Spontaneous Aminoacylation of a RNA Sequence Containing a 3′-Terminal 2′-Thioadenosine

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Albert Eschenmoser zum 80. Geburtstag gewidmet

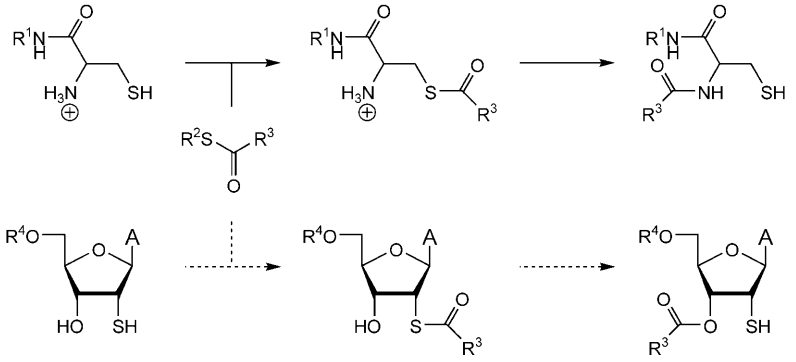
We report the synthesis of a modified 8mer RNA sequence, (C-C-C-C-A-C-C-(2′-thio)A)-RNA 5′-(dihydrogen phosphate) (**9**) containing a 3′-terminal 2′-thioadenosine (*Schemes 2* and *3*), and its spontaneous and site-specific aminoacylation with the weakly activated amino acid thioester H–Phe–SPh (**12**). This reaction, designed in analogy to the 'native chemical ligation' of oligopeptides, occurs efficiently in buffered aqueous solutions and under a wide range of conditions (*Table*). At pH values between 5.0 and 7.4, two products, the 3′-*O*-monoacylated and the 3′-*O*,2′-*S*-diacylated RNA sequences **10** and **11** are formed fast and quantitatively (*Scheme 4*). At pH 7.4 and 37°, the 3′-*O*-monoacylated product **10** is formed as major product *in situ* by selective hydrolysis of the *O*,*S*-diacylated precursor **11**. Additionally, the preparation and isolation of the relevant 3′-*O*-monoacylated product **10** was optimized at pH 5. The here presented concept could be employed for a straightforward aminoacylation of analogously modified tRNAs.

1. Introduction. – The ribosome-mediated, site-specific incorporation of unnatural amino acids into proteins is a powerful tool for creating protein analogues with specific steric, chemical, electronic, or spectroscopic properties, *in vitro* and *in vivo*. The required aminoacylated tRNA analogues are prepared semisynthetically [1], by ribozyme-catalyzed amino acid transfer from short aminoacylated RNA sequences [2], from complementary PNA amino acid thioesters [3], or by modified aminoacyl tRNA synthetases [4]. To facilitate the access to these important and valuable compounds, we try to prepare and identify biologically active tRNA analogues that are spontaneously aminoacylated by weakly activated amino acids, ideally under the conditions of *in vitro* translation reactions.

Here we describe the first example of such a reaction, which consists in a spontaneous aminoacylation of a modified 8mer RNA sequence **9** (*Scheme 3*)[1]) that carries a 2′-thioadenosine at the 3′-terminus, by the activated amino acid thioester *S*-phenyl 3-phenyl-L-thioalaninate (=(α*S*)-α-aminobenzenepropanethioic acid *S*-phenyl ester; H–Phe–SPh; **12**). Conceptually, this experiment was designed in analogy to the so-called 'native chemical ligation', which occurs between two peptides carrying an N-terminal cysteine and a C-terminal thioester. Thereby, a fast intermolecular trans-thioesterification reaction precedes an intramolecular $S \rightarrow N$ migration of the acyl group, resulting in the selective formation of a new amide bond [5]. In *Scheme 1*, the analogy between the two reactions is illustrated.

[1]) This sequence is identical to the 3′-terminal sequence of $tRNA^{Ala}$ from *E. coli*.

Scheme 1. *Formation of an Amide Bond between a Cysteine and an Amino Acid Thioester According to the 'Native Chemical Ligation'* [5] (top)*, as Compared to the Here Presented, Analogous Formation of an Ester Bond between a 2′-Thioadenosine and an Amino Acid Thioester* (bottom).



2. Syntheses. – The assembly of RNA sequences containing a 3′-terminal 2′-thioadenosine required the preparation of a suitably protected and immobilized building block **6**, which was prepared from the known, protected arabinonucleoside **1** [6] (*Scheme 2*). First, **1** was converted with $(CF_3SO_2)_2O$ in pyridine/CH_2Cl_2 into the corresponding 2′-*O*-(trifluoromethyl)sulfonyl derivative, which, after extraction, was treated with 4-methoxybenzenemethanethiol/NaH in DMSO, according to a reported method [7]. The resulting 4-methoxybenzyl thioether **2** (91% yield) was then treated with butane-1-sulfenyl chloride (prepared according to [8]) in CH_2Cl_2/AcOH according to [7], resulting in the efficient formation of the butyl disulfide derivative **3** (86% yield)[2]). After cleavage of the silyl protecting group with HF/pyridine, extraction, and 5′-*O*-dimethoxytritylation with $(MeO)_2$Tr-Cl in pyridine, the protected 2′-thioadenosine **4** was obtained (60% yield). The corresponding activated ester **5** was prepared with bis(4-nitrophenyl) heptanedioate in pyridine (68% yield). Finally, **5** was immobilized on aminoalkyl-functionalized controlled pore glass (CPG) with $^{i}Pr_2NEt$ in DMF, resulting in the solid support **6** with a loading of 30 μmol/g.

From **6**, 2′-*O*-[(triisopropylsilyl)oxy]methyl(=2′-*O*-tom)-protected ribonucleoside phosphoramidites [9], and a commercially available phosphate building block[3]), the 8mer RNA sequence $(^{-}O)_2P(=O)O-r(CCCCACCA_{d2'}(SSBu)^{2'})$ (**8**), carrying a monophosphate group at the 5′-end and a 2′-dithioprotected 2′-deoxyadenosine at the 3′-end, was assembled by automated synthesis under standard conditions [9], but employing a milder oxidation agent (20 mM I_2 in THF/pyridine/H_2O 7:2:1 instead of 100 mM I_2 in THF/pyridine/H_2O 78:20:2; *Scheme 3*). To avoid partial decomposition of the product sequence, the usually employed deprotection conditions had to be adapted. First, the cyanoethyl protecting groups were removed by washing the crude, immobilized sequence **7** with $^{i}Pr_2NH$/MeCN 1:9 for 20 min at a flow rate of 2.5 ml/

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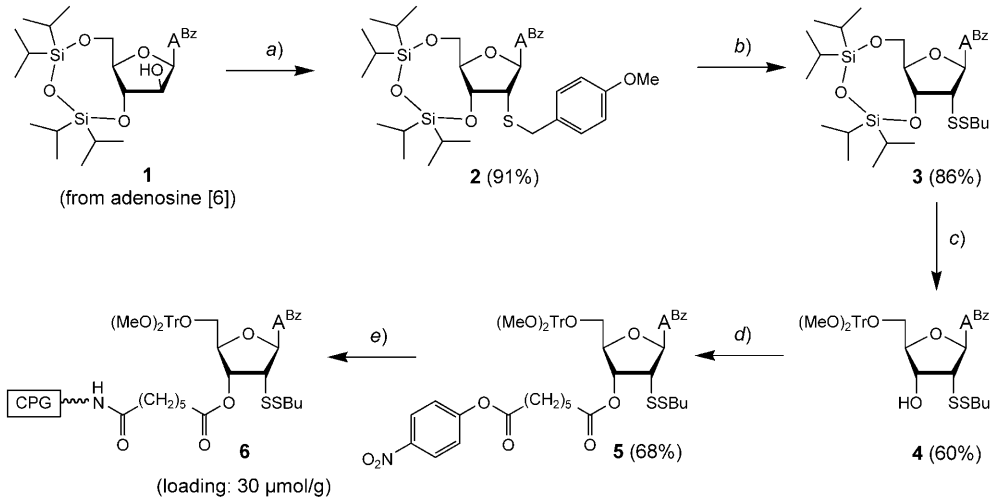
[2]) We also prepared the corresponding phenyl and *tert*-butyl disulfide derivatives. However, the phenyl disulfide group was much too labile and was removed during the oligonucleotide assembly, and the *tert*-butyl disulfide group was too stable and could never be completely cleaved under our preferred conditions.

[3]) 3-[(4,4′-dimethoxytrityl)oxy]-2,2-bis(ethoxycarbonyl)propyl 2-cyanoethyl diisopropylphosphoramidite.

Scheme 2



a) 1. $(CF_3SO_2)_2O$, pyridine, CH_2Cl_2, $-15°$ to $10°$; 2. 4-methoxybenzenemethanethiol, NaH, DMSO, $10°$. *b*) BuSCl, AcOH, CH_2Cl_2, $4–25°$. *c*) 1. HF·pyridine, pyridine, $25°$; 2. $(MeO)_2TrCl$, pyridine, $25°$. *d*) Bis(4-nitrophenyl) heptanedioate, DMAP (=*N*,*N*-dimethylpyridin-4-amine), pyridine, $25°$. *e*) 1. Long-chain-alkylamino-CPG, $^iPr_2NEt$, DMF, $25°$; 2. Ac_2O, Py, $25°$.

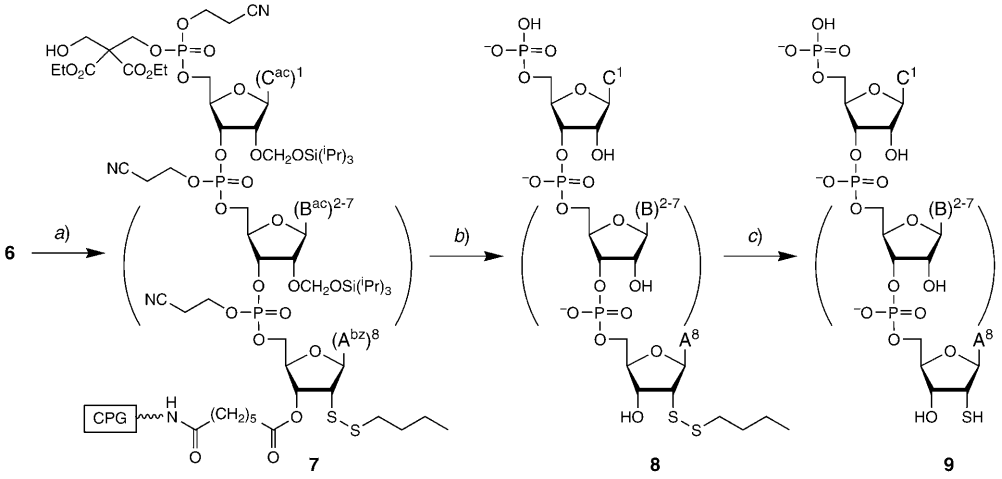
min, then removal of the nucleobase protecting groups and cleavage from the solid support was achieved with 12M NH_3 in MeOH during 6 h at $25°$, and finally, the 2′-*O*-tom protecting groups were removed by 1M $Bu_4NF·3H_2O$/0.5M AcOH in THF during 3 h at $25°$. Even under these optimized conditions (required for a complete deprotection), some by-products (*ca.* 30%) resulting from partial cleavage of the disulfide protecting groups were observed. After a double chromatographic purification (1. anion-exchange HPLC; 2. reversed-phase HPLC) and desalting, the pure product **8** was obtained in a yield of 25% (based on solid support **6**). The LC-ESI-MS of this product showed only one signal at *m/z* 2611 amu (calc. for **8**, 2611 amu). The HPLC and MS traces of purified **8** are shown in *Fig. 1*,*a* and *e*, respectively.

3. Aminoacylation Studies. – The free thiol-substituted RNA sequence **9** was formed *in situ* by reductive cleavage of the remaining disulfide protecting group of **8** ($c=0.08$ mM)[4]) with tris(2-carboxyethyl)phosphine (=2,2′,2″-phosphinidynetris[acetic acid]=TCEP; $c=1–10$ mM, depending on pH) in various aqueous buffers (pH 7.4–3.7) at $25°$ (for conditions, see *Table*). This reaction was complete within 30 min, and **9** was obtained in a purity of >95% according to HPLC and MS analyses (*Fig. 1*,*b* and *f*, resp.).

In a first set of experiments, the pH dependency of the aminoacylation reaction was investigated (see *Table* and *Scheme 4*). Crude **9** in buffered aqueous solutions (pH 7.4–

[4]) Such a low concentration was chosen to simulate the aminoacylation of analogously modified tRNAs, which are usually available and required only in very small quantities

Scheme 3



a) Assembly on an oligonucleotide synthesizer with 2′-*O*-tom-protected ribonucleosides and 3-[(4,4′-dimethoxytrityl)oxy]-2,2-(ethoxycarbonyl)propyl 2-cyanoethyldiisopropylphosphoramidite according to [9] but employing 20 mM I_2 in THF/pyridine/H_2O 7:2:1 as oxidizing agent. *b*) 1. $^iPr_2NH$/MeCN 1:9, 20 min; 2. 12M NH_3, MeOH, 25°; 3. $Bu_4NF \cdot 3H_2O$, AcOH, THF; 4. HPLC purification. *c*) TCEP (= tris(2-carboxyethyl)-phosphine) in different aq. buffers, see *Table*.

3.7, obtained from **8** and TCEP as described above) was treated with 12 equiv. of **12**[5]) (added as concentrated solution in DMF) at 25°, and the composition of the reaction mixtures was analyzed by reversed-phase HPLC after 10 and 50 min (for an example, obtained at pH 7.4, see *Fig. 1*, *c* (10 min) and *d* (50 min), resp.). Under all conditions, a fast formation of two slower migrating (less polar) products was observed, which was in agreement with the formation of the monoacylated product **10** and the diacylated product **11**, respectively. The HPLC analysis was carried out at pH 3.5, where both products **10** and **11** were hydrolytically stable[6]). Additional experiments at 37° and with only 3 or 6 equiv. of **12** were carried out at pH 7.4 and 5.0 (see *Table*). The two aminoacylated products **10** and **11** were isolated by HPLC, and their structure was confirmed by ESI-MS (*Fig. 1*, *g* and *h*)[7]).

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[5]) Prepared from commercially available *N*-[(*tert*-butoxy)carbonyl]-L-phenylalanine 4-nitrophenyl ester according to [10].

[6]) All reversed-phase HPLC analyses were carried out at pH 3.5 with a 0.1M Et_3N/H_3PO_4 buffer. At pH values between 4.0 and 5.5, partial hydrolysis of the diacylated product **11**, and at pH values between 5.5 and 7.5, partial hydrolysis of both acylated products **10** and **11** was observed.

[7]) Our analytical methods (HPLC, MS, and hydrolysis studies, see below) do not show whether **10** is the 3′-*O*- or the 2′-*S*-monoacylated product; however, since esters are thermodynamically much more stable than analogous thioesters, and since it is well-known that the migration of acyl groups between the 2′-*O* and 3′-*O* positions of ribonucleosides is an extremely fast reaction [11], we concluded that **10** is the 3′-*O*-monoacylated derivative.

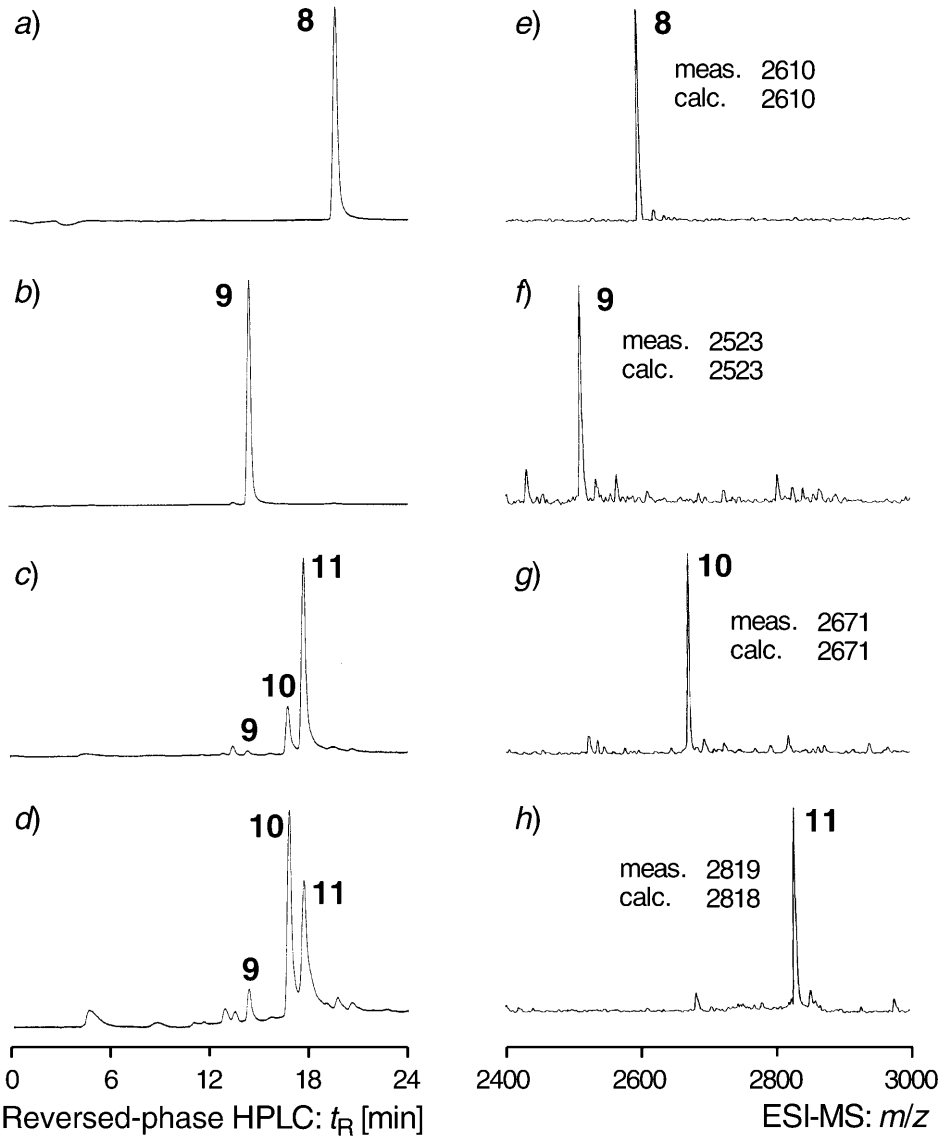


Fig. 1. a)–d) *Reversed-phase HPLC traces* (detection at 260 nm) *of a representative aminoacylation reaction carried out at pH 7.4 and 25°*: a) *disulfide-protected starting RNA sequence* **8**, b) *30 min after treatment with TCEP,* c) *10 min and* d) *50 min after addition of 12 equiv. of H–Phe–SPh* (**12**). *e*)–f) *Deconvoluted ESI-MS* (neg. mode) *of RNA sequences* **8**–**11** (for procedures, see *Exper. Part*).

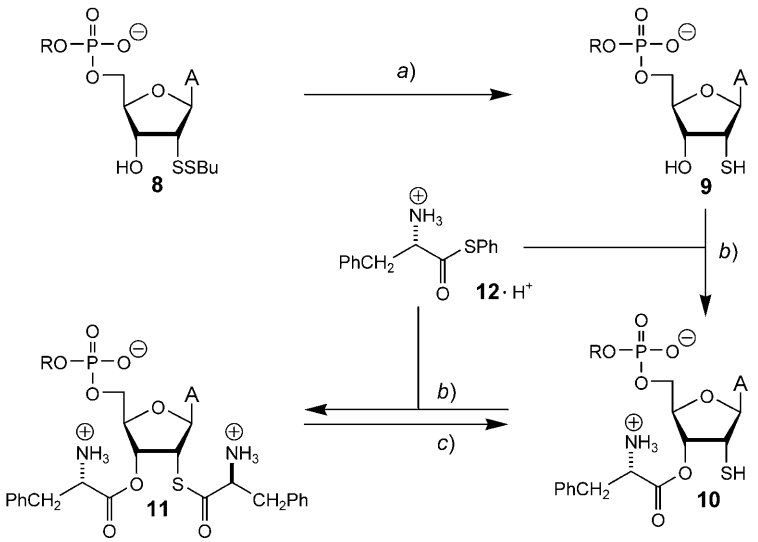
At pH values between 4.7 and 5.5, and at 25°, clean and almost quantitative formation of the monoacylated product **10** together with the diacylated product **11** was observed after 50 min. At higher pH values of 6.5 and 7.4, some unidentified by-products were formed after 50 min (*ca.* 10%), but the reactions were complete already after

Table. *Release and Aminoacylation of the 2′-Thioadenosine-Modified RNA Sequence* **9**: *Exploratory Studies under Different Conditions*[a])

Conditions[b])			*t* 10 min[c])			*t* 50 min[c])		
pH	**12** [equiv.]	*T* [°]	**9** [%]	**10** [%]	**11** [%]	**9** [%]	**10** [%]	**11** [%]
7.4	12	25	1	18	73	5	37	46
6.5	12	25	3	19	71	3	24	64
5.5	12	25	7	19	70	<1	10	86
5.0	12	25	10	18	70	<1	10	87
4.7	12	25	12	18	68	<1	10	87
3.7	12	25	43	20	36	18	26	55
7.4	6	25	2	24	64	6	78	10
7.4	12	37	1	17	72	5	41	42
5.0	6	25	27	29	43	1	33	65
5.0	3	25	54	20	23	15	35	47
5.0	12	37	2	27	67	1	32	64
5.0	6	37	8	34	53	1	35	60
5.0	3	37	30	29	34	4	41	50

[a]) For the detailed procedure, see *Exper. Part.* [b]) Incubation of **8** ($c = 0.085$ mM; 40 µl) with TCEP ($c = 1.0$ mM at pH 7.4 and 6.5; $c = 2.5$ mM at pH 5.5, 5.0, and 4.7; $c = 10$ mM at pH 3.7) in aq. buffers (50 mM each; pH 7.4, *Tris*·HCl; pH 6.5, H_3PO_4/NaOH; pH 5.5, 5.0, and 4.7, AcOH/NaOH; pH 3.7, HCOOH/NaOH) at 25° for 30 min, followed by addition of **12** (indicated equiv. rel. to **8**) in DMF (4 µl). [c]) *t* = Incubation time; quantification by reversed-phase HPLC at 260 nm; t_R (**9**) 14 min, t_R (**10**) 17 min, t_R (**11**) 18 min; % values rel. to the sum of all detected peaks; for an example of such HPLC traces from a reaction carried out at pH 7.4 and 25°, see *Fig. 1*, *c* and *d*.

Scheme 4



a) TCEP in different aq. buffers, see *Table*. *b*) Addition of *S*-phenyl 3-phenyl-L-thioalaninate (H–Phe–SPh; **12**) in DMF to buffered aq. solutions, see *Table*. *c*) NaN_3 in aq. buffer (pH 5.0), see *Fig. 4*.

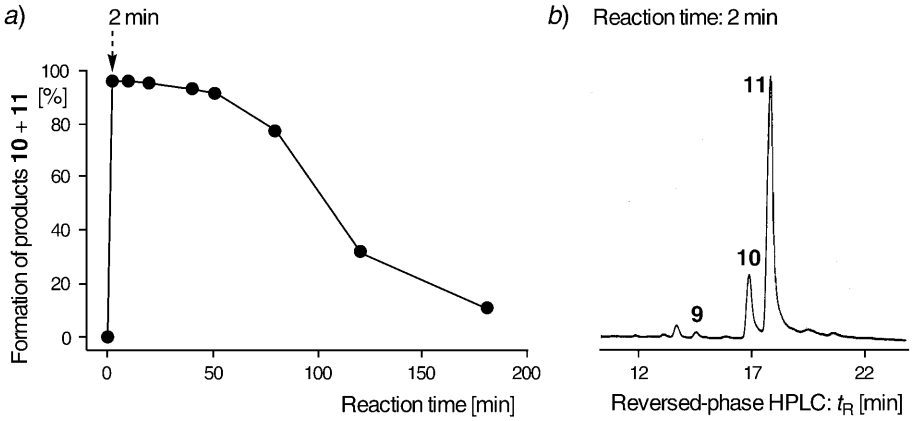


Fig. 2. *Aminoacylation reaction carried out at pH 7.4 and 37° with* **9** *and 12 equiv. of H–Phe–SPh* (**12**): a) *Plot of the amount of aminoacylated products* (**10**+**11**) *relative to the sum of all assigned RNA sequences* (**9**+**10**+**11**; data extracted from HPLC traces). b) *Reversed-phase HPLC trace* (detection at 260 nm) *of the product mixture obtained after 2 min reaction time.*

10 min at 25° (*Fig. 1*, *c* and *d*). With only 3 equiv. of **12**, an almost quantitative aminoacylation could be observed after 50 min at pH 5.0 and 37°. Under all these conditions, the parent RNA sequence $(^{-}O)_2P(=O)O$–r(CCCCACCA) was completely inert, indicating that the thiol group is involved in, and required for the aminoacylation reaction and that the activated amino acid is not interfering with the integrity of RNA sequences.

We then focused on two different reaction conditions: the first one typical for *in vitro* translations, and the second one for the purpose of preparing and isolating aminoacylated RNA sequences. In *Fig. 2*, *a*, the time course of a reaction carried out with **9** ($c = 0.08$ mM) and 12 equiv. of **12** at pH 7.4 and 37° is shown. The composition of the reaction mixture was analyzed by reversed-phase HPLC, and the relative amount of products **10** and **11** was plotted against reaction time (*Fig. 2*, *a*). After 2 min already, almost quantitative formation of the mono- and diacylated RNA sequences **10** and **11** was observed (*Fig. 2*, *b*). Only after *ca.* 50 min, they slowly were converted back to the starting material **9** by hydrolytic cleavage. The plateau between 2 and 50 min indicates the transient nature of the aminoacylated species **10** and **11**, which are constantly hydrolyzed and reacylated, until the activated amino acid is consumed.

The rate constants for the cleavage of the ester bond of **10** and the thioester bond of **11** at 37° and pH 7.4 (0.1M *Tris*·HCl) were determined by kinetic studies and are given in *Fig. 3*, *b*. Under the same conditions, but at 25°, the rate constant for the hydrolysis of **10** to **9** was $k = 0.02$ min^{-1}, which is about twice the value of an adenosine nucleotide esterified with L-Phe [12][8]). To simulate the conditions of an *in vitro* translation reac-

[8]) Under these conditions, the hydrolysis rate of the activated amino acid **12** is $k = 0.007$ min^{-1} and 0.07 min^{-1} at 25° and 37°, respectively.

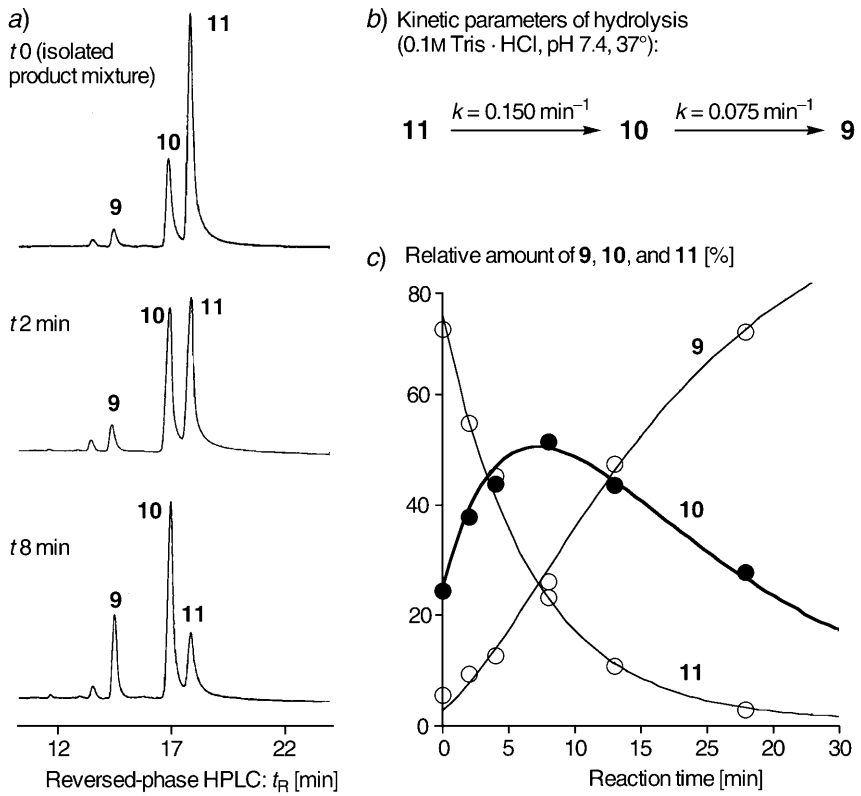


Fig. 3. a) *Reversed-phase HPLC traces* (detection at 260 nm) *obtained by incubating an isolated aminoacylation product mixture in 0.1*M *aqueous* Tris·*HCl* (pH 7.4) *at 37°*. b) *Independantly determined kinetic parameters of hydrolysis for the diacylated product* **11** *and the monocylated product* **10**. c) *Measured* (points) *and calculated* (lines) *composition of a hydrolysis reaction mixture obtained according to* a) *at pH 7.4 and 37°* (for details see *Exper. Part*).

tion, we incubated a desalted mixture of reaction products from an acylation reaction[9]) at pH 7.4 (aqueous 0.1M *Tris*·HCl buffer) and 37°. Aliquots were removed at different time intervals and analyzed by reversed-phase HPLC (for examples, see *Fig. 3*,*a*). In *Fig. 3*,*c*, the time-dependent composition of the reaction mixture is shown together with the curves calculated from the individually determined hydrolysis rate constants (shown in *Fig. 3*,*b*). The hydrolysis of the diacylated **11** to the monoacylated **10** occurs twice as fast as the hydrolysis of the latter to **9**; this results in a predominant occurrence of the relevant monoacylated RNA sequence **10** after a short incubation at 37° and pH 7.4 (*Fig. 3*,*c*).

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[9]) The isolated product mixture was obtained from an acylation reaction of **9** with **12**, carried out at pH 5.0 and at 37°; after 30 min, the reaction mixture was diluted with H_2O and desalted on a size-exclusion cartridge by elution with H_2O. The oligonucleotide-containing eluate was stabilized with 1% AcOH and concentrated to a smaller volume by lyophilization.

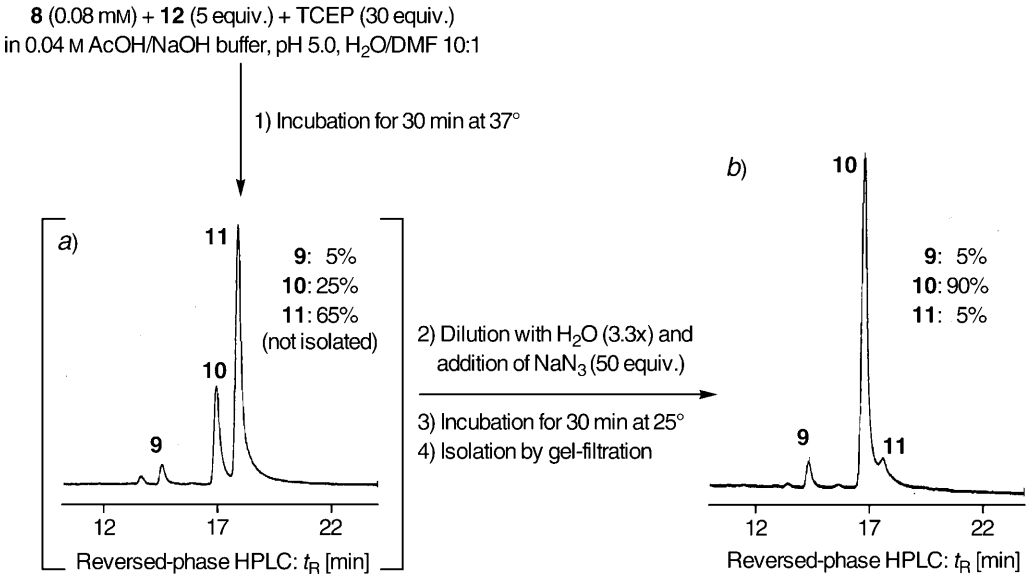

Fig. 4. *Conditions for the preparation and isolation of the monoacylated RNA sequence* **10** *and reversed-phase HPLC traces* (detection at 260 nm) *of* a) *the intermediate and* b) *the final product mixture*

An efficient acylation reaction between **9** and **12** was observed at pH 5.0 (*Table*), where aminoacylated RNAs are considerably stable [12]; therefore, we decided to optimize the preparation of **10** at this pH value. To minimize the formation of by-products, we carried out the deprotection and aminoacylation reactions simultaneously. In *Fig. 4*, the HPLC trace of such a reaction product is shown, which was obtained by incubation of **8** (0.08 mM) with TCEP (30 equiv.) and **12** (5 equiv.) at pH 5.0 and 37° for 30 min. This HPLC trace reveals the clean and almost quantitative formation of the aminoacylated products **10** and **11**. For the selective cleavage of the thioester bond (reaction **11** → **10**), a variety of different conditions (pH values, nucleophiles) were investigated. The best result was obtained by treating the reaction mixture after 30 min with NaN_3, keeping it for 30 min at 25°, and isolating the products by desalting on a size-exclusion cartridge, which removes all non-oligonucleotide components. This protocol furnished a product mixture containing 90% of the desired monoacylated RNA sequence **10**, together with 5% of **9** and 5% of **11** (*Fig. 4, b*). Lyophilization of this aqueous eluate is possible without cleavage of the ester bond if 1% AcOH is added[10]).

Discussion. – In conclusion, we found that the intriguing concept of 'native chemical ligation' of oligopeptides can also be applied to the straightforward acylation of 2′-thionucleotides with activated amino acids. Mechanistically, it can be assumed that after formation of the 2′-thioester derivative, a fast and almost irreversible migration of

[10]) It was possible to isolate product **10** in pure form by reversed-phase HPLC (see *Exper. Part*). However, such a purification is not feasible or desirable with analogously prepared aminoacylated tRNA sequences (76mers, available in rather small quantities), for which this method was developed.

the acyl group to the 3′-*O* position occurs (see *Footnote 7*); subsequently, the liberated 2′-SH group can then undergo another acylation reaction, resulting in formation of the *O*,*S*-diacylated product.

At pH 7.4 and 37°, the biologically relevant *O*-monoacylated product is formed as major product *in situ* by hydrolysis of the *O*,*S*-diacylated precursor. The *O*-monoacylated product can be prepared efficiently by acylation of the modified RNA sequence at pH 5 and 37°, followed by selective cleavage of the concomitantly formed *O*,*S*-diacylated product with NaN_3; its isolation is conveniently carried out by desalting on size-exclusion cartridges, stabilization of the eluate with 1% AcOH, and lyophilization.

The here presented method could be applied to the straightforward preparation and isolation of aminoacylated RNA sequences, or to translation systems in which a thiol-modified tRNA acts as a catalyst, respectively. Currently, we are investigating, whether translation systems tolerate these thiol-modified tRNAs.

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Experimental Part

1. *General.* Reagents and solvents (highest purity) from various suppliers were used without further purification. *Tris* = 2-amino-2-(hydroxymethyl)propane-1,3-diol. *S*-Phenyl 3-phenyl-L-thioalaninate (H–Phe–SPh; **12**) was prepared according to [10] and 2′-*O*-tom-protected RNA phosphoramidites according to [9]. The phosphoramidite building block for the introduction of the 5′-terminal monophosphate group was from *Glen Research.* Size-exclusion cartridges: *NAP-10* columns from *Pharmacia*, elution according to the manufacturer's instruction; *Sepak* cartridges from *Waters*, elution according to [13]. Workup implies distribution of the reaction mixture between the indicated aq. and org. phases, drying ($MgSO_4$) of the org. layer, and evaporation. Column chromatography: (CC) silica gel (SiO_2, *Fluka*); for purification of $(MeO)_2$Tr-protected nucleosides, the SiO_2 was pre-equilibrated with 2% Et_3N added to the packing solvent. TLC: precoated silica gel plates from *Merck,* stained by dipping into a soln. of anisaldehyde (10 ml), conc. H_2SO_4 soln. (10 ml), and AcOH (2 ml) in EtOH (180 ml) and subsequent heating. Reversed-phase HPLC: *Waters Xterra RP*$_{18}$ (5 μm; 4.6×250 mm); flow 1 ml/min; eluent *A*: 0.1M $(Et_3NH)_3PO_4$ in H_2O (pH 3.5) or 0.1M $(Et_3NH)OAc$ in H_2O (pH 5.5); eluent *B*: MeCN; elution at 25°, detection at 260 nm; unless otherwise stated, a gradient *A* → *A*/*B* 1:1 (30 min) was used; t_R in min. Ion-exchange HPLC: *Pharmacia Source 15Q* (4.6×100 mm); flow 1 ml/min; eluent *A*: 10 mM AcOH/NaOAc (pH 5.0) in MeOH/H_2O 1:1; eluent *B*: AcOH/NaOAc, 1M NaCl (pH 5.0) in MeOH/H_2O 1:1, elution at 25°, detection at 260 nm; a gradient *A* → *A*/*B* 1:1 (30 min) was used. ESI-MS (pos. mode): *SSQ 710* (*Finnigan*); measurements in MeCN/H_2O/AcOH 50:50:1. ESI-MS (neg. mode): *Q-TOF-Ultima* (*Micromass*/*Waters*), measurements in aq. $[Me_2N(Bu)H]HCO_3$ (12.5 mM, pH 6.3)/MeCN 1:1; the samples were introduced by a syringe pump; deconvolution by MaxEnt1 software; in *m*/*z*.

2. *Nucleoside Derivatives.* N^6*-Benzoyl-2′*-S*-(4-methoxybenzyl)-3′,5′*-O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2′-thioadenosine* (**2**). At −15°, a soln. of **1** (260 mg, 0.44 mmol; prepared according to [6]) in CH_2Cl_2/pyridine 1:1 (2 ml) was treated with $(CF_3SO_2)_2O$ (0.11 ml, 0.60 mmol). Workup after 5 h at 10° gave the crude 2′-*O*-(trifluoromethyl)sulfonyl derivative of **1** (330 mg; TLC (hexane/AcOEt 1:9): R_f 0.50) as brown-orange foam, which was dissolved in DMSO (8 ml). For the next step, a suspension of 50% NaH in mineral oil (106 mg, *ca.* 2.2 mmol) and 4-methoxybenzenemethanethiol (343 mg, 2.2 mmol) were added to DMSO (7 ml). After 10 min at 25°, the mixture was cooled to 10°, treated with the soln. of the crude 2′-*O*-(trifluoromethylsulfonyl derivative of **1** (330 mg; see above) in DMSO (8 ml), and stirred for 10 min at 10°. After addition of AcOEt (100 ml), extraction (1. 10% aq. citric acid soln., 2. sat. aq. $NaHCO_3$ soln.) and CC (SiO_2 (10 g), hexane/AcOEt 9:1 → 1:1), **2** (300 mg, 91%) was obtained. Yellow foam. TLC (hexane/AcOEt 1:9): R_f 0.71. ^{1}H-NMR ($CDCl_3$): 0.99–1.12 (*m*, ^{i}Pr$_2$Si); 3.75 (*s*, MeO); 3.78–3.92 (*m*, CH_2(5′)); 4.01–4.19 (*m*, H–C(4′), H–C(2′)); 4.93 (*t*, *J* = 6.9, H–C(3′)); 5.31 (*s*, CH_2S); 6.22 (*d*, *J* = 3.2, H–C(1′)); 6.71 (*d*, *J* = 8.5, 1 arom. H); 7.12 (*t*, *J* = 8.5, 2 arom. H); 7.54 (*t*, *J* = 7.6, 2 arom. H); 7.62 (*t*, *J* = 7.4, 1 arom. H); 8.05 (*d*, *J* = 7.6, 2 arom. H); 8.14 (*s*, H–C(2)); 8.80 (*s*, H–C(8)); 9.22 (br. *s*, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.0, 13.3, 13.5, 13.8 (4*d*, Me_2*C*H);

17.3, 17.4, 17.5, 17.7, 17.8, 17.9 (6*q*, *Me*$_2$CH); 35.5 (*t*, CH$_2$S); 52.4 (*d*, C(2′)); 55.7 (*q*, MeO); 61.6 (*t*, C(5′)); 70.8 (*d*, C(3′)); 84.6 (*d*, C(4′)); 90.6 (*d*, C(1′)); 114.2 (*d*, arom. C); 123.8 (*s*, C(5)); 128.3, 129.3 (2*d*, arom. C); 129.7 (*s*, arom. C); 130.2, 133.2 (2*d*, arom. C); 134.2 (*s*, arom. C); 141.9 (*d*, C(8)); 149.9 (*s*, C(6)); 151.3 (*s*, C(4)); 153.1 (*d*, C(2)); 159.1 (*s*, MeO*C*), 165.1 (*s*, Ph*C*O). ESI-MS: 750.31 ([*M* + H]$^+$).

N^6-*Benzoyl-2′-(butyldithio)-2′-deoxy-3′,5′*-O-*(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine* (**3**). Simultaneously, two solns., one containing BuSCl (250 mg, 2 mmol; prepared according to [8]) in CH_2Cl_2 (10 ml), and the other **2** (300 mg, 0.4 mmol) in CH_2Cl_2 (10 ml), were added dropwise to a cooled (4°) mixture of CH_2Cl_2/AcOH 1:1 (30 ml). After addition, the ice bath was removed and the mixture stirred for 48 h at 25°. Workup and CC (SiO_2 (10 g), hexane/AcOEt 4:1 → 1:4) gave **3** (250 mg, 86%). Light yellow foam. TLC (AcOEt/CH_2Cl_2 1:4): R_f 0.59. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.84 (*t*, *J* = 7.0, *Me*CH$_2$); 1.02–1.17 (*m*, ^{i}Pr$_2$Si); 1.20–1.40 (*m*, CH$_2$); 1.51–1.63 (*m*, CH$_2$); 2.63 (*t*, *J* = 7.4, CH$_2$S); 4.01 (*dd*, *J* = 3.5, 12.5, H–C(5′)); 4.12 (*dd*, *J* = 3.5, 12.5, H′–C(5′)); 4.16–4.23 (*m*, H–C(4′)); 4.31 (*dd*, *J* = 3.2, 7.3, H–C(2′)); 5.23 (*t*, *J* = 7.3, H–C(3′)); 6.43 (*d*, *J* = 3.2, H–C(1′)); 7.55 (*t*, *J* = 7.3, 2 arom. H); 7.63 (*t*, *J* = 7.3, 1 arom. H); 8.05 (br. *d*, *J* = 7.4, 2 arom. H); 8.24 (*s*, H–C(8)); 8.79 (*s*, H–C(2)); 9.14 (br. *s*, HN-C(6)). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.1 (*q*, *Me*CH$_2$); 13.2, 13.5, 13.8, 13.9 (4*d*, Me$_2$*C*H); 17.3, 17.5, 17.6, 17.8, 17.9, 18.0, 18.1, 18.2 (8*q*, *Me*$_2$CH); 21.9 (*t*, Me*C*H$_2$); 31.3 (*t*, *C*H$_2$CH$_2$); 38.7 (*t*, CH$_2$S); 56.7 (*d*, C(2′)); 62.1 (*t*, C(5′)); 71.9 (*d*, C(3′)); 84.3 (*d*, C(4′)); 90.5 (*d*, C(1′)); 128.3 (*s*, C(5)); 129.3 (*d*, arom. C); 133.2 (*d*, arom. C); 134.1 (*s*, arom. C); 142.5 (*d*, C(8)); 150.1 (*s*, C(6)); 151.6 (*s*, C(4)); 153.1 (*d*, C(2)); 165.0 (*s*, Ph*C*O). ESI-MS: 718.32 ([*M* + H]$^+$).

N^6-*Benzoyl-2′-(butyldithio)-2′-deoxy-5′*-O-*(4,4′-dimethoxytrityl)adenosine* (**4**). A soln. of **3** (206 mg, 0.28 mmol) in CH_2Cl_2 (2.2 ml) was treated with a 35% HF soln. in pyridine (2 ml; prepared from a 70% HF soln. in pyridine (1 ml) and pyridine (1 ml)) for 1 h at 25°. After workup and evaporation, the residue was dissolved in pyridine (1.4 ml) and treated with $(MeO)_2$TrCl (114 mg, 0.34 mmol) for 1 h at 25°. Workup and CC (SiO_2 (6 g), CH_2Cl_2 → CH_2Cl_2/AcOEt 1:1) gave **4** (134 mg, 60%). Colorless foam. TLC (hexane/AcOEt 7:3): R_f 0.34. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.80 (*t*, *J* = 7.3, *Me*CH$_2$); 1.17–1.30 (*m*, CH$_2$); 1.38–1.40 (*m*, CH$_2$); 2.37–2.54 (*m*, CH$_2$S); 3.15 (br. *d*, *J* ≈ 2.2, OH); 3.39 (*dd*, *J* = 4.0, 10.4, H–C(5′)); 3.49 (*dd*, *J* = 4.4, 10.4, H′–C(5′)); 3.79 (*s*, 2 MeO); 4.30 (*t*, *J* = 3.0, H–C(4′)); 4.70–4.75 (*m*, H–C(2′), H–C(3′)); 6.28 (*d*, *J* = 8.6, H–C(1′)); 6.80 (*d*, *J* = 8.0, 4 arom. H); 7.18–7.34 (*m*, 8 arom. H); 7.42 (*dd*, *J* = 1.2, 8.1, 2 arom. H); 7.56 (*t*, *J* = 7.4, 1 arom. H); 7.64 (*t*, *J* = 7.3, 1 arom. H); 7.98 (*s*, H–C(8)); 8.06 (*d*, *J* = 7.3, 2 arom. H); 8.62 (*s*, H–C(2)); 9.06 (*s*, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.9 (*q*, *Me*CH$_2$); 21.9 (*t*, Me*C*H$_2$); 31.1 (*t*, *C*H$_2$CH$_2$S); 39.3 (*t*, CH$_2$S); 55.7 (*q*, MeO); 59.7 (*d*, C(2′)); 63.9 (*t*, C(5′)); 73.6 (*d*, C(3′)); 85.7 (*d*, C(4′)); 88.3 (*d*, C(1′)); 113.6 (*d*, arom. C); 123.8 (*s*, C(5)); 127.4, 128.3, 128.6, 129.3, 130.5 (5*d*, arom. C); 133.2, 135.9 (2*s*, arom. C); 142.6 (*d*, C(8)); 144.8 (*s*, arom. C); 150.0 (*s*, C(6)); 152.3 (*s*, C(4)); 153.2 (*d*, C(2)); 159.0 (*s*, arom. C); 165.0 (*s*, Ph*C*O). ESI-MS: 778.30 ([*M* + H]$^+$).

N^6-*Benzoyl-2′-(butyldithio)-2′-deoxy-5′*-O-*(4,4′-dimethoxytrityl)adenosine 3′-(4-Nitrophenyl Heptanedioate)* (**5**). A soln. of **4** (134 mg, 0.18 mmol) in pyridine (2 ml) was treated with DMAP (12 mg, 0.08 mmol) and bis(4-nitrophenyl) heptanedioate (420 mg, 1.04 mmol) for 14 h at 25°. Evaporation and CC (SiO_2 (10 g), hexane/AcOEt 3:2 → 1:9) gave **5** (122 mg, 68%). Colorless foam. TLC (hexane/AcOEt 7:3): R_f 0.53. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.80 (*t*, *J* = 7.4, *Me*CH$_2$) 1.16–1.29 (*m*, CH$_2$); 1.38–1.45 (*m*, CH$_2$); 1.50–1.57 (*m*, CH$_2$); 1.74–1.87 (*m*, 3 CH$_2$); 2.29–2.51 (*m*, 2 CH$_2$); 2.66 (*t*, *J* = 7.3, CH$_2$S); 3.45–3.54 (*m*, CH$_2$(5′)); 3.79 (*s*, 2 MeO); 4.22–4.27 (*m*, H–C(4′)); 4.78 (*dd*, *J* = 5.5, 9.1, H–C(2′)); 5.77 (*dd*, *J* = 0.8, 5.6, H–C(3′)); 6.36 (*d*, *J* = 9.1, H–C(1′)); 6.80 (*d*, *J* = 8.9, 4 arom. H); 7.04–7.10 (*m*, 3 arom. H); 7.10–7.16 (*m*, 2 arom. H); 7.21–7.33 (*m*, 9 arom. H); 7.41 (*dd*, *J* = 1.5, 8.2, 2 arom. H); 7.56 (*t*, *J* = 7.2, 1 arom. H); 7.93 (*s*, H–C(8)); 8.07 (*d*, *J* = 7.5, 2 arom. H); 8.26 (*td*, *J* = 2.0, 9.0, 2 arom. H); 8.64 (*s*, H–C(2)); 9.05 (br. *s*, NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.9 (*q*, *Me*CH$_2$); 21.8 (*t*, Me*C*H$_2$); 24.7, 24.8, 28.9, 31.1, 34.2, 34.4 (6*t*, CH$_2$); 39.3 (*t*, CH$_2$S); 55.6 (*q*, MeO); 57.3 (*d*, C(2′)); 63.8 (*t*, C(5′)); 75.5 (*d*, C(3′)); 84.4 (*d*, C(4′)); 87.4 (*d*, C(1′)); 88.9 (*s*, arom. C); 113.7 (*d*, arom. C); 122.8 (*s*, C(5)); 125.6, 127.5, 128.3, 128.4, 128.6, 129.3, 130.5, 133.2 (9*d*, arom. C); 134.0, 135.8 (2*s*, arom. C); 142.2 (*d*, C(8)); 144.8, 145.7 (2*s*, arom. C); 150.1 (*s*, C(6)); 152.5 (*s*, C(4)); 153.3 (*d*, C(2)); 155.8 (*s*, arom. C); 159.1 (*s*, arom. C); 164.9, 171.4, 172.6 (3*s*, CO). ESI-MS: 1041.29 ([*M* + H]$^+$).

N^6-*Benzoyl-2′-(butyldithio)-2′-deoxy-5′*-O-*(4,4′-dimethoxytrityl)adenosine 3′-[6-(CGP-Amino)heptanoate]* (**6**). A suspension of LCAA-CPG (1.3 g; 500 Å, *Millipore*), **5** (122 mg, 0.12 mmol), and ^{i}Pr$_2$NEt (1.3 ml) in DMF (5.2 ml) was shaken for 16 h at 25°. After filtration, the solid was washed with DMF and CH_2Cl_2, suspended in pyridine (1.2 ml) and Ac_2O (0.8 ml), and shaken for 2 h at 25°. After filtration, the solid was washed with DMF and CH_2Cl_2, and dried to give **2**. Loading: 30 μmol/g.

3. *(C-C-C-C-A-C-C-[2′-(butyldithio)-2′-deoxy]A)-RNA 5′-(Dihydrogen Phosphate)* (**8**). The sequence was assembled from 60 mg of **6** by using the standard conditions for the assembly of 2′-*O*-tom-protected ribonucleoside phosphoramidites [9], but employing a modified oxidizing reagent: 20 mM I_2 in THF/pyridine/H_2O 7:2:1.

After the final detritylation, the solid support **7** was washed with $^{i}Pr_2NH$/MeCN 1:9 for 20 min (flow rate 2.5 ml/min). Cleavage from the solid support and deprotection was carried out with 12M NH_3 in MeOH (1 ml) for 6 h at 25°. The supernatant was removed by centrifugation and evaporated; the residue was treated with 1M $Bu_4NF \cdot 3\ H_2O$ in THF (1 ml) and 0.5M AcOH for 3 h at 25°. Then the mixture was diluted with 1M aq. *Tris*·HCl (1 ml; pH 7.4) and concentrated to 1 ml. After desalting on a *NAP* cartridge, the crude product was purified by ion-exchange HPLC. The product-containing fractions were concentrated to *ca.* 40% of their initial volume and purified by reversed-phase HPLC (pH 5.5, *A*/*B* 9:1 → *A*/*B* 7:3 (30 min)). The pooled, product-containing fractions were evaporated to 1/3 of the volume and finally desalted on a *Sepak* cartridge: 30 *OD* of pure **8** (25% yield based on **6**). Reversed-phase HPLC (pH 3.5; *Fig. 1*,*a*): t_R 19.8 (100%). ESI-MS (neg mode; *Fig. 1*,*e*): 2611 (calc. 2611).

4. *Experiments Described in the* Table: *General Procedure for Deprotection of* **8** *and Aminoacylation of* **9**. To 0.19 mM aq. **8** (27 µl; concentration determined spectrophotometrically at 260 nm) was added an aliquot of the indicated buffer ($c = 0.1$M; 27 µl) followed by an aq. TCEP soln. (6 µl; $c = 10$, 25, or 100 mM, resulting in 1.0, 2.5, or 25 mM solns., resp., see *Table*; prior to the addition, the pH of these TCEP stock solns. was adjusted with aq. NaOH soln.) in the corresponding buffer ($c = 50$ mM) and incubated at 25°. After 30 min, an aliquot of 20 µl was withdrawn and analyzed by reversed-phase HPLC (elution at pH 3.5). To the remaining reaction mixture, a soln. of **12** in DMF (4 µl; $c = 10$, 5, or 2.5 mM, resulting in 12, 6, or 3 equiv., resp., see *Table*) was added and incubated at the indicated temp. Aliquots of 20 µl were withdrawn after 10 and 50 min and analyzed by reversed-phase HPLC (pH 3.5). For experiments at 37°, the crude **9** was pre-incubated at 37° before the addition of **12**. Identical experiments with the parent RNA sequence $(^{-}O)_2P(=O)O-r(CCCCACCA)$ were carried out; according to HPLC and ESI-MS analyses, no aminoacylated or other products were formed.

Isolation and Characterization of **9**. To 0.19 mM aq. **8** (81 µl), 0.1M aq. AcOH/NaOH (81 µl; pH 5.0) was added, followed by 25 mM aq. TCEP (18 µl; in 0.05M AcOH/NaOH, pH 5.0). After 30 min incubation at 25°, the product soln. was desalted on a *NAP* cartridge. Data of **9**: Reversed-phase HPLC (pH 3.5): t_R 14.0 (100%); *Fig. 1*,*b*. ESI-MS (neg. mode): 2523 (calc. 2523); *Fig. 1*,*f*.

5. *Experiment Described in* Fig. 2. To 0.19 mM aq. **8** (27 µl) was added aq. *Tris*·HCl buffer (pH 7.4, $c = 0.1$M; 27 µl), followed by 10 mM aq. TCEP (6 µl; in 0.05M *Tris*·HCl buffer, pH 7.4). After 30 min at 25°, an aliquot of 20 µl was withdrawn and analyzed by reversed-phase HPLC (elution at pH 3.5). To the remaining reaction mixture, 10 mM **12** in DMF (4 µl) was added, and the soln. was incubated at 37°. Aliquots were taken after 2, 10, 20, 40, 50, 80, 120, and 180 min, and analyzed by reversed-phase HPLC (pH 3.5; see *Fig. 2*,*b*, for an example obtained after a reaction time of 2 min). By integration, the ratio **9**/**10**/**11** was determined in each chromatogram and the ratio (**10** + **11**)/(**9** + **10** + **11**) was plotted against the reaction time (*Fig. 2*,*a*).

6. *Hydrolysis Studies Described in* Fig. 3. 6.1. *Hydrolysis of* **10**. An aq. soln. of pure **10** ($c = 8$ µM, 900 µl; obtained by HPLC purification and desalting as described above) was incubated for 2 min at 25° or 37°, resp. Then, 1M aq. *Tris*·HCl (pH 7.4; 100 µl) was added, and the incubation was continued at 25° or 37°. At different time intervals, aliquots were withdrawn, treated with AcOH (→ pH 3) and analyzed by reversed-phase HPLC (pH 3.5). The integral ratios between signals of **10** and **9** were translated into individual pseudo-first-order rate constants (*Fig. 3*,*b*): k (37°) = 0.075 min^{-1} and k (25°) = 0.02 min^{-1}.

6.2. *Hydrolysis of* **11**. Desalted **11**/**10** 3:1 (total $c \approx 8$ µM; 900 µl; obtained at pH 5.0 as described above) was incubated for 2 min at 37°. Then, 1M aq. *Tris*·HCl (pH 7.4; 100 µl) was added, and the incubation was continued at 37°. At different time intervals (2, 4, 8, 12, and 24 min), aliquots were withdrawn, treated with AcOH (→ pH 3), and analyzed by reversed-phase HPLC (pH 3.5). The disappearance of the signal of **11** was translated into a pseudo-first-order rate constant (*Fig. 3*,*b*): k (37°) = 0.15 min^{-1}.

6.3. *Graph Shown in* Fig. 3,c. From the rates of hydrolysis k_1 (**11** → **10**) = 0.15 min^{-1} and k_2 (**10** → **9**) = 0.075 min^{-1}, the line graphs shown in *Fig. 3*,*c* were obtained according to the following equations: $[\mathbf{11}]_t = [\mathbf{11}]_0 \cdot \exp(-k_1 \cdot t)$; $[\mathbf{10}]_t = [([\mathbf{11}]_0 \cdot k_1)/(k_1 - k_2)] \cdot [\exp(-k_2 \cdot t) - \exp(-k_1 \cdot t)]$; $[\mathbf{9}]_t = [\mathbf{11}]_0 \cdot (1 + [k_1 \cdot \exp(-k_2 \cdot t) - k_2 \cdot \exp(-k_1 \cdot t)]/[k_2 - k_1])$. The experimentally determined amounts of **9**, **10**, and **11**, obtained at different times (see above), are also shown in the graph (as points).

7. *Hydrolysis of* **12**. A soln. of **12** in DMF ($c = 10$ MM; 50 µl) was added to 1M aq. *Tris*·HCl (pH 7.4; 950 µl; pre-incubated at 25° or 37°). At different time intervals, aliquots were withdrawn, treated with AcOH (→ pH 3), and analyzed by reversed-phase HPLC (pH 3.5; gradient *A* → *B* in 30 min; full loop injection). The dissapearance of **12** (t_R 19.3 min) was translated into individual pseudo-first-order rate constants: k (25°) = 0.007 min^{-1}, k (37°) = 0.07 min^{-1}.

8. *Experiment Described in* Fig. 4. *Simultaneous Deprotection of* **8** *and Aminoacylation of* **9**, *Selective Cleavage of* **11** *to* **10**, *and Isolation and Characterization of* **10**. To 0.19 mM aq. **8** (81 µl), 1M aq. AcOH/NaOH (81 µl; pH 5.0) was added, and the mixture was incubated at 37° for 2 min. Then, 4 mM **12** in DMF (18 µl) was added,

followed by 25 mM aq. TCEP (18 µl; in 0.05M AcOH/NaOH, pH 5.0). After 30 min incubation at 37°, a 20-µl aliquot was analyzed by reversed-phase HPLC (pH 3.5; see *Fig. 4,a*). To the remaining soln., 1.5 mM aq. NaN_3 (480 µl) was added and incubated for 30 min at 25°. The mixture was diluted with H_2O (340 µl), desalted on a *NAP* cartridge, and characterized by reversed-phase HPLC (pH 3.5; *Fig 4,b*). Pure product **10** was obtained by reversed-phase HPLC at pH 3.5, concentration of the product-containing fraction to *ca.* 60% of its initial volume, desalting on a *NAP* cartridge, followed by immediate addition of AcOH (1% final concentration), and lyophilization. Data of **10**: Reversed-phase HPLC (pH 3.5): t_R 16.9 (100%). ESI-MS (neg. mode): 2671 (calc. 2671); *Fig. 1,g*.

Isolation and Characterization of **11**. To 0.19 mM aq. **8** (81 µl), 0.1M aq. AcOH/NaOH (81 µl; pH 5.0) was added. Then, 25 mM **12** in DMF (18 µl) was added, followed by 25 mM aq. TCEP (18 µl; in 0.05 M AcOH/NaOH, pH 5.0). After 30 min incubation at 25°, a 20-µl aliquot was analyzed by reversed-phase HPLC (pH 3.5), which showed **11** and **10** in a 9:1 ratio. The remaining product soln. was desalted on a *NAP* cartridge and stabilized immediately by addition of AcOH (final concentration 1%). Data of **11**: Reversed-phase HPLC (pH 3.5): t_R 16.9 (**10**; 10%) and 18.0 (**11**; 90%). ESI-MS (neg. mode): 2818 (**11**, 90%; calc. 2819) and 2671 (**10**, 10%; calc. 2671); *Fig. 1,h*.

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