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Fluorescent Guanidinium-Azacarbazole for Oxoanion Binding in Water

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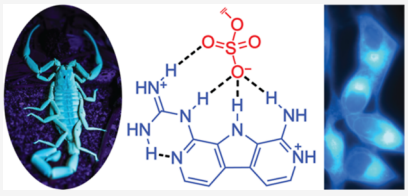
ABSTRACT: Oxoanions such as carboxylates, phosphates, and sulfates play important roles in both chemistry and biology and are abundant on the cell surface. We report on the synthesis and properties of a rationally designed guanidinium-containing oxoanion binder, 1-guanidino-8-amino-2,7-diazacarbazole (GADAC). GADAC binds to a carboxylate, phosphate, and sulfate in pure water with affinities of 3.6×10^4, 1.1×10^3, and 4.2×10^3 M^{-1}, respectively. Like 2-azacarbazole, which is a natural product that enables scorpions to fluoresce, GADAC is fluorescent in water (λ_{abs} = 356 nm, λ_{em} = 403 nm, ε = 13,400 M^{-1} cm^{-1}). The quantum yield of GADAC is pH-sensitive, increasing from Φ = 0.12 at pH 7.4 to Φ = 0.53 at pH 4.0 as a result of the protonation of the aminopyridine moiety. The uptake of GADAC into live human melanoma cells is detectable in the DAPI channel at low micromolar concentrations. Its properties make GADAC a promising candidate for applications in oxoanion binding and fluorescence labeling in biological (e.g., the delivery of cargo into cells) and other contexts.



■ INTRODUCTION

In 1861, Adolph Strecker synthesized guanidine by degrading the nucleobase guanine.[1,2] Today, a guanidino group is a component of important drugs (e.g., streptomycin, cimetidine, guanfacine, and metformin)[3,4] and is well known for its ability to bind strongly to oxoanions.[5−12] This ability arises in part from its positive charge upon protonation and its branched shape, which allows for the formation of strong bidentate hydrogen bonds with oxoanions.[13]

Early oxoanion binders were macrocycles that showed a higher affinity for inorganic phosphate than did their acyclic counterparts.[14] Since then, organic chemists have refined the number and display of guanidino groups, tuned their pK_a values, and added other hydrogen bond donors within a variety of scaffolds.[5−12] For example, the installation of an adjacent amide or pyrrole moiety can increase the affinity of a guanidinium-containing compound for oxoanions.[15,16] Likewise, decreasing the pK_a value of a guanidino group can enhance affinity[17] because hydrogen bond donors and acceptors with more closely matched pK_a values form stronger hydrogen bonds.[18−20] In that regard, a common strategy has been to employ an *N*-acylguanidinium group, which has a pK_a of 8.3 compared to that of 13.7 for an *N*-alkylguanidinium group.[15,21−23]

Typically, the affinity of a guanidinium-containing compound for oxoanions is measured in an organic solvent or (more rarely) in an aqueous/organic mixture. For example, bisguanidiniums designed and synthesized by Anslyn and co-workers bind to inorganic phosphate in DMSO ($K_a \approx 10^3$ M^{-1}).[24] No measurable affinity is apparent, however, when the water content is increased to 50% v/v.

In addition to binding oxoanions, guanidinium-containing compounds have also found utility as protein transduction domains. The human immunodeficiency virus type 1 (HIV-1) Tat peptide, a segment of the HIV-1 protein Tat, contains a high number of guanidinium-containing arginine residues.[25] Those guanidium groups can associate with oxoanions on the cell surface and facilitate internalization. After this discovery, arginine-rich cell-penetrating peptides (CPPs) became widely used for the cellular delivery of various cargoes, including proteins.[26−35] Arginine-rich membrane-permeable peptides can, however, elicit serious toxicity in mice.[36]

Inspired by CPPs, several groups have synthesized guanidinium-containing compounds that trigger cellular uptake. For example, Selwood and co-workers have synthesized small-molecule carriers (SMoCs), one of which contained four guanidino groups and mimicked the amphipathic α-helical nature of some CPPs.[37,38] The addition of multiple SMoCs to the proteins, geminin and Tev, enhanced their cellular uptake. In another study, Schmuck and co-workers appended multiple dimers of a guanidiniocarbonyl pyrrole (GCP) onto avidin and observed that the GCP-labeled avidin entered cells, whereas avidin labeled with dimers of arginine did not.[39] Although these synthetic guanidinium-containing scaffolds promoted the cellular uptake of cargo, the affinity of each for oxoanions is modest. Moreover, each cargo had to be conjugated to a fluorophore to discern delivery with fluorescence microscopy.

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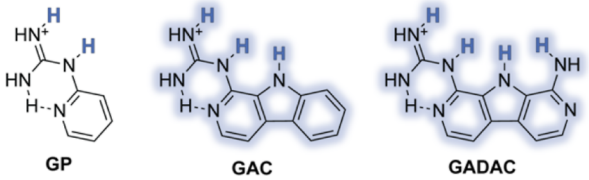
To advance this work, we report on the design, synthesis, and assessment of a novel carbazole derivative that contains a single guanidino group, 1-guanidino-8-amino-2,7-diazacarbazole (GADAC; Scheme 1). We find that GADAC binds tightly to oxoanions in pure water, enters live human cells spontaneously, and fluoresces therein. These unique attributes indicate that GADAC has utility in a variety of contexts.

Scheme 1. Hydrogen Bond Donors in 2-Guanidinopyridine (GP), 1-Guanidino-2-azacarbazole (GAC), and 1-Guanidino-8-amino-2,7-diazacarbazole (GADAC)



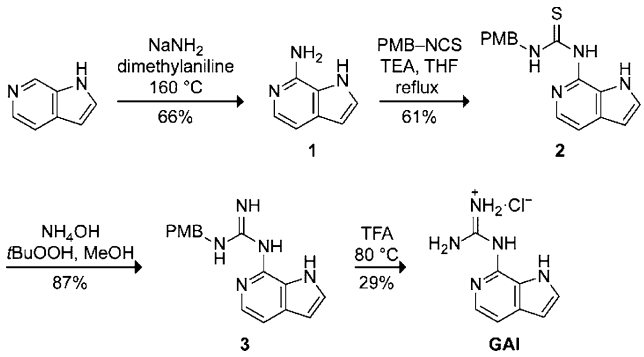
■ RESULTS AND DISCUSSION

Design. We designed GADAC to have a preorganized structure that presents four hydrogen bond donating groups to an oxoanion—two from a guanidinium group, one from a carbazole N−H, and one from a 2-aminopyridine N−H (Scheme 1). An intramolecular hydrogen bond between the guanidinium group and a second pyridino nitrogen is intended to restrict aryl C−N bond rotation and maintain planarity.[40−42] The two pyridino groups are intended to lower the pK_a values of the guanidinium and amino groups and, thereby, enhance their ability to donate hydrogen bonds to oxoanions.[17−20]

We also designed GADAC to be fluorescent for biological applications. The heterocycle 2-azacarbazole (or "β-carboline") is a natural product that is partially responsible for the ability of scorpions to fluoresce under ultraviolet (UV) light.[43] Azacarbazoles and diazacarbazoles have been used as fluorescent probes[44−47] and in organic light-emitting diodes.[48−50]

Synthesis. We began our synthetic efforts by optimizing the late-stage guanidinylation conditions on a model compound, 7-guanidino-6-azaindole (GAI) (Scheme 2). We synthesized aminoazaindole **1** from 6-azaindole via a Chichibabin reaction[51−53] in 66% yield, with exclusive selectivity for amination at the 7-position. We attempted to guanidinylate the amino group of aminoazaindole **1** with a commonly used reagent, *N,N′*-di-Boc-methylisothiourea. Instead of guanidinylation, we observed that the Boc protecting group migrated to the amino group in the major product (Figure S1). To avoid this unexpected byproduct, we converted the amino group into a thiourea. We selected *p*-methoxybenzyl-isothiocyanate (PMB−NCS) instead of the more common carbamate-protected isothiocyanate to effect this transformation, again to avoid migration of the carbamate to the amino group. We were successful in producing thiourea **2**, which we subsequently converted to PMB−guanidine **3** under mild conditions by treatment with ammonium hydroxide and *t*-butyl hydroperoxide.[54] We removed the PMB group by treatment with TFA at 80 °C to produce GAI.

Scheme 2. Synthetic Route to GAI



Before synthesizing GADAC, we used our optimized synthetic route to access 1-guanidino-2-azacarbazole (GAC; Scheme 1), which is a key control compound for our studies. More precisely, access to 2-guanidinopyridine (GP; Scheme 1), which is commercially available, GAC, and GADAC would enable us to assess the contribution of two, three, and four hydrogen bond donors to oxoanion binding (Scheme 1).[7] In addition, we sought to determine the effect of adding a guanidino group to an azacarbazole on its fluorescence.

The synthetic route to GAI was applicable to GAC, and we, thus, synthesized amine **4**, followed by thiourea **5**, PMB−guanidine **6**, and, finally, GAC (Scheme 3). We discovered that the guanidinylation of the azacarbazole core did not eliminate fluorescence—GAC glows violet under a 366 nm handheld UV lamp (Scheme 3). Unexpectedly, GAC is not appreciably soluble in water. In addition, we observed significant signal broadening of the carbon *ipso* to the guanidino group and, to a lesser extent, the other carbons of this ring through ^{13}C NMR spectroscopy in DMSO-d_6. GAI also showed broadening of the peak corresponding to the *ipso* carbon in DMSO-d_6 but did not show broadening in D_2O. Whereas the broadening of NMR peaks can have many causes (e.g., proton exchange), the broadening of these carbon peaks in GAC and GAI is consistent with an intramolecular hydrogen bond occurring between a guanidino hydrogen and the pyridine nitrogen in DMSO. Such a hydrogen bond would restrict the conformational rotation of the guanidino group, but its strength would be diminished in a protic solvent such as water. An intramolecular hydrogen bond has been observed previously in a similar scaffold, 2-guanidinopyridine, through ^{1}H NMR spectroscopy and X-ray crystallography.[40]

After optimizing the guanidinylation route with GAI and GAC, we sought to synthesize GADAC. To accomplish this goal, we first needed to synthesize 2,7-diazacarbazole **8** (Scheme 4). We attempted a Pictet−Spengler-inspired route using 6-azaindole. We successfully produced 6-azatryptamine (**S4**), but the electronics of the ring system were not conducive to closing the third ring with formaldehyde (Scheme S1). We then explored the use of organometallic couplings to form 3-amino-3′-chlorobipyridine **7**, which could be cyclized to 2,7-diazacarbazole **8** (Scheme 4). We reacted *N*-Boc-3-amino-4-bromopyridine and 3-chloro-4-pyridineboronic acid using a range of palladium catalysts (Table S1). We expected this reaction to be of moderate yield because of the difficulty in coupling two heterocycles and because (halopyridyl)boronic acids are susceptible to protodeboronation and other side reactions.[55] Despite these challenges, we were able to obtain bipyridine **7** in 33% yield by using Pd(dppf)Cl_2 as a catalyst. Further, we recovered up to 70% of the unreacted bromopyridine, allowing us to re-expose the bromopyridine to the reaction conditions to build a stock of bipyridine **7**.

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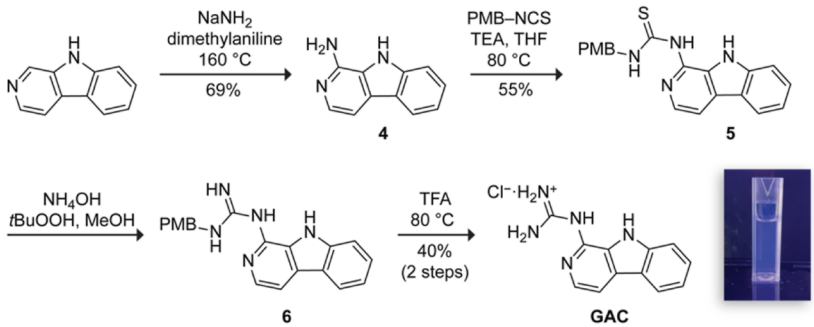
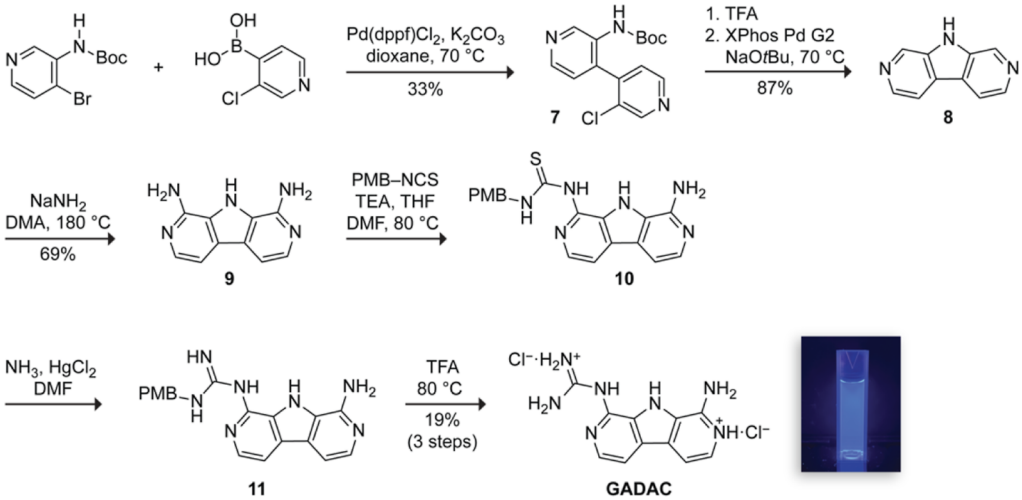
Scheme 3. Synthetic Route to GAC and Photograph of GAC (300 μM in Ethanol) under a Handheld 366 nm UV Lamp



Scheme 4. Synthetic Route to GADAC and Photograph of GADAC (5 μM in Water) under a Handheld 366 nm UV Lamp



The Boc group on bipyridine **7** was subsequently removed by treatment with TFA, and the product was exposed to XPhos Pd G2 and sodium *tert*-butoxide to produce 2,7-diazacarbazole **8**. A Chichibabin reaction[51−53] was then performed on diazacarbazole **8**, resulting exclusively in the 1,8-diamino isomer **9**. Subsequently, diamine **9** was treated with PMB−NCS to generate thiourea **10**. Interestingly, even with a 10-fold excess of PMB−NCS, the reaction added only one thiourea moiety. This reaction must also be monitored carefully to avoid product degradation upon prolonged reaction times. We attempted the previously discussed peroxide-mediated guanidinylation with thiourea **10**, but this reaction was unsuccessful. Instead, we turned to a mercury(II)-promoted reaction to obtain PMB−guanidine **11**. Due to the fragility of thiourea **10** and insolubility of PMB−guanidine **11**, these compounds were used in subsequent reactions without purification. The PMB group was removed by treatment with TFA at 80 °C to produce GADAC, which (like GAC) is fluorescent under a 366 nm handheld UV lamp (Scheme 4).

Fluorescence Characterization. We obtained the excitation and emission spectra of GAC and GADAC in both methanol and water and were gratified to observe that both compounds retained the fluorescence of the azacarbazole core (Figures S2−S4). We observed that compounds **4**, **8**, and **9** were also fluorescent (Figures S5 and S6). The aminopyridine moiety induces a redshift in GADAC compared to β-carboline and GAC (Figure 1). This redshift is beneficial for biological imaging, as low wavelengths of light can damage biomolecules.

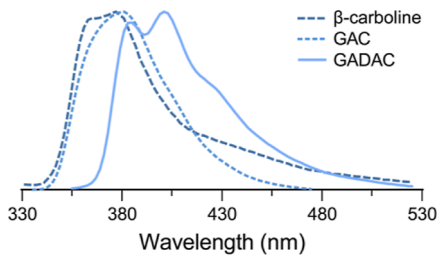


Figure 1. Normalized fluorescence emission spectra of β-carboline (λ_{ex} = 315 nm, λ_{em} = 375 nm), GAC (λ_{ex} = 315 nm, λ_{em} = 380 nm), and GADAC (λ_{ex} = 340 nm, λ_{em} = 400 nm) in methanol.

Given the number of basic nitrogens in GAC and GADAC, we were interested in studying their fluorescence properties in different protonation states. We monitored the fluorescence of these compounds in water at a range of pH values and observed that the fluorescence intensity did indeed correlate with the protonation state.

For GAC, we observed that the fluorescence emission maximum was 390 nm at neutral pH and 466 nm at low pH, resulting presumably from protonation of the pyridino nitrogen, as was observed previously with β-carboline.[56] From this transition, we determined the pK_{a1} of GAC to be 2.57 ± 0.03 (Figure S7). The fluorescence intensity of GAC at 390 nm is maintained through mid-range pH values but decreases by six-fold at pH 11.5 in comparison to pH 4.0 (Figure 2). Previously, a 2-guanidinopyridine was shown to have a pK_a of 9.4.[57] We used ^{1}H NMR spectroscopy to determine that the guanidinium group of GAI has a pK_a value

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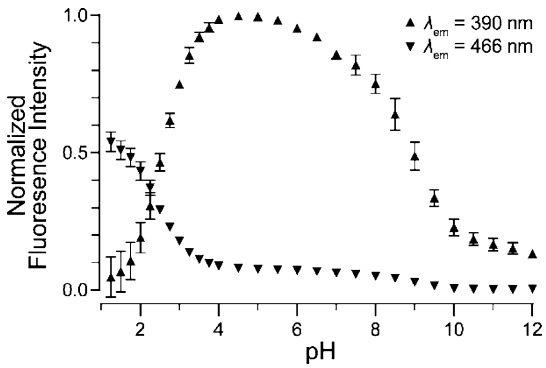

Figure 2. pH-Dependence of the normalized fluorescence emission intensity (λ_{ex} = 315 nm) of GAC in water containing NaCl (10 mg/mL).

of 9.43 ± 0.01 (Figure S8). Thus, the decrease in GAC fluorescence at high pH is likely due to the deprotonation of its guanidinium group, which has a pK_{a2} value of 8.96 ± 0.05 (Figure S7). In addition to characterizing the response of the fluorescence of GAC to pH, we also determined the extinction coefficient of GAC in water to be ε = 6400 ± 30 M^{-1} cm^{-1} (Figure S11).

As with GAC, the fluorescence intensity of GADAC at its emission maximum of 403 nm trends downward at low pH (Figure 3A). Yet, unlike β-carboline and GAC, no corresponding increase at a new wavelength was observed, even at pH < 2. The decrease in fluorescence is likely caused by the formation of the trication upon protonation of both pyridine nitrogens and the guanidino group. (The pK_{a1} of GADAC was too low to measure accurately.) Like that of GAC, the fluorescence intensity of GADAC decreases by 30-fold at pH 11.5 compared to pH 4.0. We determined the pK_{a2} of GADAC from these data to be 6.42 ± 0.04 (Table 1; Figure S9). The pK_{a2} of GADAC likely arises from the deprotonation of the pyridino nitrogen, as it is similar to the pK_a = 6.86 of 2-aminopyridinium itself.[21] Interestingly, no change in fluorescence was observed at a higher pH that would lead to guanidinium deprotonation.

To determine if this change in fluorescence intensity was due to an increase in the number of photons being emitted or absorbed, we determined the absorbance of GADAC across the pH range (Figure 3B). We found that whereas the absorbance is affected by pH, the change is minimal and not correlated to changes in fluorescence intensity, given that the

Table 1. pK_a Values and Photophysical Properties of GADAC

	pK_a		pH 4.0[c]	pH 7.4[d]
pK_{a1}	Not determined	λ_{abs}	356	356
pK_{a2}	6.42 ± 0.04[a]	λ_{em}	403	428
	6.20 ± 0.10[b]			
pK_{a3}	8.92 ± 0.07[b]	ε	13,400 ± 20	12,200 ± 40
		Φ	0.526 ± 0.015	0.119 ± 0.004

[a]Measured with fluorescence spectroscopy. [b]Measured with absorbance spectroscopy. [c]In 10 mM sodium citrate buffer containing NaCl (150 mM). [d]In 10 mM sodium phosphate buffer containing NaCl (150 mM). ε, M^{-1} cm^{-1}.

absorbance increases back to baseline values at high pH. Since two inflection points are observable in the pH-dependent absorbance data, we calculated the pK_{a2} and pK_{a3} values of GADAC (Figure S10). We found pK_{a2} to be 6.18 ± 0.10, which is congruent with the value obtained from the pH-dependent fluorescence data (Table 1); we found pK_{a3} to be 8.92 ± 0.07, which is nearly identical to the pK_a of the guanidino group in GAC.

To quantify further the relationship between absorbance and emission at different protonation states, we determined the extinction coefficient (Figure S12) and quantum yield of GADAC at pH 4.0 and pH 7.4. Here, pH 4.0 ensures nearly full aminopyridinium protonation, while pH 7.4 is relevant for applications in biological contexts. We found that ε = 12,200 M^{-1} cm^{-1} and Φ = 0.12 at pH 7.4 (Table 1). These values differ at pH 4.0, where ε = 13,400 M^{-1} cm^{-1} and Φ is 0.53. Although the extinction coefficient at pH 4.0 is slightly higher than that at pH 7.4, it is not markedly so, meaning that the increase in fluorescence seen at low pH is due to an increase in quantum yield. The dramatic increase in the quantum yield of GADAC occurs upon protonation of the aminopyridine moiety, as the positive charge on the pyridino nitrogen increases electron-donation into the ring system by the amino group. Any additional functionality that would block aminopyridine protonation, including a guanidino group on the second amino moiety, would likely diminish fluorescence. Overall, the photophysical properties of dicationic GADAC are comparable to those of DAPI, EDANS, coumarin 151, and calcein blue,[58,59] suggesting utility in a biological context.

Oxoanion Affinity. We determined the binding affinity of GADAC to three commercially available oxoanions as proxies for biological oxoanions, such as the phosphoryl groups of

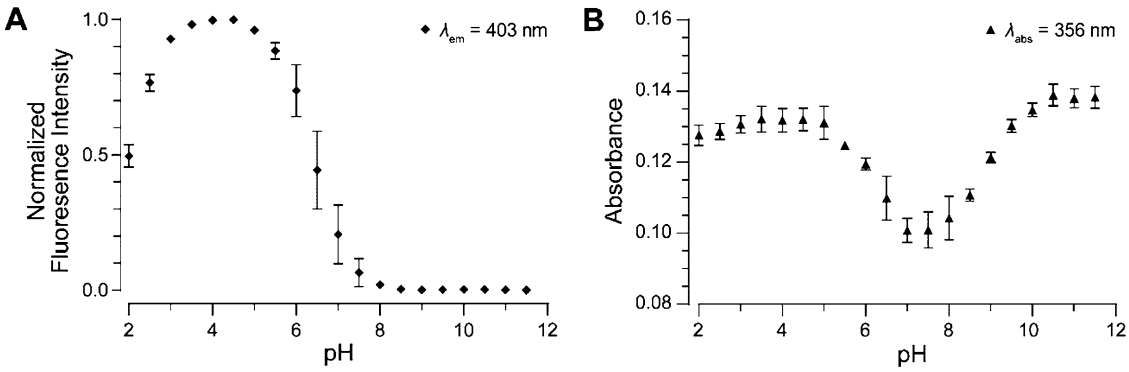

Figure 3. pH-Dependence of the photophysical properties of GADAC in water containing NaCl (10 mg/mL). (A) Normalized fluorescence emission intensity (λ_{ex} = 340 nm). (B) Absorbance (λ_{abs} = 356 nm).

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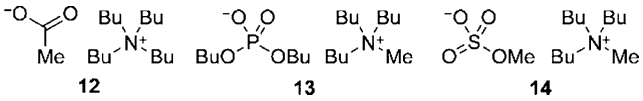
phospholipids and the carboxyl or sulfuryl groups of proteoglycans. We used acetate **12**, phosphate **13**, and sulfate **14** for these binding studies (Scheme 5). We used GP as a model guanidinium with no additional hydrogen-bond donors. We hypothesized that the binding affinity for an oxoanion would increase in the order: GP < GAC < GADAC. Obtaining the binding affinity of GAC and comparing it to the binding of GP informs the extent to which the carbazole NH contributes to the binding of anions. We expected GADAC to have the highest affinity due to its additional hydrogen bond-donating amino group (Scheme 1).

Scheme 5. Oxoanions Used in Binding Titrations



To perform the binding affinity titrations, we introduced sequential amounts of stock solutions of an oxoanion to solutions of GP, GAC, and GADAC and measured their fluorescence spectra in protic solvents. Both GP and GAC were chloride salts, whereas GADAC was a dication and dichloride salt. We observed measurable changes in fluorescence correlated to oxoanion concentration and determined binding affinities from these changes with supramolecular.org software (Figures 4 and S13).[60−62] The fluorescence emission spectra of GAC shows two maxima in an organic solvent. This property is also seen in β-carboline in the presence of acetic acid.[63]

We first explored the binding capabilities of GP, GAC, and GADAC against acetate **12** (Table 2, Figure S13). The fluorescence signal of GP showed no change when titrating against acetate **12** in isopropanol, indicating no binding. In contrast, GAC displayed a high binding affinity of $(5.3 \pm 0.3) \times 10^4$. Thus, the additional carbazole hydrogen bond donor contributes significantly to the binding of oxoanions with GAC. We attempted to assess the binding of GADAC to acetate **12** in isopropanol but observed higher-order binding that impeded our efforts. Hence, we switched to a more competitive and biologically relevant solvent, water. In water, GAC showed no change in fluorescence intensity while GADAC displayed a high binding affinity of $(3.6 \pm 0.1) \times 10^4$ (Figure 4). Thus, the amino hydrogen bond donor also greatly contributes to the ability of GADAC to bind oxoanions.

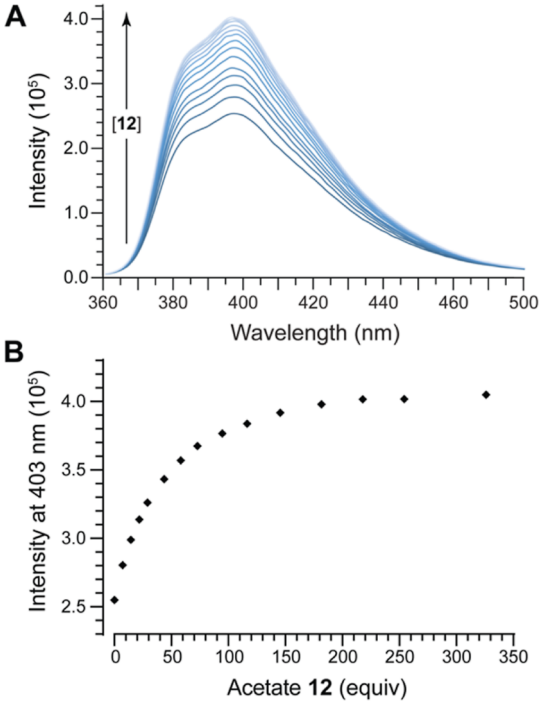


Figure 4. Representative fluorescence spectra for the titrations of GADAC with acetate **12** in water. Spectra for all binding titrations and links to open-source BindFit data are provided in Figure S13. (A) Fluorescence emission spectra of GADAC titrated with acetate **12** (λ_{ex} = 340 nm). (B) Change in intensity of GADAC at 403 nm, unfitted.

Table 2. Binding Affinities (M^{-1}) of GP, GAC, and GADAC to Acetate 12

solvent	compound	affinity
isopropanol	GP	no binding
	GAC	$(5.3 \pm 0.3) \times 10^4$
water	GAC	no binding
	GADAC	$(3.6 \pm 0.1) \times 10^4$

We also investigated the ability of GAC and GADAC to bind to phosphate **13** and sulfate **14** (Figure S13). Before interpreting data from binding experiments with sulfate **14**, we insured that its binding to GADAC is reversible in water, that is, the alkylation and sulfation reactions that have been observed with alkyl sulfates at elevated temperatures[64] are not operative here (Scheme S2; Figure S14). Titration data for GAC against all three anions in isopropanol and methanol can be found in the Supporting Information (Table S2). The binding affinities of GADAC to phosphate **13** and sulfate **14** in water were found to be $(1.1 \pm 0.0) \times 10^3$ and $(4.2 \pm 0.3) \times 10^3$, respectively. These, affinities, though high, are less than that of GADAC to acetate. This decrease is consistent with acetic acid having a higher pK_a that matches more closely the pK_a values of GADAC (Table 1).[18−20] The high affinity of GADAC for all three oxoanions in water is promising for biological applications and provides GADAC with a distinct advantage compared to previously reported guanidine-based oxoanion binders that have relatively low affinity for oxoanions in aqueous/organic mixtures.

Epifluorescence Microscopy of GADAC in Live Human Cells. After finding that GADAC is fluorescent and binds strongly to oxoanions in water, we tested its cell permeability with epifluorescence microscopy. CPPs and Selwood's and Schmuck's guanidinium-containing small molecules penetrate cells when labeled with a fluorophore.[15,37,64,65] We expected GADAC to be similarly capable of cell penetration, given its affinity for oxoanions like those on the cell surface, positive charge, and relative lipophilicity (Figure S15). Further, we anticipated that GADAC could be detected by the commonly used DAPI filter without the need for a pendant fluorophore. To test these hypotheses, we incubated M21 melanoma cells with a range GADAC concentrations (0−14 μM) in serum-supplemented medium for 1 h at 37 °C and then imaged the treated cells. Gratifyingly, we observed that GADAC was able to penetrate the cells and maintain its fluorescence at concentrations as low as 1 μM (Figure S16).

Because the fluorescence of GADAC was not distributed evenly within the cells (Figure S16), we asked whether GADAC tends to accumulate in specific organelles. To answer

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this question, we performed co-staining experiments in M21 cells. We observed stronger co-localization of GADAC with dyes selective for the endoplasmic reticulum (Figure S17), mitochondria (Figure S18), and nuclei (Figure S19) compared to a dye selective for acidic organelles such as lysosomes (Figure S20), despite the higher quantum yield of GADAC upon protonation in that acidic environment.

Next, we wondered whether GADAC reaches the cytosol via an energy-independent process such as direct transduction or an energy-dependent process such as endocytosis and subsequent endosomal escape. It has been reported that endocytosis is negligible at temperatures below 10 °C.[66] As seen in Figure 5, the uptake of GADAC into M21 cells at 4 and 37 °C is similar, which provides further evidence that GADAC crosses the plasma membrane directly in our experimental conditions. Thus, GADAC can penetrate cells and remain fluorescent within the cytosol and various organelles.

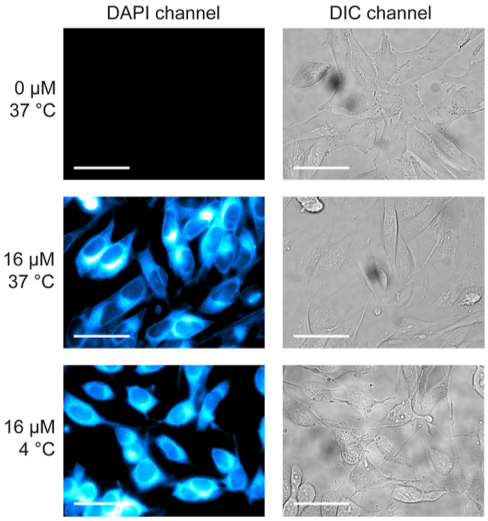

Figure 5. Images of the uptake of GADAC into live M21 cells after a 3 h incubation at 37 or 4 °C in serum-supplemented medium and washing. DAPI channel (left) with λ_{ex} = 357/44 nm and λ_{em} = 447/60 nm; DIC channel (right). The DAPI channel epifluorescent images are normalized with each other (that is, acquired at the same light intensity, exposure, and gain parameters). Scale bars, 50 μm.

CONCLUSIONS

We have designed and synthesized the diazacarbazole GADAC and demonstrated its affinity for oxoanions in water, on the order of 10^4 M^{-1} for a carboxylate and 10^3 M^{-1} for an organic phosphate and sulfate. GADAC displayed tighter binding to oxoanions in pure water than did previously reported guanidinium-containing structures in aqueous/organic mixtures,[15,67] and is further rarefied in binding to oxoanions in pure water with only a single guanidino group. Moreover, its carbazole core imbues GADAC with intrinsic fluorescence. GADAC can penetrate mammalian cells via a mechanism that involves direct transduction and enables imaging in the absence of additional fluorophores. Future efforts will seek to exploit these attributes in the transport of cargo into human cells.

ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.3c00982.

Experimental procedures and compound characterization data (PDF)

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