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Letter

A Postsynthetic Strategy for Oligonucleotides Containing 2-Fluoro-adenine and Isoguanine Nucleobases

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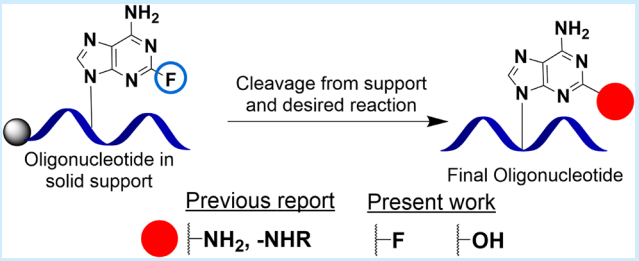
ABSTRACT: We describe a strategy for the synthesis of DNA, RNA and 2′-*O*-methyl (2′-*O*-Me) oligonucleotides containing 2-fluoro-adenine (A^F) and isoguanine (iG) nucleobases. To the best of our knowledge, this is the first nonenzymatic route for the synthesis of oligonucleotides containing 2-fluoro-adenine. We also describe the hydrolytic strategy for the conversion of 2-fluoro-adenine into isoguanine. Unlike 2,6-diaminopurine, 2-fluoro-adenine and isoguanine modifications are thermally destabilizing in the context of DNA, RNA, and 2′-*O*-methyl oligonucleotide duplexes.



Multiple oligonucleotide-based therapeutic agents, including antisense oligonucleotides, small interfering RNAs (siRNAs), and splice-switching oligonucleotides, have been approved for clinical use.[1,2] Chemical modifications of these oligonucleotides impart desirable pharmacokinetic properties like nuclease resistance, target specificity, and durability and also limit side effects. Modification of the adenine base is of interest because certain adenine derivatives, such as 2,6-diaminopurine (DAP), thermodynamically stabilize nucleic acid duplexes.[3] Positions 2 and 6 of DAP have been used as sites for conjugation of various functional groups.[4] At the nucleoside level, adenine modifications are of interest as adenine analogues that are agonists or antagonists of various adenosine receptors have therapeutic potential.[5]

We recently described a facile route for the introduction of DAP and 2-aminoadenine nucleobase conjugates into oligonucleotides without the need for amino group protection. These modifications were installed after oligonucleotide synthesis from oligonucleotides containing a 2-fluoro-adenosine precursor.[6] This strategy avoids multiple and tedious protection and deprotection steps and purification challenges. In the present work, we synthesized DNA, RNA, and 2′-*O*-methyl (2′-*O*-Me) oligonucleotides containing 2-fluoro-adenine and isoguanine nucleobases (Figure 1). 2-Fluoro-adenosine had previously been introduced into oligonucleotides only enzymatically, and site-specific incorporation is challenging when enzymatic synthesis is used.[7,8]

The chemistry of isoguanine and isoguanosine has been reviewed.[9] The conversion of a DAP analogue into isoguanine was previously reported,[10] and Seela et al. developed synthetic routes to isoguanine-containing oligonucleotides starting from an imidazole analogue and halogenated purine moiety.[11] Synthetic routes have also been described using xanthosine

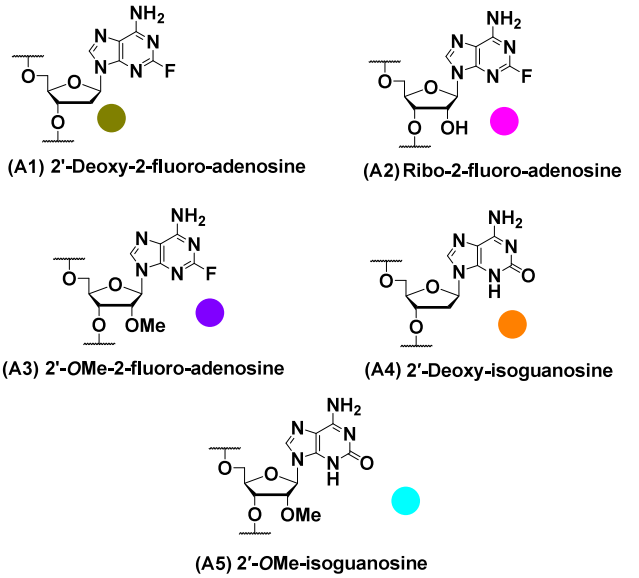


Figure 1. Structures of 2-fluoro-adenosine and isoguanosine on DNA, RNA, and 2′-*O*-Me backbones. Colored circles adjacent to modifications are those used in the bead diagrams in Tables 1 and 2.

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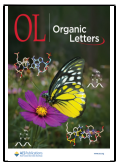

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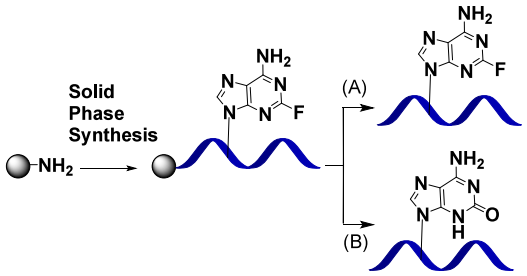
or DAP as starting materials.[12−14] These strategies involve multiple and tedious synthetic steps due to different reactivities of the 2-oxo group and the 6-amino group.[11,14−18] Incorporation of isoguanosine during solid-phase synthesis requires long coupling times,[19] and yields are low when several isoguanosine residues are incorporated into an oligonucleotide.[16,17,20−22] Incorporation of isoguanosine into oligonucleotides has also been achieved enzymatically, but enzymatic synthesis is inefficient. For example, incorporation of 2′-deoxyisoguanosine opposite an isocytosine was only 52% efficient compared to the incorporation of 2′-deoxyguanosine.[23] Some improvement in yield was reported when 5-methyl-isocytosine was used in the template.[24] Furthermore, synthesis of 2′-deoxyisoguanosine phosphoramidite from 2′-deoxyadenosine has been described,[25] and using this synthetic material nonenzymatic transcription of an isoG-isoC base pair has also been described.[26]

In the first part of this work, to confirm that 2-fluoro-adenosine could be stably incorporated into DNA oligonucleotides, we synthesized control DNA **ON1** (5′-CGCGAATTCGCG-3′) and two modified deoxyoligonucleotides, **ON2** and **ON3**, containing one and two 2-fluoro-adenines (**A1**), respectively (Scheme 1A). The exocyclic amine

Scheme 1. Preparation of Oligonucleotides Containing 2-Fluoro-adenosine or Isoguanosine[a]



[a](A) For the preparation of DNA oligonucleotides containing **A1**, the oligonucleotide was removed from the support and the phenoxyacetyl (PAC) protecting group was removed from the G-nucleotides in the sequences using 28−30% NH_4OH at 25 °C for 12 h. For the preparation of 2′-*O*-Me oligonucleotides containing **A3** or RNA oligonucleotides containing **A2**, oligonucleotides were treated with 28−30% NH_4OH at 25 °C for 12 h followed by $Et_3N{\cdot}3HF$ at 45 °C for 1.5 h. (B) For the preparation of oligonucleotides containing isoguanosine, oligonucleotides were removed from the support and deprotected as described above, and 2-fluoro-adenosine was converted into 2′-deoxy-isoguanosine (**A4**) or 2′-*O*-Me-isoguanosine (**A5**) by treatment with 0.5 M NaOH at 65 °C for 5 h.

of the G-phosphoramidite used was protected with the phenoxyacetyl (PAC) group instead of isobutyryl for facile removal with the milder deprotection condition employed (Table S1). Purification was more complicated when the isobutyryl group was used for the protection of guanosine (Figure S1). The solid support-bound oligonucleotides were treated with an aqueous (28−30%) ammonia solution at 25 °C for 12 h. These mild conditions resulted in the removal of both exocyclic protecting groups and cleavage of oligonucleotides from the solid support. The 2-fluoro group was retained under these conditions as confirmed by ^{19}F NMR and mass spectrometry (Figure 2A,B, Figure S2, and Tables S2 and S3). The yields and purities of the 2-fluoro-adenine-containing oligonucleotides were comparable to those of the DNA oligonucleotide (Figure S3).

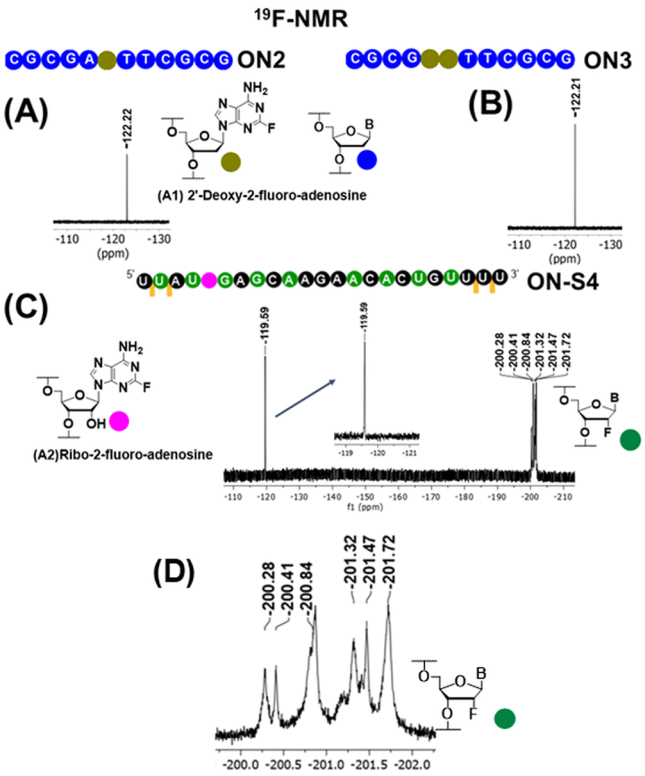


Figure 2. ^{19}F-NMR spectra of (A) **ON2** with a single (**A1**), (B) **ON3** with two (**A1**) residues, and (C) **ON-S4** with a single (**A2**) recorded at 564.67 MHz in D_2O. (D) Enlarged −200 ppm region of panel C showing 2′-F signals of ribosugar. In the bead diagram for **ON-S4**, black beads indicate 2′-*O*-Me nucleotides.

To understand the structural properties and biophysical attributes of 2-fluoro-adenine-containing oligonucleotides, RNA and 2′-*O*-Me oligonucleotides with one or two 2-fluoro-adenine residues were synthesized (Scheme 1A). The oligonucleotides were purified and characterized by LC-MS (Table 1, Table S2, and Figure S2).

The ^{19}F NMR spectra of an oligonucleotide that contains nine 2′-F RNA residues and a single 2-fluoro-adenosine (**A2**) were recorded as well as those of 2′-*O*-Me residues (**ON-S4**). These spectra clearly show distinct peaks for the 2′-F and **A2**, which both lie in the minor groove of the duplex (Figure 2C and Figures S4−S6). The distinct ^{19}F NMR pattern of the 2-fluoro-adenosine will be useful for analyses of protein−nucleic acid interactions.

The midpoints of thermal melting transitions (T_ms) of RNA and 2′-*O*-Me duplexes with and without 2-fluoro-adenosine modifications were evaluated (Table 1). Compared to fully self-complementary RNA duplex **4**, duplex **5**, which has two RNA A:G mismatches, was destabilized by −6.1 °C. Duplex **6**, which has two **A2**:G mismatch pairs, was destabilized by −5.7 °C compared to control duplex **4**. Thus, there was not a significant difference in thermal stability between the duplex with the A:G mismatches and that with the **A2**:G mismatch. Duplex **7**, which has two A:G mismatches and **A2**:U pairs, was destabilized by −7.7 °C relative to the control duplex.

RNA duplexes **8**−**10** were designed to evaluate the destabilization due to the **A2**:U pair. Duplexes **9** and **10**,

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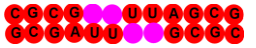
Table 1. Melting Temperatures of Control Duplexes and Duplexes Containing 2-Fluoro-adenosine

Duplex	Sequences[a]	T_m (°C)[b]	ΔT_m (°C)[c]
4		64.5	NA
5		58.4	-6.1
6		58.8	-5.7
7		56.8	-7.7
8		63.0	NA
9		61.0	-2.0
10		59.0	-4.0
11		66.3	NA
12		61.3	-5.0
13		57.8	NA
14		56.0	-1.8

[a]The top strand is shown in the 5′ to 3′ direction, and the bottom strand is shown in the 3′ to 5′ direction. In the bead diagram, ribonucleotide residues are colored red, 2′-*O*-Me nucleotides black, ribo-2-fluoro-adenosines (**A2**) pink, and 2′-*O*-Me-2-fluoro-adenosines (**A3**) violet. [b]T_m is the average of two heating curves and two cooling curves of 2 μM duplex in PBS. [c]ΔT_m was calculated from the difference between the modified duplex and the appropriate parent duplex. NA indicates not applicable.

which have one and two **A2**:U pairs, respectively, had melting temperatures that were 2 and 4 °C lower, respectively, than that of control duplex **8**. Thus, each **A2**:U pair caused a 2 °C destabilization in the RNA duplex. In the context of 2′-*O*-Me, the **A3**:U pair destabilized the 2′-*O*-Me by 5 °C (duplex **11** vs duplex **12**). In the 2′-*O*-Me:RNA hybrid duplex, an **A3** in the 2′-*O*-Me strand destabilized the duplex by 1.8 °C (duplex **13** vs duplex **14**).

The considerable destabilization in the 2′-*O*-Me:2′-*O*-Me duplex could be due to the possible steric clash of a 2′-*O*-Me group with 2-F within the duplex. The 2-fluoro-adenine in the DNA and RNA duplexes resulted in only slight destabilization. The presence of fluorine should not result in the release of significant water from the minor groove upon formation of the duplex state. N3(A) and O2(U) are the main hydrogen bond acceptors, and fluorine is small enough to be accommodated without disturbing the water structure around the base pair. It is likely that the stability decrease due to incorporation of a 2-fluoro-adenine results from a change in enthalpy (caused by alterations in stacking and hydrogen bond polarization).

We also conducted thermal melting experiments with therapeutically relevant siRNAs containing **A2**. Irrespective of whether the complementary strand was unmodified RNA or the modified siRNA strand, the 2-fluoro-adenine destabilized the duplex by 1−2 °C per modification (Table S4). Due to the very slight destabilization, thermodynamic parameters, determined using a reported method,[27] were comparable for duplexes with and without **A2**, and hence, our calculations are not included here.

The crystal structures of RNA duplexes **5**−**7** were determined at resolutions between 1.03 and 1.3 Å (Figure 3 and Table S5). The distances from the hydrogens at position 2 of adenine to the N2 hydrogens of guanine ([A]H2···(H)$_2$N2[G]) in unmodified duplex **5** and from the fluorine atoms on **A2** to the N2 hydrogens on guanine ([2-F-A]F2···(H)$_2$N2[G]) in the duplexes with one or two 2-fluoro-adenosine residues were similar. Thus, fluorine does not function as a hydrogen bond acceptor. This is consistent with the finding that the **A2**:G pair is not more stable than the A:G

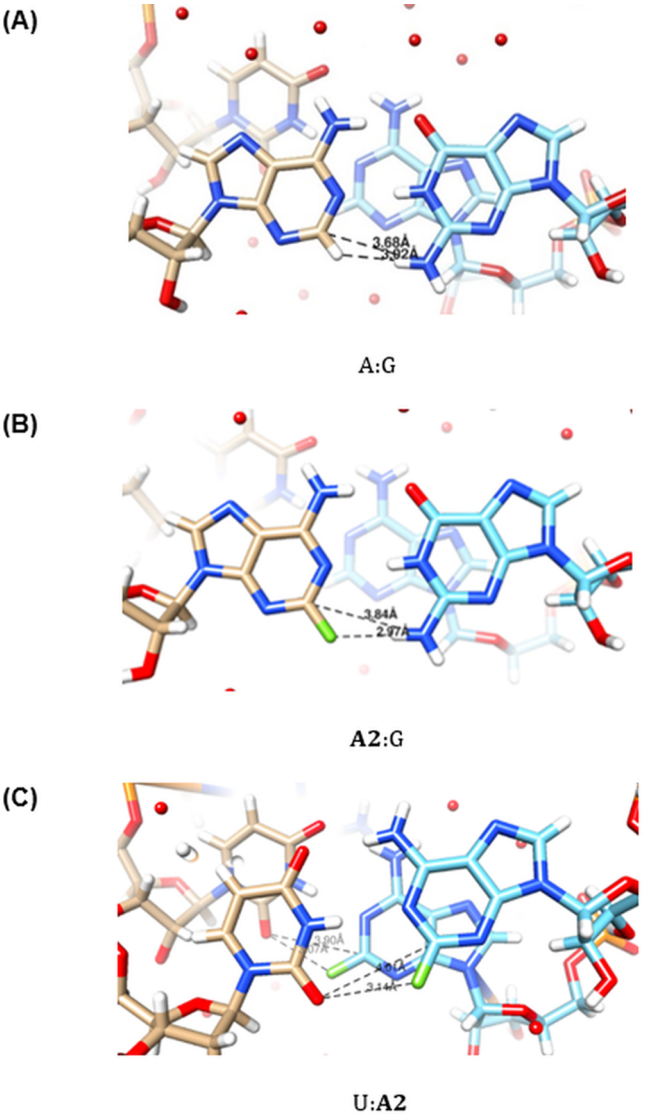


Figure 3. Crystal structures of RNAs with and without 2-fluoro-adenosine (**A2**). (A) Region of an A:G mismatch in duplex **5**. The structure was determined at 1.03 Å resolution. (B) Region of an **A2**:G mismatch in duplex **6**. The structure was determined at 1.12 Å resolution. (C) Region of an U:**A2** pair in duplex **7**. The structure was determined at 1.30 Å resolution.

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mismatch. High-resolution data of duplex **7** revealed minimal changes in hydrogen bonding distances and stacking as a result of incorporation of **A2** in a pair with uridine. This was expected as the van der Waals radius of fluorine (1.3 Å) is only slightly larger than that of hydrogen (1.2 Å). Detailed crystallographic data can be found in Table S5.

In the second part of the work, to introduce isoguanine postsynthetically, oligonucleotides containing one or two 2-fluoro-adenine residues on a solid support were treated with 0.5 M NaOH for 5 h at 65 °C, which converted the fluoro group at position 2 into a carbonyl (Scheme 1B). DNA and 2′-*O*-Me oligonucleotides containing one or two isoguanine modifications were synthesized. The identities and purities of the oligonucleotides were confirmed (Table S2 and Figure S7).

To evaluate the effect of the isoguanine modification on duplex stability, thermal melting analyses were performed (Table 2). The extent of destabilization relative to the A:T pair depended on the position and whether the strands were DNA or 2′-*O*-Me. The **A4**:T pair was more destabilizing than the **A2**:U pair (duplex **15** vs duplex **1** and duplex **22** vs duplex **11**, respectively). Two adjacent **A4**:T pairs, whether on opposite strands or the same strand, caused considerable destabilization (duplexes **16** and **17**). It is known that isoguanosine can tautomerize, allowing it to function as a hydrogen bond acceptor or donor.[15,28] Isoguanosine can only form a single hydrogen bond with thymine or uridine (Figure 4), whereas it can form two hydrogen bonds with guanine. Despite differences in the numbers of potential hydrogen bonds, we did not observe clear correlations between the type of base pairs and the extent of destabilization for duplexes **18**−**21**. This may be due to the variations in sequence, location, neighboring base pair identities, and number of modifications. Detailed structural studies will be needed to determine how isoguanine interacts within a duplex.[13]

Table 2. Melting Temperatures of Isoguanine-Containing Duplexes

Duplex	Sequences[a]	T_m (°C)[b]	ΔT_m (°C)[c]
1		58.0	NA
11		66.3	NA
13		57.8	NA
15		52.3	-5.7
16		40.8	-17.2
17		36.0	-22.0
18		72.8	NA
19		64.3	-8.5
20		65.5	NA
21		56.3	-9.2
22		59.8	-6.5
23		54.0	-3.8

[a]The top strand is shown in the 5′ to 3′ direction, and the bottom strand is shown in the 3′ to 5′ direction. In bead diagrams, 2′-deoxyribonucleotides, 2′-*O*-Me nucleotides, and ribonucleotides are colored blue, black, and red, respectively. Orange and cyan indicate 2′-deoxy-isoguanosine and 2′-*O*-Me-isoguanosine, respectively. [b]T_m is the average of two heating curves and two cooling curves of 2 μM duplex in PBS. [c]ΔT_m was calculated from the difference between the modified duplex and the appropriate parent duplex. NA indicates not applicable.

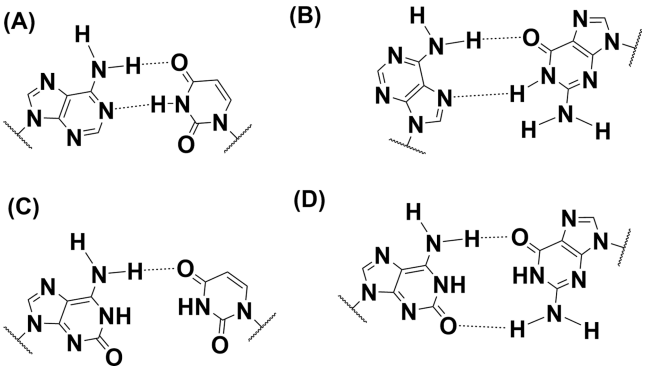

Figure 4. Hydrogen bond formation in (A) A:U, (B) A:G, (C) isoguanine:U, and (D) isoguanine:G base pairs.

In summary, here we describe straightforward routes for retention of 2-fluoro-adenine and for postsynthetic conversion of 2-fluoro-adenine into isoguanine in oligonucleotides with DNA, RNA, and 2′-*O*-Me backbones. Thermal melting studies of duplexes containing 2-fluoro-adenine or isoguanine showed that both are thermally destabilizing modifications when paired with thymine or uridine. The crystal structure of self-complementary 2-fluoro-adenine-containing RNA oligonucleotides determined at atomic resolution afforded insight into the origins of the destabilizing effect of the 2-fluoro-adenine modification.

Fluoro modifications are used extensively in therapeutic nucleic acids. For example, all siRNA conjugates approved for clinical use contain 2′-fluoro sugars. Fluorine is hydrophobic and can alter the pharmacokinetics of drugs favorably. The method described here will facilitate synthesis of 2-fluoro-adenine (A^F)-containing nucleotides for therapeutic applications and for studying protein−nucleic acid interactions and nucleic acid−nucleic acid interactions using ^{19}F NMR. In our previous work, we reported the conjugation of various delivery agents for therapeutic oligonucleotides though 2-fluoro-adenosine.[6] We have now confirmed that diagnostic agents like biotin can also be efficiently conjugated to oligonucleotides that have a 2-fluoro-adenosine residue (Figures S8 and S9).

The gene silencing activities of siRNAs containing isoguanosine have been previously reported, but these oligonucleotides were synthesized using a route more complicated than that described here.[29] Mismatched pairs

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have been used extensively in the past for allele-specific inhibition of translation by oligonucleotides, and the present method can be used to study the effects of isoguanine mismatches.[30] Similarly, incorporation of isoguanosine within the seed region of an siRNA might mitigate off-target effects, as does the destabilizing glycol nucleic acid modification.[31] The present strategy of isoguanosine synthesis can also be extended to other unnatural analogues of isoguanine (iG) proposed by Seela et al.[32]

In summary, our strategies for the incorporation of 2-fluoroadenine (A^F) and isoguanine (iG) nucleobases into oligonucleotides with different 2′ modifications avoid multiple and tedious protection and deprotection steps and purification challenges during the synthesis. This results in good yields of oligonucleotides containing these modifications, which are of interest for nucleic acid-based therapeutic and diagnostic applications.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.6c01203.

Experimental section and characterization of oligonucleotides (PDF)

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Author Contributions

‖M. Madaoui and J. Kundu contributed equally to this work.

Notes

The authors declare no competing financial interest.

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■ DEDICATION

The authors dedicate this publication to the loving memory of Professor Frank Seela, Professor of Organic Chemistry, Osnabrück University, Germany.

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