RNA Cleavage Catalyzed by Amphoteric Bis(acyl)guanidinium Derivatives

by **Michael W. Göbel**[a]), **Christo D. Roussev***[a])[b]), and **Ute Scheffer**[a])

[a]) Institut fur Organische Chemie und Chemische Biologie, Johann Wolfgang Goethe Universitat, Frankfurt am Main, Max-von-Laue-Str. 7, DE-60438 Frankfurt am Main

[b]) Department of Electrochemistry of Biocatalysts and Metal-Air Systems, Institute of Electrochemistry and Energy Systems, Bulgarian Academy of Sciences, bl. 10, BG-1113 Sofia (phone: +359-2-9792736; fax: +359-2-8722544; e-mail: CDRoussev@yahoo.com)

Described herein are two series (twelve compounds each) of very closely related guanidinium-based receptors and their ability to catalyze hydrolytic cleavage of a unique RNA substrate – oligo-dT flanked *trans*-**A**ctivation **R**esponsive region of the HIV-1 mRNA, **TAR** RNA. The significant difference in activities of otherwise very similar compounds is discussed, and direct and indirect evidences supporting our interpretation are presented. The results indicate that improvements in catalytic efficiency could be achieved with little modification of the structure of a relatively weak catalyst, and that a crucial feature could be a finely calibrated interplay between anion-binding and proton-donating abilities.

Introduction. – Detailed understanding of the enzyme mechanisms would provide us, in theory, with the opportunity to test and employ the known principles of small-molecule catalysis in design of artificial, biomimetic reagents with, hopefully, high efficiency for sequence-specific cleavage of RNA or DNA, for example. Beside the pure scientific challenge to compete with and/or mimic Nature, the availability of such man-made nucleases offers potential treatment of a large number of acute diseases at the gene level [1][2]. Many compounds with different functionalities have been tested for their abilities to promote the cleavage of RNA, DNA, or phosphodi(tri)ester models[1]), and a common strategy for the design of artificial nucleases is to incorporate, into a synthetic framework, functional groups known to be present in enzyme active sites and essential for maintaining catalytic activity.

The guanidinium functionality possesses unique anion-binding and multiple H-bond forming properties [4], and it is widely represented in natural molecular recognition and catalytic motifs [4][5]. The active site of staphylococcal nuclease (SNase)[2]) [7], *e.g.*, contains two guanidinium groups (of Arg35 and Arg87) [8] essential for binding [7][9] of the substrate and for catalysis [10], respectively. In an attempt to mimic this important feature of SNase, we [11–15] and others [4][5][16] have designed and studied various bis(alkyl)guanidinium derivatives (general structure shown in *Fig. 1*) as anion receptors and/or catalysts of phosphate transfer.

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[1]) Most commonly used model substrates include dinucleoside monophosphates (NpN), 2-hydroxy ethyl/propyl *p*-nitrophenyl phosphate (HPNP), and various alkyl and aryl phosphates. See, *e.g.*, [3].

[2]) SNase is a very remarkable DNA and RNA hydrolyzing enzyme, which hydrolyze DNA 10^{16}-fold faster than the background reaction [6].

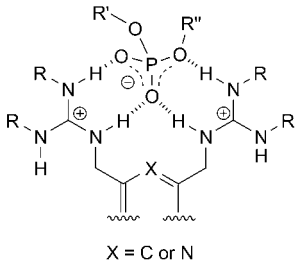

Fig. 1. *General presentation of the phosphodiester receptors studied.* Of course this is not the only possible binding manner of the simplified phosphodiester shown (for a thorough disscusion, see [16a]). The participation (if any) of the central N-atom, when present, has never been invoked in the context of the artificial nucleases investigated so far.

Such geometry and interatomic distances are considered to provide a H-bonding cleft suitable for binding phosphate anions (*Fig. 1*). The increased electrophilicity upon binding could facilitate nucleophilic attack at P-atom, and such electrostatic interaction(s) may contribute to transition-state stabilization as well. None of them, however, requires a proton transfer, though, for the displacement of a typical poor leaving group such as a sugar OH, the assistance from a properly placed electrophile is essential. In many known biocatalysts, this is a *metal ion*, but for enzymes, which do not use an active site metal, a *general acid* is involved. The simple alkyl-guanidinium group (pK_a *ca.* 14) [17] remains protonated over almost the whole pH range and is not generally expected to serve as a general acid, even when the high pK_a value of the leaving group [18] and/or the predicted pK_a values [19][20] of the phosphorane-like transition state are considered. Alternatively, or in combination, the local dielectric constant and/or specific microsolvation could drastically perturb the pK_a values of the functional groups involved in more complex structures, like some conjugates or enzyme active sites [21]. The ability of the alkyl-guanidinium group to act either as an electrostatic or as a general-acid catalyst of phosphate transfer is discussed in [4] and is not the aim of this study.

Results and Discussion. – We set out to test the efficiency of the acyl-guanidinium (pK_a *ca.* 8) [17] function as a general acid/base, when it replaces alkyl-guanidinium in structures with the same geometry (*Fig. 1*). As part of ongoing research on development of *metal-free* artificial nucleases, here we report a series of acyl-guanidinium compounds (*Fig. 2*) and compare their efficiencies in accelerating the hydrolysis of natural RNA substrate(s) under physiological conditions (37°, *Tris*-buffer; pH 7.0). We attempted to tune further the pK_a value of the acyl-guanidinium group by embedding it into different heterocycles, expecting at the same time to gain some information about the contribution (if any) of the central N-atom, when the phenyl ring is replaced by pyridine.

The RNA substrate of our choice for this study was the *trans*-activation-responsive region of the HIV-1 mRNA, TAR RNA. [4][12] Specifically, we used an oligo-dT flanked TAR-RNA chimera [26], and RNA cleavage was analyzed on an *ALFexpress*™ automatic DNA sequencer using labeled RNA probes with a Cy5, a fluorescent carbocyanine dye with an absorption maximum at 643 nm and λ_{em} at 667 nm (see *Exper. Part* and [27]). A DNA spacer of ten deoxythymidines was placed between Cy5 dye and the RNA part to ensure better resolution of the cleavage products and the addition of four deoxynucleosides (T4) at the 3′-end aimed to improve the separation of the

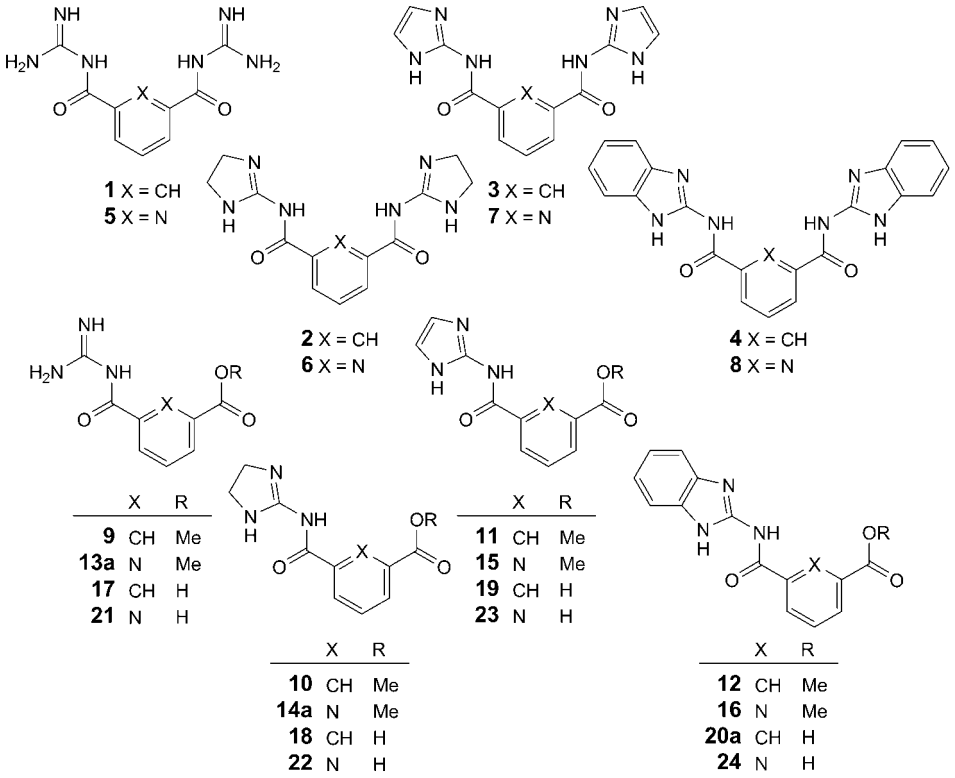

Fig. 2. *Compounds* **1**, **2**, **4**, *and* **7** *described before.* The phosphate anion-binding properties of **1** and **2** [22] were studied before, and the ability of **1** [23] and **7** [24] to catalyze a phosphate transfer were investigated with model DNA and RNA substrates. Compound **4** was synthesized and tested as a UV light absorber [25]. The attempts to isolate (or synthesize) these compounds failed in our hands (see *Discussion*).

substrate and the longest degradation product [27]. Any DNase-like activity would be detected if present, but we observed no cleavage within the oligo-dT regions with any compound tested, with or without Zn^{II} ions (see below).

A common drawback, related to the use of similar RNA assays, is a possible contamination by natural RNase(s). Reports on nonenzymatic RNA cleavage are generally treated with caution, even when careful sterilization and ultra-filtration procedures are described. To rigorously exclude any such artifacts, we carried out in parallel the 'normal' TAR RNA assays, experiments using the corresponding 3′ → 5′-*enantio*-(L)-TAR-RNA as a control [27]. When compounds are described as active, less active, or inactive, this indicates more or less similar activity against both RNA substrates, as well as towards a previously described linear RNA substrate [15] (data not shown). It is noteworthy that none of the active catalysts, described herein, was found to cleave the TAR RNA substrate(s) in a base-specific manner, but all of them showed a certain affinity toward the substrate(s) secondary structure, *i.e.*, predominate

cleavage occurs at the most exposed bulge and loop regions [4][12][15] (unpublished results; work in progress).

The hydrolytic activity (*Table*[3])) of the bis-substituted isophthalic acid derivatives **1**–**4** (*Fig. 2*) increases from the simplest bis(acyl)guanidinium, **1**, to the ethylene-bridged bis[(imidazolinylamino)carbonyl] compound **2**, reaching a maximum with compound **3**. It drops back slightly with bis[(benzimidazolylamino)carbonyl] compound **4**.

We could partially explain the observed order of activity for the isophthaloyl set **1**–**4**, if we tentatively assume that their pK_a values decrease in the order **2** ≥ **1** ≥ **3** ≥ **4**[4]), and this correlates with increasing anion binding affinity and proton donating ability *i.e.:*

- *higher* pK_a, stronger binder, bad proton donor, accordingly weak cleavage activity: **1** and **2**
- *moderate* pK_a, good binder, good proton donor, accordingly better activity: **3**, the most active compound in this study;
- *lower* pK_a, weaker binder, better proton donor, accordingly lower activity, compound **4**.

Similar pK_a-dependence 'rule' could be valid for the compounds **5**–**8** (*Fig. 2*) from the pyridine-2,6-dicarbonyl set as well, but obviously the replacement of the phenyl ring with a pyridine ring has a significant influence on their behavior and hydrolytic activity, respectively. Their activities do not follow any obvious order: the *only* active compound among them was found to be the bis[(imidazolylamino)carbonyl] derivative **7**, and none of the corresponding mono-methyl esters **13**–**16** showed any activity (data not given).

Our interpretation is consistent with a possible charge/proton delocalization within the cleft structure when a central N-atom is present (*Fig. 3*), assuming the mono-protonated species to be the main and the active ionic form at pH 7.0 [31].

Such a charge distribution would reduce, if not inhibit, the unique phosphate anion-binding ability of the guanidinium group, in the sense of less-pronounced or absent 'arginine fork' [4][32]. Additionally, possible charge/lone pair repulsion would reduce the chance of a phosphate anion to enter effectively the cleft. Furthermore, the steric hindrance and the intrinsic pK_a value in each case reflect both the anion-binding

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[3]) As a control, we followed the blank reactions with both (L and D) RNA substrates used for over than six months (we still are following them), and the extrapolated half-life times so far are *ca.* 2×10^3 h (unpublished results). To use this value for correlation, however, is not really correct, since not all the phosphodiester bonds are equally exposed and/or energetically equivalent, especially after single and/or multiple cleavage. Similarly, it would not be correct to multiply the half-life time of UpU (*e.g.*, [28] and refs. cit. therein), with the number of phosphodiester bond in the substrates used, and if one does so the number of the rate acceleration would be VERY impressive.

[4]) We were able to measure only one pK_a value for a very few compounds, namely **2**, **6**, and **7**, and the values found were 5.9, 7.6, and 7.0, respectively. They do not correlate very well with previously reported experimental [23] or calculated values (Solaris V4.67 (© 1994-2003 *ACD*). We used direct titration (*c.f.* [29]), and probably the ΔE values of second/first protonation/deprotonation are insignificant. Other measurements failed most likely as a result of lowering the solubility (microprecipitation) with the change of the pH (see *Discussion*). Furthermore, the compound **4** was synthesized as a UV-light absorber [25], and presumably some of the related ones could possess similar properties.

Table. *Relative Activities*[a]) *of the Isophthalic Acid Derivatives* **1**–**4** *and* **9**–**12**, *and the Pyridine-2,6-dicarboxylic Acid Derivatives* **7** *and* **15** *as a Cleavage Percentages of the Substrate (Cy5-(dT)$_{10}$-GGCCAGAUCUGAGCCUGGGAGCUCUCUGGCC-(dT)$_4$) at 37° in* Tris-*buffer, pH 7.0, after 20 h Incubation.* None of the other compounds synthesized, *i.e.*, **13**–**16**, **17**–**24**, was found to show any detectable activity.

Compound 'bis'	Cleavage [%] of TAR RNA at different catalyst conc. [μM]			Compound 'mono'	Cleavage [%] of TAR RNA at different catalyst conc. [μM]		
	125 μM	250 μM	500 μM		125 μM	250 μM	500 μM
1	n. d.	2.3	4.7	**9**	n. d.	n. d.	3.5
2	n. d.	5.3	10	**10**	n. d.	n. d.	2.7
3	9.7	15.5	16.6	**11**	n. d.	3.3	6.5
4	8	10.8	15.4	**12**	n. d.	3.5	6.1
7	n. d.	7.1	12.7	**15**	n. d.	n. d.	n. d.

[a]) The percentage given accounts for the disappearance of the substraste(s), not for the number of the phosphodiester bonds cleaved. Method we used allowed us to detect any sequence-specific/selective cleavage, if present, the exact positions of scissions, and to characterize the cleavage products. From mechanistic point of view, it would be incorrect to draw conclusions for the particular phosphodiesterase activity of the catalysts used, since one cannot simply divide the overall (total) rate by 31 (in case of our substrate) to obtain the rate for a single phosphodiester bond. They are not equally exposed and solvated, neither are they equal in energy and accessibility. Therefore, we have not converted the cleavage data into rate constants, and we only attempted to compare the cleavage rate enhancement over the estimated half-life time of the substrate[3]). For example, the average cleavage of 15% for 20 h in the presence of the catalyst should correspond to rate acceleration by *ca* 10^3 times .

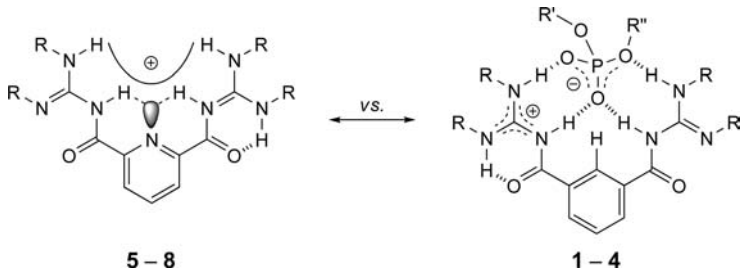

Fig. 3. *Various tautomeric forms of the acyl-Guanidiniums* (see, *e.g.*, [30])

affinity and proton donating ability, and as a result, the derivative **7** is perhaps the only system, which combines appropriate general acid catalysis and effective binding.

With respect to enzymes and binding strength, it was suggested a long time ago [21] that faster enzymes bind their substrates relatively weakly, and, with respect to our current knowledge about the nonenzymatic hydrolysis of RNA with native phosphodiester linkages, the results from all studies support the hypothesis that the decomposition of the intermediate (cleavage of the P–O(5′) bond) is the rate-limiting step (see, *e.g.*, [33][34]). In other words, leaving-group departure and release of the catalyst for turnover requires real proton transfer, while increasing the electrophilicity of the ground-state does not, and even more, the presence of a low-barrier H-bond or

strong electrostatic interaction could be reasons for the inhibition of overall catalytic event, thus generating an 'anticatalyst' [28]. In some cases, such a strong binding might induce micro-aggregates formation and/or micro-precipitation, which eventually could be detected (see below). On the other hand, the observed cleavage could be much higher in case of aggregate formation due to the different microenvironment within the particles, in case they include the substrate.

We tested whether the cleavage rate correlates with a possible micro-precipitation (aggregate formation), if present, by following the RNA-substrate hydrolysis in the presence of **3** and **7** at different concentrations used (*Table*) and by simultaneously monitoring the tentative aggregate formation, which is directly related to changes in the diffusion times with concentration and could be easily monitored by *Fluorescence-Correlation Spectroscopy* (FCS) (for details, see *Exper. Part* and [27]).

Surprisingly or not, we found a very good correlation (*Fig. 4*): the cleavage activity of the pyridine-2,6-dicarbonyl derivative **7** was almost concentration-dependent (with the highest activity of 24% substrate cleavage at 1 mM) without any micro-precipitation or aggregate formation observed (*Fig. 3*), while the activity the corresponding isophthaloyl derivative **3** depended strongly on the concentration (*Fig. 4*), and it dropped drastically with aggregate formation at higher concentrations.

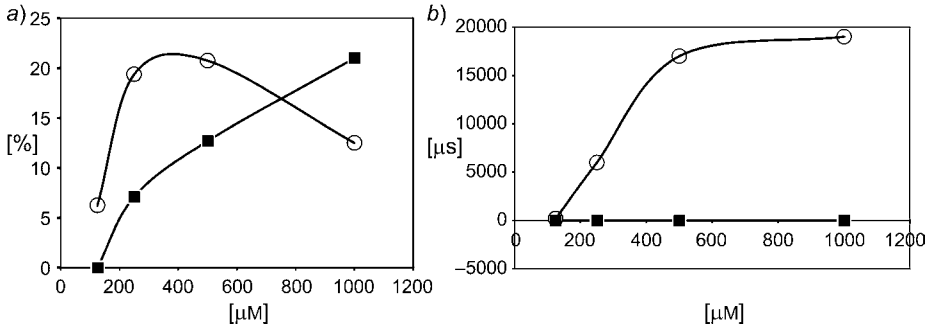

Fig. 4. *a*) *Cleavage data* vs. *concentration of the pyridinium bis-aminoimidazolium derivative* **7** (■) *and the corresponding isophthaloyl derivative* **3** (○). *b*) *Diffusion times, respectively, aggregate formations* [27] vs. *same concentrations as above.* As can be seen, the micro precipitation of **3** starts at *ca.* 250 μM, and it is very well presented at 1 μM, while for **7** there is no change of the diffusion time with increasing concentration, indicating absence of aggregate formation or micro precipitation.

The *only* difference is the presence of the central N-atom and our interpretation is consistent with the possible charge delocalization within the scaffold structure as we mentioned before (*Fig. 3*). Beside the difference in catalytic activity/aggregate formation, the influence of such a proton/charge distribution/network (if present) should be expressed during the syntheses of the receptors **1**–**8** as well, especially if its formation is favored. And indeed, we observed much higher reactivity of the corresponding methyl pyridine-2-carboxylates **13**–**16**, when compared with the isophthaloyl derivatives **9**–**12** toward external nucleophilic substitution. The second aminolysis, for example, during synthesis of the bis-substituted **5** and **6** (*Fig. 2*) was *ca.* 60 times faster than the reaction for **1** and **2** (see *Exper. Part* and [22][23]). Furthermore, we were never able to isolate sufficient amounts of **13** and/or **14** even in

the presence of more than twofold excess of dimethyl pyridine-2,6-dicarboxylic acid, and usually only quite tiny amounts of the mono-substituted **13** and **14** could be detected (TLC, UV or after ninhydrin treatment, HPLC). When some H_2O was added, under such basic conditions, a fast hydrolytic reaction occurred, and the acid **21** could be easily isolated, while boiling of the mono-methyl isophthalates **9**–**12** with 1N NaOH for couple of hours did not lead to complete hydrolysis and in some cases, *i.e.*, **11** and **12**, the hydrolysis was negligible.

The difference in reactivity, of course, could be due to different intrinsic characters of the N-atom, difference in aromaticity, polarizibility, charge accepting/donating abilities, and *etc.*, rather than formation/existence of delocalized proton/charge distribution *via* the cleft structure as tentatively proposed.

On the other hand, such a charge/proton network could favor proton transfer from the attacking nucleophile to the leaving group in the transition state (*Fig. 5*) and if it is true, a simple construct like **7** could fulfill the role of the 'dream' catalyst, once positioned appropriately in a close proximity.

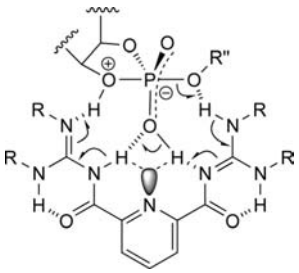

Fig. 5. *Possible way to product(s) distribution* (similar would be the reverse reaction pathway)

The basic structure (*Fig. 5*) is relatively flexible and can match at least in part both negative charges of the phosphorane-like transition state; but most likely only one O-atom could be effectively 'captured' in the acidic environment in the middle. In the active site of metalloenzymes such as SNase [9] or alkaline phosphatase [35], divalent metal ions such as Ca^{II} and Zn^{II} were found to cooperate with the guanidinium groups during phosphate transfer. Recently, *Anslyn* and co-workers [36] reported on RNA-dimer hydrolysis promoted by an artificial metallonuclease with two appended guanidinium 'arms', and a Zn^{II} center effectively replacing the central N-atom (*Fig. 5*). Similarly, we investigated the possible cooperation of Zn^{II} with receptors **1**–**8**. We found no detectable differences in the cleavage rates and/or specificity in the presence of Zn^{II} at various concentrations (data not given).

Binding strength is directly connected with turnover. The RNase-like activity of the receptors **2**, **3**, **4**, and **7** demonstrates a high affinity for the phosphate anions of the RNA backbone, and product inhibition, rather than turnover is, therefore, the most likely result. Our only (indirect) evidence for turnover at this stage is that the catalysts were still active in the presence of equimolar phosphate buffer. Under these conditions, their activity was, however, reduced by *ca.* 20% (data not shown).

It is worth noting that the benzimidazolyl derivatives **4**, **8**, **12**, and **16** are only sparingly soluble in water at room temperature so that the assays with these compounds

were carried out in the presence of 20% (*v*/*v*) MeOH. We did not study medium effects in any detail, but as a control we tested all other compounds in 20% MeOH solution. No significant differences in activity were observed (data not given), when compared with those in pure aqueous media.

Conclusions. – The different activities of very closely related structures such as **1**–**8** to promote RNA hydrolysis demonstrate the complexity of the factors responsible for overall catalysis by similar simple nucleases. The results indicate that improvements in catalytic efficiency could be achieved with little modification of the structure of a relatively weak catalyst, and that a crucial feature could be a finely calibrated interplay between anion binding- and proton-donating abilities. We postpone further mechanistic speculations until more data are available and we hope to attract the attention of the scientists exploring properties of guanidinium-based receptors with more flexible or rigid scaffolds, and to inspire new ideas on future development in the field of artificial nucleases and/or specific anion receptors.

Experimental Part

General. All commercial solvents and reagents were used as purchased except 2-amino-1*H*-imidazole (AI), which was used as the free base, obtained from the commercial sulfate (*Aldrich*, *Fluka*). 2-Amino-4,5-dihydro-1*H*-imidazole was synthesized by coupling of ethylenediamine and CNBr in MeOH according to the standard procedure. Anal. TLC: alumina plates precoated with silica gel *60* F_{254} (*Merck*) and developed with: system *A* (CH_2Cl_2/MeOH/aq. NH_3 8:2:0.4) or system *B* (CH_2Cl_2/MeOH/abs. AcOH 7:3:1). Flash chromatography (FC): silica gel *Merck-60* (SiO_2; 0.040 ± 0.063 mm) eluted with the same (or similar) mobile phase as described above. Filtration: *Celite 535* (*Fluka*). NMR Spectra: *Brucker AVANCE* 250 MHz; chemical shifts (δ) in ppm rel. to TMS as external standard. ESI-MS: *Fisons Instrument VG Platform II* with or/and without addition of NH_3 or acid.

RNA-Cleavage Assays. For additional information on this section or more details, please contact directly Dr. *Ute Scheffer* at umscheffer@aol.com or at the University address *via* regular mail.

Materials and Methods. Chemicals for polyacrylamide gel electrophoresis (PAGE) were purchased from *Roth* (DE-Karlsruhe). *NAP-10* Columns, blue dextran 2000, and the *N*-hydroxysuccinimide active ester of Cy5 (PA15101) were purchased from *Amersham Biosciences* (SE-Uppsala). Amino linker was obtained from *Glen Research* (Sterling, VA, USA), recombinant ribonuclease inhibitor *RNasin*® (N2511) was from *Promega*, RNase A from *Sigma-Aldrich*, and RNase T1 from *MBI Fermentas.*

The sample of an L-TAR RNA substrate was a generous gift from Prof. *S. Pitsch* (Institut de Chimie Moleculaire et Biologique, Ecole Polytechnique Federale de Lausanne (EPFL), BCH-LCAN, CH-1015 Lausanne), and we thank to his group for their cooperation.

Inactivation of RNases [27c]. RNA Cleavage was performed in an RNase-free environment. Glassware was warmed for 6 h at 180°. During all experimental steps, gloves were worn, and sterile techniques were used. Plastic ware and tubes were treated with diethyl pyrocarbonate (DEPC) to ensure that they were RNase-free. Solns. were prepared by mixing molecular-biology-grade powdered reagents in DEPC-treated ultra pure H_2O.

Ultrafiltration. To remove possible RNase contamination from catalysts, an ultrafiltration step (*Microcon YM3*, *Millipore*) was carried out as described by the manufacturer. After ultrafiltration, the filtrate was used for RNA-cleavage experiments. The retentate was discarded.

Purification of Cy5-Labeled Oligonucleotides. The Cy5-labeled oligonucleotides were purified by denaturing PAGE (16% monomer, 7M urea) to remove prematurely terminated products. After PAGE, the bands of interest were excised; the gel fragments were transferred to a nuclease-free tube and submerged with elution buffer (500 mM $AcONH_4$, 0.1% SDS, and 2 mM EDTA) and incubated under vigorous shaking overnight at r.t. To remove the gel fragments, '*Quantum Prep Freeze'N Squeeze*' spin

columns (DE-*BioRad*, Munich) were used. Desalting of the Cy5-labeled oligonucleotides was carried out in two steps: after *YM3* ultrafiltration, the retentate was diluted to 1 ml with DEPC-treated H_2O and loaded onto a *NAP-10* column. The pooled fractions were lyophilized to dryness, and the pellet was dissolved in DEPC-treated H_2O to give a conc. of *ca.* 0.5 μg/μl.

RNA-Cleavage Assay. The 10-μl assays had the indicated catalyst concentration (0.1 ± 10 μM) and 120 ± 140 nM of the appropriate Cy5-labeled RNA. A 50-mM *Tris*-HCl buffer containing 0.01% SDS was used, and cleavage reactions were carried out at pH 7.0, unless otherwise specified. The SDS prevents nonspecific binding of the RNA to the tube walls. Incubation was performed at 37° for 20 h. The final conc. of ribonuclease inhibitor (RNasins), if applied, was 1 unit per μl.

PAGE. Prior to electrophoresis, one volume of loading buffer (5 mg/ml blue dextran in formamide) was added to each sample, and 10-μl aliquots were loaded on the gel. The oligonucleotide fragments were separated by denaturing PAGE (16% monomer, 7M urea) on a DNA-sequencing device (*ALFexpress*, *Amersham Biosciences*). Running conditions were 1500 V and 60 mA maximum at constant 30 W, 55°, 2-s sampling interval, and 350-min running time. Electropherograms were analyzed with the AlleleLinks 1.01 software package (SE-*Amersham Biosciences*, Uppsala). The peak areas under the curves were added, and the percentage of degraded RNA was calculated. Multiple cleavage reactions were disregarded in this system. All data were averaged over a minimum of five experiments.

Fluorescence-Correlation Spectroscopy (FCS). Measurements were carried out with a *ConfoCor 2* FCS instrument (DE-*Carl Zeiss*, Jena) equipped with an *Axiovert 200 M* microscope containing a laser-adapted *Zeiss C-Apochromat 40 x/1.2 W corr* H_2O-immersion objective. Cover slips (24×60 mm, DE-*Roth*, Karlsruhe) served as sample carriers, and, for calibration of the instrument, free Cy5 dye was used. Fluctuation measurements, which are conducted in real time to give an autocorrelation curve, and further analyses, such as determination of the average number and the diffusion time of the fluorescent particles in the confocal volume were performed with the Fluorescence Correlation Microscope ConfoCor 2 Software version 3.2 SP1.

Sample Measurements. FCS Measurements were performed under conditions comparable to those used for RNA-cleavage experiments. The RNA substrates were replaced by a mixture of a Cy5-labeled $T_{20}U$ probe and an unlabeled DNA oligonucleotide [26]. A final volume of 30 μl contained 25 nM Cy5-labeled $T_{20}U$, 175 nM unlabeled DNA oligonucleotide, 125 – 1000 μM of **3** and **7**, resp. in 50 mM *Tris* · HCl, 0.01% SDS buffer (pH 7.0). The incubation step (20 h, 37°) was omitted, and all FCS measurements were performed at 37°. A He/Ne-laser at 633 nm was used as the excitation source. Each sample (30 μl droplet) was measured either five times for 45 s or ten times for 10 s.

Determination of pK_a Values. Spectrophotometric titrations were carried out with a 50-μM soln. of the catalysts in 100 mM NaH_2PO_4. The pH and the absorbance at the appropriate wavelength (determined from a difference spectrum of the acidic and basic form of the catalyst) were measured after each addition of 2 μl of 5N NaOH ([32]).

Synthesis, Isolation, and Characterization of the (Acyl)guanidinium Derivatives Tested to Promote TAR-RNA (HIV 1) Hydrolysis in this Study. Materials, Reagents, and General Guidelines. All commercial solvents and organic bases 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), Et_3N, EtN^iPr_2, *N*-ethylmorpholine, *N*-methylmorpholine, pyridine) were used as purchased. After considerable experimental efforts, we found that a 'key to success' for all reactions described here in terms of solubility, efficiency, workup and purification is the use of the guanidinium reagents (guanidine itself (Gua), 2-amino-4,5-dihydro-1*H*-imidazole (dhAI, from, 2-amino-1*H*-imidazole (AI), 2-amino-1*H*-benzimidazole (ABnIm)) as free bases. Although the syntheses of **1** – **24** do not look that challenging at first glance, as we thought at the beginning, we were not able to reproduce the described (one-sentence experimental) procedure in [23] for **7**, starting with the finding, that the *p*-toluenesulfonate salt of AI was virtually not soluble in any appropriate solvent (CH_2Cl_2, MeCN, THF, and very poorly soluble even in pure pyridine) in presence or absence of any of the org. bases mentioned above. AI as a free base is a very well soluble in some aprotic polar org. solvents as MeCN, *e.g.*, and many protic solvents such as MeOH. It could be easily prepared by mixing equimolar aq. solns. of commercial AI sulfate (*Aldrich*, *Fluka*) and $NaHCO_3$. After evaporation under reduced pressure (bath temp. under 40°) the pink-reddish solid was left for at least 24 h under high vacuum on liophilizator (*Caution!* AI is VERY light-, air-, and moisture-sensitive (especially as a free base and in soln.), and it is recommended to use dark flasks or the usual ones wrapped with foil). Pure AI

is obtained (prior to use) by dry extraction in dry MeCN by thorough sonification (3×50 ml of MeCN for 2 g of starting sulfate) of the solid residue. After filtration (*Celite*), the combined yellowish-to-red filtrates are evaporated to give a mobile liquid of pure AI in quant. yield. (*Caution!* all the procedures should be carried out under inert, dry atmosphere (N_2, Ar)).

Free bases of guanidine (Gua) and 2-amino-4,5-dihydro-1*H*-imidazole (dhAI) could be obtained by treatment of their salts with equimolar soln. of Na in MeOH. Accordingly, a soln. of guanidine hydrochloride (*Fluka*) in MeOH or 2-amino-4,5-dihydro-1*H*-imidazole (dhAI, prepared by coupling of ethylenediamine and CNBr in MeOH according to the standard procedure) was treated with freshly prepared equimolar soln. of Na in MeOH. After 1 h stirring, the soln. was filtered (normal filter paper, no vacuum, especially in the case of Gua, where a jelly precipitate results), evaporated, dried by co-evaporation from MeCN/MeOH, dissolved in dry MeOH and filtered again from the residual salt. If necessary, the procedure is repeated. In case the filtration is very slow or impossible, one could centrifuge the mixture and simply decant the supernatant. Free guanidine is virtually not soluble in MeCN, while 2-amino-4,5-diyhdro-1*H*-imidazole is partially soluble, but both compounds are well soluble in MeOH, which is far enough for the aminolysis reaction(s) further.

The commercial ABnIm (*Lancaster*) is available as free base.

General Synthetic Procedure I. Compounds **3**, **4**, **7**, and **8** were synthesized by aminolysis of the corresponding bis-acyl chlorides of the isophthalic and pyridine-2,6-dicarboxylic acids with the appropriate guanidine derivatives AI (free base) and ABnIm, resp., in the presence of org. base. We obtained better results with relatively weak bases such as pyridine (pK_a *ca.* 5) and *N*-methylmorpholine (pK_a *ca.* 8), rather than with Et_3N or EtN^iPr_2. To a soln. of the guanidinium derivative (3 equiv.) in MeCN (or acetone for ABnIm) and 5 equiv. of pyridine was added slowly (on cooling, ice-bath), a soln. of the bis-acyl chloride (1 equiv.) in CH_2Cl_2. Then, the mixture was stirred at r.t. for *ca.* 2–4 h, and the reaction was monitored with TLC (system *A* and/or *B*). After completion, the reaction was quenched with MeOH or aq. pyridine, whereupon satisfactory amounts of the mono-substituted compounds **11**, **12**, **15**, **16**, **19**, **20**, and **23**, and **24** could be isolated along with the target bis-derivative, regardless of the amount of the guanidine reagent used (for details of the workup procedure(s), see below).

General Synthetic Procedure II. Compounds **1**, **2**, **5**, and **6** were synthesized by aminolysis of the dimethyl esters of the isophthalic and pyridine-2,6-dicarboxylic acids with the appropriate guanidine derivatives, 2-amino-4,5-dihydro-1*H*-imidazole and guanidine, resp. Accordingly, to a soln. of the corresponding dimethyl ester in a minimum volume of MeCN or MeOH/MeCN was added a soln. of 3 equiv. (up to 5 equiv.) of the corresponding amino compound(s) (as a free base; see bellow) in the same solvent. Then, the mixture was heated and stirred at 60–80° under light vacuum, and the reaction was monitored by TLC (system *A* and/or *B*). After completion (*ca.* 1 h for the pyridine-2,6-dicarboxylic acid and 4–6 h for the isophthalic acid derivatives), the mixture was evaporated to dryness and worked up as described below. The yields for **1** and **2** were usually around 15–20% [21][22], while those of the reactions for **5** and **6** are almost quant.

General Workup and Purification(s). Pure empirically, and after considerable experimental work, we found fistr, all bis(acyl)guanidinium derivatives **1**–**8** in general, are *not soluble* in CH_2Cl_2, MeCN, and even MeOH as *free bases*! Therefore, after evaporation of the mixture to dryness, the solid residue was suspended in CH_2Cl_2 and thoroughly extracted by sonification several times. Usually, it is hard to filter, so the suspension is centrifuged, decanted and again resuspended. Consequently, the same procedures were repeated with MeCN as an extracting agent and MeOH (if the solubility allows). Appropriate extracts (TLC; System *A* or *B*) are evaporated, pre-adsorbed on SiO_2 (if necessary), and chromatographed for mono-Me derivatives **9**–**12**, **15**, and **16**.

The solid residue was suspended in a small volume of MeOH, treated with MeOH/HCl until a clear soln. resulted and then it was evaporated to dryness, which gave pure or almost pure bis(acyl)guanidinium derivative (*i.e.*, **1**–**8**) as a HCl salt (very hygroscopic in general). The small impurities are usually traces of starting guanidinium reagent and/or org. base used, and they could be easily removed by FC on SiO_2 (after pre-adsorbtion of the sample) using system *B*.

Isophthaloyl Derivatives as HCl Salts. Amino{[3-(carbamimidoylcarbamoyl)benzoyl]amino}methaniminium Chloride (**1**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 12.19 (br. *s*, 2 CONH); 8.7 (*t*, $J = 1.8$, H–C(2), isophth); overlaps with the absorbance at 8.66 (*s*, 8 H, Gua); 8.49 (*dd*, $J = 8$, 1.8, H–C(4) and H–C(6),

isophth); 7.83 (*t*, *J* = 7.8, H–C(5), isophth). ESI-MS (pos.): 248.8 (100, $[M+H]^+$), 249.9 (13.6, $[M+2\ H]^+$).

*4,5-Dihydro-2-{[3-(4,5-dihydro-1*H*-imidazol-2-ylcarbamoyl)benzoyl]amino}-1*H*-imidazol-3-ium Chloride* (**2**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 13 (br. *s*, 2 CONH); 9.1 (*s*, 2 NH, dhIm); 8.67 (*t*, *J* = 1.57, H–C(2), isophth); 8.45 (*dd*, H–C(4) and H–C(6), isophth); 7.84 (*t*, *J* = 7.8, H–C(5), isophth); 3.8 (*s*, 4 CH_2, dhIm). ESI-MS (pos.): 301.1 (100, $[M+H]^+$), 302.1 (51.7, $[M+2\ H]^+$), 303.2 (33.3, $[M+3\ H]^+$), 337.2 (5.3, $[M+Cl]^+$).

*2-{[3-(1*H*-Imidazol-2-ylcarbamoyl)benzoyl]amino}-1*H*-imidazol-3-ium Chloride* (**3**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 13.3 (br. *s*, 4 H), 8.77 (*s*, H–C(2), isophth); 8.47 (*dd*, *J* = 8.1, 1.8, H–C(4) and H–C(6), isophth); 7.79 (*t*, *J* = 7.8, H–C(5), isophth); 7.34 (*s*, 4 CH of Im). ESI-MS (pos.): 296.9 (100, $[M+H]^+$), 298 (17.3, $[M+2\ H]^+$), 332.9 (8.1, $[M+Cl]^+$).

*2-{[3-(1*H*-Benzimidazol-2-ylcarbamoyl)benzoyl]amino}-1*H*-3,1-benzimidazol-3-ium Chloride* (**4**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 13.3 (br. *s*, 4 H), 8.87 (*t*, *J* = 1.57, H–C(2), isophth); 8.46 (*dd*, *J* = 7.9, 1.8, H–C(4) and H–C(6), isophth); 7.83 (*t*, *J* = 7.8, H–C(5), isophth); 7.67 (*q*, *J* = 4.5, 1.4, 2 H–C(4) and 2 H–C(7), BnIm), 7.37 (*q*, *J* = 4, 1.4, 2 H–C(5) and 2 H–C(6), BnIm). ESI-MS (pos.): 397.3 (100, $[M+H]^+$), 398.1 (25.5, $[M+2\ H]^+$), 433 (11, $[M+Cl]^+$).

Pyridine Derivatives as HCl Salts. Amino({[6-(carbamimidoylcarbamoyl)pyridin-2-yl]carbonyl}-amino)methaniminium Chloride (**5**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 12.16 (br. *s*, 2 CONH); 8.86 (br. *d*, *J* = 41, 8 H, Gua); 8.56 (*d*-like, *J* = 8.4, H–C(3) and H–C(5), Py); 8.44 (*q*-like, *J* = 7.3, 1.2, H–C(4), Py). ESI-MS (pos.): 250 (100, $[M+H]^+$), 251 (14, $[M+2\ H]^+$), 285.8 (28.3, $[M+Cl]^+$).

*4,5-Dihydro-2-({[6-(4,5-dihydro-1*H*-imidazol-2-ylcarbamoyl)pyridin-2-yl]carbonyl}amino)-1*H*-imidazol-3-ium Chloride* (**6**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 12.77 (*s*, 2 CONH); 9.31 (*s*, 2 NH, dhIm); 8.56 (*d*-like, *J* = 6.9, H–C(3) and H–C(5), Py); 8.46 (*q*-like, *J* = 7.8, 1.3, H–C(4), Py); 3.86 (*s*, 4 CH_2, dhIm). ESI-MS (pos.): 301.9 (100, $[M+H]^+$), 303 (43, $[M+2\ H]^+$) 304.2 (23.5, $[M+3\ H]^+$), 338.1 (12, $[M+Cl]^+$).

*2-({[6-(1*H*-Imidazol-2-ylcarbamoyl)pyridin-2-yl]carbonyl}amino)-1*H*-imidazol-3-ium Chloride* (**7**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 13.21 (br. *s*, 4 H); 8.58 (*d*-like, *J* = 6.6, H–C(3) and H–C(5), Py); 8.48 (*q*-like, *J* = 1.2, 7.6, H–C(4), Py); 7.45 (*s*, 4 CH, Im). ESI-MS (pos.): 298 (100, $[M+H]^+$), 299 (17.3, $[M+2\ H]^+$), 333.9 (10, $[M+Cl]^+$).

*2-({[6-(1*H*-Benzimidazol-2-ylcarbamoyl)pyridin-2-yl]carbonyl}amino)-1*H*-3,1-benzimidazol-3-ium Chloride* (**8**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 13.7 (br. *s*, 4 H); 8.65 (*d*-like, *J* = 8.1, H–C(3) and H–C(5), Py); 8.52 (*t*-like, *J* = 7.8, H–C(4), Py); 7.79 (*q*, *J* = 1.5, 4.5, 2 H–C(4) and 2 H–C(7), BnIm), 7.45 (*m*, 2 H–C(5) and 2 H–C(6), BnIm). ESI-MS (pos.): 398 (100, $[M+H]^+$), 399 (25.1, $[M+2\ H]^+$), 434 (18.2, $[M+Cl]^+$).

Monomethyl Isophthalates. Amino{[3-(methoxycarbonyl)benzoyl]amino}methaniminium Chloride (**9**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 12.1 (br. *s*, 1 CONH); 8.66 (br. *s*, 4 H, Gua (overlaps with H–C(2) of isophth)); 8.6 (*s*, H–C(2), isophth); 8.45 (*d*, *J* = 8.4, H–C(6), isophth); 8.26 (*d*, *J* = 8.1, H–C(4), isophth); 7.77 (*t*, *J* = 7.8, H–C(5), isophth); 3.9 (*s*, MeO). ESI-MS (pos.): 221.8 (100, $[M+H]^+$), 222.9 (12.3, $[M+2\ H]^+$).

*4,5-Dihydro-2-{[3-(methoxycarbonyl)benzoyl]amino}-1*H*-imidazol-3-ium Chloride* (**10**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 12.95 (br. *s*, CONH); 9.14 (*s*, 2 NH, diIm); 8.6 (*t*, *J* = 1.7, H–C(2), isophth); 8.43 (*dt*, *J* = 8.7, 1, H–C(6), isophth); 8.26 (*dt*, *J* = 8.1, 1.4, H–C(4), isophth); 7.77 (*t*, *J* = 7.8, H–C(5), isophth); 3.92 (*s*, MeO), 3.8 (*s*, 2 CH_2, diIm). ESI-MS (pos.): 247.8 (100, $[M+H]^+$), 248.9 (15.8, $[M+2\ H]^+$).

*2-{[3-(Methoxycarbonyl)benzoyl]amino}-1*H*-imidazol-3-ium Chloride* (**11**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 13.1 (br. *s*, 2 H); 8.62 (*s*, H–C(2), isophth); 8.36 (*d*, *J* = 8.1, H–C(6), isophth); 8.26 (*d*, *J* = 7.8, H–C(4), isophth); 7.79 (*t*, *J* = 7.8, H–C(5), isophth); 7.33 (*s*, 2 CH, Im); 3.93 (*s*, MeO). ESI-MS (pos.): 246 (100, $[M+H]^+$), 247 (14.8, $[M+2\ H]^+$), 491.3 (6.1, $[2\ M+H]^+$).

*2-{[3-(Methoxycarbonyl)benzoyl]amino}-1*H*-3,1-benzimidazol-3-ium Chloride* (**12**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 12.48 (br. *s*, 2 H); 8.77 (*t*, *J* = 1.6, H–C(2), isophth); 8.4 (*dt*, *J* = 7.8, 1.57, H–C(6), isophth); 8.16 (*dt*, *J* = 7.7, 1.4, H–C(4), isophth); 7.69 (*t*, *J* = 7.6, H–C(5), isophth), 7.5 (*q*, *J* = 4.5, 1.4, H–C(4) and H–C(7), BnIm); 7.23 (*q*, *J* = 4.5, 1.4, H–C(5) and H–C(6), BnIm); 3.93 (*s*, MeO). ESI-MS (pos.): 296 (100, $[M+H]^+$), 297 (18.7, $[M+2\ H]^+$), 591.4 (11.3, $[2\ M+H]^+$).

*Methyl Pyridine-2-carboxylates. 2-({[6-(Methoxycarbonyl)pyridin-2-yl]carbonyl}amino)-1*H*-imidazol-3-ium Chloride* (**15**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 12.9 (br. *s*, 2 H); 8.23–8.1 (*m*, H–C(3), H–C(4) and H–C(5), Py); 7.12 (*s*, 2 CH, Im); 3.7 (*s*, MeO). ESI-MS (pos.): 246.8 (100, $[M+H]^+$), 247.8 (13.1, $[M+2\,H]^+$).

*2-({[6-(Methoxycarbonyl)pyridin-2-yl]carbonyl}amino)-1*H*-3,1-benzimidazol-3-ium Chloride* (**16**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 11.9 (br. *s*, 1 H), 8.45–8.33 (*m*, H–C(3), H–C(4), and H–C(5), Py); 7.52 (*q*, *J* = 1.6, 4.7, H–C(4) and H–C(7), BnIm); 7.17 (*m*, H–C(5) and H–C(6), BnIm); 3.99 (*s*, MeO). ^{1}H-NMR ((D$_4$)MeOD, 250 MHz): 8.57 (*d*, *J* = 8.7, H–C(3), Py); 8.46 (*d*, *J* = 6.5, H–C(5), Py); 8.33 (*t*, *J* = 7.8, H–C(4), Py); 7.75 (*q*, *J* = 1.6, 4.7, H–C(4) and H–C(7), BnIm); 7.5 (*m*, H–C(5) and H–C(6), BnIm); 4.09 (*s*, MeO). ESI-MS (pos.): 296.9 (100, $[M+H]^+$), 297.9 (17.6, $[M+2\,H]^+$), 593.2 (6, $[2\,M+H]^+$).

Benzenecarboxylic Acid HCl Salts. Amino[(3-carboxybenzoyl)amino]methaniminium Chloride (**17**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 13.4 (br. *s*, COOH); 11.9 (br. *s*, CONH); 8.6 (br. *s*, 4 H, Gua (overlaps with H–C(2), C$_6$H$_4$)); 8.57 (*s*, H–C(2), C$_6$H$_4$); 8.39 (br. *t*, *J* = 6.6, H–C(4), C$_6$H$_4$); 8.25 (*d*, *J* = 7.8, H–C(6), C$_6$H$_4$); 7.75 (*t*, *J* = 7.6, H–C(5), C$_6$H$_4$). ESI-MS (pos.): 207.7 (100, $[M+H]^+$), 208.8 (10.7, $[M+2\,H]^+$), 414.9 (4.2, $[2\,M+H]^+$).

*2-[(3-Carboxybenzoyl)amino]-1*H*-imidazol-3-ium Chloride* (**19**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 12.55 (br. *s*, 2 H); 8.68 (*s*, H–C(2), C$_6$H$_4$); 8.28 (*d*, *J* = 7.9, H–C(6), C$_6$H$_4$); 8.13 (*d*, *J* = 8, H–C(4), C$_6$H$_4$); 7.65 (*t*, *J* = 7.7, H–C(5), C$_6$H$_4$); 6.99 (*s*, 2 CH, Im). ESI-MS (pos.): 231.9 (100, $[M+H]^+$), 232.9 (13.5, $[M+2\,H]^+$), 266.4 (5.5, $[M+Cl]^+$).

Pyridine-2-carboxylic Acid HCl Salts. Amino{[(6-carboxypyridin-2-yl)carbonyl]amino}methaniminium Chloride (**21**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 13.25 (br. *s*, COOH); 12 (br. *s*, CONH); 8.94 (br. *s*, 2 H, Gua); 8.64 (br. *s*, 2 H, Gua); 8.4 (*m*, H–C(3), H–C(4), and H–C(5), Py). ESI-MS (pos.): 208.6 (100, $[M+H]^+$).

*6-(4,5-Dihydro-1*H*-imidazol-2-ylcarbamoyl)pyridine-2-carboxylic Acid* (**22**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 13 (br. *s*, 1 H); 12.1 (br. *s*, 1 H); 8.37 (*m*, H–C(3), H–C(4), and H–C(5), Py); 3.86 (*s*, 2 CH$_2$, dhIm). ESI-MS (pos.): 235 (100, $[M+H]^+$), 469 (19, $[2\,M+H]^+$).

*6-(1*H*-Imidazol-2-ylcarbamoyl)pyridine-2-carboxylic Acid* (**23**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 13.3 (br. *s*, 1 H); 12.9 (br. *s*, 1 H); 8.4–8.34 (*m*, H–C(3), H–C(4), and H–C(5), Py); 7.1 (*s*, 2 CH, Im). ESI-MS (pos.): 232.9 (100, $[M+H]^+$), 233.8 (13.1, $[M+2\,H]^+$), 464 (27, $[2\,M+H]^+$).

*2-{[(6-Carboxypyridin-2-yl)carbonyl]amino}-1*H*-3,1-benzimidazol-3-ium Chloride* (**24**). ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 12.5 (br. *s*, 1 H), 8.54–8.37 (*m*, H–C(3), H–C(4), and H–C(5), Py); 7.67 (*q*, *J* = 1.5, 4.5, H–C(4) and H–C(7), BnIm); 7.32 (*m*, H–C(5) and H–C(6), BnIm). ESI-MS (pos.): 283 (44.6, $[M+H]^+$), 565.3 (100, $[2\,M+H]^+$), 566.3 (34, $[2\,M+H]^+$).

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Supporting Information Available. Synthetic procedures, characterization of the compounds, conditions for the cleavage assays, and FCS studies are available.

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