Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 4589–4593
Bioorganic & Medicinal Chemistry

Inhibition of 15-lipoxygenase-catalysed oxygenation of arachidonic acid by substituted benzoic acids

Wendy R. Russell,[a,*] Lorraine Scobbie,[a] Garry G. Duthie[a] and Andrew Chesson[b]

[a]*Molecular Nutrition Group, Rowett Research Institute, Aberdeen, AB21 9SB, Scotland, UK*
[b]*University of Aberdeen, Aberdeen, AB24 4FA, Scotland, UK*

Received 5 April 2007; revised 7 February 2008; accepted 12 February 2008
Available online 15 February 2008

Abstract—Elevated levels of phospholipases, prostaglandin synthases and lipoxygenases in colonic cells at various stages of malignancy indicate a strong link between dietary lipids and colon cancer. Lipoxygenase-catalysed arachidonic acid metabolism plays a key role in colorectal carcinogenesis and has the potential to be modulated by phenolic compounds. Plant-based foods are rich sources of phenolic compounds and in the human colon they are predominantly available as simple phenolics such as the benzoic acids. Benzoic acids were determined in faecal waters from four volunteers consuming a western-style diet. Structure–activity relationships were established for the lipoxygenase-catalysed oxygenation of arachidonic acid using an oxygen electrode. All compounds studied inhibited this reaction (21–73%; $p < 0.001$) and many of the structural features could be rationalised by computational modelling. No correlation was observed with the ability to act as reductants, supporting the hypothesis that their mode of inhibition may not be by a direct redox effect on the non-haem iron.

1. Introduction

Aberrant arachidonic acid metabolism is involved in many aspects of inflammatory disease and in particular in the development of colorectal cancer. In the human colon, arachidonic acid is metabolised predominantly by prostaglandin H synthase and arachidonate lipoxygenase to generate prostanoids and leukotrienes, respectively. Several lines of evidence suggest that the lipoxygenase enzymes and their metabolites contribute towards the pathogenesis of colon cancer, but the physiological function of these enzymes is, as yet, far from clear. Increased expression of 15-lipoxygenase-1 was detected in human colorectal tumours and was primarily localized in the epithelium.[1] In human colorectal cancer cells, the expression of 15-lipoxygenase was only observed during apoptosis and cell differentiation and inhibition resulted in the enhancement of apoptosis,[2] but conflicting results suggest that these metabolites may also induce apoptosis.[3] More recently, 12/15-lipoxygenase metabolites were shown to up-regulate a secretory phospholipase A2,[4] and thioredoxin reductase,[5] important enzymes involved in inflammation. These observations support the hypothesis that lipoxygenase metabolites have the potential to modulate inflammatory events in the colon.

Regular consumption of fruits and vegetables is known to markedly decrease the risk of developing colon cancer.[6] Phytochemicals present in fruits and vegetables and particularly the products of the phenylpropanoid pathway are widely studied for their potential health benefits. The flavonoids are a well-studied, highly abundant group of phytochemicals. However, the X-ray structure of lipoxygenase in complex with quercetin (a predominant flavonoid) revealed that it was 3,4-dihydroxybenzoic acid that occupied the active site.[7] Therefore, in addition to the benzoic acids that are widely available parent compounds in plant-based foods, it is likely that since many of the flavonoids will also be metabolised to benzoic acids, these will make an important contribution to a diet rich in fruits and vegetables. It has been shown that it is phenolics, such as the benzoic and cinnamic acid derivatives, that are the major products of microbial metabolism and are most prevalent in the human colon.[8] If these compounds can directly inhibit or enhance lipoxygenase-catalysed oxygenation of arachidonic acid, they have the potential

Keywords: Colorectal cancer; Colon cancer; Lipoxygenase; Inflammation; Phytochemicals; Nutrition; Benzoic acids; Phenolics; Diet.
* Corresponding author. Tel.: +44 1224 716619; fax: +44 1224 716629; e-mail: W.Russell@rowett.ac.uk

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.02.041

to modulate inflammatory events in the colon. In this study, we have focused on the effect of aromatic substitution in the benzoic acids on 15-lipoxygenase-catalysed oxidation of arachidonic acid.

2. Results and discussion

2.1. Presence of substituted benzoic acids in the human colon

The presence and concentration of substituted benzoic acids in the colon were determined by the analysis of fresh faecal samples collected from four human volunteers consuming an unrestricted western-style diet (Table 1). All three monohydroxylated benzoic acids were detected in some of the volunteers in concentrations up to 3.6 μmol dm^{-3}. The only dihydroxylated benzoic acid detected was 2,5-dihydroxybenzoic acid at a concentration of 4.5 μmol dm^{-3} and the highest concentrations were observed for the mono- and di-methoxylated benzoic acids at concentration up to 148.6 and 7.5 μmol dm^{-3}, respectively. Products of the arachidonic acid pathway are generally reported to be present in concentrations in the nmol dm^{-3} range, and the ratio of arachidonic acid to substrate used in the inhibition experiments is 1:30. Since the concentration of substituted benzoic acids in the faecal waters is well in excess of this ratio, it is likely that the effects observed will be physiologically relevant.

2.2. 15-Lipoxygenase inhibition

Soybean 15-lipoxygenase was selected to assess the inhibitory affect of substituted benzoic acids on the oxygenation of arachidonic acid, as the required quantities of human 15-lipoxygenase were not available to perform these studies. Comparison of the three-dimensional structures of soybean 15-lipoxygenase with mammalian 15-lipoxygenase[9,10] demonstrated that these enzymes have similar topology and analogous active sites.[11,12] Soybean 15-lipoxygenase has also been shown to bind 3,4-dihydroxybenzoic acid and the X-ray crystal structure was available for direct comparison of binding studies with the experimental data.[7] This 15-lipoxygenase catalysed the insertion of oxygen into arachidonic acid and oxygen uptake correlated well with the concentration of arachidonic acid (Fig. 2A). Caffeic acid exhibited a dose-dependent inhibition, with full inhibition being achieved when 25 μmol was added (Fig. 2B). By using the oxygen electrode to determine the extent of enzyme inhibition, instead of measuring product formation, it is clear that it is the reaction involving oxygen insertion into arachidonic acid that is inhibited, rather than a switch in product formation. The presence of benzoic acid and its substituted analogues prior to the addition of 15-lipoxygenase did not effect the oxygen level. All of the substituted benzoic acids studied significantly ($p < 0.001$) inhibited the oxygenation of arachidonic acid by between 21% and 73% (Table 2). 3,4-Dihydroxybenzoic acid **9** was the best inhibitor and previous studies of the X-ray structure of this compound within the active site showed that the C4-OH and carboxyl group were integrated into a hydrogen bonded network with the enzyme.[7] The presence of an additional hydroxyl group at C5 significantly ($p = 0.015$) decreased this inhibition. This demonstrates that, although 3,4,5-trihydroxycinnamic acid **10** has the potential to hydrogen bond in the same manner as 3,4-dihydroxycinnamic acid **9**, the additional hydroxyl imposes a restricting factor.

Table 2. Inhibition (%) of arachidonic acid oxygenation by substituted benzoic acids

Compound	Inhibition (%)	Reaction stoichiometry
1	51.6 ± 4.2	n.r.
2	48.4 ± 0.7	n.r.
3	46.0 ± 3.4	n.r.
4	47.6 ± 4.8	n.r.
5	47.0 ± 5.6	1.41 ± 0.03
6	47.7 ± 3.0	n.r.
7	49.9 ± 2.2	1.50 ± 0.03
8	21.2 ± 0.1	0.80 ± 0.03
9	73.3 ± 1.2	1.30 ± 0.11
10	58.1 ± 0.3	n.r.
11	60.5 ± 1.0	2.14 ± 0.04
12	45.4 ± 2.7	n.r.

Data are presented as mean ± standard deviations ($n = 3$). Benzoic acids were compared at concentrations (15 μmol) which are equivalent to 36.2% inhibition by the known lipoxygenase inhibitor; caffeic acid. Inhibition was significant ($p < 0.001$) for all compounds, as determined by the *t*-TEST. Reducing equivalents are given as reaction stoichiometries for galvinoxyl radical reduction. Not reduced (n.r.).

2.3. Structural model

To compare the binding of the substituted benzoic acid inhibitors, an active site was generated from the X-ray structure of 15-lipoxygenase (soybean) containing 3,4-dihydroxybenzoic acid.[7] When the trihydroxylated analogue was fitted to the generated site, an optimum fit

Table 1. Substituted benzoic acids determined in faecal waters from four volunteers consuming an unrestricted western-style diet

Benzoic acid	Volunteer 1 (μmol dm^{-3})	Volunteer 2 (μmol dm^{-3})	Volunteer 3 (μmol dm^{-3})	Volunteer 4 (μmol dm^{-3})
2-Hydroxybenzoic acid	n.d.	0.40 ± 0.81	0.85 ± 1.48	0.87 ± 1.51
3-Hydroxybenzoic acid	3.62 ± 5.78	0.23 ± 0.45	0.70 ± 0.69	0.78 ± 0.70
4-Hydroxybenzoic acid	0.71 ± 1.42	n.d.	n.d.	0.15 ± 0.26
2,5-Dihydroxybenzoic acid	4.45 ± 3.87	n.d.	n.d.	n.d.
3-Methoxy-4-hydroxybenzoic acid	148.62 ± 130.84	9.85 ± 11.79	51.10 ± 23.15	22.75 ± 4.20
3,5-Dimethoxy-4-hydroxybenzoic acid	7.46 ± 9.21	0.76 ± 0.55	1.62 ± 1.12	n.d.
3,4,5-Trihydroxybenzoic acid	n.d.	0.76 ± 0.66	1.91 ± 2.21	0.48 ± 0.05

Data are presented as mean concentrations ±standard deviations ($n = 3$). Not detected (n.d.).

could only be achieved when the carboxyl group was angled away from Gln514 (an integral component of the 3,4-dihydoxybenzoic acid-lipoxygenase H-bonding network). However, the 5-hydroxyl group was in close proximity to Gln514 (4.561 Å compared to 3.217 Å) and this still allowed the 3-hydroxyl group to point towards the iron (4.761 Å compared to 4.406 Å) and a potential H-bonding interaction with the C-terminus (5.648 Å compared to 4.438 Å). Replacement of the hydroxyl group at C3 with a methoxyl group resulted in a further decrease in inhibition. This was despite the model of the methoxylated analogue adopting a similar alignment to the X-ray structure of the 3,4-dihydroxylated compound and demonstrated the importance of the hydroxyl at C3. For all the dihydroxylated compounds **5**–**10**, the extent of inhibition was as follows: 3,4 > 3,5 > 2,5 > 2,4 > 2,3 > 2,6. For these compounds, the 2-OH substituent appeared to be least favourable, with the compound containing two hydroxyls ortho to the carboxylic acid group (2,6-dihydroxybenzoic acid **8**) having the least effect on inhibition. When the 2,6-dihydroxylated compound **8** was fitted to the active site, optimum fits are generated only when the hydroxyls point away from the iron in an opposing direction to the 3,4-dihydroxybenzoic acid. This feature was also observed but to a lesser extent with the other 2-hydroxylated dimers **5**–**7**. The 3,5-dihydroxylated **10** compound aligns in a similar manner to 3,4-dihydroxylbenzoic acid **9** and is a better inhibitor than the dihydroxylated compounds containing 2-hydroxyl substituents. The monohydroxylated compounds all exhibited a similar inhibitory effect.

2.4. Redox chemistry

Other studies have shown that when the lipoxygenase active site is occupied by arachidonic acid, the bisallylic methylene group at C13 is located at close proximity to the non-haem iron, which is located adjacent to a bend close to the bottom of the cavity.[12] This can allow hydrogen abstraction or alternatively π-electron transfer from the double bond. To assess whether inhibition was a direct consequence of a redox reaction, stoichiometric reducing equivalents were determined by measuring the extent by which the compounds reduced the synthetic radical galvinoxyl by electron paramagnetic resonance (EPR) spectroscopy.[13,14] For all the compounds studied, their ability to act as a reductant had no direct correlation with biological effect suggesting that direct redox reaction is unlikely to be the predominant mode of inhibition.

3. Conclusion

In this study, we have shown that the hydroxylated benzoic acids are present in the colon at concentrations in the μmol dm^{-3} range. All the benzoic acids studied significantly ($p < 0.001$) inhibited lipoxygenase-catalysed oxygenation of arachidonic acid and the ratio of the benzoic acid substrates to arachidonic acid is likely to be physiologically relevant. With the exception of the 2,6-hydroxylated substrate, all the benzoic acids inhibited oxygenation to a greater extent (45.4–73.3%; Fig. 1) than the known lipoxygenase inhibitor; caffeic acid (36.2%; Fig. 2) at equivalent concentrations (15 μmol). Optimal inhibition was achieved by the 3,4-dihydroxylated benzoic acid and the position of the hydroxyl group had a greater effect than the extent of hydroxylation. The results of the EPR studies suggest that there was no direct structural correlation with redox chemistry. This is consistent with the observations from X-ray structures which have shown that inhibitors moor close to the non-haem iron affecting the iron geometry[15] but not necessarily the redox chemistry of the reaction directly. Since lipoxygenases appear to play a key role in modulating some of the inflammatory events leading to colon cancer, these important and abundant dietary plant compounds and major metabolites of the flavonoids are likely to affect these processes. However, the impact with regard to potential health benefits of these compounds will not be ascertained until the exact role of this important group of enzymes in diseases such as colon cancer has been clarified.

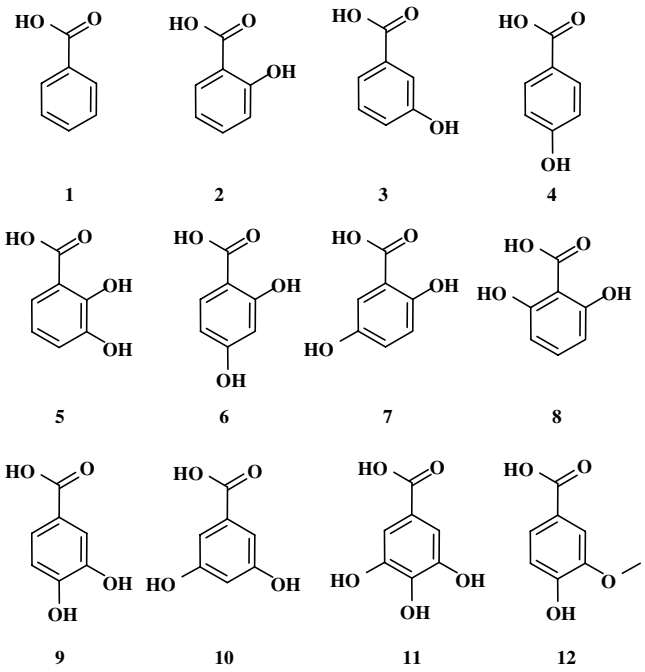

Figure 1. Structures of substituted benzoic acids: benzoic acid (1), 2-hydroxybenzoic acid (2), 3-hydroxybenzoic acid (3), 4-hydroxybenzoic acid (4), 2,3-dihydroxybenzoic acid (5), 2,4-dihydroxybenzoic acid (6), 2,5-dihydroxybenzoic acid (7), 2,6-dihydroxybenzoic acid (8), 3,4-dihydroxybenzoic acid (9), 3,5-dihydroxybenzoic acid (10), 3,4,5-trihydroxybenzoic acid (11), and 4-hydroxy-3-methoxybenzoic acid (12).

4. Experimental

4.1. General

15-Lipoxygenase (soybean P1) was purchased from Cayman Chemicals [Ann Arbor, MI] and was supplied in Tris–HCl buffer (1.5×10^8 U; pH 7; 8 cm^3). The enzyme was sub-aliquoted and stored at −80 °C. Arachidonic

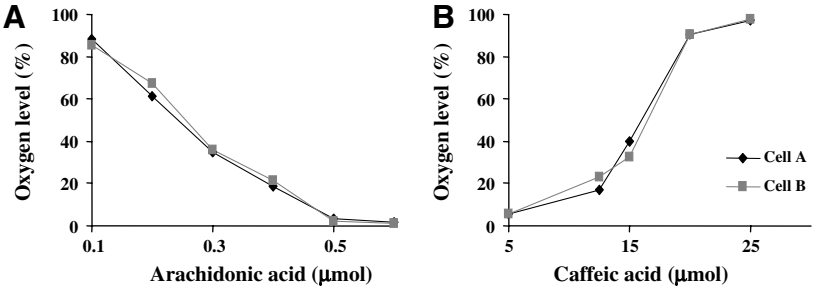

Figure 2. (A) Optimisation of arachidonic acid concentration for maximum 15-lipoxygenase-catalysed oxygenation. (B) Dose-dependent inhibition of 15-lipoxygenase-catalysed oxygenation of arachidonic acid (0.5 μmol) with caffeic acid. Experiments were repeated at five concentrations using two separate oxygen electrode cells.

acid was purchased from Sigma and was dissolved in ethanol to give a final concentration of 10 mmol dm^{-3}. All manipulation of arachidonic acid was made under nitrogen and solutions were stored in the dark at −80 °C where possible. Substituted benzoic acids were purchased from Aldrich (Compounds **1**, **3**, **5**–**8**, and **10**), Sigma (Compounds **2**, **4**, **11**, and **12**) and Fluka (Compound **9**) [Gillingham, England].

4.2. Substituted benzoic acids in faecal samples

To determine the presence and concentration of the metabolites in the colon, fresh faecal samples were collected weekly from four human volunteers consuming an unrestricted western-style diet ($n = 3$). Separation of solid matter from faecal waters was obtained by centrifugation at 50,000*g* for 2 h. Both the pellet and the supernatant were then freeze-dried and the water content was calculated. Both fractions (~100 mg) were suspended in HCl (0.2 mol dm^{-3}; 10 cm^3) and extracted into ethyl acetate (5 cm^3), separating the layers by centrifugation (3000 rpm; 5 min). This process was repeated three times and the organic layers were combined, filtered through Na_2SO_4 and evaporated to dryness under reduced pressure at a temperature of less than 40 °C. The extracts were then re-dissolved in methanol (0.2 cm^3) and filtered through a 0.2 μm PVDF membrane. Separation of the phenolic compounds was by HPLC, employing two gradient elution methods using acetonitrile (AcCN) and trifluoroacetic acid (0.05% v/v; pH 2.3). Method 1: 11–14% AcCN (35 min), 14–50% AcCN (5 min), 50% AcCN (10 min), and 50-11% AcCN (5 min). Method 2: 11–40% AcCN (40 min), 40–50% AcCN (10 min), and 50–11% AcCN (5 min). Detection was at 215 and 280 nm and the metabolites were quantified by internal standardisation and use of response factors calculated from pure compounds.

4.3. Substrate inhibition of arachidonic acid oxygenation

The oxygen electrode [Rank Brothers, England] was assembled as described in the manufactures instructions and the instrument isolated from other electronic equipment by connection through an uninterruptible power supply system. For validation of the method, all initial experiments were performed using two separate electrodes (A and B). The electrode response and stability were tested at the beginning of each experiment by the addition of sodium dithionite, which gave a reproducible and stable signal for maximum oxygen depletion from the system. Buffer (Tris–HCl; pH 7.4; 100 mmol dm^{-3}; 2.95 cm^3) was placed in the cell and an aliquot (60 μl) removed and replaced with arachidonic acid (60 μl, 10 mmol dm^{-3}). The plunger was assembled and the sensitivity set to full scale (100). Once a stable baseline was achieved, 15-lipoxygenase (5 μl; 93,750 U) was added. The signal fell rapidly and reproducibly to a value of 0.50 ± 0.00 ($n = 4$) in 100 s for both electrodes. To determine the conditions for optimum oxygenation of arachidonic acid, aliquots of buffer were replaced by arachidonic acid (10–60 μl; 10 mmol dm^{-3}), 15-lipoxygenase (5 μl; 93,750 U) was added and the signal monitored for 100 s (Fig. 2A). Caffeic acid, a known inhibitor of lipoxygenase, was used to validate the method. Aliquots of buffer were replaced with caffeic acid (10–50 μl; 0.5 mol dm^{-3} in DMSO) and the reaction was performed in the presence of arachidonic acid (50 μl; 10 mmol dm^{-3}) as detailed above (Fig. 2B). Once the experimental conditions were established, aliquots of buffer were replaced by the substituted benzoic acids (30 μl; 0.5 mol dm^{-3} in DMSO) and arachidonic acid (50 μl; 10 mmol dm^{-3}). 15-Lipoxygenase (5 μl; 93,750 U) was added and the signal recorded for 100 s.

4.4. Electron paramagnetic resonance spectroscopy

Aliquots (3 cm^3) of galvinoxyl [Aldrich] (0.5 mmol dm^{-3} in methanol) were mixed with substrates (measured at various concentrations to determine linearity, typically 0.1–0.5 mmol dm^{-3} in methanol) and transferred to an EPR quartz cell. Spectra (X-band) of unreacted galvinoxyl were recorded after five minutes on a Bruker E106 spectrometer, equipped with a TM_{110} cavity. The following instrument settings were used: modulation frequency, 100 kHz; centre field, 3480.40 Gauss; sweep width, 60 Gauss; time constant, 40.96 ms; power, 1.01 mW; and a suitable receiver gain setting, typically 1×10^4. The galvinoxyl concentrations remaining were calculated by the integration of the signal and comparison with the control. From these concentrations, the stoichiometry of the reaction was calculated.

4.5. Computational study

The structural co-ordinates of the crystal structure of 3,4-dihydroxybenzoic acid bound to the active site of

15-lipoxygenase (soybean) were downloaded from the Brookhaven Protein Database.[7] Using the Cerius[2] ligand fit package [Accelrys; Cambridge, England] running on an SGI Octane workstation [SGI], the protein and ligand were defined and a binding site was generated with a resolution of 0.5 Å and hydrogen and heavy atom radii of 2 and 2.5 Å, respectively. Energy minimised structures of the compounds were then selected as potential ligands and were fitted and scored using ligand Monte Carlo parameters with polar hydrogens set at 30° and RMS and score thresholds of 1.5 and 20, respectively. Autocharge, flexible fit and ligand permeation were allowed and soft interaction potential energy was selected.

Acknowledgments

Funding is gratefully acknowledged from the Scottish Executive Environment and Rural Affairs Department (SEERAD).

References and notes

1. Ikawa, H.; Kamitiani, H.; Calvo, B. F.; Foley, J. F.; Eling, T. E. *Cancer Res.* **1999**, *59*, 360–366.
2. Kamitiani, H.; Geller, M.; Eling, T. *J. Biol. Chem.* **1998**, *273*, 21569–21577.
3. Shureiqi, I.; Jiang, W.; Zuo, X. S.; Wu, Y. Q.; Stimmel, J. B.; Leesnitzer, L. M.; Morris, J. S.; Fan, H. Z.; Fischer, S. M.; Lippman, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, 9968–9973.
4. Kuwata, H.; Yamamoto, S.; Takekura, A.; Murakami, M.; Kudo, I. *Biochim. Biophys. Acta* **2004**, *1686*, 15–23.
5. Yu, M. K.; Moors, P. J.; Cassidy, P.; Wade, M.; Fitzpatrick, F. A. *J. Biol. Chem.* **2004**, 28028–28035.
6. Riboli, E.; Norat, T. *Am. J. Clin. Nutr.* **2003**, 559S–569S.
7. Borbulevych, O. Y.; Jankun, J.; Selman, S. H.; Skrypczak-Jankun, E. *Proteins* **2004**, 13–19.
8. Jenner, A. M.; Rafter, J.; Halliwell, B. *Free Radical Biol. Med.* **2004**, 763–772.
9. Boyington, J. C.; Gaffney, B. J.; Amzel, L. M. *Science* **1993**, *260*, 1482–1486.
10. Gillmor, S. A.; Villascnor, A.; Fletterick, R.; Sigal, E.; Browner, M. F. *Nat. Struct. Biol.* **1997**, *4*, 1003–1009.
11. Skrzypczak-Jankun, E.; Zhou, K.; McCabe, N. P.; Selman, S. H.; Jankun, J. *Int. J. Mol. Med.* **2003**, *12*, 17–24.
12. Kuhn, H.; Walther, M.; Kuban, R. J. *Prostaglandins Other Lipid Mediat.* **2002**, 263–290.
13. McPhail, D. B.; Hartley, R. C.; Gardner, P. T.; Duthie, G. G. *J. Agric. Food Chem.* **2003**, 1684–1690.
14. Russell, W. R.; Scobbie, L.; Chesson, A. *Bioorg. Med. Chem.* **2005**, 2537–2546.
15. Pham, C.; Jankun, J.; Skrzypczak-Jankun, E.; Flowers, R. A.; Funk, M. O. *Biochemistry* **1998**, 17952–17957.