164. Hydrolytical Cleavage of TAR-RNA, the *trans*-Activation Responsive Region of HIV-1, by a Bis(guanidinium) Catalyst Attached to Arginine

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(25.VII.96)

Guanidinium compounds imitating the bis(arginine) structural motif of staphylococcal nuclease (*e.g.* **3**) are known to be powerful catalysts for phosphoryl transfer reactions in dipolar aprotic solvents. Compound **3** also accelerates the hydrolysis of RNA (H_2O, pH 7). However, due to diminished substrate affinity in H_2O, the rate effects are less pronounced in aqueous than in aprotic solution. To test if a synthetic ribonuclease may be derived from the bis(guanidinium) moiety of **3** by the addition of RNA-binding substructures, the TAR sequence of HIV-1 was chosen as a target. The arginine residue of compound **4** serves as an extremely simplified mimic of tat, a protein responsible for boosting the viral transcription by complex formation with TAR. Here, we present the synthesis of **4** and its ability to bind and to cleave efficiently the truncated TAR sequence **1**. In addition, the synthesis of an acridine arginine conjugate, **19**, is reported in preliminary form. Compound **19** associates with **1** and completely blocks the cleavage induced by **4**.

1. Introduction. – The *trans*-activation responsive element (TAR) is a sequence of 59 nucleotides located at the 5′-end of all HIV-1 mRNAs [1]. TAR forms a stem-loop structure containing a 3-nucleotide bulge (*Fig. 1*), and it associates with the viral regulatory protein tat. The tat-TAR interaction has been shown to be essential for the efficient transcription of the viral genome [2–4]. Therefore, the development of agents which may interfere with the protein-RNA recognition is a potential strategy to control the proliferation of HIV [5–13]. Although the conventional antisense approach [14] is complicated in the case of TAR by the stability of the loop structure, selective recognition by oligonucleotides has been achieved using complementary hairpin loops [15]. In addition to arginine derivatives (see below) and intercalators [16] [17], aminoglycoside antibiotics [8] and tetrahydropyrimidines produced by *Streptomyces parvulus* are efficiently bound by TAR [9].

The RNA affinity of the tat-protein is mainly caused by a sequence of basic amino acids [18] (-R-K-K-R-R-Q-R-R-R-). While the lysine residues contribute to nonspecific electrostatic interactions, the principal contact mediating tat-TAR recognition is formed by a single arginine placed in the middle of the basic region [19] [20]. Model peptides containing only the cationic amino acids bind to TAR quite efficiently [19] [21–23]. Even arginine and guanidinium show considerable affinity with K_d's of *ca.* 1.5 mM [22] [24]. The cation, however, should not be modified [24]. The presence of additional alkyl residues at the guanidinium group is known to abolish the RNA interaction. Based on NMR spectra of the truncated TAR sequence **1** associated with arginine amide **2**, a first structural model of the complex was proposed [25] [26]. Further biochemical data [27–32] and a recent comprehensive NMR study [33] have led to a modification and extension of these ideas. The guanidinium ion of arginine binds to N(7) of guanosine(26) (G(26)),

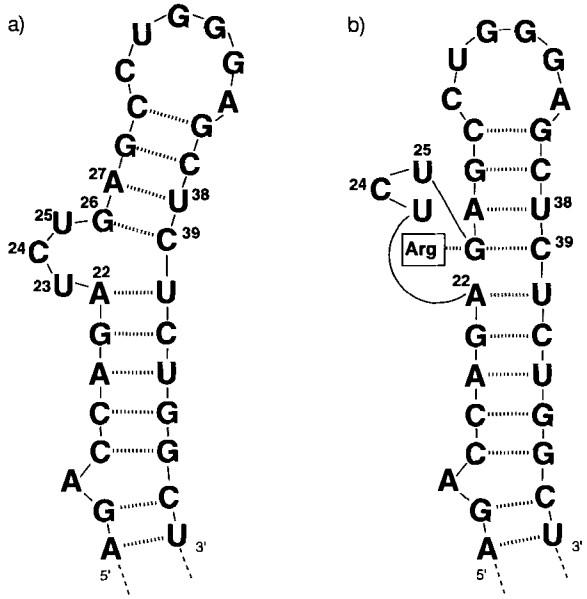

Fig. 1. a) *Wild-type sequence of HIV-1 TAR.* b) *Schematic representation of the structural changes imposed on TAR when complexed by arginine derivatives*

most probably in a *Hoogsteen*-like orientation which resembles a C^+–G–C base triplet. Three phosphodiester groups located close to G(26) are essential for complex formation. In contrast to the original model [19] ('arginine fork'), they are not directly bound to the guanidinium ion. The stacked arrangement of the three bulge nucleotides is disrupted upon complexation and U(23) moves in direct proximity of A(27) and G(26). Comparable structural changes were induced by arginine amide and a 37-mer model peptide of tat.

To interfere with viral proliferation, it would be favorable to combine RNA complexation with a nonreversible cleavage step. This goal has been achieved by the use of ribozymes [34]. However, the simplicity of the guanidinium-RNA interaction suggests that small synthetic molecules could be applied for the same purpose. Indeed, conjugates of basic peptides with Cu^{II} complexes [35] or psoralen [36] are able to destroy TAR by oxidation or photochemical cross-linking (see also [10] [11] [32]). Because these mechanisms may induce DNA damage too, further studies with more selective cleaving agents are justified. Today, synthetic and semisynthetic catalysts are known which cut RNA strictly by hydrolysis. They are based on enzyme conjugates [37–39], imidazoles [40–45], amines [46–48], metal- [49–57] or guanidinium ions [58] [59].

The knowledge of enzyme structures may give a clue how artificial catalysts could be constructed. Our work relies on the bis(guanidinium) motif found in the active site of staphylococcal nuclease which binds and activates the substrate by forming ion-pair complexes with two arginine side chains. By adopting this strategy [60] [61], we have prepared a series of simple bis(guanidinium) compounds as potential catalysts (*e.g.* **3**; *Fig. 2*): in dipolar aprotic solvents nucleophilic substitutions of a model phosphodiester are accelerated by factors up to 4800 [62] [63]. Similar results have been obtained by other

scientists [59] [64] [65]. With RNA substrates, **3** is supposed to facilitate the intramolecular nucleophilic attack of 2′-OH at P-atom, leading to strand cleavage and the formation of 2′,3′-cyclic phosphates. In aqueous solution, however, **3** is not expected to be an excellent catalyst, because a serious loss of ion-pair stability and substrate association will result from the displacement of DMF by H_2O. The introduction of further RNA binding groups, therefore, should provide gains in reaction rates as well as increased selectivity. It is tempting to ask whether a single arginine would be sufficient to convert **3** into a TAR-cleaving artificial nuclease. Subsequently, we describe the synthesis of **4** and its interaction with the truncated TAR sequence **1**. Only the arginine part of **4** is considered to occupy the tat-binding site of **1**. The other cationic groups should not fit due to their heterocyclic structure [24]. With 11-aminoundecanoic acid, a flexible spacer of sufficient length was chosen allowing the bis(guanidinium) moiety of **4** to approach all regions of the RNA sequence **1**. A first step towards high-affinity ligands for TAR was accomplished by preparing the acridine arginine conjugate **19**.

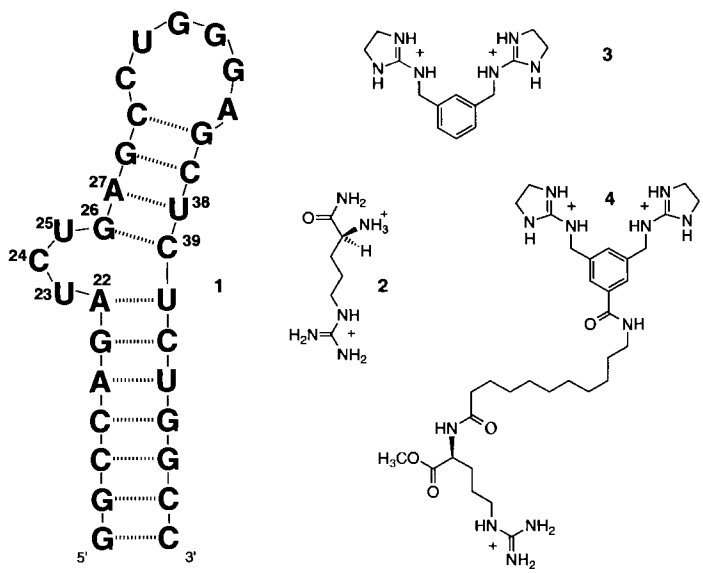

Fig. 2. *Sequence of the TAR model* **1**. Structures of compounds **2**–**4**.

2. Results. – The synthesis of cleaving agent **4** is summarized in *Fig. 3*: commercial 3,5-dimethylbenzoic acid was converted to the methyl ester **6** (97%) and brominated with *N*-bromosuccinimide (NBS; →**7**, 84%). While the yields remained low in CCl_4, a clean reaction could be achieved using methyl formate as a solvent [66]. The amino groups were then introduced by nucleophilic substitution with $NaN(CHO)_2$ (→ **8**, 75%) [67]. Acid-catalyzed hydrolysis of the amides occurred simultaneously with ester cleavage to produce amino acid **9** (98%). Compound **9** was converted to the Boc-derivative **10** (86%), activated *via* ester **11** (92%), and coupled with 11-aminoundecanoic acid (→ **12**, 72%). In the next step, a condensation of **12** with L-arginine methyl ester led to **13** (88%). Finally,

after removal of the Boc groups, the heterocyclic cations of **4** were introduced employing the zwitterionic reagent **14** (77%; isolated as a picrate salt). Zwitterion **14** has already been used for the synthesis of related bis(guanidinium) compounds [61]. The reagent is prepared by oxidation of the corresponding thiourea, modifying a known procedure [68].

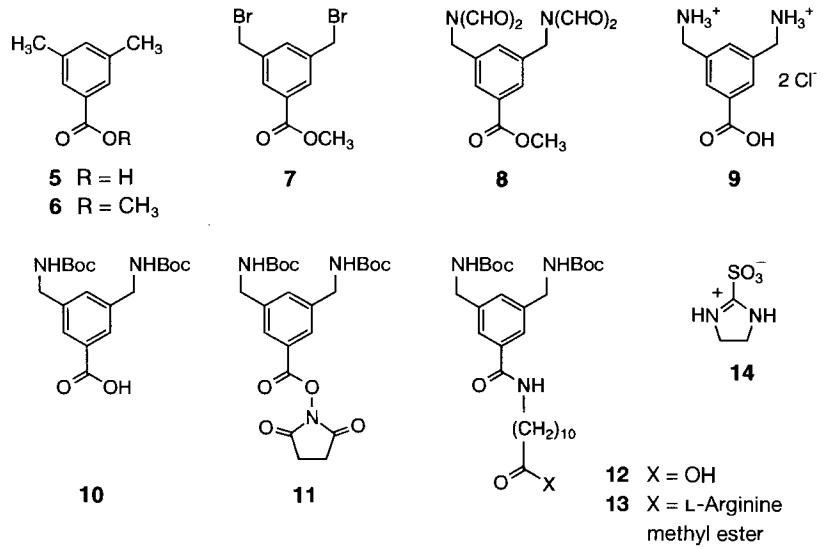

Fig. 3. *Synthesis of the bis(guanidinium) arginine conjugate* **4**. (**5** → **6**): MeOH, conc. H_2SO_4, 2 d reflux, 97%; (**6** → **7**): 3.5 equiv. NBS, AIBN, HCOOMe, light, 20 h reflux, 84%; (**7** → **8**): 2.8 equiv. $NaN(CHO)_2$, MeCN, 4 h reflux, 75%; (**8** → **9**): 1,4-dioxane, conc. aq. HCl, 2 h reflux, 98%; (**9** → **10**): 2.5 equiv. $(Boc)_2O$, CH_2Cl_2, 1M aq. NaOH, 5 d r.t., 86%; (**10** → **11**): 1.3 equiv. DCC, *N*-hydroxysuccinimide, THF, 1 d r.t., 92%; (**11** → **12**): 1 equiv. 11-aminoundecanoic acid, 2 equiv. Et_3N, MeOH, THF, 30 min 0°, 6 h r.t., 72%; (**12** → **13**): a) 1.5 equiv. DCC, *N*-hydroxysuccinimide, THF, 30 min 0°, 14 h r.t.; b) 2 equiv. L-arginine methyl ester, 4 equiv. Et_3N, MeOH, THF, 14 h r.t., 88%; (**13** → **4**): a) 8.5M methanolic HCl, 14 h r.t.; b) 3 equiv. **14** [61], 5 equiv. Et_3N, MeOH, H_2O, 14 h r.t.; c) 3.5 equiv. picric acid, MeOH, H_2O, precipitation, 77%.

Recently, we have introduced the acridine alcohol **15** [69]. Compared with 9-amino-acridines, this compound is very stable against hydrolysis. This property makes **15** an ideal candidate for the construction of acridine-arginine conjugates. Our preliminary results are summarized in *Fig. 4*. Alcohol **15** was activated as a tosylate (→ **16**) and transformed immediately into **17** by a *Gabriel*-type reaction (48% for both steps). After removal of the CHO groups, the amine **18** (68%) could be coupled with the $N(\alpha)$-Boc derivative of L-arginine (→ **19**, 85%). In the future, the protected amino residue of **19** should allow the incorporation of bis(guanidinium) catalysts in analogy with **4**.

The TAR analogue **1**, produced by *in vitro* transcription, has been characterized in the past by different methods including CD [22] (see also [30]) and NMR spectroscopy [25] [26] (see also [33]). The RNA contains the +18 to +44 region of the wild-type TAR sequence. In addition, two GC base pairs were introduced at the ends for increasing the efficiency of the enzymatic preparation. This modification is known not to influence the tat-TAR interaction. While the purified samples of **1** could be used directly for UV and CD spectroscopy, all other experiments were done with 5′-end-labeled material ($\gamma^{32}P$). We

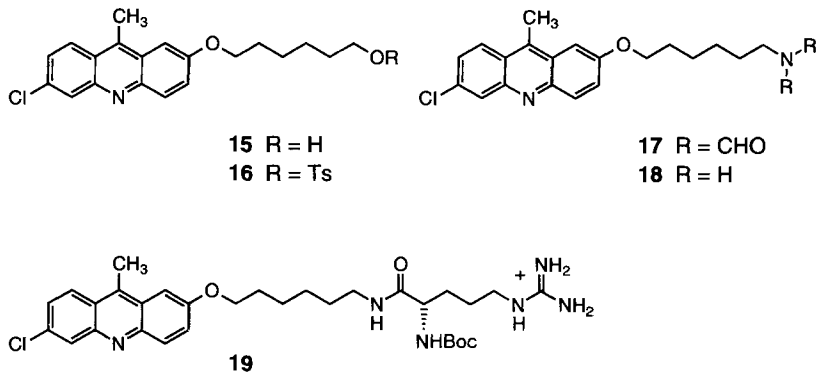

Fig. 4. *Synthesis of the acridine arginine conjugate* **19**. (**15** → **16**): 1 equiv. **15** [69], 3.3 equiv. TsCl, $CHCl_3$, pyridine, DMAP, 2 d, 40°; (**16** → **17**): 5 equiv. $NaN(CHO)_2$, MeCN, 24 h reflux, 48% (both steps); (**17** → **18**): conc. HCl, H_2O, 3 h reflux, 68% (hydrochloride salt); (**18** → **19**): a) 1.3 equiv. N(α)-Boc-L-arginine, 1.3 equiv. *N*-hydroxysuccinimide, 1.3 equiv. pyridinium *p*-toluenesulfonate, 1.3 equiv. DCC, DMF, pyridine, 24 h, r.t.; b) 1 equiv. **18** (free base) in pyridine and 20 equiv. Et_3N was added, 24 h, r.t.; c) chromatography on silica gel (AcOEt/EtOH/H_2O/AcOH 16:4:4:1), 85%.

have confirmed the identity of our RNA by degradation studies: base-induced cleavage produced a pattern consistent with a 31-mer. The sequence of **1** was then verified by partial digestion using the ribonucleases T1, A, CL3, and U2. Since the double-helical segments are, to some extent, protected against enzymatic degradation, these experiments can be used at the same time to map the bulge and loop regions. Interestingly, some cleavage between nucleotides 19 and 20 was observed in experiments with ribonuclease T1. When the thermal denaturation of **1** was examined by UV spectroscopy, a sharp and reversible transition could be observed ($T_m = 78°$).

The CD spectrum of **1** displayed a strong positive maximum at 265 nm, characteristic for double-helical nucleic acids belonging to the *A*-type of conformation (*Fig. 5*). The structural reorganization of the RNA hairpin, when titrated with L-arginine, was expressed by a slight decrease in ellipticity (23%). This effect went into saturation without further spectroscopic changes at high concentrations of arginine. The obtained K_d (2.3 ± 0.5 mM, 5°) is in good agreement with the reported value of 1.5 mM [22]. L-Arginine amide **2**, a dicationic molecule, binds distinctly better than arginine itself ($K_d = 80 \pm 10$ μM; see also [24]). Titration of **1** with compound **4** resulted in more pronounced band reductions at 265 nm ($\geq 50\%$). Again, the data could be fitted to a binding isotherm, supporting a complex stoichiometry of 1:1 ($K_d = 210 \pm 30$ μM).

Initial cleavage experiments using the nonsubstituted bis(guanidinium) compound **3** were carried out with oligo(adenylic acids) (12-mer–18-mer) and analyzed by HPLC. The substrate concentrations were monitored by comparison of the peak area marked in *Fig. 6* (A_{12-18}) with those of a nondegradable internal standard (deoxy-A_{24}). This procedure allowed a rough estimation of reaction rates. Although **3** (20 mM) promoted the RNA hydrolysis reproducibly, the observed rate acceleration at 60° and pH 7.0 (50 mM 2-[4-(2-hydroxyethyl)-1-piperazino]ethanesulfonic acid (HEPES), 1 mM ethylenediaminetetraacetic acid (EDTA)) did not exceed a factor of *ca.* 3. The low catalytic efficiency is probably caused by the weakness of nonspecific guanidinium-phosphate interactions in aqueous solution.

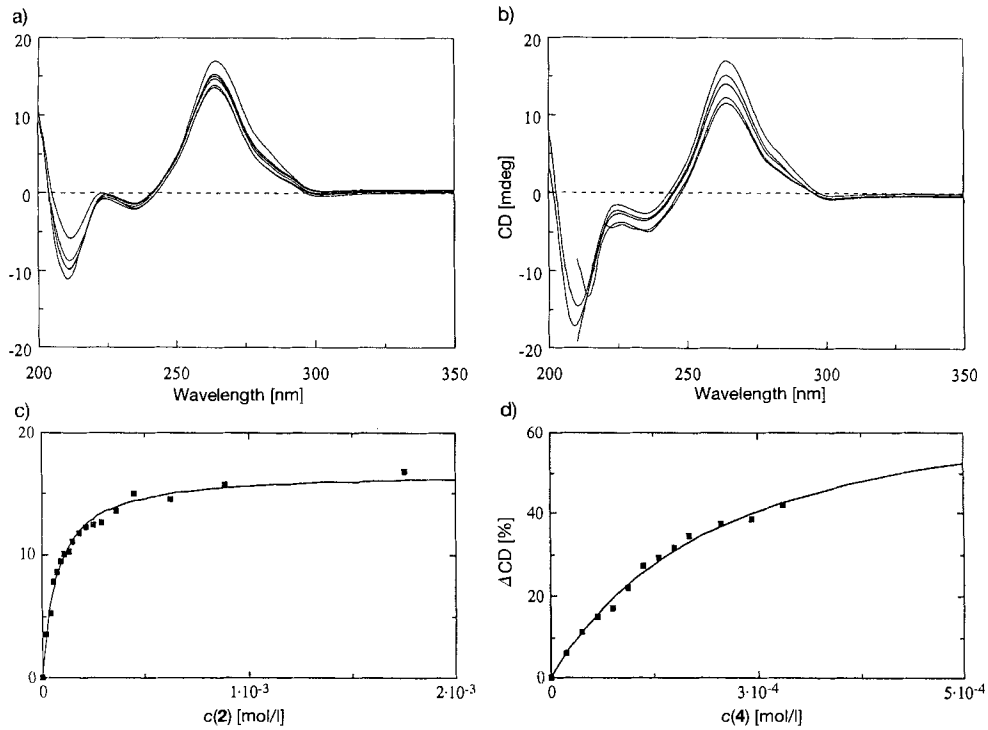

Fig. 5. *CD Spectra* (5°, pH 7.0) *of truncated TAR-RNA* **1** *with* a) *increasing amounts of arginine amide* **2** *and* b) *increasing amounts of* **4**. The calculated binding isotherms are shown in *c* and *d*.

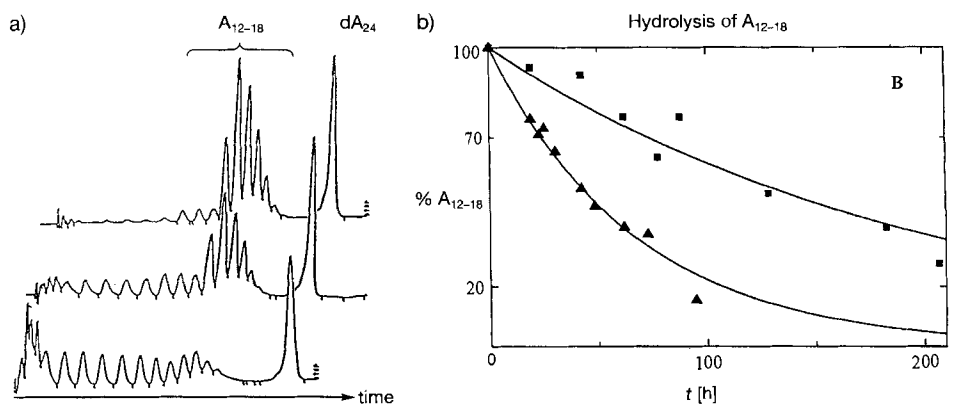

Fig. 6. *Cleavage of* $A_{12\text{-}18}$. *a*) HPLC Analysis of the degradation process; *b*) calculated pseudo-first-order kinetics. ▲: Hydrolysis in the presence of **3**, the calculated half-life is 46 h. ■: Control reaction. The calculated half-life is 140 h (60°, pH 7.0).

Within 12 h at 37° and pH 7, the TAR model **1** remained almost stable. Some minor degradation occurred in the loop and bulge regions (*Fig. 7*). In the presence of **3** (0.3 mM), the cleavage at these sites was not significantly enhanced. Neither arginine amide **2** nor a mixture of **2** and **3** caused any effect at 0.3 mM concentrations (data not shown). However, when the structural features of **2** and of **3** were combined in the conjugate **4**, 12 h of incubation (0.3 mM) resulted in pronounced degradation of RNA. Cleavage occurred in the loop (U(31)) and bulge regions (C(24)) as well as in the lower stem (C(19)). At least 30 mM solutions were necessary to produce comparable effects by **3**. High concentrations of yeast $tRNA^{Phe}$ blocked the degradation of **1** by **4**, probably by non-specific binding of the catalyst (not shown). Complete inhibition of RNA cleavage was also observed in the presence of the acridine arginine conjugate **19** (0.3 mM). This compound associated tightly with the TAR model **1** and induced a distinct shift of the electrophoretic band (*Fig. 7*).

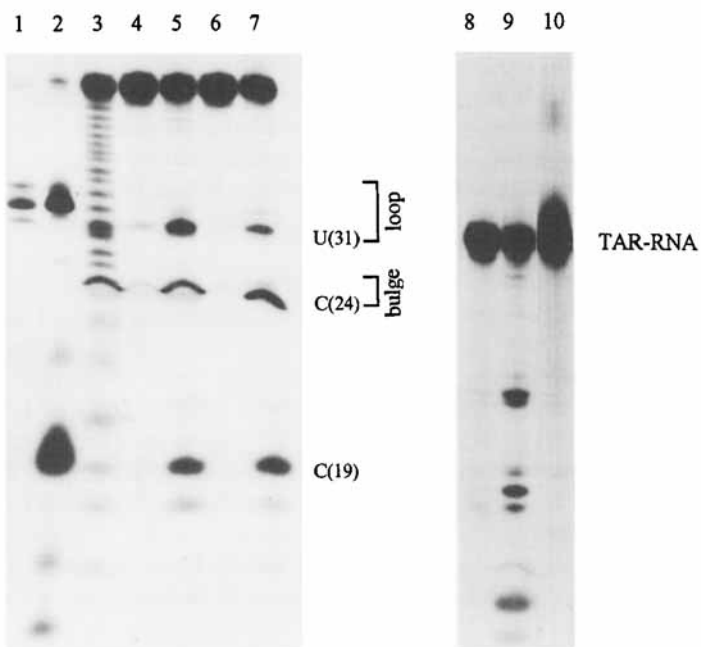

Fig. 7. *Cleavage pattern of TAR-RNA* **1**. Lane *1* and *2*: digestion with RNase T1; lane *1*: EtOH precipitation; lane *2*: EtOH precipitation omitted, short fragments are visible; lane *3*: alkaline hydrolysis; lane *4–10*: cleavage pattern after incubation for 12 h at 37° and pH 7.0; lane *4*: control without any cleaving agent; lane *5*: 300 μM **4**; lane *6*: 300 μM **3**; lane *7*: 30 mM **3**; lane *8*: control without any cleaving agent; lane *9*: 300 μM **4**; lane *10*: 300 μM **4** and 300 μM **19**.

3. Discussion. – The experiments with oligo(adenylic acids) have demonstrated that the bis(guanidinium) compound **3** promotes RNA hydrolysis with moderate efficiency. To convert **3** into a TAR-cleaving artificial nuclease, **4**, the catalyst was linked to a single arginine residue by a long alkyl chain. The arginine moiety should act as a simplified

mimic of tat, a protein which binds the TAR sequence with high affinity and specificity. The association of **4** with the truncated RNA model **1** was analyzed by CD spectroscopy. K_d (210 μM, 5°) proved to be in the same range as for arginine amide **2**.

Cleaving reactions were then carried out using ^{32}P-labeled **1** at 37° and pH 7.0. While the control sample remained stable, significant hydrolysis was observed after 12 h of incubation with 0.3 mM **4**. To obtain comparable amounts of degradation in the presence of **3**, concentrations as high as 30 mM had to be used. Thus, the catalytical power of the bis(guanidinium) group is increased *ca.* 100-fold by attaching it to a simple TAR-binding residue. This augmentation is caused by enhanced local concentrations of the bis(guanidine) and not the structural changes the association of arginine derivatives imposes on **1**: no hydrolysis was observed, when arginine amide **2** or a mixture of **2** and **3** were added in the same concentrations as **4** (0.3 mM).

If the binding modes of arginine amide **2** and the conjugate **4** are identical, **2** should act as a competitive inhibitor of RNA cleavage. Unfortunately, no convincing results could be obtained, because arginine amide itself (a dication!) induced considerable cleavage of **1** when used in the high concentration range (10 mM). In contrast, 0.3 mM of the acridine-arginine conjugate **19** completely abolished the RNA hydrolysis induced by **4**. The fact that stable RNA samples could be obtained reproducibly from numerous transcription experiments excludes that natural ribonucleases were imported accidentally during the purification and labeling of **1**. A contamination of catalyst **4** can be ruled out by the inhibition experiments mentioned above. While **19** associates with RNA **1** (gel shift!) and, thereby, blocks the binding of **4**, the compound is not expected to inhibit natural ribonucleases.

For the discussion of the preferred cleavage sites, it is important to remind that a long spacer was used for the construction of **4**. If the arginine moiety of **4** binds to G(26) of the TAR model **1**, all parts of the RNA molecule are in reach of the bis(guanidinium) catalyst. Any observed site selectivities, therefore, refer to intrinsic reactivities of the RNA sequence. They are not caused by different binding modes of **4**. The main cleavage sites were found in the loop and in the bulge region as anticipated. To our surprise, further reaction occurred at C(19) which is expected to participate in a double helical structure. Since a co-linear orientation of nucleophile (C(2′)–OH) and leaving group (C(5″)–OH) substantially facilitates the formation of 2′,3′-cyclic phosphates for stereoelectronic reasons, the cleavage sites should be restricted to the bulge and loop regions of **1**: this conformational requirement is incompatible with standard duplex structures [70] [71].

As mentioned above, C(19) also is a position where anomalous cleavage by ribonuclease T1 could be observed. The resulting short fragment is only visible, when the EtOH precipitation step of the digested mixture is omitted. While we cannot rule out that T7 RNA polymerase may erroneously incorporate a few percent of G instead of C, the possibility of a predominant mismatch was excluded by carefully repeating the syntheses of all DNA and RNA molecules involved in this work. In addition, the thermal stability of **1** (T_m = 78°) is in accord with a fully base-paired structure. A melting temperature of 67° was reported for a related TAR model lacking two GC base pairs in the lower stem [72] (see also [31]). The CD titration with **4** gives further evidence of an intrinsic lability of this region: while the data support the formation of a 1:1 complex with a similar strength as the complex of arginine amide, the stronger decrease of ellipticity at full saturation

suggests that the bis(guanidinium) moiety of **4** destabilizes to some extent the lower double-helical part. Further experiments with the wild-type sequence will exhibit, if this effect is specific for the truncated TAR model **1**, or if it is of general significance. Considering the simple structure of **4**, the cleavage results obtained with the TAR model **1** are promising. Future work will show if antiviral compounds may be derived from bis(guanidinium) conjugates binding to TAR with improved affinity and specificity.

The authors wish to express their gratitude to Mrs. *Michaela Scherr*, University of Frankfurt, for valuable advice concerning enzymatical RNA preparation. We also thank Mr. *David Gérard*, University of Geneva, for the preparation of compound **19**. Financial support of our work by the following organizations is gratefully acknowledged: *Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung, Fonds der Chemischen Industrie*, (Frankfurt, Germany), *Deutsche Forschungsgemeinschaft*. We thank Prof. *J. R. Williamson*, Massachusetts Institute of Technology, for disclosing the coordinates of his model of TAR.

Experimental Part

1. *General.* In all experiments handling nucleic acids, special precautions were undertaken to avoid contamination with nucleases. All plastic ware was autoclaved (30 min, 121°), and gloves were worn during the experiments. Glasware was washed with conc. H_2SO_4, rinsed with sterile water, and baked at 180° overnight. All buffers were prepared from sterile water (DEPC treated and autoclaved) and autoclaved once again. HPLC: *Beckman* high-pressure gradient system including 2 pumps *110B*, controling-unit *421A*, UV detector *163* and a *Shimadzu* integrator *C-R-3-A*; column: *Merck LiChrospher 100 RP-18*, 5 µm, 125 × 4 mm. Samples were analyzed in a linear gradient rising form of 45–53% solvent *A* during 20 min (solvent *A*: MeOH, solvent *B*: 0.005M tetrabutylammonium phosphate in H_2O (*PicA, Waters*)). M.p.: *Kofler* hot plate microscope, uncorrected. Polarimeter: *Perkin-Elmer 241*, in MeOH (HPLC-grade). FT-IR: *Perkin-Elmer 1600*; bands in wavenumbers (cm^{-1}). ^{1}H-NMR: *Varian XL 200, Bruker AM 250, Bruker WH 270*, or *Bruker AMX 400* spectrometers; chemical shifts (δ) in ppm relativ to Me_4Si (0.00 ppm) or (D_5)DMSO (2.50 ppm) as internal standards; *J* in Hz. MS: *Finnigan SSQ 7000* (ESI), and *Finnigan 4000* (EI) or *VG 7070E* (EI) spectrometers. Elemental analysis: *Heraeus HCN-Rapid* performed at the Institut für Organische Chemie, J. W. Goethe-Universität Frankfurt.

2. *Synthesis of the TAR Cleaving-Agent* **4**. *Methyl 3,5-Dimethylbenzoate* (**6**). 3,5-Dimethylbenzoic acid (50.2 g, 334 mmol) was suspended in MeOH (150 ml) and mixed with conc. H_2SO_4 (15 ml). The mixture was refluxed for 2 d. After neutralization with Na_2CO_3 (90 g), the mixture was dissolved in H_2O (1000 ml) and extracted two times with Et_2O (500 ml). The org. phases were dried ($MgSO_4$) and evaporated. Compound **6** crystallized slowly after drying under vacuum (53.3 g, 97%). Large slightly colored crystals. M.p. 30–33°. IR (film): 3007*m*, 2951*s*, 2919*s*, 1723*s*, 1609*s*, 1436*s*, 1381*m*, 1315*s*, 1218*s*, 1115*m*, 1018*s*, 881*m*, 867*m*, 768*m*. ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 2.32 (*s*, 2 Me); 3.83 (*s*, MeO); 7.27 (*s*, H–C(4)); 7.57 (*s*, H–C(2), H–C(6)). Anal. calc. for $C_{10}H_{12}O_2$ (164.20): C 73.15, H 7.34; found: C 72.97, H 7.27.

Methyl 3,5-Bis(bromomethyl)benzoate (**7**). A mixture of **6** (5.35 g, 32.5 mmol), NBS (20.3 g, 114 mmol, 3.5 equiv.), and a spatula of AIBN in dry HCOOMe (50 ml) was irradiated with visible light (100 W) while heating to reflux for 20 h. The solvent was evaporated and the residue dissolved in CCl_4 (100 ml). The precipitated succinimide was filtered off, and the brown soln. was washed with sat. aq. Na_2SO_3, until it became colorless. The residue obtained after evaporation was purified by chromatography (100 g silica gel; AcOEt/hexane 1:15). Compound **7** could be crystallized from AcOEt/hexane (8.84 g, 84%). Fine colorless needles. M.p. 92–93°. IR (KBr): 3069*w*, 2949*w*, 1727*s*, 1603*w*, 1435*s*, 1320*s*, 1232*s*, 994*s*, 771*s*, 698*s*, 628*s*. ^{1}H-NMR ($CDCl_3$, 270 MHz): 3.93 (*s*, MeO); 4.50 (*s*, 2 CH_2); 7.62 (*t*, *J* = 1.7, H–C(4)); 7.80 (*d*, *J* = 1.8, H–C(6), H–C(2)). Anal. calc. for $C_{10}Br_2H_{10}O_2$ (322.00): C 37.30, H 3.13; found: C 37.46, H 3.30.

Methyl 3,5-Bis[(diformylamino)methyl]benzoate (**8**). A suspension of **7** (3.00 g, 9.32 mmol) and $NaN(CHO)_2$ (2.45 g, 25.8 mmol, 2.8 equiv.) in dry MeCN (45 ml) was refluxed for 4 h. After removal of a white solid by filtration, the solvent was evaporated. Recrystallization from AcOEt/hexane yielded **8** (2.14 g, 75%). Colorless crystals. M.p. 138–139°. IR (KBr): 2996*w*, 2952*w*, 1714*s*, 1670*s*, 1607*w*, 1440*m*, 1310*s*, 1265*m*, 1228*s*, 1152*s*, 1000*s*, 965*m*, 774*m*, 743*m*. ^{1}H-NMR: ((D$_6$)DMSO, 270 MHz): 3.84 (*s*, MeO); 4.70 (*s*, 2 CH_2); 7.44 (*s*, H–C(4)); 7.72 (*s*, H–C(2), H–C(6)); 9.09 (*s*, 4 NCHO). Anal. calc. for $C_{14}H_{14}N_2O_6$ (306.27): C 54.90, H 4.61, N 9.15; found: C 54.94, H 4.56, N 8.86.

Hydrochloride of 3,5-Bis(aminomethyl)benzoic Acid (**9**). Benzoate **8** (2.60 g, 8.49 mmol) was dissolved in a mixture of 1,4-dioxane (150 ml) and conc. aq. HCl (35 ml) and heated for 2 h to reflux. After cooling down to r.t., a flow of air was passed through the soln. to eliminate HCl. Product **9** began to precipitate as a colorless solid. After 1 h, the solvent was evaporated and **9** was recrystallized from MeOH/Et_2O (2.22 g, 98%). Fine colorless needles. No m.p. could be detected up to 300°. IR (KBr): 3406*w*, 3001*w*, 2907*w*, 1709*s*, 1610*m*, 1480*m*, 1378*m*, 1187*s*, 1119*m*, 694*m*. ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 4.08 (*s*, 2 CH_2); 7.89 (*s*, H–C(4)); 8.11 (*s*, H–C(2), H–C(6)); 8.65 (br. *s*, 2 NH_3^+); 13.23 (br. *s*, COOH). Anal. calc. for $C_9H_{14}Cl_2N_2O_2 \cdot 0.75\ H_2O$ (266.64): C 40.54, H 5.86, N 10.51; found: C 40.74, H 5.83, N 10.55.

*3,5-Bis{[(*tert*-butoxycarbonyl)amino]methyl}benzoic Acid* (**10**). A soln. of **9** (3.70 g, 13.9 mmol) in 1M aq. NaOH (40 ml) and di(*tert*-butyl)pyrocarbonate (7.60 g, 34.7 mmol, 2.5 equiv.) in CH_2Cl_2 (40 ml) was stirred intensively for 5 d at r.t.. The soln. was diluted with AcOEt (100 ml) and the pH was adjusted to 5 with aq. H_3PO_4. After extraction with AcOEt, the org. phases were washed with brine and dried ($MgSO_4$). The solvent was evaporated and **10** was crystallized from AcOEt/hexane (4.55 g, 86%). Colorless powder. M.p. 165°. IR (KBr): 3364*m*, 2981*w*, 2931*w*, 1688*s*, 1534*s*, 1365*m*, 1291*m*, 1175*m*, 1050*w*, 951*w*, 881*w*. ^{1}H-NMR ((D$_6$)DMSO, 270 MHz): 1.38 (*s*, 2 *t*-Bu); 4.14 (*d*, J = 6.1, 2 CH_2); 7.32 (*s*, H–C(4)); 7.43 (*t*, J = 6.0, 2 NH); 7.69 (*s*, H–C(2), H–C(6)); 12.85 (*s*, COOH). Anal. calc. for $C_{19}H_{28}N_2O_6$ (380.44): C 59.98, H 7.42, N 7.36; found: C 59.93, H 7.29, N 7.11.

*2,5-Dioxopyrrolidin-1-yl 3,5-Bis{[(*tert*-butoxycarbonyl)amino]methyl}benzoate* (**11**). To a cooled soln. (−10°) of **10** (940 mg, 2.47 mmol) and *N*-hydroxysuccinimide (369 mg, 3.21 mmol, 1.3 equiv.) in dry THF (45 ml) a soln. of *N,N*-methanetetraylbis[cyclohexanamine] (DCC; 663 mg, 1.3 equiv.) in dry THF (20 ml) was added dropwise. After the addition was complete, the mixture was stirred at r.t. overnight. The precipitate was filtered off and the soln. was diluted with AcOEt (100 ml). The soln. was washed with 0.5M aq. HCl and H_2O (re-extracted with AcOEt). The org. phases were dried (Na_2SO_4) and evaporated. Compound **11** was recrystallized from AcOEt/hexane (1.09 g, 92%). Colorless crystals. M.p. 182°. IR (KBr): 3427*m*, 3317*m*, 2982*w*, 2938*w*, 2906*w*, 1745*s*, 1715*s*, 1673*s*, 1602*s*, 1539*s*, 1513*s*, 1364*s*, 1279*s*, 1166*m*. 1079*m*. ^{1}H-NMR ($CDCl_3$, 250 MHz): 1.46 (*s*, 2 *t*-Bu); 2.91 (*s*, 2 CH_2); 4.36 (*d*, 2 benzyl. CH_2); 4.92 (br. *s*, 2 NH); 7.53 (*s*, H–C(4)); 7.94 (*s*, H–C(2), H–C(6)). Anal. calc. for $C_{23}H_{31}N_3O_8$ (477.51): C 57.85, H 6.54, N 8.80; found: C 57.94, H 6.79, N 8.46.

*11-Amino-*N*-{3,5-bis{[(*tert*-butoxycarbonyl)amino]methyl}benzoyl}undecanoic Acid* (**12**). 11-Aminoundecanoic acid (966 mg, 4.8 mmol) was dissolved in dry MeOH (70 ml) by the help of 1 equiv. NaOMe. The soln. was cooled in an ice bath, and Et_3N (0.675 ml, 4.8 mmol, 1 equiv.) was added. Within 10 min, a soln. of **11** (2.29 g, 4.8 mmol) in dry THF (35 ml) and dry MeOH (15 ml) was added dropwise. The soln. was stirred for 30 min. at 0° and for 6 h at r.t. After dilution with AcOEt, the mixture was washed with 0.5M aq. HCl and H_2O (re-extracted with AcOEt). The org. phases were dried (Na_2SO_4) and evaporated. Compound **12** could be precipitated from AcOEt/hexane (1.94 g, 72%). Colorless powder. M.p. 98–101°. IR (KBr): 3360*m*, 3322*m*, 2927*m*, 2851*m*, 1687*s*, 1630*s*, 1525*s*, 1259*m*, 1168*m*. ^{1}H-NMR ((D$_6$)DMSO, 270 MHz): 1.23 (*m*, 6 CH_2); 1.38 (*s*, 2 *t*-Bu); 1.46–1.48 (*m*, 2 CH_2); 2.17 (*t*, J = 7.3, CH_2); 3.21 (*q*, J = 6.1, CH_2); 4.13 (*d*, J = 5.9, 2 benzyl. CH_2); 7.22 (*s*, H–C(4′)); 7.38 (*t*, J = 5.9, 2 NH); 7.54 (*s*, H–C(2′), H–C(6′)); 8.36 (*t*, J = 5.4, NH); 11.94 (br. *s*, 1 H, COOH). Anal. calc. for $C_{30}H_{49}N_3O_7$ (563.73): C 63.92, H 8.76, N 7.45; found: C 63.90, H 8.76, N 7.67.

Acetate Salt of N(α)-{*11-Amino-*N-{*3,5-bis{[(*tert*-butoxycarbonyl)amino]methyl}benzoyl}undecanoyl*}-L-*arginine Methyl Ester* (**13**). To a cooled suspension (0°) of **12** (1.00 g, 1.77 mmol) and *N*-hydroxysuccinimide (306 mg, 2.66 mmol, 1.5 equiv.) in dry THF (40 ml) was added dropwise a soln. of DCC (549 mg, 2.66 mmol, 1.5 equiv.) in dry THF (20 ml). After stirring at 0° for 30 min, the mixture was allowed to react at r.t. for 14 h. The colorless solid was filtered off, and a soln. of L-arginine methyl ester (924 mg, 3.54 mmol, 2 equiv.) and Et_3N (995 µl, 7.08 mmol, 4 equiv.) in dry MeOH (20 ml) was added slowly. After stirring overnight at r.t., the solvents were evaporated and the remaining oil was purified by chromatography (75 g of silica gel; AcOEt/EtOH/H_2O/AcOH 20:2:2:1). Product-containing fractions were collected and evaporated. Co-evaporation with toluene yielded **13** as a colorless oil containing traces of toluene and AcOH (1.23 g, 88%). IR (KBr): 3333*m*, 2970*m*, 2930*m*, 2856*m*, 1673*s*, 1537*m*, 1455*m*, 1366*m*, 1251*m*, 1166*m*, 952*m*. ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 1.24 (*m*, 6 CH_2); 1.39 (*s*, 2 *t*-Bu); 1.42–1.75 (*m*, 4 CH_2); 1.83 (*s*, 10 H, CH_3COO^- + AcOH); 2.12 (*t*, J = 7.4, CH_2); 2.29 (*s*, toluene, CH_3); 3.02 (*m*, CH_2); 3.22 (*q*, J = 6.5, CH_2); 3.61 (*s*, MeO); 4.14 (*d* + *m*, J = 6.0, 5 H, benzyl. H, 1 H–C(α)); 7.22 (*s*, 1 H, C4″), together with arom. H of toluene); 7.37 (*t*, J = 6.3, 2 NH); 7.56 (*s*, H–C(2″), H–C(6″)); 7.76 (br. *s*, 4 NH); 8.36 (*t*, J = 5.6, NH); 8.42 (*d*, J = 7.2, NH); 9.19 (br. *s*, NH). MS (ESI^+): calc. for $C_{37}H_{64}N_7O_8^+$: 734.9; found: 734.7.

*Internal Salt of 4,5-Dihydro-1*H*-imidazole-2-sulfonic Acid* (**14**). 15% aq. H_2O_2 (16.8 ml, 74 mmol, 3 equiv.) was added slowly drop by drop to a cooled (temp. ≤ 5°) and well stirred suspension of imidazolidine-2-thione (2.5 g, 24.5 mmol) and sodium molybdate dihydrate (18 mg, 0.075 mmol, 0.003 equiv.) in H_2O (30 ml). Later, the temp. was increased and kept at *ca.* 20° for 90 min. After dilution of the clear mixture with MeOH (50 ml) and cooling

in the refrigerator, **7** was allowed to crystallize overnight (1.56 g, 42%). Colorless powder. M.p. 142° (dec.). IR (KBr): 3204*s*, 3158*s*, 2942*m*, 1615*m*, 1286*s*, 1252*s*, 1070*s*, 1018*m*, 650*s*, 646*s*. ^{1}H-NMR ((D$_6$)DMSO, 270 MHz): 3.86 (*s*, 2 CH$_2$); 10.38 (br. *s*, 2 NH). Anal. calc. for $C_3H_6N_2O_3S$ (150.16): C 24.00, H 4.03, N 18.66; found: C 24.10, H 4.19, N 18.46.

Tris(picrate) Salt of N(α)-{*11-Amino*-N-{*3,5-bis*{[(*4,5-dihydro-1*H-*imidazol-2-yl)amino*]*methyl*}*benzoyl*}-*undecanoyl*}-L-*arginine Methyl Ester* (**4**). Compound **13** (373 mg, 0.47 mmol) was dissolved with gentle heating in 8.5M methanolic HCl (10 ml) and stirred for 14 h at r.t. The solvent was evaporated, and the remaining colorless foam was dissolved in MeOH (5 ml) and H_2O (5 ml). After addition of **14** (211 mg, 1.41 mmol, 3 equiv.) and Et_3N (0.328 ml, 2.34 mmol, 5 equiv.), the mixture was stirred for 14 h at r.t. Then, the soln. was filtered and evaporated. The oil was dissolved in MeOH (5 ml) and **4** (492 mg, 77%) precipitated after the addition of picric acid (1.55 mmol, 3.5 equiv.) and H_2O. Yellow powder. M.p. 107–109°. $[\alpha]_{589}^{20} = -4°$ (*c* = 0.105, MeOH). IR (KBr): 3282*m* (br.), 2926*w*, 2852*w*, 1669*s*, 1635*s*, 1604*s*, 1560*s*, 1432*m*, 1364*s*, 1315*s*, 1268*s*, 1162*m*, 1077*m*, 910*w*, 789*w*, 745*w*, 711*w*. ^{1}H-NMR ((D$_6$)DMSO, 270 MHz): 1.22 (*m*, 6 CH$_2$); 1.42–1.60 (*m*, 3 CH$_2$, H–C(*β*)); 1.71 (*m*, H–C(*β*)); 2.09 (*t*, *J* = 7.3, CH$_2$); 3.07 (*q*, *J* = 6.5, CH$_2$); 3.22 (*q*, *J* = 6.5, CH$_2$); 3.60, 3.61 (2*s*, 11 H, CH$_2$ (dihydroimidazol), MeO); 4.23 (*m*, H–C(α)); 4.40 (*d*, *J* = 6.0, 2 benzyl. CH$_2$); 7.00 (br. *s*, 4 NH); 7.32 (*s*, H–C(4″)); 7.41 (*t*, *J* = 5.6, 4 NH); 7.71 (*s*, H–C(2″), H–C(6″)); 8.17 (*d*, *J* = 7.5, NH); 8.45 (*t*, *J* = 5.5, NH); 8.58 (*s*, 6 H, picrate); 8.68 (*t*, *J* = 6.0, 2 NH); covered by arom. signals: very br. *s*, 4 NH). *Ion exchange procedure:* The ion exchanger (*Dowex 1* × *8*, 400 mesh, Cl^- form *Aldrich*) was washed several times with MeOH (HPLC-grade), until the filtrate became colorless. Then, the picrate salt of **4** (dissolved in MeOH) was passed through the column. The solvent was evaporated, and the remaining oil was vacuum dried. After dissolving the oil in sterile water, the soln. was passed through a filter (cellulose acetate, 0.2 μm) and stored at −20°. MS (ESI$^+$): calc for $C_{33}H_{56}N_{11}O_4^+$: 670.9; found: 670.6, calc. for $C_{33}H_{57}N_{11}O_4^{++}/2$: 335.9; found: 336.1; calc. for $C_{33}H_{58}N_{11}O_4^{+++}/3$: 224.3; found: 224.4. Anal. calc. for $C_{51}H_{64}N_{20}O_{25}$ (1357.18): C 45.13, H 4.75, N 20.64; found: C 44.36, H 4.79, N 20.66.

3. *Experiments with RNA. Hydrolysis of Oligo(adenylic Acids).* A_{12-18} (25 μM, *Pharmacia*) and dA_{24} (8 μM, internal standard) were incubated in 100-μl glass vials at pH 7.0 (50 mM HEPES × NaOH, 1 mM EDTA) in the presence of 20 mM **3** at 60°. The samples were analyzed by HPLC on a reversed-phase column with a linear gradient of 45–53% solvent *A* in 20 min (solvent *A*: MeOH, solvent *B*: 0.005M tetrabutylammonium phosphate in H_2O). The decrease of the sum of the integrals of the peak-group A_{12-18} was monitored as a function of time. Then, the data were fitted to a pseudo-first-order rate expression. The calculated half-lives are given in the legend of *Fig. 6*.

Preparation of RNA **1**. TAR Analogue **1** was synthesized enzymatically by T7 RNA polymerase (*Sigma*) using a 17-mer T7 promoter and a 48-mer template [73]. The RNA contains the +18 to +44 region of wild-type TAR. Additional nucleotides were introduced at the 5′-end (GG) for increasing the efficiency of transcription and at the 3′-end (CC) to form base pairs with the additional Gs. Primer and template for the *in vitro* transcription, prepared chemically by the phosphoamidite method, were supplied by *MWG Biotech*. After purification by polyacrylamide gel electrophoresis, the oligonucleotides were eluted with 0.05M NH_4OAc buffer and desalted on a prepacked *Sephadex-G25* column (*PD-10*, *Pharmacia*).

The *in vitro* transcription was performed at pH 8.1 (buffer: 50 mM *Tris* × HCl, 25 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT, 0.1% *Triton-X*) using equimolar concentrations of primer and template (1 μM) in the presence of 4 mM mononucleotide triphosphates and 1U/μl T7 RNA polymerase. After incubation for 90 min at 37°, the RNA was isolated by phenol extraction and EtOH precipitation. Purification was carried out as discribed above. The RNA was redissolved in sterile deionized water.

Labeling of **1**. 5′-End-labeling with (γ^{32}P) ATP (*Amersham*, spec. act. 3000 Ci/mmol) was done enzymatically by polynucleotide kinase (*Sigma*) after dephosphorylation with calf intestinal alkaline phosphatase (*Stratagene*) as described in [74]. Labeled RNA samples were then purified by gel electrophoresis (16% denaturing polyacrylamide gel). The major band was located by autoradiography (*Kodak*, *Biomax-MR*), eluted from the gel, concentrated by ultrafiltration (*Ultrafree-CL*, *Millipore*), and desalted on a prepacked *Sephadex-G25* column. RNA was stored in sterile water at −20°.

Thermal Denaturation of **1**. Absorbance *vs.* temp. heating curves were obtained using a *Varian Cary 1 Bio* spectrophotometer and a *Varian* temp. controller. The temp. was increased by a rate of 0.5°/min with the absorbance and temp. being recorded every 0.1°. In 0.1M NaCl, 0.01M sodium citrate at pH 7.0 the denaturation temp. was determined to be 78° (RNA concentration: 1.1 μM).

CD Measurements. All experiments were performed at 5° in a buffer pH 7.0 (5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 70 mM NaCl) on a *Jasco J 715* spectropolarimeter. The RNA concentration was 0.5 OD/ml (1.9 μM). Increasing amounts of arginine amide **2** or cleaving agent **4** were added. After each addition, the sample was allowed to equilibrate for 5 min. Scans were taken from 350 to 200 nm, with an average time of 125 ms at each wavelength. The

calculation of K_d was accomplished by nonlinear least-square fitting of ellipticities (determined at 265 nm from the base line corrected average of two scans) to a 1:1 binding isotherm.

Alkaline Hydrolysis. 5′-End-labeled TAR analogue **1** (10 pmol) was heated in 10 µl 0.1M Na_2CO_3/$NaHCO_3$, pH 9.1 at 90° for 8 min. After adding 3.3 µl of 100 mM AcOH and cooling down to 0°, the sample was precipitated with EtOH, dissolved in 5 µl loading buffer (8M urea, 100 mM *Tris*, 100 mM boric acid, 10 mM EDTA, 0.1% xylenecyanol, and 0.1% bromphenol blue) and analyzed on a 20% polyacrylamide gel.

RNase T1 Digestion. 5′-End-labeled TAR analogue **1** (10 pmol) was dissolved in 5 µl of buffer (50 mM *Tris* × HCl, 0.1 mM EDTA, pH 8.0) and incubated with 1 U RNase T1 (*Sigma*) for 8 min at 37°. Some samples were precipitated with EtOH, in other cases this step was omitted (see *Fig. 7*). The resulting material was mixed with 5 µl of loading buffer and loaded directly on a 20% polyacrylamide gel.

Cleavage Reactions. 5′-End-labeled TAR analogue **1** (1 µM) was incubated for 12 h with 300 µM **4** (or with other agents using the concentrations given in the text) at 37° and pH 7.0 (50 mM *Tris* × HCl, 0.1 mM EDTA). The reaction was stopped by adding 0.15 nmol of tRNA followed by EtOH precipitation. The samples were dissolved in 5 µl of loading buffer and analyzed on a 20% denaturating polycrylamide gel (420 × 200 × 1 mm). The fragment pattern was made visible by autoradiography. Comparison with the fragments from T1 digestion and alkaline hydrolysis allowed the localization of cleaving sites.

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