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Synthesis and Evaluation of a Novel Gene Reporter Molecule: Detection of β-galactosidase Activity Using ^{19}F NMR of a Fluorinated Vitamin B_6 Conjugate[+]

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Abstract: Gene therapy has emerged as a promising strategy for treatment of various diseases. However, widespread implementation is hampered by difficulties in assessing the success of transfection, in particular, the spatial extent of expression in the target tissue and the longevity of expression. Thus, the development of non-invasive reporter techniques based on appropriate molecules and imaging modalities may help to assay gene expression. We now report the design, synthesis and evaluation of a novel *in vivo* gene transfection reporter molecule 3-*O*-(β-D-galactopyranosyl)-6-fluoropyridoxol (GFPOL) using fluorinated vitamin B_6 as the ^{19}F NMR sensitive aglycone. GFPOL exhibits the following strengths as an *in vivo* ^{19}F NMR gene expression reporter: (a) large chemical shift response to enzyme cleavage ($\Delta\delta$=8.00 ppm); (b) minimal toxicity for substrate or aglycone; (c) good water solubility; (d) good blood stability; (e) pH responsiveness of aglycone.

Key Words: β-galactosidase, ^{19}F NMR, gene reporter, pyridoxol, pH.

INTRODUCTION

Gene therapy shows promise for the treatment of various disorders and clinical trials are underway. However**,** non-invasive detection of transgenes *in vivo* would be of considerable value for assessing the location**,** magnitude and persistence of expression. Generally, therapeutic genes are not readily detected, and thus, various reporter genes have been developed and are widely applied in molecular biology, *e.g.*, β-galactosidase (β-gal), β-glucuronidase, chloramphenicol acetyltransferase, and firefly luciferase [1]. Among these, the *lacZ* gene, encoding β-gal, is the most attractive reporter gene, because β-gal activity is readily assessed *in vitro* in hosts as evolutionarily diverse as bacteria, yeast, and mammals, and its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction [2]. Many chromogenic or fluorogenic substrates are well-established, but they are generally limited to histology or *in vitro* assays [3-8].

Recently, Weissleder *et al*. [9] presented a near infrared approach based on 9H-(1, 3-dichloro-9, 9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside (DDAOG), and Meade et al. [10] reported an NMR approach using 1-[2-(β-D-galactopyranosyloxy) propyl]-4, 7, 10-tris (carboxymethyl)-1, 4, 7, 10-tetraazacyclododecane) gadolinium (III) (EgadMe), to assess β-gal activity *in vivo*. These diverse substrates emphasize the promiscuity (lack of substrate specificity) of β-gal activity. However, EgadMe was found to be 500 times less sensitive to β-gal than the traditional "yellow" biochemical indicator *ortho*-nitrophenol-β-D-galactopyranoside (*ONPG*) and failed to enter cells, necessitating direct microinjection. We realized that introduction of a fluorine atom into the traditional nitrophenol aglycones could generate NMR indicator molecules with minimal perturbation to a well-established molecular structure. Indeed, we successfully demonstrated the use of *p*-fluoro-*o*-nitrophenyl β-D-galactopyranoside (*PFONPG*) to detect enzyme activity in solution and transfected tumor cells [11]. *PFONPG* exhibits virtually identical sensitivity to cleavage by β-gal as compared with *ONPG* [12]. However, the liberated aglycone *p*-fluoro-*o*-nitrophenol (*PFONP*) exhibits some cytotoxicity, likely by analogy to the well known uncoupler of oxidative phosphorylation 2, 4-dinitrophenol [13]. More recently, we showed that various analogs of the aglycone structure (halophenols) showed significant differences in rate of response to enzyme action and some of these alternate aglycones exhibit much lower toxicity [12]. We now report the design, synthesis, and evaluation of another novel *in vivo* gene transfection reporter molecule using fluorinated vitamin B_6 as a stable aglycone and sensitive ^{19}F NMR indicator.

RESULTS AND DISCUSSION

Design

^{19}F NMR signals are exquisitely sensitive to molecular changes and often also to the microenvironment, and thus, there are many reporter molecules exploiting fluorine atoms [14]. We have previously shown that 6-fluoropyridoxol (**1**, **FPOL**) exhibits exceptional sensitivity to changes in pH

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with ~10 ppm acid/base ^{19}F NMR shift [15]. We recognized that **1** could serve as a replacement for fluorophenol aglycones offering similar steric and electrostatic properties. The fluorine atom located *para* to the phenolic group should provide sensitive response to enzyme induced cleavage of the substrate **GFPOL. FPOL** exhibits little toxicity and can reveal local pH in a similar manner to the prototype aglycone **PFONP**.

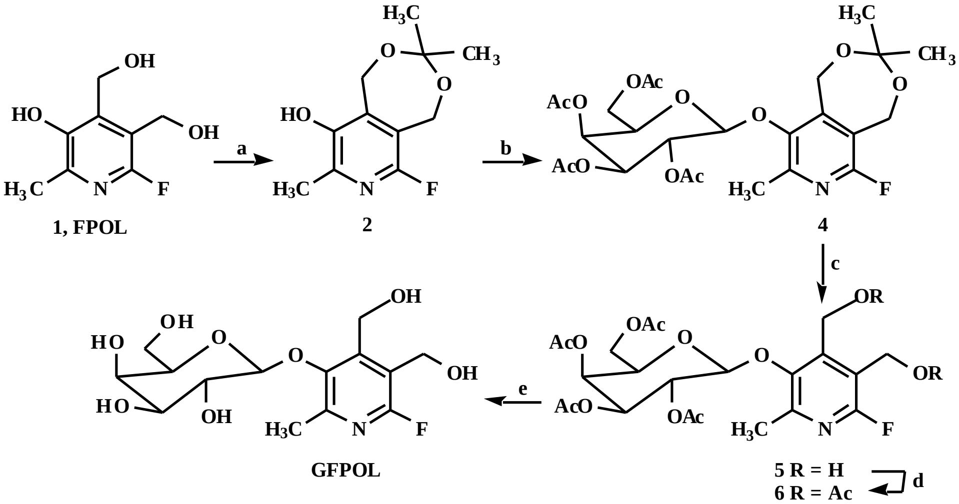
Synthesis

Condensation of **FPOL** and galactose has regioselectivity requirements and Scheme **1** outlines a synthetic route exploiting regioselective protection of the α^4, α^5- hydroxyl groups through ketal condensation. However, the ketal addition of **1** with acetone using standard conditions gave 6-fluoro-3, α^4-isopropylidenepyridoxol, as major product and the desired 6-fluoro-α^4, α^5-isopropylidenepyridoxol **2,** as minor by-product. Testing various acids as catalysts showed 2% H_2SO_4 acetone solution to provide the best yield of **2** (26%). The regioselectivity of the acetonation reaction was confirmed by analyzing ^{1}H-NMR spectra of **2** and 6-fluoro-3, α^4-isopropylidenepyridoxol, in which the 5-CH_2 signal of **2** appeared at 5.03 ppm as singlet and of 6-fluoro-3, α^4-isopropylidenepyridoxol at 4.97 ppm, but as doublet ($J_{H\text{-}5,\ HO\text{-}5}$=1.2 Hz) due to the coupling of the 5-OH. The inefficient reaction is presumably due to the unfavorable seven member ring, as opposed to alternate six member ring.

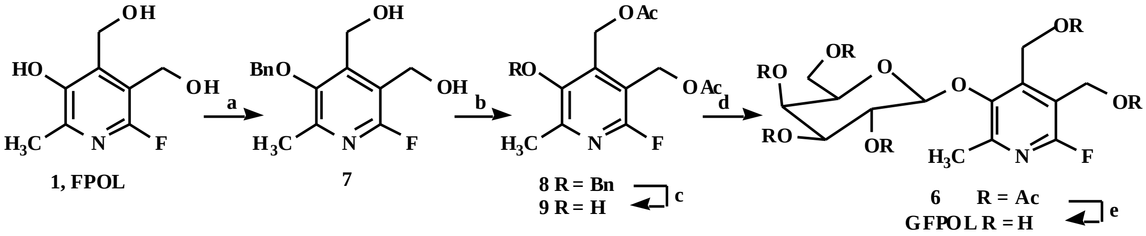
Treatment of **2** with 2, 3, 4, 6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **3** using the Koenigs-Knorr glycosylation method gave 3-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α^4, α^5-isopropylidene-6-fluoropyridoxol **4** in 85% yield. NMR verified that the galactose was in the β-configuration ($\delta_{H\text{-}1'}$ 4.64 ppm (doublet, $J_{1,\ 2}$=8.0 Hz) and $\delta_{C\text{-}1'}$ 100.03 ppm). The correlation between 2-CH_3 and H-1' of sugar ring from the NOSEY spectrum of **4** verified that 2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl residue connected at the 3 phenolic site provided further evidence that acetonation reaction did occur regioselectively on 4, 5 hydroxymethyl groups.

Cleavage of acetonide **4** for synthesis of 3-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-fluoropyridoxol **5** was achieved, however, the yields were low (≤15%), based on several hydrolysis conditions, such as 80% AcOH, 1% HCl or 90% CF_3CO_2H in MeOH, CH_2Cl_2 or 1, 4-dioxane at various temperatures (60~100 °C). A moderate amount of **1** was recoverable indicating that the β-D-galactopyranosyl $C_{1'(gal)}$-O_3 bond became weak and sensitive to acid hydrolysis presumably due to the presence of 6-fluorine atom. Acetylation of **5** to **6** facilitated purification and structural characterization by NMR. Finally, deacetylation of **6,** in NH_3/MeOH from 0°C to room temperature yielded the target molecule **GFPOL** in quantitative yield. The overall yield for **GFPOL** through this five-step route was ~3% with limiting steps in the α^4, α^5-isopropylidene group formation and hydrolysis procedures (a and c).

We thus considered alternate approaches, *e.g.*, Scheme **2**. Particularly, the acidic phenolic group *para* to the 6-fluorine atom should be ionized under mild base conditions to selectively react with benzyl bromide affording the expected 3-mono benzylated product under carefully controlled conditions. When benzyl bromide (1.1 equiv.) was added dropwise over a period of 4~5 h to the well-stirred reaction mixture of compound **1** in a biphasic dichloromethane-aqueous system (pH 10~11) using tetrabutylammonium bromide (TBAB) as the phase-transfer catalyst (PTC), 3-*O*-benzyl-6-fluoropyridoxol **7** was isolated as major product in 76% yield with small amounts of di-*O*-benzyl derivatives 3, α^4-di-*O*-benzyl-6-fluoropyridoxol (16%) and 3, α^5-di-*O*-benzyl-6-fluoropyridoxol (8%). The structure of **7** was established on the basis of the coupling characteristics of α^4, α^5-CH_2 as doublets ($J_{H\text{-}4,\ HO\text{-}4}$=6.0 Hz, $J_{H\text{-}5,\ HO\text{-}5}$=5.4 Hz) and α^4, α^5-OH as triplets in the ^{1}H-NMR spectrum. Treatment of **7** with acetic anhydride in pyridine from 0 °C to r.t. overnight gave 3-O-benzyl-α^4, α^5-di-*O*-acetyl-6-fluoropyridoxol **8** in



Scheme 1. Reagents and conditions: (a) 2% H_2SO_4, acetone, r.t. 4~5 h, 26%; (b) 2, 3, 4, 6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**3**), $Hg(CN)_2$, 4Å M.S., CH_2Cl_2, r.t., 12 h, 85%; (c) 80% AcOH, 80 °C, 4~5 h, 15%; (d) Ac_2O-Pyridine, 0 °C→r.t., 24 h, quantitative yield; (e) NH_3-MeOH, 0 °C→r.t., 24 h, quantitative yield.



Scheme 2. Reagents and conditions: (a) benzyl bromide (1.1 equiv.), CH_2Cl_2-H_2O, pH 10~11, 50 °C, TBAB, 4~5 h, 76%; (b) Ac_2O-Pyridine, 0 °C → r.t., 24 h, quantitative yield; (c) H_2, Pd/C, r.t., 12 h, quantitative yields; (d) 2, 3, 4, 6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**3**), $Hg(CN)_2$, 4Å M.S., CH_2Cl_2, r.t., 12 h, 90%; (e) NH_3-MeOH, 0 °C → r.t., 24 h, quantitative yield.

quantitative yield. The 3-benzyl protecting group was removed under 5% Pd/C hydrogenation overnight affording nucleophile α^4, α^5-di-*O*-acetyl-6-fluoropyridoxol **9** in quantitative yield. **9** was then subjected to a procedure similar to that described for the preparation of galactoside **4** giving 3-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α^4, α^5-di-*O*-acetyl-6-fluoropyridoxol **6** in 90% yield. After work up and deacetylation, the target compound **GFPOL** was obtained in 68% overall yield over five steps.

Recognizing the differential reactivity of the 3 phenolic group over the hydroxymethyl groups suggested a more direct synthesis. Direct galactopyranosylation of **1** with 2, 3, 4, 6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **3** failed as a result of the very low selectivity of the Koenigs-Knorr glycosylation reaction for the 3 phenolic group in the presence of the two free active hydroxymethyl groups. Product **5** could not be isolated, but using 2, 3, 4, 6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **3** phase-transfer catalysis gave **5** (88%) directly, and **GFPOL** (88%) through only two reaction steps (Scheme **3**).

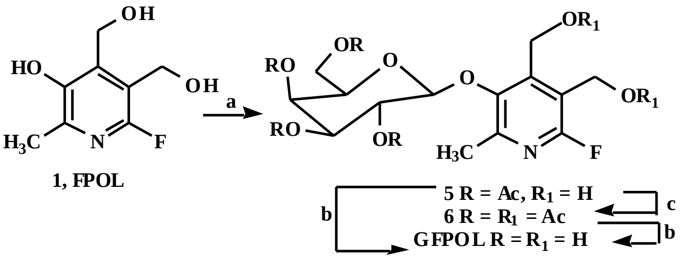
In conclusion, Scheme **3** provided a very efficient and direct method to stereo- and regioselectively synthesize 3-*O*-(β-D-galactopyranosyl)-6-fluoropyridoxol **GFPOL** in high yields. Large-scale preparation of **GFPOL** can be performed by these PTC methods.

Characteristics

As expected, **GFPOL** readily dissolves in saline, whole blood, or PBS buffer, and is much more soluble than the aglycone **1**. **GFPOL** was stable in aqueous solutions in the pH range 3 to 12 at temperatures from 25 to 37 °C for at least 5 days. Cell viability assays [16] showed that neither **GFPOL** nor **FPOL** exhibited significant cytotoxicity (Fig. **1**). For **GFPOL** viability exceeded 98% at all concentrations tested and for **FPOL** survival was > 80% up to 2 mM.

Proton decoupling was applied to simplify ^{19}F NMR spectra and sodium trifluoroacetate (NaTFA) in a capillary was used as an external standard. **GFPOL** gave a single narrow ^{19}F NMR signal at δ -3.22 ppm essentially invariant ($\Delta\delta \leq 0.06$ ppm) with pH in the range 3 to 12 and temperatures from 25 to 37 °C. Addition of β-gal (E801A) in PBS buffer (0.1M, pH=7.4) at 37 °C caused hydrolysis releasing the pH indicator aglycone **FPOL** appearing also as a single narrow ^{19}F signal shifted up-field to δ -11.21 ppm (Fig. **2**), consistent with our previous titration curve of **FPOL** [15]. Sequential ^{19}F NMR spectra showed that **GFPOL** decreased monotonically, releasing free **FPOL** with an initial rate of 4.3 μmol/min/unit (Fig. **3**). For comparison **PFONPG** gave 19 μmol/min/unit and **OFPNPG** gave 32 μmol/min/unit [12]. **PFONPG** was previously shown to exhibit very similar substrate activity to the traditional yellow biochemical indicator **ONPG** [12]. When **GFPOL** was incubated with wild type human cancer cells (prostate PC-3 or C4-2 (LNCaP lineage derived androgen independent subline or breast MCF-7) for 5 h in PBS buffer at 37 °C under 5% CO_2 in air with 95% humidity, no changes were observed in the ^{19}F NMR spectra. However, addition of **GFPOL** to cells from these lines, which had been transfected transiently or stably to express β-gal led to cleavage of **GFPOL**, as detected over a period of hours (Fig. **4**).

GFPOL provides further evidence for the ^{19}F NMR approach to assessing enzyme activity *in situ*. We are currently extending applications to imaging based on chemical shift selective excitation exploiting the large chemical shift difference between the substrate and product.



Scheme 3. Reagents and conditions: (a) 2, 3, 4, 6-tetra-O-acetyl-α-D-galactopyranosyl bromide (**3**), CH_2Cl_2-H_2O, pH 10~11, r.t., TBAB, 4~5 h, 88%; (b) NH_3-MeOH, 0 °C → r.t., 24 h, quantitative yield; (c) Ac_2O-Pyridine, 0 °C → r.t., 24 h, quantitative yield.

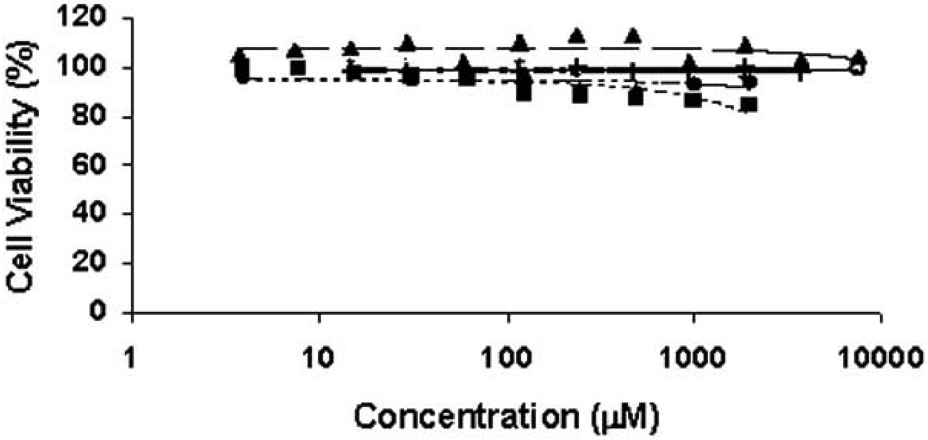


Fig. (1). Cellular toxicity of reporter molecules o C4-2 cells (GFPOL), + PC3 cells (GFPOL), · MAT-Lu cells (FPOL), ▲ MAT-Lu-LacZ cells (FPOL), ■ MAT-Lu-LacZ cells (GFPOL).

We are also initiating studies in living animals and exploring the utility of enzyme activated pH measurements.

EXPERIMENTAL

General Methods

NMR spectra were recorded on a Varian Inova 400 spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C) with $CDCl_3$, acetone-d_6 or DMSO-d_6, as solvents, and chemical shifts referenced to TMS, as internal standard. ^{19}F NMR (376 MHz) signals are referenced to dil. sodium trifluoroacetate (NaTFA) in an external capillary. 2, 3, 4, 6-tetra-O-acetyl-α-D-galactopyranosyl bromide (**3**) was purchased from Sigma. Reactions requiring anhydrous conditions were performed under nitrogen or argon. $Hg(CN)_2$ was dried before use at 50 °C for 1 h, CH_2Cl_2 was dried over Drierite. Solutions in organic solvents were dried with anhydrous sodium sulfate, and concentrated *in vacuo* below 45 °C. Column chromatography was performed on silica gel (200~300 mesh) by elution with cyclohexane-EtOAc and silica gel GF_{254} used for analytical TLC (Aldrich). Detection was effected by spraying the plates with 5% $EtOH/H_2SO_4$ (followed by heating at 110 °C for 10 min.) or by direct UV. Microanalyses were performed on a Perkin-Elmer 2400 CHN microanalyser.

β-Gal (E801A) was purchased from Promega and enzymic reactions performed at 37 °C in PBS buffer (0.1M, pH 7.4). For enzyme kinetic experiments, **GFPOL** (5.25 mg) was dissolved in PBS buffer (600 μL, pH=7.4), a PBS solution of β-gal (20 μL, E801A, 1unit/μL) was added and NMR data were acquired immediately at 37 °C.

Both PC-3 and C4-2 were plated at 5 million cells per dish (P150) and grown for 24 h. Then PC-3 and C4-2 were transfected for 48 h with a first generation adenovirus vector encoding the lacZ gene driven by the CMV promoter. PC-3 was transfected with 50 moi and C4-2 with 10 moi of Ad-CMV-lacZ. After 48 h incubation and washing, cells were trypsinized and concentrated to 10 million cells per ml. for further use (NMR).

Human breast cancer cells MCF-7 were stably co-transfected with pCMVβ (Clontech, Palo Alto, CA, USA), using TransFast^{TM} Transfection Reagent (Promega, Madison, WI, USA) comprising the *E.coli lacZ* gene located under the human cytomegalovirus (CMV) immediate-early enhancer/ promoter region and pCI-neo (Promega, Madison, WI, USA) carrying the neomycin phosphotransferase gene. For MCF-7 cells clonal selection was applied to identify those cells with highest β-gal expression.

Control wild type and transfected human prostate tumor cells (PC3 and C4-2 (LNCaP lineage derived androgen independent subline)) and human breast tumor cells MCF-7 were grown in culture dishes under standard conditions and harvested. **GFPOL** (1.84 mg) in PBS buffer (70 μL) was

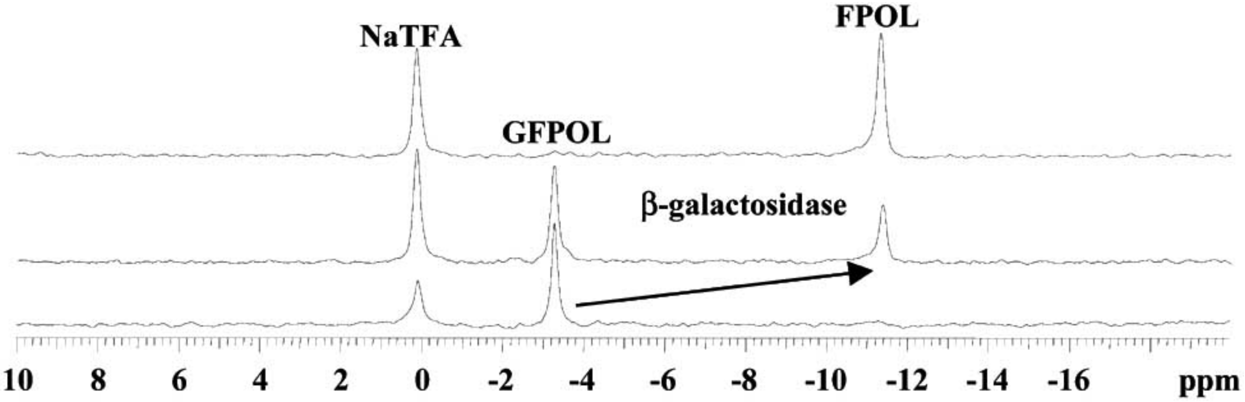


Fig. (2). ^{19}F NMR spectra of **GFPOL** during the hydrolysis by β-gal in PBS buffer at 37 °C.

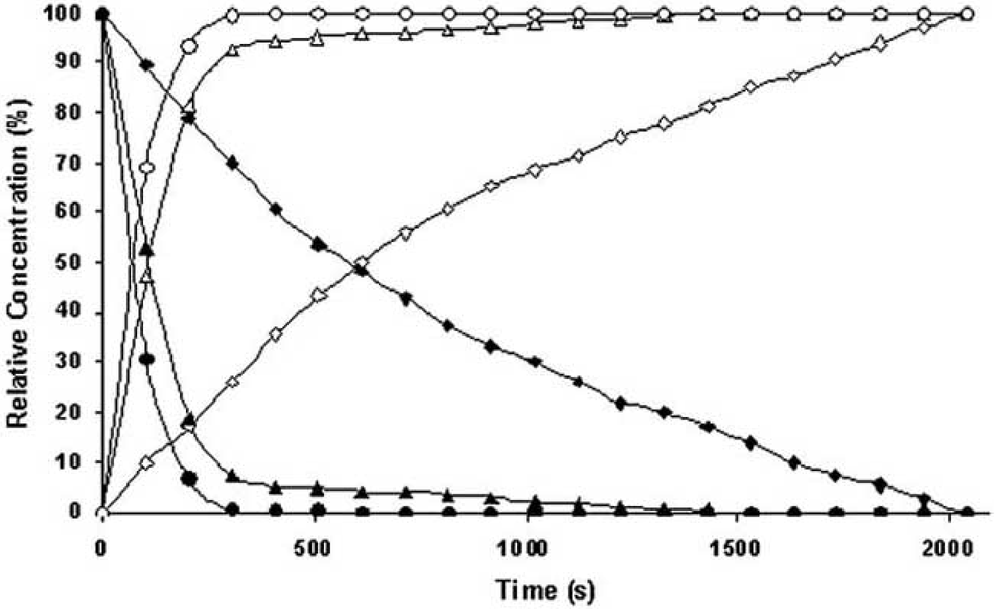


Fig. (3). Relative action of β-gal (E801A) on **GFPOL** (◆), PFONPG (Δ) and OFPNPG (O) in PBS buffer at 37 °C yielding **FPOL** (◇), PFONP (Δ) and OFPNP (○), respectivley . In each case 20 units of β-gal (E801A) were added to 15 mmol substrate in PBS at 37 °C.

added to suspension of 10^7 cells in PBS buffer (530 μL) and NMR data were acquired immediately, and again after incubation for various times up to 5 h at 37 °C (prostate tumor cells PC3 and C4-2) or at 22 °C (breast tumor cells MCF-7).

The sensitivities of human prostate cancer cells PC3, C4-2 and their LacZ transfected counterparts to **GFPOL** and **FPOL** were quantified using a colorimetric CellTiter 96 Aqueous Nonradioactive MTS Cell Proliferation Assay (Promega, Madison, WI, USA). The assays were performed in triplicate using 24-well plates seeded with 10^3 cells per well in 500 μL RPMI 1640 without phenol red and supplemented with 10% FCS and 2 mM glutamine. After 24 h incubation, the medium was replaced with fresh RPMI 1640 containing various concentrations of **GFPOL**. For the determination of IC_{50} drug concentrations, incubations with **GFPOL** (0~7.5 mM) and **FPOL** (0~2 mM) were performed for 72 h, followed by the MTS assay (Figure **1**).

Syntheses

α^4, α^5-*O-isopropylidene-6-fluoropyridoxol 2*

A suspension of 6-fluoropyridoxol **1** (0.50 g, 2.67 mmol) in anhydrous acetone (40 ml) containing 2% c.H_2SO_4 was stirred until TLC (4**:**1 cyclohexane-EtOAc) indicated complete reaction (4~5 h), then cold saturated Na_2CO_3 solution was added with vigorous stirring up to pH 8~9. The precipitate was filtered, the reaction mixture concentrated under reduced pressure followed by purification on flash silica gel column (4**:**1 cyclohexane-EtOAc) yielding the acetonide **2** (0.64 g, 26%) as a syrup, R_f 0.34(4**:**1 cyclohexane-EtOAc), δ_H: 7.45(1H, s, HO-3), 5.03(2H, s,

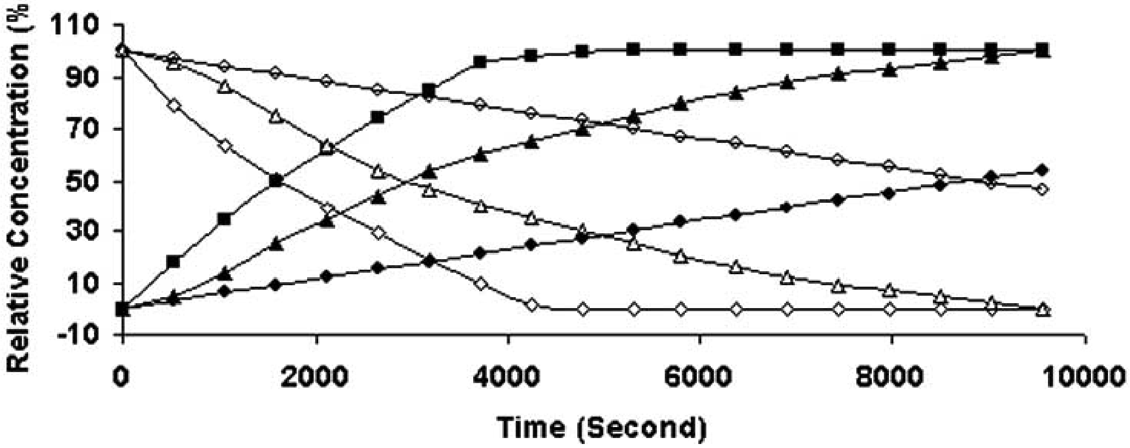


Fig. (4). Kinetic curves showing changes in **GFPOL** (open symbols) and **FPOL** (closed symbols) during incubation with human C4-2 (square) and PC-3 (triangle) prostate and MCF-7 breast (circle) cancer cells transfected to express β-gal. Prostate cells were investigated at 37 °C, while breast cells were examined at 22 °C in PBS buffer.

CH_2-5), 4.57(2H, s, CH_2-4), 2.33(3H, s, CH_3-2), 1.55(6H, s, 2×CH_3)ppm; δ_C: 154.49(s, Py-C), 152.20(s, Py-C), 144.14(d, $J_{F\text{-}C}$=14.5 Hz, Py-C), 131.37(d, $J_{F\text{-}C}$=3.8 Hz, Py-C), 114.01(d, $J_{F\text{-}C}$=32.9 Hz, Py-C), 99.51(s, **C**Me$_2$), 59.04(d, $J_{F\text{-}C}$=3.8 Hz, CH_2-5), 54.51(s, CH_2-4), 31.62(s, C(**C**H$_3$)$_2$), 17.58(s, CH_3-2)ppm.

Anal. Calcd. for $C_{11}H_{14}NO_3F$(%): C, 58.13, H, 6.21, N, 6.17; Found: C, 58.08, H, 6.16, N, 6.11.

*3-O-(2, 3, 4, 6-tetra-O-acetyl-*β*-D-galactopyranosyl)-*α^4*,* α^5*-O-isopropylidene-6-fluoropyridoxol 4*

To a solution of α^4, α^5-*O*-isopropylidene-6-fluoropyridoxol **2** (0.62 g, 2.72mmol) and Hg(CN)$_2$ (0.88 g, 3.50 mmol) in dry CH_2Cl_2 (10 mL) containing freshly activated 4Å molecular sieve (2 g) was added dropwise **3** (1.23 g, 3.0 mmol, 1.1 equiv. in CH_2Cl_2). The mixture was stirred overnight in the dark at r.t. under N_2 until TLC indicated complete reaction. The mixture was diluted with CH_2Cl_2 (30 mL), filtered through Celite, washed, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified on a silica gel column (2**:**3 cyclohexane-EtOAc) to yield **4** (1.29 g, 85%) as syrup, R_f 0.40(2**:**3 cyclohexane-EtOAc), δ_H: 4.64(1H, d, $J_{1',2'}$=8.0 Hz, H-1'), 5.25(1H, dd, $J_{2',3'}$=10.0 Hz, H-2'), 5.02(1H, dd, $J_{3',4'}$=3.6 Hz, H-3'), 5.41(1H, dd, $J_{4',5'}$=3.2 Hz, H-4'), 3.97(1H, m, H-5'), 4.21(1H, dd, $J_{5',6a'}$=4.4 Hz, $J_{6a',6b'}$= 11.2 Hz, H-6a'), 4.13(1H, dd, $J_{5',6b'}$=7.2 Hz, H-6b'), 5.10(1H, d, $J_{CH2\text{-}4a,\,CH2\text{-}4b}$=8.0 Hz, CH_2-4a), 4.67(1H, d, $J_{CH2\text{-}4a,\,CH2\text{-}4b}$=8.0 Hz, CH_2-4b), 5.14 (1H, d, $J_{CH2\text{-}5a,\,CH2\text{-}5b}$=9.6 Hz, CH_2-5a), 5.12(1H, d, $J_{CH2\text{-}5a,\,CH2\text{-}5b}$=9.6 Hz, CH_2-5b), 2.42(3H, s, CH_3-2), 2.17, 2.09, 2.08, 1.99(12H, 4s, 4×CH_3CO), 1.61, 1.59(6H, 2s, 2×CH_3)ppm; δ_C: 170.78, 170.39, 170.26, 170.11(4s, 4×CH$_3$**C**O), 155.44(s, Py-C), 153.15(s, Py-C), 145.48(d, $J_{F\text{-}C}$=15.2 Hz, Py-C), 133.16(d, $J_{F\text{-}C}$=4.0 Hz, Py-C), 116.95(d, $J_{F\text{-}C}$=32.1 Hz, Py-C), 101.41(s, **C**Me$_2$), 100.03(s, C-1'), 68.70(s, C-2'), 70.82(s, C-3'), 67.12(s, C-4'), 71.53(s, C-5'), 64.28(s, C-6'), 55.38(s, CH_2-4), 61.58(s, CH_2-5), 31.88(s, C(**C**H$_3$)$_2$), 20.90, 20.89, 20.82, 20.77(4s, 4×**C**H$_3$CO), 18.77(s, CH_3-2)ppm.

Anal. Calcd. for $C_{25}H_{32}NO_{12}F$(%): C, 53.84, H, 5.79, N, 2.51; Found: C, 53.79, H, 5.74, N, 2.49.

*3-O-(2, 3, 4, 6-tetra-O-acetyl-*β*-D-galactopyranosyl)-6-fluoropyridoxol 5*

A mixture of 3-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α^4, α^5-*O*-isopropylidene-6-fluoropyridoxol **4** (1.25 g, 2.50 mmol) in 80% AcOH (40 mL) was stirred at 80 °C for 4~5 h, until TLC (1**:**3 cyclohexane-EtOAc) showed complete reaction. The cooled mixture was neutralized with cold saturated Na_2CO_3, extracted (EtOAc, 4×30 mL), concentrated and purified by flash silica gel column (1**:**4 cyclohexane-EtOAc) yielding **5** (0.17 g, 15%) as a syrup, R_f 0.18(1**:**4 cyclohexane-EtOAc), δ_H: 4.79(1H, d, $J_{1',2'}$=8.0 Hz, H-1'), 5.55(1H, dd, $J_{2',3'}$=10.6 Hz, H-2'), 5.10(1H, dd, $J_{3',4'}$=3.6 Hz, H-3'), 5.41(1H, dd, $J_{4',5'}$=3.6 Hz, H-4'), 3.88(1H, m, H-5'), 4.24(1H, dd, $J_{5',6a'}$=4.4Hz, $J_{6a',6b'}$=12.0 Hz, H-6a'), 4.09(1H, dd, $J_{5',6b'}$=6.0 Hz, H-6b'), 5.01(2H, d, $J_{CH2\text{-}4a,\,CH2\text{-}4b}$=$J_{CH2\text{-}5a,\,CH2\text{-}5b}$=12.4 Hz, CH_2-4a, CH_2-5a), 4.62(1H, d, $J_{CH2\text{-}4a,\,CH2\text{-}4b}$=12.4Hz, CH_2-4b), 4.66(1H, d, $J_{CH2\text{-}5a,\,CH2\text{-}5b}$=12.4 Hz, CH_2-5b), 3.50(1H, m, HO-4, exchangeable with D_2O), 3.56(1H, m, HO-5, exchangeable with D_2O), 2.47(3H, s, CH_3-2), 2.23, 2.17, 2.02, 2.00(12H, 4s, 4×CH_3CO)ppm; δ_C: 170.32, 170.28, 170.18, 169.48(4×CH$_3$**C**O), 158.78(s, Py-C), 156.42(s, Py-C), 150.33(d, $J_{F\text{-}C}$=15.2 Hz, Py-C), 147.62(d, $J_{F\text{-}C}$=4.6 Hz, Py-C), 120.17(d, $J_{F\text{-}C}$=32.0 Hz, Py-C), 102.39(s, C-1'), 68.91(s, C-2'), 70.74(s, C-3'), 67.19(s, C-4'), 71.93(s, C-5'), 61.98(s, C-6'), 55.91(s, **C**H$_2$-4), 59.60(s, **C**H$_2$-5), 20.99, 20.85, 20.70, 20.67(4s, 4×**C**H$_3$CO), 19.46(s, CH_3-2)ppm.

Anal. Calcd. for $C_{22}H_{28}NO_{12}F$(%): C, 51.05, H, 5.46, N, 2.71; Found: C, 51.00, H, 5.39, N, 2.68.

As an alternative, **5** was synthesized from **1** directly by phase transfer catalysis. To a well stirred CH_2Cl_2 (10 mL)-H_2O (10 mL) biphasic mixture (pH 10~11) of **1** (0.5 g, 2.67mmol) and tetrabutylammonium bromide (TBAB; 0.1 g, 0.31mmol) as the phase-transfer catalyst, a solution of 2, 3, 4, 6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**3**)(1.21 g, 2.94 mmol, 1.1equiv.) in CH_2Cl_2 (10 mL) was added dropwise over a period of 4~5 h at r.t., and the stirring continued for an additional hour. The products were extracted (EtOAc, 4×20 mL), washed free of alkali, dried (Na_2SO_4), concentrated and the residue purified by column chromatography on silica gel (1**:**4 cyclohexane-EtOAc) to afford **5** (1.08 g, 88%) as a syrup, which was identical in all respects to the product obtained above.

*3-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-*α^4*,* α^5*-di-*O*-acetyl-6-fluoropyridoxol 6*

A solution of **5** (1.20 g, 2.64mmol) in pyridine (30 mL) was treated with Ac_2O (15 mL). The reaction mixture was stirred from 0 °C to r.t., overnight, coevaporated with toluene under reduced pressure and the residue purified by flash silica gel column chromatography (1**:**1 cyclohexane-EtOAc) to give **6** (1.56 g, 100%) as a foamy solid, R_f 0.32(1**:**2 cyclohexane-EtOAc), δ_H: 5.15(1H, d, $J_{1',2'}$ =7.8 Hz, H-1'), 5.18(1H, dd, $J_{2',3'}$=10.8 Hz, H-2'), 4.17(1H, dd, $J_{3',4'}$=6.0 Hz, H-3'), 5.41 (1H, dd, $J_{4',5'}$=5.6 Hz, H-4'), 5.31(1H, m, H-5'), 4.06(1H, dd, $J_{5',6a'}$=5.2 Hz, $J_{6a',6b'}$= 12.0 Hz, H-6a'), 4.01(1H, dd, $J_{5',6b'}$ =6.8 Hz, H-6b'), 5.28(2H, m, CH_2-5), 5.12(2H, s, CH_2-4), 2.44(3H, s, CH_3-2), 2.17, 2.12, 2.03, 2.02, 1.99, 1.96(18H, 6s, 6×CH_3CO)ppm; δ_C: 170.42, 170.41, 170.29, 170.25, 170.00, 169.68(6×CH$_3$**C**O), 158.91(s, Py-C), 156.57(s, Py-C), 152.36(d, $J_{F\text{-}C}$=16.0 Hz, Py-C), 147.05(d, $J_{F\text{-}C}$=4.6 Hz, Py-C), 115.53(d, $J_{F\text{-}C}$=32.0 Hz, Py-C), 101.97 (s, C-1'), 69.10(s, C-2'), 70.51(s, C-3'), 67.63(s, C-4'), 70.84(s, C-5'), 60.20(s, C-6'), 57.13(s, **C**H$_2$-4), 61.44(s, **C**H$_2$-5), 21.15, 20.88, 20.86, 20.84, 20.80, 20.76(6s, 6×**C**H$_3$CO), 19.83(s, CH_3-2)ppm.

Anal. Calcd. for $C_{26}H_{32}NO_{14}F$(%): C, 51.90, H, 5.37, N, 2.33; Found: C, 51.88, H, 5.31, N, 2.29.

*3-*O*-(*β*-D-galactopyranosyl)-6-fluoropyridoxol GFPOL*

Compound **6** (1.25 g, 2.1 mmol) was deacetylated with 0.5M NH_3/MeOH (30 mL) from 0 °C to r.t., giving the free galactopyranoside **GFPOL** (0.75 g) as a foamy solid in quantitative yield, R_f 0.21 (1**:**9 MeOH-EtOAc), δ_H: 4.43(1H, d, $J_{1',2'}$= 8.0 Hz, H-1'), 3.63(1H, dd, $J_{2',3'}$=8.8 Hz, H-2'), 3.39(1H, dd, $J_{3',4'}$=3.2 Hz, H-3'), 4.54(1H, dd, $J_{4',5'}$=2.8 Hz, H-4'), 4.69(1H, m, H-5'), 3.45(1H, dd, $J_{5',6a'}$=4.4 Hz, $J_{6a',}$

$_{6b'}$=10.8Hz, H-6a'), 3.07 (1H, dd, $J_{5', 6b'}$=6.0 Hz, H-6b'), 5.20~4.70(4H, br, HO-2', 3', 4', 6', exchangeable with D_2O), 4.53(2H, s, CH_2-4), 4.71(2H, s, CH_2-5), 3.39(2H, br, HO-4, 5, exchangeable with D_2O), 2.42(3H, s, CH_3-2)ppm; δ_C: 158.03(s, Py-C), 155.71(s, Py-C), 150.28(d, $J_{F\text{-}C}$=15.3 Hz, Py-C), 147.61(d, $J_{F\text{-}C}$=5.3 Hz, Py-C), 119.71 (d, $J_{F\text{-}C}$=31.3 Hz, Py-C), 105.62(s, C-1'), 70.90(s, C-2'), 73.06(s, C-3'), 68.14(s, C-4'), 75.47(s, C-5'), 60.58(s, C-6'), 53.79(s, **C**H_2-4), 54.65(s, **C**H_2-5), 19.40(s, CH_3-3)ppm.

Anal. Calcd. for $C_{14}H_{20}NO_8F$(%): C, 48.12, H, 5.77, N, 4.01; Found: C, 48.08, H, 5.75, N, 3.97.

3-O-*Benzyl-6-fluoropyridoxol 7*

To a well stirred CH_2Cl_2(15 mL)-H_2O(20 mL) biphasic mixture (pH 10~11) of **1** (1.0 g, 5.34 mmol) and TBAB (0.3 g, 0.93mmol), a solution of benzyl bromide (1.02 g, 5.88 mmol, 1.1 equiv.) in CH_2Cl_2 (15 mL) was added dropwise over a period of 4~5 h, while the reaction temperature was maintained at 50 °C, and the stirring continued for an additional hour. The products were extracted (CH_2Cl_2, 4×30 mL), washed free of alkali, dried (Na_2SO_4), concentrated and the residue purified by column chromatography on silica gel (1:2 cyclohexane-EtOAc) to afford major product 7 and small amounts of di-O-benzyl-6-fluoropyridoxol derivatives.

(1.12 g, 76%), white crystalline, R_f 0.38(1**:**2 cyclohexane-EtOAc), δ_H: 7.39(5H, m, Ar-H), 4.90(2H, s, $PhCH_2$), 4.75(2H, d, $J_{H\text{-}5, HO\text{-}5}$=5.4 Hz, CH_2-5), 4.72(2H, d, $J_{H\text{-}4, HO\text{-}4}$=6.0 Hz, CH_2-4), 3.57(1H, t, $J_{H\text{-}5, HO\text{-}5}$=5.4 Hz, HO-5, exchangeable with D_2O), 3.49(1H, t, $J_{H\text{-}4, HO\text{-}4}$=6.0 Hz, HO-4, exchangeable with D_2O), 2.44(3H, s, CH_3-2)ppm; δ_C: 157.38(s, Py-C), 155.82(s, Py-C), 149.55(d, $J_{F\text{-}C}$=4.7 Hz, Py-C), 146.97(d, $J_{F\text{-}C}$=4.0 Hz, Py-C), 119.09(d, $J_{F\text{-}C}$=31.2 Hz, Py-C), 136.33, 128.96, 128.88, 128.57(Ph-C), 55.99(s, $PhCH_2$, CH_2-4), 56.76(s, CH_2-5), 19.31(s, CH_3-2)ppm.

Anal. Calcd. for $C_{15}H_{16}NO_3F$(%): C, 64.96, H, 5.82, N, 5.05; Found: C, 64.95, H, 5.79, N, 5.04.

3, α^4-*Di*-O-*benzyl-6-fluoropyridoxol*

(0.30 g, 16%), syrup, R_f 0.30(1**:**2 cyclohexane-EtOAc), δ_H: 7.46~7.33(10H, m, Ar-H), 4.90(2H, s, $PhCH_2$-3), 4.69(2H, s, $PhCH_2$-4), 4.64(2H, d, $J_{H\text{-}5, HO\text{-}5}$=4.8 Hz, CH_2-5), 4.62(2H, s, CH_2-4), 3.37(1H, t, $J_{H\text{-}5, HO\text{-}5}$=4.8 Hz, HO-5, exchangeable with D_2O), 2.44(3H, s, CH_3-2)ppm; δ_C: 157.98(s, Py-C), 155.65(s, Py-C), 152.10(d, $J_{F\text{-}C}$=14.5 Hz, Py-C), 150.04(d, $J_{F\text{-}C}$=4.6 Hz, Py-C), 116.16(d, $J_{F\text{-}C}$=31.3 Hz, Py-C), 136.91, 136.59, 128.92, 128.76, 128.56, 128.48, 128.44(Ph-C), 77.21(s, $PhCH_2$-3), 73.48(s, $PhCH_2$-4), 56.73(s, CH_2-4), 63.30(s, CH_2-5), 19.65(s, CH_3-2)ppm.

Anal. Calcd. for $C_{22}H_{22}NO_3F$(%): C, 71.90, H, 6.04, N, 3.81; Found: C, 71.86, H, 5.99, N, 3.77.

3, α^5-*Di*-O-*benzyl-6-fluoropyridoxol*

(0.16 g, 8%), syrup, R_f 0.25(1**:**2 cyclohexane-EtOAc), δ_H: 7.39~7.30(10H, m, Ar-H), 4.77(2H, s, $PhCH_2$-3), 4.66(2H, d, $J_{H\text{-}4, HO\text{-}4}$=6.8 Hz, CH_2-4), 4.63(2H, s, CH_2-5), 4.58(2H, s, $PhCH_2$-5), 2.95(1H, t, $J_{H\text{-}4, HO\text{-}4}$=6.8 Hz, HO-5, exchangeable with D_2O), 2.46(3H, s, CH_3-2)ppm; δ_C: 158.39(s, Py-C), 156.04(s, Py-C), 151.04(d, $J_{F\text{-}C}$=14.5 Hz, Py-C), 149.72(d, $J_{F\text{-}C}$=4.6 Hz, Py-C), 120.36(d, $J_{F\text{-}C}$=32.8 Hz, Py-C), 136.78, 136.30, 128.92, 128.77, 128.60, 128.55, 128.27(Ph-C), 77.05(s, $PhCH_2$-3), 73.97(s, $PhCH_2$-5), 56.09(s, CH_2-4), 63.66(s, CH_2-5), 19.43(s, CH_3-2)ppm.

Anal. Calcd. for $C_{22}H_{22}NO_3F$(%): C, 71.90, H, 6.04, N, 3.81; Found: C, 71.88, H, 6.01, N, 3.78.

3-O-*Benzyl*-α^4, α^5-*di*-O-*acetyl-6-fluoropyridoxol 8*

Acetylation of **7** (1.10 g, 4.0 mmol) was carried out as described above for **6** to give **8** (1.43 g, 100%), white crystalline, R_f 0.44(3**:**2 cyclohexane-EtOAc), δ_H: 7.40(5H, m, Ar-H), 4.98(2H, s, CH_2-5), 4.92(2H, s, $PhCH_2$), 4.85(2H, s, CH_2-4), 2.46(3H, s, CH_3-2), 2.20, 2.17(6H, 2s, 2×CH_3CO)ppm; δ_C: 170.58, 170.46(2×CH_3**C**O), 158.48(s, Py-C), 155.98(s, Py-C), 150.35(d, $J_{F\text{-}C}$=5.0Hz, Py-C), 147.68(d, $J_{F\text{-}C}$=4.4 Hz, Py-C), 120.06(d, $J_{F\text{-}C}$=31.6 Hz, Py-C), 137.43, 129.66, 129.38, 128.88(Ph-C), 56.69(s, $PhCH_2$, CH_2-4), 57.66(s, CH_2-5), 21.45, 20.98(2s, 2×**C**H_3CO), 19.36(s, CH_3-2)ppm.

Anal. Calcd. for $C_{19}H_{20}NO_5F$(%): C, 63.13, H, 5.58, N, 3.88; Found: C, 63.09, H, 5.53, N, 3.86.

α^4, α^5-*di*-O-*acetyl-6-fluoropyridoxol 9*

A mixture of **8** (1.20 g, 3.32 mmol) and 5% Pd-C (500 mg) in MeOH (100 mL) was stirred for 24 h at r.t., under H_2 atmosphere. After filtration, the filtrate was evaporated to afford **9** in quantitative yields (white crystals), R_f 0.24(3**:**2 cyclohexane-EtOAc), δ_H: 7.56(1H, s, HO-3), 5.08(2H, s, CH_2-5), 4.91(2H, s, CH_2-4), 2.48(3H, s, CH_3-2), 2.22, 2.18(6H, 2s, 2×CH_3CO)ppm; δ_C: 170.56, 170.50(2×CH_3**C**O), 158.68(s, Py-C), 156.00(s, Py-C), 150.75(d, $J_{F\text{-}C}$=5.1 Hz, Py-C), 147.88(d, $J_{F\text{-}C}$=4.2 Hz, Py-C), 120.09(d, $J_{F\text{-}C}$=31.9 Hz, Py-C), 56.88(s, CH_2-4), 57.94(s, CH_2-5), 21.65, 20.89(2s, 2×**C**H_3CO), 19.44(s, CH_3-2)ppm.

Anal. Calcd. for $C_{12}H_{14}NO_5F$(%): C, 53.11, H, 5.20, N, 5.17; Found: C, 53.08, H, 5.17, N, 5.14.

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