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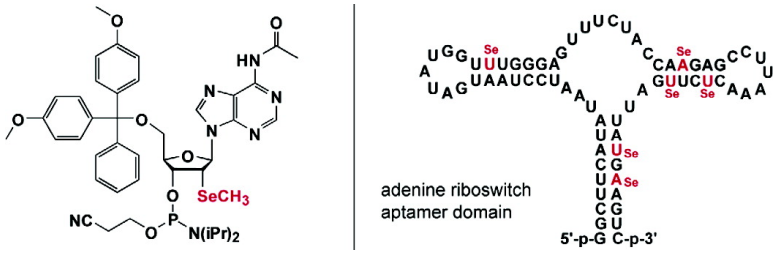

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Syntheses of RNAs with up to 100 Nucleotides Containing Site-Specific 2′-Methylseleno Labels for Use in X-ray Crystallography

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Abstract: The derivatization of nucleic acids with selenium is a new and highly promising approach to facilitate their three-dimensional structure determination by X-ray crystallography. Here, we report a comprehensive study on the chemical and enzymatic syntheses of RNAs containing 2′-methylseleno (2′-*Se*-methyl) nucleoside labels. Our approach includes the first synthesis of an appropriate purine nucleoside phosphoramidite building block. Most importantly, a substantially changed RNA solid-phase synthesis cycle, comprising treatment with *threo*-1,4-dimercapto-2,3-butanediol (DTT) after the oxidation step, is required for a reliable strand elongation. This novel operation allows for the chemical syntheses of multiple *Se*-labeled RNAs in sizes that can typically be achieved only for nonmodified RNAs. In combination with enzymatic ligation, biologically important RNA targets become accessible for crystallography. Exemplarily, this has been demonstrated for the Diels−Alder ribozyme and the *add* adenine riboswitch sequences. We point out that the approach documented here has been the chemical basis for the very recent structure determination of the Diels−Alder ribozyme which represents the first novel RNA fold that has been solved via its *Se*-derivatives.

Introduction

The most powerful approach for the three-dimensional structure determination of biomolecules is X-ray crystallography. Thereby, the extraction of structural information from X-ray diffraction patterns of a crystallized biomolecule is a complex process which demands phase determination. Phase information can be obtained by the classical multiple isomorphous replacement method (MIR) which requires at least three isomorphous crystals, one native and two heavy atom derivatives. Due to difficulties in finding such derivatized isomorphous crystals, new techniques taking advantage of anomalous scattering properties of some atoms have been developed.[1−3] These methods, termed multiwavelength anomalous dispersion (MAD) and single-wavelength anomalous diffraction (SAD), require only a single crystal for collecting a data set that is sufficient to calculate the phases and determine the three-dimensional structure. A prerequisite is that the crystal contains anomalous scattering centers such as selenium atoms (Figure 1a).

The majority of new protein crystal structures has been determined via the selenomethionyl-modified derivatives.[3] Selenomethionine incorporation and MAD phasing have become the norm primarily by the simplicity of the in vivo selenomethionine incorporation into recombinant proteins.[4,5] Selenomethionine replaces the natural methionine, yielding protein derivatives that are largely unperturbed with respect to the native structure.

In nucleic acid crystallography, *Se*-labeling is just beginning to emerge, and the high potential of selenium nucleic acid derivatives for phasing has not been generally recognized. Three years ago, pioneering work by Egli, Huang, and co-workers led to the successful MAD phasing of a short Z-form DNA duplex via phosphoroselenoate backbone modifications and of an A-form DNA duplex via 2′-methylseleno (2′-*Se*-methyl) uridine modifications.[6,7] Soon thereafter, our laboratory has been able to establish the first protocols for the preparation of short 2′-*Se*-methyluridine and 2′-*Se*-methylcytidine modified oligoribonucleotides.[8]

Extending this approach to larger, biologically attractive RNA targets, we now report an advanced methodology that allows for the preparation of high-purity RNAs with up to 100 nucleotides containing site-specifically incorporated, multiple *Se*-labels for use in X-ray crystallography. The first successful

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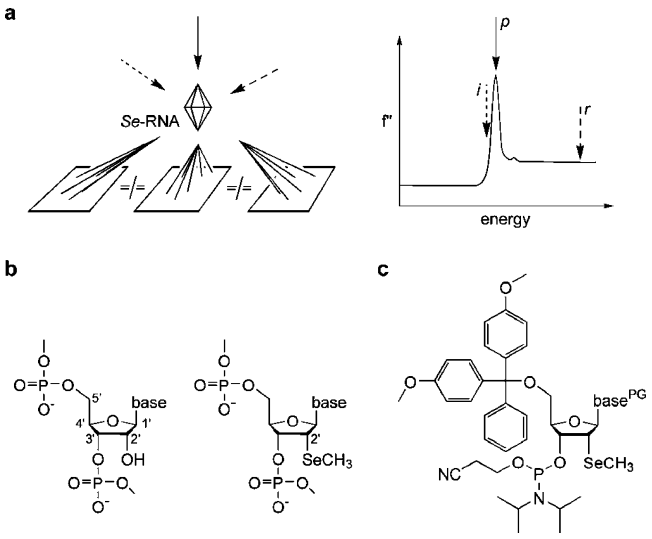

Figure 1. *Se*-modified RNA for X-ray crystallography using novel techniques for phase determination, such as MAD, SAD, or SIRAS (single isomorphous replacement with anomalous scattering). (a) In MAD data collection, for instance, the differences in intensity necessary for phasing are obtained from a single crystal containing anomalous scattering centers by varying the wavelength of the X-ray beam, indicated by different arrow-styles. The wavelengths chosen for diffraction measurements are marked in the schematic diagram of anomalous scattering (imaginary component *f″*) around the K-edge of selenium and are designated at the inflection point (i), the peak (p), and a remote (r) wavelength of higher energy.[2] (b) Chemical structure of an RNA nucleoside repetition unit and the promoted 2′-*Se*-methyl-modified counterpart. (c) General structural formula of a 2′-*Se*-methyl nucleoside phosphoramidite building block suitable for chemical RNA solid-phase synthesis; protecting group (PG).

applications of the *Se*-derivatized RNAs developed in the course of this project have been documented recently and refer to the X-ray structure of the group I intron with both exons[9] and to the recent structure determination of the Diels−Alder ribozyme solved by the SAD-technique via the corresponding 2′-*Se*-methylpyrimidine derivatives synthesized in our laboratory.[10]

Striking discoveries on the functional importance of small RNAs and specialized RNA domains have been made in recent years.[11] The investigations of phenomena such as RNA interference[12] or riboswitches,[13] are accompanied by a growing demand for X-ray structure analysis of the RNA molecules involved. If RNA is crystallized without proteins, the selenomethionine approach is excluded a priori. In addition, small RNAs do not always possess specific, noncovalent heavy metal ion binding sites appropriate for cocrystallization or soaking of the crystals in the relevant ion solutions.[14] Moreover, derivatization of pyrimidine nucleosides with 5-halogen atoms, being the common way to covalently attach anomalous scattering centers to short oligonucleotides,[15−21] is hardly reported for RNA sequences larger than 25 to 30 nucleotides. The goal of our study was to develop an alternative, namely a selenium modification method, that permits overcoming this size limitation and allows placing the anomalous scattering labels at both pyrimidine and purine nucleosides.

Results

Ribose 2′-*Se*-Methyl Modification. Three categories for the replacement of oxygen by selenium atoms are chemically reasonable in the nucleotide repetition unit of RNA. These concern phosphoroselenoates, selenonucleobases, and 2′-*Se*-modified ribose derivatives (Figure 1b,c). With respect to steric constraints, phosphoroselenoates and selenonucleobases are comparable to their natural counterparts, but they suffer from decreased stability toward hydrolysis and altered base-pairing properties, respectively. Predominantly for these reasons, we gave priority to the third category, namely to 2′-*Se*-methyl-modified nucleosides with structural features that are closely related to the naturally occurring 2′-*O*-methylated nucleosides. Incorporated within an A-form nucleic acid duplex, they support the C3′-endo type ribose puckering. The 2′-*Se*-methyl groups are directed toward the minor groove, hardly perturbing the overall duplex structure.[22] Therefore, these modifications are ideal for nucleotide replacements within the double helical regions of large and complex RNA folds.

Phosphoramidite Building Blocks. To guarantee maximal flexibility for positioning the *Se*-label within a double helical region of RNA, not only the previously reported 2′-*Se*-methylpyrimidine nucleosides[8] but also the corresponding 2′-*Se*-methylpurine counterparts are required. Here, we present the first chemical synthesis of a 2′-*Se*-methyladenosine building block for RNA solid-phase synthesis (Scheme 1). The precursor **1** was readily available as this nucleoside derivative accumulates during the preparation of standard 2′-*O*-[(triisopropylsilyl)oxy]-methyl (TOM) adenosine phosphoramidite building blocks.[23] Triflation of the ribose 2′-OH furnished intermediate **2** which was isolated and converted into the corresponding arabino nucleoside **3** by treatment with potassium trifluoroacetate. After triflation of the arabinose 2′-OH, compound **4** was reacted with sodium methylselenide, yielding key compound **5**, followed by cleavage of the 3′-*O*-TOM protecting group to give derivative **6**. By subsequent phosphitylation, the final building block **7** was obtained in good overall yield. This synthesis also provides a solid foundation for the synthesis of the corresponding guanosine building block that is currently in progress in our laboratory.

Chemical Synthesis and Deprotection of *Se*-Derivatized RNA. The preparation of RNA with 2′-*Se*-methyl-modified nucleosides relies on the 2′-*O*-TOM-methodology for strand assembly.[23,24] Importantly, the solid-phase synthesis cycle was substantially changed from standard RNA synthesis. We inserted

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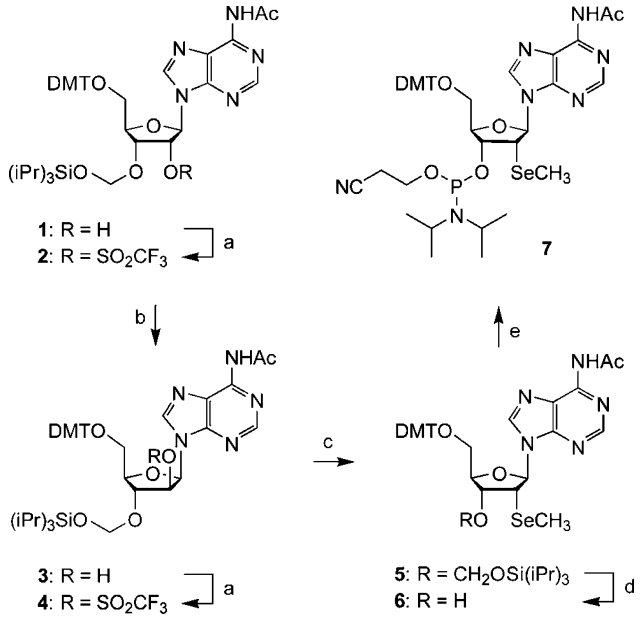
Scheme 1. Synthesis of the 2′-*Se*-Methyladenosine Phosphoramidite **7**[a]



[a] Reagents and conditions: (a) 1.5 equiv trifluoromethanesulfonyl chloride, 1.5 equiv DMAP, 2.5 equiv NEt_3, in CH_2Cl_2, room temperature, 20 min; (b) 8.3 equiv $CF_3COO^-K^+$, 2.5 equiv $(^iPr)_2NEt$, 3.3 equiv 18-crown-6-ether, in toluene, 80°C, 11 h (54% over (a) and (b)); (c) 6 equiv $NaBH_4$, 2 equiv $CH_3SeSeCH_3$, in THF, 30 min (47% over (a) and (c)); (d) 1 M TBAF, 0.5 M acetic acid, in THF, room temperature, 2.5 h, 84%; (e) 1.5 equiv (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite, 10 equiv $CH_3CH_2N(CH_3)_2$, in CH_2Cl_2, room temperature, 2 h, 91%; (DMT dimethoxytrityl, DMAP 4-(dimethylamino)pyridine; TBAF tetrabutylammonium fluoride).

an additional step after the capping−oxidation−capping operation, namely the treatment with a chemical reducing reagent (Figure 2). Such a step was lacking in our original protocols for short RNA sequences.[8] Here, we found that the repeated exposure of the growing chain to DTT was an absolute requirement for the reliable synthesis of longer RNAs containing multiple *Se*-labels (Table 1). The treatment was reflected in continuously high coupling yields (>98%) monitored via the UV-trityl assay. We speculate that selenium moieties that become oxidized during the phosphite−phosphate transformation by iodine (or *tert*-butylhydroperoxide) are regenerated in the presence of DTT. Follow-up reactions, such as syn-elimination of the seleniumoxide moiety by abstracting the ribose 1′-hydrogen, subsequent nucleobase elimination, and strand breakage are then minimized.[25]

Cleavage from the solid support and deprotection of the *Se*-modified RNAs was also performed in the presence of DTT, added in millimolar amounts to the deprotection solutions of CH_3NH_2 in ethanol/H_2O and of tetrabutylammonium fluoride (TBAF) in tetrahydrofurane (THF). The influence of DTT during the two-step deprotection of short *Se*-modified oligoribonucleotides has been studied in detail and documented previously.[8] Here, we stress the fact that the DTT treatment already during strand elongation increases the quality of the crude seleno-RNA products significantly (Figure 2). Note that this newly introduced operation does not replace the DTT treatment during RNA deprotection but has to be performed in a combined manner to give optimal results.

After the deprotection, DTT was removed by size exclusion chromatography on a Sephadex G10 column. RNAs with up to 50 nucleotides were then directly purified by anion-exchange chromatography under strong denaturating conditions (6 M urea, 80 °C). Larger RNAs were prepurified on a reversed-phase column prior to ion-exchange chromatography. The molecular weights of the purified RNAs have been confirmed by liquid chromatography (LC) electrospray-ionization (ESI) mass spectrometry (MS); this analysis also revealed that the purified products were not contaminated by N-1 strands.

Table 1 lists the chemically synthesized RNAs (**8**−**18**) containing up to seven *Se*-labels. Sequence **8** is an A_{Se}-derivative of the 22 nt C_{Se} RNA/DNA hybrid[8] that was crystallized in a ternary complex of the group I intron with both exons.[9] Sequence **13** with four *Se*-pyrimidine nucleosides and a total of 38 nucleotides was the basis for the very recent structure determination of the Diels−Alder ribozyme.[10] Sequences **9**−**12**, and **14**−**17** represent the *Se*-modified RNAs used for the enzymatic ligations described below. Finally, sequence **18** with four *Se*-labels and a total of 71 nucleotides demonstrates the upper limit of our solid-phase synthesis approach with respect to facile deprotection and purification.

Enzymatic Ligation of *Se*-Derivatized RNA. The development of high-yield enzymatic ligations of *Se*-containing RNAs has been a further aim of this project as this allows overcoming size limitations of a purely chemical approach, and consequently, the range of accessible, biologically important targets can be increased significantly.

Exemplarily, we have focused on the aptamer domain of the adenine deaminase (*add*) adenine-riboswitch[26,27] from *Vibrio vulnificus* and aimed at the preparation of the relevant *Se*-modified sequences based on single-stranded ligation via T4 RNA ligase and on splinted ligation via T4 DNA ligase, respectively. We considered two potential ligation sites for the 71 nt RNA **24** as indicated by arrows in Figure 3a (see also Table 2, Supporting Information Table 1 online; Supporting Information Figures 1−6 online). These sites were chosen because among donors (providing the 5′-phosphate terminus), 5′-terminal pyrimidines are preferred slightly over purines by the T4 RNA ligase. Among acceptors, 3′-terminal adenosines are the best acceptors, cytidines and guanosines show intermediate reactivity, and terminal uridines are poor substrates.[28] The two ligation sites chosen for the riboswitch motif[27] meet these requirements.

For ligations using T4 RNA ligase, the donor strands represented by either 31 nt or 48 nt RNAs were provided as 5′,3′-bisphosphates (**9**, **11**, **12**, **20**: Figure 3b; **16**, **17**: Figure 3d) to avoid cyclization and oligomerization of the donor and byproducts from repeated ligation of the donor with already formed ligation products. The acceptor strands (40 nt and 23 nt, respectively) were either supplied with 5′,3′-hydroxyl termini (**14**, **19**: Figure 3b; **21**: Figure 3d) or 5′-phosphate/3′-hydroxyl termini (**15**: Figure 3b; **22**: Figure 3d) obtained by chemical

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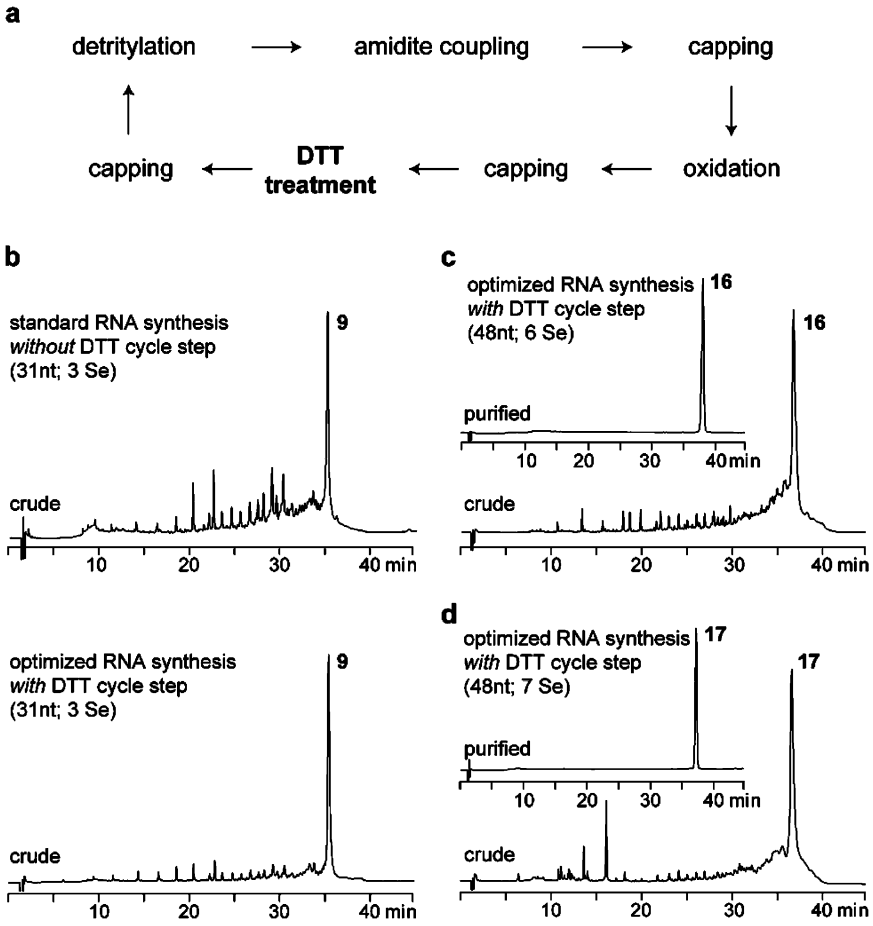

Figure 2. Chemical synthesis of *Se*-modified RNA. (a) Organization of the RNA solid-phase synthesis cycle with the newly introduced step of *threo*-1,4-dimercapto-2,3-butanediol (DTT) treatment. (b−d) HPLC-traces of crude, deprotected 2′-*Se*-modified RNAs; deprotection procedures included three steps: (1) 150 mM DTT in EtOH/H_2O, 1−3 h, room temperature; (2) CH_3NH_2 in EtOH/H_2O, 150 mM DTT, 4−6 h, room temperature; (3) 1 M TBAF in THF, 150 mM DTT, 12−16 h, room temperature. (b) Comparison of crude **9**, prepared with (bottom) and without (top) the DTT solid-phase synthesis cycle step. (c,d) Crude and purified (inset) **16** and **17**, prepared with the DTT solid-phase synthesis cycle step. Anion-exchange HPLC: Dionex DNAPac (4 × 250 mm), 80°C, 1 mL/min, 0−60% B in 45 min; A: 25 mM Tris-HCl, 6 M urea, pH 8.0; B: same as A + 0.5 M $NaClO_4$.

synthesis or with 5′-triphosphate/3′-hydroxyl termini (**23**; Figure 3d) obtained by in vitro transcription with T7 RNA polymerase.[29]

Optimized ligation conditions (as applied for the ligation of **24a** and **24b**, Figure 4a, Supporting Information Figure 1 online) account for strand concentrations of 40 μM (donor/acceptor = 1/1) and 0.20 U/μL T4 RNA ligase in Tris buffer (pH 7.8) in the presence of $MgCl_2$, DTT, and ATP, at 21°C. In general, T4 RNA ligation yields were 50−70% for ligation site S2 (31 nt + 40 nt) and higher (70−80%) for ligation site S1 (48 nt + 23 nt) (Table 2, Supporting Information Table 1 online, and Supporting Information Figures 1−6 online).

With respect to ligation site S2, we also elaborated splinted ligations using T4 DNA ligase (Figure 3a,c).[30,31] For this, the applicability of 2′-*O*-methyl RNA splints instead of DNA splints has been considered advantageous for proper annealing of the ternary ligation complex.[32] Competitive intramolecular base-pairing interactions of the two ligation fragments are more easily broken up with a 2′-*O*-methyl RNA oligonucleotide compared with a DNA oligonucleotide of the same length. Figure 3c displays the various combinations of RNA fragments for ligation of the riboswitch target using T4 DNA ligase and the 2′-*O*-methyl RNA splint **25**.

Optimized ligation conditions (as applied for the ligation of **24c**, Figure 4b) account for strand concentrations of 10 μM (donor/acceptor/splint = 1/1/1) and 0.25 U/μL T4 DNA ligase in Tris buffer (pH 7.8) in the presence of PEG 4000, $MgCl_2$, DTT, and ATP, at 37°C. The yields using T4 DNA ligase reached up to 70% (Table 2, Supporting Information Table 1 online, and Supporting Information Figures 2−6 online). Note that only minor ligation yields were observed when we used the corresponding 15 nt DNA or the chimeric splint 2′-*O*-Me-(CUCUU)d(GGTA)2′-*O*-Me(GAAAC)dT.[33,34]

The general procedure for workup of the ligation reactions included phenol−chloroform−isoamyl alcohol extraction and analysis of the reaction mixture by anion-exchange (IE) HPLC and PAGE. Subsequent purification of the desired ligation products was performed by IE-HPLC under strong denaturating conditions (6 M urea, 50−80°C). This chromatography typically gave higher yields than PAGE purification. The molecular weights of the ligation products were found virtually identical to the predicted values by LC−ESI-MS (Table 2, Supporting

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Table 1. RNAs Containing 2′-*Se*-Methyl Nucleosides Prepared by Solid-Phase Synthesis[a]

					isolated yield		molecular weight	
no.	sequence[a]	Se nt	length nt	scale μmol	OD 260 nm	nmol	calcd amu	found[b] amu
8	5′-AAGCCACA$_{Se}$CAAACC(dA)(dG)(dA)CGGCC-3′	1	22	1	30	118	7057.4	7056.3
9	5′-p-CCAAGAGCCUUAAACU$_{Se}$CU$_{Se}$UGAUUAU$_{Se}$GAAGUC-p-3′	3	31	0.5	19	54	10238.9	10238.0
10	5′-p-CCAAGAGCCUUAAACU$_{Se}$CU$_{Se}$U$_{Se}$GAUU$_{Se}$AU$_{Se}$GAAGU$_{Se}$C-p-3′	6	31	1	29	80	10469.8	10468.8
11	5′-p-CCA$_{Se}$AGA$_{Se}$GCCUUAAACUCUUGAUUA$_{Se}$UGAAGUC-p-3′	3	31	0.5	9	24	10238.9	10238.3
12	5′-p-CC$_{Se}$AAGAGCCUUAAAC$_{Se}$UC$_{Se}$UU$_{Se}$GAUUAU$_{Se}$GAAGU$_{Se}$C-p-3′	6	31	0.5	14	41	10469.8	10468.6
13	5′-GGGC$_{Se}$GAGGC$_{Se}$CGUGCCGGCU$_{Se}$CUU$_{Se}$CGGAGCAAUACUCGGC-3′	4	38	1	20	49	12569.4	12568.5
14	5′-GGCU$_{Se}$UCAU$_{Se}$AUAAUCCUAAUGAUAUGGUU$_{Se}$UGGGAGUUUCUA-3′	3	40	0.5	7	16	12992.6	12991.0
15	5′-p-GGC$_{Se}$UU$_{Se}$CAUAUAAUCC$_{Se}$UAAUGAUAUGGUU$_{Se}$UGGGAGUUUCUA-3′	4	40	1	19	42	13149.5	13148.0
16	5′-p-UGGUU$_{Se}$UGGGAGUUUCUACCAA$_{Se}$GAGCCUUAAACU$_{Se}$CUU$_{Se}$GAUUAU$_{Se}$GA$_{Se}$AGUC-p-3′	6	48	1	33	62	15954.0	15953.3
17	5′-p-UGGUU$_{Se}$U$_{Se}$GGGAGUUUCUACCA$_{Se}$AGA$_{Se}$GCCUUAAACUCU$_{Se}$UGAUUAU$_{Se}$GAA$_{Se}$GUC-p-3′	7	48	1	42	76	16031.1	16030.8
18	5′-p-GGCU$_{Se}$UCA$_{Se}$UAUAAUC$_{Se}$CUAAUGAUAUGGUU$_{Se}$UGGGAGUUUCUACCAAGAGCCUUAAACUCUUGAUUAUGAAGUC-p-3′	4	71	1	7	8	23139.5	23138.3

[a] A$_{Se}$, 2′-*Se*-methyladenosine; C$_{Se}$, 2′-*Se*-methylcytidine; U$_{Se}$, 2′-*Se*-methyluridine; dA, 2′-deoxy adenosine; dG, 2′-deoxy guanosine. [b] LC−ESI-MS.

Information Table 1 online, and Supporting Information Figures 1, 3, 4 online). Only when the acceptor strands were applied as 5′-monophosphates in T4 RNA ligation (**15**; Figure 3b), were significant amounts of adenylated **24** (5′-App-71mer) obtained as byproduct (Supporting Information Figure 4 online).

The wide range of ligation conditions tested for sequence **24** revealed the advantages and disadvantages of T4 RNA and DNA ligation approaches. Although both methodologies are amenable for large scales, T4 RNA ligations were preferred because of higher overall yields, less enzyme required, and no need for an external splint (Supporting Information Table 1 online, and Supporting Information Figures 4−6 online). For general applications, however, T4 RNA ligation encounters some restrictions. Beside the intolerance of T4 RNA ligase against ribose 2′-*Se*-methyl modifications within the last three nucleotides of the acceptor strand,[8] different nucleobase specificities of T4 RNA ligase for donor and acceptor strands are observed[8,28] (Figure 3a; S1 slightly preferred over S2). Moreover, proper annealing of the two strands to be ligated is required. In this respect, ligation of target **24** at the site S1 is ideal because the acceptor and donor strands are strongly bound to each other by formation of helices P1 and P2, thereby positioning the 3′- and 5′-ends in close proximity for reaction (Figure 3a,d; Figure 4a). Ligations with T4 RNA ligase are further confined if specific 5′- and 3′-termini are demanded in the ligation product. To avoid byproducts, the acceptor strands must be provided as 5′,3′-bisphosphates in combination with donor strands that do possess either 5′-triphosphate or 5′-hydroxyl moieties (**24a**, **24b**; Figure 4a, Supporting Information Figure 1 online). If products with 5′-phosphate and 3′-phosphate termini are requested (such as **24c**), ligations based on T4 DNA ligase with a splint are preferable (Figure 4b) as demonstrated by the ligation of acceptor **15** and donor **20** with either T4 RNA or T4 DNA ligase (Figure 4 in Supporting Information online). We note here that particular chemical groups at the 5′- and 3′-termini of an RNA sequence may be critical for the crystallization behavior. One such example refers to sequence **13** involved in the structure determination of the Diels−Alder ribozyme.[10] The ribozyme crystals grew only when the RNA had 5′- and 3′-hydroxyl groups (Figure 5), and not 5′-triphosphate and 2′,3′-cyclophosphate moieties.

Crystallization of *Se*-Derivatized RNA. To elucidate effects of the *Se*-derivatization on RNA crystallization, we performed systematic crystallization trials with the 49 nt Diels−Alder ribozyme complexed with the reaction product. Since the ribozyme consisted of two oligonucleotides (Figure 5a), we synthesized the corresponding 11-mer with two Se-labels (**29**),[8] and the 38-mer with three and four Se-labels (**27**, **13**; Figure 5a). The reaction product was covalently attached to the 5′-end of the 11-mer via a hexaethyleneglycol linker.[10] Annealing of various combinations of oligonucleotides resulted in the ribozyme carrying up to six *Se*-atoms. All of these variants were crystallized in the same conditions and produced crystals of similar shape and size (Figure 5b) diffracting at about 3 Å

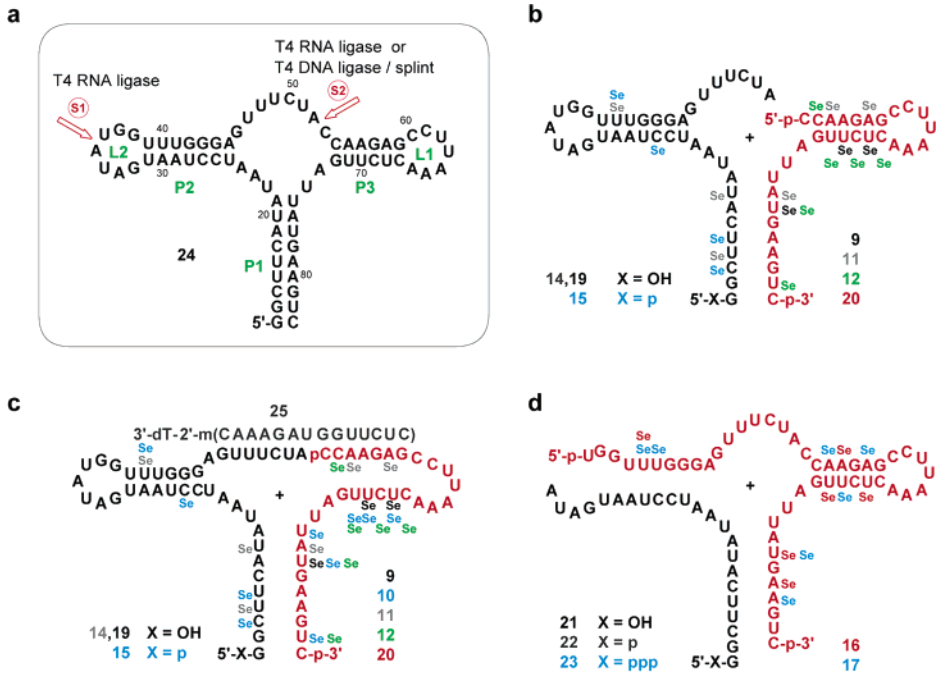

Figure 3. Enzymatic ligation of *Se*-modified RNA. (a) *add* adenine riboswitch target sequence **24** and potential sites S1 and S2 for ligation with T4 RNA ligase and T4 DNA ligase, respectively. Nucleotide numbering refers to ref 26. (b−d) Conception of the different ligation experiments: (b) T4 RNA ligations of 31 nt donor (5′-phosphate) and 40 nt acceptor (3′-OH) strands (for 3−10 *Se*-labels); (c) T4 DNA ligations of 31 nt donor and 40 nt acceptor strands with a 15 nt 2′-*O*-methyl RNA splint (for 3−10 *Se*-labels); (d) T4 RNA ligations of 48 nt donor and 23 nt acceptor strands (for 6−7 *Se*-labels).

Table 2. Selected Ligation Experiments of the *Add* Adenine Riboswitch Aptamer Domain with Multiple *Se*-Labels

							isolated yield			molecular weight	
ligation product	product sequence[a]	acceptor, donor, splint (ratio)	scale[b] nmol	ligase	ligation conditions[c]	yield %[d]	OD $^{260\,nm}$	nmol	%	calcd amu	found[e] amu
24a	5′-ppp-GGCUU CAUAU AAUCC UAAUG AUAUG GUU$_{Se}$U$_{Se}$G GGAGU UUCUA CCA$_{Se}$AG A$_{Se}$GCCU UAAAC UCU$_{Se}$UG AUUAU$_{Se}$ GAA$_{Se}$GU C-p-3′	**23**, **17** (1/1)	0.3	T4 RNA	40 μM; 0.2 U/μL; 1.0 mM ATP; 21°C; 3 h	80	0.16	0.2	66	23530.4	23530.1
24b	5′-GGCUU CAUAU AAUCC UAAUG AUAUG GUU$_{Se}$UG GGAGU UUCUA CCAA$_{Se}$G AGCCU UAAAC U$_{Se}$CUU$_{Se}$G AUUAU$_{Se}$ GA$_{Se}$AGU C-p-3′	**21**, **16** (1/1)	40	T4 RNA	40 μM; 0.2 U/μL; 1.0 mM ATP; 21°C; 2 h	80	17.7	22.0	55	23213.4	23212.9
24c	5′-p-GGC$_{Se}$UU$_{Se}$ CAUAU AAUCC$_{Se}$ UAAUG AUAUG GUU$_{Se}$UG GGAGU UUCUA CCAAG AGCCU UAAAC U$_{Se}$CU$_{Se}$UG AUUAU$_{Se}$ GAAGU C-p-3′	**15**, **9**, **25** (1/1/1)	20	T4 DNA	10 μM; 0.25 U/μL; 0.5 mM ATP; 37°C; 5 h	65	4.43	5.5	27	23370.4	23369.6

[a] A$_{Se}$, 2′-Se-methyladenosine; C$_{Se}$, 2′-*Se*-methylcytidine; U$_{Se}$, 2′-*Se*-methyluridine. [b] Amount of each strand. [c] Concentrations of each ligation fragment and of T4 RNA/T4 DNA ligase in ligation buffer (50/40 mM Tris.HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT); ATP concentration; reaction temperature; reaction time; in case of T4 DNA ligase: 5% w/v PEG. [d] Yield calculated from peak areas of HPLC-traces (UV absorbance at 260 nm). [e] ESI-MS.

resolution (Figure 5c) and belonging to the same *P*21 space group with similar cell unit parameters. Therefore, incorporation of even multiple *Se* modifications does not affect crystallization of RNA, does not change crystal quality, and requires, at most, adjustment of RNA concentration for production of large single crystals.

Discussion

The high potential and the usefulness of selenium with respect to nucleic acid X-ray structure analysis have been recognized at least by some research groups during recent years.[6−10] Although heavy-metal ion derivatization by soaking the crystals in heavy atom salt solutions is the established way to obtain crystals with anomalous scattering centers, we consider the modification with covalent 2′-*Se*-methyl groups most competitive for structure analysis of RNAs comprising between 30 and about 80 nucleotides. Heavy atom search is a time-consuming process which requires soaking the RNA crystals with dozens of compounds at various concentrations, therefore requesting many reasonably good crystals. This can be a serious obstacle. During the efforts of crystallizing the 49 nt Diels−Alder ribozyme, neither suitable heavy atom derivatives nor 5-halogenpyrimidine derivatives could be identified. Finally, crystals of the *Se*-modified RNAs developed here (Figure 5) were respon-

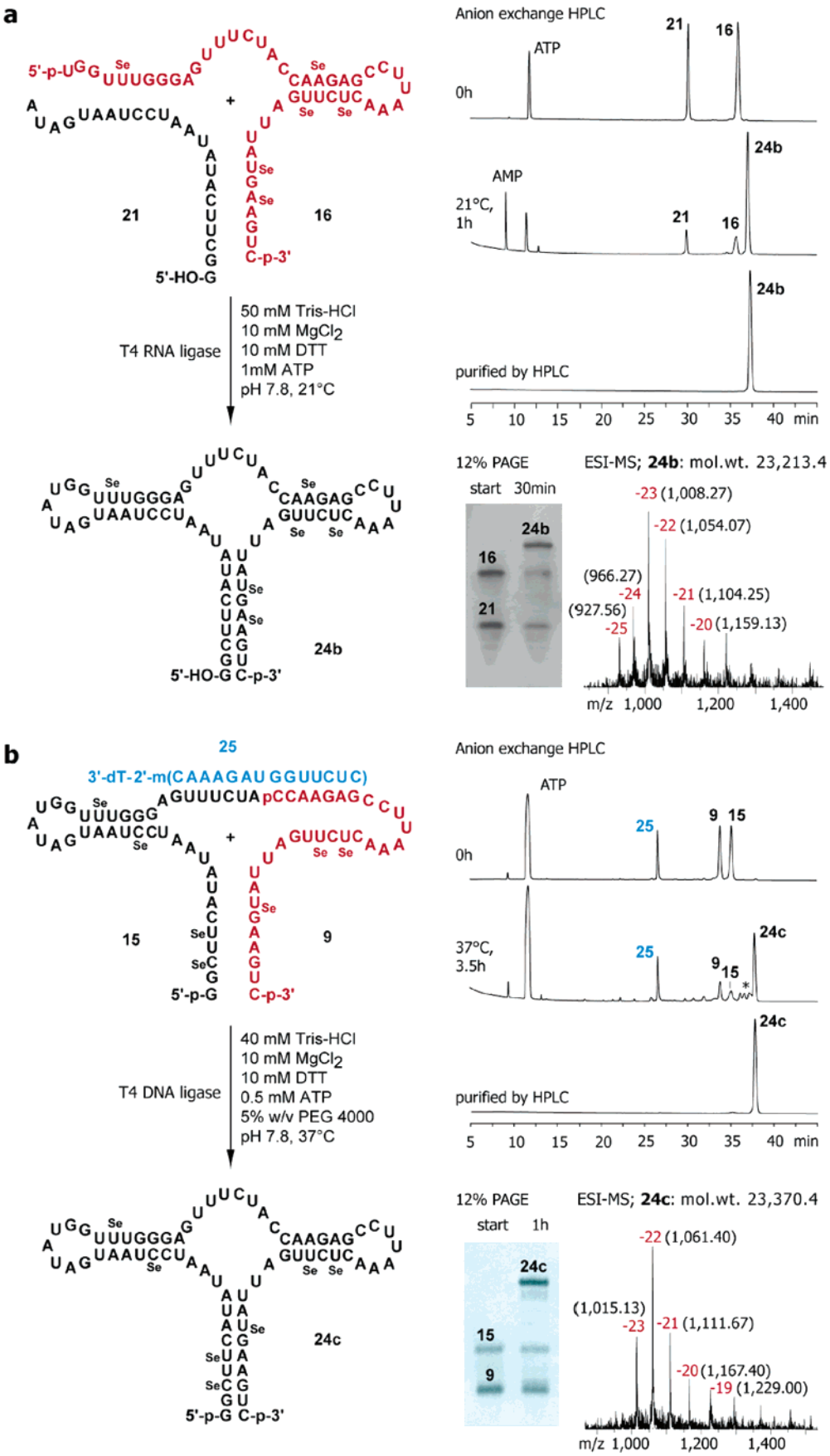

Figure 4. Representative enzymatic ligations of the *add* adenine riboswitch target sequence **24** with different 2′-*Se*-methyl nucleoside pattern. Analysis of the ligation experiments was performed by anion-exchange HPLC, denaturating PAGE, and LC−ESI-MS. (a) Ligation of **24b** with T4 RNA ligase (0.20 U/μL; c_{RNA} = 40 μM each strand; donor/acceptor = 1/1) at 21°C. (b) Ligation of **24c** with T4 DNA ligase (0.25 U/μL; c_{RNA} = 10 μM each strand; donor/acceptor/splint = 1/1/1) at 37 °C; (*slight degradation of the product at 37°C was accepted for higher reaction rates).

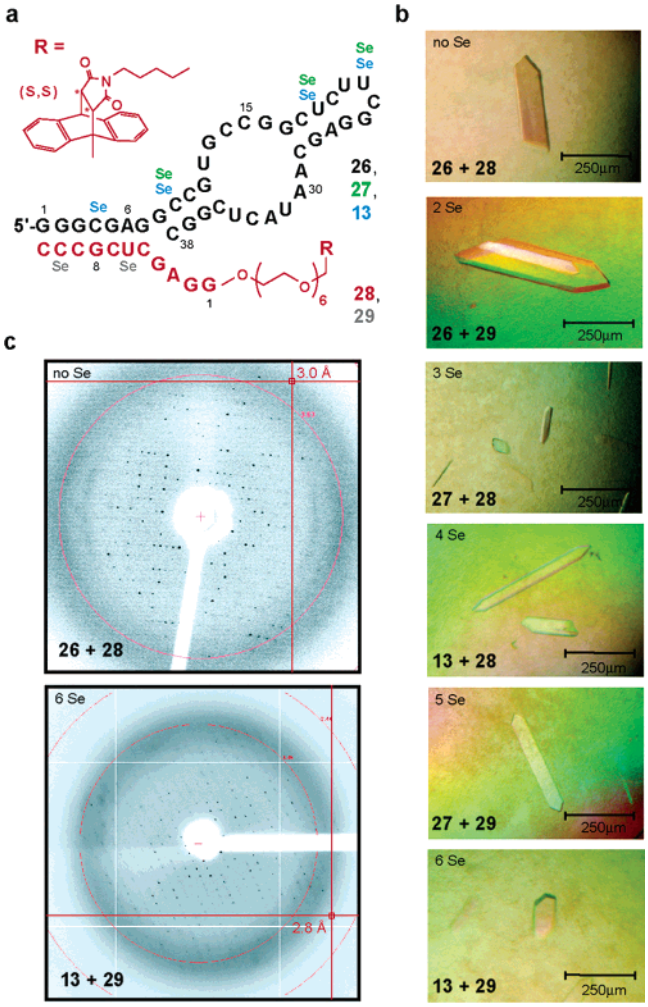

Figure 5. Crystallization of the *Se*-derivatized Diels−Alder ribozyme. (a) Sequences and secondary structure of the ribozyme. (b) Photographs of the typical crystals. Note that despite the different number of *Se*-modifications all crystals have a similar shape. (c) Diffraction patterns of the ribozyme crystals prepared either nonmodified or modified with six *Se*-atoms RNAs. Rotation images (oscillation 0.5°) were collected using in-house Rigaku X-ray generator (top) and beamline X25 at Brookhaven National Synchrotron Light Source (NSLS) (bottom). Note similar quality and resolution limits for both crystals.

sible for the structure determination.[10] Interestingly, the *Se*-modified ribozyme did not demand significant changes of the crystallization conditions compared to the ones of the native RNA. This implies that the 2′-*Se*-methyl moieties were ideally "hidden" within the minor grooves of the double helical segments. Thus far, we have experienced that positioning of the labels is most successful when the co-variant, nonconserved nucleosides within the double helices of an RNA fold are replaced by the corresponding *Se*-modified counterparts. This information on the secondary structure level is usually available by biochemical mutation experiments or by phylogenetic comparison of the RNA of interest. Such a strategy was successful for the Diels−Alder ribozyme,[10] and although we came up too late with the *Se*-modified derivatives of riboswitch RNAs, their recently determined structures[27,35] validate the positions we have chosen for *Se*-labeling as most promising for proper crystallization, in retrospect. Clearly, the risk to disrupt structurally important 2′-*O*-hydrogen bonds by replacement with 2′-*Se*-methyl groups cannot be ruled out a priori, but it is drastically reduced by this selection concept.

Asking for the number of *Se*-atoms that are needed for phasing of RNA diffraction data, one selenium should be sufficient for 20−40 nucleotides, based on estimation from phasing of *Se*-methionine protein derivatives. In the case of the Diels−Alder ribozyme, rapid radiation-induced decay of the crystals deteriorate quality of the inflection and remote data sets and did not allow MAD phasing. Therefore, the structure was solved by the SAD technique requiring a total of six selenium atoms.[10] More data on various *Se*-RNA targets will be needed for a general statement on this particular issue.

For several reasons, the *Se*-approach presented here is promising to gain a higher impact in nucleic acid crystallography than derivatization with 5-halogen pyrimidines. Because of the principal availability of all four 2′-*Se*-methyl nucleoside phosphoramidites a greater flexibility for adequate positioning within an RNA target is attained. In addition, nucleobase stacking interactions can be severely perturbed by 5-halogen-substituted pyrimidines.[22] Moreover, in contrast to 2′-*Se*-methyl nucleosides, 5-halogen pyrimidine derivatives are highly photoreactive species,[36−38] and inherent radiation damage of 5-halogen-modified nucleic acids during MAD data collection has been documented as a limitation.[19]

We believe that the recent success on the Diels−Alder structure determination based on 2′-*Se*-methyl nucleoside modifications not only demonstrates the power of this approach to solve particularly tricky problems in RNA structural biology[10] but may also initiate a more general interest and broader application of *Se*-derivatization in the field of nucleic acid crystallography.

Experimental Section

Synthesis of 2′-*Se*-Methyladenosine Phosphoramidite (7). General. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker DRX 300 MHz, or Varian Unity 500 MHz instrument. The chemical shifts are reported relative to TMS and referenced to the residual proton signal of the deuterated solvents: $CDCl_3$ (7.26 ppm), d_6-DMSO (2.49 ppm) for ^{1}H NMR spectra; $CDCl_3$ (77.0 ppm) or d_6-DMSO (39.5 ppm) for ^{13}C NMR spectra. ^{31}P-shifts are relative to external 85% phosphoric acid. ^{1}H- and ^{13}C-assignments were based on COSY and HSQC experiments. UV-spectra were recorded on a Varian Cary 100 spectrophotometer. Analytical thin-layer chromatography (TLC) was carried out on silica 60F-254 plates. Flash column chromatography was carried out on silica gel 60 (230−400 mesh). Packing of silica gel columns was performed with 1% Et_3N added to the corresponding starting eluent. All reactions were carried out under Ar atmosphere. Chemical reagents and solvents were purchased from commercial suppliers and used without further purification. Organic solvents for reactions were dried overnight over freshly activated molecular sieves (4 Å).

***N*^{6}-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-trifluoromethanesulfonyl-3′-*O*-{[(triisopropylsilyl)oxy]methyl}adenosine (2).** A mixture of **1**[23] (1.46 g; 1.82 mmol), DMAP (334 mg; 2.74 mmol), and triethylamine (635 µL; 4.56 mmol) in 35 mL of dry dichloromethane was cooled to 0°C under argon atmosphere and treated with trifluoromethanesulfonyl chloride (289 µL; 2.74 mmol). The solution was stirred for 20 min at room temperature. The reaction mixture was diluted with dichloromethane, extracted with saturated sodium bicarbonate solution, dried over Na_2SO_4, and evaporated. The crude product was purified by

(35) Batey, R. T., Gilbert, S. D.; Montange, R. K. *Nature* **2004**, *432*, 411−415.
(36) Gott, J. M.; Wu, H.; Koch, T. H.; Uhlenbeck, O. C. *Biochemistry* **1991**, *30*, 6290−6295.
(37) Xu, Y.; Sugiyama, H. *J. Am. Chem. Soc.* **2004**, *126*, 6274−6279.
(38) Zeng, Y., Wang, Y. *J. Am. Chem. Soc.* **2004**, *126*, 6552−6553.

column chromatography on SiO_2 (CH_2Cl_2/MeOH, 99.5/0.5 v/v). Yield: 1.02 g of **2** as slightly yellow foam (60%). TLC (CH_2Cl_2/MeOH, 94/6): R_f = 0.51; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.02 (m, 21H, (^{i}Pr)$_3$Si); 2.65 (s, 3H, $COCH_3$); 3.40 (dd, J = 3.9, 10.8 Hz, 1H, H1−C(5′)); 3.62 (dd, J = 2.7, 10.8 Hz, 1H, H2−C(5′)); 3.79 (s, 6H, 2× OCH_3); 4.51 (m, 1H, H−C(4′)); 4.89 (m, 1H, H−C(3′)); 4.96 (d, J = 5.0 Hz, 1H, OCH_2O); 5.11 (d, J = 5.0 Hz, 1H, OCH_2O); 6.21 (m, 1H, H−C(2′)); 6.36 (d, J = 5.1 Hz, 1H, H−C(1′)); 6.80 (d, J = 8.7 Hz, 4H, H−C(ar)); 7.28 (m, 7H, H−C(ar)); 7.40 (m, 2H, H−C(ar)); 8.10 (s, 1H, H−C(8)); 8.57 (s, 1H, H−C(2)); 8.68 (s, 1H, H−N^6) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.80 (SiCH(CH_3)$_2$); 17.71 (SiCH(CH_3)$_2$); 25.62 ($COCH_3$); 55.14 (2× OCH_3); 62.56 (C(5′)); 74.40 (C(3′)); 83.33 (C(4′)); 84.21 (C(2′)); 85.61 (C(1′)); 86.94; 89.91 (OCH_2O); 113.17, 121.97, 127.00, 127.84, 128.10, 130.03, 130.05, 135.22, 135.31, (C(ar)); 141.37 (C(2)); 144.16, 149.37, 150.83 (C(ar)); 152.65 (C(8)); 158.66 (C(ar)); 170.29 ($COCH_3$) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{44}H_{54}F_3N_5O_{10}SSi$, 930.07; found 929.5.

***N*^{6}-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-{[(triisopropylsilyl)oxy]methyl}(β-D-arabinofuranosyl)adenine (3).** To a mixture of **2** (1.02 g; 1.10 mmol) dissolved in 50 mL of toluene were added ethyldiisopropylamine (470 μL; 2.74 mmol), potassium trifluoroacetate (1.39 g; 9.12 mmol), and 18-crown-6-ether (965 mg; 3.65 mmol). The reaction mixture was stirred at 80°C for 11 h. After evaporation, the residue was dissolved in dichloromethane, extracted with half-saturated sodium bicarbonate solution, dried over Na_2SO_4, and again evaporated. The crude product was purified by column chromatography on SiO_2 (CH_2Cl_2/MeOH, 99.8/0.2−99/1 v/v). Yield: 785 mg of **3** as slightly yellow foam (89%). TLC (CH_2Cl_2/MeOH, 92/8): R_f = 0.50; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.09 (m, 21H, (^{i}Pr)$_3$Si); 2.63 (s, 3H, $COCH_3$); 3.42 (dd, J = 3.5, 10.7 Hz, 1H, H1−C(5′)); 3.66 (dd, J = 2.6, 10.7 Hz, 1H, H2−C(5′)); 3.80 (s, 6H, 2× OCH_3); 4.25 (m, 1H, H−C(4′)); 4.41 (m, 2H, H−C(3′), HO−C(2′)); 4.48 (m, 1H, H−C(2′)); 4.96 (d, J = 5.3 Hz, 1H, OCH_2O); 5.05 (d, J = 5.3 Hz, 1H, OCH_2O); 6.42 (d, J = 3.9 Hz, 1H, H−C(1′)); 6.84 (d, J = 8.4 Hz, 4H, H−C(ar)); 7.27 (m, 7H, H−C(ar)); 7.42 (d, J = 6.9 Hz, 2H, H−C(ar)); 8.43 (s, 1H, H−C(8)); 8.66 (s, 1H, H−C(2)); 8.71 (s, 1H, H−N^6) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.86 (SiCH(CH_3)$_2$); 17.74 (SiCH(CH_3)$_2$); 25.53 ($COCH_3$); 55.18 (2× OCH_3); 63.16 (C(5′)); 74.78 (C(2′)); 81.95 (C(4′)); 82.85 (C(3′)); 84.86 (C(1′)); 87.62; 89.58 (OCH_2O); 113.28, 121.38, 127.14, 127.96, 128.21, 130.07, 130.12, 135.01, 135.16 (C(ar)); 142.66 (C(2)); 143.81, 148.98, 151.20 (C(ar)); 152.17 (C(8)); 158.73 (C(ar)); 170.34 ($COCH_3$) ppm; UV/vis: λ_{max} = 271 nm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{43}H_{55}N_5O_8Si$, 798.01; found 797.5.

***N*^{6}-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-trifluoromethanesulfonyl-3′-*O*-{[(triisopropylsilyl)oxy]methyl}(β-D-arabinofuranosyl)adenine (4).** A mixture of **3** (226 mg; 0.28 mmol), DMAP (52 mg; 0.43 mmol), and triethylamine (98 μL; 0.71 mmol) in 6 mL of dry dichloromethane was cooled to 0°C under argon atmosphere and treated with trifluoromethanesulfonyl chloride (45 μL; 0.43 mmol). The solution was stirred for 20 min at room temperature. The reaction mixture was diluted with dichloromethane, extracted with saturated sodium bicarbonate solution, dried over Na_2SO_4, and evaporated. The product can be used without further purification for the next step. Yield: 255 mg of **4** as slightly yellow foam (98%). For analysis, the product was purified by column chromatography on SiO_2 (CH_2Cl_2/MeOH, 99.9/0.1−99.4/0.6 v/v). TLC (CH_2Cl_2/MeOH, 94/6): R_f = 0.51; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.07 (m, 21H, (^{i}Pr)$_3$Si); 2.65 (s, 3H, $COCH_3$); 3.52 (m, 2H, H_2−C(5′)); 3.81 (s, 6H, 2× OCH_3); 4.34 (m, 1H, H−C(4′)); 4.72 (m, 1H, H−C(3′)); 5.04 (s, 2H, OCH_2O); 5.53 (m, 1H, H−C(2′)); 6.60 (d, J = 3.3 Hz, 1H, H−C(1′)); 6.85 (d, J = 8.7 Hz, 4H, H−C(ar)); 7.29 (m, 7H, H−C(ar)); 7.48 (d, J = 7.2 Hz, 2H, H−C(ar)); 8.10 (s, 1H, H−C(8)); 8.62 (s, 1H, H−N^6); 8.67 (s, 1H, H−C(2)) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.79 (SiCH(CH_3)$_2$); 17.67 (SiCH(CH_3)$_2$); 25.62 ($COCH_3$); 55.16 (2× OCH_3); 62.67 (C(5′)); 80.47 (C(4′)); 82.89 (C(3′)); 83.20 (C(1′)); 86.29 (C(2′)); 86.62; 89.47 (OCH_2O); 113.23, 121.22, 126.94, 127.88, 128.01, 129.96, 135.49, 135.53, (C(ar)); 141.07 (C(2)); 144.38, 149.20, 150.47 (C(ar)); 152.64 (C(8)); 158.66 (C(ar)); 170.24 ($COCH_3$) ppm; UV/vis: λ_{max} = 270 nm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{44}H_{54}F_3N_5O_{10}SSi$, 930.07; found 929.5.

***N*^{6}-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxy-2′-methylseleno-3′-*O*-{[(triisopropylsilyl)oxy]methyl}adenosine (5).** Sodium borohydride (64 mg; 1.70 mmol) was placed in a sealed 25 mL two necked round-bottom flask, dried on high vacuum for 15 min to deplete oxygen, kept under argon, and suspended in dry THF (2.3 mL). Dimethyl diselenide (55 μL; 0.57 mmol) was slowly injected to this suspension, followed by drop wise addition of anhydrous ethanol; 0.4 mL was required until gas bubbles started to occur in the yellow mixture. The solution was stirred at room temperature for 1 h, and the almost colorless solution was injected into a solution of **4** (255 mg; 0.27 mmol) in dry THF (2.7 mL). The reaction mixture was stirred at room temperature for 20 min. Then, aqueous 0.2 M triethylammonium acetate buffer (5 mL, pH 7) was added, and the solution was reduced to half volume by evaporation. Dichloromethane was added, and the organic layer was washed twice with 0.2 M triethylammonium acetate buffer and finally with saturated sodium chloride solution. The organic layer was dried over Na_2SO_4, and the solvent was evaporated. The crude product was purified by column chromatography on SiO_2 (CH_2Cl_2/MeOH, 99.8/0.2−99.0/1.0 v/v). Yield: 116 mg of **5** as colorless foam (49%). TLC (CH_2Cl_2/MeOH, 94/6): R_f = 0.45; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.08 (m, 21H, (^{i}Pr)$_3$Si); 1.69 (s, 3H, $SeCH_3$); 2.64 (s, 3H, $COCH_3$); 3.44 (dd, J = 4.4, 10.4 Hz, 1H, H1−C(5′)); 3.50 (dd, J = 4.2, 10.4 Hz, 1H, H2−C(5′)); 3.80 (s, 6H, 2x OCH_3); 4.38 (m, 1H, H−C(2′)); 4.51 (m, 1H, H−C(4′)); 4.56 (m, 1H, H−C(3′)); 5.01 (d, J = 5.1 Hz, 1H, OCH_2O); 5.09 (d, J = 5.1 Hz, 1H, OCH_2O); 6.36 (d, J = 8.4 Hz, 1H, H−C(1′)); 6.81 (d, J = 8.4 Hz, 4H, H−C(ar)); 7.28 (m, 7H, H−C(ar)); 7.46 (d, J = 6.6 Hz, 2H, H−C(ar)); 8.11 (s, 1H, H−C(8)); 8.56 (s, 1H, H−C(2)); 8.62 (s, 1H, H−N^6) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 3.53 ($SeCH_3$); 11.88 (SiCH(CH_3)$_2$); 17.81 (SiCH(CH_3)$_2$); 25.58 ($COCH_3$); 44.96 (C(2′)); 55.15 (2× OCH_3); 63.58 (C(5′)); 79.77 (C(3′)); 84.48 (C(4′)); 86.61; 89.79 (OCH_2O); 91.06 (C(1′)); 113.11, 122.24, 126.89, 127.77, 128.19, 130.07, 130.10, 135.66, 135.70 (C(ar)); 142.03 (C(2)); 144.59, 149.23, 151.31 (C(ar)); 152.29 (C(8)); 158.59 (C(ar)); 170.32 ($COCH_3$) ppm; UV/vis: λ_{max} = 276 nm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{44}H_{57}N_5O_7SeSi$, 875.00; found 875.5.

***N*^{6}-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxy-2′-methylselenoadenosine (6).** Compound **5** (340 mg; 0.39 mmol) was treated with 1.7 mL of 1 M TBAF/0.5 M AcOH in THF. The solution was stirred at room temperature for 2.5 h. The solvent was evaporated, and the product was isolated by column chromatography on SiO_2 (CH_2Cl_2/MeOH, 99.5/0.5−98.0/2.0 v/v). Yield: 224 mg of **6** as colorless foam (84%). TLC (CH_2Cl_2/MeOH, 92/8): R_f = 0.49; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.95 (s, 3H, $SeCH_3$); 2.63 (s, 3H, $COCH_3$); 2.90 (s, br, 1H, HO−C(3′)); 3.45 (dd, J = 4.2, 10.5 Hz, 1H, H1−C(5′)); 3.53 (dd, J = 4.4, 10.5 Hz, 1H, H2−C(5′)); 3.80 (s, 6H, 2× OCH_3); 4.34 (m, 1H, H−C(4′)); 4.41 (m, 1H, H−C(2′)); 4.50 (m, 1H, H−C(3′)); 6.22 (d, J = 8.7 Hz, 1H, H−C(1′)); 6.82 (d, J = 8.7 Hz, 4H, H−C(ar)); 7.28 (m, 7H, H−C(ar)); 7.45 (d, J = 1.5 Hz, 2H, H−C(ar)); 8.12 (s, 1H, H−C(8)); 8.58 (s, 1H, H−C(2)); 8.79 (s, 1H, H−N^6) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 4.53 ($SeCH_3$); 25.60 ($COCH_3$); 48.80 (C(2′)); 55.20 (2× OCH_3); 63.63 (C(5′)); 72.59 (C(3′)); 85.06 (C(4′)); 86.71; 88.78 (C(1′)); 113.17, 122.19, 126.97, 127.84, 128.14, 130.04, 135.66 (C(ar)); 141.93 (C(2)); 144.47, 149.22, 151.27 (C(ar)); 152.32 (C(8)); 158.62 (C(ar)); 170.38 ($COCH_3$) ppm; UV/vis: λ_{max} = 271 nm; FT-ICR-ESI-MS (m/z): $[M+H]^+$ calcd for $C_{34}H_{35}N_5O_6Se$, 690.18341; found 690.18238.

***N*^{6}-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxy-2′-methylselenoadenosine 3′-(2-Cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (7).** Compound **6** (150 mg; 0.22 mmol) was dissolved in a mixture of ethyldimethylamine (242 μL, 2.24 mmol) in dry dichloromethane (7 mL) under argon. After 15 min at room temperature, (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite (80 mg; 0.33 mmol) was slowly added, and the solution was stirred at room temperature for 2 h. The

reaction was quenched by the addition of methanol (0.1 mL). The reaction mixture was diluted with dichloromethane, extracted with saturated sodium bicarbonate solution, and dried over Na_2SO_4, and the solvent was evaporated. The crude product was purified by column chromatography on SiO_2 (ethyl acetate/hexanes, 1/1−7/3 v/v (+1% NEt_3)). Yield: 178 mg of **7** (mixture of diastereoisomers) as colorless foam (91%). TLC (CH_2Cl_2/MeOH, 94/6): $R_f = 0.44$; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.09−1.29 (m, 24H, 2× $((CH_3)_2CH)_2N$); 1.61 (s, 3H, $SeCH_3$); 1.69 (s, 3H, $SeCH_3$); 2.34 (m, 2H, CH_2CN); 2.66 (m, 8H, $COCH_3$, CH_2CN); 3.38 (m, 2H, H1−C(5′)); 3.47−3.75 (m, 4H, $((CH_3)_2CH)_2N$, 2H, H2−C(5′), 2H, $POCH_2$); 3.78, 3.79 (2s, 12H, OCH_3); 3.90−3.99, 4.09−4.22 (2m, 2H, $POCH_2$); 4.41 (m, 4H, H−C(2′), H−C(4′)); 4.67, 4.73 (2m, 2H, H−C(3′)); 6.31 (m, 2H, H−C(1′)); 6.80, 7.22−7.45 (m, 26H, trityl-H); 8.11 (s, 1H, H−C(8)); 8.15 (s, 1H, H−C(8)); 8.49 (s, br, 2H, H-N^6); 8.54, 8.55 (2s, 2H, H−C(2)) ppm; ^{31}P NMR (121 MHz, $CDCl_3$): δ 150.8, 152.4; UV/vis (MeOH): λ_{max} = 271 nm; FT-ICR-ESI-MS (*m*/*z*): $[M+H]^+$ calcd for $C_{43}H_{52}N_7O_7PSe$, 890.29147; found 890.29005.

Solid-Phase Synthesis of 2′-*Se*-Methyl Nucleoside Containing RNAs. 2′-*O*-TOM standard nucleoside phosphoramidites and the corresponding CPG-supports (1000 Å) were obtained from GlenResearch. The phosphoramidite reagent used for 5′-phosphate labeling was prepared according to the literature.[39] Oligonucleotides with 3′-phosphates were synthesized on Amersham Custom Primer Support 3′-phosphate (Primer Support 200 at 100 μmol/g; No. 17-5214-49) or on an oximate-derivatized CPG solid support (1000 Å) according to the literature.[40]

All oligoribonucleotides containing 2′-*Se*-methyl nucleosides were synthesized on Pharmacia instrumentation (Gene Assembler Plus or Special) following modified DNA/RNA standard methods containing an additional cycle step of treatment with DTT; detritylation (2.0 min): dichloroacetic acid/1,2-dichloroethane (4/96); coupling (3.0 min): phosphoramidites/acetonitrile (0.1 M × 120 μL) were activated by benzylthiotetrazole/acetonitrile (0.35 M × 360 μL); capping (3 × 0.4 min): A: Ac_2O/sym-collidine/acetonitrile (20/30/50), B: 4-(dimethylamino)pyridine/acetonitrile (0.5 M), A/B = 1/1; oxidation (1.0 min): I_2 (10 mM) in acetonitrile/sym-collidine/H_2O (10/1/5); DTT treatment (2.0 min): DTT (100 mM) in ethanol/H_2O (2/3). A ready-to-use synthesis method file is documented in the Supporting Information online.

Solutions of standard amidites, tetrazole solutions, and acetonitrile were dried over activated molecular sieves overnight. Importantly, solutions of 2′-*Se*-methyl nucleoside phosphoramidites were only dried for 4−6 h over activated molecular sieves before consumption.

Deprotection and Purification of 2′-*Se*-Methyl Nucleoside Containing RNAs. Prior to deprotection and cleavage from the solid support the *Se*-containing RNAs were treated with DTT in ethanol/H_2O 1/1 (150 mM, 200 μL) for 1−3 h at room temperature. Then, $MeNH_2$ in EtOH (8 M, 0.60 mL), $MeNH_2$ in H_2O (40%, 0.60 mL), and DTT in EtOH/H_2O 1/1 (2 M, 95 μL; final DTT concentration 150 mM) were added, and deprotection was continued for 5−6 h. After the solution was evaporated to dryness, the 2′-*O*-silyl ethers were removed by treatment with tetrabutylammonium fluoride trihydrate (TBAF·$3H_2O$) in THF (1 M, 0.95 mL) for at least 12 h at room temperature. The reaction was quenched by the addition of triethylammonium acetate (TEAA) (1 M, pH 7.4, 0.95 mL). The volume of the solution was reduced to 1 mL, and the solution was loaded on a Sephadex G10 column (30 cm × 1.5 cm). The crude RNA was eluted with H_2O and evaporated to dryness. In contrast, deprotection solutions of RNA synthesized trityl-on were quenched by the addition of triethylammonium bicarbonate (1 M, pH 8.5, 0.95 mL), and the RNA products were eluted with 25 mM $(Et_3NH)HCO_3$ (pH 8.5).

Analysis of crude RNA products after deprotection was performed by anion-exchange chromatography on a Dionex DNAPac100 column (4 mm × 250 mm) at 80°C. Flow rate: 1 mL/min; eluant A: 25 mM Tris-HCl (pH 8.0), 6 M urea; eluant B: 25 mM Tris-HCl (pH 8.0), 0.5 M $NaClO_4$, 6 M urea; gradient: 0−60% B in A within 45 min; UV-detection at 265 nm. Crude RNA products (trityl-off) were purified on a semipreparative Dionex DNAPac100 column (9 mm × 250 mm). Flow rate: 2 mL/min; gradient: Δ5−10% B in A within 20 min. Fractions containing RNA were loaded on a C18 SepPak cartridge (Waters/Millipore), washed with 0.1−0.2 M $(Et_3NH)HCO_3$, H_2O, and eluted with H_2O/CH_3CN (6/4). RNA containing fractions were lyophilized.

RNAs synthesized trityl-on were purified by RP-HPLC prior to anion-exchange chromatography on an Amersham Resource-RPC column (6.4 mm × 30 mm) at 35°C. Flow rate: 1.5 mL/min; eluant A: 100 mM TEA in H_2O, pH 7.5, eluant B: 20 mM TEA in acetonitrile/H_2O 4/1; gradient: 25−35% B in A within 15 min.

Enzymatic Ligations. T4 RNA Ligase. Aliquots from aqueous stock solutions of the oligonucleotides to be ligated were mixed in equimolar amount, diluted with water to a concentration of 50−80 μM, heated to 90°C for 3 min, and allowed to cool to room temperature within 15 min. Ligation buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, pH 7.8 (at 25°C); 1/10 of reaction volume) was added, and the volume was adjusted by the addition of water to give a final RNA concentration of 40 μM for each oligonucleotide fragment and a final ligation buffer concentration of 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, pH 7.8 (at 25°C). Then T4 RNA ligase (New England Biolabs, 20000 U/mL in 50 mM KCl, 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol; storage solution diluted with 1× ligation buffer) was added to give a final T4 RNA ligase concentration of 0.2 U/μL. The reaction solution was incubated at 21°C for 2 h.

T4 DNA Ligase. Aliquots from aqueous stock solutions of the oligonucleotides to be ligated and the 2′-*O*-methyl RNA template oligonucleotide were mixed in equimolar amount, diluted with water to a concentration of 20−40 μM, heated to 90°C for 3 min, and allowed to cool to room temperature within 15 min. Ligation buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8 (at 25°C); 1/10 of reaction volume) and PEG 4000 (50% w/v; 1/10 of reaction volume) were added, and the volume was adjusted by the addition of water to give a final RNA concentration of 10 μM for each oligonucleotide fragment and a final ligation buffer concentration of 40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, pH 7.8 (at 25°C). Then T4 DNA ligase (Fermentas, 1 U/μL in 50 mM KCl, 20 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol) was added to give a final T4 RNA ligase concentration of 0.25 U/μL. The reaction solution was incubated at 37°C for 3−5 h.

Workup and isolation of ligation products: The reaction solutions were heated to 90°C for 3 min, extracted twice with an equal amount of phenol (water saturated)/chloroform/isoamyl alcohol (25/24/1) and once with chloroform/isoamyl alcohol (24/1). Ligation products were isolated by anion-exchange HPLC (Dionex DNAPac column, 4 × 250 mm; conditions as above). For analytical purposes, aliquots of the reaction solutions (3.5 μL in case of T4 RNA ligation; 10 μL in case of T4 DNA ligation) were diluted with 150 μL of water and treated as above. Analysis was performed by anion-exchange HPLC (conditions as above) and denaturating polyacrylamide gel electrophoresis (12% polyacrylamide, 7 M urea, 200 V, 50 min). Gels were developed by silver staining.[41]

Mass Spectrometry of 2′-*Se*-Methyl Nucleoside Containing RNAs. All experiments were performed on a Finnigan LCQ Advantage MAX ion trap instrumentation connected to an Amersham Ettan micro

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LC system. RNAs were analyzed in the negative-ion mode with a potential of −4 kV applied to the spray needle. LC: Sample (250 pmol RNA dissolved in 20 μL of 20 mM EDTA solution; average injection volume: 10−20 μL); column (Amersham μRPC C2/C18; 2.1 × 100 mm) at 21°C; flow rate: 100 μL/min; eluant A: 8.6 mM triethylamine, 100 mM 1,1,1,3,3,3-hexafluoro-2-propanol in H_2O (pH 8.0); eluant B: methanol; gradient: 0−100% B in A within 30 min; UV-detection at 254 nm.

RNA Crystallization. The Diels−Alder ribozyme was prepared by annealing of 11-mer and 38-mer RNAs (0.2 mM). The oligonucleotide mixture was heated in 5 mM Tris-HCl (pH 8.0), 30 mM NaCl, 5 mM $MgCl_2$ at 95 °C for 2 min and then cooled on ice. The RNA sample was combined with the same volume of the reservoir solution, and crystals were grown by the hanging-drop vapor diffusion method. Reservoir solution was: 30% (v/v) PEG400, 10% (v/v) 2-methyl-2,4-pentadiol, 70 mM Na-HEPES (pH 7.5), 200 mM $MgCl_2$, 200 mM NaCl, 50 mM RbCl. Crystals grew to a maximal size in approximately 20 days at +4 °C. X-ray data were collected at beamline X25 at NSLS or using in-house Rigaku X-ray generator from single crystals cooled at 100 K.

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Supporting Information Available: MS and NMR spectra of compound **7**, HPLC traces and PAGE of selected ligation experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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