Chemical modification of NSC12 leads to a specific FGF-trap with antitumor activity in multiple myeloma

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Abstract: Inhibition of FGF/FGFR signaling is a promising strategy for the treatment of malignances dependent from FGF stimulation, including multiple myeloma (MM). The steroidal derivative NSC12 (compound 1) is a pan-FGF trap endowed with antitumor activity in vivo. Chemical modifications of compound 1 were explored to investigate structure-activity relationships, focusing on the role of the bis(trifluoromethyl) 1,3-propanediol chain, the stereochemistry at C20 and functionalization of C3 position. Our studies unveiled compound 25b, the pregnane 3-keto 20R derivative of compound 1 as an effective agent, blocking the proliferation of MM cells in vitro by inhibiting FGF-dependent receptor activation and slowing MM growth in vivo. Importantly, the absence of the hydroxyl group at C3 prevents binding to estrogen receptors, which might concur to the antitumor activity observed for compound 1, leading to a specific FGF/FGFR system inhibitor, and further supporting the role of FGFR in anticancer therapy in MM.

1. Introduction

Fibroblast Growth Factors (FGFs) represent a family of structurally related peptides involved in the regulation, proliferation, differentiation, migration and survival of different cell types [1,2]. These effects are mediated by the formation of a ternary complex involving FGFs, FGF receptors (FGFRs) and cell-surface heparan sulfate proteoglycans (HSPGs) or Klotho proteins [3,4]. The signal triggered by receptor activation results in the stimulation of intracellular signaling pathways, such as the downstream MAPK cascade and PI3K-Akt pathway, which actively promote cellular proliferation and survival [5]. Aberrant activation of FGF signaling derived by protein overexpression, oncogenic mutations or gene amplifications has been associated with tumor growth and progression [6e8], with neoangiogenesis [9] and with acquired resistance towards targeted therapies [10]. Thus, the FGF/FGFR system represents a promising target for the treatment of those tumors sustained by its signaling network, including multiple myeloma (MM) [11,12].

Current pharmacological approaches that target the FGF/FGFR axis include tyrosine-kinase inhibitors (TKIs) and anti-FGFR antibodies and peptides [13e15], which suffer from limitations (bioavailability, poor physicochemical properties) and toxicological issues. The use of TKIs targeting the catalytic domain of FGFRs is associated with side effects, mainly the development of hyperphosphatemia induced by the suppression of signals triggered by hormonal FGFs [16]. Antibodies targeting a specific FGF ligand or FGFR isoform have shown promising antitumor activity in cancer cell lines and xenograft models, and some of them have progressed to the clinical trials [17], with promising outcomes especially in bladder cancer and gastroesophageal adenocarcinoma [18,19].

Binding of FGFs to their receptors can also be prevented by ligand traps, molecules that interact with growth factors in the extracellular environment and avoid the formation of the ternary signaling complexes with receptors and HSPGs [17,20]. FP-1039 is a

Abbreviations

DCM   dichloromethane
DMF   dimethylformamide
EDC   1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
TFA   trifluoroacetic acid
HFA   hexafluoroacetone
HOBt   hydroxybenzotriazole
TBTU   2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-
           tetramethylaminium tetrafluoroborate
DIPEA   N,N-Diisopropylethylamine
FBS   fetal bovine serum
FGF   fibroblast growth factor
FGFR   fibroblast growth factor receptor
H-ESI   heated electrospray ionization
HPLC   high pressure liquid chromatography
HRMS   high-resolution mass spectrometry
LiHMDS   lithium hexamethyldisilylamide
NMR   nuclear magnetic resonance
PBS   phosphate buffered saline
THF   tetrahydrofuran
TMSOTf   trimethylsilyl trifluoromethanesulfonate
UHPLC   ultra-high pressure liquid chromatography

soluble decoy receptor constituted by the extracellular domain of FGFR1 and the Fc portion of human IgG1. The anti-angiogenic and antiproliferative effects of FP-1039 were assessed in vitro and in vivo in various tumor models [21,22] and a Phase I clinical trial demonstrated the elevated tolerability of this agent when administered alone [23], or in combination with chemotherapy [24]. Attempts to develop small-molecule FGF traps led to the discovery of thrombospondin-1-mimic agent sm27 and of its mono- and bi-naphthalene analogues [25,26]. Thrombospondin-1 belongs to a class of naturally occurring molecules interacting with FGF and exerting tumor suppressive and anti-angiogenic effects. Sm27 and its analogues prevent the formation of FGF/FGFR/HSPG ternary complex by a complex mechanism, involving direct binding to the heparin-binding site of FGF2 [27]. Various FGFs are also able to interact with the soluble pattern-recognition receptor long pentraxin-3 (PTX3), a cellular macromolecule produced in response to inflammatory stimuli. In particular, the N-acetylated pentapeptide Ac-ARPCA-NH$_2$ (ARPCA) has been identified as the minimal FGF-binding peptide. PTX3 overexpression and treatment with ARPCA prevent interaction of FGFs with their receptors and tumorigenic and angiogenic activity in animal models [28,29]. In this frame, we reported the identification of NSC12 (1, Fig. 1) as the first PTX3-derived small molecule acting as a pan-FGF trap, endowed with antiangiogenic and antitumor activity in vivo after

administration by the oral route [30e32]. In addition, recent observations have shown the capacity of compound 1 to impair MM growth and dissemination by inhibiting the autocrine FGF/FGFR axis in MM cell lines and patient-derived MM cells [12].

Due to its novel mechanism of action and the antitumor efficacy in vivo, compound 1 represents a novel lead compound whose structure-activity relationships deserve to be investigated to identify regions that can be easily modulated to improve its pharmacological activity. Moreover, potential interaction with other targets which might alter interpretation of in vivo biological data need also to be taken into account. We herein describe the synthesis and biological evaluation of a series of structural analogues of compound 1 (Fig. 1). The synthetic route originally developed for compound 1 posed a major hurdle in its intrinsically low efficiency in delivering the active stereoisomer (20*S*). In particular, insertion of the bis(trifluoromethyl)1,3-propanediol side chain at position 17 needs a laborious and technically challenging reaction set up, requiring gaseous hexafluoroacetone, which has to be prepared separately and delivered. Optimization of the synthetic route allowed to explore the chemical space surrounding the lead compound, in the absence of structural information about its interaction with FGF. In the present study, structure-activity relationship investigations mainly focused on (i) the distal portion of the bis(-trifluoromethyl)1,3-propanediol side chain in position 17 and (ii) on the substituent in position 3 of the steroidal core. Since the presence of the hydroxyl group in position 3 of the steroidal nucleus of compound 1 is typical of estrogen receptor (ER) binders [33], derivatization and replacement of this group were explored with the twofold aim of (i) evaluating its relevance for compound potency and (ii) reducing the potential affinity toward the estrogen receptors. The relevance of stereochemistry at position C20 was also investigated in combination with SAR studies for position 3 by synthesizing both 20*R* and 20*S* stereoisomers. Additionally, the effect of D^5 unsaturation was evaluated for 3-hydroxy and 3-keto derivatives.

2. Chemistry

The geminal bis(trifluoromethyl)methanol group of compound 1 was replaced with different substituents, comprising a carboxylic acid (4a,b), an amide group (5a,b) and a tertiary or a primary alcohol (compound 6a,b; 7a,b respectively), prepared according to reactions depicted in Scheme 1. The key intermediate 3 is prepared *via* a Reformatsky reaction between 3**b**-acetoxy-androst-5-ene-17**b**-carbaldehyde (2) [34] and the ethyl ester of bromoacetic acid. The reaction furnishes a 1:2 mixture of 3a and 3b, epimers at C20, easily separated by column chromatography. Both ester groups of 3a,b were saponified with sodium hydroxide to obtain the carboxylic acid derivatives 4a,b. The carboxylic acids were converted to the corresponding amides 5a,b by condensation with ammonium chloride in the presence of TBTU. Tertiary alcohols 6a,b were obtained by exposure of 3a,b to an excess of methylmagnesium bromide. Primary alcohols 7a,b were obtained by reducing the **b**-hydroxy ester group of 3a,b with sodium borohydride in a refluxing mixture of tetrahydrofuran and methanol, yielding the contextual partial transesterification of position 3. Compounds 7a and 7b were then obtained treating the crude product of reduction with sodium hydroxide.

The synthesis of phenyl (11a,b,c) and pyridyl (14a,b) derivatives featured an aldol condensation of pregnenolone benzoate 8 with benzaldehyde or 2-pyridinecarboxaldehyde, respectively (Scheme 2). Epimers 9a and 9b were separated by column chromatography, and following reduction with sodium triacetoxyborohydride, three out of the four possible diastereoisomers (10a,b from 9a, in a 3:2 diasteroisomeric ratio and 10c deriving from 9b) were obtained.
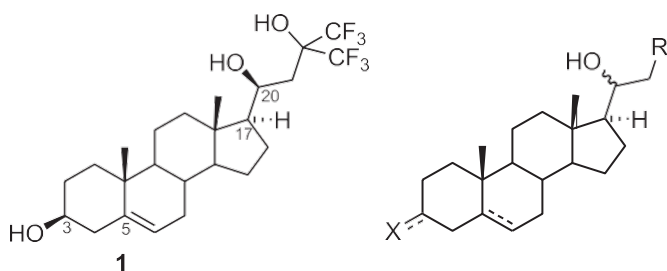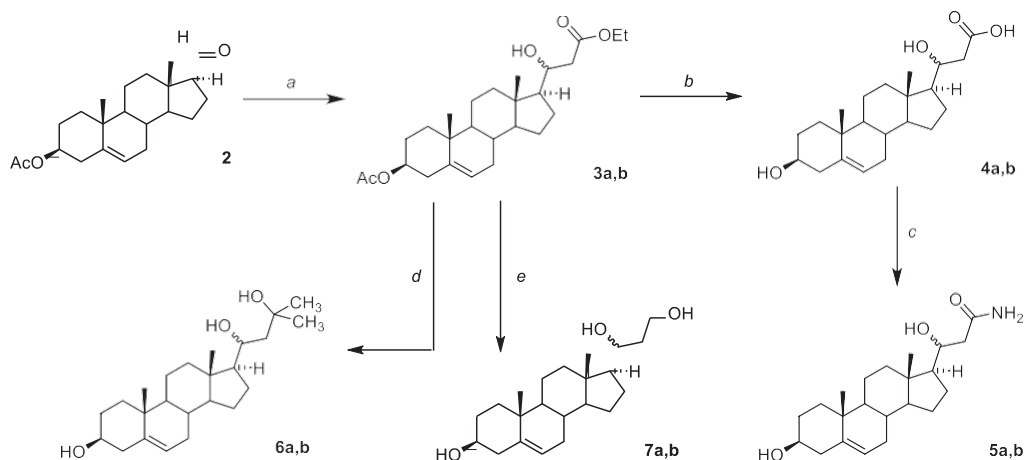

Fig. 1. Chemical structure of small-molecule FGF-trap NSC12 (1) and general formula of its derivatives described in this work.

Scheme 1. Synthesis of compounds 4a,b, 5a,b, 6a,b and 7a,b. Reagents and conditions: (a) ethyl bromoacetate, Zn^0, THF, 0 °C, 20 min, 23% yield for 3a and 47% yield for 3b; (b) NaOH, THF/H_2O, rt, 15 h, 72% yield for 4a and 86% yield for 4b; (c) NH_4Cl, TBTU, DIPEA, DMF, rt, 15 h, 25% yield for 5a and 5b; (d) MeMgBr, toluene, reflux, 3 h, 65% yield for 6a and 56% yield for 6b; (e) $NaBH_4$, THF/MeOH, reflux, 1 h; then NaOH, H_2O, 50 °C, 1 h, 24% yield for 7a and 50% yield for 7b.
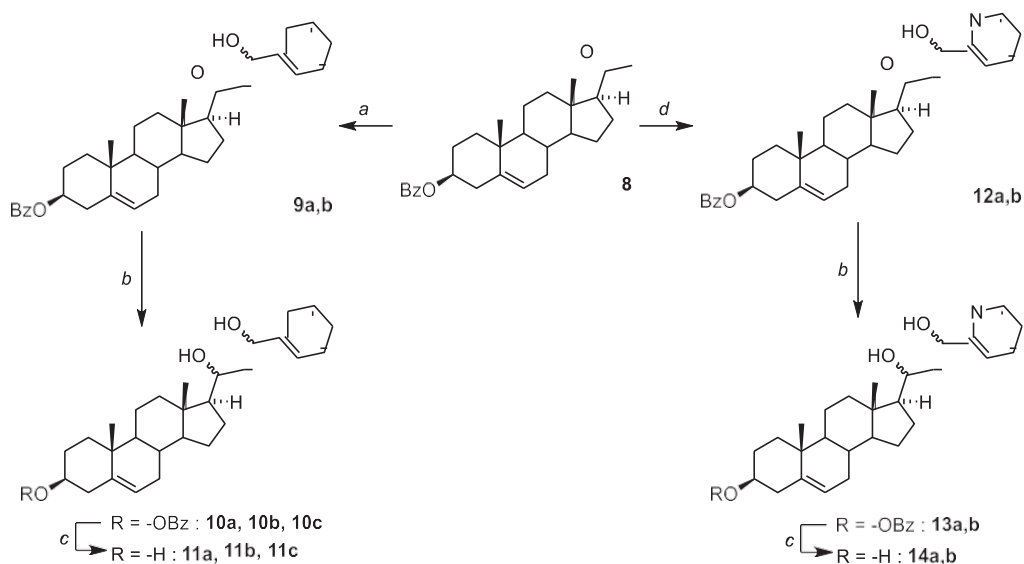

Scheme 2. Synthesis of compounds 11a,b,c and 14a,b. Reagents and conditions: (a) benzaldehyde, LiHMDS, THF, —78 °C, 1 h, 66% overall yield; (b) Na(OAc)$_3$BH, THF, rt, 1 h; (c) NaOMe, THF/MeOH, rt, 2 h, 68e88% yield; (d) 2-pyridinecarboxaldehyde, LiHMDS, —78 °C, 1 h, 82% overall yield.

Compounds 10a,b were separated by column chromatography. On the other hand, perfect coelution hampered the separation of the two epimers of compound 12 (12a,b as a 1.25:1 mixture), which therefore reacted together in the following reduction step, furnishing 13a and 13b, which were at this stage separated by column chromatography. Transesterification with sodium methoxide to remove the protecting group on position 3 furnished

compounds 11a,b,c and 14a,b.

Compounds with different substituents in position 3 of the steroidal nucleus were synthesized starting from the 3-hydroxy epimers 1 (20S) and 17 (20R) which were prepared following two synthetic approaches. Synthesis of C20 epimer 17 was performed according to ref. 31. Instead, a new convenient synthetic approach was used to prepare compound 1 (20S), involving the
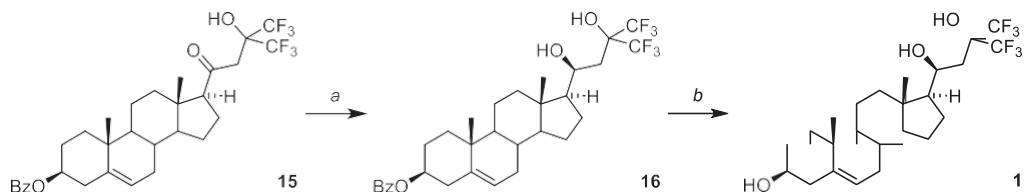
Scheme 3. New synthetic procedure developed for compound 1. Reagents and conditions: (a) H_2 (5 atm), RuCl(p-cymene)[(S,S)-Ts-DPEN], EtOAc, 50 °C, 72 h, 85% yield; (b) MeONa, MeOH/THF, 40 °C, 12 h, 92% yield.
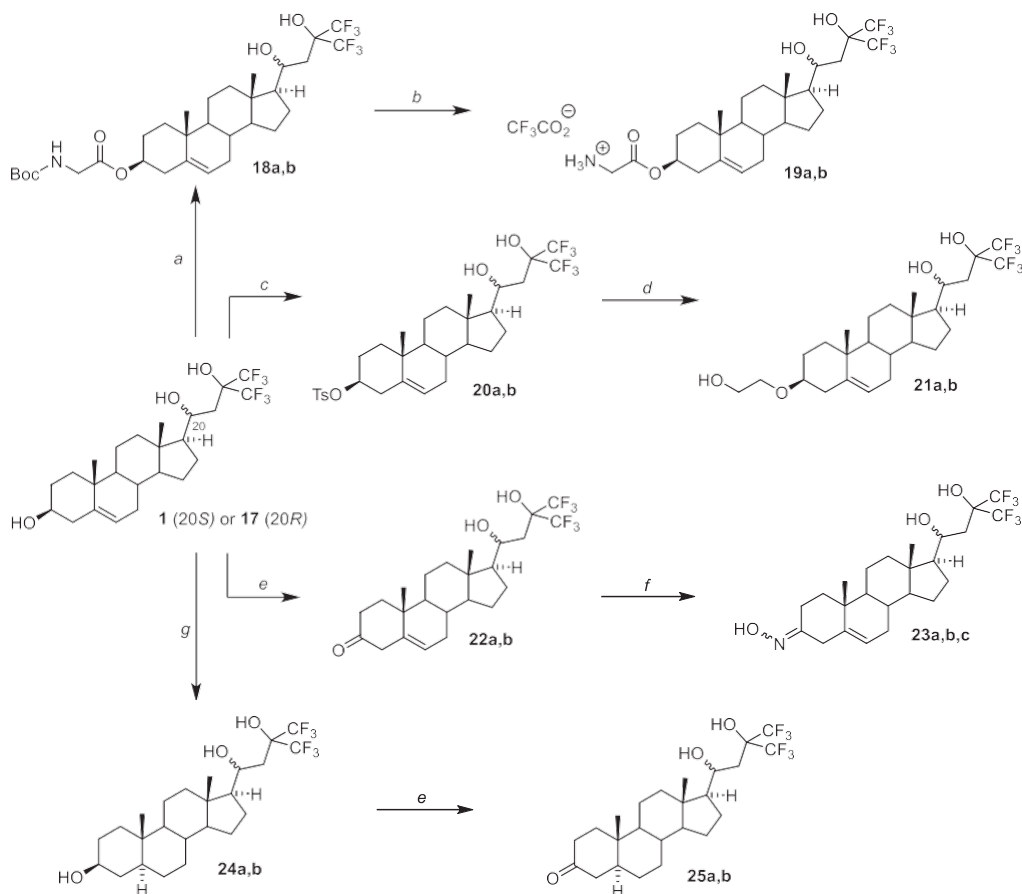
Scheme 4. Synthesis of compounds 19a,b, 21a,b, 22a,b, 23a,b,c, 24a,b, and 25a,b. Reagents and conditions: (a) *N*-Boc glycine, EDC, HOBt, Et₃N, DCM/DMF, 60 °C, 72 h, 16e26% yield; (b) TFA, DCM, rt, 3 h, quant.; (c) *p*-TsCl, Pyridine, DCM, 40 °C, 72 h; (d) ethylene glycol, dioxane, reflux, 4 h, 26e34% yield; (e) Dess-Martin periodinane, DCM/EtOAc, rt, 30 min, 60e69% yield; (f) NH₂OH·HCl, Et₃N, DCM, rt, 45 min, 78e90% yield; (g) H₂ (3 atm), 10% Pd-C, EtOH, rt, 12 h, quant.

diastereoselective catalytic hydrogenation of the **b**-hydroxy ketone 15 [31] with RuCl(*p*-cymene)[(*S,S*)-Ts-DPEN] catalyst, and subsequent methanolysis of the 3**b**-benzoate ester 16 (Scheme 3).

Due to the innate higher reactivity of the secondary alcohol in position 3, compared to the more sterically hindered C20 and C22 alcohols, functionalization of the C3 alcohol with hydrophilic substituents or its oxidation could be carried out without recurring to protecting groups (Scheme 4). The glycine derivatives 19a,b were obtained by esterification of the 3**b**-hydroxy derivatives 1 and 17 with *N*-Boc-glycine using EDC, HOBt and Et₃N, and subsequent treatment of intermediate compounds 18a,b with TFA to remove the *N*-Boc protection. To afford the 3**b**-oxyethan-2-ol derivatives 21a,b, the 3**b**-hydroxy starting materials 1 and 17 were first converted to the corresponding *p*-toluenesulfonate esters 20a,b by treating with *p*-toluenesulfonyl chloride, then subjected to nucleophilic substitution with ethylene glycol. As elucidated by Shoppee [35] and Winstein [36,37] cholesterol and similar derivatives undergo solvolysis with overall retention of 3**b** configuration with the involvement of a nonclassical carbocation that is formed by neighboring participation of the homoallylic alkene.

C3 regioselective oxidation of the 3**b**-hydroxy alcohols 1 and 17 with Dess-Martin periodinane gave the corresponding 3-oxo target compounds 22a,b that could be converted to the corresponding oximes 23a-c (*E/Z* mixture) by treatment with hydroxylamine hydrochloride.

Catalytic hydrogenation of the unsaturated alcohols 1 and 17

afforded the corresponding saturated 3**b**-hydroxy intermediates 24a,b, that were regioselectively oxidized to the corresponding 3-oxo target compounds 25a,b by treatment with Dess-Martin periodinane.

The higher reactivity of the hydroxyl group in position 3 also allowed glycosylation to be achieved following standard procedures directly on compound 17, which reacted at first with *O*-(2,3,4,6,tetraacetyl-**a,b**-D-glucopyranose)trichloroacetimidate to afford compound 26b, then the acetate protecting groups were removed by transesterification, yielding compound 27b (Scheme 5) [38]. The limited solubility of compound 1 (as opposed to 17) in either dichloromethane or acetonitrile prevented the application of the same approach to the glycosylated 20*S* epimer. This was therefore prepared starting from pregnenolone 28 (Scheme 5), which reacted with *O*-(2,3,4,6,tetraacetyl-**a,b**-D-glucopyranose)trichloroacetimidate providing compound 29 in modest, yet useful yield. Deacetylation followed by benzoylation furnished *O*-benzoylated intermediate 31, which was subjected to aldol condensation with hexafluoroacetone, according to the previously reported experimental procedure [31]. Reduction with sodium triacetoxyborohydride yielded both epimers of compound 33 (33a,b), which were separated by column chromatography. Transesterification of the minor product 33a furnished compound 27a, epimer at C20 of compound 27b, as confirmed by NMR-spectroscopy and TLC analysis.
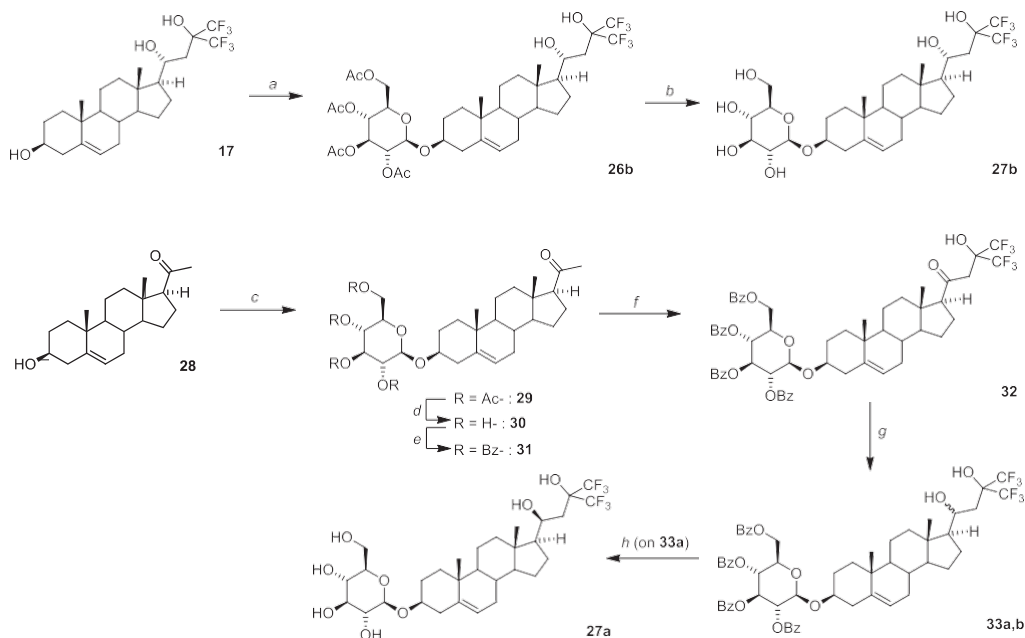
Scheme 5. Synthesis of compounds 27a,b. Reagents and conditions: (a) *O*-(2,3,4,6,tetraacetyl-**a,b**-D-glucopyranose)trichloroacetimidate, TMSOTf, DCM, —25 °C, 15 min, 15% yield; (b) NaOMe, MeOH, rt, 6 h, 46% yield; (c) *O*-(2,3,4,6,tetraacetyl-**a,b**-D-glucopyranose)trichloroacetimidate, TMSOTf, anhydrous DCM, —25 to 0 °C, 15 min; (d) NaOMe, THF, rt, 1 h, 22% yield over two steps; (e*)* BzCl, pyridine, rt, 20 min, quant.; (f) LiHMDS, HFA, THF, —78 °C, 1 h, 73% yield; (g) Na(OAc)₃BH, THF, rt, 1 h, 7% yield for 33a and 77% yield for 33b; (h) NaOMe, THF, rt, 2 h, 62% yield.

3. Results and discussion

As reported in the Introduction section, the FGF/FGFR system plays a pivotal role in MM, a hematological tumor characterized by the clonal proliferation of malignant plasma cells [39]. Previous observations from our laboratory have shown that compound 1 exerts a potent inhibitory effect on MM cells by inhibiting the autocrine loop of stimulation triggered by the extracellular interaction of endogenous FGFs with their tyrosine kinase receptors [12]. On this basis, the first phase for the characterization of newly synthesized compounds with selective FGF-trap activity consisted in the evaluation of their ability to inhibit FGFR3 phosphorylation in MM KMS-11 cells that are characterized by a t(4; 14) chromosomal translocation leading to FGFR3 overexpression [40] and consequent FGF-dependent signaling hyperactivation (Fig. 2 and Table 1). Dose-response curve obtained for reference compound 1 showed an approx. 80% inhibition of FGFR3 activation at 6.0 mM (Fig. 2A). On this basis this concentration was considered suitable for compound screening.

In an attempt to identify the structural elements and properties required for activity, we synthesized new analogues of compound 1 in which the terminal bis(trifluoromethyl)methanol portion of the C-17 side chain was replaced by similar groups, albeit with different electronic properties or steric hindrance (compounds 6 and 7), or by substituents with higher diversification (compounds 4, 5, 11 and 14). In particular, we investigated whether acidity of the terminal substituent (4), or hydrophobic interactions could improve compound potency (11 and 12). Hydrophilic substituents were inserted in position 3 of the steroidal scaffold (compounds 19, 21 and 27), while maintaining the bis(trifluoromethyl)1,3-propanediol side chain, or the C3 alcohol was replaced by a ketone and its oxime derivatives (compounds 22 and 23). The double bond in the steroidal nucleus was also reduced (compounds 24 and 25), to look for an improved complementarity with the FGF binding site. Percentages of inhibition of FGFR3 phosphorylation reported in Table 1 highlight that replacement of the bis(trifluoromethyl)1,3-

propanediol portion with other side chains at position 17 led to a decrease of activity. Among these compounds, only the carboxylic acid 4a retained a potency comparable with that of compound 1.

Compounds reported in Table 1 have several chiral carbons, and their stereochemistry differs for configuration at C20 (4**e**7, 19, 21**e**25, and 27) or at C20 and C22 carbons (11 and 14). The sign and extent of optical rotation is influenced in a complex way by the different configuration at C20 and C22 and do not provide sufficient information to assign carbon configuration. However, for these steroid derivatives, the stereochemistry at C20 of epimer couples 3, 4, 5, 6 and 7 can be tentatively attributed on the basis of the chemical shifts observed for C18 protons in other couples of epimers. In fact, we observed a diagnostic difference in the chemical shift of C18 protons in all couples of epimers with known stereochemistry at C20 (19, 21**e**25, and 27), with signals recorded at lower ppm for the 20*S* derivatives. Thus, for example, compound 3a, with its C18 methyl resonance at 0.79 ppm (CD₃OD/CDCl₃¾ 1/1) has been tentatively attributed 20*R* configuration and 3b 20*S*, having C18 resonance at 0.70 ppm (CD₃OD/CDCl₃¼1/1). On this basis, *R* configuration at C20 has been tentatively assigned to compounds 4a, 5a, 6a and 7a (all deriving from 3a), and *S* configuration to 4b, 5b, 6b and 7b, respectively. For compounds 11a-c and 14a,b the presence of an additional stereocenter prevented the application of this rule, and the stereochemistry of their C20 and C22 atoms is unknown.

Previous observations had shown significant differences in the FGF trap activity of the couple of C20 epimers 1 and 17, being the 20*S* stereoisomer (compound 1) by far the most potent [31]. To clarify whether the stereochemistry at position 20 has an impact on biological activity also for structural analogues, investigation on the structure-activity relationships for substituents at position 3 of the steroidal nucleus was accomplished by synthesizing both stereoisomers at position 20 for each 3-substituted derivative. C3-modified derivatives were prepared starting from compounds 1 and 17 by proper functionalization. Insertion of hydrophilic substituents at position 3, such as the glycine esters 19a,b, the
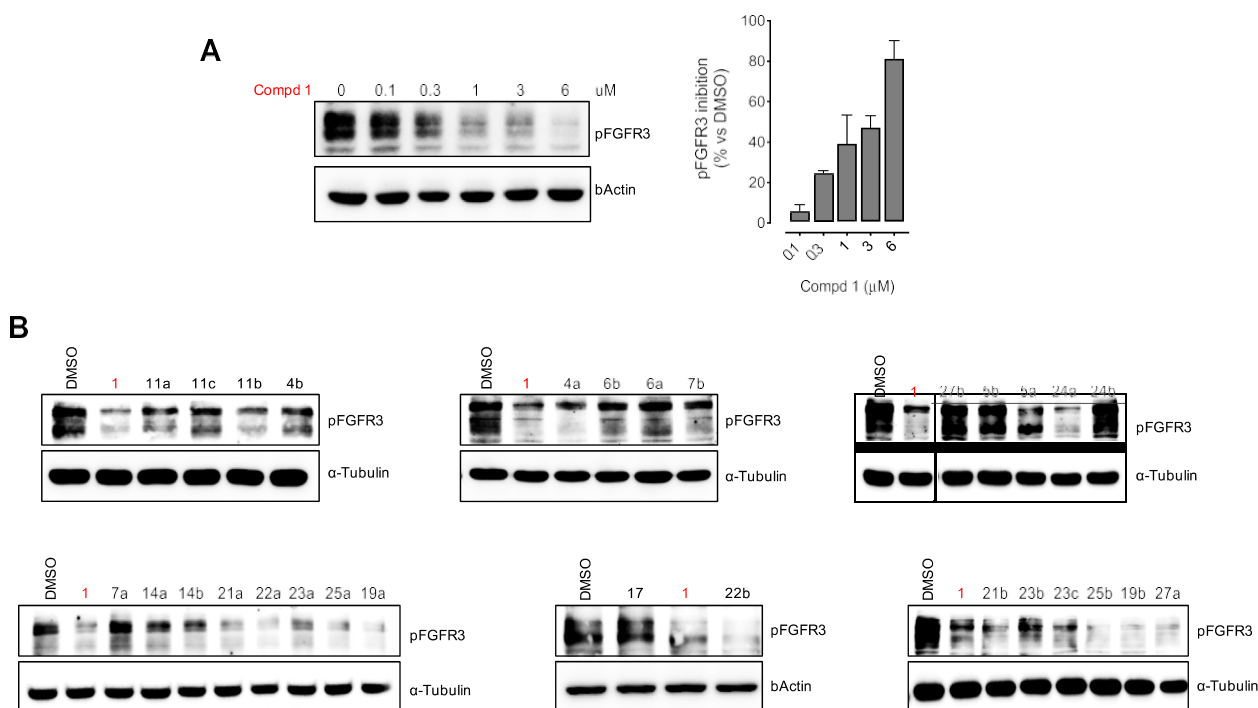
Fig. 2. Western blot analysis of FGFR3 phosphorylation (pFGFR3) in KMS-11 cells. A) Dose-response curve for reference compound 1; *left panel*: representative western blot analysis; *right panel*: densitometric analysis (mean ± SEM). B) Western blot analysis for newly synthesized compounds.

hydroxyethyloxy derivatives 21a,b and the glucosides 27a,b, resulted in compounds endowed with good inhibitory activity on FGFR3 phosphorylation, with the only exception of compound 27b which was inactive. The other compounds showed activities close to that recorded for compound 1 or higher as observed for the glycine derivatives 19a,b and glucosylated 27a which gave inhibition close to 100% at 6 mM concentration (Table 1 and Fig. 2B).

Oxidation of the hydroxyl group at position 3 led to the carbonyl derivatives 22a,b, which were then converted to oximes 23a,b,c, with 23a obtained as a mixture of *E/Z* isomers of the 20*S* stereoisomer. Attribution of *E/Z* configuration to compounds 23b and 23c was accomplished combining results of 1D and 2D ^{1}H NMR spectroscopy and quantum mechanics prediction of chemical shifts for protons on C2 and C4 of the steroidal nucleus (Supporting Information Fig. S1 and Table S1). Carbonyl derivatives 22a,b prevented FGFR3 phosphorylation to a large extent, with a percentage higher than 90% for 22a (Table 1 and Fig. 2B). On the other hand, oxime functionalization (23a,b,c) produced a decrease of potency, with only compound 23c maintaining a good inhibitory activity. Reduction of D^5 unsaturation was performed to assess the influence on potency of the A-B ring junction in the steroidal nucleus. In the case of pregnane derivatives, compound 24a was significantly potent, while the 20*R* derivative 24b did not affect FGFR3 phosphorylation. As already observed for compounds 22a,b, oxidation at position 3 provided potent compounds, with 25b being able to fully suppress FGFR3 phosphorylation (Table 1 and Fig. 2B). The inhibitory activities observed for the couples of C20 stereoisomers suggest the absence of a clear correlation between stereochemistry at C20 and potency. The significant difference in biological activity seen for 24a,b and 27a,b epimers might be related to the peculiar steroidal nucleus under consideration and/or nature of substituents.

For those compounds showing a percentage of pFGFR3 inhibition ≥5% in KMS-11 cells, we next assessed their dose-dependent antiproliferative activity in the same cells (Fig. 3 and Table 2).

All the compounds showed antiproliferative activity on KMS-11 cell line, with IC_{50} values of the same order as that calculated for the reference compound 1. Compounds 19b, 24a and 25b resulted more potent than the reference 1, in line with their high activity in the FGFR3 phosphorylation assay in which they produced an almost complete inhibition of FGFR3 phosphorylation at 6 mM concentration (Table 1 and Fig. 2B).

The two most potent compounds, the O3-glycine ester 19b and the C3-ketone 25b, were selected to be further characterized for their FGF binding properties, specificity and in vivo anti-tumor activity. In order to assess their FGF binding property, compounds 19b and 25b were investigated by surface plasmon resonance (SPR) analysis for their capacity to bind FGF2 immobilized to a BIAcore sensor chip. The SPR binding isotherms showed a Langmuir-like shape for monovalent binding for both compounds with a dissociation constant (Kd) equal to 30.0 ± 17.3 mM for compound 19b and 30.5 ± 17.0 mM for compound 25b (Fig. 4), similar to the reference compound 1 having an apparent Kd value of ~40 mM [31].

To further characterize the biological activity of compounds 19b and 25b, the two derivatives were tested for their capacity to affect cell proliferation and FGFR3 phosphorylation in KMS-11 cells in parallel with three other human MM cell lines (OPM-2, RPMI8226 and U-266 cells) that, like KMS-11 cells, are all driven by a FGF/FGFR-dependent autocrine loop of stimulation.[12] As observed in KMS-11 cells and in keeping with SPR data, compounds 19b and 25b showed a similar inhibitory activity in all the MM cell lines tested (Fig. 5).

As reported in the Introduction section, the presence of the hydroxyl group in position 3 of the steroidal nucleus that characterizes compound 1 is typical of estrogen receptor (ER) binders. In addition, a chemoinformatic approach using the web server SwissTargetPrediction [41,42] pointed out ERs a/b among the possible targets of compound 1 (Supporting Fig. S2). This prediction was confirmed by experimental data showing that compound 1 inhibits the binding of estradiol to ERs a/b when tested at low (5.0 mM) and

Table 1
Inhibition of FGFR3 phosphorylation exerted by newly synthesized compounds in KMS-11 cells.

1, 4-7, 11, 14, 17   19, 21, 27   22, 23   24, 25
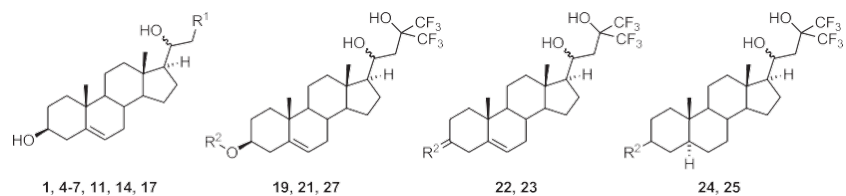
Compd.	R^1	R^2	C20 stereochemistry	pFGFR3[a] % inhibition @ 6 mM
1		–	S	81
17		–	R	inactive
4a		–	R[b]	80
4b		–	S[b]	41
5a		–	R[b]	44
5b		–	S[b]	17
6a		–	R[b]	32
6b		–	S[b]	46
7a		–	R[b]	inactive
7b		–	S[b]	57
11a		–	n.d.	49
11b		–	n.d.	68
11c		–	n.d.	28
14a		–	n.d.	26
14b		–	n.d.	34
19a	–		S	93
19b	–		R	100
21a	–		S	71
21b	–		R	85
22a	–		S	91
22b	–		R	69
(E/Z)-23a	–		S	51
(E)-23b	–		R	58
(Z)-23c	–		R	80
24a	–		S	89
24b	–		R	inactive
25a	–		S	82
25b	–		R	99
27a	–		S	100
27b	–		R	inactive

[b]Tentative attribution; see text. n.d.: not determined.

[a] Percentage of inhibition of FGFR3 phosphorylation (pFGFR3) after treatment of KMS-11 cells for 6 h with 6.0 mM concentration of tested compound.
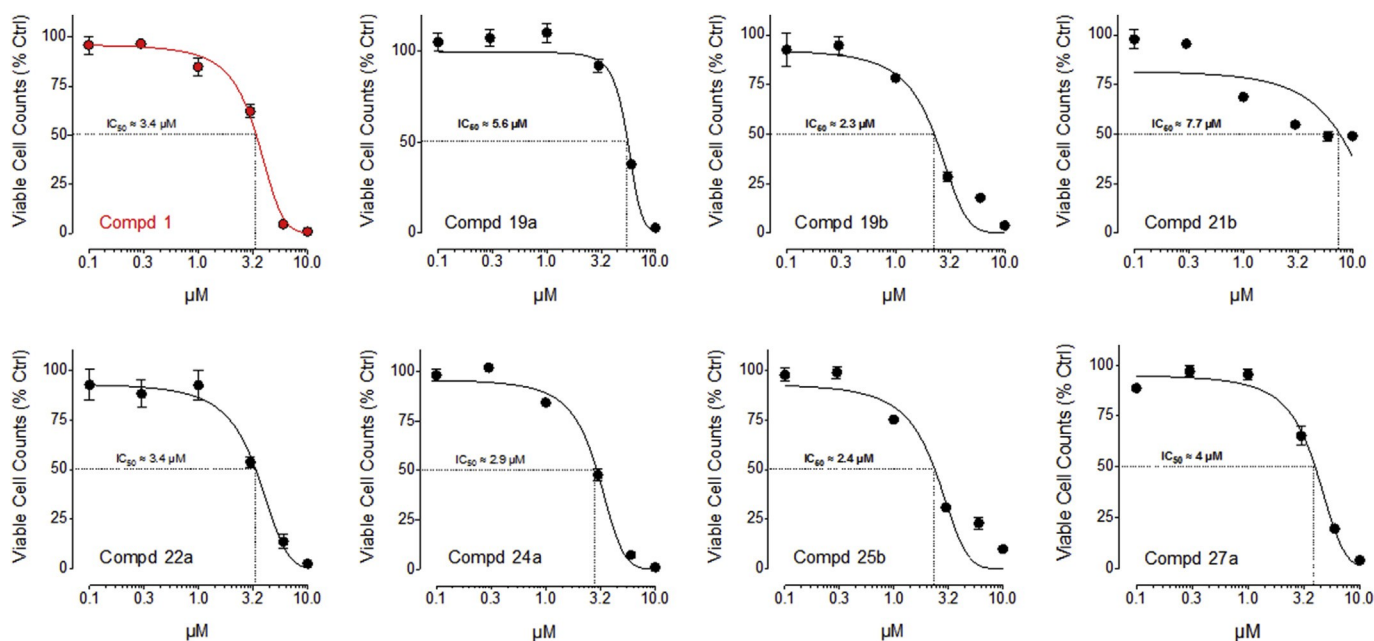
Fig. 3. Dose-response curves for antiproliferative activity in KMS-11 multiple myeloma cells treated for 48 h with tested compound. Data are mean ± SEM.

Table 2
Inhibition of KMS-11 cell proliferation (IC$_{50}$) by newly synthesized compounds.

Compd.	IC$_{50}$ proliferation[a] (mM) ± SEM
1	3.4 ± 0.1
19a	5.6 ± 0.2
19b	2.3 ± 0.2
21b	7.7 ± 1.2
22a	3.4 ± 0.2
24a	2.9 ± 0.1
25b	2.4 ± 0.2
27a	4.0 ± 0.2

[a] Concentration that inhibits by 50% the proliferation of KMS-11 cells after 48 h of incubation with increasing doses of tested compound.

high (50 mM) concentrations in an in vitro competitive binding assay (Table 3). These data indicate that compound 1 is not a selective FGF trap and that off-target effects might contribute to its antiproliferative activity. In contrast, the glycine ester derivative 19b exerted a significant effect on both ER subtypes only when tested at 50 mM concentration, whereas compound 25b did not

affect estradiol/ER binding even when tested at the highest concentration (Table 3), likely as a consequence of the presence of the carbonyl group in position 3 of the steroidal nucleus instead of the hydroxyl group typical of ER binders [33].

Finally, compounds 19b and 25b were compared to 1 for their capacity to exert an antitumor activity when tested in vivo in a KMS-11 xenograft mouse model. To this aim, KMS-11 cells were injected subcutaneously in immunodeficient mice. When tumors were palpable (10 days after grafting), animals were treated i.p. every other day with 7.5 mg/kg of the molecule under test. When compared to compound 1, derivative 25b caused a greater reduction of tumor growth, whereas compound 19b was ineffective (Fig. 6). In keeping with its antitumor activity, compound 25b reached plasma concentrations comparable to those of compound 1 when measured 90 min after i.p. injection (Table 4). In contrast, the lack of efficacy of compound 19b was paralleled by a limited plasma stability, as it resulted readily hydrolyzed to the inactive compound 17 (the 20*R* epimer of compound 1) which had been previously proved to be inactive in a model of murine Lewis lung carcinoma [31].
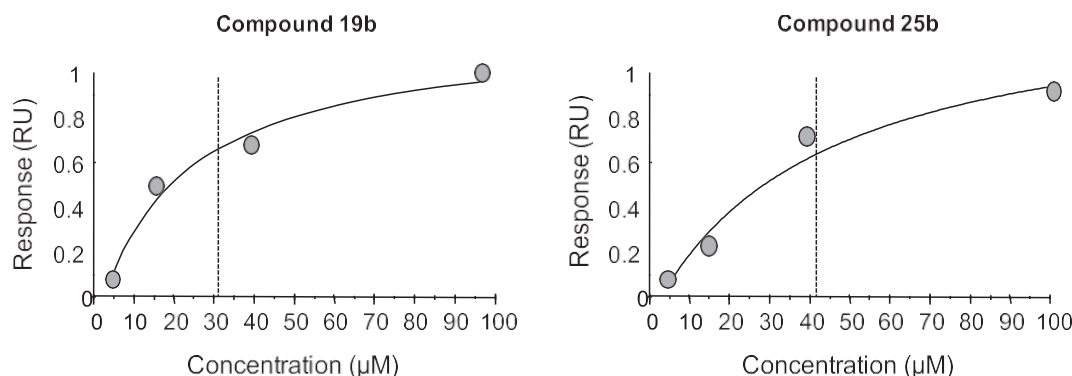

Fig. 4. SPR analysis of compounds 19b and 25b affinity and binding on FGF2 sensor chip. Representative binding isotherms are shown. Dashed vertical lines indicate the calculated Kd.
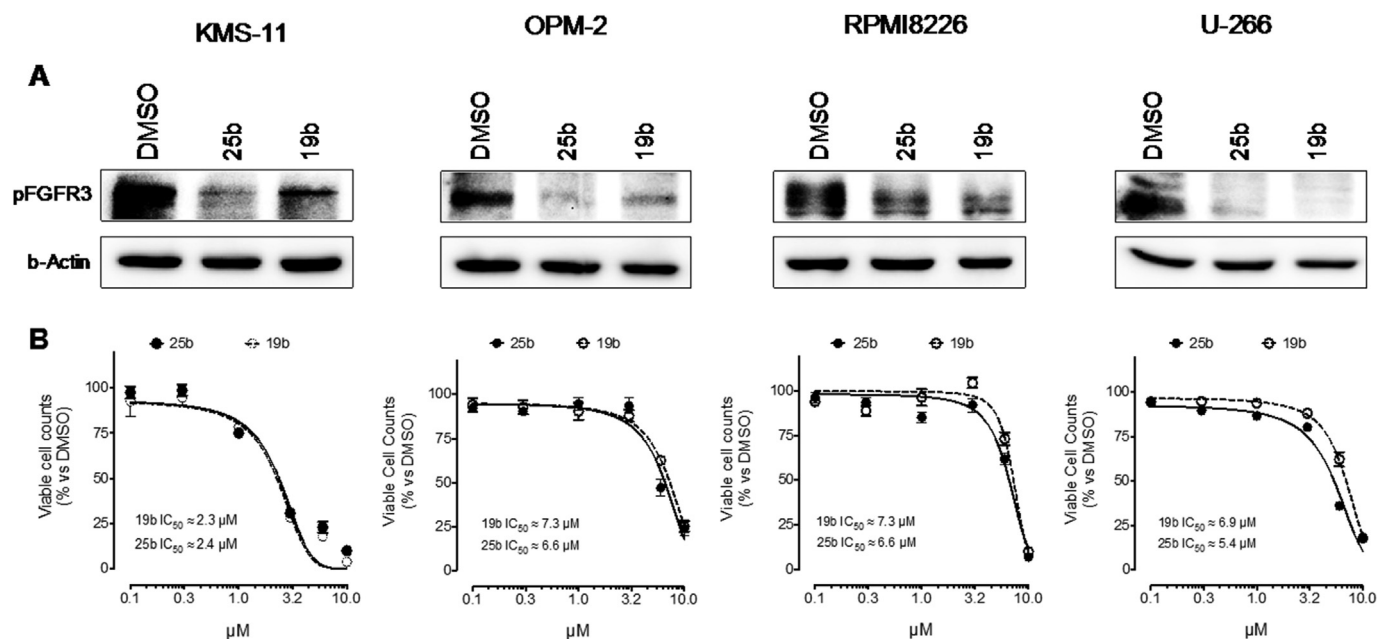
Fig. 5. A) Western blot analysis of MM cells after 6 h treatment with 6 mM of compounds 19b and 25b. B) Viable cell counts by cytofluorimetric analysis of MM cell lines treated with compounds 19b and 25b for 48 h. Data are mean ± SEM.

Table 3
Binding affinity of compounds 1, 19b and 25b to human estrogen receptors (ER) a and b.[a]

Compd.	ERa		ERb	
	5 mM	50 mM	5 mM	50 mM
1	60.8 (60.6; 60.9)	83.3 (87.7; 78.8)	74.9 (76.1; 73.8)	91.9 (91.8; 91.9)
19b	7.4 (4.9; 9.9)	50.0 (46.9; 53.1)	21.4 (18.6; 24.2)	71.3 (72.6; 70.0)
25b	0 (—9.6; 0.2)	0 (3.9; —4.7)	4.3 (0.7; 7.9)	17.6 (16.3; 18.9)

[a] % inhibition of specific radioligand ([^{3}H]estradiol) binding produced by the tested compound at the concentrations of 5.0 and 50 mM. Tests were performed in duplicate. Data are the mean of duplicates; single values are reported in parenthesis.
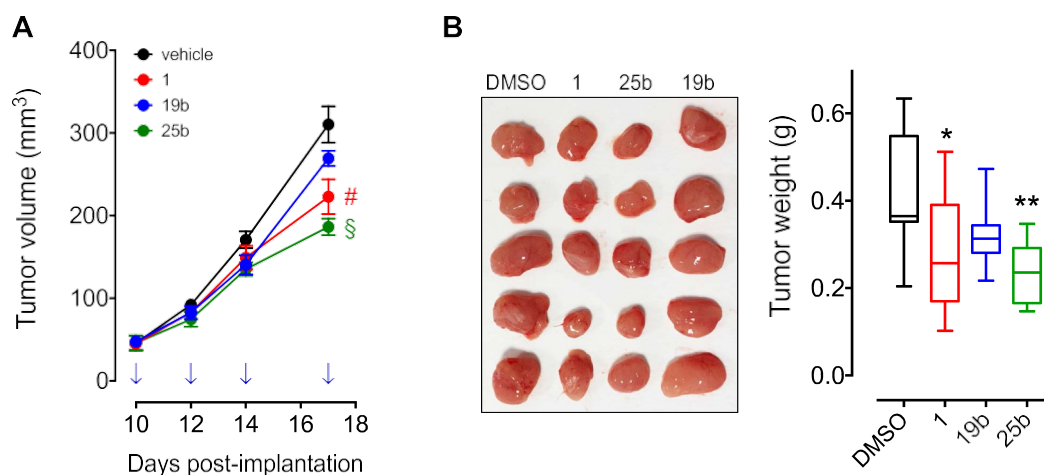

Fig. 6. A) Tumor growth of KMS-11 cells injected s.c. and treated i.p. (arrows) with compounds 1, 19b, 25b or vehicle (n ¼ 5 mice/group). B) At the end of experiment reported in A (17 days post tumor implantation), tumors were harvested, photographed and weighted. *p < 0.05, **p < 0.01, # p < 0.001 vs vehicle (DMSO), x p < 0.001 vs 19b.

4. Conclusions

Structure-activity investigation of compound 1 led to potent derivatives through modulation of the substituent in position 3 of the steroidal nucleus, while modification of side chain in position 17 did not allow to maintain significant inhibition of the FGF/FGFR signaling system in MM cells. No preferred configuration at position 20 emerged from this study, with couples of epimers showing a similar behavior in both inhibition of FGFR3 phosphorylation and the antiproliferative assay. From these premises, compounds 19b

and 25b emerged as optimized derivatives with improved binding affinity and activity in vitro. Importantly, these compounds are devoid of significant affinity for ERs which might concur to the antiproliferative activity observed for compound 1. Despite its higher activity in vitro, the limited metabolic stability of the ester derivative 19b prevented its efficacy in vivo, while compound 25b recapitulates all the structural requirements to improve over compound 1 in the KMS-11 xenograft mouse model. We propose compound 25b, the pregnane 3-keto derivative of compound 1 with opposed stereochemistry at C20 as a new pharmacological tool selective towards the FGF/FGFR axis. Its selectivity will allow investigating the impact of the FGF/FGFR signaling system under physiological and pathological conditions and will pave the way to the design of novel anticancer drugs for the therapy of FGF-dependent tumors, including MM.

5. Experimental section

5.1. Chemistry

5.1.1. General methods

All chemicals were obtained from commercial suppliers and used without further purification. Solvents were purified and stored according to standard procedures. Anhydrous reactions were conducted under a positive pressure of anhydrous N_2. Reactions were monitored by TLC, on Merck silica gel 60 F_{254} plates. Final compounds and intermediates were purified by column chromatography under "flash" conditions using Merck 230e400 mesh silica gel. Melting points were determined on a Buchi B-540 capillary melting point apparatus or on a Gallenkamp melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE 200, 300 or 400 instrument. Chemical shifts (d scale) are reported in parts per million (ppm) relative to the central peak of the solvent. ESI-MS spectra of the final products were acquired on a Thermo TSQ Quantum Access Max triple quadrupole mass spectrometer (Thermo, San Jose, CA, USA) equipped with a heated electrospray ionization (H-ESI) source. High-resolution mass spectrometry (HRMS) was performed on a Micromass Q-ToF Micro mass spectrometer (Micromass, Manchester, UK) using an ESI source or on a Thermo Scientific LTQ Orbitrap XL spectrometer (Thermo, USA). The purity of tested compounds, determined by high performance liquid chromatography (HPLC), was greater than 95%. Optical rotation analysis was performed using a PerkinElmer 241 or 341 polarimeter, the concentration c of analytes being reported as mg/mL.

5.1.2. Compound synthesis and characterization

5.1.2.1. 3b-acetoxy-21-ethoxycarbonyl-pregn-5-en-20-ol (3a,b).
Activated zinc dust (13.00 g, 198.8 mmol) was suspended in anhydrous THF (10 mL) and ethyl bromoacetate (5 mL, 45.1 mmol) was added portionwise. The solution thus obtained was slowly added *via cannula* to an ice-cooled solution of 3b-acetoxy-androst-5-ene-17b-carbaldehyde (2, 1.63 g, 4.8 mmol) in anhydrous THF (10 mL), and the resulting mixture was stirred at 0 °C for 20 min.

Water was added and the mixture extracted with EtOAc. The organic layer was washed with brine and dried over Na_2SO_4. Solvents were removed under reduced pressure and the residue was purified by silica gel column chromatography (petroleum ether/EtOAc ¼ 9:1) to afford 3a (481 mg, 23% yield) and 3b (964 mg, 47% yield). 3a: ^{1}H NMR (300 MHz, CDCl$_3$) d 5.37 (d, J ¼ 4.7 Hz, 1H), 4.65e4.54 (m, 1H), 4.17 (q, J ¼ 7.1 Hz, 2H), 3.95 (ddd, J ¼ 9.3, 7.9, 2.9 Hz, 1H), 2.62 (dd, J ¼ 16.4, 2.9 Hz, 1H), 2.46e2.26 (m, 3H), 2.02 (s, 3H), 1.98e1.76 (m, 5H), 1.76e1.35 (m, 8H), 1.27 (t, J ¼ 7.1 Hz, 3H), 1.21e0.88 (m, 8H), 0.70 (s, 3H). 3b: ^{1}H NMR (300 MHz, CDCl$_3$) d 5.37 (d, J ¼ 4.4 Hz, 1H), 4.77e4.48 (m, 1H), 4.17 (q, J ¼ 7.1 Hz, 2H), 3.94 (ddd, J ¼ 10.3, 9.1, 2.7 Hz, 1H), 2.51 (dd, J ¼ 16.4, 2.6 Hz, 1H), 2.36e2.28 (m, 3H), 2.17 (dt, J ¼ 12.6, 3.5 Hz, 1H), 2.03 (s, 3H), 1.99e1.77 (m, 3H), 1.74e1.32 (m, 9H), 1.27 (t, J ¼ 7.1 Hz, 3H), 1.21e0.91 (m, 8H), 0.79 (s, 3H).

5.1.2.2. 3b-hydroxy-21-carboxy-pregn-5-en-20-ol (4a).
Compound 3a (43 mg, 0.1 mmol) was dissolved in THF (2 mL). A solution of NaOH (36 mg, 0.9 mmol) in water (1 mL) was added and the mixture was stirred for 15 h at room temperature. HCl 1 M (1 mL) was added and solvents were removed under reduced pressure. The residue was purified by silica gel column chromatography (petroleum ether/EtOAc 3:2 þ 0.5% AcOH) to afford the title compound (26 mg, 72% yield) as a white solid; mp 235 °C (dec.). [a]$_D^{20}$ ¼ —54.17° (c ¼ 0.7, CHCl$_3$/MeOH 1:1). ^{1}H NMR (300 MHz, CDCl$_3$/CD$_3$OD) d 5.32 (d, J ¼ 5.0 Hz, 1H), 3.91 (dt, J ¼ 9.1, 3.1 Hz, 1H), 3.43 (m, 1H), 2.61 (dd, J ¼ 15.5, 3.2 Hz, 1H), 2.36e2.15 (m, 3H), 2.08e1.72 (m, 5H), 1.72e1.35 (m, 9H), 1.32e0.79 (m, 9H), 0.71 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$/CD$_3$OD) d 175.7, 141.5, 121.9, 71.8, 70.5, 57.2, 56.6, 50.8, 42.3, 42.2, 42.0, 39.4, 37.9, 37.1, 32.4, 32.2, 31.6, 26.0, 24.6, 21.4, 19.7, 12.8. ESI-HRMS [M — H]$^-$ calcd for C$_{22}$H$_{33}$O$_4$: 361.23843; found: 361.23850.

5.1.2.3. 3b-hydroxy-21-carboxy-pregn-5-en-20-ol (4b).
This compound was prepared according to the procedure described for compound 4a starting from intermediate 3b. White solid (26 mg, 86% yield); mp 225e227 °C. [a]$_D^{20}$ ¼ —22.83° (c ¼ 0.9, CHCl$_3$/MeOH 1:1). ^{1}H NMR (400 MHz, CDCl$_3$/CD$_3$OD) d 5.31 (d, J ¼ 5.0 Hz, 1H), 3.93 (dt, J ¼ 9.7, 2.7 Hz, 1H), 3.46e3.38 (m, 1H), 2.46 (dd, J ¼ 15.7, 2.8 Hz, 1H), 2.29e2.09 (m, 4H), 1.97e1.90 (m, 1H), 1.88e1.59 (m, 4H), 1.55e1.40 (m, 6H), 1.30e0.78 (m, 11H), 0.77 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$/CD$_3$OD) d 175.9, 141.8, 122.0, 72.0, 71.5, 57.1, 56.5, 51.1, 43.2, 42.5, 42.4, 40.2, 38.1, 37.3, 32.7, 32.6, 31.8, 26.1, 25.2, 21.6, 19.8, 12.5. ESI-HRMS [M — H]$^-$ calcd for C$_{22}$H$_{33}$O$_4$: 361.23843; found: 361.23898.

5.1.2.4. 3b-hydroxy-21-carbamoyl-pregn-5-en-20-ol (5a).
NH$_4$Cl (90 mg, 1.7 mmol), TBTU (98 mg, 0.3 mmol) and DIPEA (0.14 ml, 0.8 mmol) were added to a solution of 4a (0.2 mmol) in DMF (2 mL) and the resulting mixture was stirred for 15 h at room temperature. Solvents were removed under reduced pressure and the residue was purified by silica gel column chromatography (petroleum ether/EtOAc 1:1 þ 3% AcOH) followed by crystallization (EtOH/water 7:3) to afford the title compound (21 mg, 25% yield) as

colorless crystals. mp 280 °C. $[a]_D^{20}$ ¼ —44.44° (c ¼ 0.9, CHCl$_3$/MeOH 1:1). ^{1}H NMR (300 MHz, DMSO-d_6) d 7.28 (s, 1H), 6.81 (s, 1H), 5.26 (d, J ¼ 4.9 Hz, 1H), 4.57 (dd, J ¼ 10.7, 5.0 Hz, 2H), 3.81 - 3.73 (m, 1H), 3.31e3.20 (m, 1H), 2.32e2.21 (m, 1H), 2.21e1.98 (m, 3H), 1.98e1.19 (m, 13H), 1.19e0.81 (m, 8H), 0.64 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) d 173.7, 141.3, 120.4, 70.0, 68.3, 56.1, 55.9, 49.8, 42.7, 42.2, 41.0, 38.2, 36.9, 36.1, 31.4, 31.1, 24.4, 23.7, 20.4, 19.2, 12.4. ESI-HRMS [MþNa]þ calcd for $C_{22}H_{35}NNaO_3$: 384.25092; found: 384.25082.

5.1.2.5. 3b-hydroxy-21-carbamoyl-pregn-5-en-20-ol (5b).

This compound was prepared according to the procedure described for compound 5a starting from 4b. Colorless crystals (30 mg, 25% yield); mp 265 °C. $[a]_D^{20}$ ¼ —34.48° (c ¼ 1.2, CHCl$_3$/MeOH 1:1). ^{1}H NMR (300 MHz, DMSO-d_6) d 7.25 (s, 1H), 6.83 (s, 1H), 5.26 (d, J ¼ 4.9 Hz, 1H), 4.57 (dd, J ¼ 8.9, 5.1 Hz, 2H), 3.73 (qd, J ¼ 7.3, 5.7, 2.9 Hz, 1H), 3.28e3.17 (m, 1H), 2.22e1.84 (m, 6H), 1.81e1.24 (m, 10H), 1.21e0.77 (m, 9H), 0.71 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) d 174.0, 141.3, 120.4, 70.1, 70.0, 55.8, 55.8, 49.8, 42.4, 42.3, 42.0, 37.0, 36.1, 31.5, 31.4, 31.4, 25.0, 24.2, 20.5, 19.2, 11.9. ESI-HRMS [MþNa]þ calcd for $C_{22}H_{35}NNaO_3$: 384.25092; found: 384.25089.

5.1.2.6. 3b-hydroxy-21-(1-hydroxy-1-methyl)ethyl-pregn-5-en-20-ol (6a).

Compound 3a (47 mg, 0.1 mmol) was dissolved in toluene (5 mL) and cooled to 0 °C. MeMgBr (3.0 M, 0.9 mL, 0.9 mmol) was added and the mixture was slowly warmed till reflux and stirred for 2 h. It was then cooled to room temperature and diluted HCl (0.05 M) was added till neutralization. The mixture was extracted with EtOAc. The organic layer was washed with brine and dried over Na$_2$SO$_4$. Solvents were removed under reduced pressure and the residue was purified by silica gel column chromatography (DCM/MeOH 94:6) to afford the title compound (27 mg, 65% yield) as a white solid; mp 222e224 °C. $[a]_D^{20}$ ¼ —29.41° (c ¼ 0.3, CHCl$_3$/MeOH 1:1). ^{1}H NMR (300 MHz, CDCl$_3$/CD$_3$OD) d 5.32 (d, J ¼ 5.1 Hz, 1H), 3.90 (dt, J ¼ 10.9, 2.3 Hz, 1H), 3.51e3.37 (m, 1H), 2.30e2.08 (m, 2H), 2.00e1.74 (m, 5H), 1.70e0.88 (m, 24H), 0.68 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$/CD$_3$OD) d 141.5, 121.9, 72.5, 71.9, 71.7, 57.7, 57.3, 50.8, 47.6, 42.3, 42.0, 39.8, 37.8, 37.1, 32.4, 32.2, 31.7, 31.6, 27.7, 26.1, 24.5, 21.4, 19.6, 12.7. ESI-HRMS [MþNa]þ calcd for $C_{24}H_{40}NaO_3$: 399.28697; found: 399.28732.

5.1.2.7. 3b-hydroxy-21-(1-hydroxy-1-methyl)ethyl-pregn-5-en-20-ol (6b).

This compound was prepared according to the procedure described for compound 6a starting from intermediate 3b. White solid (23 mg, 56% yield); mp 231e233 °C. $[a]_D^{20}$ ¼ —43.33° (c ¼ 0.6, CHCl$_3$/MeOH 1:1). ^{1}H NMR (300 MHz, CDCl$_3$/CD$_3$OD) d 5.32e5.27 (m, 1H), 3.89 (dt, J ¼ 9.9, 6.2 Hz, 1H), 3.49e3.35 (m, 1H), 2.29e2.15 (m, 2H), 2.14e2.05 (m, 1H), 2.03e1.70 (m, 3H), 1.70e1.32 (m, 13H), 1.26 (s, 3H), 1.20 (s, 3H), 1.14e0.87 (m, 6H), 0.78 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$/CD$_3$OD) d 141.6, 121.9, 72.7, 72.1, 71.8, 57.4, 57.0, 51.0, 47.8, 43.0, 42.4, 40.1, 37.9, 37.2, 32.5, 32.4, 31.7, 31.6, 28.1, 26.2, 25.1, 21.5, 19.7, 12.5. ESI-HRMS [MþNa]þ calcd for $C_{24}H_{40}NaO_3$: 399.28697; found: 399.28729.

5.1.2.8. 3b-hydroxy-21-hydroxymethyl-pregn-5-en-20-ol (7a).

Compound 3a (105 mg, 0.3 mmol) was dissolved in THF (2 mL) and NaBH$_4$ (430 mg, 11.4 mmol) was added at room temperature. MeOH (2 mL) was then added dropwise and the resulting mixture was heated to reflux for 1 h. The reaction was cooled to room temperature, and the excess NaBH$_4$ was quenched with HCl 1 M (7 mL). Stirring was then continued for 20 min. Excess NaOH was added (pH ¼ 10) and the mixture was warmed to 50 °C until only one spot was detected by TLC analysis. After neutralization with HCl 1 M, the mixture was extracted with EtOAc. The organic layer was washed with brine and dried over Na$_2$SO$_4$. Solvents were removed under reduced pressure and the residue was purified by silica gel column

chromatography (DCM/MeOH 98:2) to afford the title compound (21 mg, 24% yield) as a white solid; mp 223e227 °C. $[a]_D^{20}$ ¼ — 46.67° (c ¼ 0.9, CHCl$_3$/MeOH 1:1). ^{1}H NMR (400 MHz, CDCl$_3$/CD$_3$OD) d 5.33 (d, J ¼ 5.0 Hz, 1H), 3.83e3.66 (m, 3H), 3.45 (dq, J ¼ 10.5, 5.0 Hz, 1H), 2.29e2.15 (m, 2H), 2.02e1.77 (m, 6H), 1.68e1.36 (m, 9H), 1.30e0.89 (m, 8H), 0.69 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$/CD$_3$OD) d 141.7, 122.1, 72.3, 71.9, 60.5, 57.5, 51.1, 42.5, 42.3, 39.8, 39.6, 38.1, 37.3, 32.6, 32.4, 31.8, 26.4, 24.8, 21.5, 19.8, 12.8. ESI-HRMS [MþNa]þ calcd for $C_{22}H_{36}NaO_3$: 371.25567; found: 371.25556.

5.1.2.9. 3b-hydroxy-21-hydroxymethyl-pregn-5-en-20-ol (7b).

This compound was prepared according to the procedure described for compound 7a starting from intermediate 3b. White solid (39 mg, 50% yield); mp 176e179 °C. $[a]_D^{20}$ ¼ —39.29° (c ¼ 0.6, CHCl$_3$/MeOH 1:1). ^{1}H NMR (300 MHz, CDCl$_3$/CD$_3$OD) d 5.15 (d, J ¼ 5.0 Hz, 1H), 3.60e3.47 (m, 3H), 3.33e3.20 (m, 1H), 2.11e2.01 (m, 2H), 1.96 (dt, J ¼ 12.6, 3.3 Hz, 1H), 1.88e1.17 (m, 13H), 1.17e0.62 (m, 9H), 0.60 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$/CD$_3$OD) d 141.6, 122.0, 72.6, 71.9, 60.3, 57.0, 57.0, 51.0, 43.0, 42.4, 40.3, 39.2, 38.0, 37.2, 32.6, 32.5, 31.7, 26.1, 25.1, 21.6, 19.7, 12.5. ESI-HRMS [Mþ Na]þ calcd for $C_{22}H_{36}NaO_3$: 371.25567; found: 371.25595.

5.1.2.10. 3b-benzoyloxy-21-(hydroxybenzyl)-pregn-5-en-20-one (9a,b).

Pregnenolone benzoate (8, 368 mg, 0.87 mmol) was dissolved in anhydrous THF (10 mL) and cooled to —78 °C under a nitrogen atmosphere. LiHMDS (1.0 M, 1.80 mL, 1.80 mmol) was slowly added, followed by benzaldehyde (0.27 mL, 2.66 mmol). The mixture was warmed to room temperature over 2 h and acetic acid (0.12 mL, 2.10 mmol) was added. The solution was diluted with DCM and washed with water. The organic layer was washed with brine and dried over Na$_2$SO$_4$. Solvents were removed under reduced pressure and the residue was purified by silica gel column chromatography (petroleum ether/EtOAc 9:1) to afford compound 9a (187 mg, 41% yield) and compound 9b (113 mg, 25% yield). 9a: ^{1}H NMR (400 MHz, CDCl$_3$) d 8.10e7.98 (m, 2H), 7.61e7.49 (m, 1H), 7.49e7.27 (m, 7H), 5.42 (d, J ¼ 5.0 Hz, 1H), 5.17 (dd, J ¼ 8.3, 3.9 Hz, 1H), 4.96e4.74 (m, 1H), 2.91e2.70 (m, 2H), 2.62e2.34 (m, 3H), 2.32e2.10 (m, 1H), 2.10e1.83 (m, 4H), 1.83e1.40 (m, 8H), 1.33e0.94 (m, 7H), 0.65 (s, 3H). 9b: ^{1}H NMR (400 MHz, CDCl$_3$) d 8.07e8.01 (m, 2H), 7.61e7.50 (m, 1H), 7.43 (t, J ¼ 7.7 Hz, 2H), 7.40e7.32 (m, 4H), 7.32e7.27 (m, 1H), 5.42 (d, J ¼ 5.0 Hz, 1H), 5.17 (dd, J ¼ 7.7, 4.4 Hz, 1H), 5.04e4.74 (m, 1H), 2.83e2.76 (m, 2H), 2.59e2.46 (m, 3H), 2.28e2.14 (m, 1H), 2.09e1.90 (m, 4H), 1.82e1.37 (m, 8H), 1.33e1.13 (m, 4H), 1.07 (s, 3H), 0.65 (s, 3H).

5.1.2.11. 3b-benzoyloxy-21-(hydroxybenzyl)-pregn-5-en-20-ol (10a).

Compound 9a (176 mg, 0.33 mmol) was dissolved in THF (5 mL) at room temperature and Na(OAc)$_3$BH (702 mg, 3.31 mmol) was added. The mixture was stirred for 30 min, then HCl 1 M (3.3 mL, 3.30 mmol) was added and the suspension was stirred for 15 min. The mixture was neutralized by slow addition of a 2 M aqueous solution of NaOH and diluted with DCM. The layers were separated and the aqueous phase was extracted with DCM. The combined organic layers were washed with brine and dried over Na$_2$SO$_4$. Solvents were removed under reduced pressure and the residue was purified by silica gel column chromatography (petroleum ether/EtOAc 4:1) to afford the title compound (145 mg, 83% yield) as a single isomer. ^{1}H NMR (300 MHz, CDCl$_3$) d 8.16e7.95 (m, 2H), 7.65e7.49 (m, 1H), 7.49e7.28 (m, 7H), 5.55e5.35 (m, 1H), 5.08 (dd, J ¼ 9.1, 2.8 Hz, 1H), 4.87 (dtt, J ¼ 12.0, 8.1, 4.5 Hz, 1H), 3.90 (ddd, J ¼ 10.4, 8.1, 2.6 Hz, 1H), 2.47 (d, J ¼ 8.0 Hz, 2H), 2.12 (dt, J ¼ 12.2, 3.3 Hz, 1H), 2.05e1.88 (m, 4H), 1.85e1.43 (m, 10H), 1.38e1.00 (m, 10H), 0.78 (s, 3H).

5.1.2.12. *3b-benzoyloxy-21-(hydroxybenzyl)-pregn-5-en-20-ol (10b,c).* This compound was prepared according to the procedure described for compound 10a starting from intermediate 9b. White solids: 10b (41 mg, 60% yield), 10c (29 mg, 40% yield). 10b: ^{1}H NMR (300 MHz, CDCl$_3$/CD$_3$OD) d 8.05e7.91 (m, 2H), 7.52 (t, *J* ¼ 7.4 Hz, 1H), 7.46e7.13 (m, 7H), 5.38 (d, *J* ¼ 4.9 Hz, 1H), 5.00 (dd, *J* ¼ 9.0, 2.8 Hz, 1H), 4.89e4.66 (m, 1H), 3.74 (dt, *J* ¼ 9.7, 5.1 Hz, 1H), 2.42 (d, *J* ¼ 7.9 Hz, 2H), 2.07e0.62 (m, 23H), 0.58 (s, 3H). 10c: ^{1}H NMR (300 MHz, CDCl$_3$) d 8.12e7.99 (m, 2H), 7.64e7.50 (m, 1H), 7.43 (dd, *J* ¼ 8.3, 6.8 Hz, 2H), 7.39e7.27 (m, 5H), 5.50e5.37 (m, 1H), 5.02e4.90 (m, 1H), 4.85 (ddd, *J* ¼ 12.1, 8.0, 4.1 Hz, 1H), 3.92 (dt, *J* ¼ 9.3, 8.1, 4.6 Hz, 1H), 2.47 (d, *J* ¼ 8.1 Hz, 2H), 2.01e0.91 (m, 25H), 0.83 (s, 3H).

5.1.2.13. *3b-hydroxy-21-(hydroxybenzyl)-pregn-5-en-20-ol (11a).* Compound 10a (143 mg, 0.27 mmol) was dissolved in THF (3 mL) and MeOH (3 mL). Excess Na (62 mg, 2.6 mmol) was added and the mixture was stirred for 2 h at room temperature. Acetic acid (0.6 mL, 10.5 mmol) was added, followed by EtOAc. The layers were separated and the aqueous phase was extracted with EtOAc. The combined organic layers were washed with brine and dried over Na$_2$SO$_4$. Solvents were removed under reduced pressure and the residue was purified by silica gel column chromatography (petroleum ether/EtOAc 65:35) to afford the title compound (101 mg, 88% yield) as a white solid; mp 190 °C. [a]$^{20}_D$¼ —31.90° (c ¼ 1.2, CHCl$_3$/MeOH 1:1). ^{1}H NMR (300 MHz, CD$_3$OD) d 7.39e7.22 (m, 4H), 7.22e7.13 (m, 1H), 5.30 (dd, *J* ¼ 4.5, 2.8 Hz, 1H), 4.95 (dd, *J* ¼ 10.1, 2.4 Hz, 1H), 3.84 (dt, *J* ¼ 9.7, 2.2 Hz, 1H), 3.53e3.37 (m, 1H), 2.32e2.05 (m, 3H), 2.05e1.70 (m, 4H), 1.66e1.39 (m, 8H), 1.31e0.85 (m, 10H), 0.77 (s, 3H). ^{13}C NMR (100 MHz, CD$_3$OD) d 146.0, 141.5, 128.7, 127.5, 126.0, 121.9, 71.7, 71.3, 70.9, 56.8, 56.5, 50.9, 46.0, 43.0, 42.3, 40.2, 37.9, 37.1, 32.5, 32.4, 31.6, 25.9, 25.1, 21.5, 19.7, 12.5. ESI-HRMS [M þ Na]^þ calcd for C$_{28}$H$_{40}$NaO$_3$: 447.28697; found: 447.28687.

5.1.2.14. *3b-hydroxy-21-(hydroxybenzyl)-pregn-5-en-20-ol (11b).* This compound was prepared according to the procedure described for compound 11a starting from intermediate 10b. White solid (23 mg, 68% yield); mp 223 °C. [a]$^{20}_D$ ¼ —50.00° (c ¼ 0.4, CHCl$_3$/MeOH 1:1). ^{1}H NMR (300 MHz, CDCl$_3$/CD$_3$OD) d 7.39e7.25 (m, 4H), 7.22 (ddd, *J* ¼ 6.1, 5.0, 2.2 Hz, 1H), 5.30 (d, *J* ¼ 4.7 Hz, 1H), 4.86 (dd, *J* ¼ 8.2, 5.3 Hz, 1H), 3.65 (dt, *J* ¼ 9.6, 2.8 Hz, 1H), 3.43 (dq, *J* ¼ 9.5, 4.5, 4.1 Hz, 1H), 2.19 (dd, *J* ¼ 9.4, 3.7 Hz, 2H), 2.11 (dt, *J* ¼ 12.5, 3.3 Hz, 1H), 1.98e1.27 (m, 12H), 1.27e0.81 (m, 10H), 0.72 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$/CD$_3$OD) d 145.4, 141.7, 129.0, 128.1, 126.8, 122.0, 74.8, 74.4, 72.0, 57.4, 57.1, 51.1, 45.8, 43.1, 42.5, 40.2, 38.1, 37.3, 32.6, 32.6, 31.8, 26.2, 25.2, 21.6, 19.8, 12.4. ESI-HRMS [M þ Na]^þ calcd for C$_{28}$H$_{40}$NaO$_3$: 447.28697; found: 447.28695.

5.1.2.15. *3b-hydroxy-21-(hydroxybenzyl)-pregn-5-en-20-ol (11c).* This compound was prepared according to the procedure described for compound 11a starting from intermediate 10c. White solid (33 mg, 78% yield); mp 209 °C. [a]$^{20}_D$ ¼ —41.67° (c ¼ 0.8, CHCl$_3$/MeOH 1:1). ^{1}H NMR (400 MHz, CD3OD) d 7.42e7.25 (m, 4H), 7.25e7.13 (m, 1H), 5.31 (d, *J* ¼ 5.0 Hz, 1H), 4.98 (dd, *J* ¼ 9.3, 2.6 Hz, 1H), 3.78 (m, 1H), 3.41 (dt, *J* ¼ 10.4, 4.8 Hz, 1H), 2.29e2.09 (m, 2H), 2.09e1.35 (m, 14H), 1.35e0.72 (m, 9H), 0.63 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) d 147.3, 141.3, 128.0, 126.4, 125.5, 120.5, 70.0, 69.0, 68.1, 56.6, 56.2, 49.7, 47.4, 42.2, 41.1, 38.6, 36.9, 36.1, 31.4, 31.2, 25.3, 23.8, 20.4, 19.2, 12.3. ESI-HRMS [M þ Na]^þ calcd for C$_{28}$H$_{40}$NaO$_3$: 447.28697; found: 447.28760.

5.1.2.16. *3b-benzoyloxy-21-(hydroxy-pyridin-2-yl-methyl)-pregn-5-en-20-one (12a,b).* Pregnenolone benzoate (8, 1.21 g, 2.89 mmol) was dissolved in anhydrous THF (20 mL) and cooled to —78 °C under a nitrogen atmosphere. LiHMDS (1.0 M, 4.40 mL, 4.40 mmol) was slowly added, followed by 2-pyridinecarboxaldehyde (1.60 mL, 16.82 mmol). The mixture was allowed to warm to —55 °C and excess saturated aqueous solution of NH$_4$Cl was added. The solution was diluted with DCM and washed with water. The organic layer was washed with brine and dried over Na$_2$SO$_4$. Solvents were removed under reduced pressure and the residue was purified by silica gel column chromatography (petroleum ether/EtOAc 4:1) to afford the mixture of the two C22 epimers (1:0.8, 1.25 g, 82% yield). ^{1}H NMR (300 MHz, CDCl$_3$) d 8.53 (dd, *J* ¼ 4.9, 1.6 Hz, 1H), 8.10e8.00 (m, 2H), 7.70 (m, 1H), 7.59e7.35 (m, 4H), 7.24e7.10 (m, 1H), 5.41 (dd, *J* ¼ 5.0, 1.8 Hz, 1H), 5.21 (m, 1H), 5.00e4.69 (m, 1H), 4.22 (d, *J* ¼ 4.8 Hz, 1H), 3.10e2.82 (m, 2H), 2.64e2.51 (m, 1H), 2.47 (d, *J* ¼ 7.8 Hz, 2H), 2.33e1.95 (m, 4H), 1.91 (dt, *J* ¼ 13.3, 3.4 Hz, 1H), 1.83e1.35 (m, 8H), 1.35e0.93 (m, 8H), 0.63-0.61 (m, 3H).

5.1.2.17. *3b-benzoyloxy-21-(hydroxy-pyridin-2-yl-methyl)-pregn-5-en-20-ol (13a,b).* This compound was prepared according to the procedure described for compound 10a starting from intermediate 12a,b. White solids: 13a (165 mg, 16% yield); 13b (217 mg, 21% yield). 13a: ^{1}H NMR (300 MHz, DMSO-d_6) d 8.48 (d, *J* ¼ 4.4 Hz, 1H), 7.96 (d, *J* ¼ 7.5 Hz, 2H), 7.78 (dt, *J* ¼ 7.6, 1.8 Hz, 1H), 7.65 (t, *J* ¼ 7.3 Hz, 1H), 7.52 (t, *J* ¼ 7.7 Hz, 2H), 7.46 (d, *J* ¼ 7.9 Hz, 1H), 7.24 (ddd, *J* ¼ 7.5, 4.8, 1.2 Hz, 1H), 5.56 (d, *J* ¼ 4.1 Hz, 1H), 5.39 (d, *J* ¼ 4.8 Hz, 1H), 4.85e4.80 (m, 1H), 4.77e4.66 (m, 1H), 4.52 (d, *J* ¼ 5.0 Hz, 1H), 3.54e3.46 (m, 1H), 2.42 (d, *J* ¼ 7.8 Hz, 2H), 2.16 (d, *J* ¼ 12.5 Hz, 1H), 2.03e1.28 (m, 12H), 1.28e0.78 (m, 10H), 0.67 (s, 3H). 13b: ^{1}H NMR (300 MHz, DMSO-d_6) d 8.46 (d, *J* ¼ 4.5 Hz,1H), 8.04e7.88 (m, 2H), 7.76 (dt, *J* ¼ 7.7, 1.8 Hz, 1H), 7.71e7.60 (m, 1H), 7.60e7.40 (m, 3H), 7.21 (ddd, *J* ¼ 7.5, 4.8, 1.2 Hz, 1H), 5.40 (d, *J* ¼ 4.8 Hz, 1H), 5.24 (d, *J* ¼ 5.3 Hz, 1H), 4.87e4.81 (m, 1H), 4.77e4.70 (m, 1H), 4.29 (d, *J* ¼ 7.3 Hz, 1H), 3.70 (q, *J* ¼ 8.9 Hz, 1H), 2.42 (d, *J* ¼ 7.8 Hz, 2H), 2.20 (d, *J* ¼ 12.6 Hz, 1H), 2.00e1.86 (m, 3H), 1.83e1.09 (m, 16H), 1.04 (s, 3H), 0.75 (s, 3H).

5.1.2.18. *3b-hydroxy-21-(hydroxy-pyridin-2-yl-methyl)-pregn-5-en-20-ol (14a).* Compound 13a (145 mg, 0.27 mmol) was dissolved in MeOH (5 mL) and sodium (24 mg, 1.03 mmol) was added at room temperature. The mixture was stirred for 2 h, then HCl 1 M (1 ml, 1.0 mmol) was added dropwise. The solution was diluted with DCM and washed with water. The organic layer was washed with brine and dried over Na$_2$SO$_4$. The solvents were removed under reduced pressure and the residue was purified by silica gel column chromatography (EtOAc/petroleum ether 2:1) to afford the title compound (82 mg, 71% yield) as a white solid; mp 225e226 °C. [a]$^{20}_D$ ¼ —73.33° (c ¼ 0.9, CHCl$_3$/MeOH 1:1). ^{1}H NMR (400 MHz, CD$_3$OD) d 8.47 (ddd, *J* ¼ 4.9, 1.8, 0.9 Hz, 1H), 7.84 (dt, *J* ¼ 7.7, 1.8 Hz, 1H), 7.56 (dt, *J* ¼ 7.9, 1.1 Hz, 1H), 7.31 (ddd, *J* ¼ 7.6, 4.9, 1.2 Hz, 1H), 5.33 (d, *J* ¼ 4.8 Hz, 1H), 4.95 (dd, *J* ¼ 7.6, 6.1 Hz, 1H), 3.60 (dt, *J* ¼ 10.0, 2.0 Hz, 1H), 3.47e3.34 (m, 1H), 2.30e2.09 (m, 3H), 1.97 (ddd, *J* ¼ 4.1, 6.1, 2.1 Hz, 2H), 1.87 (dt, *J* ¼ 13.2, 3.5 Hz, 1H), 1.82e1.36 (m, 10H), 1.27e0.88 (m, 9H), 0.73 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$/CD$_3$OD) d 163.8, 148.3, 141.4, 138.0, 123.0, 121.7, 120.9, 74.9, 74.5, 71.6, 56.8, 56.6, 50.7, 44.2, 42.8, 42.2, 39.9, 37.7, 36.9, 32.3, 32.2, 31.5, 25.8, 24.9, 21.3, 19.6, 12.4. ESI-HRMS [M þ Na]^þ calcd for C$_{27}$H$_{40}$NO$_3$: 426.30027; found: 426.30002.

5.1.2.19. *3b-hydroxy-21-(hydroxy-pyridin-2-yl-methyl)-pregn-5-en-20-ol (14b).* This compound was prepared according to the procedure described for compound 14a starting from intermediate 13b. White solid (41 mg, 64% yield); mp 200e203 °C. [a]$^{20}_D$ ¼ —22.55° (c ¼ 1.0, CHCl$_3$/MeOH 1:1). ^{1}H NMR (400 MHz, CD$_3$OD) d 8.45 (ddd, *J* ¼ 5.0, 1.8, 0.9 Hz, 1H), 7.82 (dt, *J* ¼ 7.7, 1.8 Hz, 1H), 7.56 (dt, *J* ¼ 8.0, 1.2 Hz, 1H), 7.28 (ddd, *J* ¼ 7.5, 4.9, 1.2 Hz, 1H), 5.34 (dd, *J* ¼ 4.5, 2.8 Hz, 1H), 5.00 (dd, *J* ¼ 10.0, 2.6 Hz, 1H), 3.86 (dt, *J* ¼ 10.1, 2.1 Hz, 1H), 3.50e3.35 (m,1H), 2.27e2.15 (m, 3H), 2.01e1.94

(m, 1H), 1.88 (dt, J ¼ 13.3, 3.5 Hz, 1H), 1.83e1.71 (m, 2H), 1.71e1.36 (m, 9H), 1.32e0.91 (m, 9H), 0.82 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$/CD$_3$OD) d 164.7, 148.3, 141.5, 138.1, 122.8, 121.9, 120.9, 71.7, 71.3, 71.1, 56.8, 56.8, 50.8, 45.0, 43.0, 42.3, 40.1, 37.8, 37.0, 32.4, 32.3, 31.6, 25.9, 25.0, 21.4, 19.7, 12.5. ESI-HRMS [M þ H][þ] calcd for C$_{27}$H$_{40}$NO$_3$: 426.30027; found: 426.30032.

5.1.2.20. *(20S)-3***b***-benzoyloxy-21-(bis(trifluoromethyl)hydroxymethyl)-pregn-5-en-20-ol (16).* A solution of ketone 15 (0.586 g, 1 mmol) in anhydrous EtOAc (20 mL) was hydrogenated (5 atm) at 50 °C in the presence of RuCl(*p*-cymene)[(*S,S*)-Ts-DPEN] (0.13 g, 0.2 mmol) for 72 h. After removing the solvent by distillation under reduced pressure, the crude residue was purified by silica gel flash chromatography (cyclohexane/EtOAc 90:10, as eluent) to give the desired compound 16 (0.5 g, 85% yield) as a white solid. Analytical data of the title compound correspond to those reported in the literature [31].

5.1.2.21. *(20S)-[21-(Bis(trifluoromethyl)hydroxymethyl)-20-hydroxy-pregn-5-en-3***b***-yl]* *(tert-butoxycarbonyl)glycinate* *(18a).* HOBt (46 mg, 0.34 mmol), EDC (69 mg, 0.35 mmol), Et$_3$N (86 ml, 0.62 mmol) and DMF (0.1 mL) were added to a solution of 1 (0.15 g, 0.31 mmol) in dry DCM (1 mL). After stirring for 30 min at room temperature N-Boc glycine (65 mg, 0.37 mmol) was added and the resulting mixture was stirred at 60 °C for 72 h. After cooling to room temperature, the reaction mixture was quenched with water (25 mL) and then extracted three times with EtOAc. The combined organic phases were dried (Na$_2$SO$_4$) and concentrated by distillation under reduced pressure to yield a residue that was purified by silica gel flash chromatography (cyclohexane/EtOAc 80:20 as eluent). White powder (32 mg, 16% yield). ^{1}H NMR (400 MHz, acetone-d_6) d: 7.51 (bs, 1H); 6.25 (bt, J ¼ 6.0 Hz, 1H); 5.40 (d, J ¼ 4.0 Hz, 1H); 5.29 (bd, J ¼ 6.0 Hz, 1H); 4.61-4.52 (m, 1H); 4.23-4.16 (m, 1H); 3.78 (d, J ¼ 6.0 Hz, 2H); 2.34-2.25 (m, 3H); 2.07-1.82 (m, 5H); 1.75-1.45 (m, 8H); 1.41 (s, 9H); 1.36-1.08 (m, 5H); 1.05 (s, 3H); 1.01 (ddd, J_1 ¼ 4.0, J_2 ¼ J_3 ¼ 11.0 Hz, 1H); 0.75 (s, 3H). ^{13}C NMR (100 MHz, acetone-d_6) d: 169.6, 155.9, 139.7, 125.5 (q, 2C, J ¼ 267.0 Hz), 122.2, 78.3, 76.1 (q, 1C, J ¼ 28.5 Hz), 74.1, 71.1, 57.4, 56.4, 50.0, 42.3, 41.4, 39.0, 37.9, 36.8, 36.4, 33.2, 31.6, 31.4, 27.7, 27.6, 25.1, 23.6, 20.6, 18.7, 11.8. ESI-MS (*m/z*): 640.4 [M — H]$^-$.

5.1.2.22. *(20R)-[21-(Bis(trifluoromethyl)hydroxymethyl)-20-hydroxy-pregn-5-en-3***b***-yl]* *(tert-butoxycarbonyl)glycinate* *(18b).* This compound was prepared according to the procedure described for 18a starting from intermediate 17. White powder (52 mg, 26% yield). ^{1}H NMR (400 MHz, acetone-d_6) d: 7.44 (bs, 1H); 6.25 (bt, J ¼ 6.0 Hz, 1H); 5.39 (d, J ¼ 4.0 Hz, 1H); 5.05 (bd, J ¼ 8.0 Hz, 1H); 4.60-4.52 (m, 1H); 4.22-4.14 (m, 1H); 3.77 (d, J ¼ 6.0 Hz, 2H); 2.32 (d, J ¼ 8.0 Hz, 2H); 2.16 (ddd, J_1 ¼ J_2 ¼ 3.5, J_3 ¼ 12.5 Hz, 1H); 2.06-1.96 (m, 3H); 1.91 (ddd, J_1 ¼ J_2 ¼ 3.5, J_3 ¼ 12.5 Hz, 1H); 1.86-1.80 (m, 1H); 1.79-1.45 (m, 8H); 1.41 (s, 9H); 1.36-1.07 (m, 5H); 1.05 (s, 3H); 1.01 (ddd, J_1 ¼ 5.0, J_2 ¼ J_3 ¼ 11.5 Hz, 1H); 0.85 (s, 3H). ^{13}C NMR (100 MHz, acetone-d_6) d: 169.6, 155.9, 139.7, 123.9 (q, 2C, J ¼ 286.0 Hz), 122.2, 78.4, 76.6 (q, 1C, J ¼ 28.5 Hz), 74.1, 71.7, 56.7, 56.0, 50.1, 42.4, 42.3, 39.1, 37.9, 36.9, 36.5, 32.9, 31.7, 31.6, 27.7, 27.6, 25.0, 24.2, 20.7, 18.8, 11.6. ESI-MS (*m/z*): 640.4 [M — H]$^-$.

5.1.2.23. *(20S)-[21-(Bis(trifluoromethyl)hydroxymethyl)-20-hydroxy-pregn-5-en-3***b***-yl] glycinate 2,2,2-trifluoroacetate salt (19a).* TFA (62 ml, 0.8 mmol) was added dropwise to a solution of 18a (0.032 g, 0.05 mmol) in DCM (0.4 mL) and the resulting mixture was stirred at room temperature for 3 h. After removing the solvent by distillation under reduced pressure, the crude solid residue was triturated with ether/hexane and filtered. Pink solid (23 mg, 95% yield); [a]$_D^{20}$e21.5° (c: 0.30, MeOH). ^{1}H NMR (400 MHz, DMSO-d_6) d:

8.24 (bs, 3H); 7.94 (bs, 1H); 5.94 (bs, 1H); 5.38 (d, J ¼ 3.5 Hz, 1H); 4.63-4.55 (m, 1H); 3.92 (dd, J_1 ¼ J_2 ¼ 9.5 Hz, 1H); 3.80 (s, 2H); 2.35-2.28 (m, 2H); 2.10 (d, J ¼ 15.0 Hz, 1H); 1.97-1.69 (m, 5H); 1.66-1.35 (m, 8H); 1.23-1.02 (m, 5H); 0.98 (s, 3H); 0.94 (ddd, J_1 ¼ 4.0, J_2 ¼ J_3 ¼ 11.0 Hz, 1H); 0.63 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) d: 167.1, 158.0 (q, 1C, J ¼ 29.0 Hz), 139.1, 123.6 (q, 2C, J ¼ 286.5 Hz), 123.0 (q, 1C, J ¼ 283.0 Hz), 122.5, 75.9 (q, 1C, J ¼ 28.0 Hz), 75.2, 69.1, 56.7, 55.9, 49.3, 40.9, 38.5, 37.5, 36.3, 36.1, 33.6, 31.2, 31.0, 27.2, 26.4, 24.9, 23.5, 20.3, 18.9, 12.1. ESI-HRMS [M-CF$_3$COO$^-$][þ] calcd for C$_{26}$H$_{38}$F$_6$NO$_4$: 542.2700; found: 542.2613.

5.1.2.24. *(20R)-[21-(Bis(trifluoromethyl)hydroxymethyl)-20-hydroxy-pregn-5-en-3***b***-yl] glycinate 2,2,2-trifluoroacetate salt (19b).* This compound was prepared according to the above described procedure for 19a starting from intermediate 18b. White solid (24 mg, 98% yield); mp 218e221 °C (dec). [a]$_D^{30}$ -17.5° (c: 0.31, MeOH). ^{1}H NMR (400 MHz, DMSO-d_6) d: 8.28 (bs, 3H); 7.88 (bs, 1H); 5.75 (bs, 1H); 5.37 (d, J ¼ 3.5 Hz, 1H); 4.63-4.55 (m, 1H); 3.91 (dd, J ¼ 7.5 Hz, 1H); 3.80 (s, 2H); 2.33-2.31 (m, 2H); 2.10-2.04 (m, 1H); 1.99-1.80 (m, 5H); 1.67-1.31 (m, 8H); 1.23-1.03 (m, 5H); 0.99 (s, 3H); 0.94 (ddd, J_1 ¼ 4.0, J_2 ¼ J_3 ¼ 11.0 Hz, 1H); 0.73 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) d: 167.5, 158.5 (q, 1C, J ¼ 34.0 Hz), 139.6, 124.1 (q, 2C, J ¼ 260.0 Hz), 123.4 (q, 1C, J ¼ 286.5 Hz), 122.9, 76.3 (q, 1C, J ¼ 26.5 Hz), 75.6, 70.2, 56.5, 56.0, 49.9, 42.4, 39.1, 37.9, 36.8, 36.6, 34.0, 31.8, 31.7, 27.6, 26.8, 25.3, 24.5, 20.8, 19.4, 12.1. ESI-MS [M — H]$^-$ calcd for C$_{26}$H$_{36}$F$_6$NO$_4$: 540.2. ESI-HRMS [M-CF$_3$COO$^-$][þ] calcd for C$_{26}$H$_{38}$F$_6$NO$_4$: 542.2700; Found 542.2618.

5.1.2.25. *(20S)-3***b***-tosyloxy-21-(bis(trifluoromethyl)hydroxymethyl)-pregn-5-en-20-ol (20a).* *p*-Toluenesulfonyl chloride (0.06 g, 0.31 mmol) was added to a solution of 1 (0.102 g, 0.21 mmol) in dry DCM (1 mL) and dry pyridine (90 ml) and the resulting mixture was heated at 40 °C for 72 h. After cooling to room temperature, the reaction mixture was diluted with DCM (35 mL) and the organic phase washed with 2 N HCl (2 x) and once with water. The organic phase was dried (Na$_2$SO$_4$) and concentrated by distillation under reduced pressure to yield 20a as a crude residue that was used for next step without any further purification. ^{1}H NMR (400 MHz, CDCl$_3$) d: 7.80 (d, J ¼ 8.0 Hz, 2H); 7.34 (d, J ¼ 8.0 Hz, 2H); 6.40 (bs, 1H); 5.32-5.31 (m, 1H); 4.37-4.29 (m, 1H); 4.20 (dd, J ¼ 9.5 Hz, 1H); 2.49-2.37 (m, 1H); 2.46 (s, 3H); 2.31 (ddd, J_1 ¼ 2.0, J_2 ¼ 5.5, J_3 ¼ 13.5 Hz, 1H); 2.22 (d, J ¼ 15.0 Hz, 1H); 2.00-1.64 (m, 8H); 1.61-1.40 (m, 5H); 1.29-1.15 (m, 3H); 1.08-1.00 (m, 2H); 0.97 (s, 3H); 0.90 (ddd, J_1 ¼ 5.0, J_2 ¼ J_3 ¼ 12.0 Hz, 1H); 0.69 (s, 3H).

5.1.2.26. *(20R)-3***b***-tosyloxy-21-(bis(trifluoromethyl)hydroxymethyl)-pregn-5-en-20-ol (20b).* This compound was prepared according to the procedure described for 20a starting from intermediate 17. ^{1}H NMR (400 MHz, CDCl$_3$) d: 7.78 (d, J ¼ 8.0 Hz, 2H); 7.33 (d, J ¼ 8.0 Hz, 2H); 6.53 (bs, 1H); 5.30-5.28 (m, 1H); 4.36-4.28 (m, 1H); 4.18 (dd, J ¼ 9.5 Hz, 1H); 2.48-2.41 (m, 1H); 2.45 (s, 3H); 2.31-2.25 (m, 1H); 2.04-1.89 (m, 4H); 1.81-1.61 (m, 5H); 1.54-1.36 (m, 5H); 1.32-1.09 (m, 3H); 1.05-1.00 (m, 2H); 0.96 (s, 3H); 0.92-0.83 (m, 1H); 0.78 (s, 3H).

5.1.2.27. *(20S)-3***b***-(2-hydroxyethoxy)-21-(bis(trifluoromethyl)hydroxymethyl)-pregn-5-en-20-ol (21a).* Anhydrous ethylene glycol (180 ml, 3.25 mmol) was added to a solution of crude 20a (83 mg, 0.13 mmol) in dry dioxane (0.3 mL) and the resulting mixture was refluxed for 4 h under a nitrogen atmosphere. After cooling to room temperature, the solvent was removed by distillation under reduced pressure, and the residue was taken up in DCM, washed once with water, once with a saturated aqueous NaHCO$_3$ solution and finally with brine. After drying over Na$_2$SO$_4$, the solvent was removed by distillation under reduced pressure to give a crude residue that was purified by silica gel flash chromatography

(cyclohexane/EtOAc 60:40, as eluent). White solid (18 mg, 26% yield); mp 144e146 °C. $[a]_D^{20}$e32.5° (c: 0.33, CH$_2$Cl$_2$). ^{1}H NMR (400 MHz, CDCl$_3$) d: 6.43 (bs, 1H); 5.36-5.34 (m, 1H); 4.20 (dd, J_1 ¼ J_2 ¼ 9.5 Hz, 1H); 3.73-3.71 (m, 2H); 3.60-3.57 (m, 2H); 3.24-3.16 (m, 1H); 2.38 (ddd, J_1 ¼ 2.5, J_2 ¼ 4.5, J_3 ¼ 13.0 Hz, 1H); 2.24-2.16 (m, 2H); 2.03-1.68 (m, 8H); 1.61-1.40 (m, 5H); 1.26-1.16 (m, 3H); 1.10-1.03 (m, 2H); 1.00 (s, 3H); 0.95 (ddd, J_1 ¼ 5.0, J_2 ¼ J_3 ¼ 12.5 Hz, 1H); 0.70 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$) d: 140.7, 123.5 (q, 2C, J ¼ 286.5 Hz), 121.3, 79.3, 76.7 (q, 1C, J ¼ 29.0 Hz), 71.9, 68.9, 62.1, 57.3, 56.4, 49.9, 41.7, 39.0, 38.9, 37.1, 36.8, 33.6, 31.7, 31.4, 28.3, 24.7, 23.9, 20.7, 19.3, 12.5. ESI-MS (m/z): 527.2 [M — H]⁻. ESI-HRMS [M þ NH$_4$]þ calcd for C$_{26}$H$_{42}$F$_6$NO$_4$: 546.3012; found: 546.3248.

5.1.2.28.   (20R)-3b-(2-hydroxyethoxy)-21-(bis(trifluoromethyl) hydroxymethyl)-pregn-5-en-20-ol (21b). This compound was prepared according to the procedure described for 21a starting from intermediate 20b. White solid (23 mg, 34% yield); mp 197-8 °C. $[a]_D^{20}$e24.1° (c: 0.49, CH$_2$Cl$_2$). ^{1}H NMR (400 MHz, CDCl$_3$) d: 6.35 (bs, 1H); 5.36-5.34 (m, 1H); 4.22 (dd, J ¼ 10.0 Hz, 1H); 3.73-3.70 (m, 2H); 3.60-3.58 (m, 2H); 3.24-3.16 (m, 1H); 2.38 (ddd, J_1 ¼ 2.0, J_2 ¼ 4.5, J_3 ¼ 13.0 Hz, 1H); 2.25-2.17 (m, 1H); 2.07-1.84 (m, 6H); 1.73-1.66 (m, 2H); 1.61-1.42 (m, 5H); 1.39-1.32 (m, 1H); 1.28-1.15 (m, 3H); 1.10-1.05 (m, 2H); 1.02 (s, 3H); 0.97 (ddd, J_1 ¼ 5.0, J_2 ¼ J_3 ¼ 11.0 Hz, 1H); 0.82 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$) d: 140.9, 123.7 (q, 2C, J ¼ 286.0 Hz), 121.5, 79.5, 77.3 (q, 1C, J ¼ 29.0 Hz), 73.0, 69.1, 62.3, 57.4, 56.2, 50.1, 42.6, 40.4, 39.2, 37.3, 37.0, 33.8, 31.9, 31.8, 28.5, 25.3, 24.6, 21.1, 19.6, 12.8. ESI-MS (m/z): 527.2 [M — H]⁻. ESI-HRMS [M þ NH$_4$]þ calcd for C$_{26}$H$_{42}$F$_6$NO$_4$: 546.3012; found: 546.2934.

5.1.2.29. (20S)-21-(Bis(trifluoromethyl)hydroxymethyl)-20-hydroxy-pregn-5-en-3-one (22a). Dess-Martin periodinane (0.175 g, 0.41 mmol) was added to a stirred solution of 1 (0.101 g, 0.21 mmol) in DCM (3 mL) and EtOAc (1 mL), and the resulting mixture was stirred at room temperature for 30 min. A saturated NaHCO$_3$ aqueous solution was added and the aqueous phase was extracted three times with DCM. The combined organic phases were washed once with brine, dried (Na$_2$SO$_4$) and concentrated by distillation under reduced pressure to afford a crude residue that was purified by silica gel flash chromatography (cyclohexane/EtOAc 80:20 as eluent). White solid (61 mg, 64% yeld); mp 175-6 °C. $[a]_D^{20}$ -5.6° (c: 0.29, MeOH). ^{1}H NMR (400 MHz, CD$_3$OD) d: 5.27-5.25 (m, 1H); 3.96 (dd, J ¼ 10.0 Hz, 1H); 3.34-3.28 (m, 1H); 2.64 (dd, J_1 ¼ 2.0, J_2 ¼ 16.0 Hz, 1H); 2.49 (ddd, J_1 ¼ 6.0, J_2 ¼ J_3 ¼ 14.5 Hz, 1H); 2.16-2.09 (m, 2H); 2.04-1.91 (m, 2H); 1.87-1.77 (m, 2H); 1.73 (ddd, J_1 ¼ J_2 ¼ 3.5, J_3 ¼ 12.5 Hz, 1H); 1.64-1.43 (m, 5H); 1.41-1.29 (m, 2H); 1.24-0.91 (m, 5H); 1.14 (s, 3H); 0.65 (s, 3H). ^{13}C NMR (100 MHz, CD$_3$OD) d: 211.3, 139.0, 123.8 (q, 2C, J ¼ 285.5 Hz), 122.0, 76.4 (q, 1C, J ¼ 28.5 Hz), 70.3, 70.2, 57.5, 56.3, 49.1, 41.3, 38.9, 37.0, 36.8, 36.7, 33.5, 31.5, 31.4, 25.0, 23.4, 20.8, 18.0, 11.4. ESI-MS (m/z): 481.3 [M — H]⁻. ESI-HRMS [M þ H]þ calcd for C$_{24}$H$_{33}$F$_6$O$_3$: 483.2334; found: 483.2334.

5.1.2.30. (20R)-21-(Bis(trifluoromethyl)hydroxymethyl)-20-hydroxy-pregn-5-en-3-one (22b). This compound was prepared according to the procedure described for 8a starting from intermediate 17. White solid (71 mg, 69% yield); mp 170-3 °C. $[a]_D^{20}$ -5.0° (c: 0.22, MeOH). ^{1}H NMR (400 MHz, CD$_3$OD) d: 5.37-5.34 (m, 1H); 4.08 (dd, J ¼ 10.0 Hz, 1H); 3.43-3.38 (m, 1H); 2.74 (dd, J_1 ¼ 2.0, J_2 ¼ 16.0 Hz, 1H); 2.59 (ddd, J_1 ¼ 6.0, J_2 ¼ J_3 ¼ 14.5 Hz, 1H); 2.26-2.19 (m, 1H); 2.16-1.89 (m, 5H); 1.74-1.66 (m, 2H); 1.64-1.39 (m, 6H); 1.34-1.02 (m, 5H); 1.24 (s, 3H); 0.85 (s, 3H). ^{13}C NMR (100 MHz, CD$_3$OD) d: 211.4, 139.0, 123.8 (q, 2C, J ¼ 285.5 Hz), 122.0, 76.7 (q, 1C, J ¼ 28.5 Hz), 70.7, 69.0, 56.5, 56.0, 49.4, 42.3, 39.0, 37.0, 36.9, 36.8, 33.4, 31.7, 31.5, 25.0, 24.1, 20.9, 18.0, 11.1. ESI-MS (m/z): 481.3 [M — H]⁻. ESI-HRMS [MþH]þ calcd for C$_{24}$H$_{33}$F$_6$O$_3$: 483.2334;

found: 483.2384.

5.1.2.31. (E/Z)-(20S)-21-(Bis(trifluoromethyl)hydroxymethyl)-20-hydroxy-pregn-5-en-3-one oxime ((E/Z)-23a). Et$_3$N (72 ml, 0.52 mmol) was added to a solution of hydroxylamine HCl (0.029 g, 0.41 mmol) in dry DCM (0.5 mL). After stirring at room temperature for 45 min a solution of 22a (0.050 g, 0.10 mmol) in dry DCM (1 mL) was added dropwise to the mixture and the stirring was continued for 2 h at room temperature. Water was added and the aqueous phase was extracted three times with DCM. The combined organic layers were dried (Na$_2$SO$_4$) and concentrated by distillation under reduced pressure to afford a residue that was purified by silica gel flash chromatography (DCM/MeOH 98:2 as eluent). The title compound 23a was obtained in 90% yield (E/Z-mixture, 1:1 ratio by ^{1}H NMR) as white solid. ^{1}H NMR (400 MHz, acetone-d_6) d: 9.19 (bs, 1H); 9.17 (bs, 1H); 7.39 (bs, 2H); 5.26-5.25 (m, 1H); 5.22-5.20 (m, 1H); 5.18-5.16 (m, 2H); 4.10-4.05 (m, 2H); 3.57 (dd, J_1 ¼ 1.5, J_2¼ 16.5, 1H); 3.07-3.02 (m, 1H); 2.97-2.91 (m, 1H); 2.65-2.60 (dd, J 1¼ 2.5, J_2 ¼ 16.5 Hz, 1H); 2.55 (dd, J_1¼ 1.5, J_2¼ 15.5 Hz, 1H); 2.18-2.14 (m, 3H); 2.10-2.04 (m, 1H); 1.95-1.77 (m, 6H); 1.74-1.70 (m, 2H); 1.63-1.36 (m, 15H); 1.27-0.85 (m, 12H); 1.01 (s, 3H); 1.00 (s, 3H); 0.63 (s, 6H). ^{13}C NMR (100 MHz, acetone-d_6) d: 157.0, 156.5, 140.5, 139.4, 124.0 (q, 2C, J¼ 286.0 Hz), 122.9 (q, 2C, J ¼ 283.5 Hz), 121.3, 120.6, 76.6 (q, 1C, J¼ 28.5 Hz), 71.1, 71.0, 57.4, 56.4, 49.6, 49.5, 41.4, 39.0, 38.1, 37.5, 37.45, 37.42, 37.0, 33.2, 31.5, 31.5, 31.4, 30.6, 27.3, 25.14, 25.13, 23.6, 20.7, 20.6, 20.1, 18.4, 18.3, 11.8. ESI-MS (m/z): 496.2 [M — H]⁻. ESI-HRMS [MþH]þ calcd for C$_{24}$H$_{34}$F$_6$NO$_3$: 498.2443; found: 498.2400.

5.1.2.32. (E/Z)-mixture of (20R)-21-(bis(trifluoromethyl)hydroxymethyl)-20-hydroxy-pregn-5-en-3-one oxime (23b and 23c). These compounds were prepared according to the procedure described for 23a starting from intermediate 22b. The crude (E/Z)-oxime mixture was purified by silica gel flash chromatography (cyclohexane/EtOAc 70:30 as eluent) to give the (E)-23b and (Z)-23c stereoisomers. (E)-23b: white solid (25 mg, 40% yield); mp 196e200 °C (dec). $[a]_D^{20}$e29.2° (c: 0.19, MeOH). ^{1}H NMR (400 MHz, acetone-d_6) d: 9.29 (bs, 1H); 7.45 (bs, 1H); 5.35-5.34 (m, 1H,); 5.05 (bd, J ¼ 8.0 Hz, 1H); 4.23-4.16 (m, 1H); 3.19-3.14 (m, 1H); 3.07 (dd, J_1 ¼ 2.5, J_2 ¼ 15.5 Hz, 1H); 2.67 (dd, J_1 ¼ 1.5, J_2 ¼ 15.5 Hz, 1H); 2.17 (ddd, J_1 ¼ J_2 ¼ 3.5, J_3 ¼ 12.5 Hz, 1H); 2.08-1.88 (m, 4H); 1.83-1.48 (m, 6H); 1.37-0.99 (m, 8H); 1.14 (s, 3H); 0.88 (s, 3H). ^{13}C NMR (100 MHz, acetone-d_6) d: 157.0, 140.6, 123.9 (q, 2C, J ¼ 285.5 Hz), 120.6, 76.3 (q, 1C, J ¼ 28.5 Hz), 71.7, 56.7, 56.0, 49.7, 42.4, 39.2, 37.5, 37.4, 37.0, 32.9, 31.7, 31.6, 25.0, 24.2, 20.8, 20.1, 18.3, 11.6. ESI-MS (m/z): 496.1 [M — H]⁻. ESI-HRMS [Mþ H]þ calcd for C$_{24}$H$_{34}$F$_6$NO$_3$: 498.2443; found: 498.2439. (Z)-23c: white solid; (24 mg, 38% yield); mp 198e201 °C (dec). $[a]_D^{20}$ þ15.8° (c: 0.18, MeOH) ^{1}H NMR (400 MHz, acetone-d_6) d: 9.15 (bs, 1H); 7.31 (bs, 1H); 5.26-5.25 (m, 1H); 4.90-4.87 (m, 1H); 4.11-4.04 (m, 1H); 3.56 (dd, J_1 ¼ 1.5, J_2 ¼ 16.5 Hz, 1H); 2.60 (dd, J_1 ¼ 2.0, J_2 ¼ 16.5 Hz, 1H); 2.16 (ddd, J_1 ¼ 5.0, J_2 ¼ J_3 ¼ 13.5 Hz, 1H); 2.09-2.02 (m, 2H); 1.95-1.79 (m, 3H); 1.70-1.34 (m, 6H); 1.24-0.87 (m, 8H); 1.01 (s, 3H); 0.75 (s, 3H). ^{13}C NMR (100 MHz, acetone-d_6) d: 156.5, 139.4, 123.9 (q, 2C, J ¼ 286.0 Hz), 121.3, 76.9 (q, 1C, J ¼ 28.5 Hz), 71.7, 56.7, 56.0, 49.7, 42.4, 39.2, 38.1, 37.5, 32.9, 31.7, 31.6, 30.6, 27.3, 25.0, 24.2, 20.7, 18.4, 11.6. ESI-MS (m/z): 496.1 [M — H]⁻. ESI-HRMS [MþH]þ calcd for C$_{24}$H$_{34}$F$_6$NO$_3$: 498.2443; found: 498.2441.

5.1.2.33. (20S)-21-(Bis(trifluoromethyl)hydroxymethyl)-5a-pregnan-3b,20-diol (24a). A solution of compound 1 (0.048 g, 0.1 mmol) in EtOH (0.5 mL) was hydrogenated (3 atm) over 10% Pd-C (5 mg) at room temperature for 12 h. The catalyst was removed by filtration over Celite and the filtrate was concentrate under reduced pressure to afford a crude residue that was purified by silica gel flash chromatography (EtOAc as eluent). White solid (48 mg, quantitative

yield); mp 229e235 °C. [a]$_D^{20}$ ¼ þ7.94° (c ¼ 1.3, CHCl$_3$/CH$_3$OH 1/1). ^{1}H NMR (300 MHz, CD$_3$OD/CDCl$_3$) d 4.03 (t, J ¼ 10.1 Hz, 1H), 3.50 (m, 1H), 2.17 (dd, J ¼ 15.0, 2.0 Hz, 1H), 1.87 (m, 2H), 1.76e0.88 (m, 21H), 0.80 (s, 3H), 0.69e0.59 (m, 4H). ^{13}C NMR (75 MHz, CD$_3$OD) d 129.8 (d, J ¼ 64.9 Hz), 126.0 (d, J ¼ 62.4 Hz), 122.2 (d, J ¼ 60.3 Hz), 118.4 (d, J ¼ 57.9 Hz), 71.5, 71.4, 58.5, 57.1, 55.0, 45.6, 42.4, 40.2, 38.3, 37.7, 36.2, 35.9, 34.1, 32.7, 31.6, 29.3, 26.1, 24.4, 21.7, 12.8, 12.6. ESI-HRMS [MþH]$^+$ calcd for C$_{24}$H$_{35}$F$_6$O$_3$: 485.24959; found: 485.24924.

*5.1.2.34. (20R)-21-(Bis(trifluoromethyl)hydroxymethyl)-5**a**-pregnan-3**b**,20-diol (24b).* This compound was prepared according to the procedure described for 24a starting from intermediate 17. White solid (47 mg, 98% yield); mp 199e202 °C. [a]$_D^{20}$ ¼ þ8.33° (c ¼ 1.0, CHCl$_3$/MeOH 1:1). ^{1}H NMR (400 MHz, CD$_3$OD/CDCl$_3$) d 4.15e4.02 (m, 1H), 3.53 (tt, J ¼ 11.1, 4.7 Hz, 1H), 2.07 (dt, J ¼ 12.6, 3.3 Hz, 1H), 2.05e1.86 (m, 2H), 1.80 -1.64 (m, 5H), 1.60e0.89 (m, 16H), 0.86 (s, 3H), 0.80 (s, 3H), 0.70 (ddd, J ¼ 12.1, 10.3, 4.1 Hz, 1H). ^{13}C NMR (75 MHz, CD$_3$OD) d 125.9, 125.2, 122.1, 121.4, 71.6, 71.1, 57.2, 56.2, 54.7, 45.2, 43.0, 39.8, 37.9, 37.3, 35.7, 33.5, 32.4, 31.2, 28.9, 25.6, 24.6, 21.3, 12.4. ESI-HRMS [M þ H]$^+$ calcd for C$_{24}$H$_{35}$F$_6$O$_3$: 485.24959; found: 485.24973.

5.1.2.35. (20S)-21-(Bis(trifluoromethyl)hydroxymethyl)-20-hydroxy-pregnan-3-one (25a). This compound was prepared according to the procedure described for compound 22a starting from intermediate 24a. White solid (66 mg, 65% yield); mp 200-1 °C. [a]$_D^{20}$ þ10.9° (c: 0.16, MeOH). ^{1}H NMR (400 MHz, CD$_3$OD) d: 4.06 (dd, J$_1$ ¼ J$_2$ ¼ 9.5 Hz, 1H); 2.50 (ddd, J$_1$ ¼ 5.0, J$_2$ ¼ J$_3$ ¼ 14.5 Hz, 1H); 2.39 (dd, J$_1$ ¼ J$_2$ ¼ 14.5 Hz, 1H); 2.26-2-19 (m, 2H); 2.10-2.01 (m, 2H); 1.96-1.90 (m, 2H); 1.81-1.12 (m,15H); 1.08 (s, 3H), 1.02-0.95 (m, 1H); 0.87-0.79 (m, 1H), 0.74 (s, 3H). ^{13}C NMR (100 MHz, CD$_3$OD) d: 213.3, 123.8 (q, 1C, J ¼ 285.5 Hz), 123.0 (q, 1C, J ¼ 283.5 Hz), 76.7 (q, 1C, J ¼ 28.5 Hz), 70.3, 57.6, 56.1, 53.6, 46.7, 44.0, 41.5, 39.2, 38.3, 37.5, 35.4, 35.0, 33.4, 31.5, 28.6, 25.0, 23.4, 20.9, 11.5, 10.3. ESI-MS (m/z): 483.2 [M—H]$^-$. ESI-HRMS [MþH]$^+$ calcd for C$_{24}$H$_{35}$F$_6$O$_3$: 485.2490; found: 485.2433.

5.1.2.36. (20R)-21-(Bis(trifluoromethyl)hydroxymethyl)-20-hydroxy-pregnan-3-one (25b). This compound was prepared according to the procedure described for compound 22a starting from intermediate 24b. White solid (61 mg, 60% yield); mp 180-1 °C. [a]$_D^{20}$ þ27.3° (c: 0.17, MeOH). ^{1}H NMR (400 MHz, CD$_3$OD) d: 4.08 (dd, J$_1$ ¼ J$_2$ ¼ 10.0 Hz, 1H); 2.51 (ddd, J$_1$ ¼ 7.0, J$_2$ ¼ J$_3$ ¼ 14.5 Hz, 1H); 2.39 (dd, J$_1$ ¼ J$_2$ ¼ 14.5 Hz, 1H); 2.27-2.21 (m, 1H); 2.13-1.90 (m, 5H); 1.78-1.66 (m, 3H); 1.63-1.12 (m, 12H); 1.09 (s, 3H), 1.04-0.94 (m, 1H); 0.84 (s, 3H), 0.82-0.77 (m, 1H). ^{13}C NMR (100 MHz, CD$_3$OD) d: 213.4, 123.4 (q, 1C, J ¼ 285.5 Hz), 76.7 (q, 1C, J ¼ 28.5 Hz), 70.7, 56.7, 55.7, 53.8, 46.7, 44.1, 42.5, 39.2, 38.4, 37.5, 35.4, 35.2, 33.3, 31.6, 28.6, 25.0, 24.0, 21.0, 11.2, 10.3. ESI-MS (m/z): 483.2 [M — H]$^-$. ESI-HRMS [MþH]$^+$ calcd for C$_{24}$H$_{35}$F$_6$O$_3$: 485.2490; found: 485.2490.

*5.1.2.37. (20R)-21-(Bis(trifluoromethyl)hydroxymethyl)-pregn-5-en-20-ol-3**b**-(2,3,4,6-tetra-O-acetyl)-**b**-D-glucopyranoside (26).* Compound 17 (117 mg, 0.24 mmol) and (2,3,4,6-tetra-O-acetyl-a,b-D-glucopyranosyl)trichloroacetimidate (180 mg, 0.36 mmol) were dissolved in anhydrous DCM (20 mL) and cooled to —25 °C under nitrogen atmosphere. A solution of TMSOTf in DCM (0.055 M, 440 mL, 0.024 mmol) was added and the reaction mixture was stirred for 15 min at 0 °C, then triethylamine (0.20 mL, 1.43 mmol) was added. The mixture was diluted with DCM and washed with water. The organic layer was washed with brine and dried over Na$_2$SO$_4$ and the solvents were removed under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc 3:1) to afford the title compound (30 mg, 15% yield) as a white solid. ^{1}H NMR (400 MHz, CDCl$_3$) d 6.34 (s, 1H), 5.35 (dd,

J ¼ 4.9, 2.6 Hz, 1H), 5.20 (t, J ¼ 9.5 Hz, 1H), 5.08 (t, J ¼ 9.7 Hz, 1H), 4.95 (dd, J ¼ 9.6, 8.0 Hz, 1H), 4.59 (d, J ¼ 7.9 Hz, 1H), 4.30e4.17 (m, 2H), 4.11 (dd, J ¼ 12.2, 2.5 Hz, 1H), 3.67 (ddd, J ¼ 9.9, 4.8, 2.5 Hz, 1H), 3.48 (tt, J ¼ 11.2, 4.8 Hz, 1H), 2.31e2.13 (m, 2H), 2.10e1.81 (m, 15H), 1.79e1.40 (m, 10H), 1.40e1.11 (m, 7H), 1.00 (s, 3H), 0.81 (s, 3H).

*5.1.2.38. (20R)-21-(Bis(trifluoromethyl)hydroxymethyl)-pregn-5-en-20-ol-3**b**-D-D-glucopyranoside (27b).* Compound 26 (30 mg, 0.04 mmol) was dissolved in MeOH (3 mL) and sodium (5 mg, 0.2 mmol) was added. The mixture was stirred for 6h at room temperature, then HCl 1 M (0.2 mL) was added and the mixture was diluted with EtOAc and washed with water. The organic layer was washed with brine and dried over Na$_2$SO$_4$ and the solvents were removed under reduced pressure. The residue was purified by silica gel column chromatography (CHCl$_3$/MeOH 9:1) affording the title compound as white solid (13 mg, 50% yield); mp 210 °C (dec.). [a]$_D^{20}$ ¼ —56.25° (c ¼ 0.03, CHCl$_3$/MeOH 1:1). ^{1}H NMR (300 MHz, CDCl$_3$/CD$_3$OD) d 5.33 (d, J ¼ 5.0 Hz, 1H), 4.06 (t, J ¼ 9.9 Hz, 1H), 3.81 (dd, J ¼ 12.0, 3.0 Hz, 1H), 3.70 (dd, J ¼ 11.9, 4.9 Hz, 1H), 3.64e3.48 (m, 1H), 3.48e3.33 (m, 2H), 3.22 (m, J ¼ 17.0, 9.2, 4.6, 2.2 Hz, 2H), 2.45e2.31 (m, 1H), 2.23 (t, J ¼ 12.4 Hz, 1H), 2.11e1.75 (m, 6H), 1.75e1.32 (m, 8H), 1.31e0.80 (m, 11H), 0.80 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$/CD$_3$OD) d 141.0, 125.7, 124.9, 122.8, 122.2, 101.7, 79.5, 77.1, 76.6, 74.1, 71.7, 70.8, 62.3, 57.2, 56.7, 50.7, 42.9, 39.7, 39.1, 37.8, 37.2, 33.8, 32.4, 32.2, 30.0, 25.8, 24.9, 21.3, 19.6, 12.3. ESI-HRMS [MþNa]$^+$ calcd for C$_{30}$H$_{44}$F$_6$NaO$_8$: 669.28326; found: 669.28381.

*5.1.2.39. 3**b**-pregnenolone-(2,3,4,6-tetra-O-acetyl)-**b**-D-glucopyrano-side (29).* Pregnenolone (28, 3.67 g, 11.60 mmol) was dissolved in anhydrous DCM (70 mL) and (2,3,4,6-tetra-O-acetyl-a,b-D-gluco-pyranosyl)trichloroacetimidate (3.80 g, 7.71 mmol) was added. The solution was cooled to —25 °C and TMSOTf (0.07 mL, 0.39 mmol) was added. The mixture was stirred for 15 min at 0 °C. After addition of TEA (0.2 mL, 1.43 mmol), the mixture was diluted with DCM and washed with water. The organic layer was washed with brine and dried over Na$_2$SO$_4$ and the solvents were removed under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc 85:15) affording the title compound (2.52 g, 34% yield) as a white solid. ^{1}H NMR (400 MHz, CDCl$_3$) d 5.33 (d, J ¼ 5.1 Hz, 1H), 5.18 (t, J ¼ 9.5 Hz, 1H), 5.05 (t, J ¼ 9.7 Hz, 1H), 4.93 (dd, J ¼ 9.6, 7.9 Hz, 1H), 4.57 (d, J ¼ 7.9 Hz, 1H), 4.23 (dd, J ¼ 12.2, 4.8 Hz, 1H), 4.09 (dd, J ¼ 12.2, 2.5 Hz, 1H), 3.66 (ddd, J ¼ 10.2, 4.9, 2.5 Hz, 1H), 3.47 (ddd, J ¼ 11.2, 6.6, 4.5 Hz, 1H), 2.50 (t, J ¼ 8.7 Hz, 1H), 2.31e2.11 (m, 3H), 2.10 (s, 3H), 2.05 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.98 (s, 3H), 1.90e1.80 (m, 3H), 1.71e1.34 (m, 9H), 1.28e0.99 (m, 4H), 0.96 (s, 3H), 0.60 (s, 3H).

*5.1.2.40. 3**b**-pregnenolone-**b**-D-glucopyranoside (30).* Compound 29 (2.52 g, 3.90 mmol) was dissolved in a mixture of MeOH (40 mL) and THF (40 mL). Sodium (70 mg, 3.0 mmol) was added and the mixture was stirred for 1 h at room temperature. HCl 1 M was added until neutralization was achieved. EtOAc (400 mL) was added and the mixture was washed with water. The organic layer was washed with brine and dried over Na$_2$SO$_4$ and the solvents were removed under reduced pressure. The residue was purified by silica gel column chromatography (DCM/MeOH 85:15) affording the title compound (824 mg, 44% yield) as a white solid. ^{1}H NMR (400 MHz, CD$_3$OD) d 5.37 (bs, 1H), 4.38 (d, J ¼ 7.8 Hz, 1H), 3.85 (d, J ¼ 11.9, Hz, 1H), 3.63 (m, 3H), 3.35e3.24 (m, 4H), 3.14 (t, J ¼ 9.1 1H), 2.64 (t, J ¼ 8.8 Hz, 1H), 2.44 (m, 1H), 2.28 (m, 1H), 2.20 -1.85 (m, 10H), 1.76e1.45 (m, 10H), 1.31e0.95 (m, 6H), 0.63 (s, 3H).

*5.1.2.41. 3**b**-pregnenolone-(2,3,4,6-tetra-O-benzoyl)-**b**-D-glucopyr-anoside (31).* Pregnenolone 3b-D-glucopyranoside (30, 824 mg, 1.72 mmol) was dissolved in anhydrous pyridine (10 mL) and

benzoyl chloride (2.50 mL, 21.52 mmol) was added. The mixture was stirred for 20 min at room temperature, then it was diluted with DCM and washed with water. The organic layer was washed with brine and dried over Na_2SO_4 and the solvents were removed under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc 85:15) affording the title compound (1.54 g, quantitative yield) as a white solid. ^{1}H NMR (300 MHz, CDCl3) δ 8.10e7.98 (m, 2H), 7.98e7.79 (m, 6H), 7.79e7.70 (m, 2H), 7.57e7.05 (m, 10H), 5.83 (t, J ¼ 9.6 Hz, 1H), 5.57 (t, J ¼ 9.7 Hz, 1H), 5.43 (dd, J ¼ 9.7, 7.9 Hz, 1H), 5.15 (d, J ¼ 4.8 Hz, 1H), 4.88 (d, J ¼ 7.9 Hz, 1H), 4.54 (dd, J ¼ 12.0, 3.5 Hz, 1H), 4.45 (dd, J ¼ 12.1, 5.7 Hz, 1H), 4.09 (ddd, J ¼ 9.7, 5.8, 3.6 Hz, 1H), 3.47 (tt, J ¼ 10.5, 5.0 Hz, 1H), 2.45 (t, J ¼ 8.7 Hz, 1H), 2.18e1.76 (m, 7H), 1.76e1.24 (m, 7H), 1.24e0.95 (m, 3H), 0.94e0.68 (m, 8H), 0.53 (s, 3H).

*5.1.2.42. 21-(Bis(trifluoromethyl)hydroxymethyl)-pregn-5-en-20-one-3*b*-(2,3,4,6-tetra-O-benzoyl)-*b*-D-glucopyranoside (32).*
Compound 31 (1.22 g, 1.36 mmol) was dissolved in anhydrous THF (30 mL) and the solution was cooled to —78 °C under nitrogen atmosphere. LiHMDS (1 M, 3.40 ml, 3.40 mmol) was added, followed by hexafluoroacetone (2 mL of trihydrated hexafluoroacetone were added dropwise into a flask containing warm concentrated H_2SO_4 and gaseous hexafluoroacetone was delivered to the reaction flask by an overpressure of nitrogen). The mixture was allowed to warm to —50 °C and HCl 1 M (3.4 mL) was added. The mixture was diluted with EtOAc and washed with water. The organic layer was washed with brine and dried over Na_2SO_4 and the solvents were removed under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc 85:15) affording the title compound (1.05 g, 73% yield) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.32e7.13 (m, 20H), 5.99 - 5.92 (m, 1H), 5.55 (t, J ¼ 9.4 Hz, 1H), 5.34e5.26 (m, 2H), 5.19 (s, 1H), 4.59e4.38 (m, 3H), 3.53e3.46 (m, 1H), 3.08 (d, J ¼ 15.5 Hz, 1H), 2.87 (d, J ¼ 15.5 Hz, 1H), 2.78 (t, J ¼ 8.6 Hz, 1H), 2.20 (d, J ¼ 13.2 Hz, 1H), 2.10e1.02 (m, 17H), 0.94e0.77 (m, 5H), 0.54 (s, 3H).

*5.1.2.43. (20S)-21-(Bis(trifluoromethyl)hydroxymethyl)-pregn-5-en-20-ol-3*b*-(2,3,4,6-tetra-O-benzoyl)-*b*-D-glucopyranoside (33a).*
Compound 32 (606 mg, 0.57 mmol) was dissolved in anhydrous THF (10 mL) and Na(OAc)$_3$BH (1.21 g, 5.7 mmol) was added. The mixture was stirred for 1 h at room temperature, then excess Rochelle's salt saturated solution was added and the mixture was stirred for 1 h before being diluted with DCM. The organic layer was washed with water and brine and dried over Na_2SO_4 and the solvents were removed under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc 4:1) affording the title compound (43 mg, 7% yield) and the major product of reduction (20*R*, 468 mg, 77% yield). ^{1}H NMR (400 MHz, CDCl3) δ 8.08e7.79 (m, 7H), 7.60e7.14 (m, 13H), 5.89 (t, J ¼ 9.6 Hz, 1H), 5.63 (t, J ¼ 9.7 Hz, 1H), 5.49 (dd, J ¼ 9.8, 7.9 Hz, 1H), 5.22 (d, J ¼ 5.0 Hz, 1H), 4.94 (d, J ¼ 7.9 Hz, 1H), 4.61 (dd, J ¼ 12.0, 3.4 Hz, 1H), 4.52 (dd, J ¼ 12.0, 5.9 Hz, 1H), 4.28e4.09 (m, 2H), 3.52 (tt, J ¼ 10.3, 4.9 Hz, 1H), 2.28e0.78 (m, 27H), 0.68 (s, 3H).

*5.1.2.44. (20S)-21-(Bis(trifluoromethyl)hydroxymethyl)-pregn-5-en-20-ol-3*b*-*b*-D-glucopyranoside (27a).* Compound 33a (34 mg, 0.03 mmol) was dissolved in MeOH (3 mL) and sodium (1 mg, 0.04 mmol) was added. The mixture was stirred for 2h at room temperature, then HCl 0.1 M (0.4 mL) was added and the mixture was diluted with EtOAc and washed with water. The organic layer was washed with brine and dried over Na_2SO_4 and the solvents were removed under reduced pressure. The residue was purified by silica gel column chromatography (DCM/MeOH 95:5) affording the title compound (12 mg, 62% yield) as a white solid; mp 220 °C.

$[\alpha]_D^{20}$ ¼ —46.67° (c ¼ 0.06, CHCl$_3$/MeOH 1:1). ^{1}H NMR (300 MHz, CDCl$_3$/CD$_3$OD) δ 5.39 (dd, J ¼ 4.6, 2.6 Hz, 1H), 4.40 (d, J ¼ 7.8 Hz, 1H), 4.07 (t, J ¼ 10.0 Hz, 1H), 3.87 (dd, J ¼ 11.9, 1.7 Hz, 1H), 3.75e3.52 (m, 2H), 3.46e3.21 (m, 3H), 3.16 (dd, J ¼ 8.8, 7.8 Hz, 1H), 2.45 (dd, J ¼ 3.3, 4.9 Hz, 1H), 2.38e2.13 (m, 2H), 2.13e1.38 (m, 14H), 1.38e0.80 (m, 8H), 0.71 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$/CD$_3$OD) δ 141.4, 122.4, 102.1, 79.6, 77.6, 77.2, 74.6, 71.5, 71.2, 62.5, 58.5, 57.5, 51.0, 42.3, 40.0, 39.4, 38.1, 37.5, 34.3, 32.6, 32.4, 30.3, 26.1, 24.6, 21.6, 19.7, 12.7. ESI-HRMS [M þ Na][b] calcd for C$_{30}$H$_{44}$F$_6$NaO$_8$: 669.28326; found: 669.28387.

5.2. Cell cultures

KMS-11 cells were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan) cell bank; RPMI8226, U-266, OPM-2 cells from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). All cell lines were maintained at low passage in RPMI1640 medium supplemented with 10% heat inactivated FBS and 2.0 mM glutamine, tested regularly for *Mycoplasma* negativity, and authenticated by PowerPlex Fusion System (Promega, Madison, USA).

5.3. Viable cell counting

Cells were cultured under appropriate conditions for 48 h. Propidium iodide staining (Immunostep, Salamanca, SP, EU) was used to detect PI— viable cells by flow cytometry. Absolute cell counts were obtained by the counting function of the MACSQuant® Analyzer (Miltenyi Biotec). IC$_{50}$ values are the mean of at least 2 experiments performed in triplicate.

5.4. Western blot analysis

Cells were washed in cold PBS and homogenized in NP-40 lysis buffer (1% NP-40, 20 mM TriseHCl pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 мg/mL aprotinin, 10 мg/mL leupeptin). Protein concentration in the supernatants was determined using the Bradford protein assay (Bio-Rad Laboratories). Phosphorylated FGFR3 was detected using rabbit anti-FGFR3 (phospho Y724) antibody (Abcam). b-actin or a-tubulin were used as loading controls (antibodies from Sigma-Aldrich, Saint Louis, MO). Chemiluminescent signal was acquired by ChemiDoc™ Imaging System (BioRad) and analyzed using the ImageJ software (http://rsb.info.nih.gov/ij/).

5.5. Surface plasmon resonance (SPR) analyses

A BIAcore X-100 apparatus (BIAcore Inc., Piscataway, NJ, USA) was used to set up the following experimental models. Sensorchip with immobilized FGF2 was prepared as previously described.[30] Increasing concentrations of test compounds (ranging between 5,3 and 150 мM) were injected over the FGF2-coated sensorchip and the response was recorded as a function of time tracking the SPR intensity change upon binding progression. Injection lasted for 4 min (flow rate 30 мL/min) to allow compounds association to immobilized FGF2 and was followed by 10 min of dissociation; each run was performed in 3% DMSO in PBS e 0,05% Tween 20. The equilibrium (plateau) values of the SPR sensorgrams were used to build the binding isotherms (dose-response curves). Binding isotherm points were fitted with the Langmuir equation for monovalent binding to evaluate the mass surface dissociation constant, Kd, and the scaling parameter that relates the SPR signal with the extent of binding, as the free parameters of the fitting. The errors on these parameters were assigned as a result of the fitting algorithm (95% confidence bounds). The best-fitting procedure was

performed with the SigmaPlot 11.0 software package (Systat Software Inc.).

5.6. Estrogen receptor binding

Affinity for human estrogen receptors a and b was evaluated by Eurofins (https://www.eurofinsdiscoveryservices.com/). Experimental conditions are described at https://www.eurofinsdiscoveryservices.com/catalogmanagement/viewitem/ERalpha-Human-Estrogen-NHR-Binding-Agonist-Radioligand-Assay-Panlabs/226010 for ERa and at https://www.eurofinsdiscoveryservices.com/catalogmanagement/viewitem/ERbeta-Human-Estrogen-NHR-Binding-Agonist-Radioligand-Assay-Panlabs/226050 for ERb.

5.7. Subcutaneous human xenografts

Experiments were performed according to the Italian laws (D.L. 116/92 and following additions) that enforce the EU 86/109 Directive and were approved by the local animal ethics committee (OPBA, Organismo Preposto al Benessere degli Animali, Università degli Studi di Brescia, Italy). Six-to eight-week old female NOD/SCID mice (Envigo, Udine, Italy) were injected subcutaneously with KMS-11 cells (5×10^6 cells/mouse) in 200 mL of PBS. When tumors were palpable, mice were randomly assigned to receive i.p. treat- ment with compound 1 (7.5 mg/kg), compound 19b (7.5 mg/kg), compound 25b (7.5 mg/kg) or control/vehicle DMSO. Tumor volumes were measured with caliper and calculated according to the formula $V = (D \times d^2)/2$, where D and d are the major and minor perpendicular tumor diameters, respectively. At the end of the experimental procedure, tumor nodules were excised, photographed and weighted.

5.8. Plasma level quantification of systemically administered compounds

For plasma level determination of compounds 1, 19b and 25b, a 7.5 mg/kg dose was administered i.p. to eight-week-old NOD/SCID mice (n = 4) and blood samples were collected 90 min after administration and plasma analyzed by HPLC-MS/MS. HPLC-MS/MS analysis was performed with a Thermo Accela ultra-high performance liquid chromatography (UHPLC) gradient system (Thermo, Waltham, MA, USA) coupled to a Thermo TSQ Quantum Access Max triple quadrupole mass spectrometer (Thermo Waltham, MA, USA) equipped with a heated electrospray ionization (H-ESI) ion source. Chromatographic separation occurred on a Phenomenex Synergy Fusion C_{18} 80 Å RP-column (100 mm $\times$ 2.1 mm i.d., 4 mm particle size; Phenomenex, Torrance, CA, USA) by gradient elution. Eluent A was acetonitrile; eluent B was ultra-pure water, both added with 0.1% v/v formic acid. Gradient: T(0 min), 5%A:95% B; T(1 min): 5%A:95%B; T(3 min): 95%A:5%B; T(6 min): 95%A:5%B; T(7 min): 5%A:95%B, with a 3 min re-equilibration time. Flow rate was at 350 mL min^{-1}. Mass spectrometric analyses were done in negative polarity (ESI-) and in multiple reaction monitoring (MRM) mode. H-ESI interface parameters were set as follows: probe middle (D) position; capillary temperature 270 °C; spray voltage 3.0 kV. Nitrogen was used as nebulizing gas at the following pressure: sheath gas, 35 psi; auxiliary gas, 15 arbitrary units (a.u.). Argon was used as the collision gas at a pressure of approximately 1.5 mTorr (1 torr = 133.3 Pa). Thermo Xcalibur v. 2.2 SP1 software (Thermo, Waltham, MA, USA) was employed for both data acquisition and processing.

For quantitative analysis, the following parent ion / product ions transitions were selected. 1: m/z 483.1 [M — H]⁻ / m/z 413.3 b m/z 111.0 b m/z 69.1 (tube lens (TL) 74 V; collision energies (CE) 22, 25, 74 eV, respectively; retention time (Rt) 5.00 min). 17: m/z 483.1 [M —H]⁻ / m/z 413.3 b m/z 111.0 b m/z 69.1 (TL 74 V; CE 22, 25, 74 eV; Rt 5.10 min); 19b: m/z 540.3 [M —H]⁻ / m/z 470.3 b m/z 452.3 b m/z 111.1 (TL 147 V; CE 22, 33, 25 eV; Rt 3.56 min); 25b: m/z 483.1 [M —H]⁻ / m/z 413.3 b m/z 111.0 b m/z 69.1 (TL 74 V; CE 22, 25, 74 eV; Rt 5.50 min). Calibration curves for quantification of compounds 1, 19b, 25b and 17 in mouse plasma were prepared by spiking blank mouse plasma with stock solutions of each compound in DMSO. Plasma samples were processed by protein precipitation via addition of acetonitrile containing the internal standard (19b for 1, 17, 25b and 25b for 19b at 100 nM final concentration; ratio plasma/acetonitrile: 1:2). Samples were centrifuged (13 000 rpm, 10 min, 4 °C), and 10 mL of the supernatant were injected into the HPLC-MS/MS system for quantification. Linearity was checked in the 1000e1 nM concentration range, with a LOQ equal to 1 nM. The coefficients of correlation (r2) were >0.99 for all calibration curves. The specificity of the assay was evaluated by comparison of HPLCeMS/MS traces of calibration standards at the LOQ to those of blank plasma samples. Extraction efficiency was determined by comparing the peak area ratio of spiked mouse plasma samples at three concentration levels (low, intermediate, and high) to those of extracted blank plasma spiked with the corresponding concentrations. The mean extraction recovery ranged between 90% and 95%.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

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