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Original article

Novel nonsteroidal ligands with high binding affinity and potent functional activity for the androgen receptor

Yali He, Donghua Yin [1], Minoli Perera [1], Leonid Kirkovsky, Nina Stourman, Wei Li, James T. Dalton [1], Duane D. Miller *

Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee-Memphis, 847 Monroe Avenue, Faculty Building, Room 327, Memphis, TN 38163, USA

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Abstract

While nonsteroidal androgen receptor (AR) antagonists have been known for many years, and used in the clinic for the treatment of hormone dependent prostate cancer, very little is known about nonsteroidal AR agonists. We designed and synthesized a series of chiral bicalutamide analogs, which bear electron-withdrawing groups (either a cyano or a nitro group at the 4-position and a trifluoromethyl group at the 3-position) in the aromatic A ring, and different substituents at the *para* position in the aromatic B ring of the parent molecule. We also synthesized a series of racemic bicalutamide analogs, which have a trifluoromethyl group instead of a methyl group at the R_2 position. We examined AR binding affinities of our compounds in a competitive binding assay with a radiolabeled high affinity AR ligand, ^{3}H-mibolerone, and also measured their abilities to stimulate AR-mediated transcriptional activation in a cotransfection assay. These studies demonstrated that (1) nonsteroidal ligands can be structurally modified from known nonsteroidal antiandrogens to generate ligands capable of activating AR-mediated transcriptional activation. (2) *R*-isomer analogs exhibit higher AR binding affinity and more potent functional activity than their corresponding *S*-isomers in all cases. (3) All sulphide analogs show higher AR binding affinity and more potent functional activity than their corresponding sulphone analogs, with the exception of ligand **R-8**. Those ligands which exhibit high AR binding affinity and potent functional activity for human AR may provide effective clinical uses for male fertility, male contraception, and hormone replacement therapy.

Keywords: Nonsteroidal ligands; Androgen receptor agonists; Bicalutamide derivatives

1. Introduction

The androgen receptor (AR) is an important cellular regulatory protein and plays a critical role in numerous physiological processes, including the development and maintenance of male secondary sexual characteristics such as muscle, hair and bone mass, prostate growth and spermatogenesis [1]. The AR is also thought to be involved in prostate carcinogenesis [2]. The natural steroids, testosterone **1** and 5α-dihydrotestosterone (5α-DHT) **2** are the natural ligands for the AR.

Nonsteroidal antiandrogens were extensively reported. Some of these (e.g. flutamide **3** [3,4] and bicalutamide **4** [5,6] in Fig. 1) are successfully used in the clinic for the treatment of AR dependent prostate cancer. We reported on the first nonsteroidal agonists for the human AR [7]. Recently, others have reported on new chemical classes of AR agonists [8]. Based on these previous reports and the known properties of nonsteroidal antiandrogens [9,10], nonsteroidal androgens can be designed and synthesized that will mimic the pharmacological effects of testosterone **1**, and would likely avoid many of the undesired physicochemical and pharmacokinetic properties of their steroidal counterparts, including poor oral bioavailability, rapid hepatic metabolism, and activation of other steroid receptors. Nonsteroidal AR agonists would be potentially useful for the treatment of a number of hormone dependent

* Corresponding author. Tel.: +1-901-4487529; fax: +1-901-4486828

E-mail address: dmiller@utmem.edu (D.D. Miller).

[1] Present address: College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA.

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conditions, ranging from aging or hypogonadal men requiring hormone replacement therapy [11], to male fertility, male contraception [12] and performance enhancement [13,14].

We discovered that nonsteroidal ligands with androgenic activity can be generated by means of structural modifications of known nonsteroidal antiandrogens and first reported these drugs as nonsteroidal agonists for the human AR [7]. Based on Structure–activity relationship (SAR) studies of nonsteroidal AR ligands [15–17], electron-withdrawing groups together with a branched alkyl group α to the amidic carbonyl in the aromatic A ring were required for binding and functional activity (**5**, Fig. 1). There may be a distinctive role for this electron deficient aromatic A ring with respect to direct AR receptor interaction. It is also known that the hydroxyl group (**5**, Fig. 1) plays an important role in the interaction between the AR receptor and ligand [15]. Electron-attracting substituents in the aromatic A ring can enhance the proton donor capability of the hydroxyl group by increasing the acidity of the amide moiety [15]. Chemically, the R_2 group could affect the proton donating ability of the hydroxyl group via electronic effect. We assumed that the AR binding affinity and functional activity might be increased by introducing a strong electron-withdrawing functional substituent (for example, a NO_2 group instead of a CN group) at the 4-position in the aromatic A ring, and/or by introducing an electron-attracting group (for example, a CF_3 group) at the R_2 position (**5**, Fig. 1). To optimise our parent molecule (**5**, Fig. 1) with the ultimate aim of designing a new series of human AR agonists, we designed and synthesized a series of chiral bicalutamide analogs bearing electron-withdrawing groups, either a cyano or a nitro group at the 4-position and a trifluoromethyl group at the 3-position in the aromatic A ring, and different substituents at the *para* position in the aromatic B ring of the parent molecule (**5**, Fig. 1). We also synthesized a series of racemic bicalutamide analogs, which have a trifluoromethyl group instead of a methyl group at the R_2 position. Substitution at the *meta* position in the aromatic B ring resulted in a dramatic decrease in both binding and functional activities for the AR (data were not included). Modification of the aromatic B ring only focused on the *para* position in this paper.

In summary, the present study examined the structural requirements of the aromatic A ring (R_1), aromatic B ring (R_3), functional group X-position (sulfonyl or thio), and/or a trifluoromethyl group instead of a methyl group at the R_2 position in the parent molecule (**5**, Fig. 1) for optimum activity on the human AR.

2. Chemistry

The synthesis of chiral ligands **R-3**–**R-17** is outlined in Fig. 2 (with *R*-isomers as an example). For a series of cyano ligands (cyano group at the 4-position in aromatic A ring), the starting material **R-2** (*N*-[4-cy-

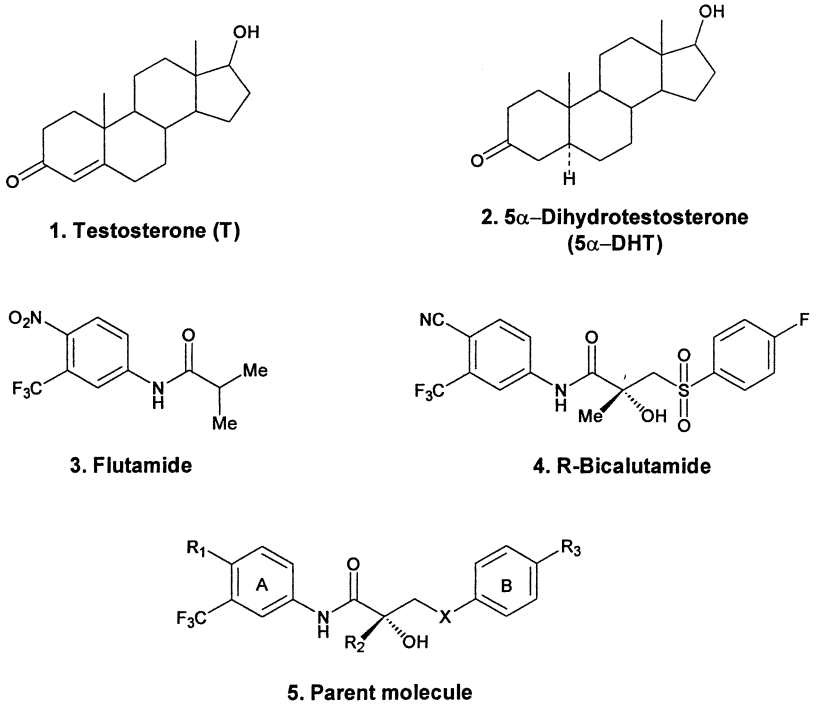


Fig. 1. Molecular structures of testosterone (T), 5α-dihydrotestosteroen (5α-DHT), flutamide, *R*-bicalutamide, and parent molecule.

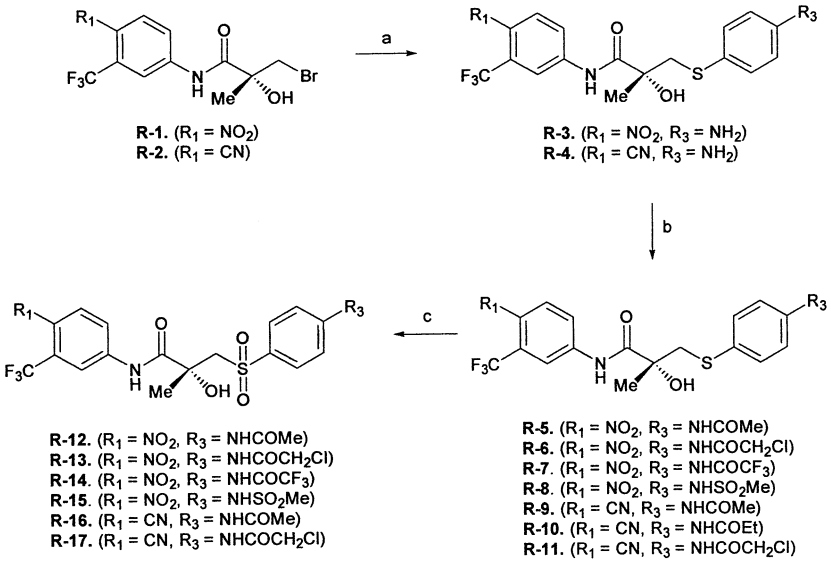


Fig. 2. (a) 1.25 equiv. NaH, 1.25 equiv. 4-aminothiophenol, anhydrous THF, 25 °C for 24 h, 75% (**R-3**), 73% (**R-4**). (b) Excess acetic anhydride, 25 °C for 10 min, 74% (**R-5**), 98% (**R-9**); 1.1 equiv. chloroacetyl chloride, 10 equiv. $CaCO_3$, anhydrous CH_2Cl_2, 25 °C for 24 h, 72% (**R-6**), 77% (**R-11**); 5 equiv. trifluoroacetic anhydride, 5 equiv. Et_3N, anhydrous THF, 0–25 °C for 24 h, 95% (**R-7**); 1 equiv. methanesulfonyl chloride, 2 equiv. Et_3N, anhydrous THF, 0–25 °C for 24 h, 60% (**R-8**); 1.5 equiv. propionyl chloride, 5 equiv. $CaCO_3$, anhydrous CH_2Cl_2, 25 °C for 72 h, 20% (**R-10**). (c) Excess peracetic acid, EtOAc, 25 °C for 1 h, nearly quantitative yield.

ano - 3 - (trifluoromethyl)phenyl] - (2*R*) - 3 - bromo - 2-hydroxy-2-methylpropanamide) was synthesized from commercially available 4-amino-2-trifluoromethyl benzonitrile and *R*-proline (L-proline for *S*-isomers) in four steps [16,18]. In the same manner as the series of cyano ligands, the starting material **R-1** (*N*-[4-nitro-3-(trifluoromethyl)phenyl] - (2*R*) - 3 - bromo - 2 - hydroxy - 2-methyl propanamide) for a series of nitro ligands (a nitro group at the 4-position in the aromatic A ring) was prepared from commercially available 4-nitro-3-(trifluoromethyl) aniline and *R*-proline (also L-proline for *S*-isomers). The starting material (**R-1** or **R-2**) was coupled with 4-aminothiophenol to afford **R-3** and **R-4**, respectively. The acetylamino compounds (**R-5** and **R-9**) were obtained by acylation of the corresponding aniline **R-3** or **R-4** with acetyl anhydride at room temperature for 10 min, while the propionylamino analog **R-10** was obtained by acylation of **R-4** with propionyl chloride in anhydrous methylene chloride at room temperature for 3 days. In the same manner as acetylamino derivatives, chloroacetylamino compounds **R-6** and **R-11** were prepared by acylation of **R-3** and **R-4** with chloroacetyl chloride, respectively. The trifluoroacetylamino analog **R-7** was prepared by reaction of **R-3** with trifluoroacetyl anhydride in anhydrous THF. The methanesulfonylamino analog **R-8** was obtained by reaction of **R-3** with methanesulfonyl chloride in anhydrous THF. Sulphide derivatives (**R-5**–**R-11**) were oxidized to their corresponding sulfonyl derivatives (**R-12**–**R-17**) by peracetic acid or chloroperbenzoic acid.

The preparation of racemic ligands **24**–**28** is outlined in Fig. 3. Commercially available starting material **18** (3-bromo-1,1,1-trifluoro acetone) was coupled with *para*-nitrothiophenol sodium salt [19] to afford **19**, which reacted with potassium cyanide and 25% sulfuric acid to provide the cyano precursor **20.** Hydrolysis of the cyano group of compound **20** in a refluxing mixture of concentrated HCl and glacial acetic acid afforded compound **21** as the precursor for the preparation of compounds **24**–**28**. After the reduction of the nitro group of **21** with tin(II) chloride, compounds **22** and **23** were prepared by acylation of the amino intermediate with acetyl chloride or chloroacetyl chloride. Coupling of **22** or **23** with commercially available 4-nitro-3-(trifluoromethyl)aniline yielded the corresponding amides, **24** and **25**, respectively. Ligand **25** was oxidized to its corresponding sulfonyl derivative **26** by peracetic acid. Coupling of **21** with commercially available 4-nitro-3-(trifluoromethyl)aniline or 4-amino-2-trifluoromethyl benzonitrile afforded compounds **27** and **28**, respectively.

3. Biological results and discussion

A competitive binding assay with ^{3}H-mibolerone, a high affinity AR binding ligand [7] was employed to examine AR binding affinities of the new analogs prepared in this study. CV-1 cells cotransfected with a human AR expression vector, an androgen-sensitive luciferase reporter vector and a control β-galactosidase vector were employed to evaluate pharmacological

activities of these ligands. For potent ligands, AR-mediated transcriptional activation increased with increasing ligand concentrations, then reached a plateau at high concentration. Only the *R*-isomer analogs and racemic analogs with high binding affinity were evaluated for their ability to stimulate AR-mediated transcriptional activation (Table 1). Control studies showed that the efficacy of transcriptional activation induced by 500 nM of each testing compound was below 1% in the absence of androgen receptor expression construct, which demonstrated that the observed increases in luciferase expression were the result of specific AR-mediated effects.

Both the 4-nitro-3-trifluoromethyl and 4-cyano-3-trifluoromethyl analogs exhibited similar AR binding affinity and functional activity. The *R*-isomer analogs exhibited higher AR binding affinity and more potent functional activity than their corresponding *S*-isomers in all cases (Table 1). These results suggested that the structural elements required for the AR binding affinity in the aromatic A ring were an electron deficient aromatic ring, and the most active isomers have the *R*-configuration. In previous studies on hydroxy flutamide analogs [7], we found that structural modification by replacing the *para*-cyano group with a nitro functional group in the aromatic A ring significantly improved the binding and functional activity. However, this SAR was not obvious in bicalutamide derivatives tested here (compare **R-5** vs. **R-9**, **R-6** vs. **R-11**, **R-12** vs. **R-16**, and **R-13** vs. **R-17**). This suggested that the mode of binding for bicalutamide analogs differs from that for hydroxyflutamide analogs.

To optimise the substitution at the *para* position of the aromatic B ring, various substituents were introduced. Comparing to **R-3** and **R-4** which bear an amino group at the *para* position in the aromatic B ring, and produced only minimal efficacies, 2.5 ± 0.3 and $1.4 \pm 0.1\%$ (mean $\pm$ S.D.), respectively, introduction of a short *para* acylamino group (for example, **R-5**–**R-7**, **R-9**–**R-14**) or *para* sulfonylamino group (**R-8** and **R-15**) in the aromatic B ring resulted in a significant increase in AR binding affinity, and in the degree of efficacy. Structural modifications at the *para* position in the aromatic B ring resulted in the most potent agonist

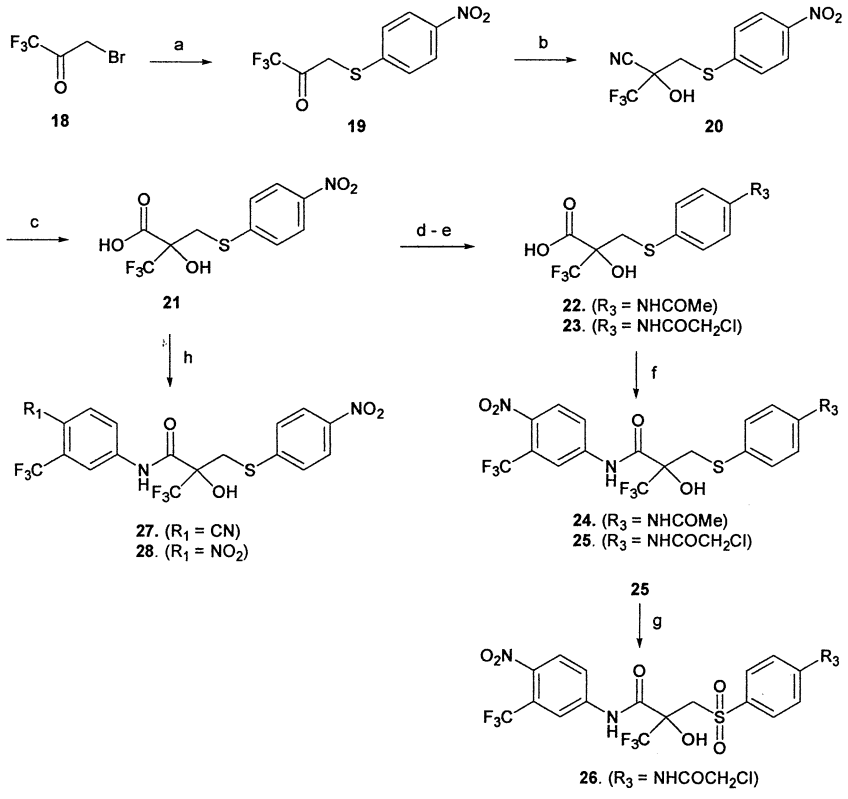
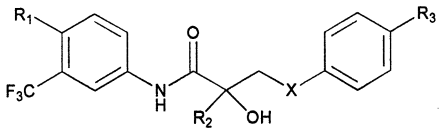


Fig. 3. (a) 1 equiv. *para*-nitrothiophenol sodium salt, anhydrous THF, 0–25 °C for 3 h, 50%. (b) 1.15 equiv. KCN, 25% (v/v) sulphuric acid aqueous solution, 0–25 °C for 20 h, 67%. (c) Concentrated HCl and glacial acetic acid (17:3, v/v), reflux for 24 h, 47%. (d) 3 equiv. tin(II) chloride, methanol and concentrated HCl (1:1), 0–25 °C for 24 h. (e) 1.5 equiv. acetyl chloride (**22**), or 1.2 equiv. chloroacetyl chloride (**23**), 10 equiv. $CaCO_3$, anhydrous MeCN, 25 °C for 48 h, 20% (**22**), 62% (**23**). (f) 1 equiv. 4-nitro-3-(trifluoromethyl) aniline, 1.25 equiv. thionyl chloride, anhydrous DMA, −15 to −10 °C then 25 °C for 3 days, 13% (**24**), 14% (**25**). g. 3 equiv. *meta*-chloro-perbenzoic acid, CH_2Cl_2, 25 °C for 1 h, 25%. (h) 1 equiv. 5-amino-2-cyanobenzotrifluoride (**27**), 1 equiv. 4-Nitro-3-(trifluoromethyl) aniline (**28**), 1.25 equiv. thionyl chloride, anhydrous DMA, −15 to −10 °C then 25 °C for 3 days, 20% (**27**), 20% (**28**).

Table 1
Structures, binding affinity (K_i) and functional activity of DHT, bicalutamide and synthesized nonsteroidal ligands



R_1	R_2	X	R_3	Ligands	K_i [a] (nM)	Efficacy [b] (% of DHT)	Potency [c] (nM)
				DHT	0.28 ± 0.02	100	1
CN	Me	SO_2	F	R [d]-Bical. [e]	11.0 ± 1.5	8.28 ± 2.66	1000
CN	Me	SO_2	F	S [f]-Bical. [e]	365 ± 10		
NO_2	Me	S	NH_2	R [d]-3	57.2 ± 1.5	2.50 ± 0.3	500
NO_2	Me	S	NH_2	S [f]-3	528 ± 26		
CN	Me	S	NH_2	R-4	91.0 ± 16.0	1.40 ± 0.1	500
CN	Me	S	NH_2	S-4	832 ± 50		
NO_2	Me	S	NHCOMe	R-5	2.94 ± 0.23	79.2 ± 5.7	100
NO_2	Me	S	NHCOMe	S-5	123 ± 27		
NO_2	Me	S	$NHCOCH_2Cl$	R-6	3.32 ± 0.36	90.0 ± 7.2	500
NO_2	Me	S	$NHCOCH_2Cl$	S-6	97.0 ± 7.0		
NO_2	Me	S	$NHCOCF_3$	R-7	2.58 ± 0.39	127.2 ± 17	500
NO_2	Me	S	$NHSO_2Me$	R-8	49.8 ± 1.4	ND [g]	ND [g]
CN	Me	S	NHCOMe	R-9	4.88 ± 0.18	ND [g]	ND [g]
CN	Me	S	NHCOMe	S-9	137 ± 30		
CN	Me	S	NHCOEt	R-10	26.6 ± 2.25	ND [g]	ND [g]
CN	Me	S	$NHCOCH_2Cl$	R-11 [h]	1.65 ± 0.10	95.5 ± 21.6	100
CN	Me	S	$NHCOCH_2Cl$	S-11 [h]	410 ± 49		
NO_2	Me	SO_2	NHCOMe	R-12	9.32 ± 0.81	45.1 ± 5.2	500
NO_2	Me	SO_2	NHCOMe	S-12	589 ± 127		
NO_2	Me	SO_2	$NHCOCH_2Cl$	R-13	6.91 ± 1.20	41.3 ± 2.9	100
NO_2	Me	SO_2	$NHCOCH_2Cl$	S-13	233 ± 74		
NO_2	Me	SO_2	$NHCOCF_3$	R-14	6.23 ± 1.13	9.8 ± 4.4	500
NO_2	Me	SO_2	$NHSO_2Me$	R-15	15.4 ± 1.7	3.3 ± 0.4	500
CN	Me	SO_2	NHCOMe	R-16	15.9 ± 0.93	30.1 ± 17.8	500
CN	Me	SO_2	$NHCOCH_2Cl$	R-17	7.04 ± 0.88	65.4 ± 6.4	500
NO_2	CF_3	S	NHCOMe	24 [i]	2.53 ± 0.14	136.3 ± 32.9	100
NO_2	CF_3	S	$NHCOCH_2Cl$	25 [i]	4.97 ± 0.56	106.3 ± 12.9	500
NO_2	CF_3	SO_2	$NHCOCH_2Cl$	26 [i]	5.04 ± 0.66	97.8 ± 9.9	500
CN	CF_3	S	NO_2	27 [i]	18.8 ± 4.6	ND [g]	ND [g]
NO_2	CF_3	S	NO_2	28 [i]	20.34 ± 1.88	ND [g]	ND [g]

Data are expressed as means ± SEM (standard error).
[a] The radioligand used in the competitive binding assay was ^{3}H-mibolerone.
[b] Maximal percentage of transcriptional activation observed for each ligand.
[c] The lowest concentration of the ligand capable of maximally stimulating AR-mediated transcription during transfection experiments.
[d] *R*-isomer analog.
[e] Bical., bicalutamide.
[f] *S*-isomer analog.
[g] ND, not determined.
[h] Ref. [7].
[i] Racemic mixture (compounds **24**–**28**).

activity. Among these substituents, the acetamido, chloroacetamido, or trifluoroacetamido sulphide derivatives (ligands **R-5**–**R-7**, **R-9**, **R-11**, **24**, and **25**) demonstrated a mean efficacy of 80–136% at 100–500 nM concentrations. However, the potency of these ligands was ca. 100–500 fold lower than that of DHT. Replacement of an acetylamino group (**R-9**) with a propionylamino group (**R-10**) or methanesulfonylamino group (**R-8**) decreased the AR binding affinity. These observations suggest that the nature of the substituent in the *para*-position in the aromatic B ring play an important role in drug (compare **R-9** vs. **R-10**).

To investigate the effects on the AR binding affinity and functional activity by introducing either a sulphide or a sulfonyl group at the X-position (**5**, Fig. 1), we designed and synthesized both sulphide and sulphone derivatives of some ligands. The results show that all sulphide analogs (**R-5**–**R-7**, **R-9**, **R-11**, and **25**) show higher AR binding affinity and more potent functional activity than their corresponding sulphone analogs

(**R-12**–**R-14**, **R-16**, **R-17**, and **26**), with the exception of **R-8** which exhibited lower binding affinity than its corresponding sulfonyl analog (**R-15**), and ligand **25** whose AR binding affinity and functional activity remained practically unchanged comparing to ligand **26**.

The effect of introducing a trifluoromethyl group instead of a methyl group at the R_2 position on AR binding affinity and functional activity was also examined (**5**, Fig. 1). A series of racemic bicalutamide analogs (**24**–**28**) was synthesized and biologically evaluated (Table 1). While the AR binding affinities of the CF_3 series of ligands were on the same order magnitude as that of the CH_3 series of ligands. Ligand **24** demonstrated the most potent functional activity, mean efficacy of 136% at concentration as low as 100 nM. These results suggested that the replacement of a methyl group by a trifluoromethyl group at the R_2 position might be helpful for the AR binding affinity. However, it has still been argued that the electronic property of R_2 and the electron-withdrawing groups on the aromatic ring A group were a crucial factor for the proton donating capability of a hydroxyl group in the interaction between the AR receptor and ligand [15]. To further attempt to optimise our analogs, we also synthesized ligands **27** and **28** in the CF_3 series of ligands. The AR binding affinity of **27** was essentially equipotent with that of **28**, which further implied that a nitro group at the 4-position in the aromatic A ring has a similar effect as the cyano group for AR binding affinity in the AR competitive binding assay. It is clear that additional structural modifications of these ligands to improve the efficacy and potency of androgenic activity are needed, and will be the subject of future research in our laboratory.

4. Conclusions

Nonsteroidal human AR ligands with high binding affinity and potent functional activity were obtained by means of structural modifications of bicalutamide, a known nonsteroidal antiandrogen used in the treatment of hormone dependent prostate cancer by using co-transfection and binding assays as guides. *R*-isomer analogs exhibit higher binding affinity and more potent functional activity for the human AR than their corresponding *S*-isomers in all cases. The ligands bearing an acetamido, chloroacetamido, or trifluoroacetamido functional group at the *para* position in the aromatic B ring represented the most active analogs in this series of ligands. All sulphide analogs showed higher AR binding affinity and more potent functional activity than their corresponding sulphone analogs, with the exception of sulphide **R-8** which showed lower AR binding affinity than sulphone **R-15** and ligand **25** whose AR binding affinity and functional activity were approximately equal to that of ligand **26**. These new AR agonist compounds are considerably different than the known steroidal AR agonists and in vivo studies need to be carried out to see if some of the undesired properties such as the lack of good oral bioavailability and metabolic breakdown of the steroidal androgen agonists are overcome, steroidal counterparts are the subjects for continuing investigation. The development of new nonsteroidal human AR agonists should provide effective clinical opportunities for therapeutical uses.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Thomas–Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin–Elmer System 2000 FTIR. Optical rotations were determined on a Autopol® III Automatic Polarimeter (Rudolph Research Model III-589-10, Fairfield, New Jersey). Proton and carbon-13 magnetic resonance spectra were obtained on a Bruker AX 300 spectrometer (300 and 75 MHz for ^{1}H- and ^{13}C-NMR, respectively). Chemical shift values were reported as parts per million (δ) relative to tetramethylsilane (TMS). Spectral data were consistent with assigned structures. Mass spectra were determined on a Bruker-HP Esquire LC System. Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA), and found values were within 0.4% of the theoretical values. Routine thin-layer chromatography (TLC) was performed on silica gel on aluminium plates (silica gel 60 F 254, 20×20 cm, Aldrich Chemical Company Inc., Milwaukee, WI). Flash chromatography was performed on silica gel (Merck, grade 60, 230–400 mesh, 60 Å). Tetrahydrofuran (THF) was dried by distillation from sodium metal. Acetonitrile (MeCN) and methylene chloride (CH_2Cl_2) were dried by distillation from P_2O_5.

(17α-Methyl-^{3}H)-mibolerone (^{3}H-MIB, 83.5 Ci mmol^{-1}) and unlabeled MIB were purchased from DuPont Research NEN Products (Boston, MA). Triamcinolone acetonide, phenylmethylsulfonyl fluoride (PMSF), TRIS base, sodium molybdate and dithiothreitol were purchased from Sigma Chemical Company (St. Louis, MO). Hydroxyapatite (HAP) was purchased from Bio-Rad Laboratories (Hercules, CA). EcoLite Plus™ scintillation cocktail was purchased from ICN Research Products Division (Costa Mesa, CA). Dulbecco's modified essential medium (DMEM) and Lipofectamine™ transfection reagent were purchased from Life Technologies (Gaithersburg, MA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, Inc. (Norcross, GA).

*5.1.1. N-[4-nitro-3-(trifluoromethyl)phenyl]-(2R)-3-[(4-aminophenyl)sulfanyl]-2-hydroxy-2-methylpropanamide (**R-3**)*

A solution of 4-aminothio phenol (7.51 g, 0.06 mol) in 60 ml of anhydrous THF was added to a suspension of previously washed 60% oil dispersion of NaH (2.30 g, 0.06 mol) in 60 ml of anhydrous THF under argon atmosphere. The resulting mixture was stirred for 22 h until a white suspension was formed. A solution of **R-1** [7] (17.84 g, 48.0 mmol) in 60 ml of anhydrous THF was added dropwise to the above suspension, and stirred for 24 h. The reaction mixture was filtered, washed with THF, and the filtrate was evaporated in vacuo to give an oil. Purification by flash column chromatography on silica gel (hexanes–chloroform, 1:1–1:2 with 0.1% ammonium as eluent) yielded 15 g (75%) of the desired compound (**R-3**) as a yellow powder: m.p. 94–95 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.36 (s, 1H, CONH), 8.41 (d, $J = 2.1$ Hz, 1H, ArH), 8.19 (dd, $J = 2.1$, 8.9 Hz, 1H, ArH), 8.07 (d, $J = 8.9$ Hz, 1H, ArH), 7.00–6.96 (m, 2H, ArH), 6.38–6.34 (m, 2H, ArH), 6.01 (s, 1H, OH), 5.09 (s, 2H, NH_2), 3.17 (d, $J = 12.8$ Hz, 1H, CHH_a), 3.07 (d, $J = 12.8$ Hz, 1H, CHH_b), 1.43 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 175.0 (C=O), 148.2 (C–N, Ar), 143.2 (C–NO_2, Ar), 141.5 (C–N, Ar), 133.4, 127.2, 123.0, 122.6 (q, $J = 32.2$ Hz), 122.1 (q, $J = 271.5$ Hz, CF_3), 119.9, 118.3 (q, $J = 6.0$ Hz), 114.2, 75.3 (CO–C–O), 47.3 (CH_2–S), 25.6 (CH_3); IR (KBr, cm^{-1}): 3496, 3397, 3357 (NH and OH), 1678 (C=O, amide), 1624 (C=C, Ar), 1598 (C=C, Ar), 1513 (C=C, Ar), 1417, 1360, 1153; ESIMS; m/z: 438.2 $(M + Na)^+$; $[\alpha]_D^{26}$ + 22.8° ($c = 0.54$, acetone); Anal. $C_{17}H_{16}F_3N_3O_4S{\cdot}0.25C_4H_8O_2{\cdot}0.2C_6H_{14}$ (C, H, N).

*5.1.2. N-[4-nitro-3-(trifluoromethyl)phenyl]-(2S)-3-[(4-aminophenyl)sulfanyl]-2-hydroxy-2-methylpropanamide (**S-3**)*

The title compound was prepared from compound **S-1** (0.85 g, 2.29 mmol) and 4-aminothiophenol (0.36 g, 2.86 mmol) in the same manner as compound **R-3**. Purification by flash column chromatography on silica gel (hexanes–chloroform, 1:1–1:2 with 0.1% ammonium as eluent) yielded 0.5 g (54%) of the desired compound as a yellow powder: m.p. 92–93 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.51 (s, 1H, CONH), 8.50 (d, $J = 2.1$ Hz, 1H, ArH), 8.29 (dd, $J = 2.1$ Hz, $J = 9.0$ Hz, 1H, ArH), 8.16 (d, $J = 9.0$ Hz, 1H, ArH), 7.07–7.03 (m, 2H, ArH), 6.44–6.40 (m, 2H, ArH), 6.10 (s, 1H, OH), 5.16 (s, 2H, NH_2), 3.18 (d, $J = 12.9$ Hz, 1H, CHH_a), 3.08 (d, $J = 12.9$ Hz, 1H, CHH_b), 1.41 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 175.0 (C=O), 148.2 (C–N, Ar), 143.3 (C–NO_2, Ar), 141.5 (C–N, Ar), 133.4, 127.3, 123.9, 122.7 (q, $J = 33.0$ Hz), 122.1 (q, $J = 272.3$ Hz, CF3), 119.9, 118.3 (q, $J = 6.0$ Hz), 114.3, 75.4 (CO–C–O), 47.3 (CH_2–S), 25.7 (CH_3); IR (KBr, cm^{-1}): 3370 (NH and OH), 1685 (C=O, amide), 1599 (C=C, Ar), 1521 (C=C, Ar), 1421, 1350, 1153; ESIMS; m/z: 438.2 $[M + Na]^+$; $[\alpha]_D^{26}$ − 23.7° ($c = 0.53$, acetone); Anal. $C_{17}H_{16}F_3N_3O_4S{\cdot}0.25C_4H_8O_2{\cdot}$ (C, H, N).

*5.1.3. N-[4-cyano-3-(trifluoromethy)phenyl]-(2R)-3-[(4-aminophenyl)sulfanyl]-2-hydroxy-2-methylpropanamide (**R-4**)*

The title compound was prepared from compound **R-2** [7] (2.55 g, 7.40 mmol) and 4-aminothiophenol (1.38 g, 11.0 mmol) by the same procedure used for compound **R-3**. The crude oily product was solidified after addition of a small amount of MeOH. Recrystallisation from MeOH afforded 2.53 g (73%) of the desired compound as white crystals: m.p. 166–167 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.44 (s, 1H, NH), 8.48 (d, $J = 1.9$ Hz, 1H, ArH), 8.23 (dd, $J = 8.6$, 1.9 Hz, 1H, ArH), 8.06 (d, $J = 8.6$ Hz, 1H, ArH), 7.05–7.01 (m, 2H, ArH), 6.48–6.43 (m, 2H, ArH), 6.08 (s, 1H, OH), 5.14 (br s, 2H, NH_2), 3.17 (d, $J = 13.0$ Hz, 1H, CHH_a), 3.07 (d, $J = 13.0$ Hz, 1H, CHH_b), 1.40 (s, 3H, Me); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 175.0, 148.2, 143.2, 136.1, 133.4, 131.4 (q, $J = 31.7$ Hz), 122.5 (q, $J = 273.6$ Hz), 122.6, 119.8, 117.4 (q, $J = 5.0$ Hz), 115.8, 114.2, 101.7 (q, $J = 2$ Hz), 75.3, 47.3, 25.6; IR (KBr, cm^{-1}): 3486, 3447, 3386, 3363, (NH, NH_2, OH), 2234 (CN), 1681 (CO), 1623, 1596, 1583, 1513, 1331; $[\alpha]_D^{27}$ + 33.0° ($c = 2$, acetone); Anal. $C_{18}H_{16}F_3N_3O_2S$ (C, H, N).

*5.1.4. N-[4-cyano-3-(trifluoromethy)phenyl]-(2S)-3-[(4-aminophenyl)sulfanyl]-2-hydroxy-2-methylpropanamide (**S-4**)*

The title compound was prepared from compound **S-2** (2.55 g, 7.40 mmol) and 4-aminothiophenol (1.38 g, 11.0 mmol) in the same manner as compound **R-4**. Recrystallization from MeOH gave 2.46 g (71%) of the desired compound as white crystals: m.p. 166–167 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.41 (s, 1H, NH), 8.47 (d, $J = 1.9$ Hz, ArH), 8.22 (dd, $J = 8.6$, 2.0 Hz, 1H, ArH), 8.05 (d, $J = 8.6$ Hz, 1H, ArH), 7.06–7.01 (m, 2H, ArH), 6.48–6.44 (m, 2H, ArH), 6.05 (s, 1H, OH), 5.22 (br s, 2H, NH_2), 3.17 (d, $J = 13.0$ Hz, 1H, CHH_a), 3.07 (d, $J = 13.0$ Hz, 1H, CHH_b), 1.39 (s, 3H, Me); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 175.0, 147.8, 143.2, 136.1, 133.3, 131.4 (q, $J = 31.7$ Hz), 122.6, 122.5 (q, $J = 273.5$ Hz), 120.2, 117.4 (q, $J = 5.0$ Hz), 115.8, 114.4, 101.7 (q, $J = 2.0$ Hz), 75.3, 47.2, 25.6; IR (KBr, cm^{-1}): 3485, 3447, 3385, 3365 (NH, NH_2, OH), 2234 (CN), 1681 (CO), 1623, 1596, 1583, 1517, 1330; $[\alpha]_D^{27}$ − 33.7° ($c = 2$, acetone); Anal. $C_{18}H_{16}F_3N_3O_2S$ (C, H, N).

*5.1.5. N-[4-nitro-3-(trifluoromethyl)phenyl]-(2R)-3-{[4-[acetylamino)phenyl]sulfanyl}-2-hydroxy-2-methylpropanamide (**R-5**)*

Compound **R-3** (0.70 g, 1.69 mol) was completely

dissolved in 10 ml of acetic anhydride, and the reaction solution was stirred for 10 min at room temperature. After the end of the reaction was established by TLC, the resulting solution was poured into 50 ml of saturated $NaHCO_3$ solution (more solid $NaHCO_3$ was added), stirred, and extracted with EtOAc (20 ml × 3). The EtOAc extract was dried over anhydrous Na_2SO_4, filtered through Celite, and evaporated in vacuo to give an oil. Purification by flash column chromatography on silica gel (hexanes–ethyl acetate, 2:1–1:1) yielded 0.56 g (74%) of the desired compound as a yellow powder: m.p. 104–107 °C, softened then melted; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.47 (s, 1H, CONH), 9.84 (s, 1H, CONH), 8.43 (d, $J = 2.1$ Hz, 1H, ArH), 8.24 (dd, $J = 2.1$ Hz, $J = 9.0$ Hz, 1H, ArH), 8.13 (d, $J = 9.0$ Hz, 1H, ArH), 7.42–7.39 (m, 2H, ArH), 7.28–7.23 (m, 2H, ArH), 6.17 (s, 1H, OH), 3.37 (d, $J = 13.2$ Hz, 1H, CHH_a), 3.20 (d, $J = 13.2$ Hz, 1H, CHH_b), 1.99 (s, 3H, $COCH_3$), 1.44 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.7 (C=O), 168.0 (C=O), 143.1 (C–NO_2, Ar), 141.5 (C–N, Ar), 137.7 (C–S, Ar), 130.3, 129.4 (C–N, Ar), 127.1, 122.9, 122.5 (q, $J = 33.0$ Hz), 122.0 (q, $J = 271.5$ Hz, CF_3), 119.2, 118.2 (q, $J = 6.0$ Hz), 75.1 (CO–C–O), 44.7 (CH_2–S), 25.6 ($COCH_3$), 23.8 (CH_3); IR (KBr, cm^{-1}): 3340 (NH and OH), 1677 (C=O, amide), 1596 (C=C, Ar), 1523 (C=C, Ar), 1397 (CH3), 1321, 1153; ESIMS; m/z: 480.2 $[M + Na]^+$; $[\alpha]_D^{26}$ + 24.2° ($c = 0.40$, acetone); Anal. $C_{19}H_{18}F_3N_3O_5S \cdot 0.5C_4H_8O_2 \cdot 0.2C_6H_{14}$ (C, H, N).

5.1.6. *N-[4-nitro-3-(trifluoromethyl)phenyl]-(2S)-3-{[4-[acetylamino)phenyl]sulfanyl}-2-hydroxy-2-methylpropanamide (**S-5**)*

The title compound was prepared from compound **S-3** (0.70 g, 1.69 mmol) and acetic anhydride (10 ml) in the same manner as compound **R-5**. Purification by flash column chromatography on silica gel (hexanes–ethyl acetate, 2:1–1:1) yielded 0.70 g (91%) of the desired compound as a light yellow powder: m.p. 105–108 °C, softened then melted; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.35 (s, 1H, CONH), 9.72 (s, 1H, CONH), 8.34 (d, $J = 2.1$ Hz, 1H, ArH), 8.15 (dd, $J = 2.1$, 8.7 Hz, 1H, ArH), 8.04 (d, $J = 8.7$ Hz, 1H, ArH), 7.35–7.32 (m, 2H, ArH), 7.21–7.16 (m, 2H, ArH), 6.11 (s, 1H, OH), 3.35 (d, $J = 13.0$ Hz, 1H, CHH_a), 3.2 (d, $J = 13.0$ Hz, 1H, CHH_b), 1.99 (s, 3H, $COCH_3$), 1.46 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.7 (C=O), 168.1 (C=O), 143.1 (C–NO_2, Ar), 141.5 (C–N, Ar), 137.7 (C–S, Ar), 130.3, 129.4 (C–N, Ar), 127.2, 122,9, 122.6 (q, $J = 33.0$ Hz), 122.1 (q, $J = 270.8$ Hz, CF_3), 119.2, 118.2 (q, $J = 6.0$ Hz), 75.1 (CO–C–O), 44.7 (CH_2–S), 25.7 ($COCH_3$), 23.8 (CH_3); IR (KBr, cm^{-1}): 3348 (NH and OH), 1677 (C=O, amide), 1596 (C=C, Ar), 1523 (C=C, Ar), 1397 (CH3), 1321, 1154; ESIMS; m/z: 480.2 $[M + Na]^+$; $[\alpha]_D^{26}$ − 24.0° ($c = 0.56$, acetone); Anal. $C_{19}H_{18}F_3N_3O_5S \cdot 0.4C_4H_8O_2$ (C, H, N).

5.1.7. *N-[4-nitro-3-(trifluoromethyl)phenyl]-(2R)-3-({4-[(2-chloroacetyl)amino]phenyl}sulfanyl)-2-hydroxy-2-methylpropanamide (**R-6**)*

Chloro acetyl chloride (0.21 g, 1.85 mmol) was added dropwise to a suspension of compound **R-3** (0.70 g, 1.69 mmol) and $CaCO_3$ (1.84 g, 16.85 mmol) in 60 ml of anhydrous CH_2Cl_2 under argon atmosphere. The resulting suspension was stirred overnight, filtered through Celite, washed with CH_2Cl_2, and the filtrate was evaporated completely in vacuo to furnish an oil. Purification by flash column chromatography on silica gel (hexanes–ethyl acetate, 2:1–1:1) yielded 0.6 g (72%) of the desired compound as a yellow powder: m.p. 58–60 °C, softened then melted; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.49 (s, 1H, CONH), 10.22 (s, 1H, CONH), 8.45 (d, $J = 2.1$ Hz, 1H, ArH), 8.25 (dd, $J = 2.1$, 9.0 Hz, 1H, ArH), 8.14 (d, $J = 9.0$ Hz, 1H, ArH), 7.46–7.42 (m, 2H, ArH), 7.33–7.28 (m, 2H, ArH), 6.20 (s, 1H, OH), 4.20 (s, 2H, $COCH_2Cl$), 3.38 (d, $J = 13.7$ Hz, 1H, CHH_a), 3.34 (d, $J = 13.7$ Hz, 1H, CHH_b), 1.45 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.7 (C=O), 164.4 (C=O), 143.1 (C–NO_2, Ar), 141.5 (C–N, Ar), 136.7 (C–S, Ar), 130.8 (C–N, Ar), 130.1, 127.1, 122.8, 122.5 (q, $J = 33.0$ Hz), 122.0 (q, $J = 270.8$ Hz, CF_3), 119.7, 118.2 (q, $J = 6.0$ Hz), 75.1 (CO–C–O), 44.5 ($COCH_2Cl$), 43.4 (CH_2–S), 25.6 (CH_3); IR (KBr, cm^{-1}): 3355 (NH and OH), 1679 (C=O, amide), 1596 (C=C, Ar), 1524 (C=C, Ar), 1400, 1322, 1153; $[\alpha]_D^{26}$ + 17.4° ($c = 0.46$, acetone); Anal. $C_{19}H_{17}ClF_3N_3O_5S$ (C, H, N).

5.1.8. *N-[4-nitro-3-(trifluoromethyl)phenyl]-(2S)-3-({4-[(2-chloroacetyl)amino]phenyl} sulfanyl)-2-hydroxy-2-methylpropanamide (**S-6**)*

The title compound was prepared from compound **S-3** (0.70 g, 1.69 mmol) and chloroacetyl chloride (0.21 g, 1.85 mmol) in the same manner as compound **R-6**. Purification by flash column chromatography on silica gel (hexanes–ethyl acetate, 2:1–1:1) yielded 0.68 g (82%) of the desired compound as a light yellow powder: m.p. 60–63 °C, softened then melted; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.50 (s, 1H, CONH), 10.32 (s, 1H, CONH), 8.45 (d, $J = 2.1$ Hz, 1H, ArH), 8.25 (dd, $J = 2.1$, 9.0 Hz, 1H, ArH), 8.14 (d, $J = 9.0$ Hz, 1H, ArH), 7.45–7.42 (m, 2H, ArH), 7.32–7.29 (m, 2H, ArH), 6.21 (s, 1H, OH), 4.20 (s, 2H, $COCH_2Cl$), 3.39 (d, $J = 13.1$ Hz, 1H, CHH_a), 3.23 (d, $J = 13.1$ Hz, 1H, CHH_b), 1.45 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.7 (C=O), 164.4 (C=O), 143.1 (C–NO_2, Ar), 141.6 (C–N, Ar), 136.7 (C–S, Ar), 130.8 (C–N, Ar), 130.1, 127.2, 122.9, 122.6 (q, $J = 33.0$ Hz), 122.1 (q, $J = 271.5$ Hz, CF_3), 119.7, 118.2 (q, $J = 6.0$ Hz), 75.2 (CO–C–O), 44.5 ($COCH_2Cl$), 43.4 (CH_2–S), 25.7

(CH_3); IR (KBr, cm^{-1}): 3349 (NH and OH), 1680 (C=O, amide), 1596 (C=C, Ar), 1525 (C=C, Ar), 1400, 1322, 1153; ESIMS; *m/z*: 514.2 $(M+Na)^+$; $[\alpha]_D^{26}$ −18.1° ($c = 0.50$, acetone); Anal. $C_{19}H_{17}ClF_3N_3O_5S \cdot 0.4C_4H_8O_2$ (C, H, N).

5.1.9. *N-[4-nitro-3-(trifluoromethyl)phenyl]-(2R)-2-hydroxy-2-methyl-3-({4-[(2,2,2-trifluoroacetyl)amino]-phenyl}sulfanyl)propanamide (**R-7**)*

To a solution of compound **R-3** (0.50 g, 1.20 mmol) in 15 ml of anhydrous THF was added 0.85 ml of Et_3N (6.0 mmol) at room temperature, and the resulting solution was cooled to 0 °C in an ice bath. Trifluoroacetic anhydride (0.85 ml, 6.0 mmol) was added dropwise to the above solution. The reaction mixture was warmed to room temperature, stirred overnight and then evaporated in vacuo to afford a residue. A solution of the residue in 20 ml of CH_2Cl_2 was washed with brine (20 ml × 2), 1N HCl solution (20 ml × 2), dried over anhydrous Na_2SO_4, filtered through Celite, and evaporated in vacuo to dryness. Purification by flash column chromatography on silica gel ($CHCl_3$–EtOAc, 6:1) yielded 0.58 g (95%) of the desired compound as a yellow amorphic powder: m.p. 50–53 °C, softened then melted; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 11.61 (s, 1H, NH), 10.51 (s, 1H, NH), 8.41 (d, $J = 2.0$ Hz, 1H, ArH), 8.22 (dd, $J = 2.0$, 9.0 Hz, 1H, ArH), 8.13 (d, $J = 9.0$ Hz, 1H, ArH), 7.51–7.48 (m, 2H, ArH), 7.36–7.34 (m, 2H, ArH), 6.25 (s, 1H, OH), 3.44 (d, $J = 13.3$ Hz, 1H, CHH_a), 3.25 (d, $J = 13.3$ Hz, 1H, CHH_b), 1.46 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.7 (C=O), 154.2 (q, $J = 36.8$ Hz, $COCF_3$), 143.1 (C–N, Ar), 141.5 (C–NO_2, Ar), 134.3, 133.1, 129.6, 127.2, 123.0, 122.5 (q, $J = 33.0$ Hz), 122.1 (q, $J = 271.5$ Hz, CF_3), 121.1, 118.1 (q, $J = 5.3$ Hz), 115.7 (q, $J = 286.5$ Hz, $COCF_3$), 75.1, 43.8, 25.7 (CH_3); IR (KBr, cm^{-1}): 3353 (OH and NH), 1712 (CON), 1599, 1543 (C=C, Ar), 1422, 1352; ESIMS; *m/z*: 534.1 $[M+Na]^+$; $[\alpha]_D^{26}$ +7.2° ($c = 0.36$, acetone); Anal. $C_{19}H_{15}F_6N_3O_5S \cdot 0.5C_4H_8O_2$ (C, H, N).

5.1.10. *N-[4-nitro-3-(trifluoromethyl)phenyl]-(2R)-2-hydroxy-2-methyl-3-({4-[(methylsulfonyl)amino]-phenyl}sulfanyl)propanamide (**R-8**)*

To a solution of compound **R-3** (0.40 g, 0.96 mmol) in 15 ml of anhydrous THF was added 0.28 ml of Et_3N (2.0 mmol) at room temperature, and the resulting solution was cooled to 0 °C in an ice bath. Methanesulfonyl chloride (0.11 g, 0.96 mmol) was added dropwise to the above mixture. The reaction mixture was warmed to room temperature, stirred overnight, and evaporated in vacuo to afford a residue. A solution of the residue in 20 ml of CH_2Cl_2 was washed with brine (20 ml × 2), 1N HCl solution (20 ml × 2), dried over anhydrous Na_2SO_4, filtered through Celite, and evaporated in vacuo to dryness. Purification by flash column chromatography on silica gel (ethyl acetate–hexanes, 1:1–2:1) yielded 0.28 g (60%) of the desired compound as a light yellow powder: m.p. 57–60 °C, softened then melted; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 9.09 (s, 1H, NH), 7.97 (d, $J = 2.2$ Hz, 1H, ArH), 7.95 (d, $J = 8.9$ Hz, 1H, ArH), 7.83 (dd, $J = 8.9$, 2.2 Hz, 1H, ArH), 7.41–7.37 (m, 2H, ArH), 7.06–7.04 (m, 2H, ArH), 6.70 (s, 1H, OH), 3.76 (d, $J = 14.1$ Hz, 1H, CHH_a), 3.15 (d, $J = 14.1$ Hz, 1H, CHH_b), 2.98 (s, 3H, CH_3SO_2), 1.56 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 173.0 (C=O), 143.2 (C–NO_2, Ar), 141.3 (C–N, Ar), 136.3, 132.7, 130.1, 127.0, 125.2 (q, $J = 33.8$ Hz), 122.0, 121.7 (q, $J = 272.3$ Hz, CF_3), 120.8, 118.1 (q, $J = 6.0$ Hz), 75.5, 45.2 (CH_2S), 39.7 (CH_3SO_2), 26.2 (CH_3); IR (KBr, cm^{-1}): 3350 (OH and NH), 1703 (C=O, amide), 1598, 1523, 1495 (C=C, Ar), 1421, 1324, 1152; ESIMS; *m/z*: 516.2 $[M+Na]^+$; $[\alpha]_D^{26}$ +12.0° ($c = 0.68$, acetone); Anal. $C_{18}H_{18}F_3N_3O_6S_2 \cdot 0.5C_4H_8O_2$ (C, H, N).

5.1.11. *N-[4-cyano-3-(trifluoromethyl)phenyl]-(2R)-3-{[4-[acetylamino)phenyl]sulfanyl}-2-hydroxy-2-methylpropanamide (**R-9**)*

The title compound was prepared from compound **R-4** (0.38 g, 0.97 mmol) and acetic anhydride (2 ml) by the same procedure used for compound **R-5**. Purification by flash column chromatography on silica gel (ethyl acetate–hexanes, 1:1) yielded 0.41 g (98%) of the desired compound as a white solid: m.p. 121–123 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.41 (s, 1H, NH), 9.85 (s, 1H, NH), 8.41 (d, $J = 1.9$ Hz, 1H, ArH), 8.18 (dd, $J = 8.6$, 1.9 Hz, 1H, ArH), 8.02 (d, $J = 8.6$ Hz, 1H, ArH), 7.42–7.39 (m, 2H, ArH), 7.27–7.23 (m, 2H, ArH), 6.17 (s, 1H, OH), 3.36 (d, $J = 13.1$ Hz, 1H, CHH_a), 3.20 (d, $J = 13.1$ Hz, 1H, CHH_b), 1.70 (s, 3H, Me), 1.44 (s, 3H, Me); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.8, 168.1, 143.1, 137.7, 136.1, 131.4 (q, $J = 31.5$ Hz), 130.3, 129.4, 122.6, 122.5 (q, $J = 271.5$ Hz), 119.2, 117.3 (q, $J = 5.25$ Hz), 115.8, 101.8 (q, $J = 2.3$ Hz), 75.1, 44.7, 25.7, 23.9; IR (KBr, cm^{-1}): 3442, 3329 (NH, OH), 2231 (CN), 1683 (CO), 1590, 1524 (C=C, Ar), 1431, 1320, 1181, 1130; $[\alpha]_D^{27}$ +27.1° ($c = 0.60$, acetone); Anal. $C_{20}H_{18}F_3N_3O_3S$ (C, H, N).

5.1.12. *N-[4-cyano-3-(trifluoromethyl)phenyl]-(2S)-3-{[4-[acetylamino)phenyl]sulfanyl}-2-hydroxy-2-methylpropanamide (**S-9**)*

The title compound was prepared from compound **S-4** (0.30 g, 0.76 mmol) and acetic anhydride (2 ml) in the same manner as compound **R-9**. Recrystallization from ethyl acetate–hexanes afforded 0.26 g (78%) of the desired compound as white solid: m.p. 122–124 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.41 (s, 1H, NH), 9.85 (s, 1H, NH), 8.41 (d, $J = 1.9$ Hz, 1H, ArH), 8.18 (dd, $J = 8.6$, 1.9 Hz, 1H, ArH), 8.02 (d, $J = 8.6$ Hz, 1H, ArH), 7.42–7.39 (m, 2H, ArH), 7.27–7.23 (m, 2H, ArH), 6.17 (s, 1H, OH), 3.36 (d, $J = 13.1$ Hz, 1H,

CHH_a), 3.20 (d, $J = 13.1$ Hz, 1H, CHH_b), 1.70 (s, 3H, Me), 1.44 (s, 3H, Me); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.8, 168.1, 143.1, 137.7, 136.1, 131.4 (q, $J = 31.5$ Hz), 130.3, 129.4, 122.6, 122.5 (q, $J = 271.5$ Hz), 119.2, 117.3 (q, $J = 5.25$ Hz), 115.8, 101.8 (q, $J = 2.3$ Hz), 75.1, 44.7, 25.7, 23.9; IR (KBr, cm^{-1}): 3442, 3329 (NH, OH), 2231 (CN), 1683 (CO), 1590, 1524 (C=C, Ar), 1431, 1320, 1181, 1130; $[\alpha]_D^{27}$ $-27.2°$ ($c = 0.58$, acetone); Anal. $C_{20}H_{18}F_3N_3O_3S$ (C, H, N).

5.1.13. N-[4-cyano-3-(trifluoromethyl)phenyl]-(2R)-3-{[4-[propionylamino)phenyl]sulfanyl}-2-hydroxy-2-methylpropanamide (**R-10**)

Propionyl bromide (0.27 g, 1.89 mmol) was added dropwise to a mixture of compound **R-4** (0.5 g, 1.26 mmol) and $CaCO_3$ (0.63 g, 6.32 mmol) in anhydrous CH_2Cl_2 (50 ml) at room temperature under argon atmosphere. The reaction mixture was stirred for 3 days. After the end of the reaction was established by TLC, the resulting mixture was filtered through Celite, washed with EtOAc, and the filtrate was evaporated completely in vacuo to dryness. Purification by flash column chromatography on silica gel (ethyl acetate–hexanes, 1:1) yielded 0.15 g (20%) of the desired compound as a white solid: m.p. 125–127 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.43 (NH), 9.78 (NH), 8.41 (d, $J = 1.6$ Hz, 1H, ArH), 8.18 (dd, $J = 1.7$, 8.6 Hz, 1H, ArH), 8.03 (d, $J = 8.6$ Hz, 1H, ArH), 7.43–7.40 (m, 2H, ArH), 7.26–7.23 (m, 2H, ArH), 6.19 (s, 1H, OH), 3.34 (d, $J = 13.2$ Hz, 1H, CHH_a), 3.19 (d, $J = 13.2$ Hz, 1H, CHH_b), 3.27 (q, $J = 7.5$ Hz, 2H, CH_2), 1.43 (s, 3H, Me), 1.05 (t, $J = 7.5$ Hz, 3H, Me); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.8, 171.8, 143.1, 137.8, 136.1, 131.4 (q, $J = 31.58$ Hz), 130.3, 129.3, 122.6, 122.5 (q, $J = 271.73$ Hz, CF_3), 119.2, 117.3 (q, $J = 5.3$ Hz), 115.8, 102.0 (q, $J = 1.9$ Hz), 75.1, 44.7, 29.4, 25.7, 9.5; IR (KBr, cm^{-1}): 3415, 3352 (NH, OH), 2231 (CN), 1680 (C=O), 1589, 1522 (C=C, Ar), 1431, 1400, 1329, 1183, 1136; $[\alpha]_D^{27}$ $+28.3°$ ($c = 0.53$, acetone); Anal. $C_{21}H_{20}F_3N_3O_3S$ (C, H, N).

5.1.14. N-[4-cyano-3-(trifluoromethyl)phenyl]-(2R)-3-({4-[(2-chloroacetyl)amino]phenyl}sulfanyl)-2-hydroxy-2-methylpropanamide (**R-11**)

Chloro acetyl chloride (0.1 ml, $d = 1.418$, 1.2 mmol) was added dropwise to a vigorously stirred suspension of compound **R-4** (0.41 g, 1.04 mmol) and $CaCO_3$ (0.56 g, 5.6 mmol) in anhydrous CH_2Cl_2 (50 ml) over a period of 1–2 min under argon atmosphere. The reaction mixture was stirred overnight, filtered through Celite, and the filtrate was completely evaporated in vacuo to give a white solid. Recrystallization from ethyl acetate–hexanes yielded 0.48 g (77%) of the desired compound as ball-shape crystals: m.p. 134.5–135 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.44 (s, 1H, NH), 10.24 (s, 1H, NH), 8.43 (d, $J = 1.8$ Hz, 1H, ArH), 8.20 (dd, $J = 8.6$, 1.9 Hz, 1H, ArH), 8.03 (d, $J = 8.6$ Hz, 1H, ArH), 7.45–7.43 (m, 2H, ArH), 7.31–7.28 (m, 2H, ArH), 6.20 (s, 1H, OH), 4.20 (s, 2H, CH_2Cl), 3.38 (d, $J = 13.1$ Hz, 1H, CHH_a), 3.23 (d, $J = 13.1$ Hz, 1H, CHH_b), 1.45 (s, 3H, Me); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.8, 164.4, 143.1, 136.7, 136.1, 131.4 (q, $J = 31.7$ Hz), 130.9, 130.1, 122.6, 122.5 (q, $J = 273.7$ Hz), 119.7, 117.3 (q, $J = 5.0$ Hz), 115.8, 101.9 (q, $J = 2.0$ Hz), 75.2, 44.5, 43.4, 25.7; IR (KBr, cm^{-1}) 3464, 3321, 3265 (NH, OH), 2238 (CN), 1679 (CO), 1609, 1597, 1522, 1429, 1327, 1174; $[\alpha]_D^{27}$ $+23.4°$ ($c = 2$, acetone); Anal. $C_{20}H_{17}ClF_3N_3O_3S$ (C, H, N).

5.1.15. N-[4-cyano-3-(trifluoromethyl)phenyl]-(2S)-3-({4-[(2-chloroacetyl)amino]phenyl}sulfanyl)-2-hydroxy-2-methylpropanamide (**S-11**)

The title compound was prepared from compound **S-4** (0.41 g, 1.04 mmol) and chloroacetyl chloride (0.1 ml, $d = 1.418$, 1.20 mmol) in the same manner as compound **R-11**. Recrystallization from ethyl acetate–hexanes gave 0.35 g (56%) of the desired compound as white crystals: m.p. 132–134 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.44 (s, 1H, NH), 10.23 (s, 1H, NH), 8.43 (d, $J = 1.7$ Hz, 1H, ArH), 8.20 (dd, $J = 8.6$, 1.9 Hz, 1H, ArH), 8.04 (d, $J = 8.6$ Hz, 1H, ArH), 7.45–7.42 (m, 2H, ArH), 7.31–7.28 (m, 2H, ArH), 6.20 (s, 1H, OH), 4.20 (s, 2H, CH_2Cl), 3.38 (d, $J = 13.2$ Hz, 1H, CHH_a), 3.23 (d, $J = 13.1$ Hz, 1H, CHH_b), 1.44 (s, 3H, Me); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.8, 164.5, 143.1, 136.7, 136.1, 131.4 (q, $J = 31.7$ Hz), 130.9, 130.1, 122.7, 122.5 (q, $J = 273.8$ Hz), 119.7, 117.3 (q, $J = 5.3$ Hz), 115.8, 101.9 (q, $J = 1.8$ Hz), 75.2, 44.5, 43.5, 25.8; IR (KBr, cm^{-1}) 3464, 3320, 3268 (NH, OH), 2239 (CN), 1679 (CO), 1609, 1598, 1521, 1429, 1327, 1174 1135; $[\alpha]_D^{27}$ $-23.4°$ ($c = 2$, acetone); Anal. $C_{20}H_{17}ClF_3N_3O_3S$ (C, H, N).

5.1.16. N-[4-nitro-3-(trifluoromethyl)phenyl]-(2R)-3-{[4-[acetylamino)phenyl]sulfonyl}-2-hydroxy-2-methylpropanamide (**R-12**)

Peracetic acid (1 ml) was added to a solution of compound **R-5** (0.40 g, 0.87 mmol) in 2 ml of EtOAc and the resulting mixture was stirred for 1 h at room temperature. After the end of the reaction was established by TLC, the reaction solution was diluted with EtOAc (150 ml), washed with saturated Na_2SO_3 solution (30 ml × 2), brine (30 ml × 2), dried over anhydrous Na_2SO_4, filtered through Celite, and evaporated in vacuo to give an oil. The oily crude product was purified by flash column chromatography on silica gel (hexanes–ethyl acetate, 3:1) to yield 0.43 g (99%) of the desired compound as a yellow powder: m.p. 101–103 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.32 (s, 1H, CONH), 10.19 (s, 1H, CONH), 8.39 (d, $J = 2.0$ Hz, 1H, ArH), 8.21 (dd, $J = 2.0$, 9.0 Hz, 1H, ArH), 8.12 (d, $J = 9.0$ Hz, 1H, ArH), 7.76–7.74 (m, 2H, ArH), 7.67–

7.64 (m, 2H, ArH), 6.36 (s, 1H, OH), 3.89 (d, $J = 14.6$ Hz, 1H, CHH_a), 3.61 (d, $J = 14.6$ Hz, 1H, CHH_b), 2.02 (s, 3H, $COCH_3$), 1.40 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 173.5 (C=O), 168.9 (C=O), 143.8 (C–NO_2, Ar), 143.2 (C–S, Ar), 141.5 (C–N, Ar), 133.6 (C–N, Ar), 129.4, 127.1, 123.0, 122.6 (q, $J = 33.0$ Hz), 122.1 (q, $J = 271.5$ Hz, CF_3), 118.2 (q, $J = 6.0$ Hz), 118.1, 73.0 (CO–C–O), 63.6 (CH_2–SO_2), 27.3 ($COCH_3$), 23.9 (CH_3); IR (KBr, cm^{-1}): 3343 (NH and OH), 1691 (C=O, amide), 1594 (C=C, Ar), 1528 (C=C, Ar), 1403, 1323, 1142; ESIMS; m/z: 512.2 $[M + Na]^+$; $[\alpha]_D^{26}$ −41.5° ($c = 0.42$, acetone); Anal. $C_{19}H_{18}F_3N_3O_7S \cdot 0.5C_4H_8O_2 \cdot 0.2C_6H_{14}$ (C, H, N).

5.1.17. *N-[4-nitro-3-(trifluoromethyl)phenyl]-(2S)-3-{[4-[acetylamino)phenyl]sulfonyl}-2-hydroxy-2-methylpropanamide (**S-12**)*

The title compound was prepared from compound **S-5** (0.30 g, 0.66 mmol) and peracetic acid (1 ml) in the same manner as compound **R-12**. Purification by flash column chromatography on silica gel (hexanes–ethyl acetate, 3:1) yielded 0.32 g (99%) of the desired compound as a light yellow powder: m.p. 100–103 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.32 (s, 1H, CONH), 10.19 (s, 1H, CONH), 8.39 (d, $J = 2.0$ Hz, 1H, ArH), 8.20 (dd, $J = 2.0$, 9.0 Hz, 1H, ArH), 8.12 (d, $J = 9.0$ Hz, 1H, ArH), 7.77–7.74 (m, 2H, ArH), 7.67–7.64 (m, 2H, ArH), 6.37 (s, 1H, OH), 3.90 (d, $J = 14.6$ Hz, 1H, CHH_a), 3.61 (d, $J = 14.6$ Hz, 1H, CHH_b), 2.02 (s, 3H, $COCH_3$), 1.41 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 173.5 (C=O), 168.9 (C=O), 143.8 (C–NO_2, Ar), 143.2 (C–S, Ar), 141.6 (C–N, Ar), 133.6 (C–N, Ar), 129.4, 127.1, 123.0, 122.6 (q, $J = 33.0$ Hz), 122.1 (q, $J = 270.8$ Hz, CF_3), 118.3 (q, $J = 6.0$ Hz), 118.1, 73.0 (CO–C–O), 63.6 (CH_2–SO_2), 27.3 ($COCH_3$), 24.0 (CH_3); IR (KBr, cm^{-1}) 3352 (NH and OH), 1692 (C=O, amide), 1594 (C=C, Ar), 1527 (C=C, Ar), 1403, 1323, 1142; ESIMS; m/z: 512.2 $[M + Na]^+$; $[\alpha]_D^{26}$ + 41.7° ($c = 0.52$, acetone); Anal. $C_{19}H_{18}F_3N_3O_7S \cdot 0.6C_4H_8O_2$ (C, H, N).

5.1.18. *N-[4-nitro-3-(trifluoromethyl)phenyl]-(2R)-3-({4-[(2-chloroacetyl)amino]phenyl}sulfonyl)-2-hydroxy-2-methylpropanamide (**R-13**)*

The title compound was prepared from compound **R-6** (0.38 g, 0.77 mmol) and peracetic acid (1 ml) by the same procedure used for compound **R-12**. Purification by flash column chromatography on silica gel (hexanes–ethyl acetate, 4:1) yielded 0.40 g (99%) of the desired compound as a yellow powder: m.p. 154–156 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.58 (s, 1H, CONH), 10.36 (s, 1H, CONH), 8.41 (d, $J = 2.1$ Hz, 1H, ArH), 8.22 (dd, $J = 2.1$, 9.0 Hz, 1H, ArH), 8.14 (d, $J = 9.0$ Hz, 1H, ArH), 7.82–7.79 (m, 2H, ArH), 7.72–7.69 (m, 2H, ArH), 6.36 (s, 1H, OH), 4.24 (s, 2H, $COCH_2Cl$), 3.90 (d, $J = 14.7$ Hz, 1H, CHH_a), 3.64 (d, $J = 14.7$ Hz, 1H, CHH_b), 1.41 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 173.7 (C=O), 165.2 (C=O), 143.2 (C–NO_2, Ar), 142.9 (C–SO_2, Ar), 141.6 (C–N, Ar), 134.8 (C–N, Ar), 129.5, 127.2, 123.1, 122.6 (q, $J = 33.0$ Hz), 122.1 (q, $J = 271.5$ Hz, CF_3), 118.6, 118.3 (q, $J = 6.0$ Hz), 73.1 (CO–C–O), 63.6 (CH_2–SO_2), 43.5 ($COCH_2Cl$), 27.2 (CH_2); IR (KBr, cm^{-1}): 3454, 3337 (NH and OH), 1690 (C=O, amide), 1593 (C=C, Ar), 1521 (C=C, Ar), 1403, 1356, 1143; $[\alpha]_D^{26}$ −47.4° ($c = 0.56$, acetone); Anal. $C_{19}H_{17}ClF_3N_3O_7S$ (C, H, N).

5.1.19. *N-[4-nitro-3-(trifluoromethyl)phenyl]-(2S)-3-({4-[(2-chloroacetyl)amino]phenyl}sulfonyl)-2-hydroxy-2-methylpropanamide (**S-13**)*

The title compound was prepared from compound **S-6** (0.44 g, 0.89 mmol) and peracetic acid (2 ml) in the same manner as compound **R-13**. Purification by flash column chromatography on silica gel (hexanes–ethyl acetate, 4:1) yielded 0.47 g (99%) of the desired compound as a light yellow powder: m.p. 155–157 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.59 (s, 1H, CONH), 10.37 (s, 1H, CONH), 8.42 (d, $J = 2.1$ Hz, 1H, ArH), 8.22 (dd, $J = 2.1$, 9.0 Hz, 1H, ArH), 8.13 (d, $J = 9.0$ Hz, 1H, ArH), 7.83–7.80 (m, 2H, ArH), 7.71–7.68 (m, 2H, ArH), 6.37 (s, 1H, OH), 4.24 (s, 2H, $COCH_2Cl$), 3.93 (d, $J = 14.7$ Hz, 1H, CHH_a), 3.63 (d, $J = 14.7$ Hz, 1H, CHH_b), 1.41 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 173.6 (C=O), 165.2 (C=O), 143.2 (C–NO_2, Ar), 142.9 (C–SO_2, Ar), 141.6 (C–N, Ar), 134.8 (C–N, Ar), 129.5, 127.1, 123.1, 122.6 (q, $J = 33.0$ Hz), 122.1 (q, $J = 271.5$ Hz, CF_3), 118.7, 118.3 (q, $J = 6.0$ Hz), 73.1 (CO–C–O), 63.6 (CH_2–SO_2), 43.4 ($COCH_2Cl$), 27.9 (CH_3); IR (KBr, cm^{-1}): 3337 (NH and OH), 1690 (C=O, amide), 1593 (C=C, Ar), 1521 (C=C, Ar), 1403, 1356, 1143; ESIMS; m/z: 546.3 $[M + Na]^+$; $[\alpha]_D^{26}$ + 47.7° ($c = 0.53$, acetone); Anal. $C_{19}H_{17}ClF_3N_3O_7S \cdot 0.35C_4H_8O_2$ (C, H, N).

5.1.20. *N-[4-nitro-3-(trifluoromethyl)phenyl]-(2R)-2-hydroxy-2-methyl-3-({4-[(2,2,2-trifluoroacetyl)amino]-phenyl}sulfonyl)propanamide (**R-14**)*

The title compound was prepared from compound **R-7** (0.10 g, 0.2 mmol) and peracetic acid (0.5 ml) by the same procedure used for compound **R-12**. Purification by flash column chromatography on silica gel (hexanes–ethyl acetate, 4:1) yielded 0.1 g (98%) of the desired compound as a yellow powder: m.p. 70–73 °C, softened then melted; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 11.51 (s, 1H, NH), 10.3 (s, 1H, NH), 8.38 (d, $J = 1.7$ Hz, 1H, ArH), 8.20 (dd, $J = 1.7$ Hz, $J = 9.0$ Hz, 1H, ArH), 8.13 (d, $J = 9.0$ Hz, 1H, ArH), 7.89–7.86 (m, 2H, ArH), 7.80–7.77 (m, 2H, ArH), 6.45 (s, 1H, OH), 3.95 (d, $J = 14.8$ Hz, 1H, CHH_a), 3.67 (d, $J = 14.8$ Hz, 1H, CHH_b), 1.40 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 173.5 (C=O), 154.3 (q, $J = 37.5$ Hz, $COCF_3$), 143.2 (C–N, Ar), 141.6 (C–NO_2, Ar), 140.9

(C–N, Ar), 136.5, 129.7, 127.2, 123.1, 122.6 (q, $J = 33.0$ Hz), 122.1 (q, $J = 267.0$ Hz, CF_3), 120.3, 118.3 (q, $J = 6.0$ Hz), 115.5 (q, $J = 287.3$ Hz, CF_3), 73.0, 63.5, 27.4 (CH_3); IR (KBr, cm^{-1}): 3344 (NH and OH), 1718 (CON), 1598, 1546 (C=C, Ar), 1410, 1353, 1321, 1151; $[\alpha]_D^{26}$ $-53.8°$ ($c = 0.58$, acetone); Anal. $C_{19}H_{15}F_6N_3O_7S$ (C, H, N).

5.1.21. *N-[4-nitro-3-(trifluoromethyl)phenyl]-(2R)-2-hydroxy-2-methyl-3-({4-[(methyl sulfonyl)amino]-phenyl}sulfonyl)propanamide (**R-15**)*

The title compound was prepared from compound **R-8** (0.19 g, 0.38 mmol) and peracetic acid (0.5 ml) in the same manner as compound **R-12**. Purification by flash column chromatography on silica gel (hexanes–ethyl acetate, 1:1–1:2) yielded 0.19 g (98%) of the desired compound as a yellow powder: m.p. 75–78 °C, softened then melted; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.44 (s, 1H, NH), 10.41 (s, 1H, NH), 8.47 (d, $J = 2.1$ Hz, 1H, ArH), 8.27 (dd, $J = 2.1$, 9.0 Hz, 1H, ArH), 8.18 (d, $J = 9.0$ Hz, 1H, ArH), 7.82–7.79 (m, 2H, ArH), 7.30–7.27 (m, 2H, ArH), 6.36 (s, 1H, OH), 3.88 (d, $J = 14.7$ Hz, 1H, CHH_a), 3.64 (d, $J = 14.7$ Hz, 1H, CHH_b), 3.09 (s, 3H, CH_3SO_2), 1.41 (s, 3H, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 173.9 (C=O), 143.3 (C–NO_2, Ar), 143.2 (C–N, Ar), 141.7, 134.6, 130.0, 127.3, 123.2, 122.7 (q, $J = 33.0$ Hz), 122.1 (q, $J = 271.5$ Hz, CF_3), 118.5 (q, $J = 6.0$ Hz), 117.4, 73.2, 63.6 (CH_2SO_2), 40.0 (CH_3SO_2), 27.2 (CH_3); IR (KBr, cm^{-1}): 3438 (OH and NH), 1701 (C=O, amide), 1598, 1524 (C=C, Ar), 1323, 1142; ESIMS; m/z: 548.1 $[M + Na]^+$; $[\alpha]_D^{26}$ $-49.2°$ ($c = 0.56$, acetone); Anal. $C_{18}H_{18}F_3N_3O_8S_2 \cdot 0.6C_4H_8O_2$ (C, H, N).

5.1.22. *N-[4-cyano-3-(trifluoromethyl)phenyl]-(2R)-3-{[4-[acetylamino)phenyl]sulfonyl}-2-hydroxy-2-methylpropanamide (**R-16**)*

The title compound was prepared from compound **R-9** (0.10 g, 0.23 mmol) and peracetic acid (0.5 ml) in the same manner as compound **R-12**. Recrystallization from ethyl acetate–hexanes afforded 0.11 g (93%) of compound **R-16** as white solid: m.p. 198–199 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.27 (s, 1H, NH), 10.20 (s, 1H, NH), 8.36 (d, $J = 1.6$ Hz, 1H, ArH), 8.15 (dd, $J = 8.6$, 1.6 Hz, 1H, ArH), 8.03 (d, $J = 8.6$ Hz, 1H, ArH), 7.75–7.72 (m, 2H, ArH), 7.65–7.62 (m, 2H, ArH), 3.88 (d, $J = 14.6$ Hz, 1H, CHH_a), 3.60 (d, $J = 14.6$ Hz, 1H, CHH_b), 2.04 (s, 3H, Me), 1.39 (s, 3H, Me); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 173.5, 168.9, 143.7, 143.1, 136.1, 133.6, 131.3 (q, $J = 31.5$ Hz), 129.4, 122.7, 122.5 (q, $J = 272.3$ Hz), 118.0, 117.3 (q, $J = 5.25$ Hz), 115.6, 101.8 (q, $J = 2.3$ Hz), 73.0, 63.6, 27.3, 24.0; IR (KBr, cm^{-1}): 3435, 3380, 3305 (NH, OH), 2243 (CN), 1686 (C=O), 1593, 1528 (C=C, Ar), 1430, 1404, 1327, 1303, 1174, 1144; $[\alpha]_D^{27}$ $-36.2°$ ($c = 0.54$, acetone); Anal. $C_{20}H_{18}F_3N_3O_5S$ (C, H, N).

5.1.23. *N-[4-cyano-3-(trifluoromethyl)phenyl]-(2R)-3-({4-[(2-chloroacetyl)amino]phenyl}sulfonyl)-2-hydroxy-2-methylpropanamide (**R-17**)*

The title compound was prepared from compound **R-11** (0.22 g, 0.47 mmol) and peracetic acid (4 ml) in the same manner as compound **R-12**. Recrystallization from ethyl acetate–hexanes gave compound **R-17** as a white solid with quantitative yield: m.p. 174–175 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.61 (s, 1H, NH), 10.33 (s, 1H, NH), 8.40 (d, $J = 1.8$ Hz, 1H, ArH), 8.17 (dd, $J = 8.6$, 1.9 Hz, 1H, ArH), 8.05 (d, $J = 8.6$ Hz, 1H, ArH), 7.81–7.78 (m, 2H, ArH), 7.70–7.67 (m, 2H, ArH), 6.38 (s, 1H, OH), 4.25 (s, 2H, CH_2Cl), 3.89 (d, $J = 14.7$ Hz, 1H, CHH_a), 3.62 (d, $J = 14.7$ Hz, 1H, CHH_b), 1.39 (s, 3H, Me); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 173.7, 165.2, 143.1, 136.1, 133.8, 131.4 (q, $J = 31.6$ Hz), 129.5, 122.8, 122.5 (q, $J = 273.6$ Hz), 118.7, 117.4 (q, $J = 5.0$ Hz), 115.9, 101.9 (q, $J = 2.0$ Hz), 73.1, 63.7, 43.5, 27.3; IR (KBr, cm^{-1}): 3477, 3357, 3325 (NH, OH), 2235 (CN), 1692 (CO), 1592, 1527, 1422, 1403, 1329, 1301, 1186, 1144; $[\alpha]_D^{27}$ $-49.4°$ ($c = 2$, acetone); Anal. $C_{20}H_{17}ClF_3N_3O_5S$ (C, H, N).

5.1.24. *1,1,1-Trifluoro-3-[(4-nitrophenyl)sulfanyl]acetone (**19**)*

3-Bromo-1,1,1-trifluoroacetone (5.0 g, 26.0 mmol) was added dropwise to a solution of *para*-nitrothiophenol sodium salt (4.6 g, 26.0 mmol) [19] in anhydrous THF (30 ml) at 0–5 °C in an ice bath. The reaction mixture was warmed to room temperature, and stirred for 3 h. After the end of the reaction was established by TLC, the mixture was filtered through Celite, washed with THF, and the filtrate was evaporated in vacuo to dryness. The crude product was separated by flash column chromatography on silica gel ($CHCl_3$–MeOH, 19:1) to yield 3.57 g (50%) of the desired compound as yellow powder: m.p. 82–84 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.11–8.14 (m, 2H, ArH), 7.55–7.58 (m, 2H, ArH), 3.42 (s, 2H, CH_2S); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 147.7, 144.5, 126.6, 125.6 (q, $J = 270.8$ Hz, CF_3), 123.7, 92.1 (q, $J = 30.6$ Hz, CCF_3), 37.2 (CH_2S); IR (KBr, cm^{-1}): 3418 (broad), 1768 (C=O), 1582, 1517 (C=C, Ar), 1344, 1205, 1170, 1108, 1022, 958, 855, 740, 699; ESIMS; m/z: 264.4 $[M]^-$; Anal. $C_9H_6F_3NO_3S \cdot H_2O$ (C, H, N).

5.1.25. *2,2,2-Trifluoro-1-hydroxy-1-{[(4-nitrophenyl)-sulfanyl]methyl}ethyl cyanide (**20**)*

An aqueous solution (25% v/v) of sulfuric acid (3.4 ml) was added dropwise to a mixture of compound **19** (3.57 g, 13.0 mmol) and KCN (1.0 g, 15.0 mmol) in 5 ml of water at 0–5 °C in an ice bath. The reaction mixture was warmed to room temperature, stirred for 20 h, diluted with water (50 ml), and extracted with Et_2O (3×150 ml). The Et_2O extracts were washed with saturated $NaHCO_3$ solution, brine, dried over

anhydrous Na_2SO_4, filtered through Celite, and evaporated completely in vacuo to afford 2.74 g (67%) of the desired compound as a light yellow solid: m.p. 62–64 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.15–8.10 (m, 2H, ArH), 7.59–7.54 (m, 2H, ArH), 7.40 (s, 1H, OH), 3.42 (s, 2H, CH_2S); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 147.7 (C–N, Ar), 144.5 (C–S, Ar), 126.6, 124.2, 123.7, 123.0 (q, $J = 379.12$ Hz, CF_3), 92.1 (q, $J = 30.75$ Hz, CCF_3), 37.2 (CH_2S); IR (KBr, cm^{-1}): 3488, 3418 (OH), 1585, 1583, 1517 (C=C, Ar), 1480, 1344, 1280, 1204, 1186, 1170, 1108, 1085.

*5.1.26. 3,3,3-Trifluoro-2-hydroxy-2-{[(4-nitrophenyl)-sulfanyl]methyl}propanoic acid (**21**)*

A mixture of compound **20** (1.5 g, 5 mmol), concentrated HCl (17 ml), and AcOH (3 ml) was heated to reflux overnight with vigorous stirring. After the end of the reaction was established by TLC, the mixture was diluted with water (100 ml) and extracted with Et_2O (4 × 100 m) which was in turn washed with saturated $NaHCO_3$ solution (4 × 100 ml). The $NaHCO_3$ solution was acidified with concentrated HCl to pH 1 and extracted with Et_2O (4 × 150 ml). The Et_2O extracts were dried over anhydrous $MgSO_4$, filtered through Celite, and evaporated completely in vacuo to dryness. Trituration in ligroin afforded 0.75 g (47%) of the desired compound as yellow powder: m.p. 92–93 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.10–8.15 (m, 2H, ArH), 7.58–7.63 (m, 2H, ArH), 3.61 (s, 2H, CH_2S); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 168.2, 146.3, 144.9, 127.3, 123.7, 123.8 (q, $J = 286.5$ Hz, CF_3), 77.4 (q, $J = 27.2$ Hz, CCF_3), 34.9 (CH_2S); IR (KBr, cm^{-1}): 3510 (broad), 3192 (broad), 1753, 1718, 1584, 1519, 1340, 1256, 1199, 1184, 1125, 1045, 982, 853, 741; Anal. $C_{10}H_8F_3NO_5S$ (C, H, N).

*5.1.27. 2-({[4-(Acetylamino)phenyl]sulfanyl}methyl)-3,3,3-trifluoro-2-hydroxypropanoic acid (**22**)*

A solution of compound **21** (2.02 g, 6.5 mmol) in MeOH (10 ml) was added dropwise to a solution of tin(II) chloride dihydrate (4.40 g, 19.5 mmol) in concentrated HCl (10 ml) cooled in an ice bath. The reaction mixture was stirred overnight, filtered through Celite, and evaporated in vacuo to give a residue. The identity was conformed by ^{1}H-NMR to be 2-({[4-(amino)-phenyl]sulfanyl}methyl)-3,3,3-trifluoro-2-hydroxypropanoic acid without further purification.

Acetyl chloride (0.7 ml, 9.8 mmol) was added dropwise to a mixture of above residue and $CaCO_3$ (6.50 g, 65 mmol) in anhydrous CH_3CN (30 ml). The resulting mixture was stirred for 48 h under argon atmosphere. After the end of the reaction was established by TLC, the mixture was filtered through Celite, washed with CH_3CN, and the filtrate was evaporated in vacuo to dryness. Purification by flash column chromatography on silica gel ($CHCl_3$–MeOH, 3:1) yielded 0.4 g (20%) of the desired compound as an ivory solid: ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.01 (s, 1H, NH), 9.97 (s, 1H, OH), 7.50–7.53 (m, 2H, ArH), 7.32–7.36 (m, 2H, ArH), 3.37 (d, $J = 13.3$ Hz, 1H, CHH_a), 3.33 (d, $J = 13.2$ Hz, 1H, CHH_b), 2.01 (s, 3H, Me).

*5.1.28. 2-[({4-[(2-Chloroacetyl)amino]phenyl}sulfanyl)-methyl]-3,3,3-trifluoro-2-hydroxy propanoic acid (**23**)*

The title compound was prepared from compound **21** (0.31 g, 1.0 mmol), tin(II) chloride dihydrate (0.68 g, 3.0 mmol), $CaCO_3$ (1.0 g, 10.0 mmol), and chloroacetyl chloride (0.1 ml, 1.2 mmol) by the same procedure used for compound **22**,. Purification by flash column chromatography on silica gel ($CHCl_3$–MeOH–AcOH, 19:1:5) yielded 0.2 g (62%) of the desired compound: ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.37 (s, 1H, NH), 10.33 (s, 1H, OH), 7.52–7.45 (m, 2H, ArH), 7.34–7.30 (m, 2H, ArH), 4.23 (s, 2H, CH_2Cl), 3.46 (d, $J = 13.8$ Hz, CHH_a), 3.35 (d, $J = 13.8$ Hz, 1H, CHH_b).

*5.1.29. N-[4-nitro-3-(trifluoromethyl)phenyl]-2-({[4-(2-acetylamino)phenyl]sulfanyl}methyl-3,3,3-trifluoro-2-hydroxypropanamide (**24**)*

Thionyl chloride (0.1 ml, 1.5 mmol) was added dropwise to a solution of compound **22** (0.40 g, 1.2 mmol) in anhydrous DMA (10 ml) at −15 to −10 °C under argon atmosphere. The reaction mixture was stirred for 1 h, and a solution of 5-amino-2-nitrobenzotrifluoride (0.25 g, 1.2 mmol) in anhydrous DMA (3 ml) was added dropwise to the above reaction solution. The resulting mixture was warmed to room temperature and stirred for 72 h. After the end of the reaction was established by TLC, the mixture was evaporated in vacuo, diluted with saturated $NaHCO_3$ solution (50 ml), and extracted with Et_2O (3 × 50 ml). The Et_2O extracts were filtered through Celite, dried over anhydrous Na_2SO_4, and evaporated in vacuo to dryness. The crude product was separated by flash column chromatography on silica gel ($CHCl_3$–MeOH, 19:1) to yield 80 mg (13%) of the desired compound as a light yellow powder: m.p. 203–204 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.78 (s, 1H, NH), 9.84 (s, 1H, NH), 8.32 (d, $J = 1.7$ Hz, 1H, ArH), 8.17 (dd, $J = 2.0$, 9.0 Hz, 1H, ArH), 8.12 (d, $J = 9.0$ Hz, 1H, ArH), 8.00 (s, 1H, OH), 7.36–7.33 (m, 2H, ArH), 7.27–7.25 (m, 2H, ArH), 3.71 (d, $J = 13.6$ Hz, 1H, CHH_a), 3.36 (d, $J = 13.6$ Hz, 1H, CHH_b), 1.97 (s, 3H, Me); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 168.1, 166.3, 142.1, 138.6, 131.8, 127.1, 126.8, 125.9, 123.7, 122.5 (q, $J = 33.8$ Hz), 122.1, 122.0 (q, $J = 270.8$ Hz, CF_3), 119.0, 118.7 (q, $J = 6.0$ Hz), 77.2 (q, $J = 26.0$ Hz, CCF_3), 38.0 (CH_2S), 23.9 (Me); IR (KBr, cm^{-1}): 3382 (broad), 1701, 1663, 1595, 1522, 1398, 1353, 1323, 1178, 1155, 1046, 840; Anal. $C_{19}H_{15}F_6N_3O_5S$ (C, H, N).

*5.1.30. N-[4-nitro-3-(trifluoromethyl)phenyl]-2-[({4-[(2-chloroacetyl)amino]phenyl}sulfanyl)methyl]-3,3,3-trifluoro-2-hydroxypropanamide (**25**)*

The title compound was prepared from compound **23** (0.33 g, 0.9 mmol) and 5-amino-2-nitrobenzotrifluoride (0.21 g, 1.0 mmol) by the same procedure used for compound **24**. Purification by flash column chromatography on silica gel ($CHCl_3$–MeOH, 19:1) yielded 70 mg (14%) of the desired compound as pale-yellow powder: m.p. 119–120 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.79 (s, 1H, NH), 10.22 (s, 1H, NH), 8.33 (d, $J = 2.0$ Hz, 1H, ArH), 8.17 (dd, $J = 2.1$, 9.0 Hz, 1H, ArH), 8.12 (d, $J = 9.0$ Hz, 1H, ArH), 8.02 (s, 1H, OH), 7.33–7.30 (m, 2H, ArH), 7.40–7.36 (m, 2H, ArH), 4.18 (s, 2H, CH_2Cl), 3.72 (d, $J = 13.3$ Hz, 1H, CHH_a), 3.39 (d, $J = 13.3$ Hz, 1H, CHH_b); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 166.3, 164.5, 142.2, 142.0, 137.6, 131.6, 128.3, 127.1, 123.9 (q, $J = 289.5$ Hz, CF_3), 123.7, 122.0 (q, $J = 274.5$ Hz, CF_3), 122.5 (q, $J = 33.0$ Hz), 119.5, 118.8 (q, $J = 6.0$ Hz), 77.3 (q, $J = 25.5$ Hz, CCF_3), 43.4 (CH_2Cl), 37.8 (CH_2S); IR (KBr, cm^{-1}): 3372 (broad), 1701, 1595, 1530, 1405, 1324, 1255, 1186, 1155, 1046, 843, 547; Anal. $C_{19}H_{14}ClF_6N_3O_5S$ (C, H, N).

*5.1.31. N-[4-nitro-3-(trifluoromethyl)phenyl]-2-[({4-[(2-chloroacetyl)amino]phenyl}sulfonyl)methyl]-3,3,3-trifluoro-2-hydroxypropanamide (**26**)*

A solution of *meta*-chloro-perbenzoic acid (0.20 g, 1.1 mmol) in CH_2Cl_2 (5 ml) was added dropwise to a solution of compound **25** (0.20 g, 0.4 mmol) in CH_2Cl_2 (10 ml) at room temperature. The reaction mixture was stirred for 1 h. After the end of the reaction was established by TLC, the mixture was diluted with CH_2Cl_2 (50 ml), washed successively with saturated Na_2SO_3 solution (3 × 30 ml), saturated Na_2HCO_3 solution (30 ml), brine (30 ml), filtered through Celite, and evaporated completely in vacuo to afford 50 mg (25%) of the desired compound as a pale yellow solid: m.p. 160–162 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.80 (s, 1H, NH), 10.64 (s, 1H, NH), 8.43 (s, 1H, OH), 8.39 (d, $J = 1.8$ Hz, 1H, ArH), 8.23 (dd, $J = 2.0$, 9.0 Hz, 1H, ArH), 8.17 (d, $J = 9.0$ Hz, 1H, ArH), 7.83–7.80 (m, 2H, ArH), 7.71–7.67 (m, 2H, ArH), 4.25 (s, 2H, CH_2Cl), 4.17 (d, $J = 14.6$ Hz, 1H, CHH_a), 3.90 (d, $J = 14.6$ Hz, 1H, CHH_b); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 165.3, 165.1, 143.5, 142.2, 142.2, 133.7, 129.7, 127.2, 123.8, 122.5 (q, $J = 32.2$ Hz), 122.0 (q, $J = 272.3$ Hz, CF_3), 118.8 (q, $J = 6.0$ Hz), 118.7, 75.5 (q, $J = 28.0$ Hz, CCF_3), 56.7 (CH_2SO_2), 43.4 (CH_2Cl); IR (KBr, cm^{-1}): 3378 (broad), 1716, 1594, 1530, 1405, 1323, 1255, 1184, 1163, 1047, 844, 546; Anal. $C_{19}H_{14}ClF_6N_3O_7S$ (C, H, N).

*5.1.32. N-[4-cyano-3-(trifluoromethyl)phenyl]-3,3,3-trifluoro-2-hydroxy-2-{[(4-nitrophenyl)sulfanyl]methyl}-propanamide (**27**)*

The title compound was prepared from compound **21** (0.31 g, 1.0 mmol) and 5-amino-2-cyanobenzotrifluoride (0.19 g, 1.0 mmol) by the procedure used for compound **24**. Purification by flash column chromatography on silica gel ($CHCl_3$–MeOH, 19:1) yielded 95 mg (20%) of the desired compound as a yellow solid: m.p. 172–174 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.84 (s, 1H, NH), 8.26 (d, $J = 1.6$ Hz, 1H, ArH), 8.22 (s, 1H, OH), 8.13 (dd, $J = 1.8$, 8.6 Hz, 1H, ArH), 8.04 (d, $J = 8.6$ Hz, 1H, ArH), 8.01–7.97 (m, 2H, ArH), 7.61–7.57 (m, 2H, ArH), 4.00 (d, $J = 13.7$ Hz, 1H, CHH_a), 3.57 (d, $J = 13.7$ Hz, 1H, CHH_b); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 166.3, 145.1, 144.6, 141.9, 136.2, 131.3 (q, $J = 27.0$ Hz), 128.4, 124.3 (q, $J = 205.5$ Hz, CF_3), 122.3 (q, $J = 270.0$ Hz, CF_3), 123.5, 123.3, 117.7 (q, $J = 4.5$ Hz), 115.5, 102.9, 77.2 (q, $J = 26.6$ Hz, CCF_3), 35.4 (CH_2S); IR (KBr, cm^{-1}) 3357 (broad), 2236, 1709, 1616, 1589, 1530, 1434, 1345, 1329, 1281, 1186, 1137, 1052, 855, 744; Anal. $C_{18}H_{11}F_6N_3O_4S$ (C, H, N).

*5.1.33. N-[4-nitro-3-(trifluoromethyl)phenyl]-3,3,3-trifluoro-2-hydroxy-2-{[(4-nitrophenyl)sulfanyl]methyl}-propanamide (**28**)*

The title compound was prepared from compound **21** (0.31 g, 1.0 mmol) and 4-nitro-3-(trifluoromethyl) aniline (0.21 g, 1.0 mmol) by the same procedure used for compound **24**,. Purification by flash column chromatography on silica gel (CH_2Cl_2–EtOAc, 19:1) yielded 0.11 g (20%) of the desired compound as a yellow solid: m.p. 143–144 °C; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 10.89 (s, 1H, NH), 8.29 (d, $J = 1.9$ Hz, 1H, ArH), 8.23 (s, 1H, OH), 8.18 (dd, $J = 2.0$, 9.0 Hz, 1H, ArH), 8.13 (d, $J = 9.0$ Hz, 1H, ArH), 8.02–7.97 (m, 2H, ArH), 7.61–7.56 (m, 2H, ArH), 4.00 (d, $J = 13.6$ Hz, 1H, CHH_a), 3.59 (d, $J = 13.6$ Hz, 1H, CHH_b); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 166.3, 145.1, 144.7, 142.3, 141.9, 128.3, 127.1, 124.3 (q, $J = 222.8$ Hz, CF_3), 123.9, 123.6, 121.9 (q, $J = 271.5$ Hz, CF_3), 118.7 (q, $J = 6.0$ Hz), 77.2 (q, $J = 27.0$ Hz, CCF_3), 35.4 (CH_2S); IR (KBr, cm^{-1}): 3482, 3352, 1710, 1617, 1599, 1543, 1520, 1346, 1323, 1279, 1181, 1044, 847, 744; Anal. $C_{17}H_{11}F_6N_3O_6S$ (C, H, N).

5.2. Biological studies

5.2.1. Preparation of cytosolic androgen receptor

Male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN), weighing ca. 250 g were castrated 24 h prior to the removal of prostates. Ventral prostates were surgically removed and immersed immediately in ice-cold homogenisation buffer consisting of 10 mM Tris, 1.5 mM disodium EDTA, 0.25 M sucrose, 10 mM sodium molybdate, and 1 mM PMSF adjusted to pH 7.4 [20]. The prostate tissue (about 0.4 g per rat) was minced, weighed, and homogenized (Model PRO 200 homogeniser, Pro Scientific, Monroe, CT) with 1

ml of the homogenisation buffer per 500 mg of prostate tissue. The homogenate was then centrifuged at 114 000 g for 1 h at 0 °C in an ultracentrifuge (Model L8-M, Beckman Instruments Inc., Palo Alto, CA) [21]. The supernatant (cytosol) containing AR protein was removed and stored at −80 °C until use.

5.2.2. AR competitive binding affinity assay

AR binding affinities of the synthesized ligands were determined by competitive binding in the presence of the high affinity AR ligand, ^{3}H-MIB. AR binding studies were performed by incubating increasing concentrations (10^{-2}–10^4 nM) of each ligand with cytosol and a saturating concentration of ^{3}H-MIB (1 nM) at 4 °C for 18 h. In preliminary experiments, the equilibrium dissociation constant (Kd) of MIB was determined under the same procedure by incubating increasing concentrations of ^{3}H-MIB (0.01–10 nM) with cytosol. We found that the minimum concentration of ^{3}H-MIB required to saturate AR sites in the cytosol preparation was 1 nM. The incubates also contained 1000 nM triamcinolone acetonide to prevent interaction of MIB with progesterone receptors [22]. For the determination of non-specific binding, separate experiments were conducted by adding 1000 nM unlabeled MIB to the incubate. Separation of bound and free radioactivity at the end of incubation were achieved by the hydroxyapatite (HAP) method, as described previously [23], and 0.8 ml of the ethanolic supernant was added to 5 ml of scintillation cocktail. Radioactivity was counted in a Beckman LS6500 liquid scintillation counter (Beckman Instruments Inc., Irvine, CA).

5.2.3. Data analysis of AR binding affinity

For competitive binding experiments, specific binding was calculated by subtracting the binding of ^{3}H-MIB observed in the presence of excess unlabeled MIB (non-specific binding) from the binding of ^{3}H-MIB observed in the absence of unlabeled MIB (total binding). Competitive displacement curves were constructed for each ligand with percent specific binding (specific binding of ^{3}H-MIB at a particular ligand concentration expressed as a percentage of the specific binding of ^{3}H-MIB in the absence of ligand) on the vertical axis and ligand concentration on the horizontal axis. The ligand concentration that reduced the percentage of specific binding by 50% (IC_{50}) was determined by computer-fitting the data for each ligand to the following equation:

$$B = B_o[1 - C/(IC_{50} + C)]$$

where B was the specific binding of ^{3}H-MIB in the presence of a particular concentration of ligand, B_o was the specific binding of ^{3}H-MIB in the absence of ligand, and C was the ligand concentration. Binding affinities of the ligands were then compared using their equilibrium dissociation constant (K_i). The K_i of each ligand was calculated using the following equation:

$$K_i = (IC_{50} \times K_d)/(L + K_d)$$

where K_d was the equilibrium dissociation constant of ^{3}H-MIB, L was the concentration of ^{3}H-MIB in the incubate, and IC_{50} was as defined above.

5.2.4. Transcriptional activation assay

To examine the AR agonistic activity of synthesized compounds, AR-mediated transcriptional activation assays were performed in transfected CV-1 cells (American Type Culture Collection, Rockville, MD) as previously described [7] with the exception of certain modifications. One day before transfection, CV-1 cells were seeded into DMEM supplemented with 10% FBS at a density of 2×10^5 cells/well in 12-well tissue culture plates. For the following steps, DMEM without phenol red was used. Transient transfection of plated cells was carried out in serum-free medium using Lipofectamine according to the manufacturer's instructions. Cells in each well were transfected with 50 ng of a human AR expression construct (pCMVhAR; generously provided by Dr Donald J. Tindall, Mayo Clinic and Mayo Foundation, Rochester, MN), 1 μg of a luciferase reporter construct (pMMTV-Luc; generously provided by Dr Ronald Evans at The Salk Institute, San Diego, CA), and 1 μg of a control β-galactosidase expression construct (pSV-β-galactosidase; Promega Corporation, Madison, WI) using 6 μl of Lipofectamine. After 10 h of transfection, cells were washed once with DMEM, and recovered in fresh DMEM supplemented with 0.2% FBS for 10–20 h. After recovery, the cells were treated with various concentrations of testing compound, 1 nM DHT, or vehicle for 48 h. All drugs were initially dissolved in 100% ethanol, and then serially diluted to the desired concentration using DMEM containing 0.2% FBS. The volumes of ethanol in final solutions were less than 0.5%. The final concentrations of testing compound were 1, 10, 100, and 500 nM. The drug containing solutions were replaced with freshly prepared ones at 24 h during the incubation. Following the treatment, the cells were washed twice with 1 × PBS, and lysed with 200 μl well^{-1} of 1 × Reporter Lysis Buffer (Promega Corporation, Madison, WI) at room temperature for 30 min. Cell lysates were then collected in Eppendorf tubes, vortex briefly, and centrifuge at 12 000 g for 2 min. Fifty microliter of supernatant from each well was used for measurement of β-galactosidase activity using a 96 well plate reader (Titertek Multiskan MCC/340, Labsystems Inc., Franklin, MA) set at 414 nm. A separate 100 μl of supernatant from each well was used for measurement of luciferase activity (Luciferase Assay System, Promega Corporation, Madison, WI) using an automated luminometer (Model AutoLumat LB953, Wallac Inc., Gaithersberg, MD).

Transcriptional activation in each well was then calculated as the ratio of luciferase activity to β-galactosidase activity to normalize the variance in cell number and transfection efficiency. Transcriptional activation induced by each testing compound was expressed as the percentage of that induced by 1 nM DHT, and the maximal percentage was the efficacy of that compound. To measure any AR-independent transcriptional activation, a parallel experiment was included in which cells co-transfected with pMMTV-Luc and pSV-β-galactosidase only were treated with 500 nM of testing compound. All experiments were performed in triplicate for at least once. Potency was reported as the lowest concentration of the testing compound that produced maximal transcriptional activation.

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