (−)-DPBQ, and (+)-DPBQ) over water. However, with increased trials, this preference decreased dramatically. It was found that birds probed DPBQ, (−)-DPBQ, and (+)-DPBQ in proportions of 71.8 ± 4.8, 71.0 ± 2.6, and 74.0 ± 6.0 respectively at 50 trials, which were still significantly higher than that of water ($p < 0.001$) (Figure 5d−f). When the glass dish of water was painted outside to match the color of DPBQ solution, birds probed DPBQ and water in almost equal proportions ($p = 0.70$) (Figure 5g). Moreover, the corresponding amounts consumed per trial also showed no significant difference between DPBQ and water (Figure 5h). The above results clearly indicated that DPBQ functions mainly as a color attractant to bird pollinators in *L. canum* nectar.

In summary, we have chemically characterized the major pigment of the dark brown nectar of a woody Labiatae as a unique symmetric proline−quinone conjugate, 2,5-di-(*N*-(−)-prolyl)-*para*-benzoquinone, and demonstrated its function in attracting birds as potential pollinators. This is the first time that both the elusive chemistry and ecological function of pigment in colored nectar have been unambiguously disclosed, which should shed more light on the evolution and ecological significance of this enigmatic floral trait.

(9) Johnson, S. D.; Hargreaves, A. L.; Brown, M. *Ecology* **2006**, *87*, 2709–2716.

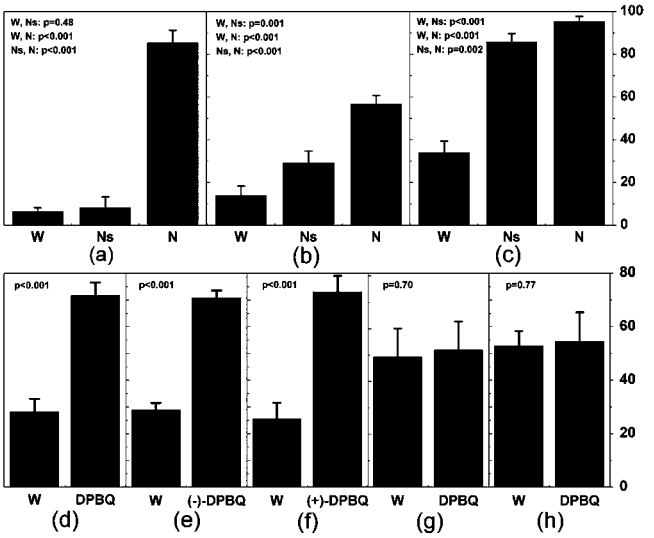

Figure 5. Preference and consumption of nectar (N), nectar sugar (Ns) DPBQ, (−)-DPBQ, (+)-DPBQ (each 500 μg/mL), and water (W) by the bird *Z. japonicus*. (a, d−f) Distribution of first choice; (b, g) Distribution of first choice (outside painted dark brown to match nectar); (c, h) Consumption per trial (outside painted to match color of nectar or DPBQ solutions).

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Supporting Information Available. Experimental procedures regarding the general experimental details, nectar materials, representative HPLC profile of nectar, isolation of DPBQ, quantification of DPBQ and results, behavioral experiment, statistical analysis, synthesis of (−)-DPBQ and (+)-DPBQ, 1D and 2D NMR spectra of DPBQ, and overlays of HPLC chromatograms and 1D NMR spectra of DPBQ, (−)-DPBQ, and (+)-DPBQ. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.