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Anthocyanins from wild carrot suspension cultures acylated with supplied carboxylic acids[1]

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Abstract

The anthocyanins accumulated by carrot cell cultures include some that are acylated. Addition of cinnamic and benzoic acid analogues to the culture medium resulted in the production of fourteen novel monoacylated anthocyanins. Four additional anthocyanins were prepared by partial acid hydrolysis, which selectively removed the xylose side chain from the branched trisaccharide part of the molecule. The carrot cells accommodate a wide range of carboxylic acids used to acylate anthocyanins and provide a system for the preparation of anthocyanins that have a variety of acyl groups attached. The compounds were characterized by ^{1}H NMR spectroscopy and mass spectrometry, and by UV-vis absorption spectroscopy at both pH 2 and 6.

Keywords: Anthocyanin; Tissue culture; Acylation; Nutraceuticals; Natural colorants

1. Introduction

Anthocyanins, which are highly colored substances found in plants, are possible viable colorants for use in food, nutraceutical, and pharmaceutical preparations [2,3]. Chemically anthocyanins are glycosides of benzopyrylium (flavylium) salts that have the unfortunate drawback in that they lose most of their color at near-neutral pH. This phenomenon is understood to be due to hydration of the flavylium nucleus at C-2, a process that interrupts the conjugation in the chromophore [4–6]. A small number of anthocyanins, characteristically those acylated on the sugar moiety with cinnamic acids, show greatly increased color retention in near-neutral solutions; that is, they exhibit a decreased rate of hydration or an increased rate of dehydration (reversal). The resultant color retention of anthocyanins is highly desirable and enhances their potential utility in the food and related industries.

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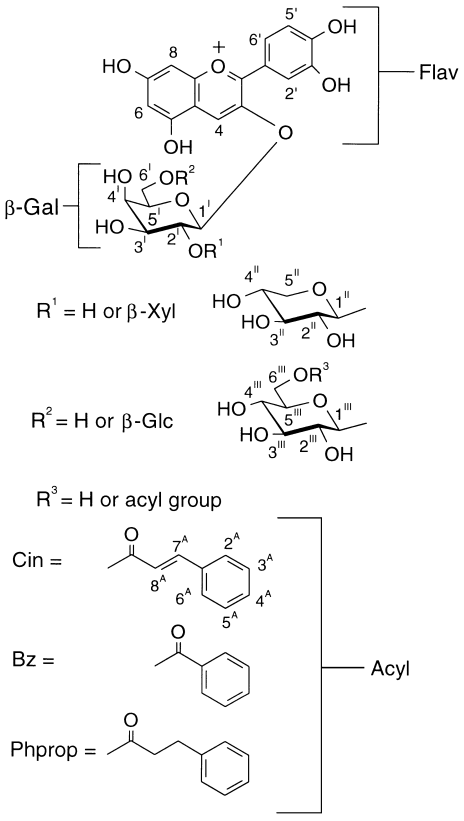
Acylation of the carbohydrate moiety is a primary method for increasing anthocyanin color retention. To improve our understanding of the ways in which these acyl groups alter the color retention of anthocyanins, it is desirable (1) to measure the effects of these acyl groups and substituents on the rate constants of the hydration–dehydration reaction, and (2) determine the physicochemical interrelationships (i.e., the distance, orientation, and any possible stereoelectronic effects) between the acyl group and the flavylium chromophore. These objectives require the availability of a series of anthocyanins that differ systematically in the acyl group present. As such a series is not available by isolation from plants in nature, a semibiosynthetic method using plant cell cultures was pursued.

When naturally occurring cinnamic acids are fed to wild carrot suspension cultures, the proportion of acylated to nonacylated anthocyanins increases. Work from these laboratories has shown that the cultures incorporate 3,4-dimethoxycinnamic acid and 3,4,5-trimethoxycinnamic acid into new anthocyanins when these acids are provided in the culture medium [7]. In these prior studies, some evidence for the presence of anthocyanins acylated with 4-hydroxybenzoic acid was also obtained, a finding that is in line with a previous report by Gläßgen et al. [8].

We now report our findings concerning the ability of carrot cell suspension cultures to biosynthesize acylated anthocyanins by providing variously substituted cinnamic and benzoic acids to the cultures. We show that the anthocyanins produced differ in their ability to retain color at pH 6 and provide further evidence that there are interactions between the acyl groups and the chromophore. A preliminary account of these findings has been reported [9].

2. Results and discussion

Isolation, purification, and characterization of the anthocyanins.—Anthocyanins were acylated with variously substituted benzoic and cinnamic acids to give compounds **5**–**24** by addition of the requisite carboxylic acids to a tissue culture of *Daucus carota* spp *carota* (wild carrot) by previously reported methods [7]. These were then isolated, purified, and characterized (see Experimental section). The nonacylated anthocyanins *β*-D-glucopyranosyl-(1→6)-[*β*-D-xylopyranosyl-(1→2)]-*β*-D-galactopyranosyl-(1→O^3)-cyanidin (**1**), *β*-D-glucopyranosyl-(1→6)-*β*-D-galactopyranosyl-(1→O^3)-cyanidin (**2**), *β*-D-xylopyranosyl-(1→2)-*β*-D-galactopyranosyl-(1→O^3)-



1	R^1 = β-Xyl	R^2 = β-Glc	R^3 = H
2	R^1 = H	R^2 = β-Glc	R^3 = H
3	R^1 = β-Xyl	R^2 = H	
4	R^1 = H	R^2 = H	
5	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-OH-3,5-(OMe)$_2$-Cin
6	R^1 = H	R^2 = β-Glc	R^3 = 4-OH-3,5-(OMe)$_2$-Cin
7	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 3,4,5-(OMe)$_3$-Cin
8	R^1 = H	R^2 = β-Glc	R^3 = 3,4,5-(OMe)$_3$-Cin
9	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-OH-Cin
10	R^1 = H	R^2 = β-Glc	R^3 = 4-OH-Cin
11	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-OMe-Cin
12	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 3-OMe-Cin
13	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 2-OMe-Cin
14	R^1 = β-Xyl	R^2 = β-Glc	R^3 = Cin
15	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-F-Cin
16	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-Cl-Cin
17	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-CF$_3$-Cin
18	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-NO$_2$-Cin
19	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-(Me$_2$N)-Cin
20	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 3,4,5-(OMe)$_3$-Bz
21	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-OH-Bz
22	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-Cl-Bz
23	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-CF$_3$-Bz
24	R^1 = β-Xyl	R^2 = β-Glc	R^3 = 4-OH-Phprop

cyanidin (**3**), and β-D-galactopyranosyl-(1→O^3)-cyanidin (**4**) were prepared to provide reference compounds to determine the effects of various acyl groups on the properties of the anthocyanins. Compounds **1** and **3** have been partially characterized [1,7,8], and **3** has been reported by Terahara et al. [10]. Compounds **5**, **7**, **9**, and **11**–**24** are all 6-*O*-acyl-β-D-glucopyranosyl-(1→6)-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranosyl-(1→O^3)-cyanidins. Of these, **5** and **9** have been characterized previously [7,8]. Compounds **6**, **8**, and **10** are 6-*O*-acyl-β-D-glucopyranosyl-(1→6)-β-D-galactopyranosyl-(1→O^3)-cyanidins.

Characterization.—(a) NMR studies. The ^{1}H NMR data for anthocyanins **1**–**24** are given in Table 1. By making use of the ^{1}H NMR chemical shifts and coupling constants for oligosaccharides in general [11], together with data that have been reported from 2D NMR spectroscopy of anthocyanins [8,12], specific protons in this series of anthocyanins were assigned. For example, the anomeric proton of each unit of the glycone was observed in regions typical for that particular sugar moiety. Ranges were as follows: D-galactose subunit δ 5.18–5.40; D-xylose subunit, δ 4.64–4.86; and D-glucose subunit δ 4.23–4.64. All these protons exhibited H-1–H-2 coupling constants between 7.1 and 7.8 Hz, showing that all the sugar linkages are in the β-D-configuration.

When xylose is absent, H-2^{I} is found upfield at less than δ 4.0 (see data for **2**, **4**, **6**, **8**, and **10**), and when xylose is present, H-2^{I} is found downfield of δ 4.169, confirming the attachment of xylose at the 2-position of the galactose residue. The connectivity between the galactose and the glucose moieties was unequivocally established by the use of NOESY spectroscopy on **1**, **7**, **15**, and **16**. A partial NOESY spectrum for **7** is shown in Fig. 1. Gläßgen et al. [8] independently showed this connectivity for **5** and the carrot anthocyanin acylated with 4-hydroxy-3-methoxycinnamic acid (ferulic acid) by ^{1}H-detected short- (HMQC) and long-range (HMBC) correlation experiments.

Comparison of the NMR data of a monoacylated compound with its nonacylated counterpart, e.g., **5** and **1**, respectively, indicates that acylation has occurred on C-6^{III} of the glucose moiety as the chemical shift of the H-6^{IIIA} of **5** is observed at δ 5.339 and that for **1** at δ 3.846. There is also a much smaller downfield shift of H-6^{IIIB} observed with the acylated compounds. For **24**, which is acylated with 3-(4-hydroxyphenyl)-propionic acid, a similar downfield shift, albeit one smaller in size, is observed for H-6^{IIIA}.

The presence of a cinnamic or benzoic acid residue on the glucose moiety has a marked effect on the chemical shift of the cyanidin H-4 proton in the acylated compounds. The chemical shifts for H-4 in nonacylated **1**–**4** are in the range of δ 9.029 to 8.981, while those for the acylated **5**–**24** appear at δ 8.474–8.953. In the anthocyanins acylated with cinnamic acids, but not in those acylated with benzoic acids or with 3-(4-hydroxyphenyl)propionic acid, there is a change in the chemical shift of H-8 from the region of δ 6.887–6.964 in the nonacylated **1**–**4** to δ 6.448–6.606 in the cinnamoylated compounds **5**–**19**. Gläßgen et al. [8] have noted a pronounced upfield shift of H-4 in carrot anthocyanins acylated with 4-hydroxy-3,5-dimethoxycinnamic (sinapic), ferulic, 4-hydroxycinnamic (4-coumaric), and 4-hydroxybenzoic acids relative to that for the nonacylated compound. These authors have interpreted this as evidence for intramolecular copigmentation (stacking). Although not noted by them, the data of Gläßgen et al. [8] also show the upfield shift of H-8 in anthocyanins

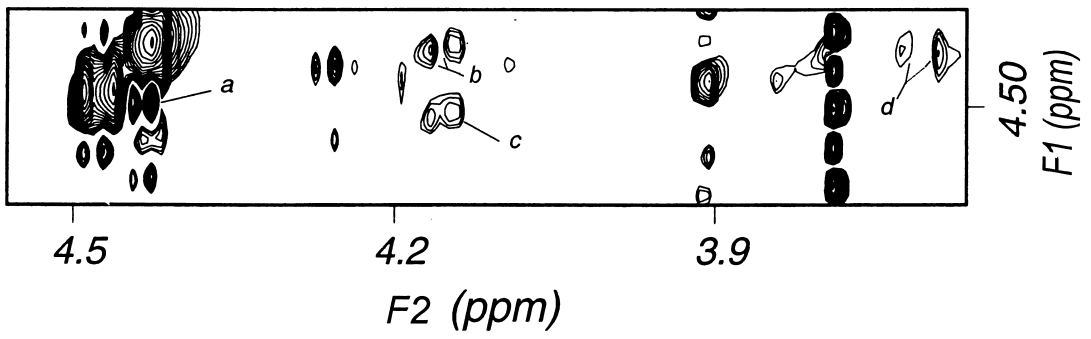


Fig. 1. Portion of the NOESY spectrum (500 ms mixing time) of **7** at 20 °C. The cross-peaks are labeled, (a) H-1^{III}–H-5^{I}, (b) H-1^{III}–H-6^{IA}, (c) H-5^{I}–H-6^{IA}, and (d) H-1^{III}–H-6^{IB}.

Table 1
^{1}H NMR data of anthocyanins **1**–**24** [a]

	H	**1** [b,c]	**2**	**3** [d]	**4**	**5** [b]	**6** [b]	**7** [b]	**8** [b]	**9**	**10**	**11**
Flav	4	8.981 br s	9.029 br s	8.976 br s	9.027 br s	8.474 d (0.9)	8.605 d (0.9)	8.478 d (0.9)	8.633 br s	8.524 br s	8.702 br s	8.524 br s
	6	6.715 d (2.0)	6.655 br s	6.643 d (1.8)	6.694 d (1.9)	6.684 d (2.0)	6.652 d (2.0)	6.695 d (2.0)	6.634 br s	6.618 br s	6.612 d (2.0)	6.609 d (2.0)
	8	6.964 br d	6.899 br d	6.893 br d	6.887 d (0.8)	6.512 dd (2.0 0.9)	6.536 d (0.9)	6.525 d (0.9)	6.548 br d	6.559 br d	6.593 d (2.0)	6.532 d (2.0)
	2′	8.090 d (2.4)	8.103 d (2.4)	8.065 d (2.4)	8.074 d (2.3)	7.913 d (2.4)	7.916 d (2.3)	7.915 d (2.3)	7.932 d (2.4)	7.934 d (2.3)	7.945 d (2,3)	7.901 d (2.4)
	5′	7.041 d (8.8)	7.018 d (8.7)	7.016 d (8.8)	7.015 d (8.8)	7.022 d (8.8)	7.033 d (8.7)	7.018 d (8.8)	7.028 d (8.8)	7.051 d (8.7)	7.030 d (8.8)	7.046 d (8.7)
	6′	8.292 dd (8.8; 2.4)	8.271 dd (8.7; 2.4)	8.290 dd (8.7; 2.4)	8.259 dd (8.7; 2.4)	8.136 dd (8.8; 2.4)	8.266 dd (8.7; 2.3)	8.186 dd (8.8; 2.3)	8.270 dd (8.8; 2.4)	8.066 dd (8.7; 2.3)	8.241 dd (8.8; 2.3)	8.110 dd (8.7; 2.4)
Gal	1^{I}	5.398 d (7.6)	5.217 d (7.7)	5.402 d (7.6)	5.241 d (7.7)	5.256 d (7.3)	5.191 d (7.3)	5.286 d (7.5)	5.218 d (7.5)	5.268 d (7.4)	5.216 d (7.6)	5.260 d (7.4)
	2^{I}	4.196 dd (7.6; 9.4)	3.956 dd (7.7; 9.7)	4.213 dd (7.7; 9.3)	3.982 dd (9.6; 7.7)	4.276 dd (9.2; 7.3)	3.994 dd (8.9; 7.3)	4.296 dd (9.3; 7.5)	3.997 dd (8.9; 7.5)	4.218 dd (9.4; 7.4)	3.966 dd (8.6; 7.6)	4.223 dd (9.3; 7.4)
	3^{I}	3.919 dd (9.4; 3.3)	3.649 dd (9.7; 3.4)	3.882 dd (9.3; 3.3)	3.650 dd (9.6; 3.4)	4.182 dd (9.2; 3.3)	3.929 dd (8.9; 3.3)	4.184 dd (9.3; 3.4)	3.925 dd (8.9; 3.3)	4.125 dd (9.4; 3.3)	~3.9	4.127 dd (9.3; 3.4)
	4^{I}	4.002 d (3.3)	3.966 d (3.4)	3.953 d (3.3)	3.942 d (3.4)	3.944 d (3.3)	3.914 d (3.3)	3.951 d (3.4)	3.909 d (3.3)	3.932 d (3.3)	~3.9	3.930 d (3.4)
	5^{I}	4.064 dd (7.3; 4.9)	~3.9	~3.8	~3.7	4.483 dd (9 6; 2.0)	4.427 dd (9.4; 1.5)	4.500 dd (9.2; 1.9)	4.446 dd (9.6; 2.0)	4.427 dd (9.4; 2.0)	~4.4	4.420 dd (9.4; 2.0)
	6^{IA}	3.997 dd (4.9; −11.2)	~3.9	~3.8	~3.7	4.215 dd (−13.1; 9.6)	4.224 dd (−12.8; 9.4)	4.222 dd (−13.3; 9.2)	4.229 dd (−13.0; 9.6)	4.195 dd (−13.0; 9.4)	4.184 dd (−12.8; 9.5)	4.192 dd (−12.9; 9.4)
	6^{IB}	3.879 dd (7.3; −11.2)	~3.9	~3.8	~3.7	3.747 dd (−13.1; 2.0)	3.734 dd (−12.8; 1.5)	3.758 dd (−13.2; 1.9)	3.732 dd (−13.0; 2.0)	3.742 dd (−13.0; 2.0)	3.743 dd (−13.2; 2.4)	3.744 dd (−12.9; 2.0)
Xyl	1^{II}	4.695 d (7.6)	—	4.684 d (7.7)	—	4.829 d (7.8)	—	4.856 d (7.8)	—	4.660 d (7.3)	—	4.694 d (7.4)
	2^{II}	3.158 dd (9.0; 7.6)	—	3.135 dd (8.9; 7.7)	—	3.193 dd (9.0; 7.8)	—	3.148 dd (8.9; 7.8)	—	3.240 dd (9.1; 7.4)	—	3.244 dd (9.1; 7.4)
	3^{II}	3.296 dd (9.0; 8.8)	—	[e]	—	3.333 dd (9.0; 9.0)	—	3.342 dd (8.9; 8.9)	—	3.298 dd (9.7; 9.1)	—	3.303 dd (9.1; 9.6)
	4^{II}	3.392 ddd (10.2; 8.8; 5.3)	—	[e]	—	3.251 ddd (10.9; 9.0; 5.2)	—	3.266 ddd (10.5; 8.9; 4.6)	—	~3.40	—	~3.41
	5^{IIA}	3.636 dd (−11.6; 5.3)	—	3.606 dd (−11.6; 5.3)	—	3.610 dd (−11.4; 5.2)	—	3.627 dd (−11.6; 4.6)	—	3.484 dd (−11.2; 5.3)	—	3.521 dd (−11.2; 5.3)
	5^{IIB}	3.019 dd (−11.6; 10.2)	—	3.007 dd (−11.3; 10.3)	—	3.121 dd (−11.4; 10.9)	—	3.144 dd (−11.6; 10.5)	—	2.810 dd (−11.2; 10.5)	— dd	2.850 dd (−11.2; 10.8)

12	13	14	15	16	17	18	19	20	21	22	23	24
8.526 br s	8.603 br s	8.583 br s	8.588 br s	8.515 br s	8.538 d (0.8)	8.554 br s	8.475 d (1.1)	8.447 br s	8.518 br s	8.953 br s	8.500 br s	8.821 br s
6.600 br s	6.552 d (1.9)	6.604 br s	6.587 br s	6.613 br s	6.603 d (2.0)	6.673 d (2.0)	6.622 d (2.0)	6.483 d (2.0)	6.632 d (2.1)	6.551 d (1.7)	6.538 d (2.0)	6.632 d (2.3)
6.483 br d	6.448 d (1.4)	6.497 br d	not seen	6.606 br d	6.514 d (0.8)	6.568 br d	6.521 dd (2.0; 1.1)	6.741 d (1.2)	6.879 br d	6.855 br d	6.818 br d	6.842 br d
7.913 d (2.2)	7.920 d (2.4)	7.915 d (2.4)	7.952 d (2.3)	7.961 d (2.3)	7.951 d (2.4)	7.958 d (2.4)	7.856 d (2.3)	7.973 d (2.3)	8.022 d (2.3)	8.023 d (2.4)	8.018 d (2.4)	8.066 d (2.3)
7.042 d (8.7)	7.037 d (8.8)	7.046 d (8.7)	7.056 d (8.8)	7.062 d (8.7)	7.044 d (8.8)	7.080 d (8.7)	7.026 d (8.7)	7.013 d (8.7)	7.056 d (8.7)	7.038 d (8.7)	7.030 d (8.7)	7.024 d (8.7)
8.095 dd (8.7; 2.2)	8.171 dd (8.6; 2.4)	8.093 dd (8.7; 2.4)	8.114 dd (8.8; 2.3)	8.100 dd (8.7; 2.3)	8.080 dd (8.8; 2.4)	8.116 dd (8.7; 2.4)	8.146 dd (8.7; 2.3)	8.219 dd (8.7; 2.3)	8.230 dd (8.7; 2.3)	8.222 dd (8.7; 2.4)	8.208 dd (8.7; 2.4)	8.257 dd (8.7; 2.3)
5.294 d (7.3)	5.392 d (7.5)	5.304 d (7.4)	5.277 d (7.4)	5.208 d (7.4)	5.254 d (7.4)	5.237 d (7.5)	5.257 d (7.3)	5.388 d (7.5)	5.232 d (7.4)	5.178 d (7.5)	5.190 d (7.4)	5.289 d (7.5)
~4.2	4.240 dd (9.4; 7.3)	4.218 dd (9.4; 7.4)	~4.2	4.221 dd (9.4; 7.4)	4.241 dd (9.4; 7.4)	4.244 dd (9.4; 7.5)	4.232 dd (9.2; 7.3)	~4.2	4.169 dd (9.2; 7.3)	4.182 dd (9.3; 7.5)	4.186 dd (9.3; 7.4)	4.203 dd (9 2; 7.5)
~4.1	4.128 dd (9.2; 3.4)	4.100 dd (9.4; 3.4)	4.091 dd (9.3; 3.3)	4.102 dd (9.4; 3.3)	4.108 dd (9.4; 3.4)	4.091 dd (9.4; 3.4)	4.152 dd (9.2; 3.3)	4.043 dd (9.4; 3.3)	3.983 dd (9.2; 3.4)	3.964 dd (9.4; 3.4)	3.974 dd (9.3; 3.4)	3.912 dd (9.2; 3.3)
3.934 d (3.3)	3.940 d (3.3)	3.935 d (3.4)	3.935 d (3.3)	3.932 d (3.3)	3.943 d (3.3)	3.972 d (3.4)	3.920 d (3.3)	3.917 d (3.3)	3.902 d (3.4)	3.896 d (3.5)	3.902 d (3.4)	3.942 d (3.3)
~4.4	4.490 dd (9.4; 2.2)	~4.4	~4.4	4.368 dd (8.7; 1.1)	4.381 dd (9.5; 2.2)	4.361 dd (9.5; 2.5)	4.410 dd (9.7; 1.9)	~4.2	4.334 dd (9.3; 2.4)	4.282 dd (8.6; 2.4)	4.271 dd (8.8; 2.6)	4.102 dd (7.9; 3.7)
~4.1	4.20 dd (−13.1; 9.3)	~4.1	~4.1	~4.1	4.178 dd (−12.9; 9.5)	4.154 dd (−12.8; 9.1)	4.190 dd (−13.0; 9.7)	4.201 dd (−13.1; 9.2)	4.108 dd (−12.7; 9.3)	4.111 dd (−12.6; 8.8)	4.093 dd (−12.5; 8.8)	3.986 dd (−11.9; 7.9)
~3.7	3.767 dd (−12.0; 1.5)	3.763 dd (−12.9; 2.3)	3.766 dd (−13.1; 3.1)	3.752 dd (−12.9; 2.0)	3.769 dd (−12.9; 2.2)	3.802 dd (−12.8; 2.3)	3.729 dd (−13.0; 1.9)	3.783 dd (−12.9; 2.0)	3.799 dd (−12.7; 2.4)	3.798 dd (−12.5; 2.6)	3.812 dd (−12.5; 2.6)	3.857 dd (−11.9; 3.7)
4.667 d (7.4)	4.731 d (7.4)	4.655 d (7.4)	4.659 d (7.4)	4.656 d (7.4)	4.686 d (7.4)	4.702 d (7.5)	4.755 d (7.3)	4.765 d (7.8)	4.678 d (7.5)	4.657 d (7.5)	4.645 d (7.4)	4.666 d (7.5)
[e]	[e]	3.229 dd (9.1; 7.4)	[e]	3.221 dd (9.1; 7.4)	3.235 dd (9.1; 7.4)	3.219 dd (9.2; 7.5)	3.267 dd (9.2; 7.3)	~3.3	3.198 dd (9.2; 7.5)	3188 dd (9.2; 7.5)	3.200 dd (9.1; 7.4)	3183 dd (9.0; 7.5)
[e]	[e]	[e]	[e]	[e]	3.300 dd (9.1; 9.1)	3.317 dd (9.2; 8.9)	3.323 dd (9.2; 9.2)	~3.3	~3.4	~3.4	3.308 dd (9.1; 9.2)	3.282 dd (9.2; 9.0)
[e]	3.672 dd (9.1 9.1)	3.640 dd (9.1; 9.0)	3.620 dd (9.2; 9.2)	3.631 dd (9.4; 9.0)	~3.38	~3.39	~3.40	~3.4	~3.6	~3.55	~3.40	~3.30
~3.5	3.622 dd (−11.6; 5.2)	~3.5	~3.4	~3.4	3.506 dd (−11.4; 4.9)	3.538 dd (−11.4; 5.2)	3.589 dd (−11.4; 4.3)	~3.6	3.594 dd (−11.6; 5.4)	~3.55	3.540 dd (−11.4; 5.3)	3.582 dd (−11.4; 5.2)
2.812 dd (−10.4; 10.4)	2.932 dd (−10.9; 10.9)	2.811 dd (−11.3; 10.9)	2.825 dd (−11.1; 10.4)	2.824 dd (−10.8; 10.4)	2.856 dd (−11.4; 10.2)	2.860 dd (−11.4; 10.4)	2.922 dd (−11.4; 10.5)	3.049 dd (−11.4; 10.6)	2.837 dd (−11.6; 10.5)	2.841 dd (−11.3; 10.6)	2.833 dd (−11.4; 10.6)	2.967 dd (−11.4; 10.4)

Table 1 contd

	H	**1** [b,c]	**2**	**3** [d]	**4**	**5** [b]	**6** [b]	**7** [b]	**8** [b]	**9**	**10**	**11**
Glc	1^{III}	4.267 d (7.7)	4.234 d (7.5)	—	—	4.474 d (7.4)	4.459 d (7.5)	4.478 d (7.5)	4.453 d (7.3)	4.442 d (7.2)	4.409 d (7.3)	4.440 d (7.1)
	2^{III}	3.178 dd (9.3; 7.7)	~3.16	—	—	~3.44	~3.44	~3.44	~3.45	~3.41	~3.41	~3.40
	3^{III}	~3.25	~3.20	—	—	~3.44	~3.44	~3.44	~3.45	~3.43	~3.43	~3.40
	4^{III}	~3.30	~3.20	—	—	3.746 dd (9 5; 9.6)	3.697 dd (9.5; 9.5)	3.747 dd (9 6; 9.4)	3.669 dd (9.4; 9.4)	3.673 dd (9.4; 9.2)	3.618 dd (9.4; 9.3)	3.661 dd (9.4; 9.1)
	5^{III}	~3.30	~3.18	—	—	~3.44	~3.44	~3.44	~3.45	~3.42	~3.42	~3.40
	6^{IIIA}	3.846 dd (−11.7; 1.8)	3.835 dd (−11.7; 2.2)	—	—	5.339 dd (−12.1; 2.8)	5.295 dd (−11.9; 2.8)	5.340 dd (−12.2; 3.0)	5.302 dd (− 12.0; 2.8)	5.253 dd (−12.0; 2.7)	5.169 dd (−11.9; 2.6)	5.252 dd (−12.0; 2.7)
	6^{IIIB}	3.633 dd (−11.7; 5.8)	3.503 dd (−11.7; 5.9)	—	—	4.115 dd (−12.2; 1.3)	4.085 dd (−11.9; 1.2)	4.134 dd (−12.2; 1.6)	4.106 dd (−12.0; 1.5)	4.113 dd (−12.0; 1.6)	4.078 dd (−12.0; 2.1)	4.115 dd (−12.0; 1.7)
Acyl	2^{A}	—	—	—	—	6.195 s	6.267 s	6.221 s	6.303 s	6.949 d (8.6)	6.904 d (8.6)	7.046 d (8.7)
	3^{A}	—	—	—	—	—	—	—	—	6.368 d (8.6)	6.356 d (8.6)	6.492 d (8.7)
	4^{A}	—	—	—	—	—	—	—	—	—	—	—
	5^{A}	—	—	—	—	—	—	—	—	6.368 d (8.6)	6.356 d (8.6)	6.492 d (8.7)
	6^{A}	—	—	—	—	6.195 s	6.267 s	6.221 s	6.303 s	6.949 d (8.6)	6.904 d (8.6)	7.046 d (8.7)
	7^{A}	—	—	—	—	7.297 d (15.9)	7.362 d (15.9)	7.310 d (15.9)	7.397 d (16.0)	7.364 d (16.0)	7.406 d (15.9)	7.404 d (16.0)
	8^{A}	—	—	—	—	6.197 d (15.9)	6.137 d (15.9)	6.308 d (15.9)	6.240 d (16.0)	6.130 d (16.0)	6.103 d (16.0)	6.178 d (16.0)
	MeO					3.413 s $3^{A},5^{A}$	3.431 s $3^{A},5^{A}$	3.415 s $3^{A},5^{A}$ 3.823 4^{A}	3.431 s $3^{A},5^{A}$ 3.762 4^{A}			3.787 s 4^{A}

12	13	14	15	16	17	18	19	20	21	22	23	24
4.359 d (7.1)	4.430 d (7.3)	4.422 d (7.6)	4.426 d (7.3)	4.431 d (7.3)	4.434 d (7.4)	4.458 d (7.5)	4.452 d (7.2)	4.452 d (7.4)	4.429 d (7.3)	4.403 d (7.4)	4.644 d (7.4)	4.278 d (7.5)
[e]	[e]	[e]	[e]	[e]	3.354 dd (9.4; 7.4)	~3.37	~3.42	~3.4	~3.40	[e]	3.312 dd (9.2; 7.4)	3.206 dd (9.3; 7.5)
[e]	[e]	3.640 dd (9 1; 9.0)	3.621 dd (9.4; 9.2)	3.631 dd (9.4; 9.0)	3.417 dd (9.8; 9.4)	3.422 dd (8.8; 9.4)	~3.42	~3.4	~3.40	~3.4	~3.40	~3.40
~3.7	~3.6	~3.4	[e]	~3.4	3.630 dd (9.8; 8.8)	3.680 dd (9.8; 8.8)	3.661 dd (9.4; 9.2)	~3.6	3.644 dd (9.6; 9.4)	~3.5	3.589 dd (9.8; 9.6)	~3.30
[e]	[e]	~3.4	~3.4	~3.4	~3.38 ddd (8.8; 2.7; 2.1)	3.490 ddd (9.8; 2.5; 2.6)	~3.42	~3.4	3.495 ddd (9.6; 3.1; 2.2)	3.484 ddd (9.5; 3.0; 3.0)	3.505 ddd (9.8; 3.3; 2.5)	~3.30
5.278 dd (−11.9; 2.6)	5.230 dd (−12.8; 3.1)	5.208 dd (−11.9; 2.7)	5.176 dd (−10.8; 2.6)	5.221 dd (−11.8; 2.6)	5.210 dd (−12.0; 2.5)	5.154 dd (−12.0; 2.5)	5.294 dd (−12.0; 2.8)	5.297 dd (−11.8; 2.3)	5.094 dd (−12.0; 2.2)	5.105 dd (−11.9; 2.3)	5.122 dd (−12.0; 2.5)	4.589 dd (−12.3; 2.0)
~4.2	~4.2	~4.1	~4.1	4.151 dd (−11.8; 1.9)	4.189 dd (−12.0; 2.1)	4.187 dd (−12.0; 2.6)	4.086 dd (−12.0; 1.5)	~4.2	4.250 dd (−12.0; 3.1)	4.282 dd (−12.2; 2.4)	4.383 dd (−12.0; 3.3)	4.083 dd (−12.3; 4.6)
6.99[f] 6.92 6.92 6.81 6.65	7.23[f] 7.13 7.13 6.71 6.59	7.135 d (7.1)	7.207 dd dd (8 6; 5.3)	7.154 d (8.5)	7.378 d (8.2)	7.465 d (8.8)	6.921 d (8.9)	7.109 s	7.759 d (8.8)	7.887dd dd (6.6; 2.0)	8.126 d (8.2)	6.861d d (8.6)
		7.046 d (7.8)	6.776 dd (8.6; 8.7)	6.971 d (8.5)	7.300 d (8.2)	7.870 d (8.8)	6.208 d (8.9)	—	6.405 d (8.8)	7.097dd dd (6.6; 2.0)	7.468 d (8.2)	6.506d d (8.6)
		7.264 dd (7.4; 7.2)	—	—	—	—	—	—	—	—	—	—
		7.046 d (7.8)	6.776 dd dd (8.6; 8.7)	6.971 d (8.5)	7.300 d (8.2)	7.870 d (8.8)	6.208 d (8.9)	—	6.405 d (8.8)	7.097dd dd (6.6; 2.0)	7.468 d (8.2)	6.506d d (8.6)
		7.135 d (7.1)	7.207 dd dd (8.6; 5.3)	7.154 d (8.5)	7.378 d (8.2)	7.465 d (8.8)	6.921 d (8.9)	7.109 s	7.759 d d (8.8)	7.887dd dd (6.6; 2.0)	8.126 d (8.2)	6.681d d (8.6)
7.385 d (16.0)	7.535 d (16.2)	7.450 d (16.1)	7.439 d (16.1)	7.445 d (16.1)	7.532 d (16.1)	7.538 d (16.1)	7.382 d (16.0)	—	—	—	—	~2.74
6.338 d (16.0)	6.521 d (16.2)	6.353 d (16.2)	6.308 d (16.1)	6.371 d (16.1)	6.524 d (16.1)	6.568 d (16.1)	6.046 d (16.0)	—	—	—	—	~2.56
3.602 s 3[A]	3.593 s 2[A]							3.623 s 3[A],5[A] 3.605 s 4[A]				

[a] Apparent, first-order couplings are indicated (br s, broad singlet; br d, broad doublet; d, doublet; dd, doublet of doublets; ddd doublet of doublets) in parentheses in Hz.
[b] Chemical shift data have been reported. See ref [1].
[c] For a comparison spectrum, see ref [8].
[d] For a comparison spectrum, see ref [10].
[e] Obscured by the MeOH-d_4 signal.
[f] Protons of the acyl moiety could not be assigned.

acylated with cinnamic acids, but not in an example acylated with 4-hydroxybenzoic acid, which is in accord with our findings. These observed upfield shifts of H-4 and H-8 show that the cinnamoyl groups are interacting with these two protons in the chromophore, while the benzoyl groups are interacting only with H-4. We have previously shown that H-2^A and H-6^A (the ortho protons) of the cinnamic acid acyl groups also have significant upfield shifts, showing their interaction with the chromophore [1]. However, the specific nature of the interactions of the acyl groups with the chromophore are not clear at this time.

Changes in the chemical shifts for H-4 and H-8 of smaller magnitude, but in the same direction as seen with the monoacylated anthocyanins, are reported by Idaka et al. [13] and Yoshida et al. [14], but not by other investigators [15–17] for anthocyanins acylated with cinnamic acids. In these latter cases different skeletal arrangements of sugars between the chromophores and acyl groups may account for the lack of effect of any of the acyl groups on either H-4 or H-8.

(b) Mass spectral studies. The molecular weights for compounds **1**–**24** were supported by the molecular ions (*m/z*) observed in both fast-atom-bombardment mass spectrometry (FABMS) and electrospray-ionization mass spectrometry (ESIMS) (see Table 2). While FABMS gave essentially only the molecular ions, ESIMS gave, in addition to the molecular ion, a series of peaks at higher *m/z* values. Except for **14**, these peaks were at $M^+ + 22$ (i.e., $M^+ - H^+ + Na^+$), at $M^+ + 40$ (i.e., $M^+ - H^+ + Na^+ + H_2O$), at $M^+ + 54$ (i.e., $M^+ - H^+ + Na^+ + CH_3OH$), and at $M^+ + 70$ (i.e., $M^+ - H^+ + K^+ + CH_3OH$). The adducts at $M^+ + 54$ and at $M^+ + 70$ did not appear when H_2O was the solvent. Compound **14** gave peaks at *m/z* 873 (M^+), 905 ($M^+ + CH_3OH$), and 927 ($M^+ - H^+ + Na^+ + CH_3OH$). The mass spectra of **16** and **22** both showed the expected distribution of ions resulting from the natural abundances of ^{35}Cl and ^{37}Cl.

(c) UV–vis spectral data. Table 2 contains data on UV-vis absorbance by the anthocyanins. In MeOH–HCl the $\lambda_{vis.max}$ of the nonacylated **1**–**4**

Table 2
Concentrations of acids added to carrot cell cultures to produce acylated anthocyanins and the spectral characteristics of the anthocyanins obtained

Compd	Added acids (mM) [a]	*m/z*	λ_{max} (nm) MeOH/HCl	λ_{max} (nm) pH 2.0	λ_{max} (nm) pH 6.0	Ratio of Abs_{max} pH 6:Abs_{max} pH 2
1		743 [c]	529, 282	513	537	0.09
2		611 [b,c]	526, 282	509	530	0.13
3		581 [b,c]	529, 281	514	538	0.09
4		449 [b]	527, 281	510	533	0.13
5	10		538, 297, 319 [d]	527	547	0.49
6		817 [b]	538, 334, 285	527	547	0.45
7	10	963 [c]	542, 287, 314 [d]	530	547	0.42
8		831 [b]	540, 286, 316 [d]	529	550	0.54
9	30	889 [c]	536, 316, 285	524	544	0.41
10		757 [b]	535, 316, 285	523	549	0.57
11	20	903 [b]	537, 286, 310 [d]	525	546	0.35
12	6	903 [b]	538, 283, 321 [d]	525	547	0.42
13	25	903 [b]	537, 326, 281	524	545	0.41
14	35	873 [b,c]	537, 282	523	546	0.38
15	15	891 [b]	538, 282	524	547	0.35
16	14	907 [b]	539, 284	525	547	0.38
17	6	941 [c]	539, 278	526	546	0.28
18	8	918 [c]	541, 285	528	549	0.20
19	20	916 [c]	535, 381, 280	525	545	0.37
20	60	937 [b,c]	539, 278	528	545	0.48
21	60	863 [b]	534, 260–280	522	542	0.31
22		881 [b]	536, 282	523	543	0.15
23	4	915 [c]	536, 283	523	542	0.14
24	100	891 [c]	531, 282	520	544	0.18

[a] Concentration of acid in Me_2SO added to cultures at days 4 and 8 at the rate of 0.01 v/v.
[b] Determined by positive-ion ESIMS.
[c] Determined by positive-ion FABMS.
[d] Peak observed as a shoulder.

were at 526–529 nm. The acylated compounds except **24** had $\lambda_{vis.max}$ at 534 nm or longer wavelengths. This bathochromic shift was generally greater when the spectra were measured in buffer at pH 2.0. Compounds **5–13** in MeOH–HCl all had either a shoulder or a peak at 310–335 nm in addition to the visible and other UV peaks. These compounds are acylated with cinnamic acids bearing an electron-donating substituent. In contrast, the anthocyanins acylated with cinnamic acids not having an electron-donating substituent, with substituted benzoic acids, and compound **24** do not show a peak or shoulder in this region.

Because anthocyanins undergo a pH-dependent hydration reaction to give colorless hemiacetals, the ratio of absorbance at pH 6:pH 2 is a semiquantitive measure of color retention by an anthocyanin at near-neutral pH. For the anthocyanins examined here, the $\lambda_{vis.max}$ at pH 6 is increased by 15–25 nm compared to those values a pH 2. The ratio of absorbance at the visible peak at pH 6:pH 2 is smallest with **1–4** and tends to be greatest with acyl groups bearing electron-donating substituents. In general, color retention at pH 6 is greater in the acylated anthocyanins compared to the non-acylated ones, is less when the acyl group is a benzoic acid than when it is a cinnamic acid, and tends to be lower in the anthocyanins those whose acyl groups bear electron-withdrawing groups. Compound **19** in MeOH–HCl has a spectrum that is different from those of **5–18**. The compound has a peak at 381 nm whose absorbance is approximately 30% that of the other peaks, which may be due to the protonated 4-(*N,N*-dimethylamino)cinnamoyl group in **19**. Support for this contention is that 4-(*N*-dimethylamino)cinnamic acid has a small peak (approximately 8% of the 265 nm absorbance) near 360 nm in MeOH–HCl and in aqueous buffer at pH 2.

Further observations on the production of novel anthocyanins.—In addition to the anthocyanins acylated with the carboxylic acids shown for compounds **5–24**, novel anthocyanins in limited amounts (data not shown) were detected on HPLC analysis of extracts of tissues fed 3,4-methylenedioxycinnamic acid, 4-methoxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, and 4-hydroxyphenylacetic acid. The anthocyanin product acylated with 4-hydroxyphenylacetic acid was not pure after chromatography on the three adsorbents routinely used in this study.

The range of carboxylic acids that these carrot cell cultures used to acylate anthocyanins is not surprising. In related examples, Tabata et al. showed that suspension cultures from four different species could each glucosylate three flavonoids, three anthraquinones, four coumarins and four hydroxylated benzoic acids [18], and Naoshima and Akakabe [19] and Akakabe et al. [20] showed enantiospecific reduction of four ketoesters, five aromatic ketones, and three heterocyclic ketones by plant cell cultures. The earlier literature on bioconversions by plant cell cultures has been reviewed by Pras [21].

Partial hydrolysis in HCl, but not in trifluoroacetic acid, of most acylated and non-acylated anthocyanins of this series preferentially removed the xylose residue. This occurred in NMR samples in CD_3OD acidified with DCl and stored in a freezer, on freeze drying solutions containing HCl, and in 5 N HCl at room temperature. The anthocyanin **24** was particularly sensitive to acid hydrolysis of the acyl function. When a sample of purified **24** was stored in 0.1% trifluoroacetic acid at −20 °C for a month, **1** was formed in significant amounts. Storage in 0.1% formic acid either at 4 °C or −20 °C did not lead to detectable deacylation of the compound during 2 weeks.

In all but two cases, the feeding of a substituted cinnamic acid to the cultures resulted in the presence of only one novel, cinnamoylated anthocyanin in the tissues. However, when either 4-(trifluoromethyl)cinnamic acid or 4-chlorocinnamic acid was provided, two novel anthocyanins were obtained. In each of the latter examples, there was found one anthocyanin acylated with the cinnamic acid (**17** and **16**, respectively) and another acylated with the corresponding benzoic acid (**23** and **22**, respectively). A specific attempt to detect the production of anthocyanins acylated with two other benzoic acids in a manner similar to the previous examples was made by feeding 4-methoxycinnamic acid and 3,4,5-trimethoxycinnamic acids. Using an HPLC system known to separate the benzoylated anthocyanins from their cinnamoylated counterparts, no benzoylated anthocyanins were detected in the tissue extracts for these latter two examples. Thus, the process in these carrot cell cultures that is responsible for the conversion of cinnamic acids to benzoic acids appears to have a greater specificity than does the mechanism for the addition of the benzoic acids to the anthocyanins.

The conversion of cinnamic acids to benzoic acids has been examined in only a few systems. Löscher and Heide [22] showed in extracts of *Lithospermum erythrorhizon* cell cultures that 4-coumaroyl-CoA and 4-hydroxybenzoyl-CoA were intermediates in the conversion, while Schnitzler et al. [23] concluded that 4-hydroxybenzaldehyde was an intermediate in the conversion by extracts from carrot cell cultures. Funk and Brodelius [24] concluded that 4-methoxycinnamic acids and 4-methoxybenzoic acids were intermediates in the formation of benzoic acids in cell cultures of *Vanilla planifolia*. Benzoic acid appears to be an intermediate in the formation of salicylic acid from *trans*- cinnamic acid in tobacco [25] and rice [26]. Thus, although the conversion of a cinnamic acid into a benzoic acid is formally analogous to β oxidation in fatty acids, there appear to be several different pathways by which the C_2 (ethylene) unit is removed.

In our studies cultures treated with unsubstituted cinnamic acid yielded the 4-hydroxylated cinnamoyl analogue (4-coumaroyl) **9** as the major product along with the new, relatively hydrophobic, nonhydroxylated anthocyanin **14** in a ratio of approximately 10:1.

The formation of the anthocyanin acylated with *p*-coumaric acid is not surprising because cinnamic acid has been identified as the precursor of *p*-coumaric acid in the hydroxycinnamic acid pathway that provides intermediates in the synthesis of anthocyanins and other compounds [27]. These acyl groups are donated to anthocyanins via 1-*O*-(4-hydroxycinnamoyl)-β-D-glucopyranoses [28]. Previous study of the apparent specificity of extracts of these carrot cell cultures that formed 1-*O*-sinapoyl-β-D-glucopyranose showed no activity with *trans*-cinnamic acid [29]. This finding suggests that the methods used by these workers may not have been sufficiently sensitive to detect the cinnamoyl ester.

3. Experimental

General procedures.—Except as described below, the culture and analytical methods used are described in Baker et al. [7]. All the acids used were obtained from Aldrich Chemical Company, Milwaukee, WI. Mass spectra were measured by FABMS in the positive-ion mode in thioglycerol [7] and by ESIMS in the positive-ion mode in 50% MeOH–H_2O, except when noted otherwise. ESIMS was performed with a Quattro-II mass spectrometer (Micro-Mass, Inc., Manchester, UK). UV-vis spectra were measured in 1% v/v concd HCl in 95% MeOH, and vis spectra were measured in 0.1 M acetate + 0.1 M phosphate adjusted to pH 2.0 and to pH 6.0 (HCl) within an hour of preparing the solution using a Hitachi Model U-2000 UV-vis spectrophotometer (Hitachi Instruments Inc., San Jose, CA). ^{1}H NMR spectra were measured using a Bruker AMX 400 spectrometer as previously described [1], except that CF_3CO_2D was substituted for DCl to avoid the observed partial hydrolysis of xylose from the compounds. This change made no observable difference to the spectrum of **17**, which demonstrates that spectra measured in either acid can be directly compared. For some compounds, the assignment of peaks to specific protons was done from 2D spectra, and in other cases by analogy to compounds where the assignments had been determined from 2D spectra. In the NOESY experiments, a total of 256 FIDs of 2K datapoints were collected with a total of 64 scans per FID with a spectral width of 3205 Hz. The data was zero-filled to 1K points in t_1 and multiplied by the sine^2 window function in both dimensions before Fourier transformation. The mixing time was 500 ms, and the transmitter offset was placed on the H_2O resonance, which was irradiated with a lower power pulse during a relaxation delay of 2 s to suppress the water signal. All processing was performed with FELIX 95 software (Biosym/Molecular Simulations) on a Silicon Graphics Indigo2 workstation.

Biological material.—The wild carrot (*Daucus carota* spp *carota*) cell clone used in these studies was WC63-1-9-1-13-1 [30], and a subline was selected from it for increased anthocyanin yield.

Culture preparation.—For each acid tested, a concentration that gave little or no growth inhibition and the best level of total and novel anthocyanin in the tissue was selected experimentally, and these data are given in Table 2. For purification of the novel anthocyanins, 1.5–2 L of culture in 100-mL portions was grown for 12 days, with the chosen concentration of the individual carboxylic acids in Me_2SO added (1% of culture volume) at days 4 and 8. The tissue was filtered on Miracloth (Calbiochem–Novabiochem Intl., San Diego, CA) washed with water, frozen in liquid nitrogen, and stored at −80 °C.

Isolation of anthocyanins.—The frozen tissue was extracted overnight in a refrigerator with 10 mL/g fresh weight of methanol containing 5% v/v formic acid. The filtered extract was concentrated approximately five-fold in vacuo at less than 30 °C to remove methanol, extracted twice with 0.5 volumes of CH_2Cl_2, and then twice with 0.5 volumes of EtOAc. The aqueous residue was chromatographed on Amberlite XAD-7 resin (Sigma Chemical Co., St. Louis, MO) (2.5×20 cm or 4×20 cm) using a gradient from 0.1% formic acid in water to 0.1% formic acid in methanol. The fractions containing the novel anthocyanin were selected by HPLC using either a μBondapak C_{18} column (3.9×300 mm, Waters Associates, Inc., Milford, MA), or a Microsorb-MV C_{18} column (4.6×250 mm, Rainin Inst. Co., Woburn, MA), and either isocratic mixtures of or gradients of (a) 5% formic acid in water, and (b) 5% formic acid plus 55% methanol plus 10% acetonitrile in water. The combined fractions were concentrated under reduced pressure and chromatographed on a C_{18} reversed-phase column (1.5×44 cm, 28–40 μm, E. Merck, Gibbstown, NJ) with a gradient from 5% formic acid in water to 5% formic acid plus 55% methanol plus 10% acetonitrile in water. The fractions containing the novel anthocyanin were combined and concentrated under reduced pressure. In some cases two novel anthocyanins were separated on the C_{18} reversed-phase column and were treated separately. The new anthocyanins were then chromatographed on a lipophilic Sephadex LH-20 column (2.5×20 cm, Pharmacia Biotech, Inc., Piscataway, NJ) with 0.1% formic acid in water as the solvent. The fractions containing the desired anthocyanin were combined, concentrated in vacuo, dissolved in 0.1% formic acid and stored frozen. Compound **4** was prepared as described above from the skin of red apples bought in the local market [31].

In the cases of **5** and **20**, the anthocyanin after chromatography on Sephadex LH-20 was rechromatographed on a PVP column (Polyclar AT >0.2 mm, 2.5×20 cm), eluted with 0.1% formic acid in water, and the fractions containing the novel anthocyanin were selected and treated as above. Compound **20**, at this stage, was still contaminated with a component absorbing at 280 nm that was removed by semipreparative chromatography on a 7.9×300 mm C_{18} μBondapak column eluted isocratically with a mixture of 0.1% formic acid and 80% methanol. The anthocyanin **21** when purified through the Sephadex LH-20 chromatography was mixed with **3**. These two compounds were then separated by semipreparative HPLC on a 7.9×300 mm C_{18} μBondapak column (Waters Associates, Inc., Milford, MA) by isocratic elution with a mixture of the solvents described above for the preparative C_{18} column. They were also well separated on the PVP column, eluting first with 0.1% formic acid in water and then a gradient to methanol containing 0.1% formic acid.

Compound **1** was prepared from either **5** or **7** that had been chromatographed on Amberlite XAD-7 and C_{18} media as described above. The anthocyanin (30 mg) in water at room temperature was treated with stirring, under nitrogen, with an equal volume of 1.8 N KOH for 7 min, followed by 5 volumes of N formic acid. The deacylated product was purified by chromatography as above on C_{18} reversed phase and on Sephadex LH-20 columns. Compound **2** was prepared from **10** by the same method. When a mixture of **9** and others, predominantly **3**, was deacylated and chromatographed as above, **1** did not separate from **3** but was easily separated on the PVP column as above.

Compound **8** was prepared from an NMR sample of **7** in CD_3OD and DCl that had been observed to lose the xylose residue. The sample was chromatographed on C_{18} reversed phase and on Sephadex LH-20 columns to give pure **8**. Compound **2** was similarly obtained from an NMR sample of **1**. Xylose was removed from **5** on freeze-drying a solution of the compound in 0.1% HCl. Xylose was removed preparatively from **5**, **7**, and **9** by partial hydrolysis in 5 N HCl at 25 °C for 60 to 72 h, and the products were chromatographed on C_{18} reversed phase and on Sephadex LH-20 columns to give pure **6**, **8**, and **10**. Compounds **9** and **10** were also easily separated on a PVP column as described above.

4. Conclusions

We have demonstrated that the systems operative in tissue cultures of *Daucus carota* spp *carota* (wild carrot) for acylating anthocyanins can tolerate a wide variety of cinnamic and benzoic acids. Incorporation of these acids into monoacylated anthocyanins **5**–**24** occurs solely at the C-6 of the glucose moiety. In all but two instances studied, the only anthocyanin isolated was the one acylated with the carboxylic acid provided. However, when the culture was fed with either 4-(trifluoromethyl)-cinnamic acid or 4-chlorocinnamic acid, the

anthocyanin acylated with the corresponding benzoic acid (compounds **23** and **22**, respectively) was also isolated.

We have used the ratio of Abs_{max} pH 6.0:Abs_{max} at pH 2.0 for compounds **5**–**24** to show that acylation enhanced the color retention of these compounds at near-neutral pH. Cinnamoylated anthocyanins in general exhibited greater color stability compared to their benzoylated counterparts. Moreover, if the acyl group bore an electron-donating substituent, the color retention of the anthocyanin was further increased. Additional investigations into their ability to retain color will be reported in the future.

^{1}H NMR studies of the monoacylated anthocyanins **5**–**24** showed that there is an interaction between the acyl group and the chromophore where the pH-dependent hydration reaction occurs that results in the loss of color. The precise nature of this interaction is being probed as a part of continued studies.

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