



Regulating the fluorescence intensity of an anthracene boronic acid system: a B–N bond or a hydrolysis mechanism?

Weijuan Ni,^a Gurpreet Kaur,^b Greg Springsteen,^a
Binghe Wang,^{b,*} and Stefan Franzen^{a,*}

^a *Department of Chemistry, North Carolina State University, Raleigh, NC 27695, USA*

^b *Department of Chemistry, Georgia State University, Atlanta, GA 30303, USA*

Received 20 April 2004

Available online 4 August 2004

Abstract

An anthracene-based fluorescent boronic acid system developed by the Shinkai group has been widely used for the preparation of fluorescent sensors for carbohydrates. Such application is based on the significant fluorescence intensity increase of this system upon binding with a carbohydrate. The mechanism through which this fluorescence intensity change happens was originally proposed to go through a B–N bond formation mechanism, which masks the nitrogen lone pair electrons. However, our own fluorescence studies suggest a possible alternative mechanism for the fluorescence change upon the formation of a boronic acid (**1a**) complex with diols. In this new proposed mechanism, complex formation induces solvolysis, which results in the protonation of the amine nitrogen if the reactions are carried out in a protic solvent such as water. This protonation prevents the photoinduced electron transfer, resulting in reduced quenching of the anthracene fluorescence. Such a solvolysis mechanism is supported by evidence from various types of experiments and theoretical calculations.

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Keywords: Anthracene-based fluorescent boronic acid; B–N bond; PET

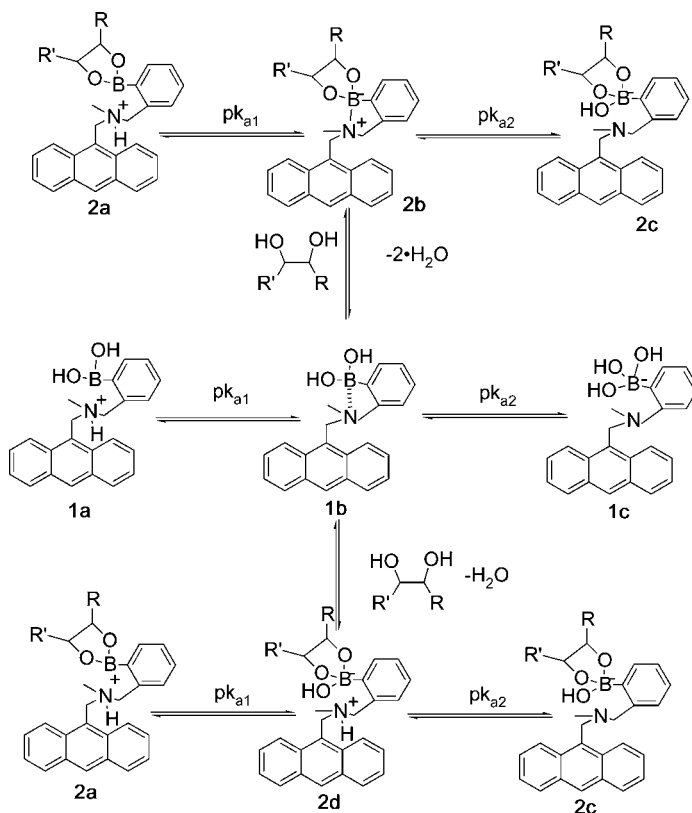
* Corresponding authors. Fax: 1-404-654-5827.

E-mail address: wang@gsu.edu (B. Wang).

1. Introduction

Due to the unique strong interactions between boronic acids and diols through reversible ester formation, there has been a great deal of interest in using boronic acids as the recognition moiety for the development of fluorescent and color sensors [1–16], carbohydrate transporters [17–22], and chromatographic stationary materials [23–26]. Various fluorescent boronic acid compounds that show fluorescent intensity/wavelength changes upon binding with diols to varying degrees have been developed [1,27–29]. Recently, our laboratory has reported for the first time that boronic acid compounds capable of binding to cell surface carbohydrates can fluorescently label cells that express such carbohydrates [15,30,31]. Critical to the development of boronic acid-based fluorescent sensors is the availability of fluorescent reporter compounds that change fluorescence intensities upon binding to a diol [10,29,32,33]. In this regard, the Shinkai group, based on what was reported by Wulff earlier [34], has made some of the most important contributions by taking advantage of the fluorescence/color modulation effect of an amino group positioned in a 1,5 relationship to the boron atom of a boronic acid moiety. One specific example is an anthracene-based fluorescent boronic acid compound (**1a**, Scheme 1), which shows significant fluorescence intensity changes upon diol binding [9,27]. It is well known that the nitrogen lone pair electrons can quench anthracene fluorescence through photoelectron transfer (PET). Therefore, compound **1a** is only weakly fluorescent at physiological pH. Due to the proximity of the amine to the boron atom, a B–N bond can be formed leading to a five-membered ring (**1b**) at neutral pH. Because diol binding often increases the Lewis acidity of the boron atom [28,35,36], carbohydrate binding has been proposed to result in the strengthening of the B–N bond in **2b**, which makes the lone pair electrons less available for fluorescence quenching through PET. The hypothesis is that B–N bond strengthening results in decreased fluorescence quenching and increased fluorescence intensities in **2b**. The same concept has been the inspiration for the development of many other fluorescence or color boronic acid reporter compounds [10,33,37]. We have also applied the Shinkai system for the preparation of sensors for mono- and oligosaccharides [17–20] and studied the effects of B–N bond formation in such systems using density functional theory (DFT) methods [38].

Although this system (**1a**) has worked very well as a fluorescent reporter compound, there are telltale signs that the mechanism through which such a system works may not have to involve B–N bond formation as originally proposed. For example, available crystal structures actually show a longer B–N bond for the ester than for the acid. The bond lengths of the ester [39] and acid [7] determined by X-ray crystallography are 1.754 and 1.669 Å, respectively. Furthermore, Copper and James [40] have shown that the fluorescence intensity of the sugar-**1b** complex is independent of the intrinsic pK_a of the boronic ester, which contradicts the B–N bond mechanism. Because of the enormous role that molecule (**1a**) has played in the field of boronic acid-based sensor design, there is the need to understand better the mechanism through which the fluorescence intensity change occurs. An understanding of the mechanism will help the future design of improved sensors.



Scheme 1. Possible mechanisms for the fluorescence intensity changes.

2. Experimental

Most reagents were purchased from Aldrich or Fisher/Acros and used as received. Anhydrous tetrahydrofuran (THF) was distilled from Na/benzophenone prior to use. Acetonitrile (CH_3CN) and dichloromethane (CH_2Cl_2) were distilled from CaH_2 .

All pH values were determined with an Accumet 1003 Handhold pH/mV/Ion Meter (Fisher Scientific). A Shimadzu RF-5301 PC fluorometer was used for the fluorescence studies. The excitation wavelength was set at 370 nm. A Shimadzu UV-1601 spectrophotometer was used for the UV absorption studies.

Synthesis of model compound **1a** followed literature procedure [9,27].

3. Experimental design, results, and discussion

To analyze the possible mechanisms through which fluorescence intensity changes can occur, one can first write the different possible forms in which the boronic acid and the boronic acid–diol complex can exist. Boronic acid can exist in the protonated

form when the pH is below its first pK_a (**1a**). There are ample literature results proving that the first pK_a is the deprotonation of the amine with the concomitant formation of a B–N bond (**1b**) [7]. The formation of (**1b**) results in a significant fluorescence intensity decrease relative to (**1a**) despite the formation of the B–N bond [9,27]. Such results indicate that B–N bond formation itself is not sufficient to “tie up” the lone pair electrons to prevent PET. With the further increase of the pH, the hydroxide comes in to displace the amino group to give **1c**, which no longer has a B–N bond.

With the addition of the diol, there are two possible scenarios, neither of which has been experimentally proven or excluded at present. In the first scenario, pK_{a1} of the ester (**2a**) is the deprotonation of the amino group and the formation of the B–N bond (**2b**) as originally proposed by Shinkai and co-workers. The second pK_a is the replacement of the B–N bond by a B–O bond leading to the formation of **2c**. In the second scenario, shown in the bottom of Scheme 1, the first pK_a is the addition of the hydroxide to the boron atom leading to the formation of **2d**. The second pK_a is still the same, leading to the formation of **2c**. The key difference between these two mechanisms is that under neutral conditions, addition of a diol will lead to the formation of **2b** in one mechanism (referred to as the B–N bond mechanism) or **2d** in the other. The latter will be referred to as the hydrolysis mechanism since going from **1b** to **2d** corresponds to a hydrolysis of the B–N bond as the boron forms a complex with a diol in **2d** when the experiments are carried out in an aqueous medium.

To analyze existing literature data and to design experiments that can help to elucidate the mechanism, one has to analyze the possible experimental outcome under different experimental conditions for these two possible mechanisms. In Table 1,

Table 1
Comparison of expected and observed phenomenon for the two different mechanisms in Scheme 1

Phenomenon	Expected results for the B–N bond mechanism	Expected results for the hydrolysis mechanism	Results observed
Fluorescence intensity change associated with the first pK_a	Observable for both the acid (1) and the ester (2)	Observable only for the acid (1), not the ester (2)	Observable only for the acid (1)
Fluorescence intensity change associated with the second pK_a	Observable for both the acid (1) and the ester (2)	Observable only for the ester (2), not the acid (1)	Observable only for the ester (2)
Magnitude of fluorescence recovery with different sugars	Different	Same	Same
The effect of carbohydrates engaged in trivalent binding	Will not induce fluorescence intensity changes	Will induce fluorescence intensity changes	Fluorescence intensity changes observed
B–N bond strength difference between the acid (1) and the ester (2)	Very large	Small	Small ^a

^a Computational results.

we have listed the expected experimental and/or computational outcomes that correspond to each of the mechanisms.

First, for the B–N bond formation mechanism (going from **2a** to **2b** to **2c**) one would expect to see a significant fluorescence intensity decrease associated with both the first and second pK_a s since the deprotonation of the amino group and breaking the B–N bond are both expected to affect the availability of the lone pair electrons. However, in the hydrolysis mechanism (going from **2a** to **2d** to **2c**) one would only expect to see a significant fluorescence intensity change associated with the second pK_a since the first pK_a does not affect the protonation state of the amino group, and therefore does not affect the availability of the nitrogen lone pair electrons in the PET process.

Second, since binding with different diols may result in the lowering of the boron pK_a to different degrees [28,36], one would expect the B–N bond strength to be different in the boronic acid complexes with different sugars. This difference in B–N strength should be reflected in fluorescence intensity changes observed with addition of sugars if the B–N bond formation mechanism is at work. However, if the hydrolysis mechanism is at work, one would not expect to see a difference in the magnitude of the maximal fluorescence recovery.

Third, there are some sugars that are known to bind to boronic acid in a trivalent fashion [5,41,42]. If the B–N bond formation mechanism is the reason for the observed fluorescence intensity change, one would not expect these trivalent sugars to cause a fluorescence intensity increase because the trivalency prevents the B–N bond formation in the sugar–boronic acid complex (**2c**).

Fourth, if the strengthening of the B–N bond is the reason for the observed fluorescence intensity changes with this system, one would expect the B–N bond in the boronic acid–diol complex (**2b**) to be much stronger than in the free boronic acid (**1b**).

With these expected differences in mind, we examined these factors one by one, through experiments, computation and analysis of literature results. Fluorescent boronic acid **1a** was synthesized following the literature procedure [27]. Density functional theory (DFT) calculations were carried out using Dmol3 as described elsewhere [38].

3.1. *pH profiles of the acid (1a) and various complexes*

We first examine the pH profile of **1a**. Shinkai's original work has already shown that there is a significant decrease in fluorescence intensity associated with the first pK_a of the boronic acid (**1a**), but not the second one [9,27]. We conducted the same experiments under similar conditions, and obtained similar results (Fig. 1). This decrease in fluorescence intensity associated with the first pK_a makes sense since it has been experimentally proven using a similar system that the first pK_a is the deprotonation of the amino group accompanied by B–N bond formation [7]. Deprotonation un masks the nitrogen lone pair electrons and makes them available for PET. It should be noted that the "affinity" of a proton for an amine group has been estimated to be in the range of 200 kcal/mol [43]. This value may vary significantly depending on solvation and pK_a , but even if this "protonation affinity" is half of that number, it is still very strong, comparable to a sigma covalent bond, and higher than the energy required to remove an electron from an amine nitrogen. On the other

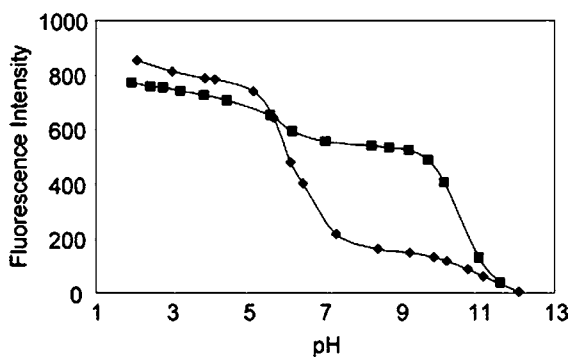


Fig. 1. pH titration profile of sensor **1** (2.25×10^{-6} M) at 25 °C in the presence (■) and absence (♦) of D-glucose (0.05 M).

hand, the B–N strength is only a few kcal/mol [38], which would not be able to “tie up” the nitrogen lone pair electrons nearly as effectively as protonation does to prevent PET. Such reasoning weighs in favor of a key role for protonation of the amine rather than B–N bond formation as the trigger that alters the fluorescence intensity.

Along these lines, it becomes easy to understand why there is only a very small fluorescence decrease associated with the second pK_a in the case of the free acid (**1b**) because breaking a ~ 3.6 kcal/mol B–N bond does not make much of a difference in the PET process. As shown in a previous study, there is only a small effect on the driving force for electron transfer or the reorganization energy. With the addition of a sugar such as glucose, the pH profile of the complex is different [9,27]. The first pK_a of the complex (**2a**) is no longer associated with a large decrease in fluorescence intensity. This observation is not in agreement with the B–N bond mechanism since one would expect to see a fluorescence intensity decrease when the amine nitrogen goes from the protonated form to the B–N bond. However, if the hydrolysis mechanism is at work, the observations can be very easily explained. In the boronic acid–diol complex, going from **2a** to **2d** does not involve the deprotonation of the amino group. Therefore, one would not expect to see a large fluorescence intensity change associated with the first pK_a of the complex (**2a**) in the hydrolysis mechanism. With the second pK_a , it is the deprotonation of the amino group that is the reason for the significant decrease in fluorescence intensity. Such a mechanism is also in agreement with the different results observed between the acid (**1a**) and complex (**2a**). The B–N interaction itself is not sufficiently strong to tie up the lone pair electrons to prevent PET, which is why the fluorescence intensity changes were observed for the first pK_a of the acid (**1a**), but not the ester (**2a**). These observations also agree with DFT calculations that indicate that fluorescence quenching will occur in the neutral B–N forms due to the reduction of the reorganization energy upon B–N bond formation (or strengthening) [38]. The hydrolysis hypothesis also explains why fluorescence intensity changes were observed for the second pK_a of the ester (**2d**) and the first pK_a of the acid (**1a**) because again it is the deprotonation step that gives rise to fluorescence intensity changes.

3.2. Effect of different sugars on the fluorescence intensity changes of **1a**

It has been known for a long time that ester formation (**2b**) lowers the pK_a of the boron species to different degrees depending on the sugar/diol used [28,36]. We have recently determined the apparent pK_a values of the phenylboronic acid–diol complex with various sugars/diols, and found that the apparent pK_a drops 2–4 pK_a units upon complexation [28]. For example, the apparent pK_a of phenylboronic acid is about 8.8. The pK_a values (or more precisely the apparent pK_a 's under the experimental conditions) of its fructose and glucose esters are 4.5 and 6.8, respectively. If the B–N bond mechanism is at work, one would expect that the fluorescence intensity of the boronic acid–diol complex to be dependent on the apparent pK_a of the boron. One would also expect to see as much change in fluorescence intensity when adding glucose to the free boronic acid and when replacing the glucose with fructose because their apparent pK_a separations (ΔpK_a) are about the same. In other words, the fluorescence intensity of the glucose complex should be much lower than that of the fructose complex because of the difference in their apparent pK_a . The same is true with the complex with other sugars. Aimed at studying the effect of various sugars on the fluorescence intensity of **1a**, we examined the binding between **1a** and fructose, glucose, sorbitol, and galactose, and found that these sugars gave about the same maximal fluorescence intensities at saturating concentrations (Fig. 2). Such results are consistent with those obtained in an earlier study by Copper and James [40] and cannot be explained by the B–N bond mechanism, but are consistent with the hydrolysis mechanism since the amine is in the same (protonated) state (**2d**) no matter which sugar/diol is added.

3.3. B–N bond strength

As has been alluded to in the pH profile studies, in order for the B–N to play a role in regulating the PET process two things have to happen.

First, the B–N bond needs to be so strong that it can “tie up” the lone pair electrons. This would mean that the B–N bond strength in the ester (**2b**) is on the same

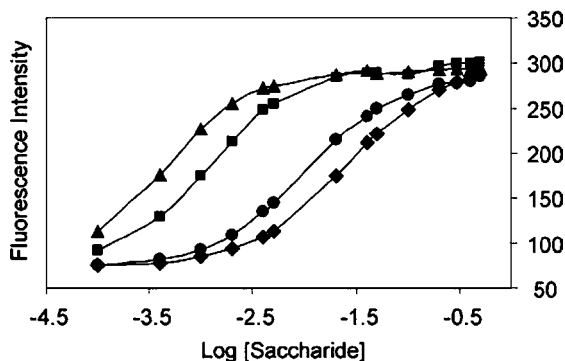


Fig. 2. The effect of sorbitol (▲), fructose (■), galactose (●), and glucose (◆) on the fluorescence intensity of **1** (2.25×10^{-6} M).

scale as the energy required transferring one nitrogen lone pair electron. The effect of protonation on the PET process serves as a good reference point. Using mass spectrometry, it has been determined that the proton affinity of an aniline amine is on the order of 215 kcal/mol [43]. However, the B–N bond strength has been estimated to be at about 3.6 kcal/mol using density functional theory [38], which is far smaller than what is required to tie up the nitrogen lone pair electrons to prevent PET.

Second, the B–N bond strength difference upon diol binding should be large compared to the B–N strength of the free acid (**1b**) in order to act as an on–off switch. Again, our theoretical calculation indicates that (1) the B–N bond in an ester (**2b**) is not much different from in the free acid (**1b**) form and (2) the change in bond strength upon ester formation is probably no more than 2 kcal/mol [38]. The first point would directly contradict a B–N bond mechanism. Even if there is an increase in B–N bond strength upon ester formation, such a small change is not expected to function as an on–off switch as described in a B–N bond mechanism. On the other hand, the computational results and the “proton affinity” experiments [43] are all consistent with the hydrolysis mechanism.

3.4. Experiments with trivalent sugars

There are several carbohydrates, including fructose and sorbitol that are known to bind boronic acids in a trivalent fashion [5,28,36,42]. If the B–N bond mechanism is at work, one would expect that those sugars that complex to boronic acid in a trivalent fashion would not induce a fluorescence intensity change upon binding since B–N bond formation would not be possible. This is also a point raised by Norrild and Sotofte [41] in a crystal structural study. Therefore, we tested the effect of fructose and sorbitol on the fluorescence intensity of **1a**. They both induced strong fluorescence intensity increases in a methanol–buffer mixed solvent (Figs. 3 and 4). It should be noted that the effect of fructose has already been tested by Shinkai and co-workers [9,27] and found to induce as much fluorescence intensity changes as the other sugars tested. This by itself already contradicts the B–N bond mechanism, although the realization of fructose binding in a trivalent fashion came after the proposed B–N bond

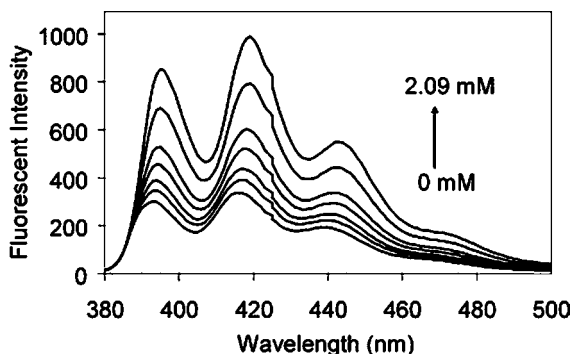


Fig. 3. Fluorescence intensity changes upon the addition of fructose to sensor **1** (2.81×10^{-6} M) in 1:1 MeOH /0.1 M aqueous phosphate buffer at pH 7.4. Fructose conc. (mM): 0, 0.04, 0.09, 0.21, 0.35, 0.88, and 2.09.

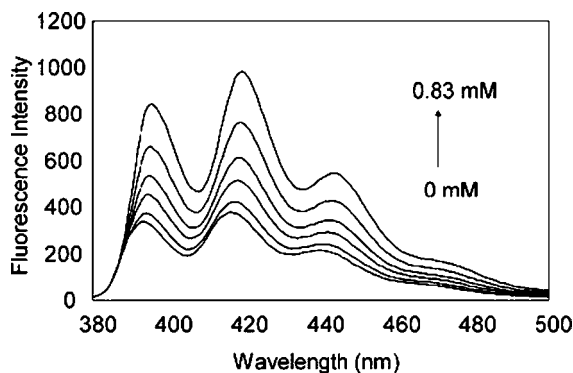


Fig. 4. Fluorescence intensity change with addition of sorbitol to sensor **1** (2.81×10^{-6} M) in 50% MeOH/0.1 M aqueous phosphate buffer at pH 7.4. Sorbitol conc. (mM): 0, 0.03, 0.10, 0.17, 0.39, and 0.83.

mechanism. Such binding results cannot be explained by the B–N bond mechanism, but are consistent with the hydrolysis mechanism.

3.5. DFT studies of model systems

We have recently conducted DFT calculations of the B–N bond strength in a variety of adducts including models for those shown in Scheme 1 and found that the B–N bond strength to be very weak (<7.2 kcal/mol) for all boronic acid and boronate ester adducts with geometry observed experimentally and the change in B–N bond strength upon formation of a boronate–ester is <1 kcal/mol [38]. The effect of the change in boron–nitrogen interaction on the electron transfer rate constant was examined in detail. Estimates of the driving force (energy change for electron transfer) and reorganization energy were obtained. The DFT studies suggest that ester formation will actually decrease the fluorescence yield in aprotic solvents, but will increase the fluorescence yield in protic solvents.

In all the points raised above and the results observed or reported in the literature, any single one is probably not enough to refute the B–N bond mechanism, but putting them all together, it forms a very strong case in support of the hydrolysis mechanism going through **2d** and against the B–N bond mechanism. The hydrolysis mechanism is essentially a pK_a switch mechanism that we have used in the design of spectroscopic and fluorescent boronic acid compounds, which changes spectroscopic and/or fluorescent properties based on the ionization state changes [29,44]. In such a situation, there are two (or more) ionizable functional groups in the boronic acid compound. In this case, it is an amino and a boronic acid group. In the absence of any diol, the pK_a of the amino group is lower than that of the boronic acid moiety. Therefore, the first pK_a of the free sensor (**1a**) is that of the amino group. However, upon addition of a diol, the intrinsic pK_a of the boronic acid is decreased, which results in the pK_a of the boronic acid moiety in the complex (**2b**) being lower than that of the amino group. In another words, the pK_a order of these two functional

groups is switched with the addition of a diol. With sensor **1a**, this switch results in the protonation of amine nitrogen, which “ties up” the nitrogen lone pair electrons and prevents PET quenching of the anthracene fluorescence.

4. Conclusions

In organic chemistry in general, it is always hard to prove a mechanism. This is no exception. However, results from the pH profile studies of the free boronic acid and its sugar complex, the effect of different sugars on the fluorescence intensity changes, the effect of trivalent sugar on the fluorescence intensity of the boronic acid, and the calculated B–N bond strength are all consistent with the hydrolysis mechanism. One can only say that based on available data, the hydrolysis mechanism proposed is a very probable mechanism. However, one cannot completely exclude the possibility that in some situation the B–N mechanism might be at work while in other situations the hydrolysis is at work. The most important thing is to recognize that there is an alternative mechanism. The understanding of this mechanism helps the future design of more effective boronic acid fluorescent reporter compounds for sensor and other applications as has been demonstrated in some of our own work [29,44].

Acknowledgments

We gratefully acknowledge the financial support of this work by the National Institutes of Health (NO1-CO-27184 and CA88343), Georgia Cancer Coalition through a Distinguished Cancer Scientist Award to B.W., and the Georgia Research Alliance through an Eminent Scholar endowment to B.W. We would also thank Dr. Markus Germann for technical help in some of our related studies.

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