Published in final edited form as:

J Med Chem. 2012 March 22; 55(6): 2649–2671. doi:10.1021/jm201482p.

Radiolabeled *cyclo*Saligenyl Monophosphates of 5-Iodo-2'-deoxyuridine, 5-Iodo-3'-fluoro-2', 3'-dideoxyuridine, and 3'-Fluorothymidine for Molecular Radiotherapy of Cancer: Synthesis and Biological Evaluation

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Abstract

Targeted molecular radiotherapy opens unprecedented opportunities to eradicate cancer cells with minimal irradiation of normal tissues. Described in this study are radioactive *cyclo*Saligenyl monophosphates designed to deliver lethal doses of radiation to cancer cells. These compounds can be radiolabeled with SPECT- and PET-compatible radionuclides as well as radionuclides suitable for Auger electron therapies. This characteristic provides an avenue for the personalized and comprehensive treatment strategy that comprises diagnostic imaging to identify sites of disease, followed by the targeted molecular radiotherapy based on the imaging results. The developed radiosynthetic methods produce no-carrier-added products with high radiochemical yield and purity. The interaction of these compounds with their target, butyrylcholinesterase, depends on the stereochemistry around the P atom. IC₅₀ values are in the nM range. *In vitro* studies indicate that radiation doses delivered to the cell nucleus are sufficient to kill cells of several difficult to treat malignancies including glioblastoma, and ovarian and colorectal cancers.

INTRODUCTION

Biological consequences of the Auger effect are confined to the proximity of the molecular site of the radionuclide decay. For this reason, the cytotoxicity of ¹²⁵I depends on the subcellular localization of this radionuclide^{1–3}. Low energy electrons from ¹²⁵I decaying in DNA produce non-repairable strand breaks and lesions^{4,5} analogous to those produced by radiations with a high linear energy transfer (LET). Approximately 90% of these DNA breaks are located within 10 nm of the ¹²⁵I decay site. Each decay of the DNA-incorporated ¹²⁵I produces several single strand breaks and a double strand break with a probability of almost one^{6–9}. 5-[¹²⁵I]Iodo-2'-deoxyuridine (¹²⁵IUdR), a thymidine analog, which is incorporated into DNA during the S phase of mitotic cells, is ~1.6 times more effective in killing mammalian cells than 5.3 MeV α particles from intracellularly localized ²¹⁰Po-citrate^{10,11}. Not surprisingly, significant research efforts are dedicated to the development of various carriers of ¹²⁵I with the goal of targeting ¹²⁵I to the cell nucleus. Investigated reagents include pyrimidines^{12–18}, DNA intercalators^{19–21}, antibodies^{22–27}, steroids^{28–30}, chemotherapeutic drugs^{31,32}, as well as peptides³³ and other reagents^{34–37}. Of

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Supporting Information Available. Preparation and analytical details for **9**, **9a**, **9b**, **10**, **10a**, **10b**, **11**, **11a**, **11b**, **20**, and **21**, HPLC analyses and conditions, stability and hydrolysis studies, cancer cell interactions with human BChE.

these, $^{125}\text{IUdR}$, which is incorporated into the DNA of proliferating cells *in vitro*³⁸ and *in vivo*³⁹, is the most effective in the production of unreparable DNA strand breaks and for this reason, it is the most radiotoxic^{40–43}.

An important factor limiting the effectiveness of $^{125}\text{IUdR}$ in the molecular radiotherapy of cancer is its short metabolic half-life in circulation. Intravenously administered $^{125}\text{IUdR}$ is destroyed within a few min after the injection. As a result, the tumor uptake is insufficient to kill cancer cells. Only cells that happen to make DNA when $^{125}\text{IUdR}$ is present can utilize this compound in place of thymidine. In view of proliferative heterogeneity of the cancer cell population, it is not surprising that even after intratumor injections only ~0.02% of the injected $^{125}\text{IUdR}$ is associated with one gram of tumor (range 0.001–0.06% ID/g)⁴⁴. Various approaches to circumvent this problem were tested, for example, local or regional administration^{43,45,46}, preparation of prodrugs^{47,48} and protein conjugates^{49,50}, as well as the co-administration of various inhibitors and antagonists to block the dehalogenation pathways^{51–54}. Recently, a targeted version of $^{125}\text{IUdR}$ and its monophosphate was synthesized and successfully evaluated as a molecular radiotherapeutic in experimental models of androgen receptor-expressing cancers such as ovarian and prostate⁵⁵.

Compounds reported herein expand the range of targetable derivatives of $^{125}\text{IUdR}$. In addition to binding butyrylcholinesterase (BChE), a tumor-associated target, these reagents have 5'-*cycloSaligenyl* monophosphate residues, which allow their intracellular retention and sustained availability to participate in the DNA synthesis, resulting in high levels of ^{125}I incorporation into the DNA. The biological behavior of this series of targetable $^{125}\text{IUdR}$ derivatives was tested in cell lines derived from difficult to treat human cancers including glioblastoma, ovarian, and colorectal cancers, which express high levels of BChE.

RESULTS AND DISCUSSION

Synthesis

Evaluated in this study *cycloSaligenyl* (*cycloSal*) monophosphates of $^{125}\text{IUdR}$, and 5-[^{125}I]iodo-3'-fluoro-2',3'-dideoxyuridine (**21b**) were synthesized in three consecutive steps as shown in Schemes 1 and 3. The non-radioactive iodo-analogs **6** – **14**, **21** and **24** were prepared first and subsequently reacted with hexamethylditin, to afford the corresponding 5-trimethylstannyl *cycloSal*-derivatives **6a** – **14a**, **21a** and **24a**. These organotin precursors furnished the target ^{125}I -iodinated *cycloSal*-phosphotriesters **6b** – **14b**, **21b** and **24b** in the course of the electrophilic iododestannylation performed in a third phase of the synthesis. All ^{125}I -radiolabelings were conducted at the non-carrier-added concentration level, i.e., using ^{125}I with specific activity of ~80,475 GBq/mmol.

Most of the time, *cycloSal*-monophosphates are attained through the phosphorus(III) route⁵⁶ and a general course of the preparation was established by the Meier's group⁵⁷. We followed this strategy in the first part of the synthesis introducing the *cycloSal*-segment by means of chlorophosphites. Thus, unprotected IUdR (**1**) along with 5-iodo-3'-*O*-levulinyl-2'-deoxyuridine (**4**) and 5-iodo-5'-*O*-trityl-2'-deoxyuridine (**5**) were coupled with cyclic chlorophosphites **15** – **17** in the presence of *N,N*-diisopropylethylamine⁵⁸ (Scheme 2) and generated corresponding phosphites were directly oxidized with *tert*-butylhydroperoxide, to give the expected diastereomeric mixtures of the *cycloSaligenyl* products. The same preparation scheme was used to introduce the *cycloSal*-moiety into thymidine, 3'-fluoro-3'-deoxythymidine (**20**) and 5-iodo-3'-fluoro-2',3'-dideoxyuridine (**21**), and to prepare *cycloSaligenyl* monophosphates **22**, **23** and **24** (Scheme 3). Direct phosphorylation of **1**, performed without protecting the 3'- or 5'-OH groups, gave access to the 5'-*O*- and 3'-*O*-*cycloSal*-5- $^{125}\text{IUdR}$ monophosphates, permitting the concurrent biological evaluation of both groups of regioisomers. Typically, when phosphorylation of **1** by chlorophosphite **15** –

17 (1.05 – 1.25 molar equivalent) was carried out at temperatures below -40°C , the mixture of 5'-*O*- and 3'-*O*-*cycloSal*igenyl phosphotriesters was formed, with only marginal regioselectivity. The 3',5'-*O,O*-diphosphorylation occurred in the range of 16– 22%. The separation of regioisomers, achieved without difficulty by flash column chromatography on a silica gel, furnished pure diastereomeric 5'-*O*-, 3'-*O*- and 3',5'-*O,O-cycloSal* products in a fair overall yield of 39% – 46%. The 5'-*O*-phosphitylation of **4**, along with the “one-pot” oxidation followed by the deprotection of 3'-levulinate group⁵⁹, led to the corresponding phosphotriesters **6** – **8** in 52 – 62% yield. Phosphitylation of 5-iodo-5'-*O*-trityl-2'-deoxyuridine (**5**) provided the expected products **12** – **14** in the overall yield of ~50%. A similar reaction applied to 5-iodo-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyuridine (**2**) failed, due to the apparent spontaneous cleavage of the DMTr group during a very slow phosphitylation step, presumably caused by the induced large steric hindrance. Phosphitylation of IUdR protected at the 3'- or 5'-position did not improve overall efficiency of the preparation, because of the necessary protection and deprotection steps, but the individual regioisomers synthesized in this way simplified the identification of the products originated from the unprotected **1**. The initial parameters used in the selection of compounds with 3-methyl*cycloSal*-, 3,5-di-(*tert*-butyl)-6-fluoro*cycloSal*- and unsubstituted *cycloSal*-ring systems were based on previous reports of similarly substituted 5'-*O-cycloSal* derivatives of 2',3'-dideoxy-2',3'-didehydrothymidine⁵⁷, showing a broad range of lipophilicity and rates of hydrolysis in aqueous buffer at physiological pH 7.3.

The organotin precursors **6a** – **14a**, **21a** and **24a** were prepared in reactions with hexamethylditin (except **6a**, which was a tri-*n*-butyl derivative) catalyzed by bis(triphenylphosphine)palladium(II) dichloride. Stannylation was carried out under nitrogen in boiling dioxane or ethyl acetate, depending on the solubility of starting iodotriester. In ethyl acetate, the dehalogenation was reduced from ~20% observed at higher temperatures, to less than 10% at 60°C . Two major products were always present. The first product, with higher mobility on TLC and proven to be the trimethylstannyl derivative, was isolated in 52% – 77% yield. The second, with lower TLC mobility, was identified as a deiodinated starting phosphotriester. All synthesized *cycloSal*-5-trimethylstannyl-2'-deoxyuridine phosphotriesters were readily amenable to no-carrier-added radioiododestannylation and excellent isolated yields of the ^{125}I -iodolabeled compounds were always achieved. Higher hydrophobicity of stannanes, as compared to the corresponding iodo-derivatives, allowed for a complete separation of the excess of trimethyltin precursors from the ^{125}I -iodinated products, even when a large volume of the crude reaction mixture (up to 1 mL) was loaded onto the HPLC column during the final purification.

We have also examined the possibility of the direct chlorophosphite coupling with radioiodinated **21b** (Scheme 3). This approach would permit a straightforward, one-step synthesis of radiolabeled *cycloSal*igenyl monophosphates derived from the already radiolabeled deoxyuridine or thymidine derivatives and could be particularly useful in the preparation of ^{18}F -containing *cycloSal*igenyl phosphotriesters. To evaluate this option, we first prepared 5- ^{125}I iodo-3'-fluoro-2',3'-dideoxyuridine (**21b**) and 5- ^{125}I iodo-5'-*O*-[*cyclo*-3,5-Di-(*tert*-butyl)-6-fluoro*Sal*igenyl]-3'-fluoro-2',3'-dideoxyuridine (**24b**) by ^{125}I -iododestannylation of the corresponding stannanes **21a** and **24a**. Subsequently, compound **21b**, which closely mimics physicochemical properties of 3'- ^{18}F fluoro-3'-deoxythymidine (^{18}FLT), was used as a surrogate of ^{18}FLT in the coupling with **17**, whereas **24b** served as the analytical standard to monitor the reaction progress and to compare products appearing in both methods. The major product isolated after the coupling of **17** with **21b** was indeed **24b** and was identical (HPLC co-injections) with **24b** previously obtained by the iododestannylation of stannane **24a**. However, the acceptable radiochemical yields (40% – 47%, reproduced in five preparations) were achieved only if (*I*) the crude *saligenyl N,N*-

diisopropylaminophosphoramidite (**18**) (Scheme 2) was initially generated by the treatment of **17** with *N,N*-diisopropylamine; and (2) the phosphorylation was carried out in the presence of 1*H*-tetrazole. Even so, these results indicate that the coupling of cyclic chlorophosphites can be effectively performed at the non-carrier-added concentrations and applied to preparations of *cycloSaligenyl*-¹⁸FLT derivatives and other radiolabeled *cycloSal*-analogs.

All ¹²⁵I-iodinations were conducted within a range of 9.25 – 444 MBq, using ~120 µg of the stannyl forerunner. The reaction mixtures were acidified with TFA in acetonitrile and hydrogen peroxide was used to oxidize sodium ¹²⁵I-iodide. The modified procedure for radio-iododestannylation, developed earlier in these laboratories for the synthesis of ¹²⁵IUdR⁶⁰, gave consistently excellent radiochemical yields of 85% – 93% and the radiochemical purity of ≥95%, across the series of *cycloSal*-phosphotriesters. Proton destannylated byproduct (3 – 8%) found in all of the crude reaction mixtures, originated mostly from the frozen tin precursor samples and was slowly increasing after the extended storage of stannanes (often exceeding six months).

To confirm the radiochemical purity and accurately measure the specific activity of labeled products, the reaction mixtures were purified on the HPLC system, with the concurrent monitoring of the radioactivity and absorbance ($\lambda = 220/280$ nm). Although the HPLC analysis performed within 24 h generally indicated products with ≥95% radiochemical purity, all radiolabeled compounds kept in a solution of aqueous acetonitrile overnight, usually at concentrations of ~37 kBq/µL, were routinely purified one more time, shortly before the intended experiments. HPLC co-injections of the ¹²⁵I-radiolabeled *cycloSal*-products and the corresponding non-radioactive iodo-analogs corroborated the identity of radioiodinated compounds. Characterization of all non-radioactive phosphotriesters was carried out by means of ¹H, ¹³C, ¹¹⁹Sn and ³¹P NMR, as well as high-resolution mass spectroscopy and thorough the HPLC analyses.

Resolution of diastereomers

The synthesis of 5'-*O*- and 3'-*O*-*cycloSaligenyl*-phosphotriesters was not stereoselective, therefore, all products were obtained as mixtures of diastereomers (*S_p* and *R_p* configurations, at approximately 1:1 ratio) at each phosphorus center. To differentiate isomers in this study, diastereomers are labeled as the *-fast* and the *-slow* in correlation to the HPLC retention time (*t_R*) of each isomer. Only tentative assignment of the *R_p*/*S_p* stereochemistry at the phosphorus atom could be made, following the already known absolute stereochemical configuration of 5-formyl-3-*tert*-butyl-*cycloSaligenyl*-2',3'-dideoxy-2',3'-didehydrothymidine monophosphate as the reference⁶¹. Thus, it could be assumed that *-fast* diastereomers should have *S_p*, and *-slow* isomers *R_p* configuration, although the unambiguous confirmation of this assignment requires further investigation.

Diastereomers of all synthesized 5'-*O*-*cycloSal*-phosphotriesters: **6** – **8** and **24** (non-radioactive compounds) and also **6b** – **8b**, **24b** (¹²⁵I-radioiodolabeled products); as well as diastereomers of all ¹²⁵I-radioiodolabeled 3'-*O*-*cycloSal*-phosphotriesters **12b** – **14b** can be separated by the reverse phase HPLC. Because of their close elution profiles, the separation of diastereomeric stannanes **6a** – **8a**, **24a** and particularly **12a** – **14a** was demanding and required numerous injections onto a tandem of two reverse phase HPLC columns. The amount of a single isolated diastereomer usually did not exceed a total of 30 mg. However, this amount was sufficient to complete all essential analyses and to prepare adequate quantities of each highly purified diastereomer of the tin precursor **6a** – **8a** (~120 µg each) suitable for the immediate radioiododestannylation. ¹²⁵I-radioiodinated diastereomers were separated in one of two ways: (1) by conducting ¹²⁵I-iododestannylation with a single diastereomer of the trimethylstannyl precursor (radioiodinated diastereomers of *cycloSal*-

triesters were repeatedly obtained in quantities of up to ~450 MBq using this method); or (2) by using a diastereomeric mixture of stannane and the separation of isomers that followed the final purification of the ^{125}I -radioiodolabeled product. In this case, however, the separation often involved multiple injections and was limited by the largest amount of the diastereomeric mixture, which could be fully resolved in a single HPLC run. Therefore, when a larger batch of the ^{125}I -labeled 5'-*O*- or 3'-*O*-cycloSal-triester diastereomer was required (185 – 370 MBq), a method employing resolved diastereomers of the trimethyltin precursor was preferred.

All 3',5'-*O*,*O*-di-cycloSal-phosphotriesters **9** – **11** with two stereogenic centers formed during the synthesis were expected to exist as mixtures of four stereoisomers, with configurations: *S_P/S_P*, *R_P/S_P*, *S_P/R_P* and *R_P/R_P*, all at the ratio of 1:1. Indeed, the HPLC and ^{31}P NMR analyses of 3',5'-disubstituted iodides **9** – **11** and stannanes **9a** – **11a** indicated mixtures of four diastereomers. These, however, were inseparable and all of the applied HPLC methods led only to a partial partition. Consequently, the ^{125}I -iodolabeled 3',5'-*O*,*O*-di-cycloSal-phosphotriesters **9b** – **11b** were isolated and purified only as mixtures of diastereomers.

Hydrolysis Pathways and Stability

In the experimentally established *cycloSal*-pronucleotide concept⁶², the *cycloSal* moiety is cleaved due to the nucleophilic attack of the hydroxide anion on the phosphorus atom and the formation of the resonance stabilized phenolate. The lower stability of the phenyl as compared to the benzyl phosphate ester moiety and activation of the phosphotriester molecule induces spontaneous cleavage of the remaining benzyl phosphate group and generates the nucleotide⁶³. In this series of *cycloSal* monophosphates however, a second hydroxyl group is present in the glycone and the potential intramolecular attack at a phosphorus center could cause the formation of the undesired 3',5'-cyclic products. Such a transformation has been observed for 3-methyl-*cycloSal*igenyl penciclovir monophosphate⁶⁴. Therefore, it was essential to examine the hydrolysis of synthesized *cycloSal*-triesters and establish whether the hydrolysis terminates with the desired monophosphates. Diastereomers of compounds **6** – **8**, **12** – **14** and **24**, as well as their radiolabeled analogs **6b** – **8b**, **12b** – **14b** and **24b**, were hydrolyzed in phosphate buffer (50 mM, pH 7.3 at 37°C) to examine the chemical stability of triesters under conditions comparable to the physiological environment (Supporting information pp. S93-S105). The release of 5- $^{127/125}\text{I}$ iodo-2'-deoxyuridine monophosphate was monitored by the HPLC and verified in the ^{31}P NMR experiments. Half-lives ($t_{1/2}$) were determined by the integration of the decreasing phosphotriesters peaks areas in chromatograms (Table 1). The hydrolytic degradations followed the pseudo-first-order kinetics and generated monophosphate of IUdR and salicyl alcohols, confirmed at the end of hydrolysis by means of co-injections of the independently prepared standard of 5- $^{127/125}\text{I}$ iodo-2'-deoxyuridine monophosphate⁵⁵ and the corresponding salicyl alcohol. Diastereomers did not hydrolyze at the same rate. The *fast* diastereomers hydrolyzed faster with the ratio of $t_{1/2}$ *slow* to $t_{1/2}$ *fast* of 1.2 – 1.5, depending on the substitution of the *cycloSal*-phenyl ring (Table 1). Since IUdR was not detected among products of the hydrolysis, the dephosphorylation of the *cycloSal*-triesters was excluded. The hydrolysis pathway of **8** was also confirmed by ^{31}P NMR experiments conducted in imidazole/hydrochloric acid buffer solution (pH 7.2) at ambient temperature as previously described⁶⁵. Identification of the hydrolysis products was based on the observed chemical shifts and proton-coupled ^{31}P NMR experiments. The data obtained for **8-fast** and **8-slow** diastereomers show the intermediate formation of benzyl phosphate diester (a quintet in the proton-coupled and a singlet at 2.18 ppm in the decoupled mode) and subsequently at the end of hydrolysis, IUdR monophosphate as a main product (a singlet at 0.08 ppm in the decoupled mode). Only traces of phenyl phosphate diester, secondary to the spontaneous

benzyl C – O bond break, were detected during the hydrolysis of **7** and **13**, with no indication of the concurrent formation of 3',5'-cyclic products.

BChE Inhibitory Activity

The inhibition potency toward human BChE of 5'-*O*- and 3'-*O*-*cycloSal*igenyl-phosphotriesters was evaluated using a multi-well assay developed in our laboratories based on the previously described procedures⁶⁶, using purified human enzyme. The significant influence of *cycloSal*-phosphotriesters stereochemistry on inhibitory potency has been identified. Only one diastereomer in each pair strongly inhibits BChE by irreversible binding to the active site of the enzyme. A similar observation was reported for other *cycloSal* triesters⁶⁷. In this study, a comparison of the half-maximal inhibitory concentrations (IC₅₀) of the tested individual *fast* and *slow* diastereomers showed that only *slow* isomers are strong inhibitors of BChE (Table 2). In contrast, *fast* diastereomers have shown weak or no inhibition of BChE. The observed BChE inhibition by diastereomeric mixtures originates almost exclusively from the *slow* isomers. For example, when human BChE was inhibited with the unresolved **8**, IC₅₀ was measured at 1,135 (43) nM, which is significantly lower compared to IC₅₀ >60 mM estimated for **8b-fast**. Under the same conditions, IC₅₀ of **8b-slow** is 50.1 (0.7) nM. Diastereomeric 3'-*O*-*cycloSal*-regioisomers do not inhibit BChE, e.g., IC₅₀ values for **14** were estimated at >1 M, and were about the same for **14-fast** and **14-slow**. Only when human BChE was incubated with **14-fast** and **14-slow** for 24 h, was some evidence observed of BChE inhibition for **14-slow** with the estimated IC₅₀ >3 mM.

Interactions of all radioactive compounds with human BChE were also evaluated using the denaturing, non-reducing (Figures 1A and 1B) and native (Figures 1C and 1D) gel electrophoresis as well as the HPLC assays (Supplementary Material pp. S106–S110). Autoradiographs of human BChE interactions with diastereomers of **6b** and **24b** are shown in Figures 1A and 1B. BChE (0.02 U) binds the *slow* diastereomers while there is no detectable binding of *fast* diastereomers. The native gel stained for BChE activity and autoradiographs shown in Figures 1C and 1D illustrate BChE activity-dependent binding of **8b-slow** and **24b-slow**.

Uptake kinetics

Each diastereomer has a distinctive time-dependent uptake in LS 174T and OVCAR-3 cancer cell lines. Both cell lines retain more of the *slow* diastereomer over time (Figure 2). More radioactivity is retained in OVCAR-3 cells compared to LS 174T cells presumably because BChE levels are higher in OVCAR-3 cells. The activity of BChE in LS 174T cells grown *in vitro* is 10.6 (0.3) U/mg total protein. OVCAR-3 cells have significantly higher levels of BChE activity 12.4 (0.6) U/mg total protein ($P = 0.017$). *In vitro* grown LS 174T and OVCAR-3 cells have doubling times of 16 h and ~40 h, respectively. The uptake profiles of **6b** and **7b** appear to have two stages. At earlier times, up to 90 min, the uptake of **6b-slow** is 6.5× and 4.6× faster than **6b-fast** in OVCAR-3 and LS 174T, respectively. In this initial phase, the radioactive content of the cells reflects the differences in BChE levels. After 90 min, the uptake ratios of **6b-slow** to **6b-fast** in both cell lines fall to ~1.7. The uptake in OVCAR-3 cells is still higher. However, the appearance of the hydrolysis products, which do not require BChE for the uptake and retention, cancels out a portion of the uptake differences. In the case of **7b-slow** and **7b-fast**, which have longer $t_{1/2}$ of hydrolysis, the uptake is linear for at least 3 h and then levels off with the ratios of *-slow-* to *-fast* uptake after 6 h of 3.5 and 3.0 in OVCAR-3 and LS 174T, respectively. The radioactivity associated with cells treated with **7b-slow** is 1.7× higher in OVCAR-3 compared to LS 174T reflecting differences in the BChE expression. Within the time frame of the experiment, the hydrolysis is not a factor in the uptake of **8b-slow** and **8b-fast**, which have $t_{1/2}$ of 2.5 days and 2 days, respectively (Table 1). Therefore, the disparity of uptake

between these diastereomers is likely in part a reflection of the active transport via the interactions with BChE. However, it appears that the hydrophobicity of **8b** facilitates passive diffusion through the cell membrane. The cell associated activity of **8b-fast** after 3 h incubation was >20× and >110× higher compared to **7b-fast** and **6b-fast**, respectively. Similarly, **8b-slow** accumulated at levels 35× and ~180× higher compared to **7b-slow** and **6b-slow**. The evaluation of **24b** was somewhat complicated by the low solubility of this compound in aqueous media. For this reason, all tests were conducted at a concentration of 18.5 kBq/mL or lower. The intracellular processing of **24b** does not parallel the metabolic fate of **6b**, **7b**, and **8b**, and for this reason, the cellular uptake is not proportional to the time of the cell exposure to the radioactive compounds. The subcellular fractionation studies described below illustrate and explain these differences. The observed differences between the *slow* and *fast* diastereomer uptake can be related directly to the BChE's activity associated with the cancer cell. Additional factors contributing to the retention of each compound appear to be the rate of hydrolysis and intracellular processing. This multifactorial dependence of the intracellular uptake and retention may be useful as a selection criterion for in vivo studies, e.g., reagents with faster rates of hydrolysis can be used in the treatment of xenografts with high proliferative rates whereas the treatment of xenografts with the lower turnover rates may benefit from reagents with prolonged intracellular retention.

Concentration-dependent uptake

The cell uptake is dependent on the extracellular concentration of the radioactive compound and it is saturable (Figures 3A and 3C). Cells were exposed to increasing concentrations of **6b-slow** and **6b-fast** for 3 h. ¹³¹IUDR added to the growth medium was used as an internal standard to verify that the cell proliferation rates were not influenced by the presence of the radioactive drugs (Figures 3B and 3D). The **6b-fast** uptake was lower compared to **6b-slow** in LS 174T and OVCAR-3 cells. B_{max} values were estimated in LS 174T cells at ~1,300 molecules/cell and ~6,800 molecules/cell for **6b-fast** and **6b-slow**, respectively. OVCAR-3 cells have higher amounts of BChE, as a result, B_{max} values were also higher at ~7,400 for **6b-fast** and >50,000 molecules/cell for **6b-slow**. The cell uptake of other compounds is also directly proportional to the extracellular concentration (Figure 4A) and time of exposure (Figure 4B).

Cell Survival and Subcellular Distribution Studies

U-87 MG glioblastoma and LS 174T colorectal adenocarcinoma cell lines, which produce colonies, were used to determine the effects of radioactive compound on the clonogenic survival, i.e., the reproductive integrity of treated cells. Surviving fractions were calculated as the ratio of the number of colonies derived from cells treated with the radioactive compound to the number of colonies derived from untreated control cells. In instances of zero or near zero clonogenic survival, the cell survival was also measured as the ratio of viable cells harvested at earlier times after treatment with radioactive drugs to the number of viable cells harvested from the control flasks treated with the vehicle only.

Glioblastoma is a good candidate for the clinical use of described reagents because proliferating cancer cells are surrounded by cells of normal brain, which are mostly quiescent. U-87 MG is an epithelial cell line derived from grade IV glioblastoma resected from the brain of a 44-year-old Caucasian female⁶⁸. U-87 cells grown *in vitro* express BChE activities at 11.1 (0.3) U/mg total protein. Treatment with **6b** *in vitro* produces significant U-87 cell killing after 24 h of exposure to this compound (Figure 5C). As anticipated, **6b** is taken up by U-87 cells and ¹²⁵I is incorporated into the DNA indicating that the intracellular processing of **6b** liberates the parent monophosphate, which then partakes in the DNA synthesis (Figures 5A and 5B). The subcellular distribution study indicates that the *slow*

diastereomer is retained within the cells and taken up into the DNA at levels approximately 50% – 90% higher compared to the *fast* isomer (Figures 5A and 5B). The cell killing is proportional to the amount of ^{125}I associated with DNA. The DNA retention of the radioactivity was also measured using the genomic tips method to confirm independently the presence of ^{125}I in DNA. The 40-h incubation of U-87 cells with 19.6 kBq/mL **6b-slow** accumulates 1.18 (0.11) mBq ^{125}I /cell in DNA. This amount of ^{125}I incorporated into the DNA of U-87 cells is well within the range of D_{37} values reported for other mammalian cells^{69,70}. For example, at 37% survival, the uptake of 0.13 mBq ^{125}I /cell and the cumulated mean lethal dose to the cell nucleus of ~80 rad was reported³.

As shown above for other cancer cell lines, the uptake by U-87 cells is also concentration-dependent (compare data in Figures 5A and 5B). The *slow* diastereomer is taken more avidly than the *fast* one. The number of cells surviving after the 24-h treatment with **6b-fast** and **6b-slow** is proportional to the extracellular concentration (Figures 5C and 6). Both compounds cause clonogenic cell death at concentrations as low as 18.5 kBq/mL (0.23 nM). In the clonogenic assay studies, U-87 cells treated with **6b-fast** or **6b-slow** failed to produce any colonies even after eight weeks in culture. Control cells treated with vehicle and cultured under identical conditions produced numerous colonies within three weeks of plating.

The radiotoxicity of the *slow* diastereomers in LS 174T cells is also greater compared to their *fast* counterparts (Figure 7). The radiotoxicity depends on the concentration, duration of the exposure to the compound, and the retention of the radioactivity. Only 60% and 38% of LS 174T cells incubated for 4 h with 114.5 kBq/mL **6b-fast** and **6b-slow**, respectively, produce colonies after 21 days in culture (Figure 7). The survival is further reduced when the extracellular concentration of the radioactive compound is increased. This is illustrated in Figure 7B for **7b-fast** and **7b-slow**. At the concentration of 185 kBq/mL, the surviving fraction of LS 174T cells treated with **7b-slow** falls to 0.63 (0.22) mBq. This clonogenic survival is proportional to the amount of ^{125}I retained within the nuclear DNA. LS 174T treated with **7b-fast** retained 0.196 (0.06) mBq ^{125}I /cell compared to cells treated with **7b-slow**, which retained 0.566 (0.333) mBq ^{125}I /cell.

The subcellular distribution of all ^{125}I -labeled reagents as a mixture of diastereomers was also analyzed in OVCAR-3 cells (Table 3). After 1 h incubation with 55.5 (0.04) kBq/mL ^{125}I -labeled reagents, the majority of the recovered radioactivity is associated with the cytoplasm. When these cells are given fresh nonradioactive medium and are allowed to continue to grow for additional 24 h, nearly the entire radioactivity is recovered with the nuclear DNA. Compound **8b** retained the highest amount of radioactivity, >230% and ~360% more than **6b** and **7b**, respectively.

The 3',5'-disubstituted derivatives had an excellent initial uptake and retention in the cytoplasm, however, this uptake did not translate into the ^{125}I levels in the DNA comparable to the amounts observed for **8b** presumably because both 3'- and 5'-hydroxyl groups were not available. Nevertheless, the DNA-associated ^{125}I was at the level of ~0.056 mBq/cell. The compound **14b**, a 3'-regioisomer of **8b**, had excellent cytoplasmic uptake at 1 h. Virtually the entire cytoplasmic content ended up in the DNA after 24 h incubation indicating that in cases where the targeted delivery may not be needed or the expression of BChE is low, the 3'-regioisomer can be as effective as the 5'-substituted derivative.

The subcellular distribution of **24b** (Figure 8) does not parallel any of the 3'-hydroxy-containing derivatives. Although IC_{50}s of **24** are comparable to IC_{50}s of **6**, the uptake and retention of both diastereomers of **24** is significantly lower. Only **24b-fast** after 1 h incubation shows ^{125}I retention in the cytoplasm comparable to other reagents. The uptake

and retention of **24b-slow** is ~20× lower in the cytoplasm, nucleus and DNA. Additionally, **24b-slow** appears to rapidly efflux from the intracellular compartments and at 24 h is practically undetectable in any of the intracellular compartments. This characteristic is clearly not compatible with therapeutic applications (Figure 9). However, a high affinity of **24b-slow** to its target, rapid clearance, and the ability to radiolabel **24b** with ^{18}F at the 3'-position are all promising features of a good imaging agent.

CONCLUSIONS

Methods developed in this study allow the synthesis of compounds with high specific activities at the no-carrier-added level. HPLC separation methods yield pure 5'-*cyclo*Sal and 3'-*cyclo*Sal diastereomers suitable for biological studies. Interactions of resolved diastereomers with BChE are distinct with the IC_{50} values in the nM range for all tested *slow* diastereomers and in the μM range or higher for the corresponding *fast* diastereomers. 3',5'-Disubstituted *cyclo*Sal derivatives cannot be resolved into the individual diastereomers with the available techniques. The wide-ranging hydrophobicity and rates of hydrolyses provide the opportunity to select reagents best suited to the proliferative rate, site of the disease, and the route of administration. Intracellular trapping and retention of the targeted *cyclo*Saligenyl derivatives of ^{125}I UDR lead to high levels of ^{125}I in the nuclear DNA in the heterogeneous cancer cell population, and produce extensive cell death. All developed reagents can be labeled with radionuclides suitable for imaging and molecular radiotherapy. Compounds **24b** are the exception; they have properties suited for ^{18}F or ^{124}I PET or ^{123}I SPECT imaging.

EXPERIMENTAL METHODS

Chemistry

Chemicals and reagents were purchased from commercial suppliers and used without further purification. Anhydrous diethyl ether was distilled from sodium wire with benzophenone as an indicator and dichloromethane was distilled from CaH_2 under nitrogen. $\text{Na}[^{125}\text{I}]\text{I}$ in 1×10^{-5} NaOH (pH 8–11) was obtained from PerkinElmer (Waltham, MA). Radioactivity was measured with Minaxi γ -counter (Packard, Waltham, MA) and a dose calibrator (Cap Intec Inc., Ramsey, NJ). Analytical TLC was carried out on precoated plastic plates, normal phase Merck 60 F₂₅₄ with a 0.2 mm layer of silica, and spots were visualized with either short wave UV or iodine vapors. Radioactive drugs on TLC and ITLC plates were analyzed on a Vista-100 plate reader (Radiomatic VISTA Model 100, Radiomatic Instruments & Chemical Co., Inc., Tampa, FL). Flush column chromatography was carried out using Merck silica gel 60 (40–60 μM) as stationary phase. Compounds were resolved and their purity confirmed by the HPLC analyses on Gilson (Middleton, WI) and ISCO (Lincoln, NE) systems using 5- μm , 250×4.6 mm, analytical columns, either Columbus C8 (Phenomenex, Torrance, CA) or ACE C18 (Advanced Chromatography Technologies, www.ace-hplc.com). Columns were protected by guard filters and were eluted at a rate of 0.8 mL/min with various gradients of CH_3CN (10–95%) in water with or without TFA (0.07%, w/v). Variable wavelength UV detectors UVIS-205 (Linear, Irvine, CA) and UV116 (Gilson) were used with the sodium iodide crystal Flow-count detector (Bioscan, Washington, DC) connected in-line at the outlet of the UV detector. Both signals were monitored and analyzed simultaneously. NMR spectra were recorded at ambient temperature in $(\text{CD}_3)_2\text{SO}$ or CDCl_3 with a Varian INOVA 500 MHz NMR instrument spectrometer (Palo Alto, CA). Chemical shifts are given as δ (ppm) relative to TMS as internal standard, with J in hertz. Deuterium exchange and decoupling experiments were performed in order to confirm proton assignments. ^{31}P NMR and ^{119}Sn NMR spectra were recorded with proton decoupling. High resolution (ESI-HR) positive ion mass spectra were acquired on an LTQ-Orbitrap mass spectrometer with electrospray ionization (ESI). Samples were dissolved in

70% methanol. Two μL aliquots were loaded into a 10- μL loop and injected with a 5 $\mu\text{L}/\text{min}$ flow of 70% acetonitrile, 0.1 % formic acid. FAB high-resolution (FAB-HR) mass spectra analyses (positive ion mode, 3-nitrobenzyl alcohol matrix) were performed by the Washington University Mass Spectrometry Resource (St. Louis, MO) and at the University of Nebraska Mass Spectrometry Center (Lincoln, NE).

All target nonradioactive compounds were found to be $\geq 97\%$ pure by the rigorous HPLC analysis, with the integration of a peak area (detected at 220 and/or 280 nm). Radioiodinated products were identified and later evaluated by means of the independently prepared non-radioactive reference compounds. The comparison of UV signals of the non-radioactive standards with the radioactive signals using radio-TLC (R_f) and radio-HPLC (t_R) analyses were employed. The 5-Iodo-3'-*O*-levulinyl-2'-deoxyuridine **4** and 3'-*O*-levulinylthymidine **19** were prepared starting from 5-iodo-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyuridine **2** and 5'-*O*-(4,4'-dimethoxytrityl)thymidine with 4-oxopentanoic acid, using the DCC/DMAP esterification method. The subsequent cleavage⁷¹ of DMTr group with ZrCl_4 in CH_3CN , led efficiently ($\sim 78\%$ yield) to uridines **4** and **19**. Thymidine and 5-iodo-2'-deoxyuridine were also used as starting compounds in the preparations of 3'-fluoro-deoxyuridines **20** and **21**. After the dimethoxytrityl protection of 5'-hydroxyl group, the configuration at the 3'-*O*-position was inverted by the mesylation/cyclization reaction sequence with methanesulfonyl chloride and 1,8-diazabicyclo[5.4.0]undecane (DBU), followed by the hydrolysis of the formed $O^2, O^{3'}$ -anhydrouridines, to give [5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxy- β -D-*threo*-pentofuranosyl]thymine and 5-iodo-[5'-*O*-(4,4'-dimethoxytrityl)-2',3'-dideoxy- β -D-*threo*-pentofuranosyl]uracile, without separation of the crude mesylates⁷². Epimerized uridines were subsequently fluorinated with DAST (*N,N'*-diethylaminosulfur trifluoride) at the 3'-*O*-position, and the 5'-*O*-DMTr groups removed with 1N HCl in acetonitrile. Analytical HPLC traces, separations of diastereomers and the related detailed HPLC analysis of all synthesized compounds **6** through **24** are provided in a supplement to this paper.

Statistical Analyses

The variables are expressed as average $\pm$ SD. Summary statistics were performed using a two-sided, unpaired Student's t-test with a significance level of $P=0.05$ using SigmaPlot/SigmaStat (Systat Software, Inc. Point Richmond, CA) and GraphPad InStat computer software (GraphPad Software, Inc., La Jolla, CA).

General Procedure A (Synthesis of Saligenyl Chlorophosphites)

To a solution of a dried salicyl alcohol derivative in Et_2O stirred at -16°C under a nitrogen atmosphere, newly distilled PCl_3 was added. After 15 min, while maintaining the same temperature, a solution of pyridine in Et_2O was added dropwise over a period of 1 h. The reaction mixture was allowed to reach ambient temperature and the stirring was continued for additional 2 h. To facilitate a complete separation of pyridinium chloride, the mixture was stored in a tightly covered reaction flask at -20°C overnight. After filtration under pressure of dry nitrogen, the solvent was evaporated in a vacuum and the resulting crude product was used in the next step of synthesis without delay. Initially crude products **15** and **16** were purified by short-path distillation under a high vacuum, giving both chlorophosphites as colorless unstable liquids, but no definite advantage has been noted in using the purified compound **15** or **16** during the synthesis of phosphotriesters with respect to achieved yields.

General Procedure B (Synthesis of Nonradioactive *cyclo*Saligenyl Phosphotriesters of 5-Iodo-2'-deoxyuridine **6** – **14**, Using Unprotected IUdR **1** and Chlorophosphites)

All reactions were performed under anhydrous conditions and a dry nitrogen or argon atmosphere. To a stirred solution of IUdR **1** and DIPEA (~ 2.5 molar excess) in DMF/THF

(2:1 mixture), cooled to or below -40°C , the THF solution of the appropriate crude chlorophosphite (1.05–1.25 molar equiv.) was added in small portions. Chlorophosphites (obtained using General Procedure A) were transferred directly from the original reaction vessel, by means of argon pressure and the syringe equipped with a long double needle. The reaction mixture was then slowly warmed to ambient temperature and further stirring continued for 30 min, to ensure completion of the reaction (TLC monitoring with DCM/MeOH 10:1.0 – 1.2 range). The reaction mixture was cooled to -40°C once again and a solution of *tert*-butyl hydroperoxide, 5 – 6 M (2.1 – 2.5 molar equiv.) in *n*-decane was added. The resulting mixture was slowly warmed to room temperature, with continued stirring for about 1 h (the reaction progress was followed by TLC). The solvent was evaporated under reduced pressure and the residue was treated with DCM. The precipitate of unreacted IUdR **1** was filtered off, washed with DCM, and dried under a high vacuum. The recovered IUdR was proven suitable for an immediate reuse. The filtrate was evaporated under reduced pressure and the residue purified by flash column chromatography on a silica gel, using a gradient of MeOH in DCM (0.7 – 1.0 : 10) and/or a gradient of MeOH in EtOAc (0.2 – 0.7 : 10), to yield each of three formed *cyclo*Saligenyl regioisomers: 5'-*O*-, 3'-*O*- and di-5',3'-*O*,*O*-substituted, separated. Diastereomers of 5'-*O*-*cyclo*Saligenyl-, as well as 3'-*O*-*cyclo*Saligenyl-phosphotriesters were later separated, by means of the HPLC, giving small quantities (~ 30 mg) of each individual isomer.

General Procedure C (Synthesis of Nonradioactive *cyclo*Saligenyl Phosphotriesters of 5-Iodo-2'-deoxyuridine **6 – **14**, Thymidines **22** – **23** and 5-Iodo-3'-dideoxy-3'-fluorouridine **24**, Using Protected IUdR **4** and **5** or Uridines **19** – **21**, and Chlorophosphites)**

Under an argon or dry nitrogen atmosphere, DIPEA and the crude saligenyl chlorophosphite, dissolved in MeCN, were added to a stirred solution of protected IUdR **4** or **5** in MeCN at -40°C . The reaction mixture was slowly warmed to ambient temperature and the reaction progress monitored by the TLC (DCM/MeOH 10 : 0.7). The reaction mixture cooled one more time to -40°C was treated with a solution of *tert*-butyl hydroperoxide 5 – 6 M (~ 2.5 molar equiv.) in *n*-decane. The mixture was warmed up slowly to room temperature and stirred for 1 – 2 h (the reaction progress was followed by TLC). The solvent was removed under reduced pressure, the residue treated with DCM (80 mL) or EtOAc (60 mL) and filtered. The filtrate was washed with the 0.3 % aqueous solution of NaHSO_3 (20 mL), brine (20 mL), dried over MgSO_4 and evaporated. Deprotection Procedures: Procedure 1: in reactions conducted with **4** the resulted solid was dissolved in pyridine (2 mL) and added to a stirred, cooled on an ice-water bath, solution of hydrazine hydrate (1.5 mL) in pyridine (3 mL), containing acetic acid (2.2 mL). The stirring continued for 5 min, and then water (40 mL) and EtOAc (50 mL) were added. The organic layer was separated and washed with the 10% aqueous solution of NaHCO_3 (20 mL), water (20 mL), dried over MgSO_4 and evaporated. Procedure 2: in reactions conducted with **5**, the crude solid was dissolved in MeCN (10 – 20 mL) and ZrCl_4 (1.2 – 1.6 molar equiv.) added. The mixture was stirred at room temperature for about 1 h (TLC monitoring). The solvent was evaporated in a vacuum, and the residue treated with EtOAc (50 mL) and water (50 mL). The organic layer was washed with brine, dried over MgSO_4 , the solvent evaporated, and the residue was purified on a silica gel column.

General Procedure D (Synthesis of Trialkyltin Precursors **6a – **14a**, **21a**, **24a**)**

The solution of appropriate iodouridine **6** – **14**, **21** or **24** (1.0 equiv), the hexamethylditin (hexa-*n*-butylditin was used in the preparation of **6a**) (1.25–1.50 equiv) and dichlorobis(triphenylphosphine) palladium(II) catalyst (0.10 equiv) in ethyl acetate or dioxane (for **6**, **7**, **12** and **13**) was refluxed (2 – 6 h) under a nitrogen atmosphere (until the starting material was detected). The reaction progress was monitored by TLC. Two major products were formed in all the reactions. The first product, with a higher TLC mobility,

isolated in 50 – 72% yield, was the trialkylstannyl derivative, and a second one (with a low TLC mobility) was a deiodinated starting compound. After cooling to ambient temperature a mixture was freed from an excess of the catalyst and partially purified by the filtration through a thin pad of silica (EtOAc/hexanes, 2:1). The resulting crude product was purified by repeating a silica gel column chromatography, using a gradient of EtOAc in hexanes (2 – 5 : 10) and/or a gradient of MeOH in DCM or CHCl₃ (0.4 – 0.7 : 10). Anhydrous samples of pure tin precursors (~ 120 µg) were stored up to eight months, with the exclusion of light, under nitrogen at –20°C; not showing the excessive decomposition (≤ 7% by the HPLC analysis) and were suitable for the immediate radio-iododestannylation. Diastereomers of 5'-*O*-*cyclo*Saligenyl-, as well as 3'-*O*-*cyclo*Saligenyl-5-trimethyltin-phosphotriesters were later separated, by the HPLC using a reverse phase columns, to give small quantities (~ 20 mg) of each individual diastereomer.

General Procedure E (Synthesis of [¹²⁵I]-Radioiodinated *cyclo*Saligenyl Phosphotriesters (6b – 14b, 21b and 24b))

Into a glass tube containing the appropriate tin precursor **6a** – **14a**, **21a** or **24a** (100 – 120 µg, 130 – 150 µmol) dissolved in MeCN (50 – 100 µL), a solution of Na¹²⁵I/NaOH (10 – 100 µL, 40 – 370 MBq) was added, followed by a 30% water solution of H₂O₂ (5 µL), and then a TFA solution (50 µL, 0.1 N in MeCN) with 2 min delay. The mixture was briefly vortexed and left for 15 min at room temperature. The reaction was quenched with Na₂S₂O₃ (100 µg in 100 µL of H₂O) and taken up into a syringe. The reaction tube was washed twice with 50 µL of H₂O/MeCN (9:1) solution. The previously withdrawn reaction mixture, plus washes were injected onto the HPLC system and separated, by means of C8 or C18 reverse phase column. The eluate from a column (1 mL fractions collected) was monitored using a radioactivity detector, connected to the outlet of UV detector (detection at 220 and 280 nm). Eluted fractions containing a product, combined and evaporated with a stream of dried nitrogen, were reconstituted in an appropriate solvent at concentrations required in subsequent experiments, and were filtered through a sterile (Millipore 0.22 µm) filter into a sterile evacuated vial. Identity of radiolabeled products was confirmed by the evaluation of UV signals of nonradioactive iodo-analogs (prepared independently, not through the iododestannylation reaction) with the radioactive signals, and/or by comparing *R_f* obtained from the radio-TLC and *t_R* from the radio-HPLC analysis. The specific activities were determined by the UV absorbance of radioactive peaks, as compared to the standard curves of unlabeled reference compounds. Radiolabeled products, if kept in a solution overnight at ambient temperature, were purified again before conducting further experiments, even though the performed HPLC analysis rarely indicated less than 95% of the radiochemical purity.

Nonradioactive *cyclo*Saligenyl Phosphotriesters

5-Iodo-5'-*O*-*cyclo*Saligenyl-2'-deoxyuridine Monophosphate (6)

Method I: General Procedure C with 3'-*O*-levulinyl IUdR **4** (1.22 g, 2.7 mmol), DIPEA (1.3 mL, 0.96 g, 7.44 mmol) and crude chlorophosphite **15** (950 mg, ~ 6 mmol), was conducted in 25 mL of MeCN and the oxidation carried out with a solution of *t*-BuOOH (0.86 mL, ≥ 4 mmol) after 40 min of phosphitylation. Before the deprotection of 3'-*O*-Lev group, a small amount of the crude product (19 mg) was purified by HPLC on Columbus C18, 100 Å (5 µm, 10 × 250 mm) column, eluted at 2.5 mL/min with 37% MeCN in water. The HPLC analysis showed two diastereomers, (47:53 ratio) *t_R* = 24.4 min, *t_R* = 24.8 min (≥ 98% pure, UV at 280 nm), ACE C18, 100 Å (5 µm, 4.6 × 250 mm) column; eluent: solvent A 10% MeCN in water, solvent B MeCN; eluted at 1.0 mL/min with a linear gradient of B from 0 – 95% over 45 min, and 95% B for 15 min. ¹H NMR (DMSO-*d*₆) δ = 11.39 (bs, 1H, NH), 8.11, 8.08 (2 s, 1H, uridine – H6), 7.26 – 7.19 (m, 1H, aryl – H4), 7.12 – 7.06 (m, 3H, aryl – H3, aryl – H5, aryl – H6), 6.08, 6.05 (2 d, 1H, H1', ²*J_{H,H}* = 7.2 Hz), 5.47 (dd, 1H,

H_A -benzyl, $^2J_{H,H} = 14.0$ Hz, $^3J_{H,H} = 7.3$ Hz), 5.45 (dd, 1H, H_B -benzyl, $^2J_{H,H} = 14.0$ Hz, $^3J_{H,H} = 7.1$ Hz), 5.42 (dd, 1H, H_A -benzyl, $^2J_{H,H} = 14.0$ Hz, $^3J_{H,H} = 7.6$ Hz), 5.40 (dd, 1H, H_B -benzyl, $^2J_{H,H} = 14.0$ Hz, $^3J_{H,H} = 7.3$ Hz), 5.17–5.09 (m, 1H, $H5''$), 4.44–4.39 (m, 2H, $H3'$, $H5'$), 4.19–4.13 (m, 1H, $H4'$), 3.33–3.29 (m, 2H, Lev – C3 – CH_2), 2.76–2.74 (m, 2H, Lev – C2 – CH_2), 2.40–2.23 (m, 1H, $H2''$), 2.22, 2.20 (2 s, 3H, Lev – C5 – CH_3), 2.21–2.16 (m, 1H, $H2'$) ppm. ^{31}P NMR (DMSO- d_6) $\delta = -8.29, -8.32$ (2 s, diastereomeric mixture) ppm. The deprotection of 3'-O-Lev group was completed in less than 10 min (a single band on TLC) and the crude product was purified on a silica gel column DCM/MeOH gradient, 10:0.7–1.0, to give **6** (735 mg, 52 %) as colorless foam; R_f value 0.42 (DCM/MeOH, 10:0.7). HPLC analysis has shown a diastereomeric mixture (4:5 ratio): $t_R = 20.2$ and $t_R = 22.2$ min (~98 % pure, UV at 280 nm), conducted on Jupiter C18 300 Å (5 μ m, 4.6 $\times$ 250 mm) column; eluent: solvent A 20% MeCN in water, solvent B MeCN; eluted at 0.8 mL/min with A for 25 min, then a linear gradient of B from 0–95% over 10 min, and finally 95% B for 10 min. 1H NMR (DMSO- d_6) $\delta = 11.68, 11.63$ (2 s, 1H, NH), 7.89, 7.78 (2 s, 1H, uridine - H6), 7.33 (tt, 1H, aryl - H4, $J = 7.5$ Hz), 7.25–7.19 (m, 2H, aryl - H6, aryl - H5), 7.17–7.12 (m, 1H, aryl - H3), 6.03, 5.98 (2 t, 1H, $H1'$, $^3J_{H,H} = 7.0$ Hz), 5.22–5.19 (m, 2H, benzyl), 5.42 (d, 1H, C3'-OH, $J = 4.6$ Hz), 4.36–4.23 (m, 2H, $H5''$, $H5'$), 4.22–4.18 (m, 1H, $H3'$), 3.94–3.89 (m, 1H, $H4'$), 2.23–2.12 (m, 1H, $H2''$), 2.10–2.05 (m, 1H, $H2'$) ppm. ^{31}P NMR (DMSO- d_6) $\delta = -9.18, -9.33$ (2 s, diastereomeric mixture) ppm. MSFAB-HR (m/z): $[M + Li]^+$ calcd for $C_{16}H_{16}N_2O_8PILi$, 528.9849, found 528.9837. Diastereomers of **6** were separated by preparative HPLC on Columbus C18, 100 Å (5 μ m, 10 $\times$ 250 mm) column, eluted at 2.6 mL/min with 20% MeCN in water. The amount of 59 mg of the diastereomeric mixture used for the separation, gave 19.1 mg of **6 fast** and 17.2 mg of **6 slow**. Analytical data of diastereomer **6 fast** are as follows: 1H NMR (DMSO- d_6) $\delta = 11.69$ (s, 1H, NH), 7.98 (s, 1H, uridine - H6), 7.35 (tt, 1H, aryl - H4, $^3J_{HH} = 7.8$ Hz, $^4J_{HH} = 1.0$ Hz), 7.27, 7.25 (2 d, 1H, aryl - H6, $J = 1.5$ Hz), 7.18 (tt, 1H, aryl - H5, $^3J_{HH} = 7.5$ Hz, $^4J_{HH} = 1.0$ Hz), 7.14, 7.12 (2 d, 1H, aryl - H3, $^3J_{HH} = 7.6$ Hz, $^4J_{HH} = 1.0$ Hz), 6.07 (dd, 1H, $H1'$, $J_{1'-2'} = 7.4$ Hz, $J_{1'-2''} = 6.5$ Hz), 5.55–5.44 (m, 2H, benzyl), 5.41 (bd, 1H, C3'-OH, $J = 4.4$ Hz), 4.39 (ddd, 1H, $H5''$, $^2J_{HH} = 11.6$ Hz, $^3J_{HH} = 7.1$ Hz, $^3J_{HP} = 3.7$ Hz), 4.29 (ddd, 1H, $H5'$, $^2J_{HH} = 11.6$ Hz, $^3J_{HH} = 6.7$ Hz, $^3J_{HP} = 4.6$ Hz), 4.18–4.16 (m, 1H, $H3'$), 3.94–3.91 (m, 1H, $H4'$), 2.19–2.16 (m, 1H, $H2''$), 2.09–2.05 (m, 1H, $H2'$) ppm. ^{13}C NMR (DMSO- d_6) $\delta = 160.77$ (C4), 150.38 (C2), 149.35 (C2-aryl), 144.33 (C6), 129.62 (C4-aryl), 126.0 (C6-aryl), 124.31 (C5-aryl), 120.93 (C1-aryl), 118.0 (C3-aryl), 109.42 (C5), 84.75 (C1'), 84.32 (C4'), 69.92 (C3'), 68.86 (C-benzyl), 67.64 (C5'), 38.72 (C2') ppm. ^{31}P NMR (DMSO- d_6) $\delta = -9.19$ ppm. Analytical data of diastereomer **6 slow** are as follows: 1H NMR (DMSO- d_6) $\delta = 11.71$ (s, 1H, NH), 7.99 (s, 1H, uridine - H6), 7.37 (tt, 1H, aryl - H4, $^3J_{HH} = 7.7$ Hz, $^4J_{HH} = 1.1$ Hz), 7.28 (dd, 1H, aryl - H6, $^3J_{HH} = 7.1$ Hz, $^4J_{H,H} = 1.5$ Hz), 7.20 (tt, 1H, aryl - H5, $^3J_{HH} = 7.6$ Hz, $^4J_{HH} = 1.0$ Hz), 7.15 (dd, 1H, aryl - H3, $^3J_{HH} = 7.6$ Hz, $^4J_{HH} = 1.0$ Hz), 6.10 (dd, 1H, $H1'$, $J_{1'-2'} = 7.5$ Hz, $J_{1'-2''} = 6.4$ Hz), 5.53–5.45 (m, 2H, benzyl), 5.43 (d, 1H, C3'-OH, $J = 4.5$ Hz), 4.35–4.27 (m, 2H, $H5''$ and $H5'$), 4.22–4.18 (m, 1H, $H3'$), 3.94–3.90 (m, 1H, $H4'$), 2.22–2.17 (m, 1H, $H2''$), 2.10–2.05 (m, 1H, $H2'$) ppm. ^{13}C NMR (DMSO- d_6) $\delta = 160.81$ (C4), 150.42 (C2), 149.39 (C2-aryl), 144.41 (C6), 129.59 (C4-aryl), 126.12 (C6-aryl), 124.29 (C5-aryl), 120.87 (C1-aryl), 118.90 (C3-aryl), 110.02 (C5), 84.83 (C1'), 84.29 (C4'), 70.13 (C3'), 68.90 (C-benzyl), 67.76 (C5'), 38.59 (C2') ppm. ^{31}P NMR (DMSO- d_6) $\delta = -9.32$ ppm.

Method II: General Procedure B conducted with IUdR **1** (1.12 g, 3.16 mmol), dissolved in 15 mL of DMF, DIPEA (1.14 mL, 0.84 g, 6.5 mmol), and newly distilled chlorophosphite **15** (604 mg, 3.83 mmol) diluted with 6 mL of dry THF and transferred in three (2 mL) portions; the oxidation with a solution of *t*-BuOOH (0.86 mL, ≥ 4.3 mmol). The time of phosphorylation 2 h, the oxidation was carried out for 1 h. All three phosphotriesters, purified on a silica gel column (DCM/MeOH gradient, 10:0.7–1.0), were collected in a form of colorless foam: 5',3'-O,O'-dicycloSal-5-IUdRMP **9** (R_f 0.72), 350 mg (16%); 3'-O-

cycloSal-IUdRMP **12** (R_f 0.58), 611 mg (37%); 5'-*O*-*cycloSal*-5-IUdRMP **6** (R_f 0.42), 759 mg (46%). The analytical data of the product **6** were identical with those reported above for **6** obtained in Method I.

5-Iodo-5'-*O*-*cyclo*(3-methylSaligenyl)-2'-deoxyuridine Monophosphate (**7**)

Method I: General Procedure C with 3'-*O*-levulinyl IUdR **4** (1.36 g, 3.0 mmol), DIPEA (1.5 mL, 1.11 g, 8.6 mmol) and the crude chlorophosphite **16** (1.04 g, ~6 mmol), was conducted in 27 mL of dry MeCN. The reaction time was extended to 4 h (TLC monitoring). The oxidation was carried on with a solution of *t*-BuOOH (0.9 mL, $\geq$ 4.5 mmol). Before the deprotection of 3'-*O*-Lev group, a small sample of the crude product (~11 mg) was purified by HPLC, on Columbus C18, 100 Å (5 μ m, 10 $\times$ 250 mm) column, eluted at 2.5 mL/min with 40% MeCN in water. The HPLC analysis showed two diastereomers, (45:55 ratio) t_R = 26.3 min, t_R = 26.6 min ($\geq$ 98% pure, UV at 280 nm), ACE C18, 100 Å (5 μ m, 4.6 $\times$ 250 mm); eluent: solvent A 10% MeCN in water, solvent B MeCN; eluted at 1.0 mL/min with a linear gradient of B from 0 – 95% over 45 min, and 95% B for 15 min. Purified 3'-*O*-Lev derivative of **7**, was further analyzed: ^1H NMR (DMSO- d_6) δ = 11.77 (bs, 1H, NH), 8.08, 8.05 (2 s, 1H, uridine – H6), 7.26 – 7.14 (m, 1H, aryl – H5), 7.10 – 7.08 (m, 2H, aryl – H6, aryl – H4), 6.09 (dd, 1H, H1', $J_{1'-2'} = 7.3$ Hz, $J_{1'-2''} = 6.4$ Hz), 5.53 – 5.42 (m, 2H, benzyl), 5.15 – 5.09 (m, 1H, H3'), 4.41 – 4.36 (m, 2H, H4', H5''), 4.15 – 4.11 (m, 1H, H5'), 3.35 – 3.33 (m, 2H, Lev – C3 – CH₂), 2.74 – 2.72 (m, 2H, Lev – C2 – CH₂), 2.41 – 2.24 (m, 4H, H2'', Lev – C5 – CH₃), 2.23 – 2.18 (m, 1H, H2'), 2.11 (s, 3H, aryl – C3 – CH₃) ppm. ^{31}P NMR (DMSO- d_6) δ = – 8.23, – 8.27 (2 s, diastereomeric mixture) ppm. MSFAB-HR (m/z): $[\text{M} + \text{H}]^+$ calcd for C₂₂H₂₅N₂O₁₀PI, 635.0292, found 635.0277. Subsequent cleavage of the 3'-*O*-Lev group was completed in < 5 min and the crude product purified on a silica gel column (DCM/MeOH, 10:0.7), to give **7** (933 mg, 58%) as colorless foam; R_f value 0.46 (DCM/MeOH, 10:0.7). The HPLC analysis showed a mixture of diastereomers: t_R = 18.2 min, t_R = 18.8 min ($\geq$ 98% pure, UV at 280 nm), ACE C18, 100 Å (5 μ m, 4.6 $\times$ 250 mm) column; eluent: solvent A 10% MeCN in water, solvent B MeCN; eluted at 1.0 mL/min with a linear gradient of B from 0 – 95% over 45 min, and 95% B for 15 min. ^1H NMR (DMSO- d_6) δ = 11.73, 11.68 (2 s, 1H, NH), 7.99, 7.86 (2 s, 1H, uridine – H6), 7.25 – 7.21 (m, 1H, aryl – H5), 7.12 – 7.07 (m, 2H, aryl – H6, aryl – H4), 6.08 (dd, 1H, H1', $^2J_{1'-2'} = 7.6$ Hz, $^2J_{1'-2''} = 6.0$ Hz), 6.05 (dd, 1H, H1', $^2J_{1'-2'} = 7.8$ Hz, $^2J_{1'-2''} = 5.8$ Hz), 5.51 – 5.39 (m, 2H, benzyl), 5.41 (d, 1H, C3'-OH', $J = 3.7$ Hz), 4.34 – 4.26 (m, 1H, H5'', H3'), 4.22 – 4.18 (m, 1H, H5'), 3.93 – 3.89 (m, 1H, H4'), 2.25 – 2.19 (m, 4H, H2'',), 2.23 (s, 3H, C3-aryl – CH₃), 2.10 – 2.05 (m, 1H, H2'') ppm. ^{13}C NMR (DMSO- d_6) δ = 162.87 (C4), 150.31 (C2), 148.55, 148.51 (C2-aryl), 141.23, 141.18 (C6), 132.86 (C5-aryl), 129.18 (C4-aryl), 126.33 (C6-aryl), 120.73, 120.66 (C1-aryl), 117.35 (C3-aryl), 109.67 (C5), 84.75, 84.65 (C1'), 84.32, 84.22 (C4'), 69.98 (C3'), 68.82 (C-benzyl), 67.64 (C5'), 38.72 (C2'), 21.11 (CH₃-aryl) ppm. ^{31}P NMR (DMSO- d_6) δ = – 8.89, – 8.93 (2 s, diastereomeric mixture) ppm. MSFAB-HR (m/z): $[\text{M} + \text{H}]^+$ calcd for C₁₆H₁₉N₂O₈PI, 536.9924, found 536.9899. Diastereomers were separated by preparative HPLC on Columbus C18, 100 Å (5 μ m, 10 $\times$ 250 mm) column; eluted with 22% MeCN in water at 2.5 mL/min flow rate. From the total of 66 mg diastereomeric **7** used for separation, 14.1 mg of **7 fast** and 12.2 mg of **7 slow** was isolated. Diastereomer **7 fast** eluted within 72 – 76 min, and **7 slow** within 79 – 81 min after the injection, and each isomer was collected in ~ 9 mL of eluent. A solvent was evaporated to dryness in a high vacuum; the product residue was reconstituted in MeCN and analyzed one more time on the analytical HPLC. Each diastereomer was found to be $\geq$ 97% pure (UV at 220 and 280 nm). Analytical data of **7 fast** are as follows: ^1H NMR (DMSO- d_6) δ = 11.70 (s, 1H, NH), 7.98 (s, 1H, uridine – H6), 7.23 – 7.20 (m, 1H, aryl – H5), 7.17 (dd, 1H, aryl – H4, $^3J_{\text{HH}} = 7.6$ Hz, $^4J_{\text{H,H}} = 1.5$ Hz), 7.12 – 7.06 (m, 1H, aryl – H6), 6.06 (dd, 1H, H1', $J_{1'-2'} = 6.6$ Hz, $J_{1'-2''} = 5.8$ Hz), 5.51 – 5.43 (m, 3H, benzyl, C3'-OH), 4.38 – 4.33 (m, 1H, H5''), 4.27 – 4.22 (m, 1H, H5'), 4.17 – 4.15 (m, 1H, H3'), 3.92 – 3.90 (m, 1H, H4'), 2.21 – 2.19 (m,

3H, C3-aryl-CH₃), 2.18 – 2.13 (m, 1H, H2''), 2.10 – 2.05 (m, 1H, H2') ppm. ³¹P NMR (DMSO-*d*₆) δ = – 8.89 ppm. Analytical data of **7** *slow* are as follows: ¹H NMR (DMSO-*d*₆) δ = 11.69 (s, 1H, NH), 7.88 (s, 1H, uridine - H6), 7.24 (dd, 1H, aryl - H5, ³J_{HH} = 7.8 Hz, ⁴J_{H,H} = 1.2 Hz), 7.09 – 7.06 (m, 2H, aryl - H6, aryl - H4), 6.07 (dd, 1H, H1', J_{1',2'} = 6.5 Hz, J_{1',2''} = 4.8 Hz), 5.49 – 5.38 (m, 3H, benzyl, C3' - OH), 4.33 – 4.25 (m, 2H, H5'', H5'), 4.21 – 4.17 (m, 1H, H3'), 3.92 – 3.89 (m, 1H, H4'), 2.24 – 2.18 (m, 4H, H2'', C3-aryl - CH₃), 2.11 – 2.06 (m, 1H, H2') ppm. ³¹P NMR (DMSO-*d*₆) δ = – 8.94 ppm. Method II: General Procedure B was conducted with IUdR **1** (2.51 g, 7.09 mmol) dissolved in 18 mL of DMF and DIPEA (1.54 mL, 1.14 g, 8.8 mmol). The crude chlorophosphite **16** (1.39 g, ~8 mmol) was dissolved in 6 mL of dry THF added in 3×2 mL portions. A solution of *t*-BuOOH (1.65 mL, ≥ 8.25 mmol) was added after 3 h of phosphitylation. The oxidation was carried out for 2 h. Phosphotriesters were purified on a silica gel column (DCM/MeOH gradient, 10 : 0.7 – 1.0) and followed by second purification (DCM/MeOH, 10 : 0.4), to achieve a complete separation of the closely eluting 3'-*O*-isomer **13**. All three products: 5',3'-*O*,*O'*-dicycloSal-5-IUdRMP **10** (*R*_f 0.77), 1.12 g (22%); 3'-*O* - cycloSal-IUdRMP **13** (*R*_f 0.64), 1.17 g (31%); 5'-*O* - cycloSal - 5 - IUdRMP **7** (*R*_f 0.47), 1.47 g (39%) were collected as colorless foams. The analytical data of product **7** were identical with those detailed above for **7** obtained according to Method I.

5-Iodo-5'-*O*-(cyclo-3,5-di-(*tert*-butyl)-6-fluoroSaligenyl)-2'-deoxyuridine Monophosphate (**8**)

Method I: General Procedure C was carried out with 3'-*O*-levulinyl IUdR **4** (1.04 g, 2.3 mmol), DIPEA (1.25 mL, 0.93 g, 7.15 mmol) and the crude chlorophosphite **17** (1.14 g, ~ 3.6 mmol) in MeCN (20 mL). The oxidation with a solution of *t*-BuOOH (1.0 mL, ≥ 5 mmol) was started after 1 h of phosphitylation. A small portion (~14 mg) of the crude product was purified by HPLC, on Columbus C18, 100 Å (5 μm, 10 × 250 mm) column, eluted at 3.0 mL/min with 43% MeCN in water. The purified 3'-*O*-Lev derivative of **8** (~7 mg), was further analyzed by HR-MS: MSFAB-HR (*m/z*): [M + Li]⁺ calcd for C₂₉H₃₇N₂O₁₀PIFLi, 757.1375, found 757.1355. The ¹³C peak was observed at 758.1393, within –2.0 ppm of the expected value of 758.1409. The HPLC analysis showed a mixture of two diastereomers: *t*_R = 22.2 min, *t*_R = 22.7 min (≥ 98% pure, UV at 280 nm), performed on ACE C18, 100 Å (5 μm, 4.6 × 250 mm) column; eluent: solvent A 50% MeCN in water, solvent B MeCN; eluted at 1.0 mL/min with a linear gradient of B from 0 – 55% over 25 min, then 55 – 95% B for the period of 20 min and 95% B for 15 min. Cleavage of 3'-*O*-Lev group was completed in <10 min (TLC monitoring) and the crude product was purified on a silica gel column (DCM/MeOH gradient, 10:0.7 – 0.9), to give **8** (930 mg, 62%) in a form of colorless foam; *R*_f value 0.52 (DCM/MeOH, 10:0.7). The HPLC analysis confirmed again the presence of two diastereomers: *t*_R = 20.9 min and *t*_R = 22.8 min (≥ 98% pure, UV at 220 and 280 nm). The analysis was conducted using Columbus C8, 100 Å (5 μm, 4.6 × 250 mm) column; eluent: solvent A 50% MeCN in water, solvent B MeCN; a column eluted at 1.0 mL/min with A for 25 min, then a linear gradient of B from 0 – 95% over 15 min, and 95% B for the period of 20 min. ¹H NMR (DMSO-*d*₆) δ = 11.11, 11.04 (2 s, 1H, NH), 7.94, 7.92 (2 s, 1H, uridine - H6), 7.28 – 7.25 (m, 1H, aryl - H4), 6.25 – 6.20 (m, 1H, H1'), 5.52 – 5.38 (m, 2H, benzyl), 4.64 – 4.57 (m, 2H, C3'-OH, H3'), 4.37 – 4.24 (m, 1H, H5'', H5'), 4.17 – 4.14 (m, 1H, H3'), 2.52 – 2.45 (m, 1H, H4'), 2.25 – 2.20 (m, 1H, H2''), 2.18 – 2.12 (m, 1H, H2'), 1.41 (s, 9H, 3 × CH₃ - *t*-Bu), 1.37 (s, 9H, 3 × CH₃ - *t*-Bu) ppm. ¹³C NMR (DMSO-*d*₆) δ = 163.27 (C4), 155.13 (C6-aryl), 150.91 (C2), 148.51 (C2-aryl), 141.18 (C6), 133.46 (C5-aryl), 130.38 (d, C3-aryl, ⁴J_{C,F} = 4.1 Hz), 127.15 (C4-aryl), 110.66 (C1-aryl), 109.77 (C5), 89.65 (C1'), 84.56, 84.45 (C4'), 71.06 (C3'), 69.22 (C-benzyl), 67.55 (C5'), 38.67 (C2'), 34.71 (1 × C-*t*Bu), 34.60 (1 × C-*t*Bu), 34.38 (d, 2 × C-*t*Bu, ⁴J_{C,F} = 3.2 Hz), 29.91 (d, 2 × CH₃-*t*Bu, ⁴J_{C,F} = 3.2 Hz), 29.86 (1 × CH₃-*t*Bu), 29.81 (1 × CH₃-*t*Bu) ppm. ³¹P NMR (DMSO-*d*₆) δ = – 8.67, – 8.93 (2 s diastereomeric mixture) ppm. MSFAB-HR (*m/z*): [M +

$[H]^+$ calcd for $C_{24}H_{32}N_2O_8PFI$, 653.0925, found 653.0930. The ^{13}C peak was measured at 654.0945, within -2.0 ppm of the expected value.

Diastereomers were isolated by preparative HPLC (≤ 6 mg of **8** per injection), on Columbus C18, 100 Å ($5 \mu m$, 10×250 mm) column; eluted at 2.5 mL/min with 47% MeCN solution in water. The separation started with 96 mg of the diastereomeric **8** and 34.4 mg of **8 fast** and 43.2 mg of **8 slow** was isolated. Diastereomer **8 fast** eluted within 26 – 27.5 min, and **8 slow** within 28.5 – 30.5 min after the injection, and each isomer was collected in ~ 5 mL of the eluate. Solvent was evaporated to dryness in a high vacuum; the product residue was reconstituted in MeCN and analyzed again on the analytical HPLC, showing the purity of $\geq 98\%$ for each diastereomer. Analytical data of **8 fast** are as follows: 1H NMR (DMSO- d_6) δ = 11.70 (s, 1H, NH), 7.98 (s, 1H, uridine - H6), 7.22 (d, 1H, aryl - H4, $^4J_{HF}$ = 9.4 Hz), 6.06 (dd, 1H, H1', $J_{1',2'} = 6.6$ Hz, $J_{1',2''} = 4.3$ Hz), 5.51 – 5.43 (m, 2H, benzyl), 5.42 (bd, 1H, C3'-OH, J = 3.6 Hz), 4.38 – 4.33 (m, 1H, H3'), 4.27 – 4.22 (m, 1H, H4'), 4.17 – 4.15 (m, 1H, H5'), 3.92 – 3.90 (m, 1H, H5''), 2.21 (s, 3H, aryl C5 - CH₃), 2.18 – 2.13 (m, 1H, H2'), 2.10 – 2.05 (m, 1H, H2''), 1.43 (s, 9H, 3 x CH₃ - *t*-Bu), 1.35 (s, 9H, 3 x CH₃ - *t*-Bu) ppm. ^{31}P NMR (DMSO- d_6) δ = -8.89 ppm. Analytical data of **8 slow** are as follows: 1H NMR (DMSO- d_6) δ = 11.69 (s, 1H, NH), 7.99 (s, 1H, uridine - H6), 7.25 – 7.22 (m, 1H, aryl - H5), 7.09 – 7.06 (m, 2H, aryl - H3, aryl - H4), 6.07 (dd, 1H, H1', $J_{1',2'} = 6.5$ Hz, $J_{1',2''} = 4.3$ Hz), 5.49 – 5.38 (m, 3H, benzyl, C3' - OH), 4.33 – 4.25 (m, 2H, H3', H4'), 4.21 – 4.17 (m, 1H, H5'), 3.92 – 3.89 (m, 1H, H5''), 2.24 – 2.18 (m, 4H, H2', aryl C5 - CH₃), 2.18 – 2.13 (m, 1H, H2'), 2.10 – 2.06 (m, 1H, H2''), 1.37 (s, 9H, 3 x CH₃ - *t*-Bu), 1.34 (s, 9H, 3 x CH₃ - *t*-Bu) ppm. ^{31}P NMR (DMSO- d_6) δ = -8.93 ppm.

Method II: General Procedure B was conducted with IUdR **1** (5.0 g, 14.1 mmol) in 30 mL of DMF and DIPEA (3.1 mL, 2.29 g, 17.7 mmol), crude chlorophosphite **17** (5.4 g, ~ 17 mmol, dissolved in 10 mL of dry THF), transferred slowly in 2×5 mL portions. Oxidation with a solution of *t* - BuOOH (4 mL, ≥ 20 mmol) was carried out for 1 h. Phosphotriesters of IUdR were purified on a silica gel column (DCM/MeOH gradient, 10 : 0.7 – 0.9). All three products were obtained in a form of colorless rigid foam: 5',3'-*O,O'*-dicycloSal-5-IUdRMP **11** (R_f 0.81), 2.81 g (21%); 3'-*O* - cycloSal-IUdRMP **14** (R_f 0.66), 3.31 g (36%); 5'-*O* - cycloSal - 5 - IUdRMP **8** (R_f 0.52), 3.95 g (43%). The analytical data of product **8** were identical with those reported above for **8** obtained using Method I.

5-Iodo-3'-O-cycloSaligenyl-2'-deoxyuridine Monophosphate (12)—This compound was obtained in two ways: (1) as the side product (611 mg, 37% yield) in the preparation of **6** using Method II, or (2) by General Procedure C conducted with 5'-*O*-trityl IUdR **5** (3.52 g, 5.90 mmol), DIPEA (1.8 mL, 1.33 g, 10.3 mmol) and the crude chlorophosphite **15** (1.3 g, ~ 8 mmol). Both synthetic pathways furnished **12** with the identical analytical data. General Procedure C was performed for 45 min in MeCN (30 mL), with the subsequent oxidation using a solution of *t* - BuOOH (2.2 mL, ≥ 11 mmol). After workup, the crude solid residue dissolved in MeCN (34 mL) was treated with ZrCl₄ (1.65 g, 7.0 mmol) and gave **12** (1.21 g, 39%) as colorless rigid foam; R_f value 0.58 (DCM/MeOH, 10:0.7). The HPLC analysis has shown a diastereomeric mixture (44:56 ratio): **12 fast**, t_R = 37.9 min and **12 slow**, t_R = 38.6 min ($\geq 98\%$ pure, UV at 280 nm); column: ACE C18, 100 Å ($5 \mu m$, 4.6×250 mm); eluent: solvent A 10% MeCN in water, solvent B MeCN; eluted at 0.8 mL/min with a linear gradient of B from 0 – 25% over 40 min, and a gradient of B from 25 – 95% B for 20 min. 1H NMR (DMSO- d_6) δ = 10.61 (s, 1H, NH), 10.58 (s, 1H, NH), 8.36 (s, 1H, uridine - H6), 8.34 (s, 1H, uridine - H6), 7.13 (m, 1H, aryl - H4), 7.06 – 6.98 (m, 3H, aryl - H3, aryl - H5, aryl - H6), 6.21 (t, 1H, H1', J = 7.0 Hz), 5.97 (t, 1H, H1', J = 7.0 Hz), 5.34 – 5.28 (m, 2H, benzyl), 4.24 – 4.22 (m, 1H, H3'), 3.82 – 3.76 (m, 2H, H4', H5'), 3.94 – 3.89 (m, 1H, H5''), 3.55 (bd, C5'-OH, J = 4.0 Hz), 2.56 – 2.44 (m, 1H, H2'), 2.34 – 2.26 (m, 1H, H2'') ppm. ^{31}P NMR (DMSO- d_6) δ = -9.74 , -9.77 ppm. MSFAB-HR (m/z): [M +

Li^+ calcd for $\text{C}_{16}\text{H}_{16}\text{N}_2\text{O}_8\text{PILi}$, 528.9849, found 528.9844 The ^{13}C isotope was observed at 529.9893 within 1.9 ppm of expected 529.9883.

5 - Iodo-3'-O-cyclo(3-methylSaligenyl)-2'-deoxyuridine Monophosphate (13)—

Compound **13** was obtained in two ways: (1) as the side product (1.17 g, 31%) during the preparation of **7** using Method II, or (2) by General Procedure C conducted with 5'-O-trityl IUdR **5** (2.34 g, 3.93 mmol), DIPEA (1.5 mL, 1.11 g, 8.6 mmol) and crude chlorophosphite **16** (1.1 g, ~6 mmol). Isolated product **13** in both synthetic pathways, showed the identical analytical data. General Procedure C was carried out in 20 mL of MeCN for 60 min, and the oxidation with a solution of *t*-BuOOH (1.6 mL, ≥ 8 mmol) subsequent to the phosphitylation. After workup, the crude solid dissolved in MeCN (40 mL) was treated with ZrCl_4 (1.0 g, 4.3 mmol) and gave **13** (842 mg, 40%) as colorless rigid foam; R_f value 0.64 (DCM/MeOH, 10:0.7). Further HPLC purification (23 mg of **13**, ~4 mg per injection) was performed on Columbus C18, 100 Å (5µm, 10 × 250 mm) column; eluent: solvent A 20% MeCN in water and solvent B MeCN; eluted at 2.5 mL/min with a linear gradient of B from 0 – 65% over 35 min. Product **13** (11.9 mg, ≥ 98% pure, UV at 280 nm) was collected within 28 – 30 min after the injection. The HPLC analysis showed a mixture of diastereomers: **13 fast**, $t_R = 21.8$ min; **13 slow**, $t_R = 22.4$ min (≥ 98% pure, UV at 280 nm); column: ACE C18, 100 Å (5µm, 4.6 × 250 mm); eluent: solvent A 20% MeCN in water, solvent B MeCN; eluted at 1 mL/min with a linear gradient of B from 0 – 60% over 45 min, and the using a linear gradient of B from 45 – 95% B for 15 min. ^1H NMR (DMSO- d_6) δ = 10.82 (s, 1H, NH), 10.65 (s, 1H NH), 8.31 (s, 1H, uridine - H6), 8.28 (s, 1H, uridine - H6), 7.23 – 7.06 (m, 3H, aryl - H4, aryl - H5, aryl - H6), , 6.20 – 6.17 (m, 1H, H1'), 5.33 – 5.27 (m, 2H, benzyl), 4.30 – 4.23 (m, 1H, H3'), 3.83 – 3.76 (m, 2H, H4', H5'), 3.94 – 3.89 (m, 1H, H5''), 3.61 (bd, C5'-OH, $J = 4.0$ Hz), 2.56 – 2.44 (m, 1H, H2'), 2.34 – 2.26 (m, 4H, H2'', aryl - C3 – CH₃) ppm. ^{31}P NMR (DMSO- d_6) δ = - 9.34, - 9.38 ppm. MSFAB-HR (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{19}\text{N}_2\text{O}_8\text{PI}$, 536.9924, found 536.9940. The ^{13}C isotope peak was measured at 537.9981; 4.3 ppm of the expected value.

5-Iodo-3'-O-[cyclo-3,5-di-(tert-butyl)-6-fluoroSaligenyl]-2'-deoxyuridine Monophosphate (14)—

Compound **14** was obtained in two ways: (1) as the side product (3.31 g 36% yield) during the preparation of **8** using Method II, or (2) by conducting General Procedure C with 5'-O- trityl IUdR **5** (3.52 g, 5.90 mmol), DIPEA (2 mL, 1.48 g, 11.5 mmol) and crude chlorophosphite **17** (2.6 g, ~ 8 mmol). Isolated product **14** from both synthetic pathways showed the identical analytical data. General Procedure C was carried on for 90 min in 30 mL of MeCN and the oxidation with a solution of *t*-BuOOH (1.5 mL, ≥ 6 mmol) was following the phosphitylation. After the workup, a crude solid dissolved in MeCN (50 mL) was treated with ZrCl_4 (1.72 g, 7.38 mmol) and gave **14** (1.65g, 43%) as colorless rigid foam (93% pure by HPLC analysis); R_f value 0.66 (DCM/MeOH, 10:0.7). Further HPLC purification (31 mg of **14**, ~5 mg per injection) was performed on Columbus C18, 100 Å (5µm, 10 × 250 mm) column; eluent: solvent A 50% MeCN in water, solvent B MeCN; eluted at 2.5 mL/min with a linear gradient of B from 0 – 40% over 15 min, and 40% B (isocratic) for 30 min. Product **14** (18.2 mg, ≥ 98% pure, UV at 280 nm) was collected within 25 – 27 min after the injection. The HPLC analysis showed a mixture of diastereomers: **14 fast**, $t_R = 31.1$ min and **14 slow**, $t_R = 33.3$ min; column: Columbus C8, 100 Å (5µm, 4.6 × 250 mm); eluent: solvent A 50% MeCN in water, solvent B MeCN; eluted at 1 mL/min with A for 45 min (isocratic) and then a linear gradient of B from 0 – 95% B over 5 min, and 95% B for 10 min. ^1H NMR (DMSO- d_6) δ = 10.89 (s, 1H, NH), 10.84 (s, 1H NH), 7.89 (s, 1H, uridine - H6), 7.84 (s, 1H, uridine - H6), 7.29 – 7.24 (m, 1H, aryl - H4), 6.27 – 6.22 (m, 1H, H1'), 5.56 – 5.44 (m, 2H, benzyl), 4.35 – 4.22 (m, 1H, H3'), 4.19 – 4.15 (m, 1H, H4'), 3.43 (bd, C5'-OH, $J = 4.0$ Hz), 2.54 – 2.46 (m, 2H, H5', H5''), 2.27 – 2.23 (m, 1H, H2'), 2.15 – 2.11 (m, 1H, H2''), 1.39, 1.35, 1.32, 1.31 (overlapped s, 18H,

aryl – C3 - 3 x CH₃ – *t*-Bu and aryl – C5 - 3 x CH₃ – *t*-Bu ppm. ³¹P NMR (DMSO-*d*₆) δ = – 8.90, – 8.77 ppm. MSFAB-HR (m/z): [M + H]⁺calcd for C₂₄H₃₂N₂O₈PF₆, 653.0925, found 653.0930. The ¹³C isotope peak measured 654.0845; –2.0 ppm of the expected value.

5'-O-[cyclo-3,5-Di-(*tert*-butyl)-6-fluoroSaligenyl]thymidine Monophosphate (22)

—General Procedure C with 3'-*O*-levulinylthymidine **19** (2.43 g, 7.14 mmol), DIPEA (3.15 mL, 2.33 g, 18 mmol) and the crude chlorophosphite **17** (3.57 g, ~ 11 mmol), was conducted in 30 mL of MeCN for 1 h and the oxidation with a solution of *t*-BuOOH (2.5 mL, ≥ 12.5 mmol) followed the phosphitylation. Before deprotection, the crude product was purified on a silica gel column (DCM/MeOH, 10:0.4). Cleavage of 3'-*O*-Lev group was completed in ≤ 5 min (a new single band on TLC) and the final product was separated on a silica gel column (DCM/MeOH gradient, 10:0.7 – 0.9), to give **22** (905 mg, 62%) as colorless rigid foam; *R*_f value 0.52 (DCM/MeOH, 10:0.7). The HPLC analysis showed a mixture of diastereomers: **22 fast**, *t*_R = 19.3 min and **22 slow**, *t*_R = 20.2 min (≥ 96% pure, UV at 280 nm); column: ACE C18, 100 Å (5μm, 4.6 × 250 mm); eluent: solvent A 50% MeCN in water, solvent B MeCN; eluted at 0.8 mL/min with solvent A for 60 min (isocratic) and then a linear gradient of B from 0 – 95% B over 30 min. ¹H NMR (DMSO-*d*₆) δ = 11.40 (s, 1H, NH), 7.53 (s, 1H, uridine - H6), 7.49 (s, 1H, uridine - H6), 7.25 (s, 1H, aryl - H4), 7.23 (s, 1H, aryl - H4), 6.24 – 6.17 (m, 1H, H1'), 5.60 – 5.45 (m, 2H, benzyl), 5.41 – 5.29 (m, 1H, H3'), 4.60 – 4.53 (m, 1H, uridine - C3' - OH), 4.48 – 4.34 (m, 3H, H4', H5', H5''), 2.47 – 2.36 (m, 1H, H2'), 2.32 – 2.26 (m, 1H, H2''), 1.73 (s, 3H, uridine - C5 - CH₃), 1.67 (s, 3H, uridine - C5 - CH₃), 1.334, 1.325, 1.321, 1.317 (overlapped s, 18H, aryl - C3 - 3 x CH₃ - *t*-Bu; aryl - C5 - 3 x CH₃ - *t*-Bu) ppm. ³¹P NMR (DMSO-*d*₆) δ = – 9.73, – 9.58 ppm. MSFAB-HR (m/z): [M + H]⁺calcd for C₂₅H₃₆N₂O₈PF₆, 542.2194, found 542.2189. The ¹³C isotope peak measured 543.2221; –2.0 ppm of the expected value.

5'-O-[cyclo-3,5-Di-(*tert*-butyl)-6-fluoroSaligenyl]-3'-deoxy-3'-fluorothymidine Monophosphate (23)

—General Procedure C with 3'-deoxy-3'-fluorothymidine **20** (454 mg, 1.86 mmol) in DMF (3 mL), DIPEA (820 μL, 0.61 g, 4.7 mmol) and the crude chlorophosphite **17** (0.65 g, ~ 2 mmol, 2 mL of THF solution) was conducted for 1 h and the phosphitylated mixture was oxidized with a solution of *t*-BuOOH (400 μL, ≥ 2 mmol). The crude product was separated on a silica gel column (DCM/MeOH gradient, 10:0.7 – 0.9) to give **23** (806 mg, 80%) as colorless rigid foam; *R*_f value 0.69 (DCM/MeOH, 10:0.7). The HPLC analysis showed a mixture of diastereomers: **23 fast**, *t*_R = 45.4 min and **23 slow**, *t*_R = 47.0 min (≥ 97% pure, UV at 280 nm); column: ACE C18, 100 Å (5μm, 4.6 × 250 mm); eluent: solvent A 50% MeCN in water, solvent B MeCN; eluted at 0.8 mL/min with solvent A for 60 min (isocratic) and then a linear gradient of B from 0 – 95% B over 30 min. ¹H NMR (DMSO-*d*₆) δ = 11.31 (s, 1H, NH), 7.47 (s, 1H, uridine - H6), 7.43 (s, 1H, uridine - H6), 7.25 (s, 1H, aryl - H4), 7.23 (s, 1H, aryl - H4), 6.22 – 6.16 (m, 1H, H1'), 5.59 – 5.46 (m, 3H, benzyl, H3'), 4.43 – 4.39 (m, 2H, H5', H5''), 3.95 – 3.91 (m, 1H, H4'), 2.17 – 2.10 (m, 2H, H2', H2''), 1.71 (s, 3H, uridine - C5 - CH₃), 1.66 (s, 3H, uridine - C5 - CH₃), 1.34, 1.31 (overlapped s, 18H, aryl - C3 - 3 x CH₃ - *t*-Bu and aryl - C5 - 3 x CH₃ - *t*-Bu) ppm. ³¹P NMR (DMSO-*d*₆) δ = – 9.46, – 9.62 ppm. MSFAB-HR (m/z): [M + H]⁺calcd for C₂₅H₃₅N₂O₇PF₆, 544.2149, found 544.2155. The ¹³C isotope peak measured 545.2183; –2.0 ppm of the expected value.

5-Iodo-5'-O-[cyclo-3,5-Di-(*tert*-butyl)-6-fluoroSaligenyl]-3'-fluoro-2',3'-dideoxyuridine Monophosphate (24)

—General Procedure C with 5-iodo-3'-fluoro-2',3'-dideoxyuridine **21** (390 mg, 1.1 mmol) in DMF (3 mL), DIPEA (480 μL, 355 mg, 2.75 mmol) and the crude chlorophosphite **17** (1.95 g, ~ 6 mmol, 4.4 mL of THF solution) was conducted for 45 min. Phosphitylation was followed by the oxidation with a solution of *t*-BuOOH (650 μL, ≥ 3.25 mmol). The crude product was separated on a silica gel column

(DCM/MeOH gradient, 10:0.6 – 0.9) to give **24** (181 mg, 76%) as colorless rigid foam; R_f value 0.73 (DCM/MeOH, 10:0.7). The HPLC analysis showed a mixture of diastereomers: **24 fast**, $t_R = 25.9$ min and **24 slow**, $t_R = 26.7$ min ($\geq 95\%$ pure, UV at 280 nm); column: ACE C18, 100 Å (5 μ m, 4.6 $\times$ 250 mm); eluent: solvent A 50% MeCN in water, solvent B MeCN; the column eluted with a linear gradient of B from 0 – 95% B over 60 min, and 95% B for 30 min at 1 mL/min. ^1H NMR (DMSO- d_6) $\delta = 11.78$ (bs, 1H, NH), 8.12 (s, 1H, uridine - H6), 8.08 (s, 1H, uridine - H6), 7.25 (s, 1H, aryl - H4), 7.24 (m, 1H, aryl - H4), 6.14 – 6.10 (m, 1H, H1'), 5.57 – 5.46 (m, 2H, benzyl), 5.37 – 5.25 (m, 1H, H3'), 4.51 – 4.32 (m, 3H, H4', H5', H5''), 2.51 – 2.43 (m, 2H, H2', H2''), 1.35, 1.34, 1.33, 1.32 (overlapped s, 18H, aryl - C3 - 3 $\times$ CH₃ - *t*-Bu and aryl - C5 - 3 $\times$ CH₃ - *t*-Bu) ppm. ^{31}P NMR (DMSO- d_6) $\delta = -9.35, -9.79$ ppm. MSFAB-HR (m/z): [M + Li]⁺ calcd for C₂₄H₃₀N₂O₇PF₂ILi, 661.0964, found 661.0953 The ^{13}C peak measured 662.0986, which was within –1.6 ppm of the expected value of 662.0998. Diastereomers were separated by the preparative HPLC (≤ 4 mg of **24** per injection), on Columbus C18, 100 Å (5 μ m, 10 $\times$ 250 mm) column, eluted at 2.2 mL/min with 57% MeCN in water. Starting with 61 mg of diastereomeric **24**, 14.1 mg of **24 fast** and 16.0 mg of **24 slow** was isolated. Diastereomer **24 fast** eluted within 37 – 39 min, and **24 slow** within 39.5 – 41.3 min after the injection. The HPLC analysis: **24 fast**, $t_R = 37.7$ min and **24 slow**, $t_R = 40.2$ min (each isomer was $\geq 98\%$ pure, UV at 280 nm); column: ACE C18, 100 Å (5 μ m, 4.6 $\times$ 250 mm); eluent: solvent A 50% MeCN in water, solvent B MeCN; eluted with a linear gradient of B from 0 – 10% B over 60 min at 0.8 mL/min. Analytical data of **24 fast** are as follows: ^1H NMR (DMSO- d_6) $\delta = 11.76$ (s, 1H, NH), 8.14 (s, 1H, uridine - H6), 7.27 (s, 1H, aryl - H4), 6.14 – 6.10 (m, 1H, H1'), 5.57 – 5.49 (m, 2H, benzyl), 5.37 – 5.29 (m, 1H, H3'), 4.53 – 4.41 (m, 3H, H4', H5', H5''), 2.53 – 2.45 (m, 2H, H2', H2''), 1.36, 1.32 (overlapped s, 18H, aryl - C3 - 3 $\times$ CH₃ - *t*-Bu and aryl - C5 - 3 $\times$ CH₃ - *t*-Bu) ppm. ^{31}P NMR (DMSO- d_6) $\delta = -9.36$ ppm. Analytical data of **24 slow** are: ^1H NMR (DMSO- d_6) $\delta = 11.72$ (s, 1H, NH), 8.08 (s, 1H, uridine - H6), 7.23 (m, 1H, aryl - H4), 6.12 – 6.09 (m, 1H, H1'), 5.53 – 5.41 (m, 2H, benzyl), 5.34 – 5.23 (m, 1H, H3'), 4.55 – 4.44 (m, 2H, H4', H5'), 3.98 – 3.94 (m, 1H, H5''), 2.51 – 2.41 (m, 2H, H2', H2''), 1.33, 1.31 (overlapped s, 18H, aryl - C3 - 3 $\times$ CH₃ - *t*-Bu and aryl - C5 - 3 $\times$ CH₃ - *t*-Bu) ppm. ^{31}P NMR (DMSO- d_6) $\delta = -9.77$ ppm.

Trialkyltin Precursors

5-Tri-*n*-butylstannyl-5'-*O*-cycloSaligenyl-2'-deoxyuridine Monophosphate (**6a**)

—General Procedure D was performed with 5-iodo-5'-*O*-cycloSaligenyl-2'-deoxyuridine monophosphate **6** (520 mg, 1.0 mmol), hexa-*n*-butylditin (750 μ L, 868 mg, 1.5 mmol) and the palladium(II) catalyst (77 mg, 0.11 mmol) in dioxane (35 mL) for 70 min. The crude product was purified by column chromatography on a silica gel, using a gradient of EtOAc in hexanes (2 – 7:10). Further drying, by repeated evaporations with anhydrous MeCN and exposure to a high vacuum, gave **6a** (360 mg, 52%) as rigid colorless foam; R_f value 0.69 (EtOAc/hexanes, 2:1). The HPLC analysis showed a mixture of diastereomers **6a fast**, $t_R = 73.9$ min and **6a slow**, $t_R = 74.4$ min ($\geq 97\%$ pure, UV at 220 and 280 nm); column: ACE C18, 100 Å (5 μ m, 4.6 $\times$ 250 mm); eluent: solvent A water, solvent B MeCN; eluted at 0.8 mL/min with a linear gradient of B from 10 – 50% over 30 min, then 50% B (isocratic) for the period of 30 min, and a linear gradient of B from 50 – 95% for 30 min. Diastereomers were not separated on a preparative scale. ^1H NMR (CDCl₃) $\delta = 8.32$ (s, 1H, NH), 8.27 (s, 1H, NH), 7.33 (s, 1H, uridine - H6), 7.31 (s, 1H, uridine - H6), 7.15 (t, 1H, aryl - H5, $J = 7.6$ Hz), 7.13 – 7.06 (m, 3H, aryl - H3, aryl - H4, aryl - H6), 6.15 – 6.12 (m, 1H, H1'), 5.39 – 5.34 (m, 2H, benzyl), 4.58 – 4.54 (m, 1H, H3'), 4.45 – 4.37 (m, 2H, H5', H5''), 4.12 – 4.09 (m, 1H, H4'), 3.23 (bd, 1H, C3'-OH, exchangeable with D₂O, $J = 4.5$ Hz), 2.46 – 2.40 (m, 1H, H2'), 2.35 – 2.25 (m, 1H, H2''), 1.56 – 1.42 (m, 3 $\times$ 2H, 3 $\times$ CH₂-*n*-Bu), 1.35 – 1.26 (m, 3 $\times$ 2H, 3 $\times$ CH₂-*n*-Bu), 1.02 – 0.98 (m, 3 $\times$ 2H, 3 $\times$ CH₂-*n*-Bu), 0.92 – 0.87 (m, 3 $\times$ 3H, 3 $\times$ 3 $\times$ CH₃-*n*-Bu) ppm. ^{31}P NMR (CDCl₃) $\delta = -8.23, -8.08$ ppm. ^{119}Sn NMR (CDCl₃) $\delta =$

– 1.97 ppm. MSFAB-HR (m/z): $[M + Li]^+$ calcd for $C_{28}H_{43}N_2O_8PSnLi$, 693.1939, found 693.1943.

5-Trimethylstannyl-5'-O-cyclo(3-methylsaligenyl)-2'-deoxyuridine

Monophosphate (7a)—General Procedure D was conducted with 5-iodo-5'-O-cyclo(3-methylsaligenyl)-2'-deoxyuridine monophosphate **7** (710 mg, 1.32 mmol), hexamethylditin (0.64 g, 1.95 mmol) and the palladium(II) catalyst (90 mg, 0.13 mmol) in dioxane (40 mL) for 2 h. Purification of the crude product on a silica gel column ($CHCl_3/MeOH$, 10:0.8) and repeated twice evaporation from dried MeCN, gave pure stannane **7a** (374 mg, 49%) as colorless foam; R_f value 0.67 ($DCM/MeOH$, 10:0.8). Purified **7a** was analyzed on HPLC, using ACE C18, 100 Å (5 µm, 4.6 × 250 mm) column; eluent: solvent A 10% MeCN in water, solvent B MeCN; eluted at 1 mL/min with a linear gradient of B from 0 – 70% over 90 min. The analysis showed a mixture of diastereomers **7a fast** $t_R = 55.2$ min, **7a slow** $t_R = 55.4$ min ($\geq 98\%$ pure, UV at 280 nm). Diastereomers were not separated preparatively. 1H NMR ($CDCl_3$) $\delta = 8.13$ (s, 1H, NH), 8.11 (s, 1H, NH), 7.33 (s, 1H, uridine - H6, $^3J_{H,Sn} = 18.4$ Hz), 7.29 (s, 1H, uridine - H6, $^3J_{H,Sn} = 18.4$ Hz), 7.17 (m, 1H, aryl - H5), 7.12 – 7.04 (m, 3H, aryl - H3, aryl - H4, aryl - H6), 6.20 – 6.15 (m, 1H, H1'), 5.41 – 5.34 (m, 2H, benzyl), 4.55 – 4.52 (m, 1H, H3'), 4.47 – 4.39 (m, 2H, H4', H5'), 4.16 – 4.11 (m, 1H, H5''), 3.57 (bd, 1H, C3'-OH, exchangeable with D_2O , $J = 4.5$ Hz), 2.45 – 2.36 (m, 1H, H2'), 2.34 – 2.27 (m, 1H, H2''), 2.21 – 2.19 (m, 3H, aryl - C3 - CH_3), 0.49 (s, 9H, 3 x CH_3 , $^2J_{Sn,H} = 29.2$ Hz) ppm. ^{31}P NMR ($CDCl_3$) $\delta = -8.37$, -8.18 ppm. ^{119}Sn NMR ($CDCl_3$) $\delta = -1.07$ ppm. MSFAB-HR (m/z): $[M + H]^+$ calcd for $C_{20}H_{28}N_2O_8PSn$, 575.0605, found 575.0593. For the ^{13}C peak measured 576.0640; -0.2 ppm.

5-Trimethylstannyl-5'-O-[cyclo-3,5-di-(tert-butyl)-6-fluorosaligenyl]-2'-deoxyuridine Monophosphate (8a)

—General Procedure D was conducted with 5-iodo-5'-O-[cyclo-3,5-di-(tert-butyl)-6-fluorosaligenyl]-2'-deoxyuridine monophosphate **8** (1.51 g, 2.3 mmol), hexamethylditin (0.98 g, 3.0 mmol) and the palladium(II) catalyst (160 mg, 0.22 mmol) in EtOAc (80 mL) for 2 h. Purification of the crude product required two silica gel columns: $DCM/MeOH$, 10:0.7 and EtOAc/MeOH gradient, 10:0 – 0.2, and final evaporation from dried MeCN, to give a pure stannane **8a** (1.22 g, 76%) as colorless foam; R_f value 0.77 ($DCM/MeOH$, 10:0.7). The HPLC analysis showed a mixture of diastereomers: **8a fast**, $t_R = 43.7$ min and **8a slow**, $t_R = 44.1$ min ($\geq 98\%$ pure, UV at 220 and 280 nm); column: ACE C18, 100 Å (5 µm, 4.6 × 250 mm); eluent: solvent A 50% MeCN in water, solvent B MeCN; eluted at 0.8 mL/min with A for 25 min, then a linear gradient of B from 0 – 95% over 20 min, and 95% B for 15 min. 1H NMR ($CDCl_3$) $\delta = 8.21$ (s, 1H, NH), 8.18 (s, 1H, NH), 7.26 (m, 1H, aryl - H5), 7.20 (s, 1H, uridine - H6, $^3J_{H,Sn} = 18.5$ Hz), 7.16 (s, 1H, uridine - H6, $^3J_{H,Sn} = 18.5$ Hz), 6.20 – 6.17 (m, 1H, H1'), 5.49 – 5.32 (m, 2H, benzyl), 4.62 – 4.56 (m, 1H, H3'), 4.54 – 4.34 (m, 2H, H4', H5'), 4.14 – 4.12 (m, 1H, H5''), 3.05 (bs, 1H, C3'-OH, exchangeable with D_2O), 2.47 – 2.41 (m, 1H, H2'), 2.34 – 2.27 (m, 1H, H2''), 1.39, 1.37, 1.35 (3 x s, 18H, aryl C3 – 3 x CH_3 - *t*-Bu, aryl C5 – 3 x CH_3 - *t*-Bu), 0.24 (t, 9H, 3 x CH_3Sn , $^2J_{Sn,H} = 29.0$ Hz), 0.22 (t, 9H, 3 x CH_3Sn , $^2J_{Sn,H} = 29.0$ Hz) ppm. ^{31}P NMR ($CDCl_3$) $\delta = -8.53$, -8.38 ppm. ^{119}Sn NMR ($CDCl_3$) $\delta = -1.17$ ppm. MSFAB-HR (m/z): $[M + H]^+$ calcd for $C_{27}H_{41}N_2O_8PFSn$, 691.1607, found 691.1640. For ^{13}C peak measured 692.1653; 1.8 ppm. Diastereomers were separated by preparative HPLC (≤ 440 µg of **8a** per injection), using a tandem of two ACE C18, 100 Å columns (5 µm, 4.6 × 250 mm); eluent: solvent A 50% MeCN in water, solvent B MeCN; eluted at 0.7 mL/min with a linear gradient of B from 0 – 10% B over 110 min. After numerous HPLC injections, 16.4 mg of **8a fast** and 23.2 mg of **8a slow** was isolated. Diastereomer **8a fast** eluted within 87.5 – 90 min and **8a slow** within 90.5 – 93 min, past the injection. After an each separation run, the isolated isomer (120 – 200 µg) was collected in ~ 2.5 mL of the eluate. The solvent was evaporated to dryness in a high vacuum; the combined product

residue was reconstituted in MeCN and analyzed again on the analytical HPLC (each diastereomer was $\geq 98\%$ pure, UV at 220 and 280 nm). Analytical data of **8a fast** are as follows: NMR (CDCl_3) δ = 7.84 (s, 1H, NH), 7.26 (m, 1H, aryl – H5), 7.20 (s, 1H, uridine – H6, $^3J_{\text{H,Sn}}$ = 18.5 Hz), 6.18 (t, 1H, H1', $^2J_{\text{H,H}}$ = 6.5 Hz), 5.46 (d, 1H, 1 x H-benzyl, $^2J_{\text{H,H}}$ = 14.0 Hz), 5.34 (d, 1H, 1 x H-benzyl, $^2J_{\text{H,H}}$ = 14.0 Hz), 4.62 – 4.58 (m, 1H, H3'), 4.44 – 4.41 (m, 2H, H4', H5'), 4.13 – 4.11 (m, 1H, H4'), 2.51 (bs, 1H, C3'-OH, exchangeable with D_2O), 2.45 – 2.41 (m, 1H, H2'), 2.31 – 2.25 (m, 1H, H2''), 1.37, 1.35 (2 x s, 18H, aryl C3 – 3 x CH_3 – *t*-Bu, aryl C5 – 3 x CH_3 – *t*-Bu), 0.23 (t, 9H, 3 x CH_3Sn , $^2J_{\text{H,Sn}}$ = 29.0 Hz) ppm. ^{31}P NMR (CDCl_3) δ = – 8.52 ppm. Analytical data of **8a slow** are as follows: ^1H NMR (CDCl_3) δ = 7.86 (s, 1H, NH), 7.27 (m, 1H, aryl – H5), 7.16 (s, 1H, uridine – H6, $^3J_{\text{H,Sn}}$ = 18.5 Hz), 6.16 (t, 1H, H1', J = 6.5 Hz), 5.47 – 5.34 (m, 2H, benzyl), 4.63 – 4.58 (m, 1H, H3'), 4.55 – 4.50 (m, 2H, H5'), 4.38 – 4.33 (m, 2H, H5''), 4.07 – 4.04 (m, 1H, H4'), 2.80 (bs, 1H, C3'-OH, exchangeable with D_2O), 2.47 – 2.42 (m, 1H, H2'), 2.35 – 2.30 (m, 1H, H2''), 1.39, 1.36 (2 x s, 18H, aryl C3 – 3 x CH_3 – *t*-Bu, aryl C5 – 3 x CH_3 – *t*-Bu), 0.24 (t, 9H, 3 x CH_3Sn , $^2J_{\text{H,Sn}}$ = 29.0 Hz) ppm. ^{31}P NMR (CDCl_3) δ = – 8.39 ppm.

5-Trimethylstannyl-3'-O-cycloSaligenyl-2'-deoxyuridine Monophosphate (**12a**)

—General Procedure D was carried out with 5-iodo-3'-O-cycloSaligenyl-2'-deoxyuridine monophosphate **12** (193 mg, 0.37 mmol), hexamethylditin (157 mg, 0.48 mmol) and the palladium(II) catalyst (26 mg, 0.037 mmol) in EtOAc (30 mL) until starting **12** was detected on TLC (~ 1.5 h). The crude product was initially separated and purified on a silica gel column (DCM/MeOH, 10:0.6). Further purification was done using the HPLC equipped with the semi preparative Columbus C18, 100 Å (5µm, 10 × 250 mm) column; eluent: solvent A 45% MeCN, solvent B MeCN; eluted at 2.5 mL/min with a linear gradient of B for 60 min. Combined fractions, after repetitive evaporation from dried MeCN, gave pure stannane **12a** (119 mg, 57%) as colorless amorphous solid; R_f value 0.64 (DCM/MeOH, 10:0.3). The HPLC analysis showed a mixture of diastereomers: **12a fast**, t_R = 47.0 min and **12a slow**, t_R = 47.4 min ($\geq 96\%$ pure, UV at 280 nm); column: ACE C18, 100 Å (5µm, 4.6 × 250 mm); eluent: solvent A 10% MeCN in water, solvent B MeCN; eluted at 1 mL/min with a linear gradient of B from 0 – 95% over 45 min, and then 95% B for 15 min. ^1H NMR (CDCl_3) δ = 8.79 (s, 1H, NH), 8.63 (s, 1H, NH), 7.49 (s, 1H, uridine – H6, $^3J_{\text{H,Sn}}$ = 18.5 Hz), 7.37 (s, 1H, uridine – H6, $^3J_{\text{H,Sn}}$ = 18.5 Hz), 7.17 (t, 1H, aryl – H4, J = 7.6 Hz), 7.14 – 7.07 (m, 3H, aryl – H3, H5, H6), 6.23 – 6.16 (m, 1H, H1'), 5.42 – 5.36 (m, 2H, benzyl), 4.31 – 4.28 (m, 1H, H3'), 4.25 – 4.21 (m, 1H, H4'), 3.97 – 3.85 (m, 2H, H5', H5'', H4'), 3.58 (bd, 1H, C5'-OH, exchangeable with D_2O , J = 4.4 Hz), 2.61 – 2.45 (m, 2H, H2', H2''), 0.32 (t, 3 x 3H, 3 x CH_3Sn , $^2J_{\text{H,Sn}}$ = 29.0 Hz) ppm. ^{31}P NMR (CDCl_3) δ = – 9.80, – 9.82 ppm. ^{119}Sn NMR (CDCl_3) δ = – 1.47 ppm. MSFAB-HR (m/z): $[\text{M} + \text{Li}]^+$ calcd for $\text{C}_{19}\text{H}_{25}\text{N}_2\text{O}_8\text{PSnLi}$, 567.0531, found 567.0549. The ^{13}C isotope measured 568.0559; – 0.9 ppm of the expected 568.0565 value.

5-Trimethylstannyl-3'-O-cyclo(3-methylSaligenyl)-2'-deoxyuridine

Monophosphate (13a)—General Procedure D was carried out with iodo-3'-O-cyclo(3-methylsaligenyl)-2'-deoxyuridine Monophosphate **13** (241 mg, 0.45 mmol), hexamethylditin (192 mg, 0.58 mmol) and the palladium(II) catalyst (32 mg, 0.046 mmol) in EtOAc (30 mL) until starting **13** disappeared on TLC (~ 2 h). The crude product was separated and purified at first on a silica gel column (DCM/MeOH gradient, 10:0.6 – 0.9). Final purification proceeded on the HPLC equipped with a semi preparative Columbus C18, 100 Å (5µm, 10 × 250 mm) column; eluent: solvent A 45% MeCN, solvent B MeCN; eluted at 2.5 mL/min with a linear gradient of B for 60 min. Combined and dried fractions, gave pure stannane **13a** (139 mg, 54%) as a colorless amorphous solid; R_f value 0.71 (DCM/MeOH, 10:0.8). The HPLC analysis showed a mixture of diastereomers: **13a fast**, t_R = 38.5 min and **13a slow**, t_R = 39.7 min ($\geq 98\%$ pure, UV at 280 nm); column: ACE C18, 100 Å (5µm, 4.6 ×

250 mm); eluent: solvent A 15% MeCN in water, solvent B MeCN; column eluted at 1 mL/min with a linear gradient of B from 0 – 25% over 50 min, then a gradient of B from 25 – 95% B over 5 min, and the 95% for 5 min. ^1H NMR (CDCl_3) δ = 8.73 (bs, 1H, NH), 7.52 (s, 1H, uridine - H6, $^3J_{\text{H,Sn}}$ = 18.6 Hz), 7.43 (s, 1H, uridine - H6, $^3J_{\text{H,Sn}}$ = 18.6 Hz), 7.18 – 7.03 (m, 3H, aryl - H4, H5, H6), 6.27 – 6.18 (m, 1H, H1'), 5.46 – 5.38 (m, 2H, benzyl), 4.30 – 4.25 (m, 1H, H3'), 4.23 – 4.21 (m, 1H, H4'), 3.97 – 3.85 (m, 2H, H5', H5''), 3.63 (bd, 1H, C5'-OH, exchangeable with D_2O , J = 4.4 Hz), 2.64 – 2.43 (m, 5H, H2', H2'', aryl - C3 - CH_3), 0.37 (t, 3 x 3H, 3 x CH_3Sn , $^2J_{\text{H,Sn}}$ = 29.0 Hz) ppm. ^{31}P NMR (CDCl_3) δ = - 9.84, - 9.77 ppm. ^{119}Sn NMR (CDCl_3) δ = - 1.41 ppm. MSFAB-HR (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{20}\text{H}_{28}\text{N}_2\text{O}_8\text{PSn}$, 575.0605, found 575.0624. The ^{13}C isotope measured 576.0624; -2.5 ppm of the expected value.

5-Trimethylstannyl-3'-O-(cyclo-3,5-di-(tert-butyl)-6-fluoroSaligenyl)-2'-deoxyuridine Monophosphate (14a)—General Procedure D was carried out with 5-

iodo-3'-O-(cyclo-3,5-di-(tert-butyl)-6-fluorosaligenyl)-2'-deoxyuridine monophosphate **14** (306 mg, 0.47 mmol), hexamethylditin (205 mg, 0.62 mmol) and the palladium(II) catalyst (34 mg, 0.048 mmol) in EtOAc (40 mL) until starting **14** disappeared on TLC (~ 3 h). The crude product was separated on a silica gel column (DCM/MeOH, 10:0.4) and the purification repeated (using EtOAc/hexanes, 10:5). Final purification was conducted using the HPLC equipped with the semi preparative Columbus C18, 100 Å (5µm, 10 × 250 mm) column; eluent: solvent A 45% MeCN, solvent B MeCN and a column eluted at 2.5 mL/min with a linear gradient of B from 0 – 95% over the period of 60 min. Combined fractions were evaporated under a vacuum and the residue dried by repetitive evaporation from dry MeCN, gave pure stannane **14a** (206 mg, 63%) as colorless ridged foam; R_f value 0.79 (DCM/MeOH, 10:0.5). The HPLC analysis showed a mixture of diastereomers: **14a fast**, t_R = 26.4 min and **14a slow**, t_R = 26.9 min (≥ 98% pure, UV at 220 and 280 nm); column: ACE C18, 100 Å (5µm, 4.6 × 250 mm); eluent: solvent A 50% MeCN in water, solvent B MeCN; eluted at 1 mL/min with a linear gradient of B from 0 – 95% over 40 min, then 95% B for 20 min. ^1H NMR (CDCl_3) δ = 8.63 (bs, 1H NH), 7.44 (s, 1H, uridine - H6, $^3J_{\text{H,Sn}}$ = 18.5 Hz), 7.40 (s, 1H, uridine - H6, $^3J_{\text{H,Sn}}$ = 18.5 Hz), 7.19 (t, 1H, aryl - H4, J = 7.6 Hz), 6.20 – 6.14 (m, 1H, H1'), 5.44 – 5.32 (m, 2H, benzyl), 4.33 – 4.29 (m, 1H, H3'), 4.27 – 4.24 (m, 1H, H4'), 3.94 – 3.82 (m, 2H, H5', H5''), 3.66 (bd, 1H, C5'-OH, exchangeable with D_2O , J = 4.5 Hz), 2.64 – 2.47 (m, 2H, H2', H2''), 1.325, 1.315, 1.312, 1.311 (overlapped s, 18H, aryl - C3 - 3 x CH_3 - *t*-Bu and aryl - C5 - 3 x CH_3 - *t*-Bu), 0.24 (t, 3 x 3H, 3 x CH_3Sn , $^2J_{\text{H,Sn}}$ = 29.0 Hz) ppm. ^{31}P NMR (CDCl_3) δ = - 9.72, - 9.62 ppm. ^{119}Sn NMR (CDCl_3) δ = - 1.43 ppm. MSFAB-HR (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{27}\text{H}_{41}\text{N}_2\text{O}_8\text{PFSn}$, 691.1607, found 691.1635. The ^{13}C isotope measured 692.1648; 1.1 ppm of the expected value.

5-Trimethylstannyl-3'-fluoro-2',3'-dideoxyuridine (21a)—General Procedure D was carried out with 5-iodo-3'-fluoro-2',3'-dideoxyuridine **21** (203 mg, 0.57 mmol), hexamethylditin (250 mg, 0.76 mmol) and the palladium(II) catalyst (40 mg, 0.057 mmol) in EtOAc (25 mL), until starting **21** disappeared on TLC (~ 90 min). Noticeable proton deiodination occurred. The crude product was separated and purified at first on a silica gel column (DCM/MeOH gradient, 10:0.3 - 0.7) and the purification was continued on the HPLC equipped with the semi preparative Columbus C18, 100 Å (5µm, 10 × 250 mm) column; eluent: solvent A H_2O , solvent B MeCN; column was eluted at the 2.7 mL/min flow rate with a linear gradient of B from 0 – 95% for the period of 45 min, and then 95% B for 15 min. Combined fractions (29 – 31) were evaporated and dried in a high vacuum, to give the pure stannane **21a** (110 mg, 49%) as colorless foam; R_f value 0.72 (DCM/MeOH, 10:0.7). The HPLC analysis showed a single peak at t_R = 27.1 min (≥ 95% pure, UV at 280 nm); column: ACE C18, 100 Å (5µm, 4.6 × 250 mm); eluent: solvent A H_2O , solvent B MeCN; eluted at 1 mL/min with a linear gradient of B from 0 – 95% over 45 min, then 95%

B for 15 min. ^1H NMR (DMSO- d_6) δ = 11.19 (s, 1H, NH), 7.63 (t, 1H, uridine - H6, $^3J_{\text{H},\text{Sn}}$ = 18.5 Hz), 6.24 – 6.21 (m, 1H, H1'), 5.34 (dd, 1H, H3', $^2J_{\text{H},\text{H}}$ = 12.0 Hz, $^2J_{\text{H},\text{H}}$ = 7.8 Hz), 5.15 (t, 1H, C5' – OH, $^2J_{\text{H},\text{H}}$ = 5.5 Hz), 4.17 (d, 1H, H4', $^2J_{\text{H},\text{H}}$ = 10.6 Hz), 3.66 – 3.53 (m, 2H, H5', H5''), 2.50 – 2.26 (m, 2H, H2', H2''), 0.19 (t, 3 x 3H, 3 x CH₃Sn, $^2J_{\text{H},\text{Sn}}$ = 28.5 Hz) ppm. ^{119}Sn NMR (DMSO- d_6) δ = – 1.22 ppm. MSFAB-HR (m/z): [M + Li]⁺calcd for C₁₂H₁₉N₂O₄FSnLi, 401.0511, found 401.0516. The ^{13}C isotope peak measured 402.0555, which was within 2.6 ppm of the expected value of 402.0545.

5-Trimethylstannyl-5'-O-[cyclo-3,5-Di-(*tert*-butyl)-6-fluoroSaligenyl]-3'-fluoro-2',3'-dideoxyuridine Monophosphate (24a)—General Procedure D was carried out with 5-iodo-5'-O-[cyclo-3,5-di-(*tert*-butyl)-6-fluorosaligenyl]-3'-fluoro-2',3'-dideoxyuridine **24** (166 mg, 0.25 mmol), hexamethylditin (112 mg, 0.34 mmol) and the palladium(II) catalyst (36 mg, 0.052 mmol) in EtOAc (14 mL) until starting **24** despaired (~45 min) on TLC (EtOAc/MeOH, 2:1). The crude product was initially separated and partially purified on a silica gel column (DCM/MeOH gradient, 10:0.2 - 0.5) and the purification was continued on the HPLC, equipped with a semi preparative Columbus C18, 100 Å (5µm, 10 × 250 mm) column; eluent: solvent A 45% MeCN, solvent B MeCN; eluted with a linear gradient of B from 0 – 95% over 60 min, at 2.5 mL/min flow rate. Combined fractions after repetitive evaporation from dry MeCN, gave pure stannane **24a** (87 mg, 49%) as colorless foam; R_f value 0.84 (DCM/MeOH, 10:0.5). The HPLC analysis showed a mixture (47 : 53 ratio) of diastereomers: **24a fast**, t_R = 37.6 min and **24a slow**, t_R = 38.2 min (≥ 95% pure, UV at 280 nm); column: ACE C18, 100 Å (5µm, 4.6 × 250 mm); eluent: solvent A 50% MeCN in water, solvent B MeCN; eluted with a linear gradient of B from 0 – 95% B over 60 min and then 95% B for 30 min at 1 mL/min. Diastereomers were not separated preparatively. ^1H NMR (DMSO- d_6) δ = 11.26 (bs, 1H, NH), 7.30 (s, 1H, uridine - H6, $^3J_{\text{H},\text{Sn}}$ = 18.5 Hz), 7.29 (s, 1H, uridine - H6, $^3J_{\text{H},\text{Sn}}$ = 18.5 Hz), 7.25 (s, 1H, aryl – H4), 7.23 (m, 1H, aryl – H4), 6.18 – 6.11 (m, 1H, H1'), 5.58 – 5.29 (m, 3H, benzyl, H3'), 4.70 – 4.32 (m, 3H, H4', H5', H5''), 2.54 – 2.42 (m, 2H, H2', H2''), 1.331, 1.324, 1.319, 1.314 (overlapped s, 18H, aryl – C3 - 3 x CH₃ – *t*Bu and aryl – C5 - 3 x CH₃ – *t*Bu), 0.183 (t, 3 x 3H, 3 x CH₃Sn, $^2J_{\text{H},\text{Sn}}$ = 29.0 Hz), 0.169 (t, 3 x 3H, 3 x CH₃Sn, $^2J_{\text{H},\text{Sn}}$ = 29.0 Hz) ppm. ^{31}P NMR (DMSO- d_6) δ = – 9.48, – 9.90 ppm. ^{119}Sn NMR (DMSO- d_6) δ = – 1.33 ppm. MSFAB-HR (m/z): [M + Li]⁺calcd for C₂₇H₃₉N₂O₇PF₂SnLi, 699.1645, found 699.1637. The ^{13}C isotope peak measured 700.1682, 0.4 ppm of the expected value of 700.1679.

[^{125}I]-Radioiodinated cycloSaligenyl Phosphotriesters

5-[^{125}I]-Iodo-5'-O-cycloSaligenyl-2'-deoxyuridine Monophosphate (6b)—General Procedure E was conducted within 20 – 433 MBq range and repeated six times, to give ~1550 MBq of **6b** in successive radioiodinations of the stannyl precursor **6a**. An average isolated yield of the product was 88%. The latest preparation was carried out with stannane **6a** (120 µg) and [^{125}I]NaI/NaOH (94 µL, 377.4 MBq). The HPLC purification of the product proceeded on Jupiter C18, 300 Å (5µm, 4.6 × 250 mm) column; eluent: solvent A 10% MeCN in water, solvent B MeCN and 0.8 mL/min elution rate of a linear gradient of B from 0 – 20% over 33 min, followed by a linear gradient of B from 20 – 95% for 5 min, and finally 95% B for the period of 15 min. The main radioactivity peak (344.1MBq, 91% yield) was eluted and collected in four fractions of the eluate (a total volume ~ 3.3 mL), within 28 – 32 min after the injection of 460 µL (~373.7 MBq) of the reaction mixture. An excess of unreacted tin precursor **6a** was separated from the radioiodinated product without difficulty, eluting ~ 20 min later (t_R = 50.6 min). If required, diastereomers of **6b** were separated using the same HPLC conditions. The diastereomer **6b fast** eluted at t_R = 29.8 min and **6b slow** at t_R = 30.8 min. When a solution of 20% MeCN in water was used as the solvent A and the column was eluted at the 0.8 mL/min flow rate with solvent A for the period of 25 min, followed by a linear gradient of B from 0 – 95% over 10 min, and finally with 95% B for 10

min; diastereomers were eluted faster and fully separated: **6b fast**, $t_R = 20.6$ min and **6b slow**, $t_R = 22.4$ min. In a single HPLC run, the complete separation (each diastereomer $\geq 98\%$ pure, Bioscan NaI(T) detector) was achieved, if the total amount of injected **6b** was ≤ 8.51 MBq. Larger batches of the single diastereomer were acquired by repeating the HPLC injections, or using a semi preparative column: Columbus C18, $100 \text{ \AA}$ ($5 \mu\text{m}$, 10×250 mm), eluted at the 2.6 mL/min flow rate with a 20% MeCN solution in water. Diastereomer **6b fast** eluted within $75 - 77$ min and **6b slow** $83 - 87$ min, past the injection, and each was collected in ~ 8 mL volume of the eluate. The solvent was evaporated to dryness in a high vacuum at 30°C , using the SpeedVac system. Log P values: **6b fast** $0.63 (\pm 0.02)$, **6b slow** $0.66 (\pm 0.03)$. The HPLC co-injections of the purified **6b** with the nonradioactive analog **6**, and parallel monitoring of the radioactivity and UV signal, confirmed the identical elution of both compounds.

5-[^{125}I]-Iodo-5'-O-cyclo(3-methylSaligenyl)-2'-deoxyuridine Monophosphate (7b)—General Procedure E was conducted within $19 - 374$ MBq range, to give ~ 1220 MBq of **7b** from six repeated radioiodinations of the stannyl precursor **7a**. An average isolated yield was 81% . The latest radioiodination was performed with stannane **7a** ($\sim 110 \mu\text{g}$) and [^{125}I]NaI/NaOH ($90 \mu\text{L}$, 344.1 MBq). The HPLC purification of the product continued on Jupiter C18, $300 \text{ \AA}$ ($5 \mu\text{m}$, 4.6×250 mm) column; eluent: solvent A 18% MeCN in water, solvent B MeCN; and 0.8 mL/min flow rate. The column was eluted with solvent A for the period of 30 min, then with a linear gradient of B from $0 - 95\%$ over 10 min, and 95% B for 20 min. The product **7b** (299.7 MBq, 87%), which eluted within $21.5 - 24.7$ min after the injection of $410 \mu\text{L}$ (~ 336.7 MBq) of the reaction mixture, was collected in three fractions (2.5 mL volume of the eluate). An excess of unreacted tin precursor **7a** was fully separated, eluting between $27.3 - 27.7$ min. The HPLC co-injections of the purified **7b** with nonradioactive analog **7**, and monitoring the radioactivity (Bioscan NaI(T) detector) and UV signal at 280 nm, verified the same HPLC mobility of both compounds. Diastereomers of **7b** were separated on Jupiter C18, $300 \text{ \AA}$ ($5 \mu\text{m}$, 4.6×250 mm) column, eluted with 20% MeCN solution in water, for the period of 45 min at the 0.8 mL/min flow rate. Single diastereomers: **7b fast** ($t_R = 25.3$ min) and **7b slow** ($t_R = 26.8$ min) were $\geq 98\%$ pure (Bioscan NaI(T)). In the course of a single HPLC run, the full separation of diastereomers was limited to the total of ~ 9.98 MBq **7b** loaded onto a column. Larger batches of resolved diastereomers were attainable through the repetitive HPLC injections, or using a larger column: Columbus C18, $100 \text{ \AA}$ ($5 \mu\text{m}$, 10×250 mm); eluent: a solution of 22% MeCN in water and the flow rate of 2.5 mL/min. Diastereomer **7b fast** eluted within $72 - 77$ min, and **7b slow** within $79 - 81$ min past the injection. The solvent was evaporated to dryness in a high vacuum, on the SpeedVac system. Log P values: **7b fast** $0.85 (\pm 0.04)$, **7b slow** $0.86 (\pm 0.04)$. The product residue was analyzed on the analytical HPLC shortly before conducting scheduled experiments and was reconstituted in an appropriate solvent.

5-[^{125}I]-Iodo-5'-O-[cyclo-3,5-di-(tert-butyl)-6-fluoroSaligenyl]-2'-deoxyuridine Monophosphate (8b)—The overall ~ 1925 MBq of **8b** was acquired in eleven radioiodinations, using one of the purified tin precursors: **8a**, **8a fast** or **8a slow**, and conducting General Procedure E within the $9.25 - 396$ MBq range. An average isolated yield of the product was 88% . The latest radiolabeling was performed with diastereomeric stannane **8a** ($\sim 115 \mu\text{g}$) and [^{125}I]NaI/NaOH ($40 \mu\text{L}$, 144.7 MBq). The HPLC purification of the product proceeded on ACE C18, $100 \text{ \AA}$ ($5 \mu\text{m}$, 4.6×250 mm) column; eluent: solvent A 50% MeCN in water, solvent B MeCN; and the flow rate of 1.0 mL/min. A column was eluted with solvent A for the period of 60 min, then with a linear gradient of B from $0 - 95\%$ over 10 min, and 95% B for 20 min. The product **8b** (128.76 MBq, 89%), which eluted within $25 - 29$ min after the injection of $350 \mu\text{L}$ (~ 139.12 MBq) of the reaction mixture, was collected in four fractions. An excess of unreacted stannane **8a** was eluting ~ 15 min later.

The mixture of purified **8b** (~444 kBq, 10 μ L) and the corresponding nonradioactive analog **8** (~15 μ g, 20 μ L) was prepared in acetonitrile, and injected onto the HPLC, using the same column and conditions as during the separation of the product. Both compounds eluted together, showing the identical retention times. Diastereomers of **8b** were separated on ACE C18, 100 $\text{\AA}$ (5 μ m, 4.6 $\times$ 250 mm) column, eluted at the 0.8 mL/min flow rate, with a 45% MeCN solution in water, for a period of 45 min. Each diastereomer: **8b fast** (t_R = 30.3 min) and **8b slow** (t_R = 32.8 min), was $\geq$ 98% pure (Bioscan NaI(Tl)). The complete separation of diastereomers in the single HPLC run was reached, if the total amount of **8b** loaded onto the column was $\leq$ 8.14 MBq. Larger batches of the individual diastereomers were obtained by the repetitive HPLC injections of purified **8b**, or by conducting the radioiodination using a single diastereomer of the tin precursor **8a fast** or **8a slow**. Fractions containing the product were combined and the solvent was evaporated in a high vacuum on the SpeedVac system. Log P values: **8b fast** 2.22 ($\pm$ 0.16), **8b slow** 2.24 ($\pm$ 0.14). The product residue was reconstituted in MeCN and analyzed again on the analytical HPLC, before conducting planned experiments.

5-[125 I]-Iodo-3'-O-cycloSaligenyl-2'-deoxyuridine Monophosphate (12b**)**—Four consecutive radioiodinations was carried out according to General Procedure E within the 9.25 – 262.7 MBq range, to give overall 360.4 MBq of **12b**. An average isolated yield of the product was 87%. The latest radiolabeling was performed with the diastereomeric stannane **12a** (~100 μ g, ~ 97% pure, UV at 280 nm) and [125 I]NaI/NaOH (65 μ L, 262.7 MBq). The HPLC purification of the product proceeded on ACE C18, 100 $\text{\AA}$ (5 μ m, 4.6 $\times$ 250 mm) column; eluent: solvent A 10% MeCN in water, solvent B MeCN; eluted at 1.0 mL/min with a linear gradient of B from 0 – 40% over 40 min, followed by a gradient of B from 40 – 95% for the period of 20 min. The radioactivity peak of **12b** (233.84 MBq, 89%) was collected in four fractions, within 21 – 25 min after the injection of the reaction mixture (~ 500 μ L, 256.1 MBq). An excess of the unreacted tin precursor **12a** was separated from the radioiodinated product, eluting ~ 5 min later (t_R = 30.2 min). Combined fractions containing **12b** were evaporated, reconstituted in MeCN (~66.7 MBq/mL) and 10 μ L volume (~666 kBq) was re-injected on the HPLC: ACE C18, 100 $\text{\AA}$ (5 μ m, 4.6 $\times$ 250 mm) column; eluent: solvent A 10% MeCN in water, solvent B MeCN; eluted at 0.8 mL/min with a linear gradient of B from 0 – 40% over 45 min, then a gradient of B from 40 – 95% for a period of 15 min. Analysis showed a mixture (44:56 ratio) of diastereomers: **12b fast**, t_R = 26.4 min and **12b slow**, t_R = 26.7 min ($\geq$ 98% pure, Bioscan NaI(Tl)). Diastereomers of **12b** were not separated preparatively. The co-injected solutions (in 50% MeCN in water) of the purified **12b** and the corresponding nonradioactive analog **12**, with parallel monitoring of the radioactivity and UV signal, has shown the identical elution mobility of both analogs. Diastereomer **12b fast** eluted at t_R = 26.3 min and **12b slow** at t_R = 26.7 min, together with **12 fast**; t_R = 26.1 min and **12 slow**; t_R = 26.4 min.

5-[125 I]-Iodo-3'-O-cyclo(3-methylSaligenyl)-2'-deoxyuridine Monophosphate (13b**)**—The total amount of prepared **13b** was 462.5 MBq, obtained in five successive radioiodinations of **13a**, carried out within 9.25 – 189 MBq range. Each reaction proceeded according to General Procedure E. An average isolated yield of the product was 73%. The latest radiolabeling was performed with the diastereomeric stannane **13a** (~100 μ g) and [125 I]NaI/NaOH (45 μ L, 174.3 MBq). The HPLC purification was achieved on Jupiter C18, 300 $\text{\AA}$ (5 μ m, 4.6 $\times$ 250 mm) column; eluent: solvent A 10% MeCN in water, solvent B MeCN. A column was eluted at 1.0 mL/min with a linear gradient of B from 0 – 95% over 45 min, then 95% B for the period of 15 min. The radioactivity peak of **13b** (123.95 MBq, 71%) was collected within three fractions (24 – 27 min) after the injection of ~ 300 μ L (160.95 MBq) of the reaction mixture. An excess of the unreacted tin precursor **13a** was separated from the radioiodinated product, eluting ~ 6 min later (t_R = 29.6 min). Combined

fractions containing the purified **13b** were evaporated, reconstituted in MeCN (~44.5 MBq/mL) and 10 μ L volume (~444 kBq), was re-injected on the HPLC column: Jupiter C18, 300 $\text{\AA}$ (5 μ m, 4.6 $\times$ 250 mm); eluent: solvent A 10% MeCN in water, solvent B MeCN; eluted at 1.0 mL/min with a linear gradient of B from 0 – 95% over 45 min, then 95% B for the period of 15 min. The analysis showed a mixture (47: 53 ratio) of **13b fast**, t_R = 23.4 min and **13b slow**, t_R = 23.7 min ($\geq$ 98% pure, Bioscan NaI(T)). Diastereomers of **13b** were not separated preparatively. Co-injected solutions (in 50% MeCN) of the purified **13b** (~660 kBq, 15 μ L) and the nonradioactive analog **13** (~17 μ g, 25 μ L) were analyzed, with monitoring the radioactivity (Bioscan NaI(T)) and UV at 280 nm, on ACE C18, 100 $\text{\AA}$ (5 μ m, 4.6 $\times$ 250 mm) column; eluent: solvent A 10% MeCN in water, solvent B MeCN; eluted at 1.0 mL/min with a linear gradient of B from 0 – 95% over a period of 60 min. The analysis confirmed the identical elution of both, the [125 I]- and [127 I]-iodoanalog.

5-[125 I]-Iodo-3'-O-(cyclo-3,5-di-(tert-butyl)-6-fluoroSaligenyl)-2'-deoxyuridine Monophosphate (14b)—General Procedure E was carried out within 18.5 – 211 MBq range, to give after four conducted radioiodinations, 466.2 MBq of **14b** in an average yield of 86%. The latest radiolabeling proceeded with stannane **14a** (~110 μ g) and [125 I]NaI/NaOH (60 μ L, 211 MBq). The HPLC purification of the crude product was achieved on Jupiter C18, 300 $\text{\AA}$ (5 μ m, 4.6 $\times$ 250 mm) column; eluent: solvent A 40% MeCN in water, solvent B MeCN. A column was eluted at 1.0 mL/min of the flow rate, with a linear gradient of B from 0 – 95% over 35 min, followed by 95% B for the period of 25 min. The product (179.1 MBq, 81%) collected within 21 – 23 min after the injection of 425 μ L (~203.5 MBq) of the reaction mixture was separated from an excess of the unreacted tin precursor **14a**, which eluted ~ 10 min later (32.8 – 33.6 min). Appropriate fractions were combined, evaporated with a stream of nitrogen, and the residue further dried in a high vacuum. The mixture of purified **14b** (~440 kBq, 10 μ L) and the corresponding nonradioactive analog **14** (~15 μ g, 20 μ L) in acetonitrile, was reinjected onto the HPLC, using the same settings as during the separation of the crude product. The analysis has shown **14b fast** at t_R = 21.2 min and **14b slow**; t_R = 21.7 min, co-eluting with diastereomers of the [127 I]-iodoanalog: **14 fast**, t_R = 20.9 min and **14 slow**, t_R = 21.6 min. Diastereomers of **14b** were most efficiently separated on ACE C18, 100 $\text{\AA}$ (5 μ m, 4.6 $\times$ 250 mm) column, eluted with 50% MeCN in water at the 0.8 mL/min flow rate. Each isomer, **14b fast** (t_R = 41.2 min) and **14b slow** (t_R = 45.7 min), was $\geq$ 98% pure (Bioscan NaI(T) detector). The complete separations of diastereomers in the single HPLC run were attained, if the total amount of **14b** loaded onto a column was $\leq$ 11.9 MBq. Larger lots of the single diastereomers were obtained in multiple injections.

5-[125 I]-Iodo-3'-fluoro-2',3'-dideoxyuridine (21b)—General Procedure E was performed in four consecutive radioiodinations, carried out within 19.2 – 148 MBq range, to give at the end 237 MBq of **21b**. An average isolated yield of the product was 91%. The largest conducted radiolabeling proceeded with the stannane **21a** (~120 μ g) and [125 I]NaI/NaOH (35 μ L, 148.4 MBq). The HPLC purification of the crude product was achieved on ACE C18, 100 $\text{\AA}$ (5 μ m, 4.6 $\times$ 250 mm) column; eluent: solvent A water, solvent B MeCN; with the 1.0 mL/min flow rate. A column was eluted with a linear gradient of B from 0 – 95% over 40 min, followed by 95% B for the period of 20 min. The product **21b** (136.5 MBq, 92%) was collected in two fractions, within 18 – 20 min after the injection of 420 μ L (~138.4 MBq) of the reaction mixture and was freed from an excess of the unreacted tin precursor **21a**, which eluted ~ 7 min later (27.1 – 27.5 min). Appropriate fractions were combined, the solvent evaporated with a stream of nitrogen and the residue dried in a high vacuum, using the SpeedVac system at 30°C. Log P value – 0.030 ($\pm$ 0.0012). The combined solutions of purified **21b** (~550 kBq, 15 μ L) and the nonradioactive analog **21** (~14 μ g, 25 μ L) were prepared in acetonitrile and injected on the HPLC: ACE C18 100 $\text{\AA}$ (5 μ m, 4.6 $\times$

250 mm) column; eluent: solvent A water, solvent B MeCN; eluted at 0.8 mL/min with a linear gradient of B from 0 – 95% over 45 min. The synchronized monitoring of the radioactivity and UV signal at 280 nm, showed the product ($\geq 98\%$ pure, Bioscan NaI(T)) **21b** eluting after $t_R = 21.8$ min, together with the [^{127}I]-iodoanalog **21** ($t_R = 21.6$ min).

5-[^{125}I]-Iodo-5'-O-[cyclo-3,5-Di-(*tert*-butyl)-6-fluoroSaligenyl]-3'-fluoro-2',3'-dideoxyuridine (cycloSal[^{125}I]UdRFMP) (**24b**)

Method I: Destannylation of Trimethyltin Precursor: The overall quantity of prepared **24b** was 390 MBq, acquired in four consecutive radioiodinations. General Procedure E was carried out within the 18.5 – 192.4 MBq range and an average isolated yield was 93%. The largest conducted radiolabeling proceeded with stannane **24a** (~120 μg) and [^{125}I]NaI/NaOH (60 μL , 192.4 MBq). The HPLC purification of the crude product was best achieved on ACE C18, 100 $\text{\AA}$ (5 μm , 4.6 $\times$ 250 mm) column; eluent: solvent A 50% MeCN in water, solvent B MeCN. The column was eluted at 1.0 mL/min of the flow rate, with a linear gradient of B from 0 – 95% over 60 min, followed by 95% B for the period of 30 min. The product **24b** (177.5 MBq, 92%) collected within 26 – 28 min after the injection of 500 μL (~188.7 MBq) of the reaction mixture, was fully separated from an excess of the tin precursor **24a**, which eluted ~ 9 min later (37.0 – 37.6 min). Fractions containing the product were combined, the solvent evaporated with a stream of nitrogen, and the residue further dried in a high vacuum. The mixture of purified **24b** (~445 kBq, 10 μL) and the nonradioactive analog **24** (~15 μg , 20 μL) was prepared in acetonitrile and analyzed on the HPLC, using the same settings as applied during the separation of the product. The analysis showed diastereomer **24b fast** at $t_R = 26.1$ min and **24b slow**; $t_R = 26.7$ min, co-eluting with the [^{127}I]-iodoanalog: **24 fast**, $t_R = 25.9$ min and **24 slow**, $t_R = 26.5$ min. Diastereomers of **24b** were separated on ACE C18, 100 $\text{\AA}$ (5 μm , 4.6 $\times$ 250 mm) column; eluent: solvent A 50% MeCN in water, solvent B MeCN. The column was eluted with a linear gradient of B from 0 – 10% over 60 min at the 0.8 mL/min flow rate. Each diastereomer, **24b fast** ($t_R = 38.1$ min) and **24b slow** ($t_R = 40.7$ min), was $\geq 98\%$ pure (Bioscan NaI(T) detector). In the single HPLC run a full separation of the both diastereomers was possible, if a total amount of **24b** loaded onto a column was ≤ 20.6 MBq and the column was eluted with 50% MeCN (isocratic) at the 0.8 mL/min flow rate. Larger lots of single diastereomers were obtained by repetitive HPLC injections or using a larger column: Columbus C18, 100 $\text{\AA}$ (5 μm , 10 $\times$ 250 mm); eluted at the 2.2 mL/min flow rate with 57% MeCN in water.

Method II: Direct Coupling of Phosphoramidite (18) with Non-carrier-added 5-[^{125}I]-Iodo-3'-fluoro-2',3'-dideoxyuridine (21b**):** This preparation was repeated five times, starting with 39.6, 50.7, 81.1, 254.2 and 353.4 MBq of purified **21b**. An average isolated yield of the purified product was 43%. Reaction tubes were flame-dried, equipped with rubber septa and magnetic micro stirrers. All preparation steps were conducted under the argon atmosphere and evaporation of solvents was accomplished with a stream of dry nitrogen. Typically, one day before the planned experiment, 2,4-bis-(*tert*-butyl)-5-fluoro-salicyl alcohol (0.05g, 0.2 mmol) was placed in the reaction tube and dissolved in dry diethyl ether (2 mL). After cooling to -17°C, phosphorus trichloride (19 μL , 0.22 mmol) was injected into the reaction mixture, using a syringe and argon pressure; followed by the slow, dropwise addition of the triethylamine solution (60 μL , 0.43 mmol) in dry diethyl ether (1 mL). The resulting mixture was left overnight at -20°C, to facilitate complete precipitation of triethylamine hydrochloride. The following day the reaction tube was centrifuged briefly and ~ 2mL of the clear reaction mixture was withdrawn, and immediately transferred into a second reaction tube. The solvent was evaporated and MeCN (1 mL) added to the residue. After cooling to -20°C, a solution of DIPA (46 μL , 0.38 mmol) in 0.5 mL of dry MeCN was added. During this addition the temperature of the reaction mixture was kept below 0°C. The resulted solution of the crude Saligenyl *N,N*-

diisopropylaminophosphoramidite was transferred via a needle, with help of argon pressure to a third reaction tube, which was immersed in the -40°C cooling bath and contained 3'-fluoro-2',3'-dideoxyuridine **21b** (50.7 MBq, $\sim 6.2 \times 10^{-4}$ μmol) and a solution (~ 0.45 M) of 1*H*-tetrazole (50 μL , 2.5×10^{-2} mmol) in dry MeCN. The resulted mixture was stirred for 5 min, sonicated briefly, and allowed to reach ambient temperature. The stirring continued for 10 min and after cooling to -40°C for a second time, a solution of *tert*-butyl hydroperoxide (5 μL , 3.25×10^{-2} mmol) in *n*-decane was added. A cooling bath was removed and when the reaction mixture reached ambient temperature (~ 20 min), the solvent was evaporated and 1 mL of ethyl acetate and 5% solution of NaHSO_3 (1 mL) were added. The mixture was vortexed, centrifuged, and an aqueous phase washed with EtOAc (3×100 μL). The organic phase, combined with washes, was partially evaporated to a total ~ 500 μL volume and injected onto the HPLC, equipped with the semi-preparative Columbus C18, 100 $\text{\AA}$ (5 μm , 10×250 mm) column; eluent: solvent A water, solvent B MeCN. The column was eluted at 2.5 mL/min flow rate, with a linear gradient of B from 0–95% over 30 min and with 95% B for 60 min. The main radioactivity peak (20.39 MBq, 40%) was collected between 34.1 – 35.2 min after the injection. The solvent was evaporated, a receiver tube further dried in a high vacuum and the residue reconstituted in dry MeCN (~ 37 kBq/ μL). The 10 μL aliquot was injected on the analytical ACE C18, 100 $\text{\AA}$ (5 μm , 4.6×250 mm) column; eluent: solvent A 50% MeCN in water, solvent B MeCN; eluted at 1.0 mL/min with a linear gradient of B from 0 – 95% over a period of 90 min. The analysis showed diastereomer **24b fast** at $t_R = 31.0$ min and **24b slow**; $t_R = 31.9$ min ($\geq 98\%$ pure, Bioscan NaI(Tl)). The HPLC co-injections of this product with the **24b**, previously prepared by the destannylation of **24a** according to Method I and also co-injections with the [^{127}I]-iodoanalog **24**, further confirmed the product identity.

Lipophilicity Measurements

The partition ratio (P) of compounds **6b** – **8b** and **21b** was determined by the shake-tube method. To a glass tube containing a dried residue of [^{125}I]-iodinated compound (740 – 1295 kBq), *n*-octanol (3.5 mL) was added and the solution was vortexed for 1 min. The three aliquots (1.0 mL each) were transferred into clean tubes and to each tube 1 mL of aqueous phosphate buffered saline (10 mM, pH 6.8) was added. The mixtures, in tightly closed tubes, were vortexed vigorously for 5 min until a homogenous suspension has formed. This was repeated twice. Organic and water layers were separated by a centrifugation at 4,000 rpm for 10 min at 20°C . Aliquots of 10, 25 and 40 μL , from each phase were carefully removed and counted in a gamma counter in a triplicate. Each P was calculated as an average of $n \geq 18$.

Chemical hydrolysis of the cycloSal phosphate triesters

Experiments to determine and compare the chemical stability and hydrolysis of the synthesized triesters were conducted in phosphate buffer, containing $\sim 15\%$ of acetonitrile. The presence of acetonitrile was necessary due to lower water solubility of the compounds **8**, **14** and **24**. Each phosphotriester **6** – **8**, **12** – **14** and **24** was dissolved in acetonitrile (1.1 – 2.4 mg / mL), just before the experiment and aliquots (100 μL) were withdrawn and transferred into vials containing phosphate buffer (550 μL , 50 mM, pH 7.3). Solutions were briefly vortexed, filtered through the membrane filter (ABF, 0.22 μm) and the initial concentration was determined from the UV - concentration standard curve for every compound. Vials were tightly covered and the mixtures equilibrated at 37°C . Samples of the identical volume (40 – 60 μL , depending on the initial concentration of the triester) were removed periodically from the hydrolyzed mixture. The reaction was terminated by the addition of acetic acid (5 μL) and each sample was analyzed by analytical HPLC. Formation of all hydrolysis products was monitored (peak area %) and the degradation profiles were

established. In a second set of experiments, the course of hydrolysis was monitored as often as possible and the chromatograms were analyzed by the integration of the decreasing areas under peaks of the phosphotriesters. Subsequently, the rate constants (k) were determined from the slopes of the degradation curves and the half-lives ($t_{1/2}$) calculated from the rate constants. The corresponding ^{125}I -iodolabeled triesters **6b** – **8b**, **12b** – **14b** and **24b** were evaluated in the same way, within the initial concentration range of 25.9 – 40.7 kBq/ μL .

Proteins, sera, buffers and cell culture reagents

Human recombinant BChE (20 mg/mL; 994 U/mg protein), insulin, human serum, and mouse serum were purchased from Sigma-Aldrich (St. Louis, MO). BChE from human serum (3.5 U/mL) was a generous gift from Dr. Oksana Lockridge (UNMC, Omaha). ATCC-formulated Eagle's Minimum Essential Medium was from the American Type Culture Collection (ATCC; Manassas, VA). Fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine, penicillin-streptomycin, and sodium pyruvate were from GIBCO® Invitrogen Cell Culture (Carlsbad, CA). NE-PER Nuclear and Cytoplasmic Extraction Reagents were from Thermo Fisher Scientific (Rockford, IL). The Genomic-tips 100/G were from QIAGEN (QIAGEN Inc., Valencia, CA). CellTiter96® AQueous One Solution Cell Proliferation Assay was from Promega Corporation (Madison, WI).

Gel Electrophoresis

All analyses were conducted on precast 4–20% or 10% Mini-PROTEAN® TGX™ polyacrylamide gels (10-well, 30 RI) using the Mini-PROTEAN electrophoresis cell (Bio-Rad, Hercules, CA). Gels were run at the constant voltage (150 V or 190 V) for 1 h. When analyses included radioactive compounds, gels were placed on the MidSci classic autoradiography film BX (MidSci, St. Louis, MO) at -80°C for 1 h to 48 h, depending on the amount of radioactivity applied. Native gels were stained for BChE activity with 1 mM butyrylthiocholine iodide as a substrate using the Karnovsky and Roots⁷³ staining procedure as described by Lockridge⁷⁴. Analyses of hBChE interactions with the radioactive compounds were also conducted on SDS-PAGE nonreducing gels.

Determination of IC₅₀

Multiwell assays employed to determine IC₅₀ were developed in this laboratory and utilized either UV or fluorescence detection. For UV-based assays, 0.05 mL/well of human BChE in 0.1 M potassium phosphate, pH 7.0 (3.5 U/mL) was placed in the desired numbers of wells of a 96-well plate. The investigated drug was diluted with DMSO to produce concentrations from 0 to 1.5 mM and aliquots of 2 μL /well of these dilutions were added to BChE-containing wells to produce the range of final drug concentrations from 0.0005 μM to 10.5 μM . Mixtures were incubated at room temperature for 30 min. The reagent consisting of BChE substrate, 1 mM (2-mercaptoethyl)-trimethylammonium iodide butyrate, and 0.5 mM 5,5'-dithio-bis(2-nitrobenzoic acid) in 0.1 M potassium phosphate, pH 7.0 (prepared fresh for each assay) was added, 0.25 mL per well. Absorption at $\lambda = 405\text{ nm}$ was read at 1 min after the addition of this reagent using the Opsys MR™ plate reader (Dynex Technologies, Chantilly, VA). The time of incubation was increased for poor BChE inhibitors. IC₅₀s reported in Table 2 were calculated using absorption values read after 1 min. Data were analyzed and IC₅₀ calculated using the four-parameter logistic equation (variable Hill Slope) provided with the GraphPad Prism software (GraphPad Software, La Jolla, CA).

Cells

NIH:OVCAR-3 human ovarian epithelial adenocarcinoma cells, LS 174T human colorectal adenocarcinoma cells, and U-87 MG glioblastoma cells were purchased from ATCC. OVCAR-3 cells were grown in RPMI-1640 medium supplemented with 0.01 mg/ml bovine

insulin, 2 mM L-glutamine and FBS to a final concentration of 20%. When sufficient numbers of OVCAR-3 cells were produced, athymic NCr-*nu/nu* female mice were injected intraperitoneally (IP) with OVCAR-3 cells suspended in 0.4 mL of serum-free RPMI-1640 medium and further propagation of these cells was from the IP xenografts. When the abdominal distention became apparent mice were killed and the peritoneal cavity was lavaged with 2 mL sterile PBS to recover nonadherent OVCAR-3 cells. Cells were centrifuged, the supernatant discarded, and the cell pellet was resuspended in complete growth medium (95%) containing DMSO (5%). Cells were stored in liquid N₂ until ready for use. LS 174T cells were grown in the ATCC-formulated Eagle's Minimum Essential Medium containing 10% FBS. Cells used in these experiments have been maintained by using 0.25% trypsin in the subculture protocol. The subcultivation ratio of 1:3 was routinely applied. The same formulation of growth medium was used for U-87 MG cells with 0.5% trypsin plus 0.53 mM EDTA in the subculture protocol. All cell culture media were supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL).

Uptake kinetics

For each drug tested, cells were plated in four 6-well plates at 2×10^5 cells/well in 3 mL growth medium. After 24 h in culture, radioactive drugs were added to wells at predetermined times. The cells were incubated with drugs up to 360 min. Each point in time was tested in triplicate. Aliquots of media (0.5 mL) were removed from each well for gamma counting to determine the radioactive concentration in each well. At the end of incubation, the radioactive medium was aspirated and disposed. Cells were washed twice with 3 mL ice-cold PBS. Aliquots of wash PBS (0.5 mL) were also taken for gamma counting. Cells were trypsinized with 1.5 mL trypsin/EDTA and 1-mL aliquot of the cell suspension was counted in a gamma counter to determine the cell-associated radioactivity.

Clonogenic assays

Colorectal adenocarcinoma—LS174T cells, 2×10^6 cells/flask, were plated in T75 flasks. After ~18 h in culture, the growth medium was removed from all flasks and replaced with either 15 mL fresh medium containing **6b-fast** 114.5 ± 0.2 kBq/mL or 15 mL fresh medium containing **6b-slow** 114.4 ± 0.4 kBq/mL. Control cells were given 15 mL fresh nonradioactive medium containing PBS in amounts identical to these added with the radioactive compounds. Triplicate 0.1-mL aliquots of medium were withdrawn from each flask and counted in a gamma counter to determine the final concentration of radiolabeled compounds. After 4 h in the incubator at 37°C, the growth medium was removed from all flasks. Monolayers were washed once with full medium and 15 mL fresh medium was added to each flask. Cells were allowed to grow undisturbed for 24 h at which time cells were trypsinized, counted, and their viability was determined. Cells were re-plated in T25 flasks at plating densities of 500 cells/flask and 200 cells/flask. Each cell density was tested in quadruplicate. Seventeen to 21 days later, colonies were washed with 5 mL ice-cold PBS, followed by 5 mL PBS/methanol (1:1; v/v), and fixed in 5 mL methanol for 10 min. Methanol was removed and flasks were left open to dry for a few hours. Crystal violet (5 mL; 0.25% in 1:1 PBS/methanol) was added to each flask to stain cells. After approximately 10 min, the dye was removed and flasks were rinsed first with tap water followed by distilled water, and were left to dry. Colonies were counted manually by two observers using the Wheaton colony counter (Wheaton, Millville, New Jersey, USA).

To determine if the reduced exposure time with higher compound concentrations produces similar radiotoxic effects, an alternative procedure with a higher concentration of the radioactive compounds and a shorter exposure time to the radioactive compounds was also employed. LS174T cells (2×10^6) were plated in T75 flasks and allowed to recover and attach for ~18 h after which time the medium was removed and replaced with medium

containing radioactive compounds **7b-fast** and **7b-slow** at 185 kBq concentration. Cells in control flasks received non-radioactive media. Triplicate 0.1-mL aliquots were taken from each flask for gamma counting. Cells were returned to the incubator for 4 h. The medium was removed from all flasks, including controls, and the cell monolayer was washed once with fresh non-radioactive medium without FBS. Five mL 2.5% trypsin-EDTA was added to each flask to dissociate the monolayer. Fresh FBS-containing medium was added to stop the action of trypsin and to form a single cell suspension. Cell numbers and cell viability were determined. All cell suspensions were centrifuged at 800 rpm for 10 min at 4°C. Fresh FBS-containing medium was added to cell pellets to produce 1×10^6 cells/mL suspension. One mL of each suspension was counted in a gamma counter to determine cell-associated radioactivity. Cells were plated in duplicate T25 flasks at densities of 1,000 cells/flask, 500 cells/flask, and 100 cells/flask. The media was changed approximately once a week. After three weeks, colonies were processed as described above.

Glioblastoma—U-87 MG cells were plated in the ATCC-formulated Eagle's Minimum Essential Medium with 10% FBS 24 h before treatment and then treated for 24 h with various concentrations of **6b-slow** and **6b-fast** ranging from 18.5 to 185 kBq/mL dissolved in PBS and added to growth media. Cells were harvested, washed, and their numbers counted in Cellometer® disposable cell counting chambers (Nexcelom Bioscience, Lawrence, MA). Cells were diluted in fresh medium to concentrations suitable for the clonogenic assay. Cells from each concentration of **6b** were plated in three T25 flasks at two cell densities, 100 cells/flask and 500 cells/flask. Control cells were given medium containing the identical amount of PBS and processed in a manner identical to cells treated with **6b**. Cells were periodically examined and their growth medium was replaced every 5–7 days. Numerous colonies formed three weeks after plating control cells. These colonies were treated and stained as described above. The **6b**-treated cells were monitored for additional four weeks, during which time, colonies did not form, not even in flasks plated at 500 cells/flask.

In a separate experiment, U-87 MG cells were treated with **6b-fast** and **6b-slow** at several concentrations ranging from 18.5 kBq/mL and 129.5 kBq/mL in triplicate. After 24 h, the radioactive medium was removed, cells were washed twice with fresh medium warmed to 37°C, and fresh medium with 10% FBS was added. Cells were allowed to grow for additional 144 h. Cells from one set of plates were harvested, counted in Cellometer® and their radioactive content determined in a gamma counter. Monolayers in the second set of cells were stained with crystal violet, washed as described above, dried, and solubilized in 35 mM sodium dodecyl sulfate/ethanol mixture (1:1, v/v). Triplicate samples were collected from each flask and absorbance was measured at $\lambda=560$ nm.

The radiotoxicity of **24b** in U-87 MG cells was also measured. To evaluate the radiotoxicity of **24b**, U-87 cells were plated in T75 flasks at $\sim 1 \times 10^6$ cells/flask in the ATCC-formulated Eagle's Minimum Essential Medium with 10% FBS. Cells were maintained in the FBS-containing media for 24 h at which time the used medium was aspirated. The used medium was replaced with fresh medium containing either **24b-fast** or **24b-slow** at the concentration of 3.7 kBq/mL. Cells were allowed to grow in the presence of radioactive drugs for 1 h. Two treatment flasks and one control flask were processed after 1 h exposure to **24b**. The cells in the remaining flasks were given fresh growth medium and were processed 24 h later. Cells were processed as follows: the radioactive medium was aspirated and saved for radioactivity determinations. Cells washed with PBS were harvested using 0.5% trypsin-EDTA. Harvested cell suspensions were centrifuged at 1,000 rpm for 15 min at 4°C. Supernatants were collected and aliquots counted in a gamma counter. Cell pellets were washed with full media, centrifuged again, supernatant collected for gamma counting, and cell pellets resuspended. Two one-mL aliquots of the cell suspension were counted in a gamma counter

to determine the total radioactive content of cells. Cell numbers and their viability were determined. Cells from each treatment concentration were replated in triplicate T25 flasks at 500 cells/flask and 100 cells/flask for the clonogenic assay. Control flasks received media containing vehicle and were processed alongside the treated cells for clonogenic assay. The remaining cell suspensions were processed for the determination of the subcellular distribution of radioactivity. The cells were collected as a pellet after centrifugation at 1,000 rpm (4°C) for 15 min, resuspended in 1 mL PBS and transferred into microcentrifuge tubes. The subcellular fractionation was conducted using NE-PER Nuclear and Cytoplasmic Extraction Reagents. The clonogenic assay cells were allowed to grow undisturbed for four weeks. The media was changed once a week. After 28 days, colonies were processed as described above. Colonies were stained with crystal violet as described above and were counted with the aid of ImageJ software (<http://rsb.info.nih.gov/ij/>). Two independent readers were employed to count the colonies. The stained colonies were also solubilized in 35 mM sodium dodecyl sulfate/ethanol mixture (1:1, v/v), triplicate samples were collected from each flask and absorbance was measured at $\lambda=560$ nm.

Subcellular fractionation

Cancer cells were plated into six T150 flasks and allowed to attach overnight. The growth medium was removed and replaced with 10 mL fresh medium containing the tested radioactive drug. Cells were exposed to the drug for 1 h after which time the radioactive medium was removed and replaced with 12 mL fresh medium. Aliquots of all radioactive growth media were counted in the gamma counter before and after the cell culture. Cells in three flasks were processed immediately. Cells in the remaining three flasks were cultured in fresh nonradioactive medium for 24 h and then processed. Cell monolayers were rinsed with 5 mL PBS, trypsinized, cells were counted, and their viability determined. Using NE-PER nuclear and cytoplasmic extraction reagents⁷⁵, the cell content was fractionated and counted in a gamma counter to determine the drug distribution in various compartments of the cancer cell. The DNA associated radioactivity was also measured using the QIAGEN Genomic-tip 100/G procedures⁷⁶ (QIAGEN Inc., Valencia, CA). To determine the effects of the duration of the exposure on the subcellular distribution, cells were grown with radioactive compounds for 40 h, 96 h, 120 h, and 144 h. Cells were plated in T75 flasks and were allowed to attach for 24 h. Radioactive compounds were added to cells with fresh media. Aliquots of media were taken for gamma counting to assure that concentrations of *fast* and *slow* diastereomers were comparable. After the prescribed time, cells were washed with nonradioactive media and either harvested and fractionated as described above, or allowed to continue to grow for up to 120 h and then harvested and processed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the U.S. Department of Defense Congressionally Directed Medical Research Programs (OC030177 to J.B.-K.), Bayer Schering Pharma Grants4Targets focus grant (J.B.-K.), and by the UNMC Department of Radiation Oncology. Mass spectrometry analyses were provided by the Washington University Mass Spectrometry Resource with support from the NIH/National Center for Research Resources (Grant 2P41RR000954). Nuclear Magnetic Resonance analyses were conducted at the UNMC Eppley Institute NMR Facility managed by E. L. Ezell. Authors gratefully acknowledge the technical assistance provided by J. Nearman.

Abbreviations used

IUdR 5-iodo-2'-deoxyuridine

BChE	butyrylcholinesterase
LET	linear energy transfer
%ID/g	percent injected dose per gram
ESI-HR	high resolution electrospray ionization

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Figure 1.

Electrophoretic analyses of interactions between radioactive compounds **6b**, **8b** and **24b**, and BChE. Autoradiograph of denaturing non-reducing 4–20% gradient SDS-PAGE gels of human BChE (0.02 U) reacted with (A) **6b-fast** and **6b-slow** and (B) **24b-fast** and **24b-slow** for 30 min (B). C. Native gel stained with the Karnovsky and Roots stain for BChE activity. D. Autoradiograph of the native gel shown in C illustrating activity-dependent interactions of **8b-slow** and **24b-slow** reacted for 30 min with increasing activities of BChE.

Figure 2.

In vitro uptake kinetics in human ovarian OVCAR-3 adenocarcinoma and human colorectal LS 174T adenocarcinoma cells. **A.** Uptake of **6b-fast** and **6b-slow** in OVCAR-3 cells. **B.** Uptake of **6b-fast** and **6b-slow** in LS 174T cells. Radioactive concentrations in **A** and **B**: 46.9 ± 1.1 kBq/mL. **C.** Uptake of **7b-fast** and **7b-slow** in OVCAR-3 cells. **D.** Uptake of **7b-fast** and **7b-slow** in LS 174T cells. Radioactive concentrations in **C** and **D**: 35.1 ± 0.3 kBq/mL. **E.** Uptake of **8b-fast** and **8b-slow** in OVCAR-3 cells. Radioactive concentrations: 16.7 ± 1.2 kBq/mL. **F.** Uptake of **8b-fast** and **8b-slow** in LS 174T cells. Radioactive concentrations of **8b-fast**: 13.6 ± 0.4 kBq/mL. Radioactive concentration of **8b-slow**: 17.5 ± 0.3 kBq/mL. Average and std dev, $n = 3$ per time.

Figure 3. Concentration-dependent uptake of **6b-fast** and **6b-slow** in human adenocarcinoma cells. **A.** Uptake of **6b** in OVCAR-3 cells. **B.** Uptake of ^{131}I UDR in OVCAR-3 cells. **C.** Uptake of **6b** in LS 174T cells. **D.** Uptake of ^{131}I UDR in LS 174T cells.

Figure 4.

Concentration- and time-dependent uptake of **8b-fast** and **8b-slow** in human adenocarcinoma cells. **A.** Uptake in LS 174T after 1 h incubation with radioactive compounds. **B.** Uptake of **8b-slow** expressed as molecules per 1,000 cells in OVCAR-3 cells after 1 h and 3 h incubation with the radioactive compound.

Figure 5.

Evaluation of **6b** in U-87 MG human glioblastoma cells. **A.** Retention of ^{125}I in DNA of U-87 MG cells treated with 27.3 kBq/mL of **6b-slow** and **6b-fast** for 24 h and allowed to grow for additional 144 h in fresh medium. **B.** Retention of ^{125}I in DNA of U-87 MG cells treated with 185 kBq/mL of **6b-slow** and **6b-fast** for 120 h. **C.** Surviving fraction of U-87 MG cells treated for 24 h with increasing concentrations of **6b-slow** and **6b-fast** (bars represent average; capped lines are std dev) and allowed to grow in fresh medium for 144 h at which time cells were counted and their viability determined.

Figure 6.

Viable U-87 MG cells recovered after 24-h treatment with **6b-slow** and **6b-fast** at two concentrations 37 kBq/mL (**A**) and 185 kBq/mL (**B**). Cells were harvested 96 h after treatment with ¹²⁵I-labeled compounds. The clonogenic death of these cells was confirmed by the lack of colony formation seven weeks after plating.

Figure 7.

Surviving fraction of LS 174T colorectal adenocarcinoma cells treated with resolved diastereomers of **6b** and **7b**. **A.** Clonogenic assay performed on cells exposed to **6b-fast** and **6b-slow** for 4 h, at a drug concentration of 114.5 kBq/mL and followed by additional 24 h culture in nonradioactive media before the cell harvest and plating for clonogenic assay. **B.** Surviving fraction of LS 174T cells treated with **7b-fast** and **7b-slow** for 4 h at a drug concentration of 185 kBq/mL. Cells were harvested immediately after the exposure to the drug and plated for the clonogenic assay.

Figure 8.

Subcellular distribution of ^{125}I in U-87 MG cells treated with **24b-fast** and **24b-slow** for 1 h and either processed immediately or after 24 h of additional culture in fresh nonradioactive medium. **A.** ^{125}I recovered in cytoplasm. **B.** Soluble ^{125}I from the nuclear fraction. **C.** ^{125}I associated with DNA.

Figure 9. Clonogenic survival of U-87 MG cells treated with **24b-fast** and **24b-slow** for 1 h at a concentration of 4.76 kBq/mL. Cells were either processed immediately for the clonogenic assay (**A**) or allowed to continue to grow with fresh medium for additional 24 h (**B**).

Scheme 1.

Synthetic Pathways to *cycloSaligenyl* Phosphotriesters **6b** – **14b**^a

^a Reagents and conditions: (**a**) (i) crude **15** for $X = Y = Z = H$, **16** for $X = Y = H, Z = CH_3$, **17** for $X = F, Y = Z = t-Bu$, prepared using General Procedure A, transferred in THF to IUdR (**1**) in DMF/THF, DIPEA, $-60^\circ C \rightarrow rt$, 2 h; (ii) $t-BuOOH$, $-40^\circ C \rightarrow rt$, 1 h; (iii) separation of 5'-, 3'-, and 5',3'-regioisomers on SiO_2 column; (**b**) $TrCl$ in pyridine, $0^\circ C \rightarrow rt$, 12 h; (**c**) (i) like **a**, but chlorophosphites **15** – **17** transferred in diethyl ether into **5** in CH_3CN , DIPEA, $-40^\circ C \rightarrow rt$ and 3 h reaction period; (ii) $t-BuOOH$, $-20^\circ C \rightarrow rt$, 1 h; (**d**) $ZrCl_4$ (2.5 equiv) in CH_3CN , 1 h, rt ; or 1N HCl/CH_3CN , $45^\circ C \rightarrow rt$, 10 min; (**e**) $DMTrCl$ in pyridine, DMAP (0.05 equiv), $0^\circ C \rightarrow rt$, 12 h; (**f**) levulinic acid, DCC, DMAP (0.05 equiv), CH_2Cl_2 /diethyl ether, rt ; (**g**) $ZrCl_4$ (1 equiv), CH_3CN , 15 min, rt ; (**h**) (i) like **c**, chlorophosphites were transferred in diethyl ether to **4** in CH_3CN , DIPEA, $-40^\circ C \rightarrow rt$, 2 h reaction period; (ii) $t-BuOOH$, $-20^\circ C \rightarrow rt$, 1 h; (**i**) $N_2H_4 \cdot H_2O$, pyridine/ $AcOH$, 2 min; (**j**) $Sn_2(CH_3)_6$ or $Sn_2(n-Bu)_6$ used in preparation of **6a**, (1.2 – 1.6 equiv) in ethyl acetate (preparation of **7a**, **8a**, **9a**, **10a**, **11a**) or in dioxane (preparation of **6a**, **7a**, **12a**, **13a**), $(Ph_3P)Pd(II)Cl_2$ (0.06 equiv), refluxed under nitrogen, 2 – 4 h; (**k**) (i) $Na^{125}I$ in $NaOH$ (9.25 – 444 MBq), 30% H_2O_2 , TFA/CH_3CN (0.1% v/v), 1 min sonication, 5 – 20 min reaction time; (ii) HPLC purification.

Scheme 2. Preparation of Chlorophosphites **15** – **17** and Phosphoramidite **18**^a

^a Reagents and conditions: (**a**) (i) PCl₃, in Et₂O, –40°C, 15 min; (ii) Et₃N or pyridine –40°C → rt., 3 h; (iii) kept under nitrogen at –20°C, 12 h; (**b**) (i) crude **17** prepared in the presence of Et₃N; (ii) (*i*-Pr)₂NH, –20°C → rt., 2 h; no purification, used immediately.

Scheme 3.

Synthesis of 5'-Sal-cycloSaligenyl Phosphotriesters **22** and **23** Derived from Thymidine and 3'-Fluoro-3'-deoxythymidine. Synthesis of 5-[^{125}I]-Iodo-5'-O-[cyclo-3,5-Di-(*tert*-butyl)-6-fluoro-Saligenyl]-3'-fluoro-2',3'-dideoxyuridine **24b** via Iododestannylation of **24a** or the Direct Coupling of Phosphoramidite **18** and 5-[^{125}I]-Iodo-3'-fluoro-2',3'-dideoxyuridine **21b**^a

^a Reagents and conditions: (a) (i) **17** prepared by General Procedure A, transferred in diethyl ether to a solution of **19** or **20** in CH_3CN , DIPEA, $-40^\circ\text{C} \rightarrow \text{rt.}$, 2 h; (ii) *t*-BuOOH, $-20^\circ\text{C} \rightarrow \text{rt.}$, 1 h; (b) for **22** from **19** $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$, pyridine/AcOH, 2 min; (c) $\text{Sn}_2(\text{CH}_3)_6$, $(\text{Ph}_3\text{P})\text{Pd}(\text{II})\text{Cl}_2$, refluxed under nitrogen, 2 h (for **21**), 4 h (for **24**); (d) (i) Na^{125}I in NaOH, 30% H_2O_2 , 2 min; (ii) TFA/ CH_3CN (0.1% v/v), 1 min sonication, 20 min reaction time; (iii) HPLC purification; (e) (i) **18** transferred in CH_3CN to **21b**, -40°C ; (ii) 1*H*-tetrazole (0.45 M solution in CH_3CN), $-40^\circ\text{C} \rightarrow \text{rt.}$, 10 min; (iii) *t*-BuOOH, $-20^\circ\text{C} \rightarrow \text{rt.}$; (iiii) a solution (5%, v/v, 1 mL) of $\text{NaHSO}_3/\text{EtOAc}$; (f) HPLC purification.

Table 1

Rates of hydrolysis and half-lives of reagents incubated in phosphate buffered saline.

	k min⁻¹	std err k min⁻¹	half-life (t_{1/2}) h	std err t_{1/2}	P	R² unweighted
6b-fast	2.25×10 ⁻³	7.0×10 ⁻⁵	5.1	0.17	<0.0001	0.950
6b-slow	1.52×10 ⁻³	4.9×10 ⁻⁵	7.6	0.25		0.933
7b-fast	5.44×10 ⁻⁴	1.0×10 ⁻⁵	21.2	0.38	<0.0001	0.997
7b-slow	4.63×10 ⁻⁴	1.0×10 ⁻⁵	25.0	0.53		0.997
8-fast	2.41×10 ⁻⁴	1.3×10 ⁻⁵	47.9	2.58	0.0006	0.992
8-slow	1.88×10 ⁻⁴	7.0×10 ⁻⁶	61.5	2.28		0.998
12-fast	2.05×10 ⁻³	5.8×10 ⁻⁵	5.3	0.17	<0.0001	0.998
12-slow	1.64×10 ⁻³	3.7×10 ⁻⁵	7.1	0.16		0.998
13-fast	9.46×10 ⁻⁴	4.5×10 ⁻⁵	12.2	0.58	0.018	0.992
13-slow	8.13×10 ⁻⁴	3.1×10 ⁻⁵	14.2	0.55		0.993
14-fast	7.66×10 ⁻⁴	1.9×10 ⁻⁵	15.1	0.37	0.001	0.997
14-slow	6.67×10 ⁻⁴	1.8×10 ⁻⁵	17.3	0.50		0.996
24-fast	2.92×10 ⁻⁴	2.0×10 ⁻⁶	39.6	0.27	<0.0001	0.999
24-slow	2.22×10 ⁻⁴	2.2×10 ⁻⁶	52.0	0.52		0.999
24b-fast	3.01×10 ⁻⁴	9.1×10 ⁻⁶	38.5	1.17	<0.0001	0.999
24b-slow	2.24×10 ⁻⁴	4.9×10 ⁻⁶	51.5	1.13		0.999

Table 2

Inhibitory activities of cycloSaligenyl-phosphotriesters towards human butyrylcholinesterase.

	IC ₅₀	
	slow	fast
6	9.2 nM [*] (0.16)	1,284 nM (256)
7	61.2 nM (1.81)	>7 mM ^{**}
8	50.1 nM (0.69)	>60 mM ^{**}
22	375.9 nM (8.43)	23.6 μM ^{**}
23	42.6 nM (0.17)	202.6 nM (0.95)
24	19.8 nM (0.12)	549.2 nM (10.98)
8 mix	1,135 nM (43.4)	

^{*}
average (*std dev*)^{**}
estimated

Table 3
Subcellular distribution of mono- and bis-cycloSaligenyl derivatives in human ovarian adenocarcinoma OVCAR-2 cell line.

	<i>cycloSal</i> position	X	Y	Z	¹²⁵ I in cytoplasm			¹²⁵ I in nucleus		
					1 hour	24 hours	cpm/10 ³ cells (<i>std dev</i>)	1 hour	24 hours	cpm/10 ³ cells (<i>std dev</i>)
6b	5'	H	H	H	20.2 (3.2)	7.3 (1.0)		5.7 (1.6)	39.4 (5.8)	
7b	5'	H	H	CH ₃	11.7 (1.7)	1.6 (0.5)		4.5 (1.0)	25.9 (7.1)	
8b	5'	F	<i>t</i> -Bu	<i>t</i> -Bu	88.3 (5.4)	3.2 (0.01)		7.9 (0.4)	92.2 (5.0)	
9b	3', 5'	H	H	H	97.6 (10.0)	37.3 (10.0)		6.4 (0.5)	18.1 (3.9)	
10b	3', 5'	H	H	CH ₃	97.1 (14.6)	13.4 (3.3)		6.0 (0.9)	24.5 (5.4)	
12b	3'	H	H	H	7.2 (0.7)	2.7 (0.3)		2.1 (0.2)	30.2 (5.3)	
6b-slow	5'	H	H	H	14.8 (6.8)	6.0 (0.7)		4.9 (2.5)	31.3 (6.0)	
7b-slow	5'	H	H	CH ₃	13.0 (1.3)	1.8 (0.6)		5.4 (0.4)	29.3 (7.0)	
8b-slow	5'	F	<i>t</i> -Bu	<i>t</i> -Bu	87.3 (7.5)	2.8 (0.7)		7.8 (0.6)	91.6 (5.7)	
13b	3'	H	H	CH ₃	6.2 (0.6)	1.4 (0.3)		2.0 (0.2)	15.7 (2.2)	
14b	3'	F	<i>t</i> -Bu	<i>t</i> -Bu	62.5 (4.3)	2.7 (0.4)		4.3 (0.3)	66.7 (8.4)	