Fluorination of Betulinines and Other Triterpenoids with DAST

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Abstract: Betulinines are lupane, des-E-lupane, 18-lupene, 20(29)-lupene and 18α-oleanane derivatives with antitumor activity. We examined fluorination of these derivatives using diethylaminosulfur trifluoride (DAST) as fluorinating agent. We prepared 19β,28-epoxy-2,2-difluoro-18α-oleanan-3-one (**3c**), 19β,28-epoxy-2,2-difluoro-18α-oleanan-3β-ol (**4a**), methyl 3β-acetoxy-30-fluorolup-20(29)-ene-28-oate (**6b**), 3β,28-diacetoxy-22-oxo-21,21-difluorolup-18-ene (**8b**) and several other fluorinated betulinines for *in vitro* cytotoxicity tests which failed to demonstrate significant anticancer activity so far.

Key words: antitumor agents, fluorine, rearrangements, steric hindrance, terpenoids

Introduction

Diethylaminosulfur trifluoride (DAST) is a widely used fluorinating reagent,[1–8] very effective for converting alcohols, ketones, aldehydes and carboxylic acids into their corresponding fluoro derivatives. Unlike other fluorinating agents, e.g. Olah reagent or SF_4, reactions with DAST can be performed in glass equipments. The problem in performing fluorination lies in the possibility of rearrangement or elimination instead of the desired reaction.

Recently, we have examined the structure-activity relationships in certain Betulinines[9] (lupane, des-E-lupane, 18-lupene, 20(29)-lupene and 18α-oleanane derivatives), and found significant antitumor activity.[9] This effort was directed towards highly polar compounds, while less polar compounds were not sufficiently examined. With a few exceptions,[10] no lupane or 18α-oleanane fluoro derivatives have been prepared until now. High antitumor activity of fluorinated compounds analogous to triterpenoids, e.g. steroid Fluoxymesteron, is well known.[11]

Herein we report on the construction of new fluoro compounds, derived from lupane, des-E-lupane, 18-lupene, 20(29)-lupene and 18α-oleanane alcohols, oxo derivatives and carboxylic acids using DAST as a source of fluorine.

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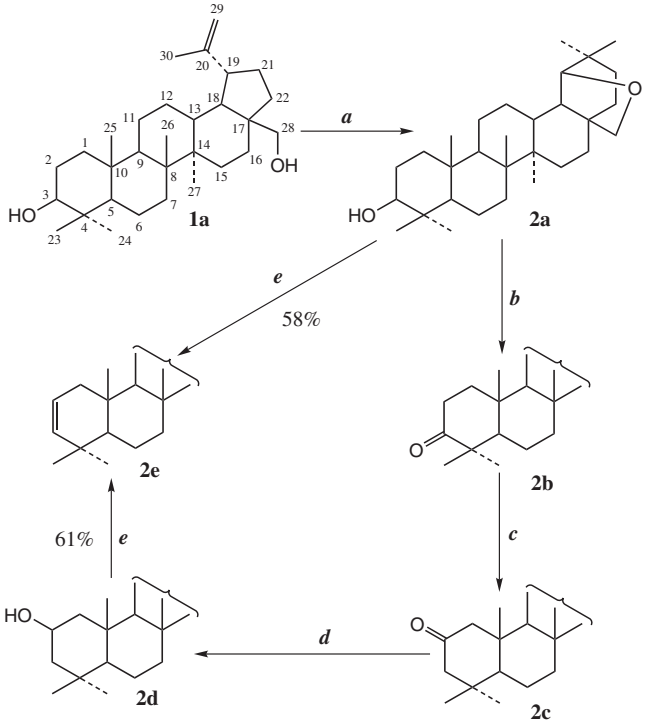
Fluorination of Alcohols

As a starting material we used betulin (**1a**), readily accessible from birch bark. This compound undergoes an acid-catalyzed rearrangement with ring E expansion to allobetuline (**2a**). For this reaction we used a 'solid acid' Montmorillonite K10. The procedure, as described in the literature,[12] did not work properly in our hands. We had to increase the amount of Montmorillonite K 10 fivefold and prolong the reaction time to 72 hours to obtain a good conversion of the starting material. Treatment of allobetuline (**2a**) with DAST under very mild conditions resulted in the formation of an elimination product, olefin **2e**, as we expected. Elimination occurred probably due to heavy steric hindrance of the alcohol group in position 3 by skeletal methyl groups at positions 23 and 24. We therefore decided to prepare alcohol **2d**, which we expected to be less sterically hindered. First of all we oxidized the hydroxy group in position 3 of the allobetuline (**2a**) with a slightly modified procedure[13] to obtain ketone **2b**. We then converted this compound into isomeric ketone **2c** by refluxing with sulfur in morpholine.[14] Reduction of the ketone **2c** with $NaBH_4$ resulted in the formation of desired alcohol **2d**. Reaction of alcohol **2d** with DAST gave olefin **2e**[12] as the single product (Scheme 1).

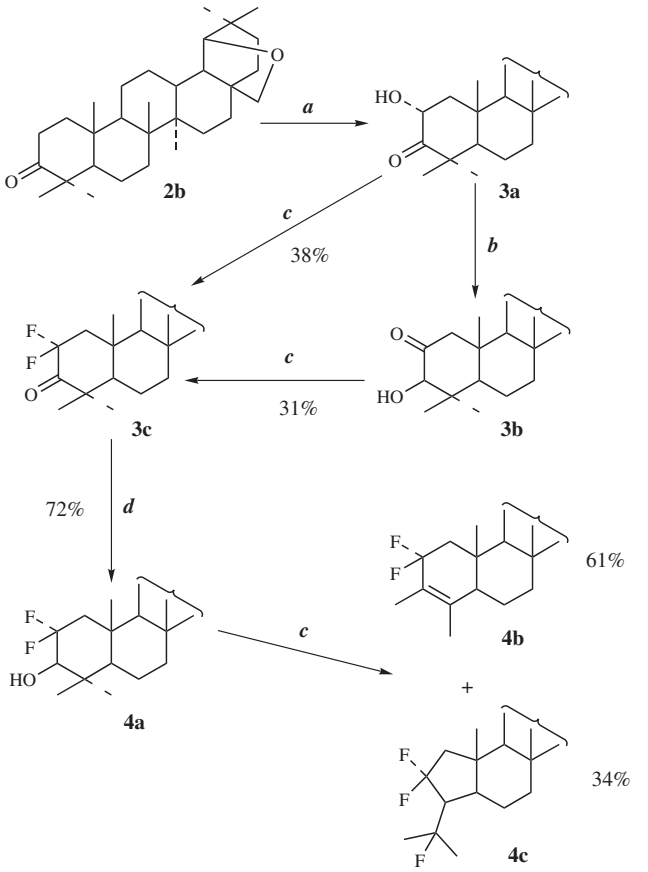
Olefin **2e** with C-2 double bond was the only product of reactions of **2a** and **2d** with DAST. We therefore used hydroxy ketones **3a** and **3b**,[15] accessible from ketone **2b**, to avoid such elimination. Both hydroxy ketones **3a** and **3b** reacted with DAST to form a single product, the difluoro ketone **3c**. Optimization of the reaction conditions led to 38% yield.

We next examined the effectiveness of DAST towards 2,2-difluoro alcohol **4a**, prepared from difluoro ketone **3c** by reduction with $NaBH_4$. Treatment of 2,2-difluoro alcohol **4a** with DAST afforded the difluoro olefin **4b**, and the trifluoro derivative **4c** as a result of rearrangement (Scheme 2).

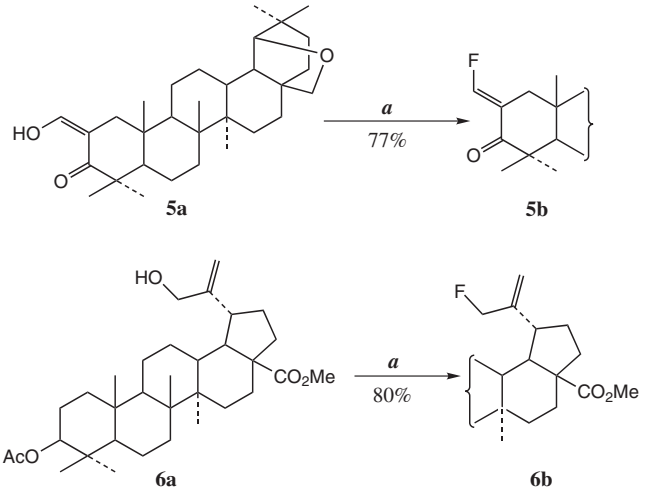
The above-mentioned unexpected reactions, rearrangements and low yields may be caused by the significant steric hindrance and rigid skeleton. We confirmed this hypothesis using unhindered enol ketone **5a** and alcohol **6a** as substrates for fluorination (Scheme 3). These reactions gave the desired fluoro derivatives **5b** and **6b** in high yields (77–80%).



Scheme 1 *Reagents and conditions*: *a.* Montmorillonite K10, $CHCl_3$, reflux; *b*. PCC, Al_2O_3, $CHCl_3$; *c*. S, morpholine, reflux; *d*. $NaBH_4$, THF, MeOH; *e*. DAST, CH_2Cl_2, –78 °C to r.t.



Scheme 2 *Reagents and conditions*: *a.* MCPBA, MeOH, H_2SO_4, $CHCl_3$; *b*. H_2SO_4, dioxane, MeOH, reflux; *c*. DAST, CH_2Cl_2, –78 °C to r.t., *d*. $NaBH_4$, THF, MeOH



Scheme 3 *Reagents and conditions*: *a.* DAST, CH_2Cl_2, –78 °C to r.t.

Fluorination of Oxo Derivatives

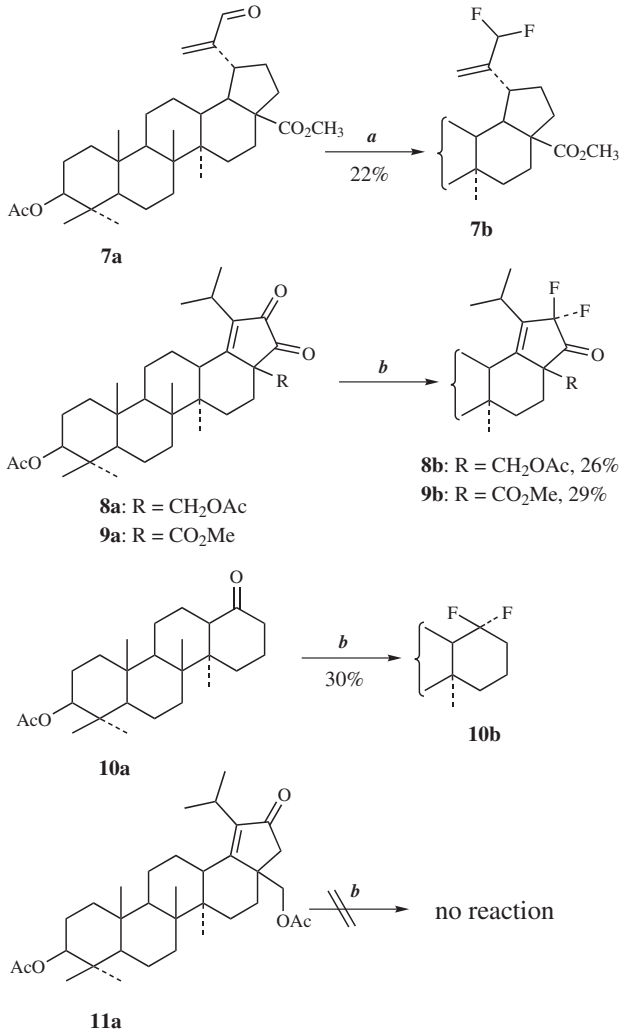
Utilization of DAST for the conversion of ketones and aldehydes into their corresponding geminal difluoro derivatives demands harder condition[2] than the substitution of a hydroxy group. Originally we used the literature conditions,[1] which recommended reflux with excess of DAST in CH_2Cl_2. We however found this procedure to be effective only towards aldehyde **7a**. Moreover, we obtained the corresponding difluoro derivative **7b** only in low yield (22%) after 48 hours of reaction time (Scheme 4).

We therefore decided to utilize other experimental conditions.[2] We performed the reaction with excess of DAST (which served also as a co-solvent) in a sealed cylindrical flask and heated to 70 °C. Under these conditions difluoro ketones **8b** and **9b** were prepared from diketones **8a** and **9a** in yields below 30%. No tetrafluorinated product was observed. In the case of heptanorketone **10a** we also succeeded and prepared the difluoro derivative **10b**. In contrast, ketones **2b, 2c** and **11a** showed no conversion under these conditions (Schemes 1, 4).

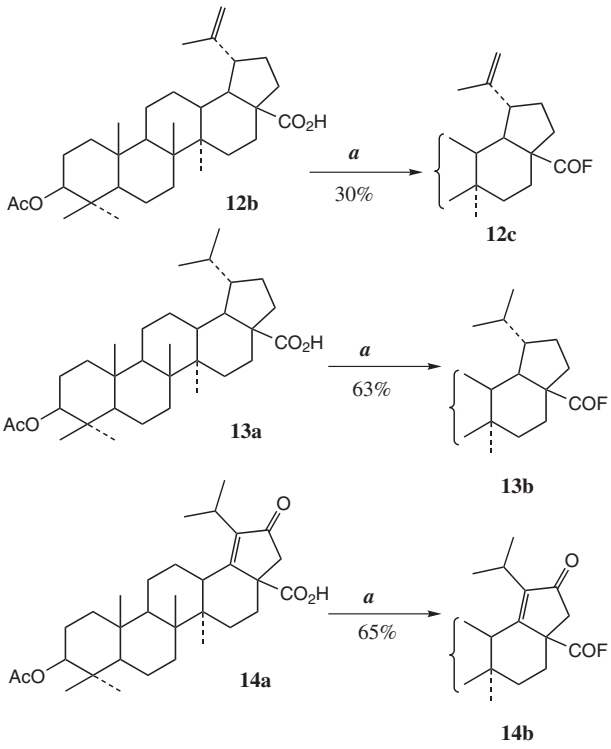
Fluorination of Carboxylic Acids

DAST is a convenient reagent for the synthesis of acyl fluorides from the corresponding carboxylic acids under very mild conditions.[1] The experimental procedure is analogous to the procedure we used for fluorination of alcohols. Reaction yields are above average (60–70%) and no by-products were observed.

Acetylbetulinic acid (**12b**) was fluorinated with DAST to acyl fluoride **12c**. We used the same procedure successfully in the reactions of the saturated derivative **13a** and 21-oxo acid **14a**, to obtain acyl fluorides **13b** and **14b**, respectively (Scheme 5).



Scheme 4 *Reagents and conditions*: *a.* DAST, $CHCl_3$, reflux; *b*. DAST, $CHCl_3$, 70 °C



Scheme 5 *Reagents and conditions*: *a.* DAST, CH_2Cl_2, –78 °C to r.t.

Conclusion

We have shown that DAST is a suitable reagent for the transformation of natural compounds with complicated structure (triterpenoids) to their corresponding fluoro derivatives. Alcohols, ketones, aldehydes and carboxylic acids can be used as substrates. The limitations of these fluorinations are the rigid skeleton of these natural compounds and the steric hindrance caused by skeletal methyl groups. The rigid skeleton induces low reactivity. Steric hindrance may cause observed rearrangements and eliminations. Having the reaction centre placed out of the rigid skeleton, the yield increased dramatically up to 80%. Conversion of ketones to difluoro derivatives demands hard conditions. In contrast, for conversion of carboxylic acids to acyl fluorides very mild conditions are sufficient.

All thirteen fluoro derivatives prepared in this work (**3c**, **4a–c**, **5b**, **6b**, **7b**, **8b**, **9b**, **10b**, **12c**, **13b**, **14b**) were tested for *in vitro* cytotoxic activity. Unfortunately, fluorinated triterpenoids exemplified in this paper failed to demonstrate significant anticancer activity on CEM leukemia cells (TCS_{50} above 250 μM). We demonstrated the decrease of cytotoxic activity by introduction of fluorine into these molecules. This fact probably originates in the high lipophilicity of those fluoro derivatives.

Melting points were determined on Kofler block and are uncorrected. Optical rotations were measured on an Autopol III (Rudolph Research, Flanders, NJ) polarimeter in $CHCl_3$. ^1H, ^{13}C and ^{19}F NMR spectra were recorded on Varian^{UNITY} Inova 400 (400 MHz for ^1H), using $CDCl_3$ as a solvent. Chemical shifts are expressed in ppm with tetramethylsilane as an internal standard for ^1H spectra and with $CClF_3$ as an internal standard for ^{19}F NMR spectra. ^{13}C NMR spectra are referenced to $CDCl_3$ (77.00 ppm). Mass spectra (EI) were measured on INCOS 50 (Finnigan MAT) mass spectrometer. IR spectra were recorded on a Perkin-Elmer 684 IR spectrometer in $CHCl_3$. TLC was performed on Kieselgel 60 F_{254} (Merck) sheets; the spots were detected by UV fluorescensce or spraying with 10% H_2SO_4 and heating to 110–200 °C. HPLC system consisted of High Pressure Pump Gilson (model 361), Inject Valve Rheodyne, Preparative Column (25 × 250 mm) with silica gel filling (Biospher 7 μm; Labio), Differential–Refractometrical Detector (Laboratorní přístroje, Praha, CR) connected with PC (software Chromulan) and Automatic Fraction Collector Gilson (model 246). Betulin (**1a**) was obtained by extraction of birch bark from our patrner papermill, Billerud, Gruvön Mill, Sweden, using literature procedure.[21] Betulinic acid (**12a**) was obtained from natural source according to literature.[18]

WARNING: DAST is a dangerous substance which can decompose explosively on temperature over 55 °C.

Fluorination of the oxo compounds was carried out in a 10 mL enclosed cylindrical flask (from Kimble–Kontes, art. no.: 747500-0010). The following compounds were prepared using literature

procedures: allobetuline (**2a**),[12] 19β,28-epoxy-18α-oleanan-3-one (**2b**),[13] 19β,28-epoxy-18α-oleanan-2-one (**2c**),[14] 19β,28-epoxy-18α-oleanan-2β-ol (**2d**),[16] 19β,28-epoxy-2α-hydroxy-18α-oleanan-3-one (**3a**),[15] 19β,28-epoxy-3β–hydroxy-18α-oleanan-2-one (**3b**),[15] enol ketone **5a**,[17] alcohol **6a**,[18] aldehyde **7a**,[9] diketone **8a**,[19] diketone **9a**,[9] ketone **10a**,[9] ketone **11a**,[22] acetylbetulinic acid (**12b**),[20] dihydrobetulinic acid (**13a**),[19] and 21-oxoacid **14a**.[9] DAST was purchased from Sigma-Aldrich.

19β,28-Epoxy-18α-oleanan-2-ene (2e)

*From Alcohol **2a***: To a stirred solution of alcohol **2a** (200 mg, 0.45 mmol) in CH_2Cl_2 (5 mL) cooled to –78 °C was added DAST (200 μL, 1.04 mmol). The reaction mixture was then allowed to warm to r.t. and the reaction was quenched by addition of H_2O (1 mL). The separated organic layer was diluted with $CHCl_3$ (50 mL), washed with H_2O (2 × 40 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The dark yellow-brown residue was filtered over a short column of silica gel (3 g, toluene) and crystallized from *i*-PrOH to give olefin **2e** (129 mg, 58%); mp 240–243 °C (Lit.[21] mp 244.5–245 °C); $[\alpha]_D^{25}$ +81 (*c* = 0.22). The ^{1}H NMR spectrum is identical with the ^{1}H NMR spectrum of an authentic sample.[12]

*From Alcohol **2d***: Alcohol **2d** (110 mg, 0.248 mmol) reacted with DAST in the same manner as alcohol **2a** to give **2e** (74 mg, 61%).

19β,28-Epoxy-2,2-difluoro-18α-oleanan-3-one (3c)

*From Hydroxy Ketone **3a***: To a stirred solution of hydroxy ketone **3a** (2.1g, 4.46 mmol) in CH_2Cl_2 (20 mL) was added DAST (2 mL, 10.35 mmol) slowly at r.t.. The reaction was quenched after 24 h by addition of H_2O (10 mL). The separated organic layer was diluted with $CHCl_3$ (100 mL), washed with H_2O (2 × 400 mL), dried ($MgSO_4$) and the organic layer was evaporated under reduced pressure. The pale brown residue was chromatographed on silica gel (toluene–Et_2O, 40:1) and crystallized from *i*-PrOH to give difluoro ketone **3c** (800 mg, 38%); mp 243–245 °C; $[\alpha]_D^{25}$ +123 (*c* = 0.52).

*From Hydroxy Ketone **3b***: Hydroxy ketone **3b** (200 mg, 0.42 mmol) reacted with DAST in the same manner as hydroxy ketone **3a** to give **3c** (63 mg, 31%).

IR ($CHCl_3$): 1743 cm^{-1} (C=O).

^{1}H NMR: δ = 0.81 (s, 3 H), 0.89 (s, 3 H), 0.94 (s, 3 H), 0.95 (s, 3 H), 0.99 (s, 3 H), 1.16 (s, 3 H), 1.23 (d, *J* = 1.6 Hz, 3 H), 2.08 (ddd, 1 H, *J* = 30.1, 15.3, 6.1 Hz, H-1α), 2.26 (dt, *J* = 15.1, 19.8 Hz, 1 H, H-1β), 3.46 (d, *J* = 7.8 Hz, 1 H, H-28a), 3.53 (s, 1 H, H-19α), 3.77 (dd, *J* = 7.8, 1.7 Hz, 1 H, H-28b).

^{13}C NMR: δ = 51.8 (dd, *J* = 23, 20 Hz, C-1), 115.8 (dd, *J* = 258, 245 Hz, C-2), 204.6 (dd, *J* = 25, 23 Hz, C-3), 54.8 (C-4), 50.9 (C-5), 19.6 (C-6), 32.3 (C-7), 40.8 (C-8), 50.1 (C-9), 37.0 (dd, *J* = 5, 2 Hz, C-10), 21.8 (C-11), 26.2 (C-12), 34.3 (C-13), 40.4 (C-14), 26.3 (C-15), 36.6 (C-16), 41.4 (C-17), 46.6 (C-18), 87.8 (C-19), 36.2 (C-20), 32.6 (C-21), 26.1 (C-22), 28.0 (d, *J* = 4 Hz, C-23), 21.0 (d, *J* = 1 Hz, C-24), 18.5 (C-25), 15.1 (C-26), 13.4 (C-27), 71.2 (C-28), 24.5 (C-29), 28.8 (C-30).

^{19}F NMR: δ = –100.10 (ddd, *J* = 262.1, 20.4, 6.1 Hz), –87.76 (dddt, *J* = 262, 30, 20, 2 Hz).

MS: *m*/*z* (%) = 476 (M$^+$, 100), 458 (8), 445 (20), 405 (80), 388 (1), 341 (1), 281 (1), 204 (16), 191 (23).

Anal. Calcd for $C_{30}H_{46}F_2O_2$: C, 75.59; H, 9.73; F, 7.97. Found: C, 75.33; H, 9.37; F, 7.13.

19β,28-Epoxy-2,2-difluoro-18α-oleanan-3β-ol (4a)

To a solution of difluoro ketone **3c** (475 mg, 0.99 mmol) in a mixture of THF (12 mL) and MeOH (12 mL) cooled in an ice-bath was added $NaBH_4$ (200 mg, 5.29 mmol). After 2 h, the reaction mixture was poured into 5% aq HCl (100 mL) and extracted with $CHCl_3$ (100 mL). The organic layer was washed with H_2O (3 ×). The organic layer was dried ($MgSO_4$), evaporated, and the residue was crystallized from butanone to give **4a** (340 mg, 72%); mp 299–301 °C; $[\alpha]_D^{25}$ +48 (*c* = 0.39).

IR ($CHCl_3$): 3608 cm^{-1} (C–OH).

^{1}H NMR: δ = 0.80 (s, 3 H), 0.89 (d, 3 H, *J* = 1.2 Hz), 0.92 (s, 3 H), 0.93 (s, 3 H), 1.00 (s, 6 H), 1.08 (s, 3 H), 2.33 (dt, 1 H, *J* = 4.6, 14.0 Hz), 3.45 (d, 1 H, *J* = 7.8 Hz, H-28a), 3.52 (s, 1 H, H-19α), 3.77 (dd, 1 H, *J* = 7.8, 1.5 Hz, H-28b), 3.35 (dd, 1 H, *J* = 22.4, 7.3 Hz, H-3α).

^{13}C NMR: δ = 46.3 (t, *J* = 20 Hz, C-1), C-2 not found, 78.5 (t, *J* = 20 Hz, C-3), 39.6 (d, *J* = 6 Hz, C-4), 55.1 (C-5), 18.0 (C-6), 33.6 (C-7), 40.9 (C-8), 51.4 (C-9), 38.4 (d, *J* = 5 Hz, C-10), 21.4 (C-11), 26.3 (C-12), 34.0 (C-13), 40.8 (C-14), 26.3 (C-15), 36.7 (C-16), 41.5 (C-17), 46.8 (C-18), 87.9 (C-19), 36.2 (C-20), 32.7 (C-21), 26.2 (C-22), 19.1 (C-23), 15.6 (C-24), 15.9 (d, *J* = 5 Hz, C-25), 15.3 (C-26), 13.4 (C-27), 71.2 (C-28), 24.5 (C-29), 28.8 (C-30).

^{19}F NMR: δ = –90.71 (dq, *J* = 245.2, 5.4 Hz, F-2α), –110.20 (dddd, *J* = 245.2, 34.7, 22.2, 13.3 Hz, F-2β).

MS: *m*/*z* (%) = 478 (M$^+$, 100), 458 (69), 447 (34), 438 (17), 427 (23), 407 (78), 342 (11), 243 (9), 220 (11), 203 (17), 191 (25).

Anal. Calcd for $C_{30}H_{48}F_2O_2$: C, 75.27; H, 10.11; F, 7.94. Found: C, 75.62; H, 9.88; F, 7.88.

Difluoro Olefin 4b and Trifluoro Derivative 4c

To a stirred solution of alcohol **4a** (170 mg, 0.35 mmol) in CH_2Cl_2 (5 mL), cooled to –78 °C was added DAST (170 μL, 0.88 mmol). The reaction mixture was then allowed to warm to r.t. and the reaction was quenched by addition of H_2O (1 mL). The separated organic layer was diluted with $CHCl_3$ (50 mL), washed with H_2O (2 × 40 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The brown residue was separated by HPLC (3.5% EtOAc in hexane) and lyophilized (*t*-BuOH) to give difluoro olefin **4b** (93 mg, 61%); mp 232–233 °C (subl.); $[\alpha]_D^{25}$ +55 (*c* = 0.36) and trifluoro derivative **4c** (56 mg, 34%); mp 235–236 °C; $[\alpha]_D^{25}$ +79 (*c* = 0.38).

4b

IR ($CHCl_3$): 1683 cm^{-1} (C=C).

^{1}H NMR: δ = 0.80 (s, 3 H), 0.83 (t, 3 H, *J* = 2.0 Hz), 0.93 (s, 3 H), 0.94 (s, 3 H), 1.00 (s, 3 H), 1.80 (dt, 3 H, *J* = 3.7, 2.5 Hz, CH_3C=C), 1.87 (q, *J* = 2.7 Hz, CH_3C=C), 2.10–2.18 (m, 3 H, Σ *J* = 31.7 Hz), 3.45 (d, 1 H, *J* = 7.8 Hz, H-28a), 3.52 (s, 1 H, H-19α), 3.77 (dd, 1 H, *J* = 7.8, 1.8 Hz).

^{13}C NMR: δ = 52.9 (t, *J* = 23 Hz, C-1), C-2 not found, 131.6 (t, *J* = 25 Hz, C-3), 128.8 (C-4), 55.6 (C-5), 22.4 (C-6), 33.2 (C-7), 40.3 (C-8), 48.1 (C-9), 41.3 (C-10), 23.5 (C-11), 26.4 (C-12), 34.1 (C-13), 40.8 (C-14), 26.2 (C-15), 36.8 (C-16), 41.5 (C-17), 46.9 (C-18), 88.0 (C-19), 36.3 (C-20), 32.7 (C-21), 26.1 (C-22), 22.2 (d, *J* = 5 Hz, C-23), 20.4 (C-24), 15.4 (C-25), 15.7 (C-26), 13.4 (C-27), 71.2 (C-28), 24.5 (C-29), 28.8 (C-30).

^{19}F NMR: δ = –80.80 (dt, *J* = 248.5, 19.0 Hz), –82.72 (dq, *J* = 248.5, 19.6 Hz).

MS: *m*/*z* (%) = 460 (M$^+$, 9), 440 (100), 420 (8), 409 (2), 389 (1), 365 (1), 316 (1), 205 (15), 191 (3).

Anal. Calcd for $C_{30}H_{46}F_2O$: C, 78.21; H, 10.06; F, 8.25. Found: C, 75.11; H, 10.01; F, 8.11.

4c

IR ($CHCl_3$): 1241 cm^{-1} (C–O–C).

^{1}H NMR: δ = 0.80 (s, 3 H), 0.93 (s, 3 H), 0.94 (s, 3 H), 0.98 (s, 3 H), 1.01 (s, 3 H), 1.49 (d, 3 H, *J* = 22.7 Hz), 1.51 (d, 3 H, *J* = 23.0 Hz), 2.12 (dd, 1 H, *J* = 17.6, 13.4 Hz), 2.83 (dddd, 1 H, *J* = 21.3, 17.8, 13.9, 12.1 Hz, H-3α), 3.45 (d, 1 H, *J* = 7.8 Hz, H-28a), 3.52 (s, 1 H, H-19α), 3.77 (dd, 1 H, *J* = 7.8, 1.6 Hz, H-28b).

^{13}C NMR: δ = 52.7 (*J* = 21 Hz, C-1), C-2 not found, 57.7 (C-3), 97.1 (d, *J* = 165 Hz, C-4), 53.5 (d, *J* = 7 Hz, C-5), 20.1 (C-6), 34.4 (C-7), 40.9 (C-8), 50.4 (C-9), 43.3 (d, *J* = 7 Hz, C-10), 24.0 (C-11), 26.4 (C-12), 34.1 (C-13), 40.8 (C-14), 26.1 (C-15), 36.8 (C-16), 41.5 (C-17), 46.9 (C-18), 87.9 (C-19), 36.3 (C-20), 32.7 (C-21), 26.2 (C-22), 26.8 (C-23), 29.1 (C-24), 15.3 (C-25), 16.2 (C-26), 13.5 (C-27), 71.2 (C-28), 24.5 (C-29), 28.8 (C-30).

^{19}F NMR: δ = –79.42 (ddd, *J* = 240.2, 21.3, 14.7 Hz), –91.59 (dm, *J* = 240 Hz, Σ *J′* = 64.6 Hz), –126.77 (m, Σ *J* = 161.5 Hz).

MS: *m/z* (%) = 480 (M^+, 90), 460 (6), 449 (33), 440 (54), 425 (82), 409 (100), 389 (4), 371 (3), 341 (2), 272 (5), 258 (8), 225 (49), 205 (61), 191 (30).

Anal. Calcd for $C_{30}H_{47}F_3O$: C, 74.96; H, 9.86; F, 11.86. Found: C, 74.69; H, 10.12; F, 12.07s.

Fluoro Ketone 5b

To a stirred solution of enol ketone **5a** (107 mg, 0.23 mmol) in CH_2Cl_2 (5 mL), cooled to –78 °C was added DAST (100 μL, 0.52 mmol). The reaction mixture was then allowed to warm to r.t. and then quenched by addition of H_2O (1 mL). The separated organic layer was diluted with $CHCl_3$ (50 mL), washed with H_2O (2 × 40 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The dark yellow residue was separated by HPLC (6% EtOAc in hexane) to give fluoro ketone **5b** (81 mg, 77%) as white crystalline solid; mp 231–233 °C (MeOH); $[\alpha]_D^{25}$ +75 (*c* = 0.17).

IR ($CHCl_3$): 1616 (C=C), 1689 cm^{-1} (C=O).

^{1}H NMR: δ = 0.81 (s, 3 H), 0.84 (d, 3 H, *J* = 0.9 Hz), 0.94 (d, 3 H, *J* = 0.9 Hz), 0.94 (s, 3 H), 1.02 (s, 3 H), 1.09 (s, 3 H), 1.10 (s, 3 H), 2.86 (dt, 1 H, *J* = 16.1, 2.1 Hz, H-1), 3.46 (d, 1 H, *J* = 7.9 Hz, H-28a), 3.55 (s, 1 H, H-19α), 3.79 (dd, 1 H, *J* = 7.8, 1.4 Hz, H-28b), 7.35 (ddd, 1 H, *J* = 82.6, 3.1, 1.5 Hz, H-31).

^{13}C NMR: δ = 38.5 (C-1), 119.7 (C-2), 207.0 (C-3), 45.5 (C-4), 53.1 (C-5), 20.1 (C-6), 32.7 (C-7), 40.4 (C-8), 48.9 (C-9), 35.8 (C-10), 21.7 (C-11), 26.4 (C-12), 34.3 (C-13), 40.8 (C-14), 26.4 (C-15), 36.7 (C-16), 41.5 (C-17), 46.8 (C-18), 87.9 (C-19), 36.3 (C-20), 32.8 (C-21), 26.2 (C-22), 28.8 (C-23), 22.0 (C-24), 15.3 (C-25), 16.0 (C-26), 13.4 (C-27), 71.3 (C-28), 24.5 (C-29), 28.7 (C-30), 156.8 (d, *J* = 278 Hz, C-31).

^{19}F NMR: δ = –121.14 (ddd, *J* = 82.4, 6.4 Hz, 4.1 Hz).

MS: *m/z* (%) = 470 (M^+, 100), 450 (15), 439 (23), 399 (69), 383 (1), 341 (1), 323 (1), 215 (14), 203 (6), 189 (7).

Anal. Calcd for $C_{31}H_{47}FO_2$: C, 79.10; H, 10.06; F, 4.04. Found: C, 79.34; H, 9.76; F, 4.53.

Methyl 3β-Acetoxy-30-fluorolup-20(29)-en-28-oate (6b)

To a stirred solution of alcohol **6a** (100 mg, 0.18 mmol) in CH_2Cl_2 (5 mL), cooled to –78 °C was added DAST (100 μL, 0.52 mmol). The reaction mixture was then allowed to warm to r.t. and then quenched by addition of H_2O (1 mL). The separated organic layer was diluted with $CHCl_3$ (50 mL), washed with H_2O (2 × 40 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The dark yellow residue was separated by HPLC (6% EtOAc in hexane) and crystallized from MeOH to give **6b** (80 mg, 80%); mp 219–221 °C; $[\alpha]_D^{25}$ –6.0 (*c* = 0.37).

IR ($CHCl_3$): 1256 (C–O–C), 1655 (C=C), 1721 cm^{-1} (C=O).

^{1}H NMR: δ = 0.83 (s, 3 H), 0.84 (s, 3 H), 0.84 (s, 3 H), 0.91 (s, 3 H), 0.96 (s, 3 H), 2.04 (s, 3 H), 2.95 (td, 1 H, *J* = 11.1, 4.4 Hz, H-19b), 3.67 (s, 3 H, CO_2CH_3), 4.47 (m, 1 H, Σ *J* = 16.0 Hz, H-3α), 4.84 (d, 2 H, *J* = 47.5 Hz, H-30), 5.02 (m, 2 H, Σ *J* = 6.3 Hz, H-29).

^{13}C NMR: δ = 38.4 (C-1), 23.7 (C-2), 80.9 (C-3), 37.8 (C-4), 55.4 (C-5), 18.2 (C-6), 34.2 (C-7), 40.7 (C-8), 50.4 (C-9), 37.1 (C-10), 21.0 (C-11), 26.7 (C-12), 38.2 (C-13), 42.3 (C-14), 29.7 (C-15), 32.0 (C-16), 56.6 (C-17), 50.1 (C-18), 42.2 (C-19), 150.1 (d, *J* = 12.2 Hz, C-20), 27.3 (C-21), 36.6 (C-22), 27.9 (C-23), 16.5 (C-24), 16.2 (C-25), 15.9 (C-26), 14.6 (C-27), 176.5 (C-28), 110.9 (d, *J* = 11.1 Hz, C-29), 85.0 (d, *J* = 169 Hz, C-30), 21.3 (3β-OCO*C*H$_3$), 171.0 (3β-O*C*OCH$_3$), 51.3 (CO_2CH_3).

^{19}F NMR: δ = –215.22 (td, *J* = 47.3, 2.3 Hz).

MS: *m/z* (%) = 530 (M^+, 63), 510 (20), 470 (100), 455 (42), 427 (14), 411 (14), 291 (13), 219 (13), 207 (19), 189 (56).

Anal. Calcd for $C_{33}H_{51}FO_4$: C, 74.68; H, 9.69; F, 3.58. Found: C, 74.34; H, 9.72; F, 3.66.

Difluoro Olefin 7b

To a stirred solution of aldehyde **7a** (200 mg, 0.36 mmol) in $CHCl_3$ (5 mL) was added DAST (500 μL, 2.6 mmol). The reaction mixture was refluxed 24 h, diluted with $CHCl_3$ (30 mL), and the $CHCl_3$ layer was washed carefully with 5% aq $NaHCO_3$ solution (40 mL) and H_2O (40 mL). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. The dark yellow-brown residue was filtered over a short column of silica gel (3 g, toluene–Et_2O, 10:1) and separated by HPLC (6% EtOAc in hexane) to give difluoro olefin **7b** (46 mg, 22%); mp 246–248 °C (MeOH); $[\alpha]_D^{25}$ +21 (*c* = 0.31).

IR ($CHCl_3$): 1255 cm^{-1} (C–O–C).

^{1}H NMR: δ = 0.83 (s, 3 H), 0.84 (s, 3 H), 0.84 (s, 3 H), 0.91 (s, 3 H), 0.97 (s, 3 H), 2.04 (s, 3 H), 3.08 (td, 1 H, *J* = 11.3, 4.3 Hz), 3.68 (s, 1 H, CO_2CH_3), 4.45 (m, 1 H, Σ *J* = 16.0 Hz, H-3α), 5.24 (s, 1 H, H-30a), 5.30 (t, 1 H, *J* = 3.2 Hz, H-30b), 6.03 (t, 1 H, *J* = 55.9 Hz).

^{13}C NMR: δ = 38.4 (C-1), 23.7 (C-2), 80.9 (C-3), 37.8 (C-4), 55.4 (C-5), 18.2 (C-6), 34.3 (C-7), 40.7 (C-8), 50.4 (C-9), 37.1 (C-10), 21.0 (C-11), 27.1 (C-12), 38.1 (C-13), 42.3 (C-14), 29.6 (C-15), 32.0 (C-16), 56.3 (C-17), 34.3 (C-18), 38.2 (C-19), 148.7 (t, *J* = 18.7 Hz, C-20), 33.0 (C-21), 36.5 (C-22), 16.5 (C-23), 27.9 (C-24), 16.1 (C-25), 15.9 (C-26), 14.6 (C-27), 176.3 (C-28), 114.4 (t, *J* = 9.9 Hz, C-29), 116.6 (t, *J* = 238.5 Hz, C-30), 171.0 (3β-O*C*OCH$_3$), 21.3 (3β-OCO*C*H$_3$), 51.3 (CO_2CH_3).

^{19}F NMR: δ = –115.72 (dd, *J* = 298, 56 Hz), –114.68 (dd, *J* = 298.5, 56 Hz).

MS: *m/z* (%) = 550 (M^+, 13), 488 (28), 473 (13), 284 (15), 249 (13), 225 (29), 205 (32), 189 (100).

Anal. Calcd for $C_{33}H_{50}F_2O_4$: C, 72.23; H, 9.18; F, 6.92. Found: C, 71.90; H, 9.08; F, 7.32.

Difluoro Ketone 8b

Diketone **8a** (500 mg, 0.9 mmol) was reacted with DAST (2 mL, 10.4 mmol) in $CHCl_3$ (1 mL) in a sealed flask. The sealed flask with the reaction mixture was positioned in the fume hood behind protective shielding, and heated to 70 °C in an oil bath. After 24 h, the mixture was cooled in an ice-bath and carefully opened before work-up. The mixture was diluted with $CHCl_3$ (50 mL), and washed carefully with aq $NaHCO_3$ solution (40 mL) and H_2O (40 mL). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. The dark brown residue was chromatographed on a column of silica gel (50 g). Elution with a mixture of toluene and Et_2O (10:1) followed by crystallization from butanone gave difluoro ketone **8b** (135 mg, 26%); mp 188–190 °C; $[\alpha]_D^{25}$ +67 (*c* = 0.23).

IR ($CHCl_3$): 1253 (C–O–C), 1608 (C=C), 1727 cm^{-1} (C=O).

^{1}H NMR: δ = 0.85 (s, 3 H), 0.86 (s, 3 H), 0.93 (s, 3 H), 0.96 (s, 3 H), 1.14 (s, 3 H), 1.20 (dd, 1 H, *J* = 7.2, 2.6 Hz), 1.25 (dd, 1 H, *J* = 7.2, 2.5 Hz), 1.98 (s, 3 H, 28-OAc), 2.05 (s, 3 H, 3β-OCOCH$_3$), 2.93 (m, 1 H, Σ *J* = 20.4 Hz, H-13β), 3.38 (septet, 1 H, *J* = 7.0 Hz, H-20), 3.96 (d, 1 H, *J* = 11.1 Hz, H-28a), 4.49 (m, 1 H, Σ *J* = 16.3 Hz, H-3α), 4.77 (d, 1 H, *J* = 11.1 Hz, H-28b).

^{13}C NMR: δ = 38.6 (C-1), 23.6 (C-2), 80.7 (C-3), 37.8 (C-4), 55.4 (C-5), 18.1 (C-6), 34.7 (C-7), 41.3 (C-8), 50.9 (C-9), 37.1 (C-10), 21.3 (C-11), 27.7 (C-12), 41.6 (C-13), 44.2 (C-14), 27.3 (C-15),

26.8 (C-16), 52.2 (C-17), 149.1 (t, J = 10.3 Hz, C-18), 138.4 (t, J = 19.5 Hz, C-19), 25.8 (C-20), 117.0 (dd, J = 252.6, 249.5 Hz, C-21), 204.2 (t, J = 24.8 Hz, C-22), 27.9 (C-23), 16.5 (C-24), 16.8 (C-25), 16.8 (C-26), 15.7 (C-27), 63.7 (C-28), 22.1 (C-29), 22.1 (C-30), 171.0 (3β-O*C*OCH$_3$), 21.3 (3β-OCO*C*H$_3$), 170.3 (28-O*C*OCH$_3$), 20.4 (28-OCO*C*H$_3$).

^{19}F NMR: δ = –97.71 (ddq, J = 301.8, 7.9, 3.0 Hz), –103.70 (d, J = 301.8 Hz).

MS: m/z (%) = 576 (M$^+$, 1), 557 (14), 517 (7), 497 (11), 474 (10), 294 (10), 259 (7), 234 (10), 217 (14), 203 (29), 189 (100).

Anal. Calcd for $C_{34}H_{50}F_2O_5$: C, 70.80; H, 8.74; F, 6.59. Found: C, 70.57; H, 8.70; F, 6.37.

Difluoro Ketone 9b

Diketone **9a** (450 mg, 0.8 mmol) was treated with DAST (1 mL, 5.2 mmol) in $CHCl_3$ (1 mL) in the same manner as described above. The dark brown residue was separated by HPLC (25% EtOAc in hexane). Lyophilization (*t*-BuOH) gave difluoro ketone **9b** as a white solid (138 mg, 29%); mp 131–133 °C; $[\alpha]_D^{25}$ +31 (c = 0.25).

IR ($CHCl_3$): 1255 (C–O–C), 1615 (C=C), 1728 cm^{-1} (C=O).

^{1}H NMR: δ = 0.84 (s, 3 H), 0.85 (s, 3 H), 0.91 (s, 3 H), 0.96 (s, 3 H), 1.03 (s, 3 H), 1.24 (dd, J = 4.4, 2.4 Hz), 2.05 (s, 3 H, 3β-OCOCH$_3$), 2.44 (dq, J = 13.4, 2.4 Hz), 2.65 (t, J = 9.3 Hz), 3.39 (septet, 1 H, J = 7.0 Hz, H-20), 3.74 (s, 3 H, CO_2CH_3), 4.48 (m, 1 H, Σ J = 16.5 Hz, H-3α).

^{13}C NMR: δ = 38.5 (C-1), 23.6 (C-2), 80.7 (C-3), 37.7 (C-4), 55.4 (C-5), 18.0 (C-6), 34.6 (C-7), 41.1 (C-8), 50.7 (C-9), 37.1 (C-10), 21.3 (C-11), 27.7 (C-12), 43.7 (C-13), 43.8 (C-14), 27.9 (C-15), 28.4 (C-16), 53.3 (C-17), 148.7 (t, J = 10.3 Hz, C-18), 138.3 (t, J = 19.1 Hz, C-19), 25.8 (C-20), 117.7 (t, J = 253.1 Hz, C-21), 204.1 (t, J = 25.0 Hz, C-22), 27.7 (C-23), 16.5 (C-24), 16.5 (C-25), 16.8 (C-26), 15.8 (C-27), 168.3 (C-28), 21.1 (C-29), 21.2 (C-30), 171.0 (3β-O*C*OCH$_3$), 21.2 (3β-OCO*C*H$_3$), 51.7 (CO_2CH_3).

^{19}F NMR: δ = –95.35 (ddd, J = 299.8, 8.4, 3.0 Hz), –103.92 (d, J = 299.8 Hz).

MS: m/z (%) = 543 (M$^+$ – 19, 100), 482 (69), 467 (5), 459 (3), 439 (8), 423 (9), 348 (2), 304 (4), 280 (31), 203 (31), 190 (74).

Anal. Calcd for $C_{33}H_{48}F_2O_5$: C, 70.43; H, 8.60; F, 6.75. Found: C, 70.14; H, 8.55; F, 6.33.

Difluoro Derivative 10b

Heptanorketone **10a** (300 mg, 0.33 mmol) was treated with DAST (300 μL, 1.33 mmol) in $CHCl_3$ (300 μL) in the same manner as describe above. After 12 h, the reaction mixture was worked up. The dark brown residue was separated by HPLC (13% EtOAc in hexane). Lyophylization (*t*-BuOH) gave difluoro derivative **10b** as a white solid (40 mg, 30%); mp 168–170 °C; $[\alpha]_D^{25}$ +101 (c = 0.18).

IR ($CHCl_3$): 1255 (C–O–C), 1721 cm^{-1} (C=O).

^{1}H NMR: δ = 0.84 (s, 3 H), 0.86 (s, 3 H), 0.88 (s, 3 H), 0.99 (s, 3 H), 1.01 (d, J = 2.0 Hz), 2.05 (s, 3 H, OCOCH$_3$), 4.49 (m, 1 H, Σ J = 16.5 Hz, H-3α).

^{13}C NMR: δ = 38.5 (C-1), 23.6 (C-2), 80.7 (C-3), 37.7 (C-4), 55.4 (C-5), 18.1 (C-6), 33.2 (C-7), 40.9 (C-8), 50.4 (C-9), 37.1 (C-10), 19.1 (C-11), 20.1 (C-12), 43.9 (dd, J = 20.9, 20.9 Hz, C-13), 42.0 (d, J = 7.6 Hz, C-14), 18.6 (d, J = 10.3 Hz, C-15), 30.2 (C-16), 34.5 (d, J = 26.0 Hz, C-17), 124.7 (d, J = 240.0 Hz, C-18), 16.4 (C-23), 27.9 (C-24), 16.5 (C-25), 15.8 (C-26), 14.5 (d, J = 7.2 Hz, C-27).

^{19}F NMR: δ = –90.20 (d, J = 236.5 Hz), –106.35 (ddt, J = 235.8, 29.8, 12.2 Hz).

MS: m/z (%) = 410 (M$^+$, 17), 350 (51), 335 (21), 307 (6), 249 (15), 228 (5), 189 (100).

Anal. Calcd for $C_{25}H_{40}F_2O_2$: C, 73.13; H, 9.82; F, 9.25. Found: C, 72.98; H, 9.52; F, 9.26.

Fluorination of Ketones 2b, 2c and 11a with DAST

The ketones (200 mg, 0.38 mmol) were reacted with DAST (300 μL, 1.33 mmol) in $CHCl_3$ (500 μL) in the same manner as describe above. The brown residue was separated by HPLC (15% vol. EtOAc in hexane). According to spectral data, only starting material was recovered.

Acyl Fluoride 12c

To a stirred solution of acid **12b** (200 mg, 0.40 mmol) in CH_2Cl_2 (5 mL) was added DAST (200 μL, 1.04 mmol) at –78 °C. The reaction mixture was then allowed to warm to r.t. and then quenched by addition of H_2O (1 mL). The separated organic layer was diluted with $CHCl_3$ (50 mL), washed with H_2O (2 × 40 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The yellow residue was separated by HPLC (3% EtOAc in hexane) and crystallized from MeOH to give acyl fluoride **12c** (130 mg, 65%); mp 211–213 °C; $[\alpha]_D^{25}$ +26 (c = 0.33).

IR ($CHCl_3$): 1256 (C–O–C), 1720 (C=O), 1823 cm^{-1}.

^{1}H NMR: δ = 0.83 (s, 3 H), 0.84 (s, 3 H), 0.85 (s, 3 H), 0.95 (s, 3 H), 0.97 (s, 3 H), 1.69 (s, 3 H), 2.04 (s, 3 H, OCOCH$_3$), 2.90 (td, 1 H, J = 10.8, 4.9 Hz, H-19), 4.47 (m, 1 H, Σ J = 16.2 Hz, H-3α), 4.64 (t, 1 H, J = 1.5 Hz, H-29a), 4.75 (d, 1 H, J = 2.1 Hz, H-29b).

^{13}C NMR: δ = 38.4 (C-1), 23.7 (C-2), 80.9 (C-3), 37.8 (C-4), 55.4 (C-5), 18.1 (C-6), 34.2 (C-7), 40.7 (C-8), 50.4 (C-9), 37.1 (C-10), 20.8 (C-11), 25.3 (C-12), 38.5 (C-13), 42.4 (C-14), 29.7 (C-15), 30.9 (C-16), 57.0 (d, J = 39 Hz, C-17), 49.1 (C-18), 46.7 (C-19), 149.3 (C-20), 30.0 (C-21), 35.6 (C-22), 27.9 (C-23), 16.5 (C-24), 16.2 (C-25), 15.9 (C-26), 14.7 (C-27), 165.2 (d, J = 374 Hz, C-28), 110.4 (C-29), 19.3 (C-30), 21.3 (3β-OCO*C*H$_3$), 172.0 (3β-O*C*OCH$_3$).

^{19}F NMR: δ = 35.90 (d, J = 11.3 Hz).

MS: m/z (%) = 500 (M$^+$, 76), 485 (4), 453 (7), 440 (28), 425 (23), 397 (8), 304 (11), 250 (15), 203 (15), 189 (100).

Anal. Calcd for $C_{32}H_{49}FO_3$: C, 76.76; H, 9.86; F, 3.79. Found: C, 76.73; H, 10.11; F, 3.28.

Acyl Fluoride 13b

Dihydro acid **13a** (131 mg, 0.25 mmol) in CH_2Cl_2 (5 mL) was fluorinated with DAST (130 μL, 0.68 mmol) in the same manner as described for acid **12b**. The yellow residue was separated by HPLC (3% EtOAc in hexane) and crystallized from *i*-PrOH to give acyl fluoride **13b** (66 mg, 63%); mp 247–251 °C; $[\alpha]_D^{25}$ +2 (c = 0.51).

IR ($CHCl_3$): 1256 (C–O–C), 1720 cm^{-1} (C = O).

^{1}H NMR: δ = 0.76 (d, J = 6.8 Hz), 0.84 (s, 3 H), 0.85 (s, 3 H), 0.86 (d, J = 6.8 Hz), 0.86 (d, J = 0.8 Hz), 0.95 (s, 3 H), 0.95 (s, 3 H), 2.05 (s, 3 H, OCOCH$_3$), 1.84 (ds, 1 H, J = 6.7, 2.9 Hz, H-20), 4.47 (m, 1 H, Σ J = 16.5 Hz, H-3α).

^{13}C NMR: δ = 38.4 (C-1), 23.7 (C-2), 80.9 (C-3), 37.8 (C-4), 55.4 (C-5), 18.1 (C-6), 36.0 (C-7), 40.7 (C-8), 50.2 (C-9), 37.1 (C-10), 20.8 (C-11), 26.7 (C-12), 38.4 (C-13), 42.6 (C-14), 29.7 (C-15), 30.8 (C-16), 57.5 (d, J = 38 Hz, C-17), 48.6 (C-18), 43.9 (C-19), 29.5 (C-20), 34.3 (C-21), 22.3 (C-22), 27.9 (C-23), 16.5 (C-24), 16.2 (C-25), 15.9 (C-26), 14.6 (C-27), 165.5 (d, J = 376.2 Hz, C-28), 14.6 (C-29), 22.8 (C-30), 21.3 (3β-OCO*C*H$_3$), 171.0 (3β-OCO*C*H$_3$).

^{19}F NMR: δ = 35.48 (d, J = 10.5 Hz).

MS: m/z (%) = 502 (M$^+$, 58), 455 (92), 442 (38), 428 (43), 399 (28), 249 (22), 204 (14), 189 (100).

Anal. Calcd for $C_{32}H_{51}FO_3$: C, 76.45; H, 10.22; F, 3.78. Found: C, 76.30; H, 9.99; F, 3.47.

Acyl Fluoride 14b

Oxo acid **14a** (200 mg, 0.39 mmol) in CH_2Cl_2 (5 mL) was fluorinated with DAST (200 μL, 1.05 mmol) in the same manner as described for acid **12b**. The yellow residue was separated by HPLC (10% EtOAc in hexane) and crystallized from MeOH to give acyl fluoride **14b** (160 mg, 65%); mp 247–251 °C; $[\alpha]_D^{25}$ –46 (0.50).

IR ($CHCl_3$): 1255 (C–O–C), 1613 (C=C), 1706 (C=O), 1831 cm^{-1}.

^1H NMR: δ = 0.85 (s, 3 H), 0.86 (s, 3 H), 0.92 (s, 3 H), 0.95 (d, *J* = 0.6 Hz), 1.08 (s, 3 H), 1.21 (d, *J* = 6.9 Hz), 1.22 (d, *J* = 7.0 Hz), 2.05 (s, 3 H, $OCOCH_3$), 2.23 (dd, 1 H, *J* = 18.7, 0.9 Hz, H-22a), 2.66 (d, 1 H, *J* = 18.7 Hz, H-22b), 2.7 (dd, 1 H, *J* = 12.6, 3.3 Hz, H-13α), 2.44 (ddd, 1 H, *J* = 13.6, 4.2, 2.5 Hz, H-16β), 3.22 (sept, 1 H, *J* = 7.0 Hz), 4.49 (m, 1 H, Σ *J* = 16.6 Hz, H-3α).

^{13}C NMR: δ = 38.6 (C-1), 23.6 (C-2), 80.6 (C-3), 37.8 (C-4), 55.4 (C-5), 18.1 (C-6), 34.8 (C-7), 41.3 (C-8), 51.0 (C-9), 37.1 (C-10), 21.0 (C-11), 27.5 (C-12), 45.5 (C-13), 54.3 (C-14), 28.8 (C-15), 33.4 (C-16), 52.6 (d, *J* = 46 Hz, C-17), 168.4 (C-18), 146.9 (C-19), 25.2 (C-20), 205.0 (C-21), 45.5 (C-22), 27.9 (C-23), 16.5 (C-24), 16.8 (C-25), 16.6 (C-26), 15.8 (C-27), 163.8 (d, *J* = 368 Hz, C-28), 20.0 (C-29), 19.9 (C-30), 21.3 (3β-OCO*C*H_3), 171.0 (3β-O*C*OCH_3).

^{19}F NMR: δ = 30.0 (d, *J* = 6.8 Hz).

MS: *m*/*z* (%) = 500 (M^+, 37), 457 (6), 440 (40), 425 (37), 411 (4), 397 (13), 305 (8), 281 (29), 251 (38), 203 (29), 189 (100).

Anal. Calcd for $C_{32}H_{47}FO_4$: C, 74.67; H, 9.20; F, 3.69. Found: C, 74.87; H, 9.31; F, 3.68.

Cytotoxic MTT Assay

Screening of cytotoxic activity was performed on highly chemosensitive T-lympoblastic leukaemia CEM cells using cytotoxic MTT assay.[9] The cells were prepared and diluted according to the expected target cell density (5000 cells/well). The cells were added by pipette (80 μL) into 96-well microtiter plates. Inoculates were allowed a pre-incubation period of 24 h at 37 °C and 5% CO_2 for stabilization. Four-fold dilutions, in 20 μL aliquots, of the intended test concentration were added at time zero to the microtiter plate wells. All tested compounds were dissolved in 10% DMSO and concentrations were examined in duplicate. Incubation of the cells with the test compounds lasted for 72 h at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed using MTT. Aliquots (10 μL) of the MTT stock solution were pipetted into each well and incubated for a further 1–4 h. After this incubation period, formazan produced was dissolved by the addition of 100 μL/well of 10% aq SDS (pH 5.5), followed by a further incubation at 37 °C overnight. The optical density (OD) was measured at 540 nm with a Labsystem iEMS Reader MF. Tumour cell survival (TCS) was calculated using the following equation: $TCS = (OD_{\text{drug-exposed well}}/\text{mean } OD_{\text{control wells}}) \times 100\%$. The TCS_{50} value, the drug concentration lethal to 50% of the tumour cells, was calculated from appropriate dose-response curves.

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