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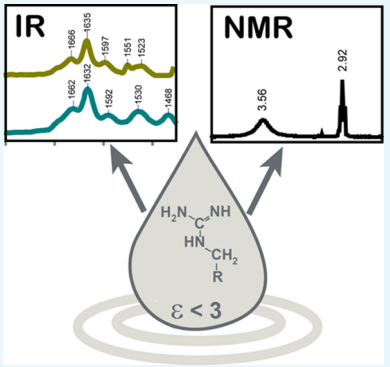
Anhydrous Monoalkylguanidines in Aprotic and Nonpolar Solvents: Models for Deprotonated Arginine Side Chains in Membrane Environments

Andrew Toyi Banyikwa,[†] Stephen E. Miller,[‡] Richard A. Krebs, Yuewu Xiao,[§] Jeffrey M. Carney,[∥] and Mark S. Braiman*

Chemistry Department, Center for Science & Technology, Syracuse University, Room 1-014, Syracuse, New York 13244-4100, United States

Supporting Information

ABSTRACT: In this study, the synthesis of crystalline dodecylguanidine free base and its spectroscopic characterization in nonpolar environments are described. IR as well as ^{1}H and ^{15}N NMR spectra of the free base dissolved in aprotic solvents are substantially different from the previously reported spectra of arginine, or other monoalkylguanidinium compounds, at high hydroxide concentrations. The current results provide improved modeling for the spectroscopic signals that would be expected from a deprotonated arginine in a nonpolar environment. On the basis of our spectra of the authentic dodecylguanidine free base, addition of large amounts of aqueous hydroxide to arginine or other monoalklyguanidinium salts does not deprotonate them. Instead, hydroxide addition leads to the formation of a guanidinium hydroxide complex, with a dissociation constant near ~500 mM that accounts for the established arginine p*K* value of ~13.7. We also report a method for synthesizing a compound containing both phenol and free-base guanidine groups, linked by a dodecyl chain that should be generalizable to other hydrocarbon linkers. Such alkyl-guanidine and phenolyl-alkyl-guanidine compounds can serve as small-molecule models for the conserved arginine−tyrosine groupings that have been observed in crystallographic structures of both microbial rhodopsins and G-protein-coupled receptors.



INTRODUCTION

Recent structural and physiological studies have provided evidence for a number of arginine side chains of integral membrane proteins situated in nonpolar environments.[1] The exact H-bonding environments, energetics, and even protonation states of all of these membrane-buried isolated arginines and arginine−tyrosine groupings remain somewhat ill-defined. Three examples are as follows.

(1) In KvAP potassium channels, voltage gating carries four cationic arginine side chains in the voltage-sensor paddles through the nonpolar region of the lipid bilayer.[2,3] (2) The conformational change involved in the activation of G-protein-coupled receptors repositions a conserved arginine in transmembrane helix III, from its initial location in a salt bridge with a neighboring aspartate (or glutamate) to the nonpolar interior of the protein, where it forms a H-bonded interaction with a conserved tyrosine from transmembrane helix V.[4,5] Prior to G-protein binding, this conserved arginine sits in a very nonpolar environment and has no obvious counteranion.

(3) In microbial rhodopsins (e.g., bacteriorhodopsin, bR), time-resolved Fourier transform infrared spectra, including ^{15}N-labeled arginines, may have provided evidence for a transient deprotonation of the guanidine group of a conserved arginine in helix III, specifically in the M and/or N intermediates.[6−8] Solid-state ^{15}N NMR spectra of M definitely also show a strong perturbation of this arginine,[9] but were interpreted as showing evidence for a new unusual H-bonding environment for guanidinium, rather than deprotonation. X-ray crystal structures of M also show that the guanidino group of this arginine transiently moves much closer to the phenolic oxygen of an adjacent conserved tyrosine.[10]

The protonation and H-bonding states of arginines in such proteins are not well understood, in part due to the difficulty in obtaining unambiguous spectroscopic signals that can differentiate protonated and unprotonated states of the arginine side chain, particularly in completely aprotic environments, or in nonpolar environments with very low concentrations of H-bonding partners. From computations, it has been concluded that even at the center of a lipid bilayer, the guanidinium group of arginine is likely to remain protonated.[11−13] However, one challenge facing computational methods is that a necessary experimental control is missing: a model system, for which the guanidine group can be proved experimentally to deprotonate and computational methods correctly predict the deprotonation.

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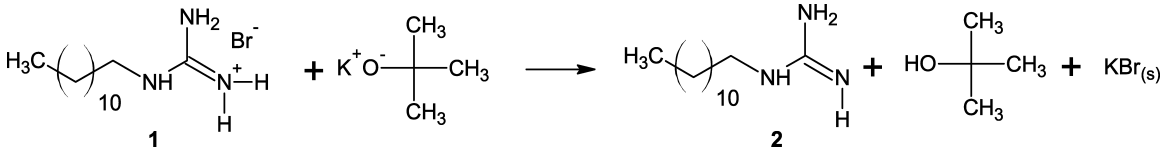
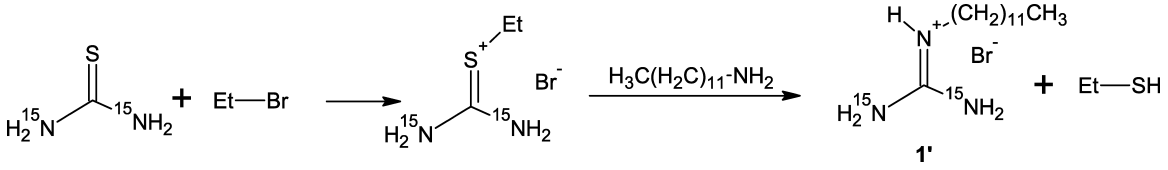
Scheme 1. General Method for Deprotonation of an Alkylguanidinium Bromide (e.g., 1) in Methanol Solution To Form the Corresponding Alkylguanidine Free Base (e.g., 2)

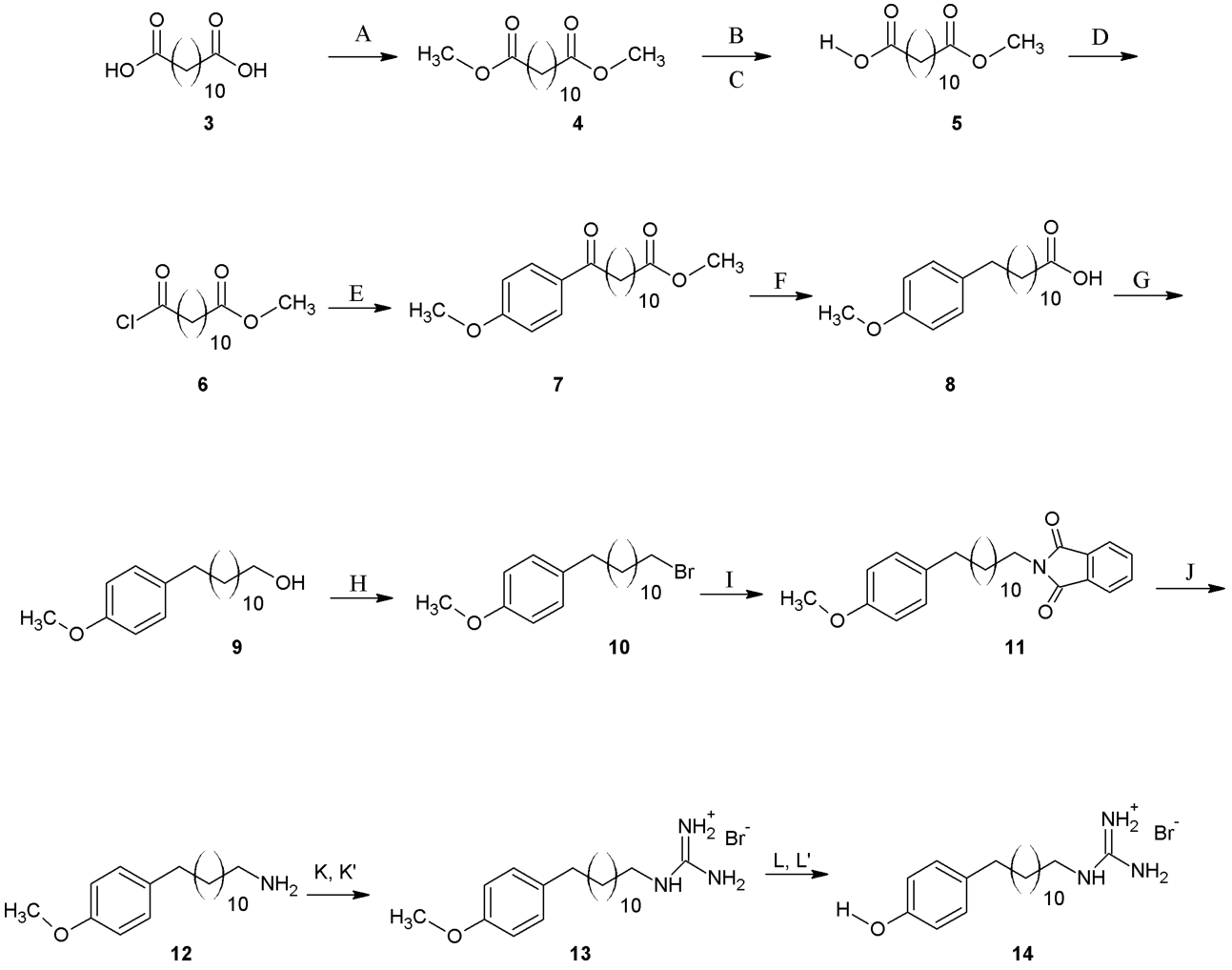


Scheme 2. General Method for Incorporation of Isotope Labels from the Commercially Available Labeled Thiourea into the Terminal Nitrogens of Monoalkylguanidines[a]



[a]The product with $R_1 = CH_3(CH_2)_{11}-$ is compound **1′**.

Scheme 3. Overall Synthesis Scheme for (*p*-Phenolyl) Dodecylguanidinium Bromide (14) and for the Corresponding $^{15}N_2$-Labeled Compound (14′)[a]



[a]Not shown here is the deprotonation step needed to form the corresponding free-base compounds **15** and **15′**. This was carried out as in Scheme 1. Reagents and conditions: (A) reflux, 8 h, H_2SO_4 (cat), methanol; (B) $Ba(OH)_2$ (1/2 equiv), 24 h, methanol; (C) 2 N HCl, ether; (D) $SOCl_2$, dichloromethane (DCM); (E) $AlCl_3$, anisole, CH_2Cl_2, DCM; (F) H_2NNH_2, *t*BuOK, 48 h, dimethyl sulfoxide (DMSO); (G) $LiAlH_4$, 5 h, THF; (H) PhP_3, CBr_4, 12 h, DCM; (I) potassium phthalimide, reflux, 2 h, dimethylformamide; (J) H_2NNH_2, reflux, 12 h, absolute ethanol; (K) *S*-methyl thiourea hydroiodide, 2 h, absolute ethanol; (K′) *S*-ethyl thiourea-$^{15}N_2$ hydrobromide, 2 h, absolute ethanol; (L, L′) 48 % HBr, reflux, 6 h.

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Experimentally, synthetic nonpolar single-helix peptides that readily insert into a lipid bilayer have been modified to include arginine in the middle of the α-helix.[14−16] However, rather than forcing its side chain to deprotonate, the result has been that the single arginine remains protonated by forcing changes in the degree of insertion and/or helix tilt angle.

Here, we describe the synthesis of anhydrous monoalkylguanidine free base, dodecylguanidine, in pure crystalline state using the general approach shown in Scheme 1. The dodecyl substituent confers sufficient solubility in nonpolar solvents to permit unambiguous identification of spectroscopic signals from the deprotonated monoalkylguanidine (**2**). The spectra signal a deprotonation, that is, a big change from the corresponding monoalkylguanidinium, much bigger than that seen in spectra previously obtained during high-pH arginine titrations in aqueous solutions[17−19] or even in previous measurements of guanidine "free bases" in aprotic solvents, where water was not rigorously excluded.[20] This suggests that in the earlier spectra the guanidinium group never deprotonated, but simply formed a tightly bound hydroxide complex that produced minor spectral perturbations.

Additional synthetic methods were developed to incorporate >90% ^{15}N isotope labels from thiourea into the guanidine's two terminal nitrogens, according to Scheme 2. This high level of isotope incorporation has permitted valuable new spectroscopic modeling: not only ^{15}N NMR chemical shift measurements, which can be used as markers for deprotonated guanidines in several different environments, but also clear identification in IR spectra of several guanidine-group vibrations that depend strongly on protonation state and H-bonding environment.

These results in aggregate indicate that in the presence of only nonpolar aprotic solvent molecules along with a small (stoichiometric) amount of water, the arginine side chain is likely to exist predominantly as a stoichiometric guanidinium hydroxide complex. However, the question remains: in such an environment, could a more lipophilic H-bond donor displace water to form a H-bond pair with a deprotonated arginine side chain? The biomolecule with requisite properties that comes to mind is the phenolic side chain of tyrosine.

Again, there is no prior model for such H-bonded neutral guanidine-phenol groupings, isolated away from other H-bonding partners. Previous spectroscopic modeling of tyr−arg side-chain interactions[22] combined synthetic neutral poly-Tyr and poly-Arg polypeptides. However, such polypeptides are insoluble in aprotic solvents. Furthermore, although both amino and carboxy termini were protected, preventing their participation in proton transfers, these model systems retain numerous peptide linkages with H-bond donor and acceptor groups.[22]

Our goal was therefore to synthesize a small molecule with a covalent hydrocarbon linkage between phenol and dodecylguanidine groups, which would permit modeling of a 1:1 H-bonded interaction between them in aprotic solvent, without any possible interference from other acid/base or H-bonding groups. The first small-molecule model compound we prepared that met these criteria was *p*-phenolyl dodecylguanidine, using the synthetic approach shown in Scheme 3.

The ultimate goal of this synthetic approach is to produce small-molecule model compounds that will adopt a conformation with a singular intramolecular phenol−guanidine H-bond in aprotic environments. Such model compounds should allow the H-bonding interactions of arginine and tyrosine to be modeled outside of proteins, by dissolving the model compounds in aprotic solvents, micelles, and artificial membranes.

IR, ^{1}H NMR, and solid-state ^{15}N NMR spectra of the newly synthesized (*p*-phenolyl) dodecylguanidinium bromide (**14**, **14′**) and the corresponding free bases (**15**, **15′**) as crystalline solids are presented below. These spectra demonstrate that in the solid state of the "free base," proton transfer is essentially complete from phenolate to guanidinium, to form zwitterions.

Our hypothesis that a different nonzwitterionic structure for **15** may prevail in membrane-like solvent environments is explored in the accompanying paper.[23] There, it is determined spectroscopically that very strong stoichiometric H-bonding of **15** occurs in aprotic solvents. Specifically, head-to-tail H-bonded homodimer complexes of **15** readily form in DMSO, with partial proton transfer from guanidine back to phenol. Similar H-bonded heterodimer complexes of dodecylguanidine (**2**) with *p*-cresol are even soluble to ∼1 mM in hexane.[23] The latter complexes exist nearly 50:50 as two main protomers in rapid equilibrium: not only dodecylguanidinium/cresolate, but also fully neutral cresol/dodecylguanidine.

Thus, monoalkylated guanidine molecules, such as **1**, complexed in aprotic solvents with phenols, either without or with a covalent linkage as in **15**, may serve as small-molecule model compounds for tyr−arg groupings that might hypothetically be buried in an overall neutral form into nonpolar environments.

RESULTS AND DISCUSSION

Spectroscopy of Small-Molecule Models for the Deprotonated Arginine Side Chain. In Figures 1−4, we present IR, ^{1}H NMR, and ^{15}N cross-polarized magic-angle spinning (CP-MAS) data measured from dodecylguanidine free base **2**. Included also are comparison to the protonated version of the same molecule (dodecylguanidine hydrobromide, **1**) as well as to the corresponding phenolyl-attached free base (**15**) and hydrobromide (**14**). The dodecylguanidine free-base spectra are all markedly different from previous spectra presented as arising from the deprotonated, or partially deprotonated, monoalkylguanidine side chain of arginine.[7,9,17−20]

IR Spectra. Figure 1 presents IR spectra of monoalkylguanidines. Upon dissolving the pure crystalline free base of dodecylguanidine (**2**) in a variety of nonpolar solvents (Figure 1A−E), the spectra show strong bands near 1670, 1645, and 1605 cm^{-1}. These three bands are downshifted by 5−10 cm^{-1} upon isotope labeling of the two terminal guanidine nitrogens (see spectra G−K in Figure S-3 in Supporting Information). These isotope-sensitive bands are therefore assigned as guanidine-group vibrations with predominantly C−N stretch character.

The bandshapes in Figure 1 are significantly different from those published previously as deprotonated monoalkylguanidine solution spectra.[6,20] In particular, a moderately strong ^{15}N isotope-sensitive band at 1555−1560 cm^{-1} in earlier work[6,20] is not reproduced in any of the dodecylguanidine spectra measured in water-free nonpolar solvents (Figure 1A−D). A very clear 1556 cm^{-1} band was previously observed when aqueous monoalkylguanidinium chloride was reacted with sodium hydroxide and then extracted into chloroform.[6,20] A still weaker version of this band at a slightly higher frequency (near 1570 cm^{-1}) has also been previously observed in an aqueous solution of arginine at elevated pH.[17] However, in the spectra of Figure 1A−D, the bands in the frequency range

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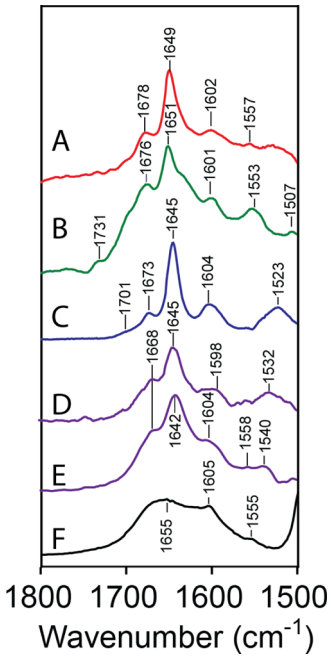

Figure 1. IR spectra of dodecylguanidine free base (**2**). Most spectra were measured as saturated solutions in dry nonpolar solvents: benzene (A); CCl_4 (B); CH_2Cl_2 (C); $CHCl_3$ (D). An additional solution spectrum was measured in water-saturated $CHCl_3$ (E). A sixth spectrum (F), provided for comparison, represents crystalline (*p*-phenolyl)-dodecyl-guanidine free base (**15**) measured as a KBr pellet. Vertical scales were matched to facilitate comparisons. (More extensive ranges of these spectra from 1800 to 1100 cm^{-1}, and also including the corresponding ^{15}N isotope-labeled compounds **2′** and **15′**, are provided in Figures S-3–S-12 in Supporting Information.)

1560–1530 cm^{-1} show little sensitivity to ^{15}N labeling of the two terminal nitrogens (see spectra G–J in Figure S-3 in Supporting Information). This is true even for the measurement in pure chloroform (Figure 1D).

The samples in Figure 1A–D represent the anhydrous crystalline dodecylguanidine free base dissolved in anhydrous aprotic solvents. The spectra previously published as deprotonated monoalkylguanidine in chloroform[6,20] did not use crystalline free base and did not utilize a rigorous procedure for excluding water. The chloroform/water extraction method used in producing the earlier samples suggests the likely presence of a water/hydroxide that is H-bonded to the guanidine/guanidinium, upshifting the 1530 cm^{-1} vibration (Figure 1D) and giving rise to the ^{15}N isotope-sensitive band at 1555–1560 cm^{-1}. A previous vibrational analysis of guanidine free base itself[24] indicates that vibrations in the 1500–1600 cm^{-1} range are likely due to C–N–H bends, which are both capable of mixing with the C–N stretches at higher frequencies and susceptible to frequency shifts due to H-bonding.

The conclusion that earlier attempts at measuring IR spectra of deprotonated alkylguanidine always included bound water and/or hydroxide is also supported by our current ^1H and ^{15}N NMR results (see below). It is further supported by the changes in the IR spectrum when small amounts of water are added to the chloroform solvent, as shown in Figure 1E. The presence of saturated H_2O concentration in the solvent used to dissolve the dodecylguandine is sufficient to upshift the 1530 cm^{-1} band to ~1540 cm^{-1} (Figure 1E). This broad 1540 cm^{-1} band then becomes sensitive to ^{15}N labeling of the two terminal nitrogens (shifting to 1523 cm^{-1} in spectrum K in Figure S-3 in Supporting Information). The 1540 cm^{-1} frequency observed in Figure 1E is not upshifted quite as far as the ~1555 cm^{-1} frequency seen previously when monoalkylguanidinium salts in water were treated with NaOH and then extracted with chloroform.[6,20] However, the presence of the dodecylguanidine during the earlier extractions[6,20] undoubtedly led to higher water content in the chloroform phase, as compared to the method used to prepare the sample for Figure 1E, which was to preequilibrate pure chloroform and water and then use the chloroform phase to dissolve crystalline anhydrous dodecylguanidine.

The ~1555 cm^{-1} band in earlier work was assigned to a mixed vibration, involving a significant component of stretching of the two terminal C–N bonds, as evidenced by its sensitivity to ^{15}N isotope labeling.[6,20] These and other publications also documented that the corresponding protonated alkylguanidinium halides never show absorption bands in the 1500–1600 cm^{-1} region when measured in solutions, whether the solvent was water, methanol, or $CHCl_3$.[21] We have confirmed this result (see also Supporting Information).

It is evident from the results in Figure 1 that the guanidine vibrations are strongly dependent on protonation state and H-bonding environment. This strong sensitivity to protonation state and H-bonding, along with the fast temporal resolution of IR spectroscopy, would seem to make IR the spectroscopic technique of choice for analyzing the environment around deprotonated guanidine. This is especially true when the guanidine group of arginine is present in a fast proton-transfer equilibrium with H-bonded partner(s), and protonated and deprotonated forms of the guanidine may be rapidly interconverting on the subnanosecond time scale.[22]

There are several additional caveats that must be placed on this conclusion. In particular, the results obtained for isolated molecules in solution do not apply in crystalline solid samples. That is, both protonated and unprotonated dodecylguanidine (**1**, **1′** and **2**, **2′**) show clear bands in the 1600–1550 cm^{-1} region, when they are measured in crystalline form as KBr pellets (see Figure S-1 in Supporting Information). The origin of these bands is unclear because they show no sensitivity to ^{15}N labeling of the two terminal nitrogens and they are not seen when these compounds are dissolved in a solvent. We conclude on the basis of this insensitivity that they may be entirely unrelated to any kind of group vibration that would occur for isolated arginine side chains in proteins. However, until the origin of these bands is fully explained by a complete vibrational assignment, including treatment of phonons in the crystal, there is a small risk of ambiguity in the interpretation of arginine bands that may be observed in this spectral region.

^1H NMR Spectra. Figure 2A,B shows ^1H NMR spectra of pure crystalline dodecylguanidine redissolved in benzene-d_6 or DMSO-d_6. Here, we will focus on the features that have not previously been observed—in particular, the broad resonance of the NH protons, at δ = 3.56 ppm (benzene) or δ = 4.72 ppm (DMSO). The integrated area of this peak in either Figure 2A or B is double that of the resonance at ~2.9 ppm, which itself corresponds to the two α-methylene protons, that is, on the carbon immediately adjacent to the guanidine group.

Therefore, the resonances at δ = 3.56 and 4.72 ppm in Figure 2A,B, respectively, correspond to the four guanidine-group NH protons, all of which are chemically equivalent on the NMR time scale. This equivalence is likely due to moderately rapid exchange between these four protons. The chemical shift value of this peak is quite variable in different solvents, as well as at

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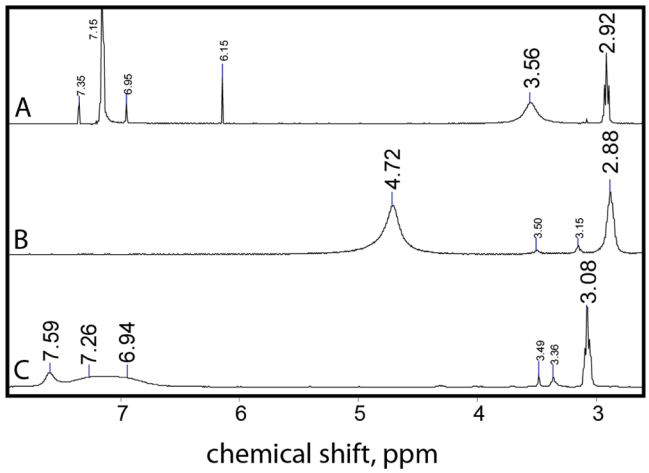

Figure 2. ^{1}H NMR spectra of dodecylguanidine free base (**2**) at a concentration of 4 mM in benzene-d_6 (A) and 80 mM in DMSO-d_6 (B). Dodecylguanidinium bromide (**1**) was measured at a concentration of 100 mM in DMSO-d_6 only (C), due to insufficient solubility in benzene-d_6. All samples were at 25 °C. This spectral region was selected because no resonances were observed further downfield, and upfield resonances included only those from the alkyl chain, DMSO solvent, and tetramethylsilane (TMS) standard. In the original data (not shown), the signal for the $-CH_2-$ group next to the guanidine (3.08−2.88 ppm) was a well-resolved triplet with J = 7.0 Hz splitting and 3−4 Hz line width. The spectra were replotted here after various degrees of Fourier smoothing, to simplify visualization of this α-methylene resonance and the broader one from the four to five N−H protons (7.59−3.56 ppm, 100−300 Hz line widths), as well as smaller peaks near 3.5 ppm, on the same vertical scale. The resonance at 7.15 ppm in A, due to residual protons on benzene-d_6, still had to be truncated at ∼10% of its full height to allow the solute resonances to be visualized. Other signals indicated with smaller fonts represent additional solvent contaminants. See text for details.

different concentrations in the same solvent, particularly in DMSO which invariably contains a small amount of water contaminant. The guanidino chemical shift decreases from 4.72 at the highest measured concentration (80 mM) and most water-free solvent (from a freshly broken ampoule) monotonically down to near ∼3.5 in very wet solvent. The bandwidth is 100 Hz in the driest DMSO (Figure 2B) and decreases as water is added or as the concentration of solute is decreased. However, even at the highest concentration and in the driest commercially available DMSO-d_6, the guanidine protons resonate as a single band, with a width as large as that (∼100 Hz full width at half-maximum) also seen in benzene-d_6 (Figure 2A). This could be a sign that a significant rate of exchange, responsible for the broadening and coalescing of these four proton resonances, can occur exclusively due to internal processes of the guanidine group. Rapid tautomerization, through internal proton transfers between neighboring imino (=NH) and amino ($-NH_2$) groups, along with rapid C−NH_2 bond rotations, might account for this exchange among these four protons.

Alternatively, the dodecylguanidine free base could conceivably form head-to-head dimers in aprotic solvents, analogous to those obtained with carboxylic acids. Such symmetric H-bonded dimers are expected to promote rapid concerted proton transfers. Transient formation and dissociation of such dimers, even at the lowest concentration that we measured in benzene-d_6 (4 mM), might also help to account for the chemical equivalence of all four guanidine protons on the nmr time scale. Measuring even lower concentrations has so far been impossible because of H/D exchange with the solvent (discussed five paragraphs below).

In contrast, the spectrum of dodecylguanidinium bromide (Figure 2C) shows three distinct, but broad, resonances for the five guanidinium-group protons, centered at δ = 7.6, 7.2, and 6.9. These have area ratios of 1:2:2 relative to the α-methylene peak at δ = 3.08 (based on fitting to Lorentzian bandshapes). The slower exchange of the guanidinium protons is possibly related to the absence of any nitrogen with a lone pair of electrons that can serve as a proton acceptor and to the very weak basicity of bromide. The absence of a good proton acceptor group in dodecylguanidinium bromide is expected to weaken its H-bonded associations, in particular those that could induce head-to-head dimerization in aprotic solvents. The lack of a proton acceptor would likewise be expected to slow either dimer-mediated or internal proton-exchange process, relative to the guanidine free base. In the free base, the imino group is expected to be an excellent proton acceptor from the other N−H groups due to the closely matched pK_a values. An additional factor that could slow internal exchange among the five guanidinium protons is the absence of C−N single bonds. The partial-double-bond character of all three C−N bonds is expected to hinder bond rotation, relative to the lower bond order of C−NHR in the dodecylguanidine free base.

The spectrum in Figure 2C is somewhat similar to the corresponding spectra of both dodecylguanidinium chloride and dodecylguanidine, measured in chloroform-d, previously published by Xiao and Braiman.[20] In the prior work, the guanidinium-group proton resonances were a bit farther upfield, probably due to the less-polar solvent (chloroform-d), as well as sharper, likely resulting from the lower solvent viscosity. Nevertheless, the chemical shift values of the α- and β-methylene groups were similar at 3.15 and 1.61 ppm compared to the current values for the dodecylguanidinium bromide salt at 3.08 and 1.43 ppm (the latter falling outside the range of Figure 2).

The previous[20] measurement of the ^{1}H NMR spectrum of what was presented as dodecylguanidine in chloroform-d is now clearly problematic, in light of the data shown in Figure 2. In that earlier work,[20] the chemical shifts for the two $-CH_2-$ groups closest to the "deprotonated" guanidino group were nearly unchanged from those of dodecylguanidinium chloride. However, as noted in that work, the earlier sample had more water present in it; the N_2-drying procedure used previously does not remove water from alkylguanidines as thoroughly as crystallization from acetonitrile does. As a result, the earlier spectrum corresponded at least in part to dodecylguanidinium hydroxide. Furthermore, a sharp peak observed at ∼7.3 ppm, assigned in that work[20] to guanidine protons, was instead likely due to unexpectedly large amounts of C^1HCl_3 present in the sample. This was formed by rapid exchange of the dodecylguanidine protons into chloroform-d, with the corresponding replacement of most of the guanidine NH protons by deuterium from the solvent.

We have now directly measured the ^{1}H/^{2}H exchange process between dodecylguanidine free base (**2**) and chloroform-d (Figure 3). In benzene solvent, at concentrations of base and chloroform of 0.010 and 0.50 M, respectively, this process occurs with a pseudo-first-order decay time of ∼3.4 min. The estimated second-order rate constant for the rate-limiting step (which is expected to be deuterium transfer from C^2HCl_3 to dodecylguanidine) is therefore ∼0.01 M^{-1} s^{-1}. Although this is

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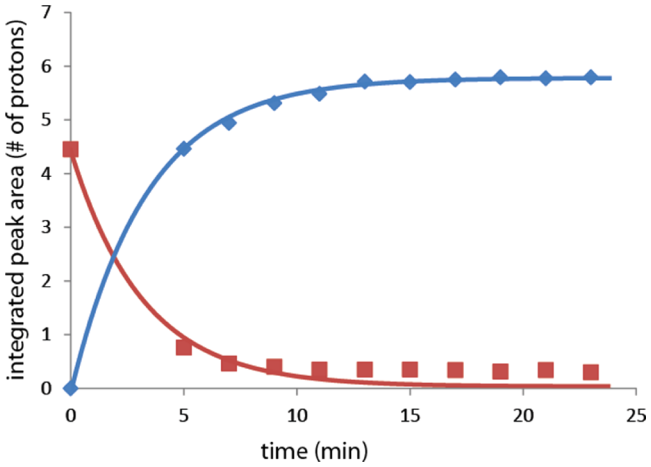

Figure 3. Time dependence of the proton exchange in a sample, initially containing 0.010 M dodecylguanidine free base (**2**) in benzene-d_6. Just after the measurement plotted as t = 0, an amount of chloroform-d was added to give a concentration of 0.50 M; the next measurement (at t = 5 min) was made as quickly as the sample could be inserted and the NMR instrument locked and shimmed. Temperature was 298 K. Integrated areas of the measured C^1HCl_3 peak at 6.1 ppm (blue diamonds) and 3.5 ppm for 100% benzene-d_6 or 3.3 ppm for chloroform-d 0.5 M in benzene-d_6 (red squares) are plotted, along with the best least-squares fits to single-exponential functions, giving $y = 4.4[1 - \exp(-t/3.34\ \text{min})]$ for the blue plot and $y = 5.8 \exp(-t/3.52\ \text{min})$ for the red plot.

quite slow for a proton-transfer rate constant, we conclude that the basicity of dodecylguanidine is sufficiently high to partially deprotonate $CHCl_3$, whose pK_a value is generally given as ∼16.

Our results even provide evidence for slower proton exchange of the dodecylguanidine free base with benzene-d_6. During the time period from 5 to 23 min in the measurement summarized in Figure 3 (raw data in Supporting Information Figure S-2), the benzene proton signal at 7.15 ppm increases by ∼11%. On the y-axis scale of Figure 3, this corresponds to a transfer of 0.4 protons, that is, ∼40% of the protons transferred from dodecylguanidine to the much lower concentration of chloroform-d in the same time period.

In contrast to the earlier published ^{1}H NMR spectra in chloroform-d solvent, the sample and measuring conditions presented in Figure 2A,B have mostly overcome the problem of exchange with solvent deuterons and therefore represent the authentic ^{1}H NMR spectrum of dodecylguanidine free base. The sharp peaks in Figure 2B at δ = 3.50 and 3.15 ppm probably represent a very small amount of OH^-, produced by the reaction of the minor contaminant of water in commercial DMSO-d_6 with the strong base dodecylguanindine. Tentative assignment to OH^- is based on the observation that addition of microliter quantities of ^2H_2O to the NMR sample increased the size of both signals (data not shown). For tetraalkylammonium hydroxides in DMSO, the signal of free hydroxide shows some dependence on alkyl chain length, varying between δ = 4.43 for tetramethyl and tetraethylammonium counterions and δ = 4.56 ppm for tetrapropylammonium.[25]

On the basis of these new model compounds in Figure 2A,B, the deprotonated guanidine group of arginine is expected to show a single NH resonance in the range 3−5 ppm in the absence of external H-bond donors. Deprotonated arginine is also expected to show a chemical shift near δ = 2.9 ppm for the $-CH_2-$ group immediately adjacent to the deprotonated guanidine. This is significantly upfield from the value of δ = 3.08 for the protonated dodecylguanidinium ion (Figure 2C).

These characteristic chemical shift values of monoalkylguanidine free base can be used to identify deprotonated arginines in biological environments. For such a purpose, our model compounds are more relevant than arginine in the presence of high $[OH^-]$, for which NMR measurements have never exhibited either the NH or $-CH_2-$ chemical shifts depicted in Figure 2A,B. This is not surprising for the NH protons, which in the presence of high $[OH^-]$ are shifted, weakened, and/or broadened to the point of undetectability. However, not even the $-CH_2-$ resonance at δ = 2.9 in Figure 2A,B has ever been observed for arginine at high hydroxide concentration. Indeed, the methylene protons adjacent to the guanidinium group vary only within the range of δ = 3.3−3.15 over the entire pH range of 0−14.[18,19] Over the more relevant pH range 10−14, where the same authors[18,19] (and others cited therein) have concluded that arginine's guanidinium group deprotonates, there is actually a drop of only about ∼0.05 ppm, from δ = 3.20 to 3.15 ppm. This is substantially smaller than the 0.2 ppm shift we observe between the different protonation states in Figure 2 and therefore does not likely correspond to a full deprotonation. Instead, the relatively small NMR chemical shift changes that occur in arginine at high pH, and that are fitted well by a titration curve with an apparent pK_a of ∼13.3, likely correspond to complexation of OH^- to guanidinium, with a complexation constant of ∼5 M^{-1}, corresponding to pK_b = 0.7. This conclusion also agrees with results presented in the sections on IR (above) and ^{15}N NMR spectroscopy (below).

Solid-State ^{15}N NMR. Figure 4A shows that in $^{15}N_2$-labeled crystalline dodecylguanidine free base **2′** there are distinct ^{15}N

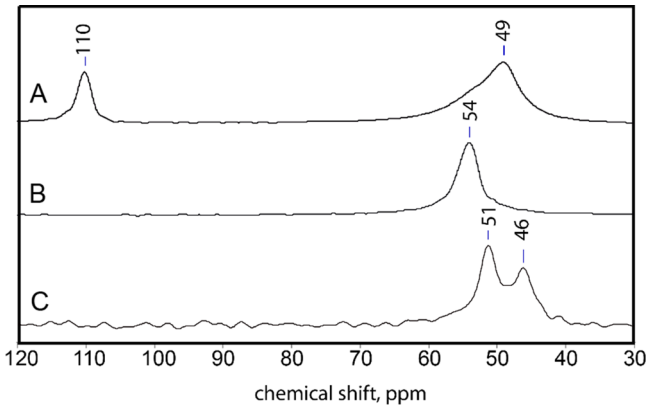

Figure 4. Solid-state cross-polarized magic-angle spinning (CP-MAS) ^{15}N NMR spectra of (A) crystalline dodecyl-guanidine-[$^{15}N_2$] free base, (B) crystalline dodecylguanidinium-[$^{15}N_2$] bromide, and (C) crystalline p-phenol-dodecyl-guanidine-[$^{15}N_2$] free base.

chemical shifts for amino and imino nitrogens, at 49 and 110 ppm, respectively. Although the 110 ppm value is well within the range that has been previously observed for imino nitrogens (e.g., in Schiff bases), such a large downfield shift of ∼60 ppm, relative to dodecylguanidinium itself (Figure 4B), has not previously been observed for ^{15}N in arginine or in any other monoalkylguanidine in aqueous media. Recent NMR titration curves of aqueous arginine with an apparent pK_a of 13.8 showed a ^{15}N resonance for the terminal nitrogens that shifted downfield only by 22 ppm.[19]

In fact, a previous model for the deprotonated guanidine group, namely, $^{15}N_2$-arginine dissolved in DMSO/water (1:1)

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at pH 13.8 and −80 °C, exhibited chemical shift values of 51.6 and 43.3 ppm for the two terminal nitrogens of the guanidino group.[9] These are very close to the single value 54 ppm that we measure for crystalline dodecylguanidinium bromide (**1′**, Figure 4B) as well as the two values we observe for crystalline 12-(*p*-phenol)-dodecylguanidine−$^{15}N_2$ (**15′**, Figure 4C). Our IR spectra of the latter compound (Spectra F and L, Figure S-3 in Supporting Information) indicate that the proton is mostly transferred from phenol to guanidine so that **15′** exists (at least in crystalline state) as a guanidinium−phenolate zwitterion. The solid-state NMR spectrum in Figure 4C is in agreement with this conclusion because it provides no evidence for an imino group, as is evident in Figure 4A. The reason for the moderate differences in the two observed chemical shifts is that compound **15′** evidently crystallizes with the two $-NH_2$ groups in measurably different environments. By contrast, compound **1′** crystallizes in such a fashion as to give nearly identical environments for the two terminal nitrogens so as to give rise to a single broad peak near 54 pm (Figure 4B).

From the results in Figure 4, we conclude that in authentic monoalklylguanidine free base the crystal symmetry is such that there are two distinct classes of terminal nitrogens: those with a C═NH bond and those with a C−NH_2 bond—and no rapid interconversion on the NMR time scale. This conclusion is consistent with the measured crystal structure of guanidine free base, in which each guanidine has two C−NH_2 groups and one clearly distinct C═NH group with a significantly shorter bond length.[18] The latter accepts a H-bond from a C−NH_2 group on a different molecule, but the proton is shared unequally.

Challenges in Preparing Anhydrous Dodecylguanidine Free Base. Previous attempts to model deprotonated arginine spectroscopically have all involved deprotonation of arginine itself or another monoalkylguanidinium salt with aqueous hydroxide solutions. Spectra of the resulting samples were obtained with[6,20] or without[9,18,19] subsequent extraction into an aprotic solvent. However, these attempts gave only solutions, or amorphous solids, with unknown levels of hydrations. Likewise, our (unpublished) attempts to make crystallizable alkylguanidine free base in a butanol−water mixture, by bubbling anhydrous NH_3 through it, were unsuccessful. One possible reason for the failure of all of these approaches to give spectroscopic results similar to those shown in Figure 1A, 2A, or 3A is that guanidinium prefers to form a bound hydroxo complex instead of deprotonating, in the presence of high hydroxide ion concentration (with or without the additional presence of water).[21,24]

On the basis of such observations, deprotonation to form guanidine by raising the pH (i.e., increasing the hydroxide concentration) is likely impossible in aqueous solutions, or even in organic solvents containing a substantial mole fraction of water. A frequently given textbook value for the pK_a of arginine's guanidine side chain, 12.5, actually understates the value of 13.4−13.6 that has historically been obtained from direct potentiometric titrations of monoalkylguanidinium salts.[29,30] More recently, direct potentiometric titrations of arginine itself, coupled with NMR measurements, have consistently yielded values of 13.6−13.8.[18,19]

However, even potentiometric and NMR titration curves with a clear break point do not suffice to establish the formation of deprotonated guanidine in the presence of high aqueous hydroxide concentrations. There is an intrinsic ambiguity to titrations in aqueous solution, which can produce similar results for either deprotonation of a cation at elevated pH or binding of OH^- to form a cation−hydroxo complex. For example, the formation of the hydroxo complex of magnesium (in a DMSO/water mixture) causes an inflection point in its titration curve at pH = 11.5 that mimics the shape expected for a deprotonation, which is clearly not occurring.[31] Potentiometric and even spectroscopic changes for guanidinium at these high pH values in water[18,19] might likewise occur as a result of OH^- binding, rather than deprotonation.

It appears, in fact, that no previous experiment can be interpreted unambiguously as having produced arginine, or any other monoalkylguanidine, as the base with only four covalently attached H atoms, either as a pure compound or in solution. In particular, previous NMR titration experiments of arginine in aqueous solution[18,19] produced changes in ^{15}N or ^{1}H chemical shifts only half as big as we see between dodecylguanidinium and crystalline dodecylguanidine, either directly in solid state (Figure 4) or dissolved into water-free aprotic solvents (Figure 2). We conclude that all previous arginine titration experiments in the presence of water are more likely to have produced a weakly bound guanidinium hydroxide complex, which appears to have a dissociation constant of ∼500 mM.

This broad generalization does not apply to arylated guanidines, nor to multiply alkylated guanidines, some of which have indeed been shown spectroscopically to fully deprotonate in aqueous solution. However, these related compounds have many inadequacies as models for the arginine side chain. Thus, all previous attempts to model the deprotonated arginine side chain spectroscopically have been, at best, crude approximations.

Our procedures for preparing crystalline dodecylguanidine free base (**2**) were based on those of previous workers, who successfully prepared guanidine itself for the first reported crystal structure of this compound.[26] They showed that to obtain the free base a guanidinium salt must be water-free prior to addition of a deprotonating agent (i.e., an external base). Furthermore, the added base must not result in the formation of water, that is, hydroxide is generally an unsuitable reagent, and this makes water an unsuitable solvent. This is because the free base guanidine itself is extremely basic and hydrolyzes water rapidly to form guanidinium hydroxide. It has long been known that to remove the hydroxide ion along with the proton attached to the guanidine group prolonged exposure of guanidinium hydroxide to an extremely dry vapor phase is required, which can be obtained only with a very strong desiccant, such as P_2O_5.[24] It is to be expected that the same problems apply to monoalkyl guanidines as to free guanidine itself. An additional problem is that prolonged exposure to hydroxide, a good nucleophile, along with a polar solvent, causes the monoalkyl guanidino group to become hydrolyzed, forming substituted ureas.[27]

Forming a stable water-free crystalline monoalkylguanidinium salt is itself somewhat challenging because these compounds are themselves quite hygroscopic. The best crystallization solvent appears to be acetonitrile, in which alkylguanidinium salts are soluble only near the boiling temperature. Cooling to room temperature then affords a highly pure water-free crystalline material, which gives a clear melting point and an elemental analysis matching as predicted.

Acetonitrile was previously used to crystallize the *p*-toluenesulfonate salts of alkylguanidines, giving elemental analyses that match the solvent-free chemical formula.[28] However, this was not an ideal guanidinium salt for our purpose because the presence of such a complex counterion

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makes the interpretation of the IR bands of the guanidinium group more difficult. Therefore, we sought to prepare a simple halide salt of dodecylguanidinium using similar crystallization procedures as described for the *p*-toluenesulfonate salts[28] and were most successful with the bromide. One advantage of the bromide salts is that they are generated directly by the reaction of amines with *S*-alkylthiouronium bromides, which in turn can easily be prepared from thiourea and bromoethane. The corresponding *S*-alkylthiouronium chlorides are not as easily prepared because chloroalkanes are less susceptible to S_N2 displacements. The *S*-alkylthiouronium iodide is as easily prepared as the bromide, but the resulting guanidinium iodides tend to be less stable than the bromides, and rapidly discolor upon storage, probably due to air oxidation of the iodide. Thus, when we prepared the iodide at step (**13**) in Scheme 3, we found it most useful to immediately exchange the counterion with bromide.

Our procedure for deprotonation of the dried crystalline monoalkylguanidinium salts followed the procedure for guanidinium itself[26] with slight modification. Instead of ethanol, the solvent used was dry methanol; and instead of preparing hydroxide-free sodium ethoxide by adding Na to ethanol, we used potassium *tert*-butoxide, by simply dissolving the commercial material in well-dried methanol, thus avoiding the hazards of sodium metal. In methanol, the *tert*-butoxide serves as a strong base (pK_a of *t*-butanol ≈17), immediately abstracting a proton to form methoxide. Unlike hydroxide, however, both methoxide and *t*-butoxide appear to be sufficiently strong bases to deprotonate guanidinium but sterically incapable of carrying out nucleophilic attack on the guanidino group.

Isotopic Enrichment of Guanidine's Nitrogen Atoms (^{15}N > 98%). We demonstrated the utility of selective isotope labeling guanidine compounds in measuring solid-state ^{15}N NMR spectra (Figure 4). The formation of the guanidine group at a late step in the synthesis of guanidine compounds (e.g., **14**) simplifies ^{15}N-enrichment at the two terminal nitrogens. The yield from the subsequent steps can be maintained at a high level, conserving the expensive enriched isotope.

In the synthesis of the alkylguanidiniums from alkylamines, *S*-methyl thiourea hydroiodide and *S*-ethyl thiourea hydrobromide can be used nearly interchangeably. The formation of the former (from thiourea and methyl iodide) is slightly easier and less expensive than the latter (from thiourea and ethyl bromide). However, the use of *S*-methyl thiourea hydroiodide in the conversion of amines to guanidines required the extra step of immediately substituting iodide with bromide because the alkylguanidinium bromides were found to be more stable than the iodides upon storage over several days, as mentioned above. When working with the expensive ^{15}N isotope label, this added step led to unacceptable loss of yield. Therefore, in the synthesis of the ^{15}N-labeled compounds (**1′** and **13′**), the need for counterion exchange was circumvented using *S*-ethyl thiourea-$^{15}N_2$ hydrobromide.

We attempted several other strategies (not shown in Scheme 3) to form guanidines from amines, such as reacting the amine with CS_2 to form a dithiocarbamate intermediate. Reaction of this intermediate with natural-abundance or ^{15}N-ammonia was then attempted, to introduce the isotope label. However, we never achieved consistent yields of guanidines that could compete with the use of *S*-alkyl thiouronium salts. The latter were easily prepared from $^{15}N_2$-thiourea, which is (at least intermittently) commercially available.

We also showed that it was also possible to isotopically enrich the amine $-NH_2$ group to form anisole-dodecylamine-^{15}N (**12′**), using commercially available potassium ^{15}N-pthalimide (Cambridge Isotope Labs). We have characterized **12′** with low-resolution mass spectrometry and IR spectroscopy to demonstrate the incorporation of the label (data not shown) but have not yet used it to prepare guanidino compounds (**13**, **14**, or **15**) with the internal nitrogen carrying an^{15}N label.

Design and Synthesis of Phenolyl-Alkylguanidine Model Compounds (15, 15′). With considerable effort, we were able to produce solutions of dodecylguanidine in aprotic solvents but never in the presence of even small (stoichiometric) amounts of water. The ubiquitous presence of water in biological systems suggests that arginine deprotonation might never be observed physiologically. It is questionable whether even the interior of a physiologically formed lipid bilayer is an environment sufficiently water-free to support formation of a deprotonated arginine side chain on its own, rather than complexed with water as the guanidinium hydroxide. Computations have indicated the likelihood of charged amino acids carrying water into the membrane interior, producing "water defects".[13]

What is less clear is whether another strong H-bond donor that is less acidic than water, for example, the side chain of tyrosine, might be able to displace water from such an alkylguanidinium hydroxide inside the most nonpolar region of a membrane or a protein. We have begun to address this question by synthesizing *p*-phenolyl-dodecylguanidine. The only previous examples of a simple hydrocarbon linking a guanidine group covalently to a phenolic compound are salts of *N*-guanyltyramine, whose synthesis was previously reported using commercially available tyramine as a starting material.[32] Our goal in synthesizing an analogue with a much longer, flexible aliphatic linkage was to provide for the possibility of an unhindered internal H-bond interaction, with or without proton transfer, between the guanidino−imino group and the phenol−OH at opposite ends of the linker. Another goal was to provide sufficient nonpolar surface area to permit this compound to be dissolved in nonpolar environments. A third goal was to choose methods that could easily be transferred to a wide variety of different-sized hydrocarbon linkers.

The types of reactions used in the synthesis (Scheme 3) have been described previously.[32−37] However, in the Experimental Section, we will highlight some changes in conditions that were important for improving yields. Other strategic routes, for example, starting with para-substituted phenols, might be expected to give a higher yield with fewer steps. However, unlike the strategy we devised, these other routes do not leave open as many options for isotope labeling of the phenol ring, which may be eventually desirable for additional spectroscopic or scattering experiments. For example, it will eventually be useful to examine the position of the phenolic proton within the H-bond by neutron scattering. For such a measurement, it will be helpful to have all of the nonexchangeable protons replaced with deuterons. This will be possible with our synthetic route because the key starting materials (anisole and dodecane dicarboxylic acid) are both commercially available with all C−H bonds deuterated.

We have not obtained any evidence that the specific dodecyl linker described here provides sufficient steric flexibility to permit an internal H-bond between phenol and guanidine. In fact, IR spectra (Figure 1D,E) and the ^{15}N NMR CPMAS spectrum (Figure 4B) of the crystalline product (**15**, **15′**) show

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that the free base of this compound exists predominantly in a zwitterionic form in this crystal. X-ray structures in the accompanying paper[23] demonstrate that these zwitterions form head-to-tail H-bonded dimers in crystals. Concentration-dependent spectroscopy supports formation of similar dimers in DMSO solution above ~30 mM. On the basis of the spectra, these dimers show indication of an approximately 10−25% contribution from the fully neutral species, that is, the nonzwitterionic protomers of **15**.

However, the accompanying paper[23] also shows that different results are observable for 1:1 mixtures of dodecylguanidine (**2**) and *p*-cresol in even less-polar solvents, for example, hexane. In such water-free environments, strong intermolecular H-bonds are formed that correspond more closely to a neutral H-bonded pair (phenol + guanidine). These pairs are in the form of heterodimers, rather than internally H-bonded monomers, which were the primary target of our synthesis of *p*-phenolyl-dodecylguanidine here. Nevertheless, those results[23] suggest the hypothesis that strong H-bonding between arginine and tyrosine side chains, when both are simultaneously forced to be buried near each other in the nonpolar aprotic region of a membrane, might constitute a rare biological environment that could bring about substantial deprotonation of an arginine side chain. To test this hypothesis rigorously, it must still be determined if there are any biphasic environments, in which phenol (tyrosine) groups can form strong H-bonds to deprotonated guanidine (arginine) groups when tethered inside the nonpolar region—even stronger than those that water molecules can form, when the latter are readily available from a nearby aqueous phase.

CONCLUSIONS

It is necessary to remove water and hydroxide rigorously from pure monoalklylguanidines to deprotonate them significantly, even when they are subsequently dissolved in otherwise nonpolar environments. The same is expected for the side chain of arginine. By following this guideline strictly, we have obtained crystalline dodecylguanidine and demonstrated that the deprotonated guanidine group's spectroscopic properties are strikingly different from those previously reported for aqueous arginine in the presence of high hydroxide concentrations. Authentic spectra of pure recrystallized dodecylguanidine, measured in dry organic solvents, are even different from those previously published using noncrystalline dodecylguanidine samples, from which tightly bound water could not be adequately removed merely by drying under N_2. All of the previously published spectra of what were thought to be deprotonated arginine or monoalkylguanidine likely correspond instead to tightly bound guanidinium hydroxide complexes.

EXPERIMENTAL SECTION

Solution-State NMR Measurements. All ^{1}H and ^{13}C NMR (300 MHz) spectra were recorded at 25 °C in benzene-d_6, chloroform-*d* (C^2HCl_3), or DMSO-d_6. Chemical shifts are presented in parts per million and are generally referenced to TMS added as an internal standard. In some of the measurements of synthesis intermediates, TMS-free solvent was used to simplify the spectra near the alkyl chain resonances. In these cases, the chemical shift scale was referenced to the residual $CHCl_3$ peak at 7.27 ppm for ^{1}H and 77.23 ppm for ^{13}C (when chloroform-*d* was used a solvent) and the DMSO residual peaks at 2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C (when DMSO-d_6 was used as solvent). ^{1}H splitting patterns are designated as singlet (s), doublet (d), triplet (t), multiplet (m), and broad (br).

Solid-State ^{15}N NMR Measurements. All solid-state ^{15}N NMR cross-polarization magic-angle spinning (CP-MAS) measurements were performed at Analytical and Technical Services at SUNY ESF using a 300 MHz Bruker spectrometer with a 7 mm CPMAS probe with the sample at 25 °C. The spinning rate was 4000 Hz; acquisition time, 0.02 s; number of data points, 484; delay between pulses, 5 s; sweep width, 400 ppm (12 165.450 Hz); ^{1}H decoupling field, 47 200 Hz; and spectra were processed with a line broadening of 40 Hz. All chemical shifts are referenced to an ammonium-$^{15}N_2$ sulfate standard (defined as 0 ppm).

IR Measurements. Spectra were obtained using a Nicolet Magna IR 860 spectrometer using demountable liquid cells with two BaF_2 windows.

Data Analysis. Band-fitting and other data manipulations were performed using GRAMS software (Thermo Galactic). NMR bands were fitted to pure Lorentzian shapes using two parameters for each band (center and width), assuming a zero baseline.

Mass Spectrometry. Low-resolution (±0.1 amu) measurements were performed at Analytical and Technical Services at SUNY ESF, on a Thermo Scientific Polaris Q trap mass spectrometer, using a solid probe with temperature ramp from 35 to 450 °C. High-resolution mass spectroscopy was performed by positive electrospray ionization on a Bruker 12 T APEX-Qe FTICR-MS and Apollo II ion source at COSMIC Lab, Old Dominion University.

Elemental Analyses. Combustion analysis for C/H/N was performed by Complete Analysis Laboratories Inc. (Parsippany, NJ).

Chemical Syntheses. Solvents used were of reagent grade. Except as noted, all materials were obtained from Aldrich Chemicals.

General Notes on Crystallization during Purifications. Frequent crystallization of intermediates turned out to be crucial for removing unreacted starting materials, as well as side products. All of the intermediates contain a long floppy alkyl chain, making them susceptible to form oils or waxes upon drying. Failure to purify these led to increasingly poor yields along the synthetic route.

Hexane turned out to be the crystallization solvent of choice for products up through compound (**12**). That is, compounds **4**, **6**, **8**−**10**, and **12** are soluble in boiling hexane but precipitate as white crystals at room temperature. Reaction conditions and solvents used in these steps were polar and produce polar contaminants via side reactions that are insoluble in hexane. Upon selective extraction of the crude solid products with hot hexane, these contaminants are left behind. Cooling of the hexane solution produces pure crystals that give excellent melting points and elemental analyses.

The guanidinium salts **13** and **14** were insufficiently soluble in hexane, or any nonpolar solvent, to permit crystallization. However, they were soluble in hot acetonitrile. As noted above, crystallization from acetonitrile helps to remove water from the very hygroscopic guanidinium group, allowing the isolation of a pure crystalline sample. It should be noted, however, that unlike dodecylguanidine free base (**2**) the free base of 12-(*p*-phenol) dodecylguanidine (**15**) (actually a zwitterion in the crystal state, as shown above by IR and NMR measurements) was not

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significantly soluble in acetonitrile and was only successfully recrystallized from methanol.

*Purification of Dodecyl Guanidinium Bromide (**1**).* As described previously,[19] the commercially available dodecylguanidinium acetate salt (obtained from Agway as the fruit-tree fungicide Syllit) was extracted from insoluble solids and recrystallized with 2-propanol and dried. It was then converted to the bromide salt by means of a simple two-phase ion-exchange procedure. That is, 1-butanol and a (10-fold excess of) saturated aqueous NaBr were added to the solid dry dodecylguanidinium acetate salt, and the contents were thoroughly mixed. The phases were allowed to separate completely and then the bottom aqueous layer was removed. Extraction of the butanol phase with saturated aqueous NaBr was repeated once more and then the remaining butanol phase was removed and dried completely. The resulting solids were recrystallized from acetonitrile, filtering out some residual NaBr when the acetonitrile solution was near its boiling point. Upon cooling to room temperature, pure crystalline dodecylguanidinium bromide (**1**) was obtained. ^{1}H NMR (DMSO-d_6) δ ppm 7.59−6.6 (br), 3.08 ppm (t, 2H), 1.43 ppm (t, 2H), 1.23 ppm (m, 18H), 0.83 ppm (t, 3H). ^{13}C (DMSO-d_6) 175.48, 31.968, 29.721, 29.692, 29.679, 29.647, 29.390, 29.295, 29.093, 26.708, 22.758, 14.575. Mp 71−73 °C. Elemental analysis theoretical 50.65% C, 9.74% H, 13.63% N Experimental 50.58% C, 9.82% H, 13.59% N.

*Synthesis of Dodecylguanidinium-$^{15}N_2$ Bromide (**1′**).* Dodecylamine, obtained from Fisher Scientific, was reacted with a 1.4-fold molar excess of *S*-ethyl-thiourea-$^{15}N_2$ hydrobromide, prepared as follows: 0.8 g (10.25 mmol) of thiourea-$^{15}N_2$ (^{15}N = 98%), obtained from Cambridge Isotope Laboratories (Cambridge, MA), and 1.56 g (14.38 mmol, i.e., 1.4 mol equiv) of bromoethane were refluxed in 2-propanol for 20 min, then concentrated by evaporation under dry N_2 at room temperature, and placed in a freezer (−20 °C) to induce crystallization. The crystals were filtered and washed with cold diethyl ether (3×) and then dried under vacuum. The mass of *S*-ethylthiourea-$^{15}N_2$ hydrobromide recovered is 1.74 g (91.86%). To reduce losses of the expensive ^{15}N isotope, this crude product was used directly for guanidinium synthesis, without further purification. Dodecylamine (0.3 g, 1.618 mmol) and crude *S*-ethylthiourea-$^{15}N_2$ hydrobromide (0.39 g, 1.3 mol equiv) were added to 6.0 mL of an ethanol/water (1:1) mixture and heated under reflux. The pH of the solution was monitored and periodically readjusted to ∼10.5 using 10 M sodium hydroxide. After 2 h, the solution was allowed to cool to room temperature and air-dried. The product **1′** was recrystallized from acetonitrile and yielded 0.15 g (30.12%).

*Synthesis of Dodecylguanidine Free Base (**2**) or Dodecylguanidine-$^{15}N_2$ Free Base (**2′**).* Dodecyl guanidinium bromide (**1** or **1′**) was dissolved in warm methanol, at a concentration of ∼1 M. The solution was cooled to room temperature and then 1 mol equiv of potassium *tert*-butoxide, also freshly dissolved as a 1 M solution in dry methanol, was added slowly and dropwise, with constant stirring. This caused the solution to become milky, due to precipitation of KBr. The solution was stirred at room temperature for another 20 min and then heated momentarily to the boiling temperature of methanol. The hot solution was centrifuged briefly, and the clear supernatant was transferred to another flask, leaving behind a pellet of white KBr. The supernatant was concentrated and cooled under a gentle stream of N_2. This caused the dodecylguanidine free base to precipitate from the cold methanol. When precipitate formation slowed greatly, the remaining supernatant was removed, along with dissolved *tert*-butanol. The residual free base (**2**) was recrystallized from fresh dry methanol and thoroughly dried under N_2. The resulting residue was further recrystallized from acetonitrile. Mp 94.5−95 °C. IR, NMR—see Figures 1 and 2. Elemental analysis theoretical 68.12% C, 13.54% H, 18.34% N; experimental 68.77% C, 12.79% H, 18.24% N. [$C_{13}H_{31}N_3$, MW 156 + 31 + 42 = 229].

*Synthesis of (p-Phenolyl)-dodecyl Guanidinium Bromide (**14**, **14′**) and Corresponding Free Bases (**15**, **15′**).* We used a 13-step synthesis, summarized in Scheme 3, for connecting phenol and guanidino groups by a hydrocarbon linker.

1,12-Dodecanedioic acid (**3**) was obtained from Fisher Scientific. Mp 127.5−129 °C. ^{1}H NMR (DMSO-d_6) δ ppm 12.00 (s, 2H), 2.2−2.1 ppm (t, 2H), 2.6−2.4 ppm (m, 4H), 1.3−1.1 (m, 16H). ^{13}C (DMSO-d_6) δ (ppm) 174.48, 33.63, 28.83, 28.70, 28.52, 24.46.

Dodecanedioic acid dimethyl ester (**4**) was synthesized by refluxing 60.0 g of 1,12-dodecanedioic acid (**3**, 260 mmol) and 8 mL of concentrated sulfuric acid in 100 mL of methanol. The progress of the reaction was monitored using IR. The reaction was deemed complete after complete disappearance of the carboxylic acid peak at ∼1700 cm^{-1} with simultaneous appearance of the ester peak at ∼1742 cm^{-1}. After cooling to room temperature, 300 mL of water was added and the product was extracted 3× with 100 mL of benzene. Combined benzene fractions were vacuum-dried, yielding 65.43 g (97.43%) of **4**. ^{1}H NMR ($CHCl_3$) δ ppm 3.64 (s 6H), 2.30−2.25 (t, 4H), 1.61−1.57 (m, 4H), 1.25 (s, 16H). ^{13}C ($CHCl_3$) δ ppm 173.867, 50.980, 33.628, 28.891, 28.755, 28.660, 24.476. Mp 61−63 °C. [M]+ calculated for $C_{14}H_{26}0_4$ was 258; found 258. Elemental analysis theoretical 65.11% C, 10.07% H. Experimental 64.97% C, 9.82% H.

Dodecanedioic acid monomethyl ester (**5**) was synthesized by adding 65.43 g (253.6 mmol) of dodecanedioic acid dimethyl ester (**4**) into 256 mL of 1 N $Ba(OH)_2$ (126.8 mmol) in methanol. The flask was capped immediately, and after thorough mixing, the flask was left at room temperature for 24 h. The precipitated barium salt was separated by suction filtration. The solid was then transferred to a separatory funnel containing 30 mL of methanol, and the contents were thoroughly mixed. Into this solution, 100 mL of 4 N aqueous HCl and 100 mL of diethyl ether were added. After thorough mixing, the phases were allowed to separate and the ether phase was removed. The remaining aqueous phase, together with precipitates of barium salt, was extracted 2× more with ether. The ether phases (300 mL in total) were combined and washed 3× with 100 mL of water and then vacuum-dried. Mass of the monomethyl ester recovered was 57.2 g (92.43%). ^{1}H NMR ($CHCl_3$) δ ppm 3.60−3.59 (s, 3H), 2.30−2.20 (m, 4H), 1.55−1.54 (m, 4H), 1.20 (s, 12H). ^{13}C ($CHCl_3$) δ ppm 180.412, 174.635, 51.684, 34.296, 34.263, 29.520, 29.385, 29.303, 29.212, 25.123, 24.841. Mp 48−49 °C. [M]+ observed was at 244, matching the value calculated for $C_{13}H_{24}0_4$. Elemental analysis theoretical 63.93% C, 9.84% H. Experimental 63.87% C, 10.01% H.

Dodecanedioic acid monochloride monomethyl ester (**6**) was synthesized by slow addition of 41.83 g of thionyl chloride (351.6 mmol, 1.5 equiv) into 300 mL of dichloromethane containing 57.2 g (234.42 mmol) of dodecanedioic acid monomethyl ester (**5**). After the addition was completed, 5.0 mL of *N*,*N*-dimethylformamide was added dropwise as a

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catalyst. The progression of the reaction was monitored by IR spectroscopy and deemed complete after complete disappearance of the COOH carbonyl absorbance at ~1700 cm^{-1}, with simultaneous appearance of the acyl chloride peak near 1800 cm^{-1}. Without extraction or purification, the acyl chloride product was used for the subsequent synthesis step.

*Synthesis of 11-(p-Methoxybenzoyl) Undecanoyl Methanoate (**7**).* Into a stirred solution of **6** in CH_2Cl_2, 30.4 g of anisole (281.12 mmol, 1.2 equiv) was added, followed by slow addition of anhydrous $AlCl_3$ (350.98 mmol, 1.5 equiv), with rapid appearance of a deep purple color and evolution of HCl gas. After gas evolution ceased with further $AlCl_3$ addition, the solution was stirred for 5 h more. Then, 200 mL of water was added with vigorous stirring, initially very slowly and dropwise, until evolution of HCl gas ceased and the deep purple color disappeared. The CH_2Cl_2 and aqueous phases were allowed to separate. Then, the bottom (CH_2Cl_2) phase was collected, washed 2× with 100 mL of water, filtered, and dried. The solid residue was recrystallized from hexane and dried, yielding 55.71 g (71.2%). ^{1}H NMR ($CDCl_3$) δ ppm 7.91−7.94 (d, 2H), 6.90−7.27 (d, 2H), 3.85 (s, 3H), 3.65 (s, 3H), 2.86−2.91 (t, 2H), 2.26−2.31 (t, 2H), 1.58−1.74 (m, 4H), 1.27−1.31 (s, 12H). ^{13}C NMR ($CHCl_3$) δ ppm 199.563, 174.605, 163.501, 130.528, 113.852, 55.647, 51.666, 38.502, 34.306, 29.645, 29.604, 29.580, 29.418, 29.320, 25.137, 24.821. M.P 60−63 °C. [M]+ calculated for $C_{20}H_{30}O_4$ was 334; found 334. Elemental analysis theoretical 71.86% C, 8.98% H. Experimental 71.74% C, 9.04% H.

*Synthesis of 12-(p-Methoxyphenyl) Dodecanoic Acid (**8**).* Compound **7** (4.96 g) was dissolved in DMSO (80.0 mL). Into this solution, potassium *tert*-butoxide (2.0 mol equiv) and excess hydrazine monohydrate (8.0 mol equiv) were added, and the mixture was refluxed for 48 h. Then, the reaction was cooled to room temperature, and 0.1 M aqueous HCl (50.0 mL) was added until the pH of the aqueous phase fell below 2.0. The product was extracted using CH_2Cl_2 (2 × 100 mL). The residual DMSO in the CH_2Cl_2 phase was removed by repeated extraction with water (3 × 50 mL). The CH_2Cl_2 phase was then removed and dried. The dried sample was crystallized from hexane to afford 3.79 g of compound **8** (yield, 79.95%). ^{1}H NMR (DMSO-d_6) δ ppm 11.99 (s, 1H), 7.09−7.06 (d, 2H), 6.83−6.80 (d, 2H), 3.7 (s, 3H), 2.5−2.49 (m, 2H), 2.20−2.14 (m 2H), 1.49−1.47 (d, 4H), 1.22 (s, 12H). ^{13}C (DMSO-d_6) δ (ppm) 174.55, 157.29, 134.19, 129.16, 113.62, 54.94, 34.25, 33.68, 31.28, 29.02, 28.89, 28.77, 28.60, 28.57, 24.52 Mp 73−75 °C. [M]+ observed was at 306, matching the value calculated for $C_{19}H_{30}0_3$. Elemental analysis theoretical 74.5% C, 9.8% H. Experimental 73.43% C, 10.23% H.

*Synthesis of 12-(p-Methoxyphenyl) Dodecanol (**9**).* Compound **8** (3.79 g) was added slowly to a stirred THF solution (20.0 mL) containing $LiAlH_4$ (4 mol equiv), leading to evolution of H_2 with each portion added. After addition was complete, the mixture was stirred 5 h more at room temperature. Then, 40 mL of water was added slowly, followed by addition of CH_2Cl_2 (50.0 mL). The resulting slurry was vacuum-filtered, and the filtrate was placed in a separatory funnel. The bottom (CH_2Cl_2) layer was collected and dried. The resulting solid was recrystallized from hexane, affording 3.22 g of compound **9** (yield, 89.03%). ^{1}H NMR ($CDCl_3$) δ (ppm) 7.05−7.02 (d, 2H), 6.78−6.75 (d, 2H), 3.73 (s, 3H), 3.6 (t, 3H), 2.51−2.45 (t, 3H), 1.53−1.48 (m, 4H), 1.24−1.20, (s, 16H). ^{13}C ($CDCl_3$) δ (ppm) 157.748, 135.262, 129.437, 113.819, 63.295, 55.447, 35.243, 33.009, 31.978, 29.834, 29.794, 29.725, 29.635, 29.479, 25.941. Mp 64−66 °C. [M]+ calculated for $C_{19}H_{32}0_2$ was 292; found 292. Elemental analysis: theoretical 78.08% C, 10.96% H, experimental 78.28% C, 10.89% H.

*Synthesis of 12-(p-Methoxyphenyl) Dodecyl Bromide (**10**).* Compound **9** (3.21 g) was dissolved in CH_2Cl_2 (50.0 mL) containing CBr_4 (1.2 mol equiv, 4.38 g) and placed in an ice bath. Into this solution, triphenylphosphine (1.2 mol equiv, 3.46 g) was added slowly over ~10 min. The solution was stirred at 0 °C for another 30 min before being stored at room temperature overnight. The sample was then concentrated by evaporating CH_2Cl_2 and then the concentrated solution was poured into 100 mL of stirred hexane and filtered. The filtrate was cooled to 0 °C, causing unreacted starting materials and unwanted byproducts to precipitate but leaving liquid brominated product in hexane. The cold hexane solution was filtered (again), and the filtrate was then concentrated by blowing dry air being cooled on ice. After complete evaporation of hexane, the pure liquid product (**10**) was removed from residual solid contaminants. The final mass obtained was 2.57 g (yield, 65.85%). ^{1}H NMR ($CDCl_3$) δ (ppm) 7.13−7.10 (d, 2H), 6.87−6.83 (d, 2H), 3.8 (s, 3H), 3.45−3.40 (t, 2H), 2.59−2.53 (t, 2H), 1.89−1.85 (t, 2H), 1.62−1.57 (t, 2H), 1.45−1.41, (t, 2H), 1.39−1.29, (s, 14H). ^{13}C ($CDCl_3$) δ ppm 157.775, 135.246, 129.444, 113.830, 55.450, 35.257, 34.296, 33.057, 31.989, 29.812, 29.791, 29.740, 29.649, 29.490, 28.988, 28.397. Mp 64−66 °C. [M]+ calculated for $C_{19}H_{31}OBr$ was 354; found 354 (with nearby equal 356 peak due to ^{81}Br isotope).

*Synthesis of 12-(p-Methoxyphenyl) Dodecyl Phthalimide (**11**).* 12-(*p*-Methoxyphenyl) dodecyl bromide (**10**, 3.23 g) was added into 30.0 mL of *N*,*N*-dimethylformamide, followed by addition of potassium phthalimide (1.5 mol equiv, 1.84 g), and the mixture was refluxed for 2 h. The sample was allowed to cool to room temperature, and 30 mL each of CH_2Cl_2 and water were added and mixed. After separation, the CH_2Cl_2 layer was removed and back-extracted twice with 30.0 mL of water to remove *N*,*N*-dimethylformamide and potassium salts and then air-dried. Into the brownish solid, hexane was added and then heated to boiling. In boiling hexane, the desired compound dissolves, leaving behind insoluble contaminants. Crystallization of the cooled supernatant yielded a white solid (**10**). ^{1}H NMR ($CDCl_3$) δ ppm of 12-(*p*-methoxyphenyl) dodecyl phthalimide 7.86−7.85 (d 2H), 7.71−7.68 (d, 2H), 7.12−7.07 (d, 2H), 6.85−6.80 (d, 2H), 3.79 (s, 3H), 3.71−3.66 (t, 3H) 2.57−2.50 (t, 3H), 1.68−1.66 (t, 2H), 1.57−1.55 (t, 2H), 1.28−1.26 (s, 16H). 13C ($CDCl_3$) δ ppm 168.738, 157.821, 135.339, 134.081, 132.458, 129.493, 123.402, 113.885, 55.508, 38.350, 35.298, 32.021, 29.853, 29.828, 29.807, 29.770, 29.733, 29.536, 29.450, 28.867, 27.128. Mp 74−80 °C.

*Synthesis of 12-(p-Methoxyphenyl) Dodecylamine (**12**).* Compound **11** was mixed with excess hydrazine monohydrate (10.0 mL) in 50.0 mL of absolute ethanol and refluxed for 24 h and then allowed to cool to room temperature. Then, 100 mL of hexane was added, followed by addition of 100 mL of 2 M NaOH. The high-pH aqueous layer was separated and reextracted with another 100 mL of hexane. Hexane fractions were combined and dried, affording 1.23 g of the crystalline solid amine (yield, 46.6%). ^{1}H NMR ($CDCl_3$) δ (ppm) 7.11−7.09 (d, 2H), 6.85−6.82 (d, 2H), 3.80 (s, 3H), 2.71−2.66 (t, 2H), 2.57−2.52 (t, 2H), 1.60−1.55 (t, 2H), 1.44−1.42 (t, 2H), 1.27 (s, 16H), 1.1−0.9 (s, 2H) ^{13}C ($CDCl_3$) δ (ppm) 157.810, 135.298, 129.463, 113.862, 55.481, 42.521, 35.274, 34.170, 31.995, 29.838, 29.755, 29.512, 27.134. Elemental analysis

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theoretical 78.62% C, 11.38% H, 4.5% N experimental 78.32% C, 11.46% H, 4.69% N. [M]+ calculated for $C_{19}H_{33}ON$ was 291; found 291.

Synthesis of 12-(p-Methoxyphenyl) Dodecyl Guanidinium Bromide (13). The amine **12** (2.17 g, 7.48 mmol) was refluxed in 40 mL of absolute ethanol for 2 h, along with 3.25 g (15.0 mmol) of *S*-methyl thiourea hydroiodide. (The 2.17 g starting mass in this step is higher than the stated yield from the previous step. Several repetitions of the earlier steps were used to obtain this starting amount. Also, the reagent *S*-methyl thiourea hydroiodide was prepared as described above for *S*-ethylthiourea-$^{15}N_2$ hydrobromide in the synthesis of dodecylguanidinium-$^{15}N_2$ bromide (**1′**), except that methyl iodide was used instead of ethyl bromide.) The pH of the solution was observed to decrease as the reaction progressed. Constantly adjusting the pH to ∼10.5 (by adding 10 M aqueous NaOH) keeps the amine group deprotonated. This makes it a better nucleophile. This procedure is an improvement of what is reported in the literature,[21] in which the reaction of alkylamine with an alkylthiourea hydroiodide was done at 40 °C for 42.5 h (vs 2 h in this synthesis). Our use of a pH-adjusted ethanol/water mixture instead of methanol, and refluxing at a higher temperature, decreased the reaction time to 2 h in our synthesis (vs 42.5 h for the previous work) and improved the yield to ∼50%, as compared to the reported 21%.[28] Subsequently, the heat was turned off and the ethanol and water were evaporated under a stream of dry air. To the solid residue, 40 mL of 50:50 (v/v) 1-butanol/2 M aqueous NaBr was added, with vortexing. This permitted the exchange of the counteranion (here, I^- being replaced by Br^-), as described above for the analogous formation of dodecylguanidinium bromide from the acetate salt. The phases were allowed to separate, and the bottom (aqueous) layer was removed. The butanol phase was dried and then the solid residue was recrystallized using CCl_4. Filtering and drying yielded 1.4 g of compound **13** (46.77%). ^{1}H NMR (DMSO-d_6) δ (ppm) 7.11−7.09 (d, 2H), 6.85−6.82 (d, 2H), 3.2−3.0 (t, 2H), 2.57−2.52 (t, 2H), 1.60−1.55 (t, 2H), 1.44−1.42 (t, 2H), 1.27 (s, 16H), ^{13}C (DMSO-d_6) δ (ppm) 157.703, 157.241, 135.299, 129.424, 113.809, 55.444, 42.200, 35.230, 31.975, 29.873, 29.754, 29.495, 28.779, 27.001. Mp 78−82 °C.

Synthesis of 12-(p-Methoxyphenyl) Dodecyl Guanidinium-$^{15}N_2$ Bromide (13′). A similar procedure was followed as described above for compound **13**, except for improvements in the workup to obtain a higher yield with the more expensive isotope-labeled reagent. The starting material, deprotonated amine (**12**), is easier to separate from the desired product when recrystallizing from acetonitrile than from CCl_4. That is, both the guanidinium salt (**13**) and unreacted amine (**12**) are extracted into hot acetonitrile. Upon cooling, only the guanidinium salt (**13**) precipitates, leaving behind the unreacted amine (**12**) in the supernatant. Therefore, after the solvent was removed by air drying, acetonitrile was added and heated to boiling. The hot solution was decanted, leaving behind only a small amount of unreacted *S*-ethyl thiourea-$^{15}N_2$ hydrobromide. The acetonitrile solution was then cooled to room temperature, leading to crystallization of the pure guanidinium salt (**13′**). The supernatant was removed, and the crystalline solid was dried thoroughly under a gentle stream of dry air. ^{1}H NMR spectrum of this product is identical to that of 12-(*p*-methoxyphenyl) dodecyl guanidinium bromide (**13**).

Synthesis of 12-(p-Phenolyl) Dodecylguanidinium Hydrobromide (14). It was found that deprotecting anisole after guanidinium synthesis is a better route than deprotecting anisole first, then converting the phenol-dodecylamine to guanidinium (**14**). This is likely because the phenol group competes with the amine as a nucleophile reacting with the *S*-methyl thiourea salt. Fortunately, the demethylation conditions described here have little adverse effect on the guanidinium group. Even extending the deprotection reaction time from 6[38] to 12 or 24 h did not result in significant degradation of the guanidinium group. Therefore, removal of the methyl group from **13** was achieved by adding 1.35 g of it to a flask containing 30 mL of 48% HBr and refluxing the contents for 6 h with continuous stirring. Then, the flask was cooled to room temperature, leading to precipitation of the product (**14**). The contents were filtered and then the collected solid was washed with 50 mL of deionized water and air-dried. The dried sample was recrystallized from boiling acetonitrile over $MgSO_4$ and then again filtered and air-dried, yielding 0.68 g of compound **14**. ^{1}H NMR (DMSO-d_6) δ (ppm) 9.08 (s, 1H), 7.48 (s, 1H), 6.6−7.4 (br), 6.92−6.95 (d, 2H), 6.66−6.63 (d, 2H), 3.08−3.06 (t, 2H), 2.49−2.42 (t, 2H), 1.60−1.4 (m, 7H), 1.23 (s, 16H), ^{13}C (DMSO-d_6) δ (ppm) 157.390, 155.844, 132.944, 129.641, 115.604, 41.373, 34.962, 31.987, 29.681, 29.629, 29.557, 29.268, 29.091, 26.698. Mp 159−162 °C. Elemental analysis theoretical 41.5% C, 5.38% H, 16.15% N experimental 41.42% C, 5.29% H 16.08% N. High-resolution mass spectroscopy exact mass $(C_{19}H_{33}N_3O)H^+$ = 320.269639u, mass observed 320.269057.

Synthesis of 12′-(p-Phenolyl) Dodecyl Guanidinium-$^{15}N_2$ Bromide (14′). 12-(*p*-Methoxyphenyl) dodecyl guanidinium-$^{15}N_2$ bromide (**13′**) was mixed with 20.0 mL of 48% HBr and refluxed for 12 h with continuous stirring. After 12 h, the solution was allowed to cool slowly to room temperature and then held at 4 °C for few minutes and filtered. The resulting brownish solid was washed with water to remove the colored impurities and air-dried. Recrystallizations in acetonitrile were repeated until both the precipitate and the acetonitrile filtrate were free of color. The ^{1}H NMR spectrum of this product is identical to that of 12-(*p*-phenol) dodecyl guanidinium hydrobromide (**14**).

Deprotonation of **14** and **14′** formed (*p*-phenolyl) dodecyl guanidine free base (**15**) and (*p*-phenol) dodecyl guanidine-$^{15}N_2$ free base (**15′**), respectively. The same procedures that were used above to deprotonate the unlabeled compound dodecylguanidinium bromide (**1**) were applied to its *p*-phenolyl-modified derivative, in both natural-abundance isotope (**14**) and ^{15}N-labeled forms (**14′**). However, a considerably longer period of heating in methanol was necessary to dissolve the initial compounds, as well as during recrystallization of the deprotonated products—up to 30 min, as compared to 1−2 min for the simple dodecylguanidine (**2**).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00281.

Figure S-1, additional IR spectra of compounds **1**, **1′**, **2**, **2′**, and **15** in KBr pellets, as well as **1** and **1′** in CCl_4 solution; Figure S-2, raw time-dependent NMR spectra corresponding to data in Figure 3; Figure S-3, IR spectra of Figure 1 in the main text, showing a more complete spectral range from 1800 to 1100 cm^{-1}, along with corresponding spectra of the $^{15}N_2$-labeled compounds (**1′**, **15′**); Figures S-4−S-12, individual spectra in Figure

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S-3A, B, C, D, E, G, I, J, and K, respectively, provided to show the full vertical regions without truncation or overlap (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: mbraiman@syr.edu.

ORCID

Stephen E. Miller: 0000-0002-6988-2465

Mark S. Braiman: 0000-0003-0968-7833

Present Addresses

[‖]Chemistry Department, Christopher Newport University, 1 Avenue of the Arts, Newport News, Virginia 23606, United States (J.M.C.).

[§]EMD Millipore, 80 Ashby Road, Bedford, Massachusetts 01730, United States (Y.X.).

[‡]Chemical Biology Laboratory, National Cancer Institute, National Institutes of Health Fort Detrick, 376 Boyle Street, Frederick, Maryland 21702-1201, United States (S.E.M.).

[†]University of Dodoma, College of Natural and Mathematical Sciences (CNMS), Department of Chemistry, P.O. Box 259, Dodoma (A.T.B.).

Notes

The authors declare no competing financial interest.

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