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Synthesis of 25-Fluoroponasterone A, a Fluorinated Analogue of 20-Hydroxyecdysone.

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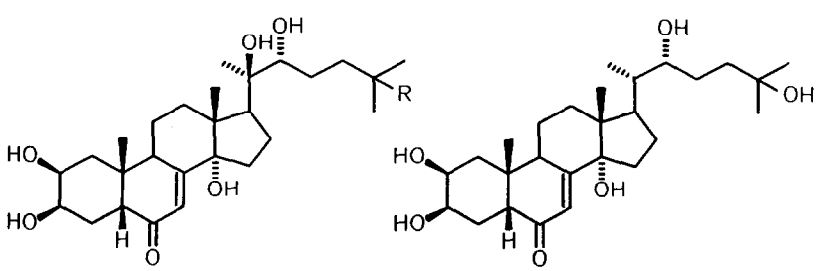
Abstract: *Very precise reaction conditions, using mild fluorination procedures combined with acetonide deprotection, were developed for preparation of 25-fluoroponasterone A from 20-hydroxyecdysone. Biological activity of the new compound was examined and the possible relationship between structure and biological activity in ecdysteroids was studied using MMP2 calculations of the conformations of the side chain of three related compounds: ecdysone, 20-hydroxyecdysone and 25-fluoroponasterone A.*

2ß,3ß,14α,20R,22R,25-Hexahydroxy-5ß-cholest-7-en-6-one, known as 20-hydroxyecdysone (**1**) is the moulting hormone of insects, crustaceans and nematodes. 20-Hydroxyecdysone and its biological precursor ecdysone (**2**) were the first identified compounds of a family of polyhydroxylated steroids named generically ecdysteroids. Over 100 related structures have been isolated from plants[1] and more than 60 from invertebrates[2], being some of these structures common in both kingdoms.



1: 20-Hydroxyecdysone, R = OH
3: 25-Fluoroponasterone A, R = F

2: Ecdysone

Figure 1. Structures of 20-hydroxyecdysone (**1**), ecdysone (**2**) and 25-fluoroponasterone A (**3**).

Regulation of moulting is the best established activity of ecdysteroids in insects, but **1** is involved in a diversity of hormonal functions, namely regeneration, metamorphosis, reproduction and differentiation[2]. These compounds also exhibited biological activity in mammals[3,4]. Some ecdysteroids interfere with the moulting process of insects, behaving as analogs or antagonists of the moulting hormone[5]. The number of ecdysteroids acting as analogs is higher than those acting as antagonists. Unfortunately, they are not very useful for pest control since insects have developed very efficient detoxification pathways. Exogenous ecdysteroids are quickly metabolised and eliminated. Moreover, the level of the most currently available ecdysteroid **1** is very accurately regulated in haemolymph and very high doses are needed to produce irreversible damage to the insect.

Our aim was to obtain synthetic analogs of 20-hydroxyecdysone that could block the metabolic paths for this compound. Such analogs should not be eliminated by the insect, and hence disrupt the normal haemolymph ecdysteroid titers. Fluorine chemistry has been a field of intensive search for compounds with enhanced biological activity, and in insect biochemistry studies on a number of fluorinated sterols, juvenile hormones and pheromones have been described[6]. Likewise, to block the biosynthesis of **1**, some side chain fluorinated sterols have also been reported[7,8] but only the 29-fluorosterols showed a remarkable activity in blocking the citrate cycle[9,10]. To our knowledge, no fluorinated ecdysteroid has been previously reported but a related compound, 26-iodoponasterone A was found to be 126 fold more active than its hydroxylated precursor, inokosterone[11]. The replacement of a hydroxyl group by a fluorine atom may introduce relatively small changes in the polarity and Van der Waals radius of the fluorinated analog[12], and since the main metabolic path for 20-hydroxyecdysone involves the C-26, C-22, C-20 or C-3 positions[13] we focused our work on that substitution near these positions. As our first choice, the synthesis of 25-fluoroponasterone A (**3**) was carried out.

Results and discussion.

Treatment of 20-hydroxyecdysone-2,3:20,22-diacetonide (**4**) with DAST (diethylamine sulfur trifluoride) in anhydrous Cl_2CH_2 yielded 25-fluoroponasterone A-2,3:20,22-diacetonide (**5**) and the corresponding 14-dehydro derivative (**6**). Reaction temperature must be accurately regulated, since under -65°C no reaction was observed, but when the temperature was allowed to increase to values over -55°C dehydration was the major reaction. If the temperature was maintained at -59 ± 1°C, the fluorinated diacetonide **5** was obtained in very high yield, however this compound was slowly transforming into **6**. Thus, the reaction progress was monitored on TLC, and it was quenched after about one hour. Whereas the usual quenching procedure for reactions with DAST is carried out by pouring the reaction mixture onto ice, this method yielded only **6** in the present case. It was necessary to destroy any excess of DAST or its acidic reaction byproducts by addition of solid $CaCO_3$ before allowing the temperature to increase, to obtain a 70% yield of **5** (80% based on transformed starting material).

Hydrolysis from **5** to **3** was also a critical step. Although the 2,3-acetonide was easily hydrolysed yielding 25-fluoroponasterone A-20,22-acetonide (**7b**), the removal of the second acetonide at C20-C22 required longer reaction times and highly acidic media. By using *p*-TsOH/MeOH or $HClO_4$/MeOH the yield of the unwanted 14-dehydro-25-fluoroponasterone A (**8b**) was quite high. Other hydrolysis systems were then assayed, using an acidic resin and different organic solvents. The best results were obtained with Amberlite XAD-15 in MeOH, which afforded **3** in 30% isolated yield after 24 hours, together with 14% of recovered **5** and the 20,22-monoacetonide **7b** (42%). The last two products could be recycled and, thus, **3** was obtained with a minimum of dehydration as side reaction. Special care has to be taken to avoid the presence of traces of acidic

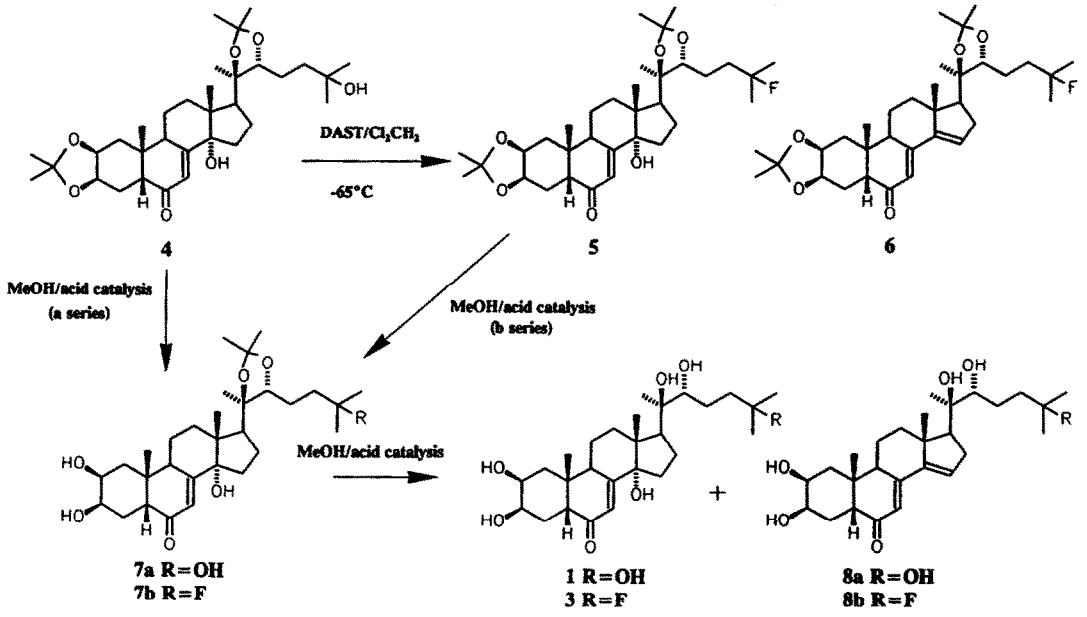


Figure 2. Reaction scheme for 25-fluoroponasterone A synthesis.

compounds before evaporation of the solvent. Since 2,2-dimethoxypropane has a higher boiling point than MeOH, a back reaction to **5** could occur.

Activity of 25-fluoroponasterone A **3** on oocyte development was assayed *in vivo* in comparison with that of **1**[14], using *Blattella germanica* as test insect. To study the inhibitory activity in oocyte growth compounds were injected in freshly ecdysed adult females, and ovaries were explanted 4 days later. To study the choriogenetic action[15] compounds were injected on day 4 and ovaries explanted 24 h later. The corresponding results indicate that both compounds show similar activities (Table 1).

Table 1. Effect of 20-hydroxyecdysone and 25-fluoroponasterone A in ovarian development and choriogenesis in *Blattella germanica*.

	Inhibition of oocyte growth (injection day 0, dissection day 4)		Induction of choriogenesis (injection day 5, dissection day 5)		
	N	BOL	N	BOL	CI
control	21	1.07±0.19	14	1.35±0.15	0
20-hydroxyecdysone#	21	0.81±0.17	11	1.53±0.17	0.34
control	16	1.14±0.22	10	1.52±0.12	0
25-fluoroponasterone A#	17	0.81±0.24	10	1.60±0.17	0.23

N=sample size. BOL= basal oocyte length (mean ± sd) at day 5. CI= chorionation index (0: no effects; 1: early chorion formation; 2: mid chorion formation; 3: late chorion formation). # 5μg/female dose.

Since very little information was available on structure-activity relationships in ecdysteroids, we undertook a conformational study of ecdysone **2**, 20-hydroxyecdysone **1** and 25-fluoroponasterone A **3**. Due to the big size of these molecules and the high number of calculations needed to study the side chain mobility, a MMP2 semiempirical method, was used. A conformational space was searched by rotation of pairs of consecutive bonds using a 20° resolution for each dihedral angle (see Fig. 3 for one example). All structures were fully optimised in all the local minima found. Five and six conformations differing in less than 16 KJ/mol were found for **2** and **1**, respectively (Table 2). In both compounds the same largely preferred conformation was found (comprising 90% of the population in **2** and 94% in **1**). Thus, 20-hydroxylation did not appear to induce any conformational changes in the side chain, but despite sharing the same conformational structure, **1** is much more active than **2**[16], the major difference between both compounds being charge distribution.

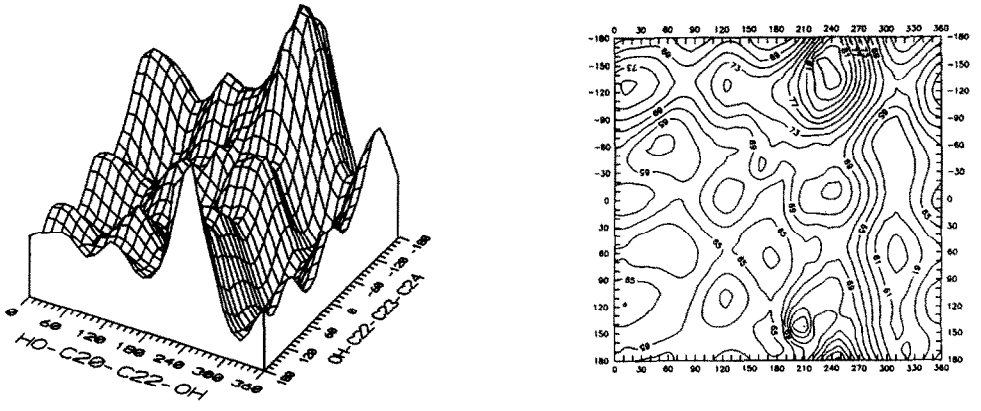
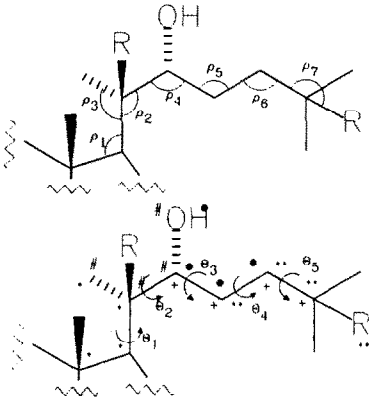


Figure 3. Conformational space for two angles of 20-hydroxyecdysone.

Table 2. Side chain preferred conformations* for ecdysone **2**, 20-hydroxyecdysone **1** and 25-fluoroponasterone A **3**



	2	**1**	**3**
E (KJ/mol)	236.4	241.8	237.6
Population (%)	89.8	93.9	90.3
3-O to 6-O distance (Å)	4.817	4.817	4.817
6-O to 22-O distance (Å)	10.672	10.707	10.696
ρ_1	120.5	122.7	122.6
ρ_2	112.5	111.7	111.7
ρ_3	112.6	112.4	112.5
ρ_4	115.3	116.1	116.1
ρ_5	112.7	112.6	112.6
ρ_6	115.7	115.7	116.2
ρ_7	107.1	107.1	107.1
θ_1	-58.6	-54.3	-53.8
θ_2	63.5	67.2	68.2
θ_3	62.1	63.8	64.1
θ_4	174.8	174.2	175.8
θ_5	177.4	177.1	178.5

*Full data for less preferred conformations potential minima will be available on request

The predicted structure for 25-fluoroponasterone A 3, displayed the same preferred conformation, comprising 90% of the population (Table 2), as in the case of the natural ecdysteroids. The optimised structures obtained with MMP2 were used to obtain the charge distribution by performing a 1SCF calculation with MOPAC. The electronic density in the side chain is similar for all three compounds, and thus, the replacement of the hydroxyl group by fluorine has apparently only affected to a minor extent the rest of the molecule (see Fig. 4). Two tests were used to study the reliability of the predicted structures, optimised with the MMP2 procedure. First, the energies of the ground state and the first excited state were calculated (performing a 1SCF calculation with a MOPAC program), and the difference was used to calculate an approximate value of the UV spectra in hexane. After correction of the bathochromic effect, a predicted value of 239 nm was obtained for the UV spectrum in MeOH, in good accordance with the experimental value, 243 nm. When the geometry was fully optimised with MOPAC, the predicted value raised to 241 nm, in better accordance with the experimental value. This improvement, though, does not seem to justify the 100 fold increase of calculation time.

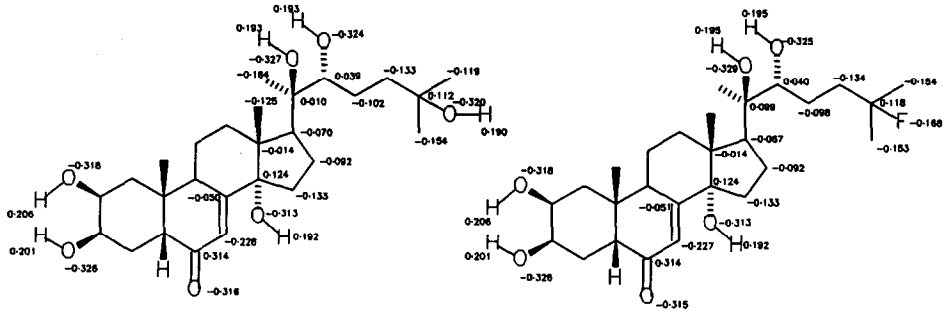


Figure 4. Charge distribution calculated with MOPAC for 20-hydroxyecdysone and 25-fluoroponasterone A.

Secondly, the structures of the preferred conformation for the three compounds were optimized with MMP2 using the dielectric constant of methanol and the obtained structures were used to calculate the vicinal coupling constants in ^{1}H-NMR. For this purpose the 3JHH program, based on an extended Karplus equation, was used. As shown in Table 3, the predicted and the experimental values were in good concordance.[17]

Table 3. Theoretical and experimental coupling constants (in Hz) for 25-fluoroponasterone A, 20-hydroxyecdysone and ecdysone.

	2[17]		1[17]		3	
	exp.	calc.	exp.	calc.	exp.	calc.
2-H_{ax}	12/3/3	10.8/4.6/2.8	12/3/3	10.8/4.6/2.8	12/3/3	10.8/4.6/2.7
3-H_{eq}	3/3/3	3.6/2.8/2.4	3/3/3	3.6/2.8/2.4	$w_{1/2}$=10	3.6/2.7/2.4
5-H	12/5	11.9/4.5	12/5	11.9/4.5	12/5	11.9/4.5
9-H	$w_{1/2}$=22	12.1/4.6	$w_{1/2}$=22	12.0/4.7	$w_{1/2}$=24	12.1/4.7
12-H_{ax}	13/5	12.5/4.3	13/5	12.4/4.4	13/5	12.4/4.4
22-H	$w_{1/2}$=16	5.7/1.8/4.3	11/2	10.6/2.0	-	10.6/2.0
21-CH_3	7.5	7.0	-	-	-	-

Table 4. Selected ^{1}H-NMR and ^{19}F-NMR spectral data in Cl_3CD. Chemical shifts in ppm referred to TMS (H) or trifluoroacetic acid (F). Coupling constants and width at half height (w½) in Hz.

	1	4	5	6	7b	3'
2-Hax	3.89ddd (12,5,3)	4.21ddd (15,6,6)	4.22ddd (15,6,6)	4.15ddd (11,6,6)	3.87d (br,14)	3.84ddd (12,3,3)
3-Heq	4.04cs (w½=8)	4.26cs (w½=8)	4.25cs (w½=6)	4.25ddd (4,4,1)	4.02cs (w½=14)	3.95cs (w½=10)
5-H	2.23dd (14,4.5)	2.21ddd (7,7,2)	2.22ddd (7,7,2)		2.23ddd (7,7,1)	2.38dd (12,5)
7-H	5.84d (2.5)	5.84d (2.1)	5.84d (2.4)	6.08d (2.4)	5.83d (2)	5.81d (2.1)
9-H	3.11sc (w½=22)	2.80ddd (11,7,2)	3.11ddd (12,7,2.5)	2.64ddd (16,11,2)	3.05ddd (15,11,2)	3.15ddd (w½=24)
15-H				5.97dd (4,2)		
16-Hax				2.27dd (7,4)		
16-Heq				2.33dd* (12,7)		
17-H	2.33cs	2.34dd (13,5)	2.33dd (13,5)	2.34dd* (12,4)	2.40dd (13,4)	2.35cs
22-H	3.44d (10)	3.64d (8)	3.60dd (8,4)	3.69dd (9,3)	3.61dd (9,4)	3.30**
18-CH_3	0.86	0.78	0.78	0.96	0.79	0.89
19-CH_3	0.98	0.97	0.96	1.05	0.97	0.97
21-CH_3	1.21	1.15	1.14	1.19	1.16	1.20
26-CH_3	1.24	1.23	1.34d (24)	1.35d (21)	1.36d (22)	1.33d (21)
27-CH_3	1.25	1.24	1.36d (20)	1.36d (22)	1.37d (20)	1.34d (21)
2'-CH_3		1.32	1.31	1.29	1.32	
3'-CH_3		1.41	1.39	1.41	1.40	
2"-CH_3		1.32	1.31	1.32		
3"-CH_3		1.48	1.48	1.48		
25-F			-64.42cs (w½=82)	-64.56cs (w½=80)	-63.7cs (w½=69)	-63.7h (J=21)

#: CD_3OD

* = assignments can be exchanged. Assignments of 26-CH_3 and 27-CH_3 signals can be exchanged in all compounds.

**= J cannot be determined due to solvent signal interference.

Table 5. ^{13}C-NMR spectral data of mentioned compounds in Cl_3CD. Chemical shifts in ppm. Coupling constants with ^{19}F in Hz.

	1#	4	5	6	7	3#
C-1	37.46	37.66	37.66	37.63	36.59	37.33
C-2*	68.72	72.21	72.21	72.10	67.65	68.49
C-3*	68.54	71.67	71.67	71.74	67.33	68.67
C-4	32.81	31.80	31.80	31.61	31.51	32.85
C-5	51.78	50.87	50.88	50.76	50.05	51.77
C-6	206.39	202.87	202.90	202.14	204.23	206.42
C-7	122.12	121.46	121.43	120.71	121.49	122.12
C-8	167.88	163.18	163.24	153.73	165.11	167.91
C-9	35.14	34.55	34.55	38.69	33.84	35.08
C-10	39.28	37.87	37.38	38.39	38.25	39.25
C-11+	21.54	20.58	20.58	20.58	20.43	21.48
C-12	32.52	26.92	26.96	26.87	26.92	32.50
C-13	48.65	47.53	47.53	47.54	47.27	48.61
C-14	85.26	85.07	85.03	148.78	84.82	85.23
C-15	31.76	31.04	31.03	128.07	30.96	31.75
C-16+	21.54	21.95	22.03	39.65	22.01	21.49
C-17	50.54	49.06	49.08	57.66	49.06	50.50
C-18	18.02	17.11	17.12	19.10	17.10	18.06
C-19	27.39	29.03	29.08	28.90	23.90	24.41
C-20	77.94	84.47	84.24	83 15	84.25	78.00
C-21	21.09	21.24	21.23	21.23	21.21	24.41
C-22	78.41	82.06	81.57	81.29	81.52	78.00
C-23	24.38	23.63	23.20/5	23.30/3	23.17/4	23.03/4
C-24	42.37	41.47	39.04/23	38.90/24	38.06/21	40.20/22
C-25	71.29	70.44	95.54/165	95.28/165	95.51/164	96.04/152
C-26$	29.12	29.30	26.05/25	26.00/25	26.08/25	26.60/26
C-27$	29.61	29.72	27.57/25	27.40/25	27.48/25	27.55/25
C-1'		107.06	107.03	107.02	106.95	
C-2'		28.61	28.61	28.51	29.04	
C-3'		26.75	26.74	26.82	29.04	
C-1''		108.35	108.35	108.30		
C-2''		26.53	26.53	26.39		
C-3''		23.67	23.67	23.32		

#: CD_3OD

*, +, $: assignment can be exchanged.

Experimental part.

Fluorination reactions were performed under a stream of dry nitrogen and were monitored by HPLC using a system consisting of two Applied Biosystems-400 pumps, an Applied Biosystems-491 dynamic injector, a diode-array UV detector Applied Biosystems-1000S with a Hewlett-Packard 3396A integrator for pump control, detection and UV spectra recording (at 242 and 330 nm). The temperature of the column [LiChroCART 5 μm 12.5x0.4 cm with LiChrospher 100 RP-18 package (Merck)] was thermostatised at 45°C with a Spark Holland SPH-99 oven. i-PrOH/H_2O was used as mobile phase with a gradient from 10:90 to 60:40 in 12 min at a flow of 1.2 mL/min.

Silica gel chromatography was performed on tlc plates (Merck) that were developed under UV light and by spraying with H_2SO_4/MeOH (15:85) and heating. Silica gel 60-200 μm (SDS, 60 A CC) was used for open column chromatography and silica gel 5-40 μm (Merck, 60H) was used for flash chromatography. All solvents were dried and purified by standard techniques. 20-Hydroxyecdysone (**2**) was isolated from *in vitro* cultivated prothalli of *Polypodium vulgare* by reported procedures with slight modifications[18,19].

^{1}H-NMR (300 MHz), ^{13}C-NMR (75 MHz) and ^{19}F-NMR (280 MHz) were recorded on a Varian Unity 300, chemical shifts are reported relative to Me_4Si and CF_3COOH, the ^{13}C-NMR multiplicities were determined by DEPT experiments and all the coupling constants are given in Hz. Mass spectra were obtained by HPLC-TSP-MS with a HP-5988A quadrupole apparatus [direct flow with $HCOONH_4$ (0.05M)/MeOH (50:50), positive mode, TSP 190-192 °C, stem 96 °C, ion source 200 °C].

Calculations were done on a HP9000-835 computer, the conformational spaces were searched with MM2/MMP2 (85) program with a 20° resolution for each dihedral angle and 324 conformers were used to adjust each surface. H-bond was considered by using the parametrisation described by Allinger[20]; as external parameters the Fock matrix for the π system and the dielectric constant of the solvent were introduced (78.35 for water and 35.63 for MeOH).

For the calculation of the UV absorption maxima and the charge density distribution MOPAC program package was used. 1SCF calculations with a PM3 type hamiltonian was used over MMP2 fully optimised geometries. In selected cases, the geometry was fully optimised with MOPAC. The theoretical coupling constants were calculated with 3JHH program[21], based on a Karplus modified equation[22].

20-Hydroxyecdysone-2,3:20,22-diacetonide (4). 15 mg (0.031 mmol) **1** were dissolved in 1 mL of 2,2-dimethoxypropane and traces of *p*-TsOH acid were added as catalyst. After 10 min a TLC control showed complete reaction of initial product. After addition of $NaHCO_3$ and five minutes of stirring, 5 mL of H_2O was added and the aqueous solution extracted with Cl_3CH (3x3mL), the joined organic layers were washed with H_2O (5 mL), dried over $MgSO_4$ and evaporated under reduced pressure, yielding **4** quantitatively.
4: UV λ_{max} (MeOH) 242 (ϵ= 10841). ^{19}F-NMR and ^{1}H-NMR see Table 4. ^{13}C-NMR see Table 5. MS [TSP, m/z (relative intensity)]: 543 (26, M+1-H_2O), 561 (100, M+1), 562 (39, M+2), 578 (40, M+NH_4), 579 (50, M+1+NH_4), 580 (35, M+2+NH_4), 593 (43, M+1+MeOH).

Hydrolysis of 20-hydroxyecdysone-2,3:20,22-diacetonide (4) and 20-hydroxyecdysone-20,22-acetonide (7a). The hydrolyses of **4** and **7a** were assayed under different conditions by combination of different solvents (Cl_2CH_2, MeOH or dioxane) with different catalysts (*p*-TsOH, $HClO_4$ or Amberlite XAD-15). In most cases the removal of the protective groups was accompanied with dehydration and 14-dehydro-20-hydroxyecdysone was the main product. Only when MeOH was used with Amberlite XAD-15 the hydrolysis rate was very slow but yielded **1** with minor quantities of dehydration product.

14-Dehydro-25-fluoroponasterone A-2,3:20,22-diacetonide (6). 8.9 mg (0.016 mmol) of **4** was dissolved in 4 mL of Cl_2CH_2 and the solution was cooled to -70 °C. After addition of 4.5 mg (0.027 mmol) of DAST in 3 mL of Cl_2CH_2 the reaction was allowed to reach room temperature (5 h), then 10 mL of a 5% aqueous solution of $NaHCO_3$ was added. After vigorous stirring, the organic layer was washed with H_2O (2x10mL), dried over $MgSO_4$ and the solvent was evaporated under reduced pressure, yielding 7.5 mg (81 %) of **6**.
6: UV λ_{max} 242 (ϵ= 8024). ^{19}F-NMR and ^{1}H-NMR see Table 4. ^{13}C-NMR see Table 5.

25-Fluoroponasterone A-2,3:20,22-diacetonide (5). 40 mg (0.071 mmol) of **4** was dissolved in 20 mL of Cl_2CH_2 and cooled to -70 °C. An excess of DAST (75 mg, 0.465 mmol) in 3 mL of Cl_2CH_2 was added and the temperature increased up to -55 °C in 75 min. After addition of solid $NaHCO_3$ and $CaCO_3$ this suspension was stirred for 30 min and then it was allowed to reach room temperature. The crude was extracted with H_2O (20 mL), the organic layer washed with H_2O (3x20mL), and dried over $MgSO_4$. The evaporation of the solvent at reduced pressure afforded an oil (44.6 mg). Chromatography of this oil on silica gel (5 g), eluting with AcOEt/Hx (1:2 and 1:1) yielded 6.8 mg of **6** (17 %), 28.2 mg of **5** (70 %) and 4.9 mg of **4** (13 %).
5: UV λ_{max} 241 (ϵ= 10032). ^{19}F-NMR and ^{1}H-NMR see Table 4. ^{13}C-NMR see Table 5. MS [TSP, m/z (Relative intensity)] : 544

(7, M+1-F), 545 (12, M+1-H_2O), 560 (16, M+NH_4-HF), 563 (96, M+1), 580 (100, M+NH_4), 581 (84, M+1+NH_4), 596 (26, M+2+MeOH).

25-Fluoroponasterone A (3). 500 mg of an acid resin (Amberlite XAD-15) and a drop of water were added to a solution of 28.2 mg (0.050 mmol) of **5** in 1 mL of MeOH. After 24 hours the catalyst was filtered and washed with MeOH (5x1mL). The solution and the washing fractions were joined and filtered through a column of basic resin (500 mg, Amberlite IRA-410) to eliminate any acidic traces. The solvent was evaporated under reduced pressure and the residue (22.1 mg) was chromatographed on 3 g of silica gel, with AcOEt/Hx (1:4, 1:2, 1:1) as mobile phase, affording unreacted **5** (3.7 mg, 14 %), 11 mg of 25-fluoroponasterone A-20,22-acetonide (**7b**, 42 %) and **3** (7.9 mg, 30%).

7: ^{1}H-NMR and ^{19}F-NMR see Table 4. ^{13}C-NMR see Table 5.

3: UV λ_{max} 240 (ϵ= 11800).^{19}F-NMR and ^{1}H-NMR see Table 4. ^{13}C-NMR see Table 5. MS [TSP, m/z (Rel. intensity)]: 380 (30, M+NH_4-$C_6H_{13}OF$), 445 (M+1-HF-H_2O), 462 (26, M-HF), 463 (100, M+1-HF), 465 (33, M+1-H_2O), 466 (11, M+2-H_2O), 478 (14, M+2+MeOH-HF-H_2O), 480 (93, M+NH_4-HF), 481 (58, M+1+NH_4-HF), 483 (92, M+1), 484 (51, M+2), 496 (20, M+1+MeOH-F), 500 (72, M+NH_4), 501 (15, M+1+NH_4), 502 (13, M+2+NH_4), 515 (20, M+1+MeOH).

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