3,5-Di-(*tert*-Butyl)-6-fluoro-*cyclo*Sal-d4TMP − A Pronucleotide with a Considerably Improved Masking Group

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A new, considerably improved *cyclo*Sal masking group has been developed. This new group combines four desirable properties and has been attached to the *anti*-HIV drug 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T, **1**) to give 3,5-(di-*tert*-butyl)-6-fluoro-*cyclo*Sal-d4TMP (**2i**). This phosphate triester has a reasonable chemical half-life, highly selectively released d4TMP, has poor – if any – inhibitory effect on butyrylcholinesterase (BChE), and achieved the TK-bypass.

Introduction

Nucleoside analogues are widely used as antiviral and/or antitumor agents. Unlike many other drugs, however, nucleoside analogues are not bioactive as such, a prerequisite for their biological action being their metabolism to the corresponding nucleoside triphosphates by cellular or viral kinases.[1] Often, one of the three kinase-catalyzed steps is the metabolism-limiting step, particularly the first conversion into thymine-nucleoside monophosphate (nucleotide; NMP), catalyzed by thymidine kinase (TK).[2] The use of nucleotides as therapeutics, however, is not possible, because they are not able to penetrate the cell membrane due to their negative charges. Moreover, nucleotides are efficiently catabolized in the blood stream by unspecific nucleotidases. In such a case, intracellular delivery of the nucleotide from a lipophilic precursor (TK-bypass) would bypass these limitations.[3] It has recently been shown that *cyclo*Sal-pronucleotides **2** are a successful class of delivery systems for antiviral nucleotide analogues.[4] Mechanistically, the delivery of the nucleotide from the *cyclo*Sal triesters is the result of a chemically induced releasing process. The *cyclo*Sal approach has been applied successfully to various nucleoside analogues, such as 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T, **1**),[5] (*E*)-5-bromovinyl-2′-deoxyuridine,[6] acyclovir,[7] 2′,3′-dideoxyadenosine,[8] and 2′-*ribo*-fluoro-2′,3′-dideoxyadenosine.[9] We have shown that the corresponding *cyclo*Sal-d4TMP triesters **2** achieved the TK-bypass through the intracellular delivery of d4TMP **3**. The cleavage mechanism is summarized in Scheme 1.

The properties of *cyclo*Sal nucleotides have been "fine-tuned" through selection of different substitution patterns in the salicyl alcohol residue **4** (*cyclo*Sal moiety).[4a] Despite the successful TK-bypass, the structural variations were in some cases accompanied by unwanted properties in the phosphate triesters, including very high resistance to hydrolysis or reduced selectivity in the d4TMP delivery. Here we report on a considerably improved *cyclo*Sal masking unit attached to d4TMP.

Chemistry and Discussion

Four properties are desirable for *cyclo*Sal-d4TMP pronucleotides:

i) They should have reasonable stability,
ii) They should deliver d4TMP highly selectively,
iii) They should achieve the TK-bypass, and
iv) They should not be inhibitors of cholinesterases such as acetylcholinesterase (AChE) or butyrylcholinesterase (BChE).

So far, the best *cyclo*Sal-d4TMP triesters have fulfilled at least two of these criteria, but never all four. From our earlier work it had become apparent that the introduction of donor substituents (e.g., methyl or *tert*-butyl groups) into triester derivatives provided reasonable stability and full retention of the *anti*-HIV activity found in wild-type CEM cells in the TK-deficient CEM cell line (Table 1).[4,5] As an example, 3-methyl-*cyclo*Sal-d4TMP (**2b**) showed a half-life $t_{1/2}$ = 16 h at pH 7.3 in 25 mM phosphate buffer and was found to retain full activity in HIV-infected CEM/TK$^-$ cells. Although its half-life is only 8 h, the same is true for the 5-methyl derivative **2c**, and retention of antiviral activity was also observed for 3,5-dimethyl-*cyclo*Sal-d4TMP (**2d**,

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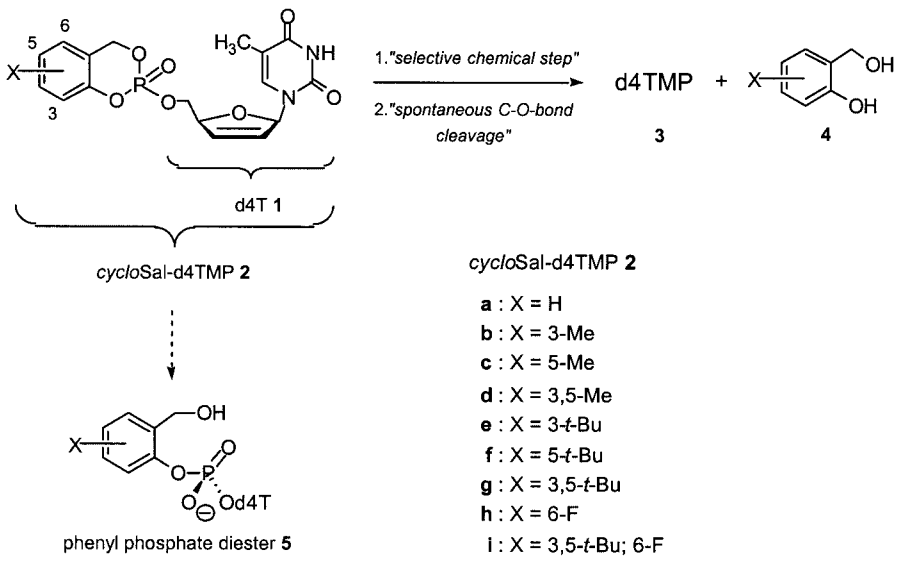


Scheme 1

Table 1. Properties of different *cyclo*Sal-d4TMP phosphate triesters **2** in relation to d4T (**1**)

	Substituent X	$t_{1/2}$ [a] [h]	Product ratio[b] **3:5**	IC_{50}[c] [μM] BChE	EC_{50} [μM][d] CEM/O HIV-1	HIV-2	CEM/TK$^-$ HIV-2	CC_{50}[e] [μM]
2a	H	4.4	99:1	0.8	0.28±0.1	0.62±0.38	0.50±0.17	46.9±22.2
2b	3-Me	16	94:6	1.2	0.087±0.012	0.12±0.03	0.093±0.023	20.9±5.9
2c	5-Me	8.0	96:4	4.6	0.18±0.03	0.34±0.22	0.18±0.06	37.1±16.9
2d	3,5-Me	29	85:15	46	0.093±0.023	0.17±0.1	0.08±0.01	17.4±4.7
2e	3-*t*Bu	96	92:8	4.2	0.18±0.11	0.65±0.07	0.33±0.11	34.4±7.5
2f	5-*t*Bu	8.6	94:6	1.4	0.14±0.02	0.90±0.14	1.5±0.7	33.9±3.0
2g	3,5-*t*Bu	73	66:34	> 50	1.1±0.14	1.2±0.0	2.0±0.0	27.0±7.4
2h	6-F	1.1	100:0	0.4	0.17±0.12	0.38±0.32	0.85±0.25	72.1±6.0
2i	3,5-*t*Bu; 6-F	6.2	100:0[f]	48	0.16±0.06	0.33±0.11	0.60±0.23	41.1±0.78
d4T (**1**)	–	–	–	–	1.68±1.12	1.35±1.63	33.3±20.8	78.6±31.6

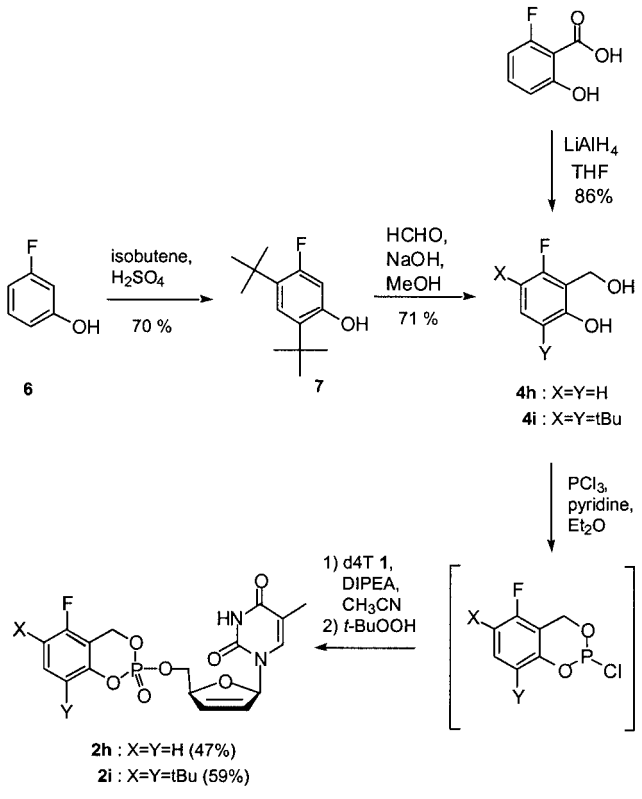
[a] Hydrolysis stability in 25 mM phosphate buffer, pH 7.3 at 37 °C. [b] Product ratio d4TMP **3**: phenyl phosphate diester **5** observed in ^{31}P NMR studies in imidazole/HCl buffer, pH 7.3. [c] Inhibitory potency: 50 % inhibitory concentration against butyrylcholinesterase from human serum. [d] Antiviral activity: 50 % effective concentration. [e] Cytostatic activity: 50 % cytostatic concentration. [f] After six weeks in the NMR tube.

$t_{1/2}$ = 29 h). Detailed studies of the hydrolysis products by ^{31}P NMR spectroscopy, however, revealed that the use of triester **2b** gave rise to the formation of phenyl phosphate diester **5** as a side product in 6 % yield in addition to d4TMP (**3**, Scheme 1), while the 5-methyl derivative gave only 4 % of the corresponding side product. On the other hand, the triester **2d** gave 15 % of the corresponding diester **5**.[10] A second hydrolysis pathway was taking place in addition to the formation of d4TMP. This unwanted pathway gained importance if the hydrolysis half-lives increased. The hydrolysis of 3,5-di-(*tert*-butyl)-*cyclo*Sal-d4TMP (**2g**) showed the formation of 34 % of diester **5** and a chemical half-life of 73 h. Consequently, high chemical stability and/or the formation of considerable amounts of diester **5** resulted in losses of antiviral activity in the CEM/TK$^-$ cell assay (Table 1). Additionally, some *cyclo*Sal triesters (**2a**–**c**, **2e**, and **2f**) were found to be inhibitors of human BChE,[11] a serine hydrolase present in different human tissues and human serum. The physiological importance of this enzyme is still unknown. In contrast, though, human acetylcholinesterase (AChE) is a highly important enzyme that belongs to the same class of hydrolases. Surprisingly, all the triesters **2** described here were devoid of any inhibitory potency against AChE (data not shown[11]). It was interesting to note that we observed a clear correlation of the inhibitory potency against BChE and the substitution pattern in the *cyclo*Sal part. While triesters **2a**, **2b**, **2f**, and particularly **2h** showed considerable BChE inhibition ($IC_{50(BChE)}$ = 0.8 μM, 1.2 μM, 1.4 μM, and 0.6 μM, respectively), double methylation in positions 3 and 5 of the *cyclo*Sal ring of triester **2d** produced a pronounced decrease in the inhibitory effect ($IC_{50(BChE)}$ = 46 μM). With increasing steric bulk of the substituents, as in compound **2g** (*tert*-butyl), no inhibition of BChE was observed at all ($IC_{50(BChE)}$ > 50 μM; Table 1).

There are therefore distinct differences in the properties of the described *cyclo*Sal-d4TMP triesters **2**. Our aim in the

study presented here was to combine all positive properties into one masking group. We therefore prepared 3,5-di-(*tert*-butyl)-6-fluoro-*cyclo*Sal-d4TMP (**2i**). As shown above, the presence of two *tert*-butyl groups produces high stability in the triester. Moreover, the *tert*-butyl groups block the interaction with BChE. The fluorine atom in position 6 was introduced in order to achieve two goals. Firstly, we knew from previous studies on 6-chloro-*cyclo*Sal-d4TMP that the presence of an acceptor group in this position prevents the formation of the unwanted phenyl phosphate diester **5**. The formation of **5** is due to a heterolytic bond cleavage of the benzyl-C−O bond, affording a benzyl cation intermediate.[10] Thus, although two *tert*-butyl groups are present, hydrolysis of **2i** should result in a highly selective delivery of d4TMP. Secondly, we had observed in the same study that the presence of a halogen atom in position 6 reduces the chemical stability of the *cyclo*Sal triester (Table 1). Because of this effect, we expected a considerable decrease in the hydrolysis half-life for the new triester **2i** in relation to the *tert*-butyl triesters (**2e**, **2f**) and the di-(*tert*-butyl) triester **2g**.

The target triester **2i** was prepared by starting from commercially available 3-fluorophenol (**6**, Scheme 2). An acid-catalyzed alkylation with isobutene was carried out first, according to a modified literature procedure,[12] to give 4,6-(*tert*-butyl)-3-fluorophenol (**7**) in 70 % yield. Treatment of this material with a basic formaldehyde solution resulted in the formation of the corresponding salicyl alcohol **4i** in 71 % yield.[13] For comparison, 6-fluorosalicyl alcohol (**4h**) was also prepared (86 % yield). The diol was readily accessible by reduction of the corresponding salicylic acid. These diols were treated with PCl_3 and subsequently with d4T according to our previously reported procedure[4a] to give the target *cyclo*Sal triesters **2h** and **2i** in 47 % and 59 % yields, respectively. As reported previously, triesters **2h** and **2i** were obtained as 1:1 mixtures (crude material) of diastereomers. The structures were unambiguously confirmed by different NMR techniques and mass spectrometry.



Scheme 2

Chemical hydrolysis of the triester **2i** in 25 mM phosphate buffer at pH 7.3 showed a half-life of 6.2 h. This half-life ($t_{1/2}$) is similar to the values for the 5-(*tert*-butyl)- (**2f**) and 5-methyl-*cyclo*Sal-d4TMP triester (**2c**) and significantly lower than that of 3-(*tert*-butyl)-*cyclo*Sal-d4TMP (**2e**) (Table 1). However, the introduction of the fluorine atom in position 6 of the *cyclo*Sal ring produced a pronounced decrease in the hydrolytic stability ($t_{1/2}$ = 1.1 h) in relation to the reference *cyclo*Sal derivative **2a** ($t_{1/2}$ = 4.4 h; Table 1). In ^{31}P NMR studies in imidazole/HCl buffer, pH 7.3 after six weeks, only the formation of d4TMP **3** was observed for both triesters (**2h**, **2i**), which shows that the introduction of the fluorine atom completely avoids the formation of the phenyl phosphate diesters **5** (Scheme 1). In contrast, the triesters **2a**−**g** also gave varying amounts of the phenyl phosphate diesters **5** (Table 1). Next, incubation studies with human AChE showed that neither **2h** nor **2i** was as inhibitory to the enzyme as the triesters reported before. Moreover, incubation with human serum confirmed that the new triester **2i** has almost no inhibitory potency towards BChE ($IC_{50(BChE)}$ = 48 μM). Interestingly, the 6-fluoro-substituted derivative **2h** was even more inhibitory ($IC_{50(BChE)}$ = 0.4 μM) than the unsubstituted reference compound **2a** ($IC_{50(BChE)}$ = 0.8 μM), thus confirming the dominating effect of the two *tert*-butyl groups. Finally, in antiviral tests with HIV-1- and HIV-2-infected wild-type CEM/O cells, triester **2i** exhibited pronounced antiviral potency, even better than the activity of the parent **1**. Most importantly, the antiviral activity was fully retained in HIV-2-infected CEM/TK$^-$ cells, so triester **2i** achieved the TK-bypass. This retention of activity has also been observed for other *cyclo*Sal-d4TMP triesters (**2a**−**e**). However, some of those triesters exhibited considerable inhibitory effects towards BChE, while triester **2i** was non-inhibitory (Table 1). Surprisingly, although the half-life of the 6-fluoro-triester **2h** was significantly lower, **2h** also showed considerable retention of activity in the TK-deficient cells. It may be noted that, except for 6-fluoro-*cyclo*Sal-d4TMP (**2h**), all other compounds shown in Table 1 had virtually identical 50 % cytostatic concentrations (CC_{50} values), irrespective of antiviral potency and degree of inhibition of BChE. The CC_{50} value is defined as the compound concentrations required to reduce the number of viable cells by 50 %. It is important to mention that the cytotoxicity of triester **2h** is identical to, while that of triester **2i** is only slightly higher than that of the parent nucleoside **1**. Keeping the retention of the antiviral activity in the thymidine kinase-deficient cells in mind, these results clearly show the advantage of the *cyclo*Sal-pronucelotide approach.

In conclusion, 3,5-di-(*tert*-butyl)-6-fluoro-*cyclo*Sal-d4TMP (**2i**) has for the first time fulfilled all the expected properties and combines four desired properties of the *cyclo*Sal approach. The masking group disclosed here is therefore the best masking group so far and will now be incorporated into various other nucleotide analogues. Work towards this is currently underway in our laboratories.

Experimental Section

General Remarks: NMR spectra were recorded with Bruker AMX 400 and Bruker DRX 500 Fourier transform spectrometers. All ^{1}H and ^{13}C NMR chemical shifts (δ) are quoted in ppm and calibrated on solvent signals. The ^{31}P NMR chemical shifts (proton decoupled) are quoted in ppm relative to H_3PO_4 as the external reference. The ^{19}F NMR chemical shifts (proton coupled) are quoted in ppm relative to $CFCl_3$ as the external reference. The spectra were recorded at room temperature. Electron impact mass spectra were measured with a VG Analytical VG/70−250S spectrometer (double focussing). FAB high-resolution (HR) mass spectra were recorded with a VG Analytical 70−250S spectrometer by use of an MCA method and polyethylene glycol as support. Merck precoated 60 F_{254} plates with a 0.2 mm layer of silica gel were used for thin layer chromatography (TLC). All preparative TLCs were performed with a chromatotron (Harrison Research, Model 7924T) on glass plates coated with 1-mm or 2-mm layers of Merck 60 PF_{254} silica gel containing a fluorescent indicator. For column chromatography, Merck silica gel 60, 230−400 mesh was used. Analytical HPLC was performed on a Merck−Hitachi HPLC system (D-7000) equipped with a LiChroCART 125−3 column containing reversed-phase silica gel Lichrospher 100 RP 18 (5 μm) (Merck, Darmstadt, Germany). The lyophilized products **2h** and **2i** did not give useful microanalytical data, most probably due to incomplete combustion of the compounds or to the presence of varying amounts of water, but were found to be pure by rigorous HPLC analysis (gradient of 5−100 % acetonitrile in water over 25 min, flow 0.5 mL/min). All reactions were carried out under dry nitrogen except for the synthesis of **4i** and **7**. Solvents used in the inert gas syntheses were commercially available dry solvents stored under argon and over molecular sieves (Fluka). Diethyl ether was dried with sodium/benzophenone and distilled under nitrogen.

General Procedure for the Synthesis of the *cyclo*Sal Phosphate Triesters 2: A solution of the salicyl alcohol derivative (1.0 equiv.) in dry diethyl ether was cooled to −20 °C. After addition of freshly distilled phosphorous(III) chloride (1.2 equiv.) and stirring at −20 °C for 5−10 min , a solution of dry pyridine (2.3 equiv.) in dry diethyl ether was added at the same temperature over a 2−3 h period. After completion of the addition, the reaction mixture was warmed to room temperature and stirred for 1−2 h. It was kept at −20 °C overnight for complete precipitation of pyridinium chloride. Filtration under nitrogen and evaporation of the filtrate under reduced pressure afforded the phosphitylating agents (saligenyl chlorophosphites) as crude products suitable to be directly used for the synthesis of the *cyclo*Sal phosphate triesters without further purification.

The general synthesis of *cyclo*Sal d4T monophosphates has been published before.[3,5,13] Diisopropylethylamine (DIPEA, 2.0 equiv.) was added to a solution of the nucleoside analogue (1.0 equiv.) in dry acetonitrile. The resulting solution was cooled to −20 °C and the appropriate chlorophosphite (2.0 equiv.) was added. The solution was warmed to room temperature, and stirring was continued for 1 h. Subsequently, *tert*-butyl hydroperoxide (3.3 equiv., solution in *n*-decane) was added at −20 °C. After the mixture had warmed to room temperature and been stirred for 1 h, the solvent was removed under reduced pressure. The resulting residue was purified by preparative TLC (Chromatotron, 1. ethyl acetate/methanol, 9:1; 2. dichloromethane/methanol gradient). Lyophilization yielded the products as colorless foams. Diastereomeric ratios were obtained from the ^{31}P NMR spectra.

6-Fluoro-*cyclo*Sal-d4T Monophosphate (2h): The crude chlorophosphite was synthesized from **4h** (0.50 g, 2.8 mmol), phosphorous(III) chloride (0.47 g, 3.4 mmol), and pyridine (0.51 g, 6.4 mmol) in diethyl ether (20 mL) as described in the General Procedure (yield 0.32 g). *Cyclo*Sal d4T monophosphate **2h** was synthesized from d4T (**1**, 163 mg, 0.725 mmol), diisopropylethylamine (187 mg, 1.45 mmol), chlorophosphite (300 mg, 1.45 mmol), and *tert*-butyl hydroperoxide (5.5 M solution in *n*-decane, 0.45 mL, 2.5 mmol) in acetonitrile (15 mL). The product was obtained as a colorless foam: yield 47 % (141 mg), mixture of two diastereomers, ratio 1.0:1.0. R_f = 0.55 (dichloromethane/methanol, 9:1). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 1.67 (s, 1 × 3 H, CH_3-thymine) , 1.72 (s, 1 × 3 H, CH_3-thymine), 4.28−4.42 (m, 2 × 2 H, 2 × H5′), 4.95−5.01 (m, 2 × 1 H, 2 × H4′), 5.44−5.65 (m, 2 × 2 H, 2 × H-benzyl), 6.04 (dd, $^{3}J_{H,H}$ = 6.3, $^{3}J_{H,H}$ = 6.3 Hz, 2 × 1 H, 2 × H2′), 6.39 (d, $^{3}J_{H,H}$ = 5.7 Hz, 1 × 1 H, 1 × H3′), 6.44 (d, $^{3}J_{H,H}$ = 5.7 Hz, 1 × 1 H, 1 × H3′), 6.79−6.82 (m, 2 × 1 H, 2 × H1′), 7.01 (dd, $^{3}J_{H,H}$ = 8.6, $^{3}J_{H,H}$ = 8.6 Hz, 2 × 1 H, 2 × H3-aryl), 7.12 (ddd, $^{3}J_{H,H}$ = 8.8, $^{3}J_{H,H}$ = 8.8, $^{4}J_{H,F}$ = 5.7 Hz, 2 × 1 H, 2 × H4-aryl), 7.19−7.22 (m, 2 × 1 H, 2 × H6-thymine), 7.44 (ddd, $^{3}J_{H,H}$ = 14.7, $^{3}J_{H,F}$ = 14.7, $^{4}J_{H,H}$ = 7.7 Hz, 2 × 1 H, 2 × H5-aryl), 11.35 (s, 1 × 1 H, 1 × NH), 11.37 (s, 1 × 1 H, 1 × NH) ppm. ^{13}C NMR (101 MHz, [D$_6$]DMSO): δ = 11.86 (1 × CH_3-thymine), 11.90 (1 × CH_3-thymine), 63.70 (dd, $^{2}J_{C,P}$ = 2.0, $^{3}J_{C,F}$ = 5.1 Hz, 1 × C-benzyl), 63.91 (dd, $^{2}J_{C,P}$ = 2.0, $^{3}J_{C,F}$ = 5.1 Hz, 1 × C-benzyl), 84.00 (d, $^{2}J_{C,P}$ = 2.6 Hz, 1 × C5′), 84.10 (d, $^{2}J_{C,P}$ = 2.6 Hz, 1 × C5′), 89.28 (2 × C1′), 109.64 (1 × C5-thymine), 109.69 (1 × C5-thymine), 111.29 (d, $^{4}J_{C,F}$ = 2.0 Hz, 1 × C3-aryl), 111.49 (d, $^{4}J_{C,F}$ = 2.0 Hz, 1 × C3-aryl), 114.34 (d, $^{3}J_{C,F}$ = 3.6 Hz, 1 × C4-aryl), 114.43 (d, $^{3}J_{C,F}$ = 3.1 Hz, 1 × C4-aryl), 127.31 (1 × C2′), 127.34 (1 × C2′), 130.76 (d, $^{2}J_{C,F}$ = 10.7 Hz, 1 × C1-aryl), 130.87 (d, $^{2}J_{C,F}$ = 10.2 Hz, 1 × C1-aryl), 132.69 (1 × C3′), 132.76 (1 × C3′), 135.70 (2 × C6-thymine), 150.18 (1 × C2-thymine), 150.21 (1 × C2-thymine), 150.69 (1 × C2-aryl), 150.72 (1 × C2-aryl), 157.70 (d, $^{1}J_{C,F}$ = 249.2 Hz, 2 × C6-aryl), 163.73 (1 × C4-thymine), 164.05 (1 × C4-thymine) ppm. ^{19}F NMR (471 MHz, [D$_6$]DMSO): δ = −116.97 (dd, $^{3}J_{H,F}$ = 9.7, $^{4}J_{H,F}$ = 5.2 Hz), −117.00 (dd, $^{3}J_{H,F}$ = 9.7, $^{4}J_{H,F}$ = 5.2 Hz) ppm. ^{31}P NMR (202 MHz, [D$_6$]DMSO): δ = −9.57, −9.72 ppm. HRMS (FAB) calcd. for $C_{17}H_{16}FN_2O_7P$ [MH$^+$] 411.0757, found 411.0767. HPLC: t_R = 13.7 min.

3,5-Di-(*tert*-Butyl)-6-fluoro-*cyclo*Sal-d4T Monophosphate (2i): The crude chlorophosphite was synthesized from **4i** (1.01 g, 3.97 mmol), phosphorous(III) chloride (0.66 g, 4.8 mmol), and pyridine (0.73 g, 9.2 mmol) in diethyl ether (30 mL) as described in the General Procedure (yield: 1.11 g). Triester **2i** was synthesized from d4T (**1**, 120 mg, 0.535 mmol), diisopropylethylamine (140 mg, 1.08 mmol), chlorophosphite (340 mg, 1.08 mmol), and *tert*-butyl hydroperoxide (5.5 M solution in *n*-decane, 0.33 mL, 1.8 mmol) in acetonitrile (12 mL). The product was obtained as a colorless foam: Yield 59 % (164 mg), mixture of two diastereomers, ratio 0.8:1.0. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 1.32 (s, 1 × 9 H, 1 × CH_3-*t*Bu), 1.34 (s, 2 × 9 H, 2 × CH_3-*t*Bu), 1.35 (s, 1 × 9 H, 1 × CH_3-*t*Bu), 1.61 (s, 2 × 3 H, 2 × CH_3-thymine), 4.35−4.37 (m, 2 × 2 H, 2 × H5′),

4.98−5.02 (m, 2 × 1 H, 2 × H4′), 5.41 (d $^2J_{H,H}$ = 15.1 Hz, 1 × 1 H, 1 × H-benzyl), 5.47 (d, $^2J_{H,H}$ = 13.9 Hz, 1 × 1 Hz, 1 × H-benzyl), 5.53 (dd, $^2J_{H,H}$ = 15.1, $^3J_{H,P}$ = 7.6 Hz, 1 × 1 H, 1 × H-benzyl), 5.56 (dd, $^2J_{H,H}$ = 13.9, $^3J_{H,P}$ = 8.2 Hz, 1 × 1 H, 1 × H-benzyl), 6.03−6.08 (m, 2 × 1 H, 2 × H2′), 6.42−6.45 (m, 2 × 1 H, 2 × H3′), 6.81−6.85 (m, 2 × 1 H, 2 × H1′), 7.22 (s, 1 × 1 H, 1 × H6-thymine), 7.23 (q, $^4J_{H,H}$ = 1.3 Hz, 1 × 1 H, 1 × H6-thymine), 7.26 (d, $^4J_{H,F}$ = 9.5 Hz, 2 × 1 H, 2 × H4-aryl), 11.36 (s, 1 × 1 H, 1 × NH), 11.37 (s, 1 × 1 H, 1 × NH) ppm. ^{13}C NMR (101 MHz, [D$_6$]DMSO): δ = 12.07 (2 × CH_3-thymine), 29.83 (1 × CH_3-*t*Bu), 29.86 (1 × CH_3-*t*Bu), 29.99 (d, $^4J_{C,F}$ = 3.1 Hz, 2 × CH_3-*t*Bu), 34.30 (d, $^3J_{C,F}$ = 3.1 Hz, 2 × C-*t*Bu), 34.62 (1 × C-*t*Bu), 34.67 (1 × C-*t*Bu), 63.86 (2 × C-benzyl), 69.34 (d, $^3J_{C,P}$ = 6.1 Hz, 1 × C5′), 69.45 (d, $^3J_{C,P}$ = 6.1 Hz, 1 × C5′), 84.44 (d, $^3J_{C,P}$ = 3.0 Hz, 1 × C4′), 84.51 (d, $^3J_{C,P}$ = 3.0 Hz, 1 × C4′), 89.49 (1 × C1′), 89.66 (1 × C1′), 109.98 (1 × C5-thymine), 110.07 (1 × C5-thymine), 111.27 (dd, $^2J_{C,F}$ = 9.2, $^3J_{C,P}$ = 4.1 Hz, 1 × C1-aryl), 111.47 (dd, $^2J_{C,F}$ = 9.2, $^3J_{C,P}$ = 4.1 Hz, 1 × C1-aryl), 125.21 (d, $^3J_{C,F}$ = 7.1 Hz, 2 × C4-aryl), 127.71 (1 × C2′), 127.75 (1 × C2′), 131.57 (1 × C3-aryl), 131.67 (d, $^4J_{C,F}$ = 4.1 Hz, 1 × C3-aryl), 133.08 (1 × C3′), 133.16 (1 × C3′), 133.70 (2 × C5-aryl), 136.05 (2 × C6-thymine), 147.32 (2 × C2-aryl), 151.08 (2 × C2-thymine), 155.11 (d, $^1J_{C,F}$ = 248.2 Hz, 2 × C6-aryl), 164.05 (1 × C4-thymine), 164.10 (1 × C4-thymine) ppm. ^{19}F NMR (471 MHz, [D$_6$]DMSO): δ = −117.37 (d, $^4J_{H,F}$ = 9.5 Hz), −117.50 (d, $^4J_{H,F}$ = 9.5 Hz) ppm. ^{31}P NMR (202 MHz, [D$_6$]DMSO): δ = −8.71, −9.07 ppm. HRMS (FAB) calcd. for $C_{26}H_{32}FN_2O_7P$ [MH$^+$] 523.2009, found 523.2092. HPLC: t_R = 20.2 min.

6-Fluorosalicyl Alcohol (4h): 6-Fluorosalicylic acid (0.55 g, 3.5 mmol) was added dropwise to a suspension of $LiAlH_4$ (0.27 g, 7.0 mmol) in THF (20 mL) over 30 min. The reaction mixture was stirred at room temperature for 2 h, then heated under reflux for 1 h. The reaction was quenched with 2 N HCl, and the product was extracted with diethyl ether. The organic layer was washed twice with water and dried with $MgSO_4$, and the solvent was removed under reduced pressure. Purification of the residue by chromatography on silica gel with a gradient of methanol in dichloromethane yielded 0.43 g (86 %) of the title compound as a colorless solid. R_f = 0.52 (dichloromethane/methanol, 9:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.48 (s, 1 H, benzyl-OH), 4.99 (s, 2 H, H-benzyl), 6.59 (ddd, $^3J_{H,H}$ = 9.5, $^3J_{H,H}$ = 8.3, $^4J_{H,F}$ = 1.1 Hz, 1 H, H4), 6.66 (d, $^3J_{H,H}$ = 8.3 Hz, 1 H, H3), 7.12 (ddd, $^3J_{H,H}$ = 8.3, $^3J_{H,F}$ = 8.3, $^4J_{H,H}$ = 6.6 Hz, 1 H, H5), 7.83 (br., 1 H, phenol-OH) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ = 51.46 (d, $^3J_{C,F}$ = 5.1 Hz, C-benzyl), 105.49 (d, $^2J_{C,F}$ = 22.4 Hz, C5), 111.29 (d, $^4J_{C,F}$ = 2.0 Hz, C3), 115.40 (d, $^3J_{C,F}$ = 18.3 Hz, C4), 128.95 (d, $^2J_{C,F}$ = 11.2 Hz, C1), 157.27 (d, $^3J_{C,F}$ = 8.1 Hz, C2), 162.15 (d, $^1J_{C,F}$ = 243.1 Hz, C6) ppm. ^{19}F NMR (471 MHz, $CDCl_3$): δ = −119.45 (dd, $^3J_{H,F}$ = 10.0, $^4J_{H,F}$ = 10.0 Hz) ppm. MS (EI) calcd. for $C_7H_7FO_2$ M = 142, found *m*/*z* = 142 (M$^+$, 54 %), 124 (76), 96 (100), 77 (5), 51 (5).

4,6-Di-(*tert*-Butyl)-3-fluorophenol (7): Isobutene gas was bubbled through 3-fluorophenol (**6**) (4.10 g, 36.6 mmol) for 5 min at 40 °C. Afterwards, concd. sulfuric acid (0.34 g, 3.5 mmol) was added and the inflow of isobutene was continued for 1 h. After the addition of water, dichloromethane was added and the phases were separated. The organic phase was neutralized with satd. sodium hydrogencarbonate solution and dried with Na_2SO_4. The solvent was removed in vacuo and the resulting residue was purified by column chromatography (petroleum ether/dichloromethane, 4:1), yielding 5.73 g (70 %) of the title compound as a colorless solid. R_f = 0.14 (petroleum ether/dichloromethane, 4:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.37 (s, 9 H, CH_3-*t*Bu), 1.42 (s, 9 H, CH_3-*t*Bu), 4.76 (s, 1 H, phenol-OH), 6.39 (d, $^3J_{H,F}$ = 13.0 Hz, 1 H, H2), 7.19 (d, $^4J_{H,F}$ = 9.7 Hz, 1 H, H5) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ = 29.78 (CH_3-*t*Bu), 30.19 (d, $^4J_{C,F}$ = 3.1 Hz, CH_3-*t*Bu), 33.88 (d, $^3J_{C,F}$ = 3.1 Hz, C-*t*Bu), 34.39 (C-*t*Bu), 104.90 (d, $^2J_{C,F}$ = 27.0 Hz, C2), 125.39 (d, $^3J_{C,F}$ = 7.1 Hz, C5), 128.08 (d, $^2J_{C,F}$ = 11.2 Hz, C4), 130.83 (d, $^4J_{C,F}$ = 3.6 Hz, C6), 152.72 (d, $^3J_{C,F}$ = 10.2 Hz, C1), 159.86 (d, $^1J_{C,F}$ = 246.7 Hz, C3) ppm. ^{19}F NMR (471 MHz, $CDCl_3$): δ = −113.34 (dd, $^3J_{H,F}$ = 13.0, $^4J_{H,F}$ = 9.7 Hz) ppm. HRMS (FAB) calcd. for $C_{14}H_{21}FO$ (M) 224.1576, found 224.1559.

3,5-Di-(*tert*-Butyl)-6-fluorosalicyl Alcohol (4i): Compound **7** (5.55 g, 24.7 mmol) and sodium hydroxide (1.18 g, 27.5 mmol) were dissolved in methanol (15 mL). Aqueous formaldehyde solution (37 %, 5.9 mL, 79 mmol formaldehyde) was added to this solution, and the resulting mixture was stirred at room temperature for 3 days. The reaction was monitored by TLC (dichloromethane/methanol, 9:1) and finally quenched by the addition of water and contd. hydrochloric acid (resulting pH 4−5). The aqueous mixture was extracted five times with dichloromethane. The combined organic layers were dried with Na_2SO_4 and the solvent was removed in vacuo. The resulting crude product was recrystallized from petroleum ether to yield 4.49 g (71 %) of the title compound as a colorless solid. R_f = 0.74 (dichloromethane/methanol, 9:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.34 (d, $^4J_{H,F}$ = 1.0 Hz, 9 H, CH_3-*t*Bu), 1.41 (s, 9 H, CH_3-*t*Bu), 5.02 (s, 2 H, H-benzyl), 7.14 (d, $^4J_{H,F}$ = 9.9 Hz, 1 H, H4), 7.92 (s, 1 H, phenol-OH) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ = 29.95 (CH_3-*t*Bu), 30.48 (d, $^4J_{C,F}$ = 3.1 Hz, CH_3-*t*Bu), 34.28 (d, $^3J_{C,F}$ = 3.1 Hz, C-*t*Bu), 34.95 (C-*t*Bu), 58.13 (d, $^3J_{C,F}$ = 10.2 Hz, C-benzyl), 112.89 (d, $^2J_{C,F}$ = 17.3 Hz, C1), 124.64 (d, $^3J_{C,F}$ = 8.1 Hz, C4), 126.94 (d, $^2J_{C,F}$ = 12.2 Hz, C5), 131.88 (d, $^4J_{C,F}$ = 3.1 Hz, C3), 154.67 (d, $^3J_{C,F}$ = 5.1 Hz, C2), 156.86 (d, $^1J_{C,F}$ = 252.2 Hz, C6) ppm. ^{19}F NMR (471 MHz, $CDCl_3$): δ = −119.74 (d, $^4J_{H,F}$ = 9.9 Hz) ppm. HRMS (FAB) calcd. for $C_{15}H_{23}FO_2$ (M) 254.1682, found 254.1681.

Hydrolysis Studies on the *cyclo*Sal Phosphate Triesters: Hydrolysis studies of *cyclo*Sal nucleotides (phosphate buffer, pH 7.3) by HPLC analysis have been described previously.[13]

^{31}P NMR Hydrolysis Studies on the *cyclo*Sal Phosphate Triesters: ^{31}P NMR hydrolysis studies of *cyclo*Sal nucleotides **2a**−**g** have been described previously.[13]

Cholinesterase Assay: For the modified cholinesterase assay used for inhibition studies of human BChE (serum) see ref.[11b].

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