Synthetic anthocyanidins and their antioxidant properties

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Abstract
Anthocyanidins were synthesized to study the effect of methoxy substitution on the B ring to their antioxidant property. Comparative FRAP studies show 2′- and 4′-methoxy substituents have higher antioxidant activities, which may be attributed to both resonance and inductive effects.

Keywords: Dyes, Green chemistry, FRAP, One pot synthesis

Background
Anthocyanidins are pigments that are associated with the bright coloration of flowers and fruits. These natural dyes belong to the flavonoid family, with their basic structure comprising of an aromatic ring (A) fused with an heterocyclic ring containing an oxygen (C), which is also bonded to a third aromatic ring (B). These compounds are normally substituted with hydroxy groups, which help stabilize the charge on the flavylum cation. When one of the phenols is substituted with glycosides, the compound is called an anthocyanin.

The natural occurrence of anthocyanins and anthocyanidins warrants their study not only for the evolutionary advantage they confer to plants, but also for their potential applications (Castañeda-Ovando et al. 2009). Besides their utility as colorants for foods and cosmetics (Campanella et al. 2010), they are also explored in materials science (Pina et al. 2012) for example, as photosensitizers for photovoltaics (Calogero et al. 2013; Gokilamani et al. 2013), and as molecular logic gates (Pina et al. 1998). Like many polyphenols, they exhibit biological activities that are beneficial to human health (Pojer et al. 2013) such as in glucose metabolism (Alzaid et al. 2013), protection against cardiovascular disease (Wallace 2011), and mediation of oxidative stress (Zafra-Stone et al. 2007). Their putative roles in human pathologies are of interest, particularly in cancer prevention (Wang and Stoner 2008; Cooke et al. 2005). Despite their biological significance, their pharmacokinetics in humans remains largely unexplored (Kay 2006). Thus, to further the utility of anthocyanins in therapeutics and gain an understanding of their activities as applied to drug design, we synthesized anthocyanidins 1–3 and studied their antioxidant properties.

There are several methods for determining and expressing antioxidant activity, particularly for natural anthocyanins extracted from plants (Thaipong et al. 2006; Huang et al. 2005; Pulido et al. 2000; Sochor et al. 2010). This paper reports the preparation and characterization of three new anthocyanidins with different substitution patterns on the B ring. The antioxidant activities of the synthetic anthocyanidins were studied using a modified ferric reducing activity of plasma (FRAP) assay (Benzie and Strain 1996, 1999).

Results and discussion
Synthesis of the flavylum cation occurs under harsh conditions (Balaban et al. 1969) and preparations of anthocyanidins have been achieved by bubbling the reaction with hydrogen chloride gas (Moncada et al. 2004), treatment with perchloric acid (Sato et al. 1999; Dorofeenko and Olekhnovich 1972), or employment of corrosive Lewis acids such as boron trifluoride etherate (Kuhner et al. 2001). Recently, milder synthesis using sulfuric acid was reported (Calogero et al. 2013), and described herein is a convenient approach to obtaining anthocyanidins, using less solvent and shorter reaction times. A summary of synthetic methods is listed...
in Table 1 and the synthesis of flavylium ring has been comprehensively reviewed elsewhere (Iacobucci and Sweeney 1983).

Scheme 1 shows the condensation of 2,4-dihydroxybenzaldehyde with different acetophenone derivatives using a minimum amount of acetic and sulfuric acid. Heating in a water bath for 30 min facilitated the reaction, which resulted in a dark viscous liquid. The products were purified by trituration with diethyl ether. When performed with minimum exposure to air, fine, brightly colored powders are obtained, which were dried further in a vacuum desiccator. The hygroscopic anthocyanidins were assumed to be bisulfate salts, and the yields were 92–95 %. While the use of concentrated sulfuric acid is still harsh, improvements such as shorter heating time, use of the renewable solvent acetic acid, minimum solvents and adjuvants used during purification, and high yields makes our procedure greener. Characterization by $^1$H and $^{13}$C NMR and HRMS confirms the products, which have nearly similar UV–Vis and IR spectra in the functional group region.

Solutions of 1–3 were prepared by first dissolving in DMF, and subsequent dilution with acetate buffer (pH 3.6). Flavylium salts are in equilibrium with their hydrates in aqueous solutions, with low pH favoring the non-hydrated pyrilium cation (Moncada et al. 2004). Once hydrated, they may undergo ring opening, then tautomerization to the enone, and further isomerization to give trans chalcones. Buffered solutions of 1–3 showed no variation in the UV spectra when kept in the dark, and when kept cold for at least 1 week, hinting on their stability.

A modified FRAP assay was used to study the antioxidant properties of 1–3. Freshly prepared FRAP reagent was admixed with antioxidants at room temperature, which showed rapid development of color characteristic of the formation of the Fe$^{2+}$ complex. Spectrophotometric measurements were taken 2 min after mixing and all studies were performed in triplicate. The initial color change was fast, however the redox reaction continued for longer than 15 min, similar to what has been observed in polyphenol antioxidants (Pulido et al. 2000). Varying the location of the methoxy substituent on the C ring offers slight differences in the reducing power of the synthesized flavylium salt, with 1 showing the highest antioxidant activity (Fig. 1). This may be attributed to the added stability conferred by conjugation with the B ring substituents (Calogero et al. 2013). It can be reasoned that the higher activity of 1 compared to 3 is due to inductive effects of the proximal 2′ methoxy to the

| Conditions | Yield (%) | References |
|------------|-----------|------------|
| Salicylaldehyde, acetophenone, HBF₄, HOAc, acetic anhydride, 60 °C, 12 h | 40–58, 23–78 | Katritzky et al. (1998), Gomes et al. (2009) |
| Salicylaldehyde, acetophenone, BF₃ etherate, neat | 81 | Kühnert et al. (2001) |
| Salicylaldehyde, acetophenone, H₂SO₄, HOAc, overnight | 40–88 | Calogero et al. (2013) |
| Salicylaldehyde, acetophenone, EtOAc, HCl gas, 0 °C, 3 days | 56–75, 55–84 | Mora-Sournille et al. (2013), Mas (2003) |
| Salicylaldehyde, acetophenone, HF₆, HOAc | 89 | Kueny-Stotz et al. (2008) |
| Salicylaldehyde, acetophenone, HCl gas, formic acid | 56 | Moncada et al. (2004), Michaelidis and Wizinger (1951) |
| Salicylaldehyde, benzaldehyde, ethyl chloroformate, HClO₄, 1–12 h | 49–95 | Sato et al. (1999) |
| Salicylideneacetophenone, HBF₄OEt₂ or HOTf in Et₂O | 62–67 | Fichtner et al. (2001) |
| Phenol, ary lethynylketone, HF₆, HOAc, r.t. | 82–99 | Kueny-Stotz et al. (2007) |

Table 1 Reported syntheses of anthocyanidins

Scheme 1 Synthesis of anthocyanidins 1–3

1: $X₂ = H, X₃ = OMe (97\%)$
2: $X₁ = H, X₂ = OMe, X₃ = H (99\%)$
3: $X₁ = OMe, X₂ = H, X₃ = H (98\%)$
flavylium oxygen, which is absent in the 4′ methoxy (see Additional files 1, 2). The resonance effect is absent for the 3′ methoxy, resulting in least stable derivative (2).

The solution chemistry of anthocyanidins is complex (Pina et al. 2012) and analogous anthocyanidins under similar pH exist in equilibrium between the flavylium ion, deporotonated quinoidal base, and as the hydrated hemiketal (Brouillard et al. 1982; Sweeny and Iacobucci 1983). The FRAP assay is non-specific for any antioxidant present under the reaction conditions that could reduce $\text{Fe}^{3+}$, which takes into account the chemistry flavylium ions undergo in solution. Under similar assay conditions, ascorbic acid gives higher FRAP value (2.7) and shows a higher antioxidant activity than anthocyanidins 1–3. FRAP values are normally obtained after 4 min at 37 °C, or 6 min at room temperature. No significant variation of the FRAP value was observed between 4 and 6 min for our experiments, which are 2.2, 2.0, and 2.1 mM for 1, 2, 3, respectively, based on equivalent FeSO$_4$ standard. In comparison, purified anthocyanin extracts from fruit show reducing power one-third that of ascorbic acid, however these comparisons are not straightforward because the reducing power is dose-dependent even for ascorbic acid (Sun et al. 2014).

**Conclusion**

In conclusion, we demonstrate a greener synthesis of anthocyanidins, which allows facile purification by trituration. This facilitates the study of the effects of various substituents on the different rings to the properties of anthocyanidins. In this case, we show that altering the location of the methoxy substituent on the B ring results in slight variations in the resultant antioxidant activity, as measured by the FRAP assay. The methoxy substituent on the 2′ position of the B ring stabilizes the radical formed in the 7-OH position by conjugation, and by inductive effects due to the proximity of the the methoxy group to the pyrilium oxygen. These results demonstrate the feasibility of tailoring the redox properties of synthetic anthocyanidins.

**Experimental**

All starting materials and solvents were purchased from commercial sources. NMR analyses were performed using a Bruker 400 MHz Avance, and IR analyses were performed using a Bruker Alpha ATR-IR. High-resolution mass spec were obtained from The City College of New York Mass Spectrometry Facility, and the counter anion was not included in the molecular ion peak calculations.

**General procedure for FRAP**

Freshly prepared FRAP solution was prepared by mixing acetate buffer at pH 3.6 (10.0 cm$^3$, 20 mM), TPTZ solution (1.0 cm$^3$, 10 mM), and FeCl$_3$ solution (1.0 cm$^3$, 10 mM) in a vial. Stock solutions of the anthocyanidins (35.0 mg) were prepared in DMSO (100 cm$^3$, 1 mM). All solutions were sparged with N$_2$ prior to each experiment. For each experiment, the stock was diluted to 0.5 mM with acetate buffer and equilibrated for 3 min. The
experiment was initiated in a new vial containing de-ionized water (900 μL) and TPTZ solution (9.0 cm³). To this was added the diluted anthocyanins (300 μL), mixed, and immediately transferred to a cuvette. Data capture was started exactly 2 min after the reaction was initiated. The blank was prepared similarly, but adding only buffer instead of the stock anthocyanin solution. Each experiment was repeated at least three times.

General procedure for anthocyanins
To a 25-cm³ round bottomed flask was added 2,4-dihydroxybenzaldehyde (414 mg, 3.00 mmol) and the corresponding methoxycetophenone isomer (0.413 cm³), and sulfuric acid (0.500 cm³) was added. The mixture was equipped with an air condenser and heated in a boiling water bath for 30 min. The solid product was obtained by triturating the oil with diethyl ether (2.0 cm³). Purification was achieved by dissolving the crude in acetic acid and triturating with ether at room temperature. The precipitate was washed with diethyl ether before drying in a vacuum desiccator.

7-hydroxy-2-(2-methoxyphenyl)chromenylium hydrogen sulfate (1, C₁₆H₁₄O₇S) Rust-colored powder, 0.994 g (95 %). M.p.: 100–107 °C (decomposed); 1H NMR (400 MHz, MeOH-d₄) δ = 9.1 (d, 1H, J = 8.7 Hz), δ = 8.5 (d, 2H, J = 9.1 Hz), δ = 8.4 (d, 1H, J = 8.7 Hz), δ = 8.2 (d, 1H, J = 9.0 Hz), δ = 7.5 (d, 1H, J = 2.0 Hz), δ = 7.4 (dd, 1H, J = 8.9, 2.2 Hz), δ = 7.3 (d, 2H, J = 9.1 Hz), δ = 4.0 (s, 3H); 13C NMR (100 MHz, MeOH-d₄) δ = 173.7, 170.8, 168.7, 160.1 155.2, 143.2, 133.6, 122.83, 122.76, 120.6, 117.2, 113.7, 103.8, 57.0 ppm; HRMS (ESI) m/z 253.0889 (M⁺), calcd for C₁₆H₁₃O₃ 253.0865.

7-hydroxy-2-(3-methoxyphenyl)chromenylium hydrogensulfate (2, C₁₆H₁₄O₇S) Dark red powder, 0.966 g (92 %). M.p.: 122–155 °C (decomposed); 1H NMR (400 MHz, MeOH-d₄) δ = 9.3 (d, 1H, J = 8.5 Hz), δ = 8.5 (d, 1H, J = 8.5 Hz), δ = 8.3 (d, 1H, J = 9.0 Hz), δ = 8.1 (d, 1H, J = 8.2 Hz), δ = 8.0 (s, 1H), δ = 7.64 (m, 1H), δ = 7.62 (d, 1H, J = 1.8 Hz), δ = 7.5 (dd, 1H, J = 9.0, 2.1 Hz), δ = 7.4 (dd, 1H, J = 8.3, 1.9 Hz), δ = 4.0 (s, 3H); 13C NMR (100 MHz, MeOH-d₄) δ = 171.9, 170.6, 160.9, 160.2, 155.1, 133.1, 131.0, 130.5, 122.5, 121.8, 121.4, 120.5, 113.4, 113.1, 102.3, 55.0 ppm; HRMS (ESI) m/z 253.0890 (M⁺)⁺, calcd for C₁₆H₁₃O₃ 253.0865.

7-hydroxy-2-(4-methoxyphenyl)chromenylium hydrogensulfate (3, C₁₆H₁₄O₇S) Orange-red powder, 0.990 g (95 %). M.p.: 157–190 °C (decomposed); 1H NMR (400 MHz, MeOH-d₄) δ = 9.1 (d, 1H, J = 8.7 Hz), δ = 8.5 (d, 2H, J = 9.1 Hz), δ = 8.4 (d, 1H, J = 8.7 Hz), δ = 8.2 (d, 1H, J = 9.0 Hz), δ = 7.5 (d, 1H, J = 2.0 Hz), δ = 7.4 (dd, 1H, J = 8.9, 2.2 Hz), δ = 7.3 (d, 2H, J = 9.1 Hz), δ = 4.0 (s, 3H); 13C NMR (100 MHz, MeOH-d₄) δ = 173.7, 170.8, 168.7, 160.1, 155.2, 143.2, 133.6, 122.83, 122.76, 120.6, 117.2, 113.7, 103.8, 57.0 ppm; HRMS (ESI) m/z 253.0889 (M⁺), calcd for C₁₆H₁₃O₃ 253.0865.

Additional files

Additional file 1. Checklist for compound characterization.
Additional file 2. Compound characterization data.

Abbreviation
FRAP: ferric reducing/antioxidant power assay.

Authors’ contributions
HSB drafted the manuscript and performed synthesis. PC performed synthesis and characterization. AT performed FRAP assays.

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Compliance with ethical guidelines
The authors declare that they have no competing interests.

References
Alzaid F, Cheung H-M, Preedy VR, Sharp PA (2013) Regulation of glucose transporter expression in human intestinal Caco-2 Cells following exposure to an anthocyanin-rich berry extract. PLoS One 8(11):e78932. doi:10.1371/journal.pone.0078932
Balaban AT, Schroth W, Fischer G (1969) Pyrylium salts part I. Syntheses. In: Katritzky AR, Boulton AJ (eds) Advances in heterocyclic chemistry, vol 10. Academic Press, pp 241–326. doi:10.1016/S0065-2725(08)60499-7
Benzie IFF, Strain JJ (1999) The ferric reducing/antioxidant power assay: a measure of antioxidant power: the FRAP assay. Anal Biochem 239(1):70–76. doi:10.1006/abio.1996.0292
Benzie IFF, Strain JJ (1999) [2] Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. In: Lester P (ed) Methods in enzymology, vol 299. Academic Press, pp 15–27. doi:10.1016/S0076-6879(99)99005-5
Bouillard R, Iacobucci GA, Sweeny JG (1982) Chemistry of anthocyanin pigments. 9. UV-visible spectrophotometric determination of the acidity constants of apigeninidin and three related 3-deoxyflavylum salts. J Am Chem Soc 104(26):7585–7590. doi:10.1021/ja00390a033
Calogerou G, Sinopoli A, Citro I, Di Marco G, Petrov V, Diniz AM, Parola AJ, Pina F (2013) Synthetic analogues of anthocyanins as sensitizers for dye-sensitized solar cells. Photochem Photobiol Sci 12(5):883–894. doi:10.1039/C3PP25347C
