Dual Recognition of Double-Stranded DNA by 2′-Aminoethoxy-Modified Oligonucleotides**

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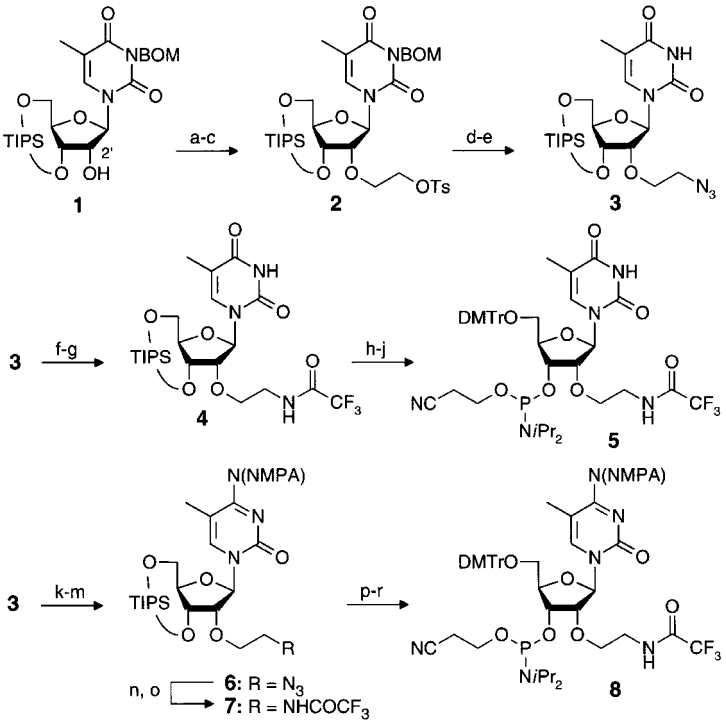
Molecular recognition of duplex DNA by proteins plays a central role in biology. Although there are no clear and simple rules or codes to describe sequence-specific recognition, hydrogen bonds with the nucleic bases as well as with the phosphodiester backbone are of critical importance.[1] Efforts to mimic such recognition with smaller synthetic biopolymers have been described.[2] However, in all these cases, specific contacts with the DNA duplex are only provided by hydrogen bonds with the bases, and potential interactions with the DNA backbone are left unexploited. Here we report that 2′-aminoethoxy-modified oligonucleotides can interact simultaneously with the bases and the phosphodiester backbone at each base-pair step of the DNA target, which provides for a dramatic increase in the binding affinity as well as in the association rate constant.

The sequence-specific recognition of duplex DNA by pyrimidine oligonucleotides involves the formation of triple-helical structures, which are stabilized by Hoogsteen hydrogen bonds between the bases on the DNA target and the pyrimidine third strand.[3] Examination of a molecular model of a triple helix with a RNA third strand indicates that the 2′-hydroxyl groups of the RNA and the phosphate groups of the DNA second strand are in close proximity.[4] Thus, it appeared conceivable that the attachment of a short amino alkyl group at the 2′ position of the ribose of the third strand would allow the protonated amino group to form a specific intermolecular contact with a proximal phosphate group of the DNA duplex. Such charge–charge interactions should strongly enhance the affinity of the modified oligonucleotide for double-strand DNA.

To test this hypothesis, we first examined the properties of a 2′-aminoethoxy-modified oligonucleotide with regard to triplex formation. The synthesis of the 2′-aminoethyl-modified monomeric tymidine and C5-methylcytidine building blocks is summarized in Scheme 1. Alkylation of protected ribothymidine **1**[5] with methyl bromoacetate followed by reduction of the ester group and tosylation gave the tosylate **2**. Hydrogenolytic removal of the BOM group and subsequent replacement of the tosyloxy group by an azide substituent yielded the azidoethyl derivative **3**, which was further transformed into the desired protected 2′-aminoethoxy-thymidine and C5-methylcytidine phosphoramidites **5** and **8** by standard procedures. A pentadecameric oligonucleotide containing five 2′-aminoethoxy-modified thymidines was synthesized, and its affinity for a double-stranded DNA target measured by UV-melting experiments (Figure 1A). A large increase in melting temperature T_m of 17.5 °C was observed compared to the unmodified DNA oligonucleotide control of identical length and sequence, corresponding to an increase of +3.5 °C per modification (Figure 1B). The magnitude of the difference in T_m (ΔT_m) was not significantly affected by changes in KCl concentration, replacement of KCl by NaCl, use of other buffers, or addition of $MgCl_2$ or spermine. Moreover, the modified oligonucleotide remains sensitive to a single base-pair mismatch.[6]



Scheme 1. Synthesis of protected 2′-aminoethoxy phosphoramidites thymidine (**5**) and C5-methylcytidine (**8**): a) $BrCH_2CO_2Me$ (5 equiv), NaH (2.2 equiv), DMF, 1.5 h, −5 °C, 98%; b) $LiBH_4$ (4 equiv), MeOH/THF (2/8), 1.5 h, 5 °C, 84%; c) TsCl (1.5 equiv), NEt_3 (1.6 equiv), DMAP (10 wt%), CH_2Cl_2, 8 h, 22 °C, 89%; d) H_2, Pd/C (20 wt%), HCl (0.02 equiv), THF/MeOH (1/1), 7 h, 22 °C, 88%; e) NaN_3 (3 equiv), DMF, 3 h, 65 °C, 92%; f) $SnCl_2$ (3.5 equiv), MeOH, 1 d, 22 °C, 70%; g) $(CF_3CO)_2O$ (1.2 equiv), pyridine, 2 h, 22 °C, 57%; h) TBAF (2.1 equiv), THF, 15 min, 22 °C, 100%; i) DMTrCl (1.2 equiv), pyridine, 16 h, 22 °C, 85%; j) $(iPr_2N)_2POCH_2CH_2CN$ (2.2 equiv), diisopropylammonium tetrazolide (2.4 equiv), CH_2Cl_2, 1 h, 22 °C, 85%; k) triazole (22.5 equiv), $POCl_3$ (2.5 equiv), NEt_3 (23 equiv), CH_3CN/CH_2Cl_2 (1/1), 3 h, 22 °C; l) concd NH_3 (aq)/dioxane (1/1), 1 h, 22 °C, 84% over two steps; m) *N*-methyl-2,2-dimethoxypyrrolidine (1.8 equiv), pyridine, 3 h, 22 °C, 95%; n) H_2, Pd/C (10 wt%), MeOH/THF (1/1), 3 h, 22 °C; o) CF_3COOEt (10 equiv), NEt_3 (7 equiv), MeOH, 1 h, 22 °C, 96% over two steps; p) TBAF (1.0 equiv), THF, 1 h, 22 °C, 100%; q) DMTrCl (1.2 equiv), pyridine, 1 h, 22 °C, 85%; r) $(iPr)_2N_2POCH_2CH_2CN$ (2.2 equiv), diisopropylammonium tetrazolide (2.4 equiv), CH_2Cl_2, 2 h, 40 °C, 87%. TIPS = 1,1,3,3-tetraisopropyldisiloxane, BOM = benzyloxymethyl, N(NMPA) = *N*-methylpyrrolidinamidine, DMF = *N,N*-dimethylformamide, THF = tetrahydrofuran, DMAP = 4-dimethylaminopyridine, TBAF = tetra-*n*-butylammonium fuoride, DMTr = 4,4′-dimethoxytriphenylmethyl, Ts = toluene-4-sulfonyl.

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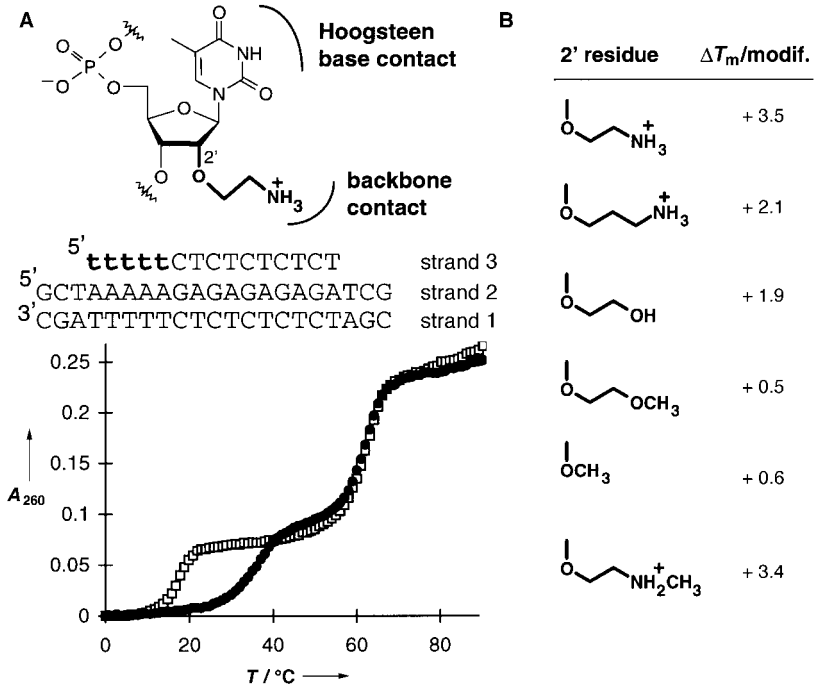

Figure 1. Affinity of modified oligonucleotides for duplex DNA. A) A pentadecameric oligonucleotide containing five 2′-aminoethoxy-modified thymidines (boldface, lower-case letters, strand 3) was hybridized to its duplex DNA target (strands 1 and 2) in a solution with 180 mM KCl, 20 mM Na^+, 10 mM phosphate (pH 7.0), and 0.1 mM ethylenediamine tetraacetate (EDTA). Thermal denaturation resulted in two UV transitions. The first transition corresponds to the melting of the triple helix (dissociation of strand 3), and the second to melting of the DNA duplex (dissociation of strands 1 and 2). The T_m values for the triplex helices with modified oligonucleotides (●) were compared to that obtained for an unmodified DNA oligonucleotide control (□). A_{260} = absorption at 260 nm. B) Structure – affinity relationships for different 2′-substituted oligonucleotides. The T_m values were obtained by mathematical curve fitting assuming a two-state model for each UV transition; calculated ΔT_m/modifications are within 0.1 °C uncertainty. The purity and identity of each oligonucleotide were determined by capillary electrophoresis and matrix assisted laser desorption time of flight (MALDI-TOF) mass spectroscopy.[13] A pK_a of 8.7 was determined for the 2′-aminoethoxy modified thymidine, indicating that the 2′ side chain is protonated under physiological conditions.

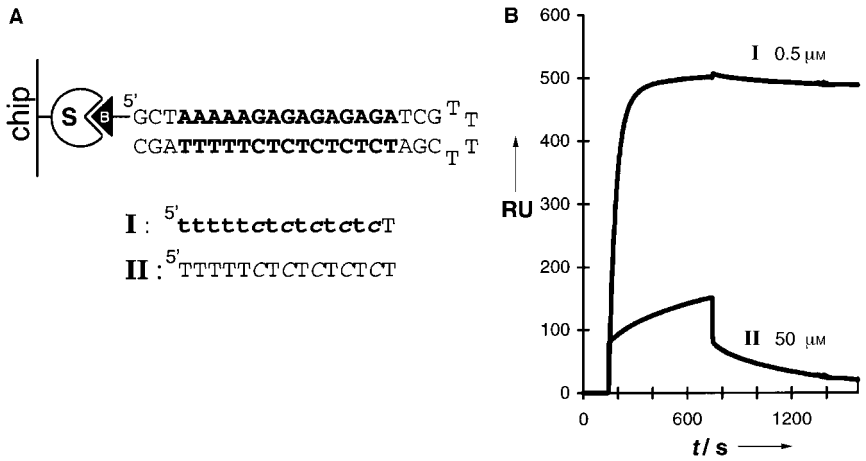

Figure 2. Real-time detection of triplex association and dissociation with the surface plasmon resonance sensor BIAcore 2000 system (Pharmacia Biosensor). A) A 5′-biotinylated DNA hairpin containing the duplex target sequence (boldface) was immobilized on a chip coated with streptavidine (S). Binding of the fully modified 2′-aminoethoxy oligonucleotide **I** to the hairpin was compared to the DNA oligonucleotide control **II**. Binding buffer: 150 mM NaCl, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES, pH 7.4), 3.4 mM EDTA, 0.05 % Tween 20. Both oligonucleotides contained C5-methylcytidines (italics) instead of natural cytosine bases owing to their more favorable DNA-binding properties under physiological conditions.[14] B) Sensograms showing changes (in resonance units, RU) upon association and dissociation of the third oligonucleotide strand to the double-stranded DNA hairpin.

To explore the molecular basis of this pronounced enhancement in binding affinity, we investigated the affinity of a series of other related 2′-O-modified oligonucleotides for double-stranded DNA (Figure 1 B). Extension of the spacer between the protonated amine group and the 2′-oxygen atom by one additional methylene group (2′-aminopropoxy substituent)[7] resulted in a significant decrease in triplex stability. This indicates that the protonated aminoethoxy side chain is ideally positioned to interact specifically with the nearby phosphate group, and that the enhanced triplex stability is not the result of a mere nonspecific electrostatic effect. Replacement of the amino group by an uncharged hydrogen-bond donor (2′-hydroxyethoxy substituent) also led to a considerable increase in binding affinity as compared to an unmodified DNA third strand, but the magnitude of the effect was significantly smaller than that observed for the 2′-aminoethoxy-modified oligonucleotide. This finding is consistent with the proposed intermolecular contact, as the positively charged amino moiety should interact with the negatively charged phosphate backbone more strongly than the uncharged hydroxyl group. Moreover, complete removal of the hydrogen bond donor capacity of the side chain (2′-methoxy-ethoxy substituent)[8] reduced the affinity of the modified oligonucleotide for the DNA duplex to a level similar to that of the 2′-methoxy analogue. This effect is unlikely to be the result of an unfavorable steric interaction between the added methyl group and the major groove of the DNA, as 2′-aminoethoxy and 2′-monomethylaminoethoxy derivatives display the same binding enhancement with respect to the oligonucleotide control.

To gain further insight into the origins of this large improvement in binding affinity, we determined the kinetic and thermodynamic parameters for the binding of a fully modified 2′-aminoethoxy oligonucleotide to a double-stranded DNA target. Measurement of hybridization kinetics by real-time surface plasmon resonance[9] revealed that the association rate constant of the fully modified 2′-aminoethoxy oligonucleotide **I** was more than a 1000 times greater than that of the unmodified oligonucleotide control **II** (Figure 2 and Table 1). The magnitude of the increase in association rate suggests

Table 1. Association (k_a) and dissociation rate constants (k_d), calculated dissociation binding constants K_D, and dissociation free energies ΔG (25 °C) for the 2′-aminoethoxy-modified oligonucleotide **I** and DNA control **II**.[a]

Oligo-nucleotide	k_a [$M^{-1}s^{-1}$]	k_d [s^{-1}]	K_D [M]	ΔG [kcal mol^{-1}]
I	$3.6 \pm 1.6 \times 10^4$	$3.3 \pm 1.3 \times 10^{-5}$	0.91×10^{-9}	−12.2
II	33 ± 19	$1.4 \pm 0.2 \times 10^{-3}$	4.2×10^{-5}	−5.9

[a] By a quantitative DNase I footprint experiment,[16] K_D was determined to be 4.8×10^{-9} M for oligonucleotide **I** (buffer: 140 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM spermine, 20 mM 3-(*N*-morpholine)propanesulfonic acid (MOPS), pH 7.2), which is similar to that obtained by the surface plasmon experiment.

that the formation of specific contacts between the 2′-aminoethoxy side chains and the phosphate backbone of the DNA duplex contributes actively to the nucleation-zipping association process.[10] Consistent with the stabilizing effect of additional specific interstrand contacts, the rate constant for dissociation of the third strand indicated that the modified oligonucleotide **I** dissociates 40 times more slowly from its target duplex than the oligonucleotide control **II**. Remarkably, the equilibrium dissociation constant calculated from the rate constants for modified oligonucleotide **I** (10^{-9} M) is 46 000-fold smaller than that obtained with **II**, which represents a gain of 6.3 kcal mol^{-1} in binding energy.

A model of a triple helix containing a fully modified 2′-aminoethoxy third strand was derived from molecular-dynamics (MD) simulations, and revealed that the protonated 2′-aminoethoxy residue is indeed capable of interacting with an adjacent phosphate group of the second strand without compromising the overall triplex architecture (Figure 3).[11] Throughout the MD trajectory, several observations pointed toward a specific interaction. The protonated aminoethoxy side chain (in a low-energy (+)-*gauche* conformation) always interacts with the *pro-R*-oxygen atom of residue $i-1$, but not of residue i, regardless of whether the orientation is originally *trans* or *gauche*. Similar MD experiments performed with a 2′-aminopropoxy-modified third strand indicated that in this case the amino group did not exhibit any preference for an interaction with either the i or $i-1$ *pro-R* oxygen atom and, in addition, could make contacts with its own 2′-oxygen atom.

Overall, these results are consistent with the specific effects displayed by the 2′-aminoethoxy-modified oligonucleotides for DNA binding, and support the existence of a novel interstrand contact with stringent geometrical requirements between the protonated aminoethoxy side chain and one phosphodiester oxygen atom of the DNA backbone. Moreover, they confirm the energetically important role of backbone contacts often seen in protein–DNA complexes.

Modulation of gene expression by oligonucleotide-directed formation of triple helices has been limited in part by weak affinity, slow association rate, and lack of nuclease resistance of the unmodified oligonucleotides. 2′-Aminoethoxy-modified oligonucleotides, for the first time, offer an efficient solution to these problems by combining high affinity, fast rate of association, and high nuclease resistance (data not shown and reference [12]). Therefore, they should compete effectively for DNA binding with proteins such as transcription factors, and eventually provide powerful compounds for the regulation of gene expression.

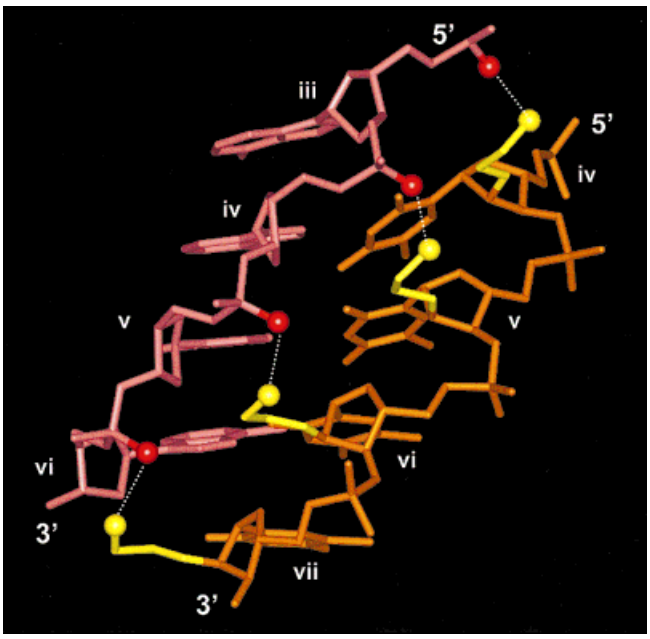

Figure 3. Molecular model showing the interaction of the 2′-aminoethoxy side chains (yellow) with the *pro-R*-phosphate oxygen atoms (red); with a N–O distance of 2.8 Å. For clarity, only four residues of the second (pink) and third strand (orange) are shown. This model was obtained from the average MD structure after 1-ns simulation performed on a T_{10}:A_{10}:**t**$_{10}$ triple-helical complex with a fully modified 2′-aminoethoxy third strand (boldface, lower-case letters). A distance-dependent dielectric function was used to simulate an aqueous environment, and counterions were not included; an approximation was judged sufficient for the geometrical factors analyzed here.[15]

Experimental Section

Kinetic measurements**:** A biotinylated DNA hairpin (2 μM) in binding buffer was applied to a chip coated with streptavidine (SA-5, Pharmacia Biosensor) and extensively washed. The association curve was obtained by injection of the oligonucleotide in binding buffer at a rate of 5 μL min^{-1} for 10 min at 25 °C. The oligonucleotide solution was then substituted by binding buffer alone, and the dissociation curve was recorded for 10 min. The jump (in resonance units, RU) between the association and the dissociation curve corresponds to a change in refractive index between the two solutions. To completely remove the third strand oligonucleotide from the chip, a denaturing step (10 mM NaOH, 1 min) followed by a washing step with binding buffer was performed after each measurement. Rate constants were evaluated with a nonlinear curve fitting program (BIA Evaluation, Pharmacia Biosensor) assuming first-order kinetics.

A range of oligonucleotide concentrations was tested (oligonucleotide **I**: 0.1–1.0 μM, oligonucleotide **II**: 10–100 μM) as well as different amounts of immobilized biotinylated hairpin. The rate constants reported correspond to the average of at least twelve measurements at these various concentrations. Control experiments indicated that oligonucleotide **I** or **II** did not bind to the streptavidine chip alone.

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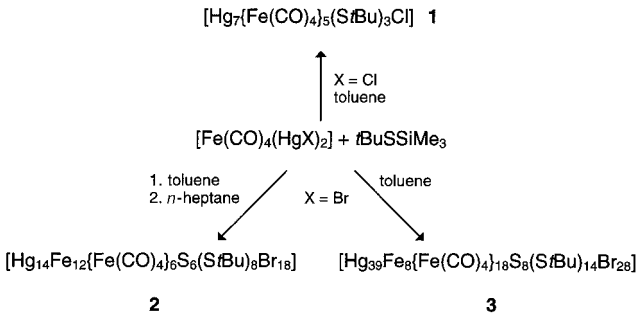
New Iron – Mercury Clusters: $[Hg_7\{Fe(CO)_4\}_5(S\mathit{t}Bu)_3Cl]$, $[Hg_{14}Fe_{12}\{Fe(CO)_4\}_6S_6(S\mathit{t}Bu)_8Br_{18}]$, and $[Hg_{39}Fe_8\{Fe(CO)_4\}_{18}S_8(S\mathit{t}Bu)_{14}Br_{28}]$**

Dieter Fenske* and Marco Bettenhausen

The reaction of coinage-metal salts with phosphanes and silylated chalcogen derivatives has already led to a great number of new compounds.[1] For example, the reaction of PR_3 complexes (R = organic group) of copper and silver halides with silylated derivatives of selenium and tellurium led to the isolation and characterization of many Cu – Se clusters such as $[Cu_{146}Se_{72}(PPh_3)_{30}]$,[2] and a large number of Ag – Te clusters such as $[Ag_{48}(n\mathrm{BuTe})_{24}Te_{12}(PEt_3)_{14}]$.[3] This method was applied recently for the synthesis and subsequent structure determination of $[Cd_{32}Se_{14}(SePh)_{36}(PPh_3)_4]$ and $[Hg_{32}Se_{14}(SePh)_{36}]$.[4] These compounds have similar structures to the previously described $[Cd_{32}Se_{14}(SR)_{36}(L)_4]$ clusters (R = organic group; L = H_2O, DMF).[5] The compound $[Hg_{32}Se_{14}(SePh)_{36}]$ can be formed by the treatment of $[Fe(CO)_4(HgCl)_2]$ with $PhSeSiMe_3$. The synthesis of $[Fe(CO)_4(HgCl)_2]$ was described in 1928 by Hock and Stuhlmann.[6] Recently the phosphane-bridged mercury cluster $[\{(HgP\mathit{t}Bu)_4\}_3]$ was synthesized by the treatment of $[Fe(CO)_4(HgOAc)_2]$ with $\mathit{t}BuP(SiMe_3)_2$;[7] this cluster is not accessible from the reaction of $HgCl_2$ with silylated phosphanes.

In this work we present the reactions of $[Fe(CO)_4(HgX)_2]$ (X = Cl, Br) with the silylated sulfur derivative $\mathit{t}BuSSiMe_3$. Only a few examples of metal-rich chalcogen-bridged mercury complexes, such as the adamantane-like compounds $[Hg_4(SPh)_6(PPh_3)_4](ClO_4)_2$, $[Hg_4(SPh)_5(\mu_2\text{-Br})Br_4](PPh_4)_2$,[8] and the complex $[Hg_4(\mathit{t}BuS)_4(\mu_2\text{-Cl})_2(Cl)_2(py)_2]$[9] have been reported. Clegg and Sola et al.[10] have isolated the compound $[Hg_7(SC_6H_{11})_{12}Br_2]$, and characterized it by X-ray crystallography.

Treatment of a suspension of $[Fe(CO)_4(HgX)_2]$ (X = Cl, Br) in toluene with $\mathit{t}BuSSiMe_3$ affords a yellow-red solution within a few days. Crystals of the iron – mercury clusters **1** – **3**, which were characterized by X-ray crystallography, were grown directly from this solution or by layering with *n*-heptane (Scheme 1).[11]



Scheme 1. Synthesis of the iron – mercury clusters **1** – **3**.

The reaction of $[Fe(CO)_4(HgCl)_2]$ with $\mathit{t}BuSSiMe_3$ leads to a yellow solution from which orange needles of **1** (Figure 1) crystallize. Compound **1** consists of an Hg_3Fe_2 (Hg5-Fe4-Hg6-Fe5-Hg7) and an Hg_4Fe_3 fragment (Hg1-Fe1-Hg2-Fe2-Hg3-Fe3-Hg4). These Hg – Fe chains are linked together by three sulfur atoms from the *t*BuS groups and a chlorine atom. The chlorine atom is in the center of the molecule with Hg – Cl bond lengths of 298.2(7) – 359.6(7) pm. These values lie significantly above the sum of the ionic radii of Hg^{2+} and Cl^- (Cl – Hg1 302.5(7), Cl – Hg5 303.5(7), Cl – Hg7 298.2(7) pm) or in the range of the van der Waals radii (Cl – Hg2 3596(7), Cl – Hg3 330.4(7), Cl – Hg4 337.0(7), Cl – Hg6: 348.7(7) pm). Thus, the Hg – Cl bonds are very weak. In

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