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## Phosphorus, Sulfur, and Silicon and the Related Elements

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t713618290>

### SYNTHESIS AND REACTIVITY OF NUCLEOSIDE SULFILIMINES

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Online publication date: 11 August 2010

**To cite this Article** Slaitas, A. and Yeheskiely, E.(2004) 'SYNTHESIS AND REACTIVITY OF NUCLEOSIDE SULFILIMINES', *Phosphorus, Sulfur, and Silicon and the Related Elements*, 179: 1, 153 — 171

**To link to this Article:** DOI: 10.1080/10426500490257113

**URL:** <http://dx.doi.org/10.1080/10426500490257113>

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## SYNTHESIS AND REACTIVITY OF NUCLEOSIDE SULFILIMINES

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(Received May 8, 2003; accepted July 26, 2003)

*Treatment of 3'- and 5'-protected deoxycytidine and deoxyadenosine with bis-[ $\alpha,\alpha$ -bis(trifluoromethyl)benzyloxy]diphenylsulfur resulted in the formation of the corresponding 4-N- and 6-N-diphenylsulfilimine derivatives in good yields. The 4-N- and the 6-N-dimethylsulfilimines of these nucleosides were obtained using DMSO/trifluoroacetic anhydride. These dimethyl- and diphenylsulfilimine derivatives were found to have characteristic UV absorbances at 284–296 nm. Stability tests of the nucleoside dimethylsulfilimine and diphenylsulfilimine under the conditions commonly used in oligonucleotide chemistry by the H-phosphonate approach were executed, and possible applications of these compounds have been discussed.*

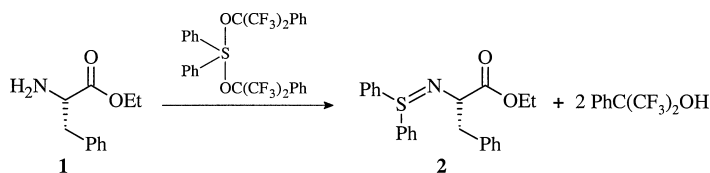
**Keywords:** H-phosphonate; nucleoside; nucleotide labeling; sulfilimine; synthesis

In the course of our research directed towards investigating the potential use of sulfuranes as peptide-coupling agents,<sup>1</sup> amino acid sulfilimines were isolated. The sulfilimines were formed during the condensation step, when bis-[ $\alpha,\alpha$ -bis(trifluoromethyl)-benzyloxy]diphenylsulfur (BTBDS) was added to the amino component **1**, prior to treatment with the carboxyl residue (the major product was diphenylsulfilimine **2**, Scheme 1) or when BTBDS was introduced to a mixture comprising of both the amino and carboxyl constituents. As a part of our on-going research focused on the development of synthetic methods for the preparation of modified PNAs,<sup>2–5</sup> peptides,<sup>6</sup> and nucleopeptides,<sup>7–12</sup> later to be tested for their biological activity, we were interested in preparing

We thank the Swedish Natural Science Research Council for support.

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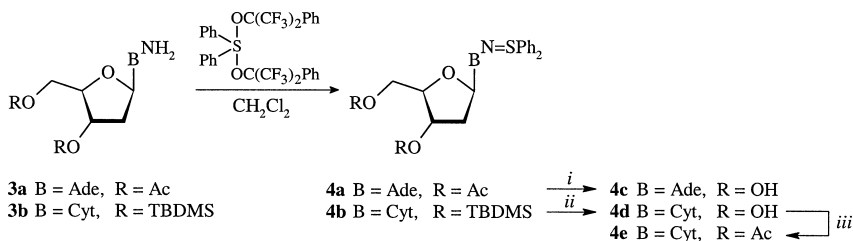
nucleoside sulfilimines and studying their properties and evaluating their possible use in nucleic acid chemistry.



**SCHEME 1** Formation of a sulfilimine from an amine and BTBDS.

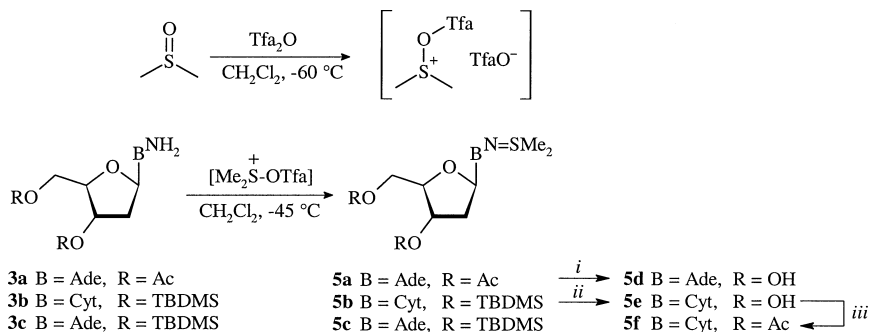
## RESULTS AND DISCUSSION

In order to explore the properties of an aromatic as well as aliphatic nucleoside sulfilimines, we embarked on the preparation of the diphenyl- and the dimethylsulfilimine derivatives of suitably-protected deoxycytidine and deoxyadenosine. Our intention is to use the information obtained from investigating these compounds as a basis for a broader study on the properties of nucleoside sulfilimines. For the synthesis of the nucleoside diphenylsulfilimine, BTBDS was the reagent of choice, while for making the nucleoside dimethylsulfilimines a protocol employing dimethylsulfoxide/trifluoroacetic anhydride was followed.<sup>13</sup> This method relies on the activation of dimethylsulfoxide (DMSO) followed by addition of an amine. The sulfoxide can be activated by a variety of reagents such as  $P_2O_5$ ,  $BF_3$ ,  $SO_3$ <sup>14</sup> or trifluoroacetic anhydride ( $Tfa_2O$ ). For the synthesis of dimethylsulfilimines **4a** and **4b**,  $Tfa_2O$  was chosen, since it was reported to perform better than  $P_2O_5$ ,  $BF_3$  and  $SO_3$ .<sup>13</sup>



**SCHEME 2** Reagents: (i)  $NH_3/MeOH$ ; (ii)  $Et_3N \times 3 HF/THF$ ; (iii)  $Ac_2O/Py$ .

Hence, treatment of 3',5'-O-protected deoxynucleosides **3a**<sup>15</sup> and **3b**<sup>16</sup> with *bis*-[ $\alpha,\alpha$ -bis(trifluoromethyl)benzyloxy]diphenylsulfur in  $CH_2Cl_2$  resulted in the formation of diphenyl-sulfilimines **4a** and **4b**



**SCHEME 3** Reagents: (i)  $\text{NH}_3/\text{MeOH}$ ; (ii)  $\text{Et}_3\text{N} \times 3 \text{ HF}/\text{THF}$ ; (iii)  $\text{Ac}_2\text{O}/\text{Py}$ .

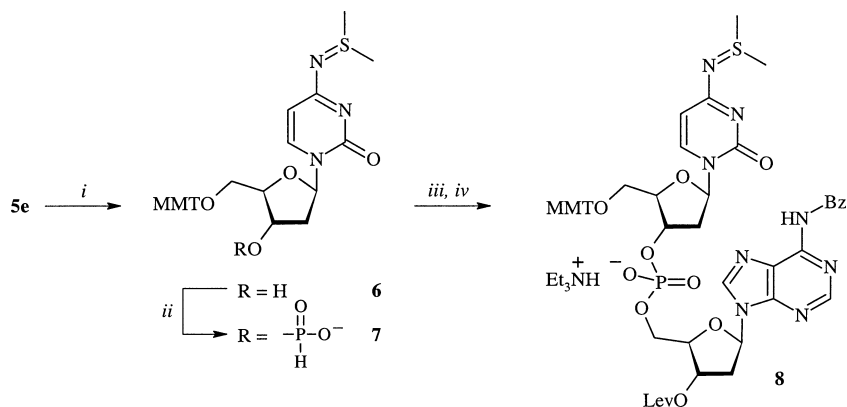
in 82% and 88% yield, respectively (Scheme 3). It should be mentioned, that protection of the free hydroxyl functions of the nucleosides during the BTBDS and the DMSO/trifluoroacetic anhydride reactions is mandatory, since BTBDS can cause elimination of alcohols to produce alkenes,<sup>17,18</sup> and the DMSO/trifluoroacetic anhydride mixture can bring about their oxidation.<sup>19,20</sup> Next, compound **4c** was obtained by ammonolysis of diacetylated **4a** in anhydrous  $\text{NH}_3/\text{MeOH}$ . Fluoride-ion assisted cleavage of the *t*-butyldimethylsilyl (TBDMS) groups from **4b** resulted in desilylated compound **4d**, which was further acetylated with acetic anhydride in pyridine to give, after work-up and purification, **4e** (92%).

Nucleoside dimethylsulfilimines **5a**, **5b**, and **5c** were prepared from **3a**, **3b**, and **3c**<sup>16</sup> in 70%, 62% and 63% yield, respectively (Scheme 3) by the reactions of the properly *bis*-*O*-protected nucleosides with DMSO/trifluoroacetic anhydride in  $\text{CH}_2\text{Cl}_2$ . Dimethylsulfilimine derivative **5a** was further ammonolyzed as mentioned above for bis-acetylated **4a**, while **5f** was prepared by removal of the TBDMS group from **5b** with  $\text{Et}_3\text{N} \times 3 \text{ HF}$  for 18 h (70% isolated yield), followed by acetylation with acetic anhydride in pyridine, which proceeded in quantitative yield. The dimethylsulfilimine group was completely intact during the desilylation and the acetylation reactions.

While engaged in the synthesis of **4a–e** and **5a–f**, we found that the sulfilimines have characteristic UV absorbance maxima at 284–296 nm. At this wavelength range, the nucleobases have an absorbance minimum.<sup>21</sup> This UV absorbance simplified purification and analysis of the above-mentioned compounds, since it enabled a fast and clear identification of sulfilimine-containing compounds in the reaction mixtures.

In the next stage after the preparation of the completely protected nucleoside sulfilimines, we focused our attention on testing their stability during oligonucleotide synthetic conditions for the H-phosphonate

approach. In the course of their preparation, it became apparent that dimethylsulfilimines **5a–f** are less stable than their diphenyl counterparts **4a–e**. Consequently, a successful application of the former derivatives in the assembly of dimer **8** will indicate that diphenylsulfilimine will also survive this procedure. Therefore, deoxycytidine dimethylsulfilimine **5e** was selected as starting material in the preparation of dinucleotide **8** as outlined in Scheme 4.



**SCHEME 4** Reagents: (i) MMT-Cl/Py (ii)  $\text{H}_3\text{PO}_3$ /Py/PivCl (iii) 3'-O-Lev-dA<sup>Bz</sup>, PivCl/Py/MeCN or OXP (iv)  $\text{I}_2$ /Py/ $\text{H}_2\text{O}$ .

First, tritylation of compound **5e** with monomethoxytrityl chloride (MMT-Cl) in pyridine afforded compound **6** (49%). Then, phosphorylation in pyridine using phosphorous acid and pivaloyl chloride (PivCl) led, after hydrolysis, to the formation of H-phosphonate monoester **7** (56% isolated yield). Next, condensation of monomer **7** with 6-*N*-benzoyl-2'-deoxy-3'-*O*-levulinoyladenine (3'-*O*-Lev-dA<sup>Bz</sup>)<sup>22</sup> under the mediation of *bis*-(2-oxo-3-oxazolidinyl)phosphoryl chloride (OXP)<sup>23</sup> afforded, after oxidation with iodine/water and purification by column chromatography, dimer **8** (87%). In addition, dinucleotide **8** was also synthesized by treating phosphonate **7** and 3'-*O*-Lev-dA<sup>Bz</sup> in acetonitrile and pyridine with pivaloyl chloride, which is the most common coupling agent in oligonucleotide synthesis by the H-phosphonate method.<sup>24</sup> After 15 min, oxidation with iodine in pyridine/water for 5 min afforded phosphate diester-containing dimer **8**. There was a complete conversion of starting material **7** into **8** as judged by  $^{31}\text{P}$  NMR spectroscopy and HPLC analysis of crude compound **8** (using dimer **8** previously prepared from the OXP reaction as a reference).

Stimulated by the outcome of the phosphorylation and oxidation steps, which are two of the most crucial points in solid-phase

oligonucleotide synthesis, we then explored the stability of the dimethylsulfilimine group under conditions for another essential step, i.e. detritylation.<sup>24</sup> This is a repeated mild acidolysis of the di- or monomethoxytrityl groups that serve as temporary protection of the 5'-hydroxy functions of the nucleosides (at the extension point of the oligonucleotide chain). In addition, the iodine oxidation<sup>24</sup> was further investigated in more detail. Moreover, although a capping<sup>25</sup> (acetylation) protocol is not regularly used in the H-phosphonate methodology, acetylation is often performed in order to protect the hydroxyl functions of the nucleosides and is also standard in the amidite approach. It was therefore included in the stability studies. The results of these tests are summarized in Table I.

The data in Table I show that the dimethylsulfilimine function of nucleosides **5a** and **5f** was not stable in 2% dichloroacetic acid (DCA) in 1,2-dichloroethane (DCE). Some degradation was observed already after 30 min for both compounds **5a** and **5f**. This implies that the dimethylsulfilimine group on deoxyadenosine and deoxycytidine may not survive the repeated detritylation steps. Nonetheless, it may be stable during several detritylations, necessary for the preparation of a short oligonucleotide fragment. In contrast with the observed lability under acidic conditions of compound **5a**, the dimethylsulfilimine of derivative **5c** was not degraded when exposed to the conditions required for the oxidation of the phosphonate diester function into a phosphate diester<sup>24,26</sup> or to the capping solution (Ac<sub>2</sub>O/*sym*-collidine/DMAP/MeCN) commonly used in some solid-phase nucleic acid synthesis protocols.<sup>27</sup> On the other hand, when cytidine dimethylsulfilimine **5b** was dissolved in the above mentioned iodine solution, it was intact for the first 5 min. However, slightly longer treatment led to cleavage of the sulfilimine

**TABLE I** Stability of Adenosine and Cytidine Dimethylsulfilimines<sup>a</sup>

Time (min)	2% DCA/DCE		0.01 M Iodine <sup>b</sup>		Capping mixture <sup>c</sup>	
	dA <b>5a</b>	dC <b>5f</b>	dA <b>5c</b>	dC <b>5b</b>	dA <b>5c</b>	dC <b>5b</b>
5	99	~100	~100	~100	~100	90
15	99	97	~100	90	~100	90
30	92	96	~100	88	~100	79
60	90	94	99	81	~100	76
120	83	91	99	77	~100	72

<sup>a</sup>The values given in the table correspond to the percentage of the intact sulfilimine as determined by straight-phase HPLC (mean error  $\pm$  3%).

<sup>b</sup>As a solution in collidine/water/MeCN.

<sup>c</sup>Prepared by mixing equal volumes of Capping A and B solutions, containing Ac<sub>2</sub>O/collidine/MeCN 2:3:5 (v/v/v) and 6% 4-(*N,N*-dimethylamino)pyridine (DMAP) in MeCN (w/v), respectively.

(10% within the 15 min) as indicated in Table I. Moreover, exposure of **5b** to the capping mixture proved to have a deleterious effect on the sulfilimine integrity. In this case, 10% of nucleoside **5b** was affected within 5 min. The information summarized in Table I suggests that only properly protected dimethylsulfilimine derivatives of deoxyadenosine may endure oligonucleotide synthesis protocols that require repeated oxidation steps or oxidation times longer than 5 min. This is also correct for methods that endorse repeated capping step.

Next, the diphenylsulfilimino group in compounds **4a**, **4b**, and **4e** was subjected to the same conditions as described in Table I for the dimethylsulfilimine function. The figures obtained from these stability tests are given in Table II, and they indicate that, in contrast to their dimethyl counterparts, compounds **4a** and **4b** were stable during treatment with 2% DCA in DCE for 120 min. Prolonged exposure (24 h) to this mixture showed that the deoxycytidine derivative **4d** remained unaffected, while  $\text{Ac}_2\text{dA} = \text{SPh}_2$  (**4a**) was less stable and was partially degraded (9%).

The diphenylsulfilimine moiety was also found to be much more stable in the oxidation mixture of  $\text{I}_2$ /collidine in aqueous acetonitrile. After 2 h, the deoxycytidine derivative **4b** was still completely intact in contrast with **4a** which was partially cleaved (6%) as depicted in Table II. Furthermore, no cleavage of the diphenylsulfilimine function in **4a** and **4b** was observed in a mixture of  $\text{Ac}_2\text{O}$ /collidine/DMAP in MeCN for at least 2 h.

The stability of the diphenylsulfilimino group towards the conditions listed in Table II suggests that it will endure the assembly of longer

**TABLE II** Stability of Adenosine and Cytidine Diphenylsulfilimines<sup>a</sup>

Time (min)	2% DCA/DCE		0.01 M Iodine <sup>b</sup>		Capping mixture <sup>c</sup>	
	$\text{Ac}_2\text{dA-}$ $\text{SPh}_2$ <b>4a</b>	$\text{Ac}_2\text{dC-}$ $\text{SPh}_2$ <b>4e</b>	$\text{Ac}_2\text{dA-}$ $\text{SPh}_2$ <b>4a</b>	$\text{Si}_2\text{dC-}$ $\text{SPh}_2$ <b>4b</b>	$\text{Ac}_2\text{dA-}$ $\text{SPh}_2$ <b>4a</b>	$\text{Si}_2\text{dC-}$ $\text{SPh}_2$ <b>4b</b>
5	~100	~100	~100	~100	~100	~100
15	~100	~100	~100	~100	~100	~100
30	~100	~100	~100	~100	~100	~100
60	~100	~100	99	~100	~100	~100
120	~100	99	94	~100	~100	~100

<sup>a</sup>The values given in the table correspond to the percentage of the intact sulfilimine as determined by straight-phase HPLC (mean error  $\pm 3\%$ ).

<sup>b</sup>As a solution in collidine/water/MeCN.

<sup>c</sup>Prepared by mixing equal volumes of Capping A and B solutions, containing  $\text{Ac}_2\text{O}$ /collidine/MeCN 2:3:5 (v/v/v) and 6% DMAP in MeCN (w/v), respectively.

nucleic acid fragments on a solid support using the H-phosphonate approach. A successful application of nucleoside diphenylsulfilimines in the phosphoroamidite chemistry is also very likely, provided they would survive the repeated phosphorylation step.

In the following stage, the stability of  $dA = SMe_2$ ,  $dC = SMe_2$ ,  $dA = SPh_2$  and  $dC = SPh_2$  (**5d**, **5e**, **4c** and **4d**, respectively) in an aqueous environment and during ammonolysis, which is the final step in oligonucleotide synthesis,<sup>24</sup> employed to remove the protecting groups from the nucleobases and detach the oligonucleotide fragment from the solid-support, was examined. Exposure to 33% aq.  $NH_3$  for 16 h at 21°C showed that **4c**, **4d**, and **5d** were un-degraded while **5e** lost 12% of the dimethylsulfilimine group. Upon treatment with 33% aq.  $NH_3$  at elevated temperature, the diphenylsulfilimine derivatives **4c** and **4d** remained unaffected, but compounds **5d** and **5e** were almost completely converted into the corresponding deoxyadenosine and deoxycytidine, respectively. In anhydrous  $NH_3$  in methanol, at 21°C, all four nucleoside sulfilimines **5d**, **5e**, **4c**, and **4d** were found to be stable for at least 2 h.

The increased stability of the diphenylsulfilimines **4c** and **4d**, compared to the dimethyl- **5d** and **5e**, is also evident in water and 50 mM TRIS buffer (pH 9.0).

The diphenylsulfilimines were stable in aqueous solutions at 21°C for 16 hours, while the dimethyl analogues were substantially degraded under the same conditions. In  $H_2O$  at elevated temperature however, only  $dA = SPh_2$  (**4c**) was stable, while compound  $dC = SPh_2$  (**4d**) lost 37% of the sulfilimino group, and derivatives **5d** and **5e** were detected only in trace amounts.

The outcome of the stability tests in aqueous solutions is consistent with the results of the tests reported above which show the increased stability of the diphenylsulfilimine function compared to the dimethylsulfilimine. This outcome leads to the conclusion that the diphenylsulfilimine group would not be cleaved at the final stage of nucleic acids synthesis, i.e. the deprotection of the nucleobases and detachment from the solid-support which is brought about by ammonolysis either at ambient temperature or at 60°C.

This stability to ammonolysis and to the above-mentioned conditions depicted in Table II permits retaining the diphenylsulfilimine group during and after oligonucleotide synthesis, a fact that can make them useful for the introduction of different functionalities into the nucleic acid fragment which can be accomplished by substitution and/or extension of the sulfilimine aromatic ring(s) accordingly.

Finally, since it was reported that 2-mercaptoethanol ( $\beta$ -ME) could cause cleavage of sulfilimines in the presence of acid,<sup>28</sup> we were curious



**TABLE III** Stability of 3',5'-Unprotected Nucleoside Sulfilimines in Aqueous Solutions<sup>a</sup>

No.	Compound	50 mM TRIS (pH 9.0), 21°C, 16 h	H <sub>2</sub> O, 21°C, 16 h	H <sub>2</sub> O, 60°C, 16 h	33% aq. NH <sub>3</sub> , 21°C, 16 h	33% aq. NH <sub>3</sub> , 60°C, 16 h
1	dA = SMe <sub>2</sub> <b>5d</b>	90	96	0	98	1
2	dC = SMe <sub>2</sub> <b>5e</b>	59	77	3	88	7
3	dA = SPh <sub>2</sub> <b>4c</b>	~100	~100	98	~100	~100
4	dC = SPh <sub>2</sub> <b>4d</b>	99	99	64	~100	98

<sup>a</sup>The values given in the table correspond to the percentage of the intact sulfilimine as determined by reversed-phase HPLC (mean error  $\pm$  3%).

to determine the effect of  $\beta$ -ME solution on **4a**, **4b**, **5b**, and **5c**. We found that the dimethylsulfilimines **5b** and **5c** underwent a rapid conversion (within 5 min) into their corresponding starting materials **3b** and **3c**, while the diphenylsulfilimines were much more stable. After 2 h, the deoxycytidine derivative **4b** was still completely intact in contrast with **4a**, which was partially cleaved (10%).

## CONCLUSIONS

Nucleoside diphenyl- and dimethylsulfilimines were synthesized in good yields. The stability tests clearly show that the nucleoside diphenylsulfilimines are significantly more stable than those with the dimethylsulfilimines group. These tests suggest that nucleoside dimethylsulfilimines are not suitable for the solid-phase synthesis of long oligonucleotide fragments by the H-phosphonate approach. However, they may be used in the synthesis of short fragments, such as dimers, and possibly trimers and tetramers, either in solution or on a solid-support with reasonably good yields.

Unlike the dimethylsulfilimine nucleosides, it should be possible to use the diphenylsulfilimine derivatives in the solid-support assembly of long oligonucleotides. Incorporating one or several nucleoside diphenylsulfilimine can be advantageous due to the fact that they are unaffected by ammonolysis and that the sulfilimine moiety absorbs at 290–300 nm. Thus, they could be used as UV tags for oligonucleotides via pin-pointing their location in the presence of unlabeled fragments. Another possible advantage is that by modifying one or both the phenyl rings accordingly, diarylsulfilimine-containing nucleotides could be used to introduce functionalities, such as, for example an intercalator, at a predetermined position in a nucleic acid fragment.

## EXPERIMENTAL

*General Remarks:* Solvents were purchased from Merck Eurolab. Dry solvents (except THF) were obtained by treatment with the appropriate molecular sieves (3 Å for MeCN and methanol, 4 Å for DMSO, CH<sub>2</sub>Cl<sub>2</sub>, 1,2-dichloroethane, and pyridine). The THF was distilled over Na/K-benzophenone ketyl.

2'-Deoxynucleosides were purchased from Pharma-Waldof GmbH (Germany). *sym*-collidine (2,4,6-trimethylpyridine), BTBDS, H<sub>3</sub>PO<sub>3</sub> and Piv-Cl were purchased from Aldrich; 2-mercaptoethanol from Sigma; MMT-Cl, OXP, TBDMS-Cl, and Tfa<sub>2</sub>O from Fluka; Ac<sub>2</sub>O, DCA, and Et<sub>3</sub>N × 3 HF from Lancaster, DMAP from Novabiochem, and iodine from May and Baker Ltd. All reagents were used as supplied, except Piv-Cl and DCA which were distilled through a short Vigreux column.

Flash chromatography was performed on Merck Silica gel 60 (40–63 μm).

TLC was performed using analytical Merck silica plates with F<sub>254</sub> indicator and visualized under 254 nm UV or by staining with H<sub>2</sub>SO<sub>4</sub>/MeOH. The following mobile phase systems were used: CHCl<sub>3</sub>/MeOH 9:1 (v/v)—System A, CHCl<sub>3</sub>/MeOH 4:1 (v/v)—System B, ethyl acetate/iPrOH 9:1—System C.

Straight-phase HPLC was performed on a Jasco HPLC system equipped with Jasco Diode Array detector (MD-910). The columns were as follows: Analytical (5 μm, ThermoHypersil, 250 × 4.6 mm) or preparative silica column (5 μm, Hypersil, 150 × 21.2 mm). The HPLC experiments were executed at ambient temperature. The mobile phase consists of 2% MeOH and 0.2% Et<sub>3</sub>N in CH<sub>2</sub>Cl<sub>2</sub> with a flow rate of 1 mL/min and observed at 285 nm.

The RP HPLC was performed on a C<sub>18</sub> column (5 μm, Jupiter, 250 × 4.6 mm) at 30°C. Buffer A—50 mM TRIS (pH 9.0), buffer B—70% MeCN in water, gradient 0–60% B in 60 min.

UV absorbance spectra were recorded on Jasco Diode Array detector (wavelength range 220–400 nm, acquisition time 800 ms).

Nominal mass spectra were recorded on a Micromass LCT mass spectrometer (ESI-TOF). High-resolution mass spectral (HRMS) measurements were obtained in positive or negative ion mode using leucine enkephalin as an internal mass standard.

NMR spectra were recorded on a Bruker Avance DRX 400 spectrometer at 400.13 and 100.61 MHz for <sup>1</sup>H and <sup>13</sup>C, respectively. The chemical shifts are reported in ppm using residual solvent signal as an internal reference.

### 3',5'-di-*O*-Acetyl-deoxyadenosine Diphenylsulfilimine (4a)

A solution of 3',5'-di-*O*-acetyl-deoxyadenosine (0.31 g, 0.93 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was added under a stream of nitrogen and stirring to a solution of BTBDS (0.75 g, 1.11 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (1.0 mL). After 1 hour, the reaction mixture was transferred into a separation funnel containing CH<sub>2</sub>Cl<sub>2</sub> (20 mL) and 0.25 M phosphate buffer (20 mL, pH 7.0). The organic phase was washed with water and brine (10 mL each) and then was dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under reduced pressure yielding colored oily substance. Purification by flash column chromatography (silica) using 2% methanol and 0.1% Et<sub>3</sub>N in CH<sub>2</sub>Cl<sub>2</sub> afforded pure compound **4a**.

Yield 0.39 g (82%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>): 8.23 (1H, s), 7.97 (1H, s), 7.92-7.87 (4H, m), 7.51-7.44 (6H, m), 6.44 (1H, dd, *J* = 5.8, 8.3), 5.42 (1H, dt, *J* = 2.2, 6.2), 4.41 (1H, dd, *J* = 5.3, 12.9), 4.36-4.32 (2H, m), 2.96-2.89 (1H, m), 2.59 (1H, ddd, *J* = 2.3, 5.8, 14.1), 2.12 (3H, s), 2.10 (3H, s).

<sup>13</sup>C NMR (CDCl<sub>3</sub>): 170.90, 170.76, 162.31, 152.79, 149.53, 137.53, 132.21, 130.10, 128.15, 125.32, 84.69, 82.72, 75.15, 64.30, 37.95, 21.37, 21.23.

HRMS calculated for C<sub>26</sub>H<sub>26</sub>N<sub>5</sub>O<sub>5</sub>S *m/z*: 520.1655. Found: 520.1673.

HPLC: *t*<sub>R</sub> 3.4 min (2% MeOH and 0.2% Et<sub>3</sub>N in CH<sub>2</sub>Cl<sub>2</sub>).

*λ*<sub>max</sub> (2% MeOH and 0.2% Et<sub>3</sub>N in CH<sub>2</sub>Cl<sub>2</sub>) 296 nm.

### 3',5'-bis-*O*-TBDMS-deoxycytidine Diphenylsulfilimine (4b)

Compound **4b** was synthesized and purified as described above for compound **4a** using 3',5'-bis-*O*-TBDMS-deoxycytidine (0.66 g, 1.45 mmol) and BTBDS (1.11 g, 1.65 mmol).

Yield 0.82 g (88 %).

<sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.86 (1H, d, *J* = 7.5), 7.74-7.69 (4H, m), 7.49-7.43 (6H, m), 6.33 (1H, dd, *J* = 5.4, 6.1), 6.02 (1H, d, *J* = 7.5), 4.39 (1H, dt, *J* = 5.7, 5.5), 3.91 (1H, dd, *J* = 2.6, 11.3), 3.85-3.83 (1H, m), 3.76 (1H, dd, *J* = 2.1, 11.3), 2.38-2.32 (1H, m), 2.11-2.05 (1H, m), 0.92 (9H, s), 0.86 (9H, s), 0.10 (6H, d, *J* = 2.3), 0.04 (6H, s).

<sup>13</sup>C NMR (CDCl<sub>3</sub>): 172.24, 155.89, 139.67, 136.74, 136.65, 132.29, 132.20, 130.19, 130.15, 128.56, 128.31, 100.04, 87.33, 85.83, 77.71, 62.35, 42.46, 26.34, 26.16, 18.77, 18.39, -4.16, -4.51, -5.08, -5.14.

HRMS calculated for C<sub>33</sub>H<sub>50</sub>N<sub>3</sub>O<sub>4</sub>SSi<sub>2</sub> *m/z*: 640.3061. Found: 640.3065.

HPLC: *t*<sub>R</sub> 3.3 min (2% MeOH and 0.2% Et<sub>3</sub>N in CH<sub>2</sub>Cl<sub>2</sub>).

*λ*<sub>max</sub> (2% MeOH and 0.2% Et<sub>3</sub>N in CH<sub>2</sub>Cl<sub>2</sub>) 297 nm.

### Deoxyadenosine Diphenylsulfilimine (4c)

Compound **4a** (142 mg, 0.28 mmol) was dissolved in saturated  $\text{NH}_3/\text{MeOH}$  (12 mL). After 2 h, when TLC analysis (System A) indicated that the starting material was consumed and the solvent and ammonia were removed under reduced pressure. The remaining solid was dissolved in methanol, precipitated from diethyl ether, filtered, and washed with diethyl ether. Chromatographically pure compound **4c** was then obtained and used without additional purification.

Yield 72.6 mg (60%).

$^1\text{H}$  NMR ( $\text{CD}_3\text{CN}$ ): 8.02 (1H, s), 7.96 (1H, s), 7.93–7.88 (4H, m), 7.62–7.55 (6H, m), 6.33 (1H, dd,  $J = 5.6, 9.0$ ), 5.81 (1H, bs), 4.57 (1H, bd,  $J = 5.4$ ), 4.05 (1H, dd,  $J = 2.3, 3.7$ ), 3.78 (1H, dd,  $J = 2.2, 12.6$ ), 3.71–3.64 (1H, m), 3.42 (1H, bs), 2.88 (1H, ddd,  $J = 5.4, 9.1, 14.5$ ), 2.26 (1H, ddd,  $J = 1.5, 5.7, 13.3$ ).

$^{13}\text{C}$  NMR ( $\text{CD}_3\text{CN}$ ): 162.32, 151.23, 148.69, 140.10, 137.70, 137.60, 132.52, 132.46, 130.39, 127.78, 127.69, 89.64, 87.35, 72.92, 67.08, 63.36, 40.46.

HRMS calculated for  $\text{C}_{22}\text{H}_{22}\text{N}_5\text{O}_3\text{S}$   $m/z$ : 436.1443. Found: 436.1441.

RP-HPLC  $t_R$  26.8 min.

$\lambda_{\text{max}}$  (MeOH): 294.4 nm ( $\epsilon = 21800$ ).

### Deoxycytidine Diphenylsulfilimine (4d)

Compound **4d** can also be prepared as described above for compound **4c** using 99 mg (0.20 mmol) of 3',5'-di-*O*-acetyl-deoxycytidine diphenylsulfilimine (**4e**) and 10 mL saturated  $\text{NH}_3$  in methanol.

Yield 51 mg (62%).

$^1\text{H}$  NMR ( $\text{CD}_3\text{CN}$ ): 7.84–7.81 (4H, m), 7.65 (1H, d,  $J = 7.4$ ), 7.59–7.54 (6H, m), 6.10 (1H, t,  $J = 6.7$ ), 6.02 (1H, dd,  $J = 0.3, 7.5$ ), 4.34 (1H, bd,  $J = 5.1$ ), 3.84 (1H, q,  $J = 3.5$ ), 3.71–3.68 (1H, m), 3.66 (1H, m), 3.51 (1H, bs), 3.40 (1H, bs), 2.18 (1H, dd,  $J = 1.4, 2.8$ ), 2.16 (1H, t,  $J = 2.1$ ).

$^{13}\text{C}$  NMR ( $\text{CD}_3\text{CN}$ ): 171.89, 155.28, 140.81, 137.11, 137.05, 132.54, 132.50, 130.38, 127.85, 127.78, 99.69, 87.68, 87.19, 71.47, 67.08, 62.27, 40.36.

HRMS calculated for  $\text{C}_{21}\text{H}_{22}\text{N}_3\text{O}_4\text{S}$   $m/z$ : 412.1331. Found: 412.1347.

RP-HPLC  $t_R$  25.2 min.

$\lambda_{\text{max}}$  (MeOH): 293.7 nm ( $\epsilon = 17500$ ).

### 3',5'-di-*O*-Acetyl-deoxycytidine Diphenylsulfilimine (4e)

Compound **4b** (0.34 g, 0.53 mmol) was dissolved in dry THF (12 mL), and  $\text{Et}_3\text{N} \times 3 \text{ HF}$  (0.87 mL, 5.33 mmol) was added. The reaction was

followed by TLC (System A), and, upon completion, the solvent was removed under reduced pressure. The remaining oily residue containing deoxycytidine diphenylsulfilimine(**4d**) was co-evaporated with dry pyridine ( $2 \times 3$  mL), re-dissolved in pyridine (3 mL) and  $\text{Ac}_2\text{O}$  (0.16 mL, 1.6 mmol) was then added with stirring. After completion, the reaction was quenched with methanol (1 mL), and the solvent removed at reduced pressure. The residue was dissolved in ethyl acetate (20 mL), extracted with saturated aqueous  $\text{NaHCO}_3$  ( $2 \times 10$  mL), water (10 mL) and brine (10 mL) and then was dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated. Compound **4e** was purified by short column chromatography using 2% methanol in chloroform.

Yield 0.24 g (92%).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 7.75–7.71 (4H, m), 7.53–7.46 (6H, m), 7.44 (1H, d,  $J = 7.5$ ), 6.39 (1H, dd,  $J = 5.6, 8.3$ ), 6.08 (1H, d,  $J = 7.5$ ), 5.19 (1H, dt, 2.2, 6.7), 4.37–4.29 (2H, m), 4.24–4.22 (1H, m), 2.57 (1H, ddd,  $J = 2.1, 5.6, 14.2$ ), 2.10 (3H, s), 2.09 (3H, s), 2.06 (1H, dd, 6.2, 7.9).

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 172.20, 170.94, 170.83, 155.62, 138.12, 136.48, 136.43, 132.40, 132.33, 130.25, 130.22, 128.49, 128.32, 126.29, 100.90, 86.33, 82.21, 74.90, 64.48, 38.64, 21.39, 21.24.

HRMS calculated for  $\text{C}_{25}\text{H}_{26}\text{N}_3\text{O}_6\text{S}$   $m/z$ : 496.1542. Found: 496.1526.

HPLC:  $t_R$  3.1 min (2% MeOH and 0.2%  $\text{Et}_3\text{N}$  in  $\text{CH}_2\text{Cl}_2$ ).

$\lambda_{\text{max}}$  (2% MeOH and 0.2%  $\text{Et}_3\text{N}$  in  $\text{CH}_2\text{Cl}_2$ ) 296 nm.

### 3',5'-di-*O*-Acetyl-deoxyadenosine Dimethylsulfilimine (**5a**)

Anhydrous DMSO (0.114 mL, 1.61 mmol) in  $\text{CH}_2\text{Cl}_2$  (1.8 mL) was mixed in an oven-dried flask equipped with a thermometer and a magnetic stirring bar. The reaction flask was cooled to  $-65^\circ\text{C}$ , and neat trifluoroacetic anhydride (0.125 mL, 0.90 mmol) was then added drop-wise (the temperature of the reaction mixture should not exceed  $-55^\circ\text{C}$ ). During the addition of  $\text{Tfa}_2\text{O}$ , a white precipitate was formed (sulfonium reagent).

Meanwhile, 3',5'-di-*O*-acetyl-deoxyadenosine (0.30 g, 0.90 mmol) was dissolved in a mixture of dry  $\text{CH}_2\text{Cl}_2$  and DMSO (1.8 mL and 0.26 mL, respectively). This solution was slowly added to the pre-formed sulfonium reagent (temperature kept below  $-45^\circ\text{C}$ ). After addition, the reaction was continued for another 60 min., cooled down to  $-60^\circ\text{C}$ , where upon  $\text{Et}_3\text{N}$  (0.28 mL) was added. Finally, the reaction mixture was allowed to warm up to  $0^\circ\text{C}$  and transferred into a separation funnel containing  $\text{CH}_2\text{Cl}_2$  (20 mL) and 0.25 M phosphate buffer (10 mL, pH 7). The organic phase was further washed with water (10 mL) and brine (10 mL) and was then dried ( $\text{Na}_2\text{SO}_4$ ) and evaporated.

Compound **5a** was purified by flash column chromatography as follows, the column was first eluted with 10% iPrOH in ethyl acetate (elution of by-products) and then with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> containing 0.1% Et<sub>3</sub>N (elution of the product).

Yield 0.25 g (70%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>): 8.26 (1H, s), 7.91 (1H, s), 6.43 (1H, dd, *J* = 5.9, 8.1), 5.43 (1H, dt, *J* = 2.3, 6.3), 4.44-4.40 (1H, m), 4.36-4.31 (1H, m), 2.96-2.88 (1H, m), 2.86 (6H, s), 2.60 (1H, ddd, *J* = 3.1, 6.6, 14.8), 2.13 (3H, s), 2.11 (3H, s).

<sup>13</sup>C NMR (CDCl<sub>3</sub>): 170.93, 170.78, 162.35, 152.85, 149.16, 137.25, 124.84, 84.68, 82.70, 75.12, 64.29, 37.94, 33.60, 21.38, 21.24.

HRMS calculated for C<sub>16</sub>H<sub>22</sub>N<sub>5</sub>O<sub>5</sub>S *m/z*: 396.1342. Found: 396.1349.

HPLC: *t*<sub>R</sub> 4.4 min (2% MeOH and 0.2% Et<sub>3</sub>N in CH<sub>2</sub>Cl<sub>2</sub>).

λ<sub>max</sub> (2% MeOH and 0.2% Et<sub>3</sub>N in CH<sub>2</sub>Cl<sub>2</sub>) 289 nm.

### 3',5'-bis-*O*-TBDMS-deoxycytidine Dimethylsulfilimine (**5b**)

Compound **5b** was prepared as described above for compound **5a** using 3',5'-bis-*O*-TBDMS-deoxycytidine (1.00 g, 2.2 mmol), DMSO (0.28 mL, 3.95 mmol), and Tfa<sub>2</sub>O (0.30 mL, 2.2 mmol).

Yield 0.70 g (62%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.80 (1H, d, *J* = 7.5), 6.34 (1H, dd, *J* = 5.5, 6.1), 5.78 (1H, d, *J* = 7.5), 4.39 (1H, dt, *J* = 5.5, 5.9), 3.90 (1H, dd, *J* = 2.5, 11.3), 3.84-3.83 (1H, m), 3.75 (1H, dd, *J* = 1.9, 11.3), 2.77 (3H, s), 2.76 (3H, s), 2.38-2.32 (1H, m), 2.10-2.04 (1H, m), 0.91 (9H, s), 0.87 (9H, s), 0.08 (6H, s), 0.04 (6H, s).

<sup>13</sup>C NMR (CDCl<sub>3</sub>): 171.79, 156.02, 139.48, 99.54, 87.33, 85.73, 70.66, 62.34, 42.45, 32.97, 32.92, 26.31, 26.16, 18.75, 18.40, -4.16, -4.50, -5.12, -5.17.

HRMS calculated for C<sub>23</sub>H<sub>46</sub>N<sub>3</sub>O<sub>4</sub>SSi<sub>2</sub> *m/z*: 516.2748. Found: 516.2750.

HPLC: *t*<sub>R</sub> 3.3 min (2% MeOH and 0.2% Et<sub>3</sub>N in CH<sub>2</sub>Cl<sub>2</sub>).

λ<sub>max</sub> (2% MeOH and 0.2% Et<sub>3</sub>N in CH<sub>2</sub>Cl<sub>2</sub>) 292 nm.

### 3',5'-bis-*O*-TBDMS-deoxyadenosine Dimethylsulfilimine (**5c**)

Compound **5c** was prepared as described above for compound **5a** using 3',5'-bis-*O*-TBDMS-deoxyadenosine (0.30 g, 0.63 mmol), DMSO (0.16 mL, 2.25 mmol), and Tfa<sub>2</sub>O (0.174 mL, 1.25 mmol).

Yield 0.21 g (63%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>): 8.28 (1H, s), 8.07 (1H, s), 6.45 (1H, t, *J* = 6.4), 4.62-4.58 (1H, m), 4.00 (1H, dt, *J* = 3.3, 3.4), 3.87 (1H, dd, *J* = 4.2, 11.1),

3.78 (1H, dd,  $J = 3.2, 11.1$ ), 2.87 (6H, s), 2.62–2.55 (1H, m), 2.44–2.38 (1H, m), 0.93 (9H, s), 0.92 (9H, s), 0.10 (6H, s), 0.08 (6H, s).

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 152.38, 149.06, 137.95, 124.69, 88.06, 84.39, 72.24, 63.21, 41.78, 33.77, 26.42, 26.18, 18.86, 18.42,  $-4.27$ ,  $-4.41$ ,  $-4.95$ ,  $-5.07$ .

HRMS calculated for  $\text{C}_{24}\text{H}_{46}\text{N}_5\text{O}_3\text{SSi}_2$   $m/z$ : 540.2860. Found: 540.2863.

HPLC:  $t_R$  4.7 min (2% MeOH and 0.2%  $\text{Et}_3\text{N}$  in  $\text{CH}_2\text{Cl}_2$ ).

$\lambda_{\text{max}}$  (2% MeOH and 0.2%  $\text{Et}_3\text{N}$  in  $\text{CH}_2\text{Cl}_2$ ) 292 nm.

### Deoxyadenosine Dimethylsulfilimine (5d)

Compound **5d** was prepared as described above for compound **4c** starting from 64.2 mg of compound **5a** and 10 mL of saturated ammonia in methanol.

Yield 39.2 mg (60%).

$^1\text{H}$  NMR ( $\text{CD}_3\text{CN}$ ): 8.07 (1H, s), 7.83 (1H, s), 6.29 (1H, dd,  $J = 5.7, 9.2$ ), 6.13 (1H, d,  $J = 9.7$ ), 4.57 (1H, d,  $J = 5.1$ ), 4.06 (1H, dd,  $J = 2.1, 3.5$ ), 3.79 (1H, dd,  $J = 0.8, 12.8$ ), 3.67 (1H, dt,  $J = 1.9, 12.4$ ), 3.47 (1H, bs), 2.88 (1H, ddd,  $J = 5.3, 9.2, 14.5$ ), 2.79 (6H, s), 2.25 (1H, dt,  $J = 1.4, 5.7$ ).

$^{13}\text{C}$  NMR ( $\text{CD}_3\text{CN}$ ): 162.65, 151.47, 148.15, 139.38, 125.33, 89.70, 87.44, 72.99, 63.43, 40.48, 32.31, 32.27.

HRMS calculated for  $\text{C}_{12}\text{H}_{18}\text{N}_5\text{O}_3\text{S}$   $m/z$ : 312.1130. Found: 312.1132.

RP-HPLC  $t_R$  13.3 min.

### Deoxycytidine Dimethylsulfilimine (5e)

To a solution of compound **6b** (0.60 g, 1.17 mmol) in dry THF (25 mL) was added in one portion neat  $\text{Et}_3\text{N} \times 3 \text{ HF}$  (1.9 mL, 11.7 mmol). The reaction was followed by TLC (system A) and, upon completion (16 h), the mixture was concentrated at reduced pressure. The residue was quenched with 2 M TEAB buffer (3 mL, pH 7.5) and lyophilized. Chromatographically pure compound **5e** was obtained as a white powder by trituration from acetone, and was used without additional purification.

Yield 0.24 g (70%).

$^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ): 7.84 (1H, d,  $J = 7.5$ ), 6.30 (1H, t,  $J = 6.8$ ), 5.91 (1H, d,  $J = 7.5$ ), 4.38 (1H, dt,  $J = 3.3, 6.2$ ), 3.93 (1H, q,  $J = 3.6$ ), 3.76 (2H, dq,  $J = 3.4, 12.0$ ), 2.82 (6H, s), 2.32 (1H, ddd,  $J = 3.6, 6.1, 13.5$ ), 2.19–2.10 (1H, m).

$^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ): 139.51, 100.21, 87.69, 86.30, 71.20, 61.91, 40.81, 31.01, 30.94.

HRMS calculated for  $\text{C}_{11}\text{H}_{18}\text{N}_3\text{O}_4\text{S}$   $m/z$ : 288.1018. Found: 288.1017.

RP-HPLC  $t_R$  9.6 min.

**3',5'-di-*O*-Acetyl-deoxycytidine Dimethylsulfilimine (5f)**

To a suspension of compound **5e** (55.8 mg, 0.19 mmol) in dry pyridine (1 mL) was added acetic anhydride (47  $\mu$ L, 0.49 mmol). After completion (determined by TLC analysis in System A), the reaction mixture was quenched with methanol (1 mL) and concentrated, and the remaining solid was dissolved in  $\text{CH}_2\text{Cl}_2$  (2 mL). Purification on a silica column, which was eluted with 2% MeOH in  $\text{CH}_2\text{Cl}_2$ , gave pure compound **5f** as a white foam.

Yield 71.7 mg (99.5%).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 7.39 (1H, d,  $J = 7.5$ ), 6.43 (1H, dd,  $J = 5.6, 8.4$ ), 5.85 (1H, d,  $J = 7.5$ ), 5.22-5.19 (1H, m), 4.37-4.29 (2H, m), 4.25-4.23 (1H, m), 2.78 (6H, s), 2.58 (1H, ddd,  $J = 2.0, 5.6, 14.2$ ), 2.11 (3H, s), 2.10 (3H, s), 2.06 (1H, dd,  $J = 6.1, 7.8$ ).

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 171.71, 170.94, 155.75, 100.45, 86.19, 82.19, 74.91, 64.47, 46.17, 38.58, 32.89, 32.82, 21.40, 21.24, 9.04.

HRMS calculated for  $\text{C}_{15}\text{H}_{22}\text{N}_3\text{O}_6\text{S}$   $m/z$ : 372.1229. Found: 372.1226.

HPLC:  $t_R$  4.7 min (2% MeOH and 0.2%  $\text{Et}_3\text{N}$  in  $\text{CH}_2\text{Cl}_2$ ).

$\lambda_{\text{max}}$  (2% MeOH and 0.2%  $\text{Et}_3\text{N}$  in  $\text{CH}_2\text{Cl}_2$ ) 289 nm.

**5'-*O*-Monomethoxytrityl-deoxycytidine Dimethylsulfilimine (6)**

To a suspension of compound **5e** (80 mg, 0.28 mmol) in dry pyridine (2 mL) was added in one portion monomethoxytrityl chloride (112 mg, 0.35 mmol). The completion of the reaction was determined by TLC (System A). The reaction mixture was quenched with methanol (1 mL) and concentrated, and the remaining solid was dissolved in  $\text{CH}_2\text{Cl}_2$  (10 mL). The organic layer was washed with sat. aq.  $\text{NaHCO}_3$  (5 mL) and brine (5 mL) and then was dried ( $\text{Na}_2\text{SO}_4$ ). Purification was performed by flash column chromatography (silica) using first EtOAc/ $i$ PrOH 9:1 containing 0.1%  $\text{Et}_3\text{N}$  as eluent, then  $\text{CH}_2\text{Cl}_2$ /MeOH 9:1 + 0.1%  $\text{Et}_3\text{N}$ . This gave pure compound **6** as a white foam.

Yield 76 mg (49%).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 7.64 (1H, d,  $J = 7.5$ ), 7.46-7.43 (4H, m), 7.35-7.23 (8H, m), 6.88-6.84 (2H, m), 6.44 (1H, t,  $J = 6.2$ ), 5.66 (1H, d,  $J = 7.5$ ), 4.52-4.49 (1H, m), 4.05 (1H, q,  $J = 3.9$ ), 3.81 (3H, s), 3.47-3.41 (2H, m), 2.77 (3H, s), 2.77 (3H, s), 2.59-2.52 (1H, m), 2.24-2.18 (1H, m).

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 171.73, 159.06, 156.08, 144.51, 144.35, 139.19, 135.47, 130.81, 128.79, 128.36, 127.50, 113.63, 99.94, 87.38, 85.84, 85.72, 72.19, 63.81, 55.66, 42.00, 32.89, 32.78, 30.11.

HRMS calculated for  $\text{C}_{31}\text{H}_{34}\text{N}_3\text{O}_5\text{S}$   $m/z$ : 560.2219. Found: 560.2216.



### 5'-O-Monomethoxytrityldeoxycytidine Dimethylsulfilimine 3'-H-phosphonate (7)

Phosphorous acid (370 mg, 4.5 mmol) was co-evaporated with dry pyridine ( $3 \times 3$  mL) and then re-dissolved in dry pyridine (2.7 mL). To this solution was added pivaloyl chloride (0.28 mL, 2.3 mmol), resulting in the formation of the pyrophosphorous acid reagent.

The reagent (0.27 mL) was then added to the solution of compound **6** (50.6 mg, 0.09 mmol) in pyridine (0.27 mL). The reaction was followed by TLC (System B), and upon, completion, was quenched with 1 M TEAB buffer (1 mL, pH 7.5). Next,  $\text{CH}_2\text{Cl}_2$  was added (15 mL) and the organic mixture was washed with 2 M TEAB (3 mL, pH 7.5). Drying ( $\text{Na}_2\text{SO}_4$ ) and evaporation of the organic phase gave crude compound **7**, which was purified by straight-phase HPLC, using 8% methanol in  $\text{CH}_2\text{Cl}_2$  with 0.2%  $\text{Et}_3\text{N}$  as eluent.

Yield 36.5 mg (56%).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 7.52 (1H, d,  $J = 7.4$ ), 7.40-7.37 (2H, m), 7.25-7.16 (8H, m), 6.86 (1H, d,  $J = 620.5$ ), 6.80 (2H, d,  $J = 8.9$ ), 6.41 (1H, t,  $J = 6.8$ ), 5.55 (1H, d,  $J = 7.5$ ), 4.86 (1H, bs), 4.20 (1H, bs), 3.75 (3H, s), 3.33 (2H, bs), 3.00 (6H, q, 7.3), 2.72 (3H, s), 2.71 (3H, s), 2.16-2.10 (1H, m), 1.30 (9H, t,  $J = 7.3$ ).

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 171.53, 158.98, 156.45, 144.25, 138.98, 135.37, 130.83, 128.83, 128.31, 127.43, 113.61, 100.50, 87.31, 85.97, 85.21, 74.39, 64.00, 55.61, 45.90, 40.68, 32.88, 32.78, 27.97, 9.28.

$^{31}\text{P}$  NMR ( $\text{CDCl}_3$ ): 4.98 (d,  $J = 612.4$ ).

HRMS calculated for  $\text{C}_{31}\text{H}_{35}\text{N}_3\text{O}_7\text{PS}$   $m/z$ : 624.1933. Found: 624.1941.

HPLC:  $t_R$  12.0 min (8% MeOH and 0.2%  $\text{Et}_3\text{N}$  in  $\text{CH}_2\text{Cl}_2$ ).

$\lambda_{\text{max}}$  (2% MeOH and 0.2%  $\text{Et}_3\text{N}$  in  $\text{CH}_2\text{Cl}_2$ ) 290 nm.

### Dinucleotide 8 (Using OXP as a Coupling Agent)

Compound **7** (12.8 mg, 18  $\mu\text{mol}$ ) and 6-*N*-benzoyl-3'-*O*-levulinoyl-deoxyadenosine (12.3 mg, 27  $\mu\text{mol}$ ) were dried by co-evaporation with dry pyridine ( $3 \times 1$  mL) and then were dissolved in pyridine (1 mL). To this solution was added OXP (9.2 mg, 36  $\mu\text{mol}$ ) under stirring. After 16 hours, a 0.01 M solution of iodine in pyridine/water 98:2 (0.36 mL) was added, and oxidation was continued for 5 min. The reaction mixture was diluted with  $\text{CH}_2\text{Cl}_2$  (15 mL) and transferred to a separation funnel containing 10% aq.  $\text{Na}_2\text{S}_2\text{O}_3$  (5 mL) and 1 M TEAB (pH 7.5, 5 mL). Drying ( $\text{Na}_2\text{SO}_4$ ) and evaporation of the organic phase gave crude compound **8**, which was purified by straight-phase HPLC, using 10% methanol in  $\text{CH}_2\text{Cl}_2$  with 0.2%  $\text{Et}_3\text{N}$  as eluent.

Yield 18.4 mg (87%).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ ) selected signals: 8.85 (1H, s), 8.80 (1H, s), 8.01 (2H, d,  $J = 7.4$ ), 7.59–7.56 (2H, m), 7.50–7.46 (2H, m), 7.40–7.38 (4H, m), 7.28–7.23 (5H, m), 7.19–7.15 (2H, m), 6.81–6.78 (2H, m), 6.63 (1H, dd,  $J = 5.8, 8.8$ ), 6.42 (1H, t,  $J = 5.7$ ), 5.61 (3H, s), 5.53 (1H, d,  $J = 7.6$ ), 5.49 (1H, d,  $J = 5.4$ ), 5.07–5.04 (1H, m), 4.96–4.91 (1H, m), 4.27–4.24 (2H, m), 4.09–4.07 (2H, m), 3.42–3.33 (2H, m), 2.20 (3H, s).

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ) selected signals: 172.47, 171.58, 258.96, 156.05, 152.94, 152.29, 149.85, 144.49, 144.22, 142.71, 139.27, 135.36, 134.16, 132.99, 130.88, 129.20, 128.89, 128.86, 128.38, 128.28, 127.39, 123.58, 113.55, 99.99, 87.34, 85.87, 85.22, 84.92, 84.31, 63.79, 55.61, 53.36, 46.18, 38.23, 32.82, 30.24, 30.24, 28.37, 9.04, 8.51.

$^{31}\text{P}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  -0.42.

HRMS calculated for  $\text{C}_{53}\text{H}_{54}\text{N}_8\text{O}_{13}\text{PS}$   $m/z$ : 1073.3269. Found: 1073.3311.

HPLC:  $t_R$  4.1 min (10% MeOH and 0.2%  $\text{Et}_3\text{N}$  in  $\text{CH}_2\text{Cl}_2$ ).

## Dinucleotide 8 (Using Pivaloyl Chloride as a Coupling Agent)

Dinucleotide **8** was also prepared by treating a solution of phosphonate **7** (3.2 mg, 4.4  $\mu\text{mol}$ ) and 6-*N*-benzoyl-3'-*O*-levulinoyldeoxyadenosine (2.2 mg, 4.9  $\mu\text{mol}$ ) in acetonitrile/pyridine (4/1 v/v, 150  $\mu\text{L}$ ) with pivaloyl chloride (1.1  $\mu\text{L}$ ). After 15 min., a solution of iodine in pyridine/water (98/2 v/v, 88  $\mu\text{L}$ , 0.01 M) was added, and oxidation was continued for 5 min. Reaction work-up was performed as described above. The remaining solid was dissolved in  $\text{CDCl}_3$  and its  $^{31}\text{P}$  NMR spectrum was recorded.

$^{31}\text{P}$  NMR ( $\text{CDCl}_3$ ): -0.42 as the only product).

An aliquot was injected into HPLC (10% MeOH/ $\text{CH}_2\text{Cl}_2$  + 0.2%  $\text{Et}_3\text{N}$ )— $t_R$  4.1 min.

## Stability Tests

### 2% DCA Test—General Procedure

Compound **5a** (1.61 mg) was dissolved in 2% (v/v) DCA in DCE (407  $\mu\text{L}$ ). Aliquots (10  $\mu\text{L}$ ) were taken at 0, 5, 15, 30, 60, and 120 min and quenched with the HPLC mobile phase (490  $\mu\text{L}$ ). From this solution, 20  $\mu\text{L}$  were injected into the Straight-Phase HPLC and analyzed. The mobile phase consists of 2% MeOH and 0.2%  $\text{Et}_3\text{N}$  in  $\text{CH}_2\text{Cl}_2$  with a flow rate of 1 mL/min and observed at 285 nm. The peak area of compound **5a** at 0 min was assigned to 100% (response standard), and the subsequent areas (5–120 min) were compared to the standard.

### **Capping Test—General Procedure**

Compound **5c** (2.57 mg) was dissolved in Capping solution A [6% DMAP in MeCN (w/v), 238  $\mu$ L], and an equal volume of Capping solution B [ $\text{Ac}_2\text{O}$ :collidine:MeCN 2:3:5 (v/v/v)] was added. The analysis was performed by HPLC as described above.

### **Iodine Test—General Procedure**

Compound **5c** (1.00 mg) was dissolved in an iodine solution (463  $\mu$ L of 0.01 M). This solution was prepared by dissolving 0.103 g  $\text{I}_2$  in 26 mL dry MeCN and then adding 2.4 mL collidine and 12 mL water. Aliquots (10  $\mu$ L) were taken at 0, 5, 15, 30, 60, and 120 min, and quenched with the HPLC mobile phase (490  $\mu$ L) which contained 0.1% dimethyl phosphite (to remove the free iodine). The Straight-Phase HPLC analysis was performed as described above.

### **Aqueous Buffers and Ammonia Tests—General Procedure**

Compound **4c** (1.13 mg) was dissolved in TRIS buffer (pH 9.0, 260  $\mu$ L 50 mM). After 16 h, an aliquot (5  $\mu$ L) was withdrawn and quenched into the HPLC mobile phase (50  $\mu$ L). In the ammonia tests, the aliquots were first centrifuged under reduced pressure to ensure the removal of volatile substances, i.e. ammonia and methanol, and then were redissolved in the mobile phase (55  $\mu$ L). Aliquots (5  $\mu$ L) were analyzed by reversed-Phase HPLC.

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