Tunable Transition Metal–Ligand Complexation for Enhanced Elucidation of Flavonoid Diglycosides by Electrospray Ionization Mass Spectrometry

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A tunable ESI-MS/MS strategy for differentiation of flavone and flavanone diglycoside isomers based on metal complexation with auxiliary ligands is reported. The addition of a metal salt and an auxiliary ligand to a flavonoid solution results in the formation of $[M(II)(\text{flavonoid-H})(\text{auxiliary ligand})]^+$ complexes, where $M(II)$ is a transition metal. A series of auxiliary ligands with electron-withdrawing substituents were synthesized to tailor the relative metal binding affinities of the ligands and thus directly influence the stabilities, and consequently the dissociation pathways, of the complexes. Upon collisionally activated dissociation, the complexes yield fragmentation patterns in which the abundances of key diagnostic ions are enhanced, thus facilitating isomer differentiation. (J Am Soc Mass Spectrom 2007, 18, 422–431) © 2007 American Society for Mass Spectrometry

Differentiation of isomers remains a challenging analytical problem and confirming structures of isomers is difficult even when they can be adequately separated from mixtures. Tandem mass spectrometry has been used extensively for structural elucidation of isomers [1–12] and there continues to be interest in applying existing tandem mass spectrometric methods, such as MS^n [9, 13, 14], and developing new ones, such as STEP [15, 16], a statistically based method that relies on comparison of abundances of fragment ions obtained for different collision activation conditions, to differentiate isomers. Our group has focused on the development of metal complexation strategies to enhance the differentiation of isomers [17–23] because metal complexation often alters the dissociation pathways of ions, thus giving additional diagnostic fragment ions. We have reported several metal complexation strategies that have been useful for distinguishing flavonoid isomers and, in this study, we expand this approach by developing a “tunable” collisionally activated dissociation (CAD) method based on the use of rationally selected auxiliary ligands to form metal complexes. The concept of this tunable CAD strategy is applied for the differentiation of flavonoid isomers.

Flavonoids are phytochemicals found in almost all plants, including fruits and vegetables. Several studies have indicated their antioxidant, chemopreventive, antiviral, antibacterial, and radical-scavenging properties

[24–28]; moreover, they have been implicated in cardiovascular protection [29–36]. Although all flavonoids are structurally similar, many exist as glycosides and there are subtle differences in their structure that lead to important changes in their biological activity. These differences include hydroxylation position of the aglycon, glycosylation site, sequence of glycosylation, and interglycosidic linkages of the glycan portion. It is because of these subtle differences that there is a need for sensitive analytical techniques to elucidate these similar compounds.

Mass spectrometry has become an ideal tool for the analysis of flavonoids, and two recent reviews provide an excellent background of the techniques used and the analyses performed [37, 38]. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) has been used extensively to differentiate several sets of flavonoid isomers over the past decade. For example, for a series of flavonoid diglycosides, CAD has been used to pinpoint the intersaccharide linkages (rutinosides: $1 \rightarrow 6$ disaccharides versus neohesperidosides: $1 \rightarrow 2$ disaccharides) in flavonoid-*O*-glycosides based on relative abundances of the fragment ions obtained from protonated flavonoids [7]. However, in general it has been noted that flavonoids do not protonate particularly well because they are acidic, phenolic compounds. Although flavonoids deprotonate efficiently, the resulting fragmentation patterns often do not give diagnostic ions adequate for differentiation of isomers. Metal complexation is an alternative ionization strategy that has been explored in our group for improved analysis of flavonoids [17–23], and we have explored metal complexation both with and without the use of an auxiliary

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ligand to produce stable complexes. For example, using an auxiliary ligand, typically a pyridyl-type chelating agent, complexes of the type $[M(II) (\text{flavonoid} - H) \text{ auxiliary ligand}]^+$ are formed. Metal complexation not only provides greater ion abundances but also enhances the structural differentiation of isomeric flavonoids.

In one of our previous studies involving flavonoid diglycosides, the impact of the auxiliary ligand on the abundances of metal complexes and the resulting fragmentation pathways of the complexes was evaluated [21]. It was demonstrated that the nature of the auxiliary ligand affected the types of fragment ions and their abundances in the CAD mass spectra. Specifically, it was found that as the alkyl substituents of the ligands changed (that is, 1,10-phenanthroline versus 4,7-diphenyl-1,10-phenanthroline), the abundances of several diagnostic fragment ions observed for flavone rutinoid and neohesperidoside isomeric pairs varied systematically. This result revealed that it was possible to tune the CAD spectra by careful selection of the auxiliary ligand. However, the only suitable commercially available auxiliary ligands were those with electron-releasing alkyl substituents, thus limiting the scope of the study. Electron-releasing substituents increase the metal binding energies of pyridyl ligands and therefore in some cases destabilize the desired $[M(II) (\text{flavonoid} - H) \text{ pyridyl ligand}]^+$ complexes. Pyridyl ligands have intrinsically high metal binding energies, both in solution [39–43] and in the gas-phase [44, 45], arising from their ability to chelate metal ions by their nitrogen atoms. Addition of electron-withdrawing substituents should temper the metal binding energies and thus expand the options for creating tunable auxiliary ligands for the differentiation of isomers, such as flavonoids, by metal complexation strategies.

In this study, we explore the effect of incorporating electron-withdrawing substituents into the pyridyl ligands for metal complexation, evaluated in terms of the success of differentiating flavonoid isomers. The halogens are interesting substituents because of their inductive electron-withdrawing effects. For assessment of the method, 12 isomers of 7-*O*-flavonoid diglycosides are evaluated, including three isomeric pairs from the flavone class (both $1 \rightarrow 2$ versus $1 \rightarrow 6$ disaccharide linkages) and three pairs from the flavanone class ($1 \rightarrow 2$ versus $1 \rightarrow 6$) (Figure 1).

Experimental

Chemical Reagents

The flavonoids rhoifolin, isorhoifolin, neodiosmin, diosmin, and narirutin were purchased from Indofine (Somerville, NJ, USA). Fortunellin, linarin, poncirin, and didymin were purchased from Extrasynthese (Genay, France). Naringin, neohesperidin, hesperidin, CoBr_2 , CuBr_2 , 2,2'-bipyridine, 6,6'-dibromo-2,2'-bipyridine, 4,4'-dimethyl-2,2'-bipyridine, 1,10-phenanthroline, and 5-

nitro-1,10-phenanthroline were purchased from Sigma (St. Louis, MO, USA).

Ligand Syntheses

4,4'-Dibromo-2,2'-bipyridine was synthesized from 2,2'-bipyridine according to a literature procedure [46, 47] and was purified using silica gel flash chromatography. ^1H NMR (CDCl_3) δ = 7.509 (2H, dd, J = 5.2 Hz, 1.6 Hz), 8.486 (2H, d, J = 5.2 Hz), 8.608 (2H, d, J = 1.6 Hz).

4,4'-Bis(bromomethyl)-2,2'-bipyridine was synthesized from 4,4'-dimethyl-2,2'-bipyridine according to a literature procedure [48] and was purified using silica gel flash chromatography. ^1H NMR (CD_3Cl_3) δ = 7.507 (2H, d, J = 8 Hz), 7.671 (2H, t, J = 7.6 Hz, 8 Hz), 8.378 (2H, d, J = 7.6 Hz).

5,6-Dibromo-1,10-phenanthroline, 3,5,6-tribromo-1,10-phenanthroline, and 3,5,6,8-tetrabromo-1,10-phenanthroline were synthesized from 1,10-phenanthroline according to a literature procedure [49]. The phenanthrolines were purified using successive recrystallizations. 5,6-Dibromo-1,10-phenanthroline: ^1H NMR (CDCl_3) δ = 7.740 (2H, dd, J = 8.4 Hz, 8.0 Hz), 8.782 (2H, dd, J = 8.8 Hz, 1.2 Hz), 9.226 (2H, dd, J = 4.4 Hz, 1.2 Hz). 3,5,6-Tribromo-1,10-phenanthroline: ^1H NMR (CDCl_3) δ = 7.748 (1H, dd, J = 8.8 Hz, 4.2 Hz), 8.765 (1H, dd, J = 8.8 Hz, 1.6 Hz), 8.928 (1H, d, J = 2 Hz), 9.201 (2H, m). 3,5,6,8-Tetrabromo-1,10-phenanthroline: ^1H NMR (CDCl_3) δ = 8.920 (2H, d, J = 2.2 Hz), 9.190 (2H, d, J = 2 Hz).

A Varian 400 MHz instrument (Varian Inc., Palo Alto, CA, USA) was used for all NMR spectroscopy.

Metal Complexation/Mass Spectrometry

Stock solutions of 4×10^{-4} M metal salts and auxiliary ligands were prepared in methanol. Solutions containing the flavonoid, metal salt, and auxiliary ligands were prepared in approximately 1:1:1 ratios at 1×10^{-5} M.

The experiments were performed using a Thermo Finnigan (San Jose, CA, USA) LCQ Duo quadrupole ion trap (QIT) mass spectrometer equipped with an electrospray ionization (ESI) source. The flow rate of the solutions was $5 \mu\text{L min}^{-1}$. The heated capillary temperature was 200 °C and the ESI voltage was set at +4.5 kV. The ionization times were set to 5–25 ms and each spectrum was an average of 20 scans. The lens and octapole voltages, sheath gas flow rate, and capillary voltage were optimized for maximum intensity of the ion corresponding to $[M(II) (\text{flavonoid} - H) L]^+$, where M is the metal and L is the auxiliary ligand. During MS/MS experiments, these parent ions were isolated and the CAD voltage was adjusted so that the parent ion intensity decreased to roughly 10% of the base peak. An isolation window sufficient to exclude all but the carbon-12 isotope was used for most experiments, and an isolation window sufficient to include all bromine isotopes was used for confirmation of the presence of the brominated ligands in selected fragment ions.

Figure 1. 7-O-Flavonoid diglycosides used in the study. Molecular weight is indicated in parentheses.

Molecular Modeling

Preoptimization and generation of coordinates was performed with CAChe Worksystem Pro using MM3/AM1. Final optimization was performed with the Gaussian'03 software package at the B3LYP/6-31G* level.

Results and Discussion

A series of six brominated pyridyl ligands were used in the present study (Figure 2), three based on the 2,2'-bipyridine skeleton (4,4'-dibromo-2,2'-bipyridine; 6,6'-dibromo-2,2'-bipyridine; and 4,4'-bis(bromomethyl)-

Figure 2. Ligands used in the study. The “O” symbols represent atoms discussed in the molecular modeling part of the study.

2,2'-bipyridine) and three based on the 1,10-phenanthroline skeleton (5,6-dibromo-1,10-phenanthroline; 3,5,6-tribromo-1,10-phenanthroline; and 3,5,6,8-tetrabromo-1,10-phenanthroline), with the overarching goal of using them as “tunable” auxiliary ligands for complexation and structural characterization of isomers, as demonstrated specifically for flavonoids. Complexation with 4,4'- and 6,6'-dibromo-2,2'-bipyridine was compared to allow evaluation of the influence of the position of the electron-withdrawing groups. 4,4'-Bis(bromomethyl)-2,2'-bipyridine was synthesized to investigate the effect of moving the bromine groups further from the aromatic ring. Three different brominated 1,10-phenanthroline compounds were synthesized to allow evaluation of the impact of the number of bromine groups on formation and dissociation of complexes. 5-Nitro-1,10-phenanthroline was used to evaluate the effect of an alternative electron-withdrawing substituent. For the experiments de-

scribed in the following sections, ESI-MS/MS was used to analyze solutions containing either 2,2'-bipyridine or a brominated ligand in a solution containing one of 12 different flavonoids and either a copper or cobalt salt.

Complexation with Bromobipyridines

In the ESI mass spectra of each solution containing one flavonoid with 4,4'-dibromo-2,2'-bipyridine and either CuBr₂ or CoBr₂, the abundances of the resulting complexes are comparable. The spectra are dominated by the [M(II) (flavonoid – H) 4,4'-dibromo-2,2'-bipyridine]⁺ complexes, without interference from the formation of numerous other metal–ligand species (Figure 3). Division of the total ion current among several different metal-containing products has been one shortcoming noted in some previous metal complexation studies,

Figure 3. Full-scan spectrum of a solution of rhoifolin/Cu(II)/4,4'-dibromo-2,2'-bipyridine showing the flavonoid/metal/ligand complex as the dominant species.

Figure 4. Representative spectra for CAD of flavonoid/metal/4,4'-dibromo-2,2'-bipyridine with a flavone rutinoside/neohesperidoside and a flavanone rutinoside/neohesperidoside, comparing the Cu and Co complexes. (-D) indicates the loss of disaccharide moiety, (-R) indicates the loss of rhamnose moiety, (-L) indicates the loss of ligand, and X indicates a cross-ring cleavage of the sugar moiety.

thus having a negative impact on ultimate detection limits.

Because both copper and cobalt resulted in the formation of equally abundant flavonoid complexes, the CAD spectra were acquired for all the complexes of the flavonoids used in the study. Figure 4 shows this comparison for one representative flavonoid from each of the four classes summarized in Figure 1 (that is, one flavone neohesperidoside, one flavone rutinoside, one flavanone neohesperidoside, and one flavanone rutinoside). All of the copper complexes dissociate predominantly by the loss of the disaccharide moiety, corresponding to the neutral loss of 308 Da (left panel of Figure 4). The copper complex of isorhoifolin exhibits two additional fragment ions arising from cross-ring cleavages of the disaccharide (cross-ring cleavages are designated by X in the figures) in conjunction with loss of the 4,4'-dibromo-2,2'-bipyridine ligand, yielding the fragment ions at m/z 523 and 495. The cobalt complexes exhibit more intricate fragmentation pathways with unique ions for each flavonoid (right panel of Figure 4). The most common ones include the loss of the terminal rhamnose group (-146 Da), loss of the disaccharide moiety (-308 Da), loss of the auxiliary 4,4'-dibromo-2,2'-bipyridine ligand (-314 Da), loss of the aglycon group (-A), and other combined losses thereof. There is at least one diagnostic fragment ion for each of the isomers, and in most cases there are at least two. In addition, there are two diagnostic cross-ring cleavages involving the disaccharide group for each of the flavone and flavanone rutinosides (that is, isorhoifolin and didymmin in Figure 4). The resulting ions are observed at m/z 876 (-74, X1) and m/z 846 (-104, X2) for the isorhoifolin complex and m/z 892 (-74, X1) and m/z 862 (-104, X2) for the didymmin complex. Proposed cleavages that are

consistent with the observed fragment ions are shown in Scheme 1 for these cross-ring cleavages. There is another notable series of cross-ring cleavages for the flavone rutinoside isomers (fragment ions labeled XN in the right panel of Figure 3B for the isorhoifolin complex, also seen for the analogous complexes of diosmin and linarin). Some of these cross-ring cleavages occur in conjunction with loss of the 4,4'-dibromo-2,2'-bipyridine ligand.

It is interesting that these extensive cross-ring fragmentations are observed for the flavone rutinoside complexes but not the flavanone rutinoside complexes. We speculate that this striking difference in the degree of cross-ring cleavages is related to the ability of the rutinoside moiety to interact with the metal center in the complexes, which may influence the migration of the metal or auxiliary ligand chelate upon activation, as well as the strength of the glycosidic bond. Previous

Scheme 1. Proposed cleavages for a flavonoid/Co/4,4'-dibromo-2,2'-bipyridine cobalt complex using isorhoifolin as an example.

computational modeling of [Co(II) (flavonoid – H) (2,2'-bipyridine)]⁺ complexes by Zhang and Brodbelt [50] revealed that the most favorable metal coordination sites involved the carbonyl oxygen atom and adjacent deprotonated oxygen atom of the aglycon. Moreover, it was shown that for the rutinoides, the disaccharide folds toward the metal center, thus increasing the interaction of the disaccharide with the metal [50]. This factor presumably could enhance the possibility of migration of the metal/auxiliary ligand chelate in the complexes containing the 4,4'-dibromo-2,2'-bipyridine ligands and/or modify the electron density in the glycoside bonds, thus promoting cross-ring cleavages instead of glycosidic bond cleavage. It was also found that the glycosidic bond energies and point charges for the aglycon–disaccharide bonds were greater for the flavonoid rutinoides than for the flavonoid neohesperidosides [50]. This effect may be further exaggerated for the complexes containing the 4,4'-dibromo-2,2'-bipyridine ligand, given that the electron-withdrawing bromine atoms should temper the metal binding energy of the auxiliary ligand, which could translate into alternate fragmentation pathways to glycosidic bond cleavage, such as cross-ring cleavages.

The analytical utility of two other brominated bipyridine ligands, 6,6'-dibromo-2,2'-bipyridine and 4,4'-bis(bromomethyl)-2,2'-bipyridine, was also evaluated. The only complexes observed by ESI-MS for solutions containing a flavonoid, a metal salt, and 6,6'-dibromo-2,2'-bipyridine were ligand/metal complexes of the type [M(I) 6,6'-dibromo-2,2'-bipyridine + H₂O]⁺. Molecular modeling was used to evaluate the 4,4'-dibromo-2,2'-bipyridine/Co(II) and 6,6'-dibromo-2,2'-bipyridine/Co(II) complexes to assess the potential hindrance to flavonoid binding. The dihedral angles of the N—C—C—N bonds on the ligand (labeled with a "O" in Figure 2) were found to have a minimum energy at 0° for both complexes, and this is consistent with previous modeling studies of other flavonoid metal complexes containing pyridyl ligands [50]. The C—Br bond distances were 1.9248 Å for the 6,6'-dibromo-2,2'-bipyridine complex. These distances, coupled with the dihedral angle that shows that the bromine atoms are in the same plane with Co, indicate that the proximity of the bromine atoms to the cobalt ion causes substantial steric hindrance, which prevents simultaneous coordination of 6,6'-dibromo-2,2'-bipyridine and a flavonoid to the metal ion, thus making 6,6'-dibromo-2,2'-bipyridine a poor auxiliary ligand.

In contrast to the ESI-MS results for the 6,6'-dibromo-2,2'-bipyridine/flavonoid/metal solutions, abundant flavonoid complexes were observed for solutions containing 4,4'-bis(bromomethyl)-2,2'-bipyridine and a transition metal. However, the CAD spectra were dominated by the loss of the rhamnose moiety and the loss of the disaccharide group. These CAD spectra did not allow differentiation of the various isomers, and thus

Figure 5. Representative CAD mass spectra of the [3,5,6,8-tetrabromo-1,10-phenanthroline (flavonoid – H) Co(II)]⁺ complexes containing the following flavonoids: (a) rhoifolin, (b) isorhoifolin, (c) poncirin, and (d) didymine. (-D) indicates the loss of disaccharide moiety, (-R) indicates the loss of rhamnose moiety, and (-A) indicates the loss of aglycon.

4,4'-bis(bromomethyl)-2,2'-bipyridine proved to be an unsatisfactory auxiliary ligand.

Complexation with Phenanthroline Ligands

The complexation of each of the bromophenanthrolines (structures shown in Figure 2) was studied with each of the 12 flavonoids. Figure 5 illustrates the CAD mass spectra of the complexes containing the 3,5,6,8-tetrabromo-1,10-phenanthroline ligand, four representative flavonoids, and cobalt. The dominant dissociation route for all four flavonoid complexes in Figure 5 is the loss of the rhamnose moiety, as well as elimination of the disaccharide group, two pathways that are commonly observed for other types of flavonoid/metal complexes. The presence of other unique fragment ions in each CAD spectrum allow each pair of isomers to be readily distinguished.

The types of fragment ions observed for all of the cobalt complexes containing each of the bromophenanthroline ligands as compared to unmodified 1,10-phenanthroline and each of the flavonoids are summarized in Table 1. In general, the types of fragmentation pathways are similar to those noted earlier for the complexes containing the 4,4'-dibromo-2,2'-bipyridine ligand, including the loss of the rhamnose group, the elimination of the disaccharide moiety, and the loss of the aglycon and rhamnose groups. The most notable difference between the fragmentation patterns of the flavonoid rutinoides and the neohesperidosides are two cross-ring cleavages (-74 Da, -104 Da) that were also observed for the analogous flavonoid rutinoides complexes containing the 4,4'-dibromo-2,2'-bipyridine ligand. In addition, the flavanone neohesperidosides are differentiated from the flavones by the characteristic loss of the aglycon moiety.

Table 1. Summary of fragment ion distributions observed in the CAD spectra for selected Co/ flavonoid/ligand complexes grouped by flavonoid diglycoside class^a

4,4'-dibromo-2,2'-bipyridine/Co	(-R)	(-D)	(-A)	(-A,-R)	(-L)	X1	X2
Flavone neohesperidoside (rhoifolin)	54	100		19			
Flavone rutinoside (isorhoifolin)	100	68			80	10	14
Flavanone neohesperidoside (poncirin)	95	100	8	58			
Flavanone rutinoside (didymin)	100	83			29	5	7
1,10-phenanthroline/Co	(-R)	(-D)	(-A)	(-A,-R)	(-L)	X1	X2
Flavone neohesperidoside (rhoifolin)	15	100		6			
Flavone rutinoside (isorhoifolin)	14	100					4
Flavanone neohesperidoside (poncirin)	38	100		32			
Flavanone rutinoside (didymin)	16	100		8			
5,6,-Dibromo-1,10-phenanthroline/Co	(-R)	(-D)	(-A)	(-A,-R)	(-L)	X1	X2
Flavone neohesperidoside (rhoifolin)	73	100		23			
Flavone rutinoside (isorhoifolin)	94	100				15	21
Flavanone neohesperidoside (poncirin)		100					
Flavanone rutinoside (didymin)		100					
3,5,6-Tribromo-1,10-phenanthroline/Co	(-R)	(-D)	(-A)	(-A,-R)	(-L)	X1	X2
Flavone neohesperidoside (rhoifolin)	100	89		15			
Flavone rutinoside (isorhoifolin)	100	61		4		12	18
Flavanone neohesperidoside (poncirin)	79	100	5	26			
Flavanone rutinoside (didymin)	94	100		8		4	11
3,5,6,8-Tetrabromo-1,10-phenanthroline/Co	(-R)	(-D)	(-A)	(-A,-R)	(-L)	X1	X2
Flavone neohesperidoside (rhoifolin)	100	68		14			
Flavone rutinoside (isorhoifolin)	100	36			5	8	14
Flavanone neohesperidoside (poncirin)	100	56	8	20			
Flavanone rutinoside (didymin)	100	72		6		11	6
5-Nitro-1,10-phenanthroline/Co	(-R)	(-D)	(-A)	(-A,-R)	(-L)	X1	X2
Flavone neohesperidoside (rhoifolin)	38	100		14			
Flavone rutinoside (isorhoifolin)	31	100			8	6	12
Flavanone neohesperidoside (poncirin)	100	100		66			
Flavanone rutinoside (didymin)	48	100			10	6	14

^a(-D) indicates loss of disaccharide moiety; (-R) indicates loss of rhamnose moiety; (-L) indicates loss of ligand; (-A) indicates loss of aglycon; and X1 and X2 represent the cross-ring cleavages of (-74) and (-104), respectively ($\pm 2\%$).

The summary in Table 1 reveals that the number of different fragment ions increases as the number of electron-withdrawing bromine atoms on the 1,10-phenanthroline auxiliary ligand increases. The number of fragmentation pathways correlates with the relative stabilities of the metal complexes. Complexes in which one of the two coordinated molecules (that is, flavonoid versus auxiliary ligand) has a significantly greater metal binding energy than that of the other tend to dissociate by just a few routes, thus typically being less useful for confident differentiation of isomers. Among the three brominated phenanthroline ligands, the 3,5,6,8-tetrabromo-1,10-phenanthroline ligand is expected to have the lowest metal binding energy as a result of the influence of the four electron-withdrawing bromine atoms on the metal-coordinating nitrogen atoms. This makes the metal binding energy of the 3,5,6,8-tetrabromo-1,10-phenanthroline ligand closer to the metal binding energy of a typical flavonoid, thus increasing the

stability of the 3,5,6,8-tetrabromo-1,10-phenanthroline/ flavonoid complexes and resulting in a greater array of competitive dissociation routes. In fact, of the three bromophenanthroline ligands, the tetrabromo ligand (3,5,6,8-tetrabromophenanthroline) is the only one that allows successful differentiation of all of the flavonoid isomers, in terms of providing at least one unique diagnostic fragment ion for each isomer.

To further assess the impact of electron-withdrawing groups on the tunability of the auxiliary ligands, 5-nitro-1,10-phenanthroline was evaluated because the nitro group is one of the most electron-withdrawing groups available. Abundant complexes of the type $[M(II)(\text{flavonoid} - H) 5\text{-nitro-1,10-phenanthroline}]^+$ were formed with the flavonoids, and the resulting CAD spectra also displayed the enhanced diagnostic cross-ring cleavages observed with the brominated ligands (Table 1). This result further underscores the strategy of adding electron-withdrawing groups to alter the dissociation pathways of the targeted complexes.

Figure 6. Comparison of the CAD mass spectra of $[\text{Co(II)}(\text{isorhoifolin-H})\text{L}]^+$ complexes containing each brominated ligand, where L is the brominated ligand. A square and a circle are used to represent the cross-ring cleavages resulting in losses of 74 and 104 Da, respectively. A diamond is used to represent loss of the ligand in conjunction with a cross-ring cleavage of the sugar. (-D) indicates the loss of disaccharide moiety, (-R) indicates the loss of rhamnose moiety, (-L) indicates the loss of ligand, and X indicates a cross-ring cleavage of the sugar moiety.

Comparison of All Ligands

Figure 6 shows the CAD mass spectra for a series of cobalt/isorhoifolin complexes, each containing one of six different auxiliary ligands. Figure 6 comprehensively illustrates how the bromination of the auxiliary ligands affects the tunability of the CAD patterns. The CAD mass spectra for the complexes containing either 2,2'-bipyridine or 1,10-phenanthroline are also shown for comparison. A quick inspection of the CAD mass spectra reveals notable differences in the relative abundances of specific fragment ions, in addition to the emergence of new diagnostic fragment ions as the identity of the auxiliary ligand changes. For all of the complexes, the elimination of the rhamnose moiety (-R) and the loss of the disaccharide group (-D) is consistently observed, and the relative abundances of the two resulting fragment ions vary significantly for the series of CAD spectra. As the degree of bromination increases for the phenanthroline ligands, the ratio of (-R)/(-D) increases (Figure 6c–e). Another notable difference is the increase in the abundance of the two cross-ring cleavages X1 (-74, labeled with a square) and X2 (-104, labeled with a circle) upon bromination of the auxiliary ligands. Also noteworthy is the increase in the abundance of the fragment ion of 414 Da (Figure 6f, labeled with a diamond) for the complex containing 4,4'-

dibromo-2,2'-bipyridine, which results from the loss of the ligand in conjunction with a cross-ring cleavage of the sugar. Although the CAD spectra of complexes containing 2,2'-bipyridine or 1,10-phenanthroline yielded some of the same diagnostic fragments, there was an enhancement in the abundance of these fragment ions using the brominated ligands.

Overall, Figure 6 and Table 1 clearly demonstrate the tunability of the CAD patterns promoted by the use of different auxiliary ligands. This tunability is related to the relative metal binding energies of the auxiliary ligand and flavonoids. Based on the significant differences in fragmentation pathways of pairs of isomeric complexes observed earlier in Figure 4 and compared to the performance of the other auxiliary ligands, 4,4'-dibromo-2,2'-bipyridine was judged to be the best overall auxiliary ligand for the differentiation of the series of flavonoids in the present study.

Electron-Withdrawing Group Effect

The results presented in this work show that the tunability of dissociation of flavonoid complexes is accomplished by adding electron-withdrawing substituents to the auxiliary ligands used to coordinate the transition metal and stabilize the flavonoid complexes. To under-

stand the impact of the electron-withdrawing substituents on the stabilities and dissociation pathways of the flavonoid complexes in the gas phase, it is instructive to consider the influence of electron-withdrawing substituents on the binding energies of pyridyl ligands in solution. Although extensive solution data are not available, numerous binding constants have been measured for pyridyl ligands with Co(II) and Fe(II), two representative transition metals, in aqueous solution. Fe(II) was not a metal used in the present ESI-MS study, although the binding constants for Fe(II) complexes were compiled because this is the only metal for which brominated ligands have been studied.

The binding constants for cobalt/pyridyl ligand complexes in solution decrease in the order: 5,6-dimethyl-1,10-phenanthroline (log K 7.47) > 5-methyl-1,10-phenanthroline (log K 7.14) > 1,10-phenanthroline (log K 7.08) > 5-nitrophenanthroline (log K 6.3) [43], thus showing the impact of the addition of electron-releasing versus electron-withdrawing substituents on 1,10-phenanthroline. The binding constants for Fe(II)/pyridyl complexes decrease in the order: 5,6-dimethyl-1,10-phenanthroline (log K 6.37) > 5-methyl-1,10-phenanthroline (log K 6.00) > 1,10-phenanthroline (log K 5.85) > 5-bromo-1,10-phenanthroline (log K 5.65) > 5-nitrophenanthroline (log K 5.06) [43]. This trend indicates that the metal/ligand binding energies decrease as the substituents on the ligand become more electron withdrawing. A similar inductive effect would be expected for the metal/ligand binding energies in the gas phase. The variations in the metal/ligand binding energies may influence the energetics of dissociation pathways and also affect the preferred coordination sites of the flavonoid complexes. The ligand also has an impact on the way the disaccharide moiety of the flavonoid interacts with the metal. As mentioned previously, modeling studies have indicated that for the rutinoid isomers, the disaccharide folds toward the metal center [50]. The addition of bromo substituents changes the steric environment of the auxiliary ligands, which may influence the conformations of the complexes and thus the degree of interaction of the disaccharide group with the metal center [50].

Conclusions

The fragmentation pathways of flavonoids can be tuned by using a metal complexation strategy that incorporates auxiliary ligands containing electron-withdrawing substituents. Upon collisional activation, the resulting [Co(II) (flavonoid – H) auxiliary ligand]⁺ complexes dissociate by pathways that are characteristic of flavone and flavanone 7-O-diglycosides (that is, loss of rhamnose and disaccharide groups), in addition to those that allow differentiation of isomers that differ only in the type of intersaccharide linkage. In this work, the pathways that distinguish flavone and flavanone disaccharides with 1 → 2 and 1 → 6 linkages have been enhanced by adding electron-withdrawing substituents

to the auxiliary ligands. The addition of electron-withdrawing groups to the auxiliary ligands reduces their metal-ligand binding affinities and consequently alters the stabilities and conformations of the [M(II) (flavonoid – H) L]⁺ complexes, thus resulting in the enhancement of diagnostic fragmentation pathways.

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