# Design, Synthesis, and Biological Evaluation of Nonsteroidal Cycloalkane[d]isoxazole-Containing Androgen Receptor Modulators

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Ⓢ *Supporting Information*

ABSTRACT: We report here the design, preparation, and systematic evaluation of a novel cycloalkane[d]isoxazole pharmacophoric fragment-containing androgen receptor (AR) modulators. Cycloalkane[d]isoxazoles form new core structures that interact with the hydrophobic region of the AR ligand-binding domain. To systematize and rationalize the structure—activity relationship of the new fragment, we used molecular modeling to design a molecular library containing over 40 cycloalkane[d]isoxazole derivatives. The most potent compound, 4-(3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazol-3-yl)-2-(trifluoromethyl)benzonitrile (**6a**), exhibits antiandrogenic activity significantly greater than that of the most widely used antiandrogenic prostate cancer drugs bicalutamide (**1**) and hydroxyflutamide (**2**) in reporter gene assays measuring the transcriptional activity of AR (decreasing approximately 90% of the total AR activity) and in competitive AR ligand-binding assays (showing over four times higher potency to inhibit radioligand binding in comparison to bicalutamide). Notably, **6a** maintains its antiandrogenic activity with AR mutants W741L and T877A commonly observed and activated by bicalutamide and hydroxyflutamide, respectively, in prostate cancer patients.
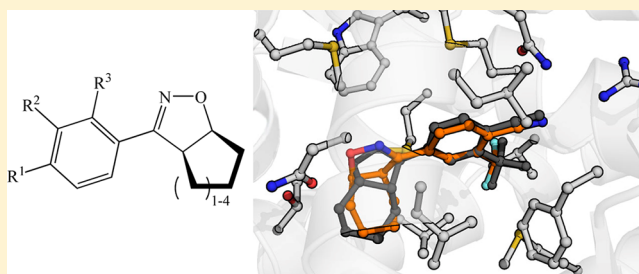
INTRODUCTION

Androgens are especially important for the maintenance and development of the male sexual characteristics.[1] The regulatory effects of the androgens (testosterone and its metabolite 5α-dihydrotestosterone, DHT) are mediated through the androgen receptor (AR), which is a ligand-regulated transcription factor and a member of the nuclear receptor superfamily.[2] The AR is prominently expressed in male reproductive organs but moderately also in female genitalia and ovary as well as tissues such as skin, sebaceous and sweat glands, hair follicles, cardiac and skeletal muscle, and brain.[3] AR has a supreme role in development and progression of prostate cancer,[4,5] which is the second most common cause of cancer-related deaths among men in developed countries.[6] Competitive antiandrogens are needed for maximal androgen blockade in treatment of both early stage and progressed prostate cancer.[7,8]
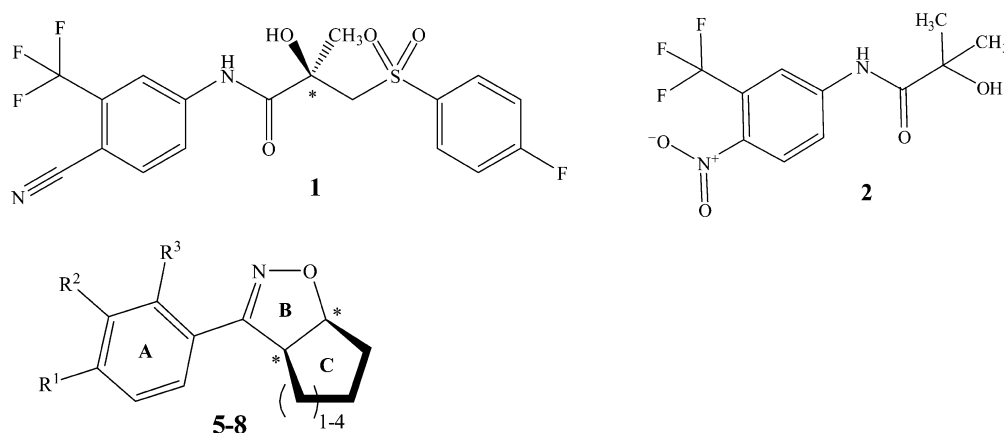
Nonsteroidal selective androgen receptor modulators (SARMs) have been developed to overcome side effects related to the clinical use of steroidal compounds.[4,9−29] The basic idea of the SARMs is that they modulate the transcriptional activity of AR in a tissue-selective fashion.[9−11] Ligand binding induces conformational changes in the ligand binding domain (LBD) of the AR, which modulates its surface topology and the protein—protein interactions between the receptor and other cellular proteins.[26,30] This all offers tools to ligand-selective gene regulation, due to potential changes in recognition of AR DNA-binding sites and/or in interactions with coregulators whose expression levels vary between tissues.[4] Few nonsteroidal antiandrogens have been used clinically for treatment of prostate cancer,[4,10,31,32] but during the treatment, they eventually lose their ability to inhibit the AR, and the cancer turns into a "refractory form", i.e. "castration resistant" prostate cancer (CRPC). This is suggested to result from, for example, mutations in AR, altered expression of AR or its coregulator proteins, aberrant AR posttranslational modifications, and gene fusions resulting in abnormal androgen regulation of oncogenic transcription factors and intracrine androgen production.[33−37] Therefore, there is a continuous need for novel AR antagonists which can tolerate these mutations.[10,31,38−42]

In the present work, a new class of nonsteroidal antiandrogenic compounds have been designed, synthesized, and biologically evaluated by utilizing computer-assisted molecular modeling, flexible molecular libraries,[43,44] and cell-based transcription[45] and binding assays with AR.[46] The developed lead compounds bind to and inhibit the activity of the AR as potently as or better than the most widely used nonsteroidal antiandrogenic drug bicalutamide (**1**)[47] and hydroxyflutamide (**2**, Figure 1).[48] The novel cycloalkane[d]-isoxazole pharmacophoric fragment-containing compounds

Figure 1. The skeletal structure of novel cycloalkane[*d*]isoxazole-containing androgen receptor modulators (**5−8**) and the structures of nonsteroidal androgen antagonists **1** and **2**. Chiral centers are marked with *. Due to cis-geometry of the cycloalkenes, only *R,R*- and *S,S*-enantiomers are formed in the 1,3-dipolar cycloaddition. Ring C points out of the plane formed by rings A and B, giving the molecule a twisted shape (see Figure 2 for a better view of the 3D-structure).

maintained their antagonistic activity with AR mutants commonly observed in prostate cancer patients.

■ RESULTS AND DISCUSSION

Molecular Design. The structures of the earlier described estrogen receptor (ER) agonists[44] were used as a template for the design of the new AR-binding compounds. The aromatic ring (ring A, Figure 1) and the 4,5-dihydroisoxazole ring (ring B) forming the main body of the ER active compounds were left intact, while we focused on modifications at the substituents of the phenyl ring and the size of the novel cycloalkane moiety (ring C) fused with the heterocycle. The new compounds were designed to fill the AR ligand-binding pocket efficiently (Figure 2), to be rigid enough to avoid entropic loss upon binding, and to increase receptor selectivity. These goals were sought with a
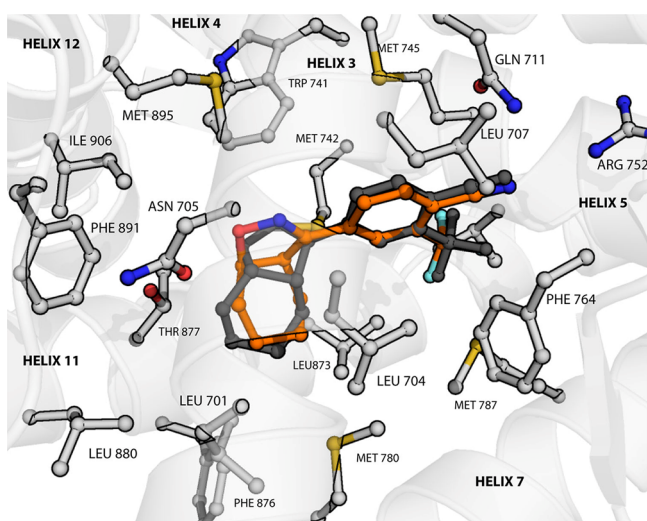

Figure 2. *R,R* (orange) and *S,S* (dark gray) isomers of compound **6a** bound in the AR ligand-binding pocket. The receptor structure shown in the figure is from the energy minimization with *R,R*-isomer. The cyano group makes hydrogen bonds with Arg752 and Gln711. The cyclohexane ring of the ligand fills the other corner of the pocket contacting hydrophobic residues Leu701, Leu704, Trp741, and Met 742. Compound **6a** lacks an aliphatic hydroxyl group, which in the case of DHT connects Asn705 and Thr877 with hydrogen bonds, thus stabilizing the helix 12.

cycloalkane ring that fuses carbons 4 and 5 of isoxazole (ring B) to form cycloalkane[*d*]isoxazoles (Figure 1).

Syntheses. All the present isoxazoles were synthesized starting from appropriate aromatic aldoximes **3** and alkenes **4**, as shown in Scheme 1, using methods described previously in detail by us.[43] Slight modifications were made in an effort to reduce nitrile oxide dimerization and, by that means, to improve yields. The dimerization reaction was mainly a problem with the aldoximes having the aromatic ring substituted with an electron-withdrawing group (nitro or cyano). The method was modified in such a way that the aldoxime dissolved in dichloromethane and triethylamine was added simultaneously dropwise to a dichloromethane/aqueous sodium hypochlorite biphasic mixture containing a 10-fold excess of the cycloalkene dipolarophile. We also tried cycloaddition with *N*-chlorosuccinimide and chloramine-T instead of the hypochlorite, but yields were lower. The in situ-generated nitrile oxide underwent 1,3-dipolar cycloaddition and led to isoxazoles **5−8** (**d−t**). With nitrile-substituted aldoximes, the cycloaddition gave less than 3% yields, but with fluorine substituted precursors, the desired nitrile products **5−8** (**a−c**) were achieved with satisfactory yields. In the latter case, the aromatic fluorine of the cycloaddition product was substituted with a cyano group by performing a cyano-defluorination reaction with KCN.[49,50] After preparation, all the compounds were run through a semipreparative HPLC system.[43] For each successful separation, the enantiomer with a shorter retention time is marked in the tables as ′ and the other enantiomer as ″.

Biological Evaluation. Our structure−activity relationship (SAR) studies were carried out with luciferase reporter gene activity and [³H]R1881 competitive receptor-binding assays. The biological activities of the compounds were compared with those of **1** and **2**. The measured activity differences were considered statistically significant when $p < 0.05$. The reporter assays measured the transcriptional activity of AR. We used a firefly luciferase (FLuc) gene construct driven by an AR-regulated rat probasin promoter fragment which was cotransfected with an AR expression construct into COS-1 cells. When the cells were exposed to an AR agonist testosterone, the AR was activated, resulting in increased transcription of the reporter gene and thereby augmented synthesis and activity of luciferase. Addition of antiandrogens

Scheme 1[a]
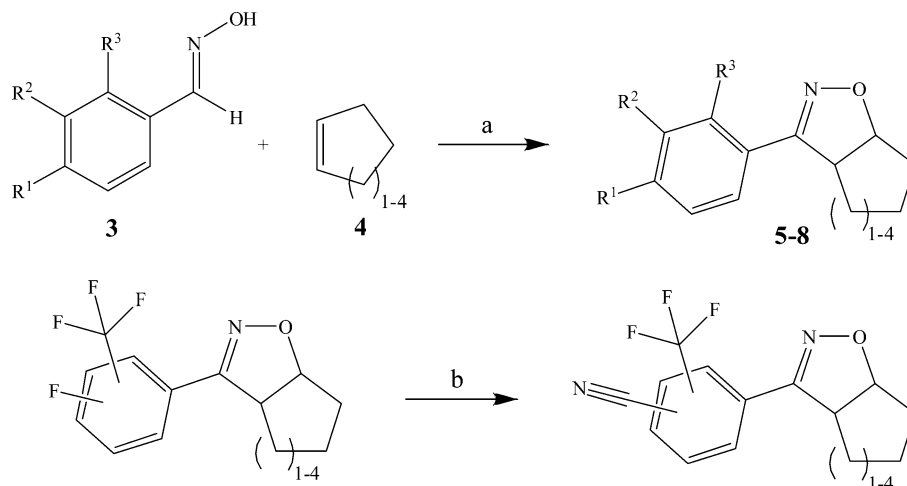
[a]Synthesis of cycloalkane[d]isoxazole-containing androgen receptor modulators: R^1 = H, Cl, CN, F, OMe, NO_2; R^2 = H, Cl, CF_3, F, OMe; R^3 = H, Cl, CF_3, OMe; or R^1 = H, R^2 and R^3 form a naphthalen-1-yl with the Ph. Reagents and conditions: (a) NaOCl, TEA, CH_2Cl_2, 0 °C to rt, 24 h. (b) KCN, DMSO, 80−150 °C, overnight.

together with the agonist inhibited the AR activity leading to a decreased luciferase activity, which was directly proportional to the inhibitory activity of the antiandrogen.[45] The results obtained from these assays with wild type hAR are listed in Tables 1−4.

Well known antiandrogens **1** and **2** (Table 1) reduced the total luciferase activity by 86−87% when 10 μM antiandrogen and 0.1 μM testosterone concentrations were used. The in vitro activity screening revealed several compounds (**6a**, **6c″**, **6d**, **6e′**, **7a**, **7d**, **8b′**, **8e′**, and **8e″**) expressing antagonistic behavior equal or stronger (Tables 1−4) than that of **1** and **2**. Compound **6a** (R^1 = CN, R^2 = CF_3, R^3 = H, Figure 1) reduced the total luciferase activity approximately 90% and **6e′**, **8e′**, and **8e″** (R^1 = NO_2, R^2 = H, R^3 = CF_3) showed over 95% reduction of the activity. Moreover, **6a** and **6d** (R^1 = NO_2, R^2 = CF_3, R^3 = H) were tested using AR LBD mutants W741L and T877A commonly observed in prostate cancer patients,[51−53] and both compounds were found to maintain their antagonist behavior (Figure 3).

We did not detect any agonistic activity using a 10 μM concentration of the ligands with AR construct. In addition, we evaluated the possible partial agonist function of **6a** and **6d** using various concentrations (from 1 nM to 10 μM) of the ligands but did not see any activation of AR (Figure S1 in Supporting Information). We also wanted to rule out the possibility that the observed AR antagonistic behavior of our compounds was due to misinterpreted inhibition of the enzymatic activity of FLuc, which has been shown to be a potential error source causing "false positives" in high-throughput screens.[54,55] However, none of the compounds showed any inhibitory effect at 10 μM concentration when incubated with purified-recombinant FLuc enzyme in vitro.
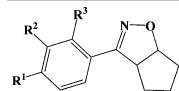
To further characterize the compounds' biological activity log IC_{50} values were determined. Relative binding inhibition (RBI) was measured in COS-1 cells transfected with an AR expression vector and exposed to synthetic AR agonist [3H]R1881 in the absence (with vehicle, ethanol) and presence of AR antagonist. Dose−response curve fitting (e.g., in Figure 4) was performed with GrafPad Prism[56] using least-squares fit with logarithmic scale and variable slope.

Results from the whole cell binding studies showed that our most efficient compound **6a** inhibits the radioligand binding with greater affinity than **1** and **2**. The fitted curve of **6a** indicates that it has over inhibition potency 4-fold higher than that of **1** and 8-fold higher than that of **2**. Furthermore, **7a** which is a cycloheptane-containing analogue of **6a** also binds to AR significantly stronger than that of **1** and **2** and shows similar antiandrogenic activity decreasing the total luc-reporter activity by ca. 87%. Overall, compounds bearing 4-NO_2 or 4-CN group and 3-CF_3 or 2-CF_3 group attached to the aromatic moiety and cyclohexane- or cycloheptane[d]isoxazole pharmacophoric fragment have the best affinities for the hAR. The electron-withdrawing group in ring A (Figure 1) seems to be crucial for AR binding as well as for their antagonistic behavior. Similar aromatic substituents are found also in **1** and **2**.[4]

We also evaluated several other aromatic substituents to characterize the main interactions between the receptor and the ligand (Table 2). Compounds bearing a halogen, hydroxyl, or methoxyl group as a substituent on ring A showed only weak affinity for AR. We were not able to fit inhibition curves for all the compounds presented here (marked with N.D. in Tables 1−4), but a few of these compounds showed inhibition in the competitive binding assay at 10000 molar excess compared to radioligand (inh% in Tables 1−4). Also the naphthalen-1-yl group (**6t′**) showed weak affinity. Compounds having an aromatic fluorine together with a trifluoromethyl group (**5f**, **5g′**, **5g″**, **6f**, **6g′**, **6g″**, **7f**, **7g′**, **7g″**, **8f′**, **8f″**, **8g′**, and **8g″**) showed significant affinity to AR. Interestingly, compounds which have *para*-nitro (**6i**) or *para*-cyano (**6h′** and **6h″**) group without trifluoromethyl show very weak ability to inhibit the binding of [3H]R1881. On the basis of these results, the polar trifluoromethyl substituent seems to be essential for AR binding affinity and antagonist activity. We wanted also to determine the influence of the position of cyano group in the ring A with compound **6a** (*para*-cyano) and enantiomers **6c′** and **6c″** (*meta*-cyano). Although both isomers of **6c** are very active, racemic mixture **6a** has significantly higher potency to inhibit radioligand binding. For most ligands, the results of FLuc reporter and whole cell binding assays seem to correlate very well, i.e., the most active compounds also appear to bind to

Table 1. In Vitro Biological Evaluation of Bicalutamide, Hydroxyflutamide, Testosterone, and Cyclopentane[d]isoxazole Derivatives

	References	Transcriptional AR antagonism[a]		Relative binding inhibition (RBI)[b]			
Entry	Compound	Ant. %[c]	SD$_{ant}$[c]	logIC$_{50}$[d]	logSE[d]	Inh. %[e]	SD$_{inh}$[e]
1	(R)-Bicalutamide	13.5	0.8	-6.92	0.06	96.6	1.1
2	Hydroxyflutamide	13.0	1.1	-6.65	0.06	94.8	0.8
	Testosterone	100	1.8	-8.34	0.05	0.1	0.04
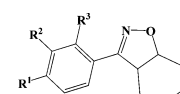
				Transcriptional AR antagonism		Relative Binding Inhibition (RBI)			
Entry	R^1	R^2	R^3	Ant. %	SD$_{ant}$	logIC$_{50}$	logSE	Inh. %	SD$_{inh}$
5a'[f]	CN	CF$_3$	H	44.9	3.6	-6.21	0.05	87.6	0.3
5a''[f]	CN	CF$_3$	H	18.9	0.5	-6.65	0.04	91.8	1.7
5b[f]	CN	H	CF$_3$	29.3	3.7	-6.35	0.04	91.7	1.7
5d'	NO$_2$	CF$_3$	H	19.2	6.8	-5.60	0.06	69.7	4.5
5d''	NO$_2$	CF$_3$	H	22.9	4.9	-6.52	0.04	90.9	0.9
5e	NO$_2$	H	CF$_3$	21.8	4.1	-6.57	0.05	93.6	2.1
5f	F	CF$_3$	H	54.6	2.3	-5.83	0.04	84.5	4.9
5g'	F	H	CF$_3$	23.2	0.5	-5.87	0.06	86.5	6.6
5g''	F	H	CF$_3$	37.4	7.3	-5.81	0.03	85.0	3.3

[a]AR antagonism activity was determined by FLuc gene construct driven by an AR-regulated rat probasin promoter fragment which was cotransfected with an AR expression construct (pSG5-hAR) into COS-1 cells. [b]Relative binding inhibition (RBI) was measured using a whole COS-1 cell assay in which the cells were transfected with pSG5-hAR, exposed to synthetic AR agonist [3H]R1881 in the absence (with vehicle, ethanol) and the presence of a wide range of compound concentrations. [c]The relative transcriptional activity of AR in the presence of 100 nM testosterone (set as 100), sample concentrations 10 μM. Results are presented as mean together with standard deviation (SD$_{ant}$). [d]Relative binding inhibition (log IC$_{50}$) was measured using competitive whole cell binding assay in the presence of 1.34 nM the [3H]R1881 with a wide range of compound concentrations to obtain their binding inhibition curves and to determine each chemical's log IC$_{50}$ value (log M) with standard error (log SE). [e]Inhibition percentage (inh %) at the highest measured concentration (10000-fold molar excess compared to [3H]R1881) are presented together with standard deviation (SD$_{inh}$). Nonspecific binding was measured, and complete inhibition of the specific binding of the radioligand was set as 100%. [f]Symbol ′ = enantiomer with a shorter retention time in the chiral separation and ″ = the enantiomer with a longer retention time (retention times reported in Experimental Procedures). Entry number without a symbol means that we were unable to separate the enantiomers and that a racemic mixture was tested.
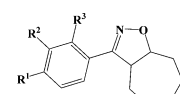
the receptor with the highest potency. In many cases, the enantiomers of the same compound differ significantly in the potency of AR activation (e.g., **5a′** compared to **5a″**).

To evaluate cycloalkane[d]isoxazole interactions, we systematically studied how the size of the C ring affects the binding affinity of the compounds possessing both *meta*-trifluoromethyl and *para*-cyano groups as aromatic substituents. Ligand docking suggested that the C ring contacts hydrophobic side chains of Leu701, Leu704, Trp41, and Met742 (Figure 2). In this series of compounds the cyclohexane (**6a**) and cycloheptane (**7a**) derivatives bind significantly stronger than the other members of the cycloalkane homology series. Cyclopentane-containing ligands **5a′** and **5a″** bind significantly weaker than **6a** and are the weakest binders of all the homologues. On the other hand, the cycloheptane derivative **7a** expresses affinity to AR stronger than that of cyclooctane-containing **8a**. Reduction of the aliphatic ring size probably

Table 2. In Vitro Biological Evaluation of Cyclohexane[d]isoxazole Derivatives

				Transcriptional AR antagonism[a]		Relative binding inhibition (RBI)[b]			
Entry	R^1	R^2	R^3	Ant. %[c]	SD$_{ant}$[c]	logIC$_{50}$[d]	logSE[d]	Inh. %[e]	SD$_{inh}$[e]
6a[f]	CN	CF$_3$	H	9.2	1.7	-7.56	0.10	99.2	0.5
6b'[f]	CN	H	CF$_3$	27.5	4.1	-5.75	0.06	78.8	0.6
6b''[f]	CN	H	CF$_3$	20.3	1.7	-5.94	0.05	83.6	0.9
6c'	H	CN	CF$_3$	17.8	3.8	-7.10	0.03	97.4	0.4
6c''	H	CN	CF$_3$	5.4	1.7	-7.19	0.03	97.6	0.2
6d	NO$_2$	CF$_3$	H	10.6	1.5	-6.92	0.04	95.1	1.5
6e'	NO$_2$	H	CF$_3$	2.9	0.5	-6.89	0.05	95.1	1.3
6e''	NO$_2$	H	CF$_3$	75.9	3.7	-6.58	0.04	93.8	0.7
6f	F	CF$_3$	H	53.4	5.4	-5.96	0.04	88.5	4.2
6g'	F	H	CF$_3$	49.7	5.1	-5.94	0.06	84.6	0.7
6g''	F	H	CF$_3$	27.0	6.1	-6.01	0.04	87.7	3.0
6h'	CN	H	H	110.7	3.1	N.D.[g]	N.D.	-5.4	6.8
6h''	CN	H	H	115.8	10.0	N.D.	N.D.	-5.8	1.0
6i	NO$_2$	H	H	48.0	6.7	N.D.	N.D.	15.0	4.5
6j'	F	H	H	101.6	7.9	N.D.	N.D.	-3.1	6.9
6j''	F	H	H	99.3	2.6	N.D.	N.D.	-4.2	3.1
6k	H	F	H	100.3	2.1	N.D.	N.D.	-3.5	7.2
6l''	H	H	F	101.2	10.6	N.D.	N.D.	17.0	7.0
6l'	H	H	F	103.6	11.5	N.D.	N.D.	-5.6	4.3
6m'	Cl	H	H	40.9	2.4	N.D.	N.D.	30.5	5.4
6m''	Cl	H	H	91.4	7.7	N.D.	N.D.	-5.6	3.0
6n'	H	Cl	H	95.2	4.5	N.D.	N.D.	20.3	9.9
6n''	H	Cl	H	106.7	5.8	N.D.	N.D.	-8.6	5.7
6o'	H	H	Cl	101.5	2.0	N.D.	N.D.	30.2	3.8
6o''	H	H	Cl	101.2	4.0	N.D.	N.D.	66.3	6.5
6p'	OMe	H	H	100.5	5.8	N.D.	N.D.	0.0	3.3
6p''	OMe	H	H	99.2	2.4	N.D.	N.D.	2.2	3.3
6q'	H	OMe	H	101.8	2.5	N.D.	N.D.	2.9	1.1
6q''	H	OMe	H	102.7	3.3	N.D.	N.D.	-0.7	5.9
6r	H	H	OMe	100.0	1.7	N.D.	N.D.	2.6	1.0
6s'	H	H	H	103.2	6.8	N.D.	N.D.	-6.8	4.7
6s''	H	H	H	100.5	5.2	N.D.	N.D.	-3.6	4.7
6t'	3-Naphthalen-1-yl			40.2	7.1	N.D.	N.D.	68.4	1.9
6t''	3-Naphthalen-1-yl			103.3	8.1	N.D.	N.D.	50.6	8.5

[a–f]See Table 1. [g]N.D. indicates a negligible functional potency that could not be detected.

**Table 3. In Vitro Biological Evaluation of Cycloheptane[d]isoxazole Derivatives**

				Transcriptional AR antagonism[a]		Relative binding inhibition (RBI)[b]			
Entry	R^1	R^2	R^3	Ant. %[c]	SD$_{ant}$[c]	logIC$_{50}$[d]	logSE[d]	Inh. %[e]	SD$_{inh}$[e]
7a[f]	CN	CF$_3$	H	12.6	0.7	-7.36	0.03	98.7	0.6
7b'[f]	CN	H	CF$_3$	29.8	3.6	-5.94	0.04	85.4	1.5
7b''[f]	CN	H	CF$_3$	27.2	4.8	-6.00	0.05	87.4	2.7
7d	NO$_2$	CF$_3$	H	10.1	0.9	-6.98	0.03	97.8	0.5
7e'	NO$_2$	H	CF$_3$	17.4	1.5	-6.51	0.05	90.5	3.1
7e''	NO$_2$	H	CF$_3$	25.5	1.5	-6.41	0.05	89.5	2.0
7f	F	CF$_3$	H	43.8	2.4	-4.96	0.02	55.7	5.8
7g'	F	H	CF$_3$	28.8	2.0	-6.09	0.04	88.8	3.1
7g''	F	H	CF$_3$	15.6	0.8	-6.18	0.03	92.2	0.7

[a–f]See Table 1.

**Table 4. In Vitro Biological Evaluation of Cyclooctane[*d*]isoxazole Derivatives**

Entry	R^1	R^2	R^3	Transcriptional AR antagonism[a]		Relative binding inhibition (RBI)[b]			
				Ant. %[c]	SD_{ant}[c]	$logIC_{50}$[d]	logSE[d]	Inh. %[e]	SD_{inh}[e]
8a[f]	CN	CF_3	H	23.9	1.7	−6.53	0.07	85.8	1.3
8b′[f]	CN	H	CF_3	9.4	0.3	−6.19	0.04	87.3	2.9
8b″[f]	CN	H	CF_3	65.5	9.0	−5.70	0.07	76.0	4.8
8d	NO_2	CF_3	H	27.8	1.0	−6.15	0.04	85.7	3.8
8e′	NO_2	H	CF_3	3.1	1.7	−6.13	0.09	85.9	5.5
8e″	NO_2	H	CF_3	2.2	0.8	−6.11	0.05	89.4	3.5
8f′	F	CF_3	H	57.7	9.9	−5.23	0.05	66.3	9.8
8f″	F	CF_3	H	56.6	1.4	−5.55	0.04	76.0	0.3
8g′	F	H	CF_3	19.8	0.5	N.D.[g]	N.D.	49.9	5.9
8g″	F	H	CF_3	35.6	7.8	−4.88	0.05	50.2	5.9

[a]–[f]See Table 1. [g]See Table 2.
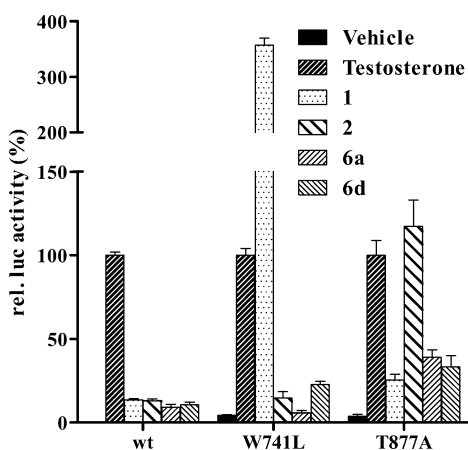
Figure 3. Comparison of **6a**, **6d**, bicalutamide (**1**), and hydroxy-flutamide (**2**) on the transcriptional activity of WT, W741L, or T877A AR as assessed by luciferase reporter gene assay. COS-1 cells were transfected with pSG5-hAR, pSG5-hARW741L, or pSG5-hART877A with pProbasin(−285/+32)-Luc reporter, and the cells were treated with vehicle (ethanol), 100 nM testosterone, or 10 μM **1**, **2**, **6a**, or **6d** for 18 h before harvesting the cells for reporter analyses. Results are shown as relative luciferase (rel luc) activity, with reporter activity in the presence of testosterone set as 100%. Columns present the mean ± SD.

leads to a loss of favorable contacts with the receptor, whereas a larger ring size may be too bulky to optimally fill the binding pocket (Figure 2). In addition, it is possible that changing the number of the carbons in the aliphatic C ring alters its orientation relative to the main body formed by the A and B rings, thus also modifying the overall shape of the molecule. Hydrogen bonds formed by the *para*-nitro or -cyano group of the A ring are important for binding affinity. These groups are supposed to bind to the hydrogen-bonding network formed by residues Arg752 and Gln711 (Figure 2). Comparison of *para*-cyano- and *meta*-trifluoromethyl-containing ligands to *para*-nitro- and *meta*-trifluoromethyl-containing ligands shows that the binding affinity significantly increases in most cases when substituting the nitro group with a cyano group (**5**−**8a** in comparison to **5**−**8d**). Altogether, having *para*-cyano together with a *meta*-trifluoromethyl group at the phenyl ring together with a novel cyclohexane[*d*]isoxazole pharmacophoric fragment
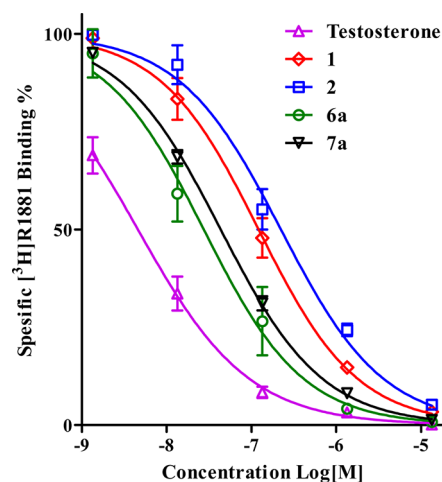
Figure 4. Competition of [^{3}H]R1881 binding from AR in COS-1 cells. Inhibition of specific [^{3}H]R1881 binding by each compound is expressed relative to the value in the absence of competition set to 100. Data are presented as mean ± SEM. Curve fitting and log IC_{50} calculations were performed using GraphPad Prism software.

is essential for the maximal binding and activity of the novel AR antagonists.

ECD Spectra and Absolute Configurations. The HPLC-separated enantiomers of compounds **5a**, **5d**, **6e**, and **7e** were selected as examples for the characterization of the absolute configurations by using electronic circular dichroism (ECD) spectroscopy and time-dependent density functional theory calculations. Compound **5a** possesses an aromatic substitution similar to the most potent ligand **6a** (4-CN, 3-CF_3), and **5d** is their 4-NO_2 analogue (Table 1). On the other hand, compound **6e** was chosen for the experiment because the difference of the transcriptional antagonistic effect between the enantiomers **6e′** and **6e″** is particularly significant (Table 2). The measured CD spectra of the ′ and ″ forms and the theoretical spectra of the *S,S*-enantiomers of the four compounds are shown in Figures S2−S5 (Supporting Information). In each sample, the two enantiomers show clear mirror images in the CD spectra. Comparison of the spectra suggests that **5a′** and **5d′** are *S,S*-enantiomers, whereas **6e′** and **7e′** have *R,R*-configurations. In the case of **5a**, **5d**, and **6e**, the prediction of the absolute configuration can be considered reliable. In general, the calculated spectra are red-shifted by 10−30 nm compared to experimental spectra. For **7e**, also the relative intensities of the measured and calculated bands show notable deviation, making the prediction less reliable but still indicative. Compound **6e′**, which according to our ECD prediction possesses the *R,R*-configuration, is a remarkably stronger transcriptional antagonist compared with the *S,S*- enantiomer **6e″** (Table 2). Thus, this also suggests that binding of (*R,R*)-**6a** (which differs from **6e** only by the para substitution) to the LBP of AR shown in Figure 2 represents the mode that leads to AR antagonism higher than that of the alternative configuration.

■ CONCLUSIONS

In this report, we have described the cycloalkane[*d*]isoxazole moiety as a novel pharmacophoric fragment interacting with the hydrophobic region of the AR ligand-binding pocket. Our SAR studies show that the key features for maximal biological activity are an aromatic ring bearing an electron-withdrawing group (*para*-cyano together with *meta*-trifluoromethyl) togeth-

er with the novel cyclohexane[*d*]isoxazole moiety. The most potent structure, **6a**, fills the AR ligand-binding pocket by making favorable hydrophobic contacts with the receptor. The best ligand **6a** shows four times higher potency to inhibit radiolabeled ligand binding compared with that of **1**. Our reporter assays measuring transcriptional activity of AR indicate that the compound exhibits strong antiandrogenic activity with a potency significantly greater than that of the most widely used antiandrogenic prostate cancer drugs **1** and **2**. It is also notable that the lead compound maintained its antiandrogenic activity with AR mutants W741L and T877A that are activated by **1** and **2**, respectively, and commonly observed in prostate cancer patients. To summarize, our novel compounds form a library of highly potential AR antagonists. The cyclohexane[*d*]isoxazole pharmacophoric fragment interacts with the AR LBD and can thus be considered as a unique lead for further development of AR modulators with a high potency. The next logical step will be the characterization of the in vivo behavior of the compounds.

■ MATERIALS AND METHODS

Reagents and Equipment. Aldoximes **3** were synthesized from the corresponding benzaldehydes and hydroxylamine hydrochloride.[18] All the other starting materials, reagents, and solvents were commercial products. DMSO was distilled prior to use. Preparative thin layer chromatography was performed using 2.0 mm thick silica gel plates with fluorescent indicator UV$_{254}$ (Macherey-Nagel). HPLC purifications and chiral separations were performed on a Shimadzu chromatography system using a Regis Technologies (*R*,*R*)-Whelk-O 2 (25 cm × 10 mm i.d.) column in *n*-hexane/*i*-PrOH/AcOH at flow rate 5 mL/min, and the compounds were detected by UV absorption at 254 nm. The purity of >95% of the compounds was confirmed by GC/EI-MS data recorded on a Hewlett-Packard (Palo Alto, CA) gas chromatography mass spectrometer. High-resolution electrospray mass spectra were obtained on an Applied Biosystems/MDS Sciex QSTAR XL spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer operating at 500.13 and 125.77 MHz, respectively. All *J* coupling constants are given in hertz. The CD spectra were acquired with a Jasco J-700 spectropolarimeter and processed using the J-700 program for Windows. The experiments were carried out at rt using quartz cells (Hellma GmbH, Germany) with Suprasil windows and an optical path length of 0.5 cm. The HPLC-separated pure enantiomers were dissolved in acetonitrile to 100 μM concentration, and the spectra were recorded in the wavelength range of 190−450 nm.

Synthesis of Cycloalkane[*d*]isoxazoles (compounds 5d−g, 6d−g, 6i−t, 7d−g, and 8d−g). *Procedure A (Scheme 1).* To a vigorously stirred solution of alkene **4** (10 mmol) in 3 mL of DCM was added 5% NaOCl solution (5 mL) at 0 °C. Then aldoxime **3** (1 mmol) in 2 mL of DCM and TEA (1.08 mmol) were added dropwise, the temperature was allowed to rise to rt, and the mixture was stirred for 24 h. The water layer was separated and washed twice with 5 mL of DCM. Then the organic layers were combined and washed with 2 M HCl and saturated NaHCO$_3$ and H$_2$O and evaporated to give the crude product, which was purified by TLC using DCM as an eluent.

3-(4-Nitro-3-(trifluoromethyl)phenyl)-4,5,6,6a-tetrahydro-3aH-cyclopenta[d]isoxazole (5d). Yield 16%, a yellow wax; ^{1}H NMR δ 8.13 (d, 1 H, *J* = 1.6), 7.95 (dd, 1 H, *J* = 8.5, 1.6), 7.90 (d, 1 H, *J* = 8.5), 5.32 (m, 1 H), 4.01 (m, 1 H), 2.19 (m, 1 H), 1.97−1.83 (m, 2 H), 1.82−1.75 (m, 2 H), 1.50 (m, 1 H); ^{13}C NMR δ 156.5, 148.2, 134.8 (3 s), 131.0 (d), 126.5 (q), 126.1 (d), 124.8 (dq), 122.1 (q), 90.0, 51.4 (2 d), 36.1, 31.8, 23.9 (3 t). Chiral separation in *n*-PrOH/AcOH 98/2/0.5, retention times 19.4 min (**5d′**) and 22.1 min (**5d″**). HRMS (ESI+) calcd for C$_{13}$H$_{12}$F$_3$N$_2$O$_3$ [M + H]$^+$ 301.0800, found 301.0794 (**5d′**) and 301.0812 (**5d″**).

3-(4-Nitro-2-(trifluoromethyl)phenyl)-4,5,6,6a-tetrahydro-3aH-cyclopenta[d]isoxazole (5e). Yield 13%, yellow viscous oil ^{1}H NMR δ 8.61 (d, 1 H, *J* = 2.2), 8.41 (dd, 1 H, *J* = 8.5, 2.2), 7.70 (d, 1 H, *J* =

8.5), 5.31 (dd, 1 H, *J* = 8.9, 5.6), 4.13 (dd, 1 H, *J* = 8.9, 8.4), 2.19 (dd, 1 H, *J* = 12.2, 6.8), 1.70−1.45 (m, 5 H); ^{13}C NMR δ 156.2, 148.0, 135.4 (3 s), 132.9 (d), 130.6 (q), 126.6 (d), 122.6 (q), 122.5 (dq), 88.5 (d), 54.9 (dq), 35.7, 30.6, 23.1 (3 t). HRMS (ESI+) calcd for C$_{16}$H$_{18}$F$_3$N$_2$O$_3$ [M + H]$^+$ 301.0800, found 301.0812.

3-(4-Fluoro-3-(trifluoromethyl)phenyl)-4,5,6,6a-tetrahydro-3aH-cyclopenta[d]isoxazole (5f). Yield 70%, a yellow wax; ^{1}H NMR δ 7.94 (dd, 1 H, *J* = 7.0, 1.4), 7.88 (dd, 1 H, *J* = 9.0, 6.8, 1.4, 1 H), 7.24 (dd, 1 H, *J* = 9.4, 9.0), 4.28 (dd, 1 H, *J* = 8.5, 5.0), 4.03 (m, 1 H), 2.19 (m, 1 H), 1.95−1.87 (m, 2 H), 1.85−1.73 (m, 2 H), 1.53 (m, 1 H); ^{13}C NMR δ 160.7 (dd), 157.0 (s), 132.6 (dd), 126.6 (d), 126.0 (qd), 122.6 (q), 119.1 (qd), 117.8 (dd), 88.9, 52.1 (2 d), 36.1, 31.7, 23.8 (3 t). HRMS (ESI+) calcd for C$_{13}$H$_{12}$F$_4$NO [M + H]$^+$ 274.0855, found 274.0858.

3-(4-Fluoro-2-(trifluoromethyl)phenyl)-4,5,6,6a-tetrahydro-3aH-cyclopenta[d]isoxazole (5g). Yield 75%, a yellow wax; ^{1}H NMR δ 7.43−7.38 (m, 2 H), 7.24 (ddd, 1 H, *J* = 8.2, 7.9, 2.6), 5.20 (m, 1 H), 4.02 (m, 1 H), 2.11 (m, 1 H), 1.72−1.65 (m, 2 H), 1.64−1.57 (m, 2 H), 1.47 (m, 1 H); ^{13}C NMR δ 163.0 (dd), 157.2 (s), 133.9 (dd), 131.3 (qd), 125.3 (d), 123.3 (qd), 119.4 (dd), 115.0 (ddq), 87.9, 55.7 (2 d), 36.2, 30.7, 23.4 (3 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 8.8 min. (**5g′**) and 10.2 min. (**5g″**). HRMS (ESI+) calcd for C$_{13}$H$_{12}$F$_4$NO$_2$ [M + H]$^+$ 274.0855, found 274.0858 (**5g′**) and 274.0852 (**5g″**).

3-(4-Nitro-3-(trifluoromethyl)phenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6d). Yield 1%, a yellow wax; ^{1}H NMR δ 8.15 (d, 1 H, *J* = 1.7), 8.02 (dd, 1 H, *J* = 8.5, 1.7), 7.93 (d, 1 H, *J* = 8.5), 4.61 (m, 1 H), 3.31 (m, 1 H), 2.33 (m, 1 H), 2.00 (m, 1 H), 1.80 (m, 1 H), 1.78−1.63 (m, 2 H), 1.54 (m, 1 H), 1.32−1.21 (m, 2 H); ^{13}C NMR (in acetone-*d$_6$*) δ 163.1, 149.3, 135.9 (3 s), 133.1, 127.5 (2 d), 127.0 (dq), 124.8, 124.5 (2 q), 82.9, 44.4 (2 d), 27.3, 25.8, 23.0, 21.3 (4 t). HRMS (ESI+) calcd for C$_{14}$H$_{14}$F$_3$N$_2$O$_3$ [M + H]$^+$ 315.0957, found 315.0941.

3-(4-Nitro-2-(trifluoromethyl)phenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6e). Yield 4%, yellow wiscous oil ^{1}H NMR: δ 8.62 (d, 1 H, *J* = 2.1), 8.45 (dd, 1 H, *J* = 8.3, 2.1), 7.76 (d, 1 H, *J* = 8.3), 4.69 (m, 1 H), 3.40 (m, 1 H), 2.15 (m, 1 H), 1.82 (m, 1 H), 1.72−1.62 (m, 2 H), 1.58 (m, 1 H), 1.34−1.22 (m, 2 H); ^{13}C NMR δ 161.8, 149.5, 135.6 (3 s), 133.1 (d), 130.7 (q), 126.9 (d), 123.9 (q), 122.6 (dq), 81.3 (d), 48.1 (dq), 25.6, 25.4, 22.1, 20.3 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 90/10/0.5, retention times 13.2 min (**6e′**) and 14.5 min (**6e″**). HRMS (ESI+) calcd for C$_{14}$H$_{14}$F$_3$N$_2$O$_3$ [M + H]$^+$ 315.0957, found 315.0943 (**6e′**) and 315.0959 (**6e″**).

3-(4-Fluoro-3-(trifluoromethyl)phenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6f). Yield 55%, a yellow wax; ^{1}H NMR δ 7.94−7.88 (m, 2 H), 7.25 (dd, 1 H, *J* = 9.3, 9.2), 4.54 (m, 1 H), 3.72 (m, 1 H), 2.26 (m, 1 H), 1.97 (m, 1 H), 1.78 (m, 1 H), 1.77−1.59 (m, 2 H), 1.54 (m, 1 H), 1.38−1.15 (m, 2 H); ^{13}C NMR δ 162.3 (s), 160.8, 138.7 (2 d), 132.6, 126.6 (2 dd), 126.1 (qd), 122.6 (q), 116.3 (dd), 81.4, 45.0 (2 d), 26.7, 25.3, 22.5, 20.4 (4 t). HRMS (ESI+) calcd for C$_{14}$H$_{14}$F$_4$NO [M + H]$^+$ 288.1012, found 288.1017.

3-(4-Fluoro-2-(trifluoromethyl)phenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6g). Yield 51%, a yellow wax; ^{1}H NMR δ 7.52 (dd, 1 H, *J* = 8.4, 5.5), 7.47 (dd, 1 H, *J* = 8.7, 2.6), 7.30 (ddd, 1 H, *J* = 8.5, 8.4, 2.6), 4.63 (m, 1 H), 3.31 (m, 1 H), 2.10 (m, 1 H), 1.81 (m, 1 H), 1.68 (m, 1 H) 1.59−1.52 (m, 3 H), 1.38−1.20 (m, 2 H); ^{13}C NMR δ 163.1 (dd), 162.7 (s), 133.8 (dd), 131.4 (qd), 125.3 (d), 123.4 (qd), 119.3 (dd), 115.0 (ddq), 80.8, 48.4 (2 d), 25.8, 25.4, 22.3, 20.5 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 7.2 min (**6g′**) and 9.6 min (**6g″**). HRMS (ESI+) calcd for C$_{14}$H$_{14}$F$_4$NO [M + H]$^+$ 288.1012, found 288.1007 (**6g′**) and 288.1002 (**6g″**).

3-(4-Nitrophenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6i). Yield 6%, a yellow viscous oil; ^{1}H NMR δ 8.25 (m, 2 H), 7.88 (m, 2 H), 4.58 (m, 1 H), 3.30 (m, 1 H), 2.30 (m, 1 H), 2.00 (m, 1H), 1.81 (m, 1 H), 1.75−1.64 (m, 2H), 1.55 (m, 1 H), 1.33−1.21 (m, 2H). ^{13}C NMR δ 162.7, 148.8, 136.0 (3 s), 127.9, 124.5, 81.9, 44.3 (4 d), 26.7, 25.3, 22.4, 20.4 (4 t). HRMS (ESI+) calcd for C$_{13}$H$_{15}$N$_2$O$_3$ [M + H]$^+$ 247.1183, found 247.1077.

3-(4-Fluorophenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6j). Yield 11%, a yellow wax; ^{1}H NMR δ 7.70 (m, 2 H), 7.09 (m, 2 H), 4.50 (m, 1 H), 3.23 (m, 1 H), 2.26 (m, 1 H), 1.96 (m, 1 H), 1.77 (m, 1 H), 1.71−1.60 (m, 2H), 1.54 (m, 1 H), 1.30−1.20 (m, 2H); ^{13}C NMR δ 164.1 (d), 163.1 (s), 129.2 (dd), 126.1 (d), 116.3 (dd), 80.9, 45.0 (2 d), 26.8, 25.4, 22.7, 20.6 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 90/10/0.5, retention times 6.8 min (6j′) and 7.6 min (6j″). HRMS (ESI+) calcd for $C_{13}H_{15}FNO$ [M + H]$^+$ 220.1138, found 220.1130 (6j′) and 220.1140 (6j″).

3-(3-Fluorophenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6k). Yield 11%, a yellow wax; ^{1}H NMR δ 7.47 (d, 1 H, J = 7.9), 7.43 (ddd, 1 H, J = 9.0, 2.5, 1.5), 7.37 (ddd, 1 H, J = 8.1, 8.1, 5.9), 7.09 (ddd, 1 H, J = 8.3, 8.1, 2.5), 4.52 (m, 1 H), 3.23 (m, 1 H), 2.27 (m, 1 H), 1.98 (m, 1 H), 1.77 (m, 1 H), 1.72−1.61 (m, 2H), 1.55 (m, 1 H), 1.30−1.20 (m, 2H); ^{13}C NMR δ 163.5, 163.3, 132.0 (3 d), 130.6, 123.0, 117.2, 114.1 (4 dd), 81.1, 44.8 (2 d), 26.8, 25.4, 22.6, 20.5 (4 t). HRMS (ESI+) calcd for $C_{13}H_{15}FNO$ [M + H]$^+$ 220.1138, found 220.1141.

3-(2-Fluorophenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6l). Yield 25%, a yellow viscous oil; ^{1}H NMR δ 7.85 (ddd, 1 H, J = 7.6, 7.5, 1.7), 7.38 (m, 1 H), 7.17 (ddd, 1 H, J = 7.6, 7.5, 0.9), 7.11 (dd, 1 H, J = 11.3, 8.4), 4.54 (m, 1 H), 3.44 (m, 1 H), 2.19 (m, 1 H), 1.90 (m, 1 H), 1.78 (m, 1 H), 1.64−1.55 (m, 2H), 1.54 (m, 1 H), 1.30−1.20 (m, 2H); ^{13}C NMR δ 160.9, 160.7 (2 d), 132.0, 129.9, 124.9 (3 dd), 118.0 (d), 116.8 (dd), 80.9, 46.3 (2 d), 25.9, 25.6, 22.5, 20.6 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 90/10/0.5, retention times 6.1 min (6l′) and 6.9 min (6l″). HRMS (ESI+) calcd for $C_{13}H_{15}FNO$ [M + H]$^+$ 220.1138, found 220.1140 (6l′) and 220.1138 (6l″).

3-(4-Chlorophenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6m). Yield 25%, a colorless wax; ^{1}H NMR δ 7.64 (m, 2 H), 7.37 (m, 2 H), 4.50 (m, 1 H), 3.22 (m, 1 H), 2.27 (m, 1 H), 1.95 (m, 1 H), 1.77 (m, 1 H), 1.72−1.60 (m, 2H), 1.54 (m, 1 H), 1.28−1.20 (m, 2H); ^{13}C NMR δ 163.4, 136.3 (2 s), 129.5, 128.5 (2 d), 128.3 (s), 81.0, 44.7 (2 d), 26.8, 25.4, 22.7, 20.6 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 9.0 min (6m′) and 10.8 min (6m″). HRMS (ESI+) calcd for $C_{13}H_{15}ClNO$ [M + H]$^+$ 236.0842, found 236.0838 (6m′) and 236.0849 (6m″).

3-(3-Chlorophenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6n). Yield 11%, a colorless wax; ^{1}H NMR δ 7.68 (dd, 1 H, J = 1.7, 1.6), 7.59 (ddd, 1 H, J = 7.5, 1.7, 1.6), 7.37 (ddd, 1 H, J = 7.5, 1.7, 1.7), 7.31 (ddd, 1 H, J = 8.4, 7.5), 4.51 (m, 1 H), 3.23 (m, 1 H), 2.27 (m, 1 H), 1.97 (m, 1 H), 1.77 (m, 1 H), 1.71−1.60 (m, 2H), 1.54 (m, 1 H), 1.27−1.22 (m, 2H); ^{13}C NMR δ 163.3, 135.2 (2 s), 130.4, 130.3 (2 d), 128.3 (s), 127.3, 125.4, 81.1, 44.7 (4 d), 26.7, 25.4, 22.6, 20.5 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 8.7 min (6n′) and 9.6 min (6n″). HRMS (ESI+) calcd for $C_{13}H_{15}ClNO$ [M + H]$^+$ 236.0842, found 236.0842 (6n′) and 236.0833 (6n″).

3-(2-Chlorophenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6o). Yield 24%, a colorless wax; ^{1}H NMR δ 7.53 (dd, 1 H, J = 7.6, 1.8), 7.43 (dd, 1 H, J = 8.1, 1.2), 7.35 (ddd, 1 H, J = 8.1, 7.5, 1.8), 7.30 (dd, 1 H, J = 7.6, 7.5, 1.2), 4.67 (m, 1 H), 3.70 (m, 1 H), 2.04 (m, 1 H), 1.83 (m, 1 H), 1.71 (m, 1 H), 1.57 (m, 1 H), 1.59−1.43 (m, 2H), 1.35 (m, 1 H), 1.26 (m, 1 H); ^{13}C NMR δ 163.6, 133.5 (2 s), 131.5, 131.2, 130.6 (3 d), 129.5 (s), 127.3 (d), 80.6, 47.2 (2 d), 26.2, 25.2, 22.4, 20.7 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 7.9 min (6o′) and 17.2 min (6o″). HRMS (ESI+) calcd for $C_{13}H_{15}ClNO$ [M + H]$^+$ 236.0842, found 236.0845 (6o′) and 236.0838 (6o″).

3-(4-Methoxyphenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6p). Yield 22%, a yellow wax; ^{1}H NMR δ 7.61 (m, 2 H), 6.89 (m, 2 H), 4.43 (m, 1 H), 3.80 (s, 3 H), 3.19 (m, 1 H), 2.23 (m, 1 H), 1.93 (m, 1 H), 1.72 (m, 1 H), 1.67 (m, 1 H), 1.58 (m, 1 H), 1.51 (m, 1 H), 1.25−1.18 (m, 2 H); ^{13}C NMR δ 164.0, 161.4 (2 s), 128.8 (d), 122.4 (s), 114.6, 80.5 (2 d), 55.7 (q), 45.1 (d), 26.9, 25.5, 22.9, 20.7 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 19.4 min (6p′) and 25.1 min (6p″). HRMS (ESI+) calcd for $C_{14}H_{18}NO_2$ [M + H]$^+$ 232.1338, found 232.1338 (6p′) and 232.1338 (6p″).

3-(3-Methoxyphenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6q). Yield 77%, a yellow wax; ^{1}H NMR δ 7.29 (m, 1 H), 7.27 (d, 1 H, J = 7.9), 7.20 (d, 1 H, J = 7.7), 6.92 (d, 1 H, J = 8.2), 4.47 (m, 1 H), 3.81 (s, 3 H), 3.21 (m, 1 H), 2.24 (m, 1 H), 1.96 (m, 1 H), 1.73 (m, 1 H), 1.65 (m, 1 H), 1.59 (m, 1 H), 1.51 (m, 1 H), 1.30−1.18 (m, 2 H); ^{13}C NMR δ 164.3, 160.2, 131.1 (3 s), 130.1, 120.0, 116.7, 111.9, 80.9 (5 d), 55.8 (q), 45.0 (d), 26.9, 25.5, 22.8, 20.6 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 14.5 min (6q′) and 16.7 min (6q″). HRMS (ESI+) calcd for $C_{14}H_{18}NO_2$ [M + H]$^+$ 232.1338, found 232.1334 (6q′) and 232.1332 (6q″).

3-(2-Methoxyphenyl)-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6r). Yield 21%, a yellow wax; ^{1}H NMR δ 7.61 (dd, 1 H, J = 7.6, 1.6), 7.34 (ddd, 1 H, J = 8.4, 7.5, 1.8), 6.94 (ddd, 1 H, J = 7.6, 7.5, 1.0), 6.90 (dd, 1 H, J = 8.4), 4.52 (m, 1 H), 3.81 (s, 3 H), 3.58 (m, 1 H), 2.02 (m, 1 H), 1.77 (m, 1 H), 1.72 (m, 1 H), 1.53−1.41 (m, 3 H), 1.29−1.18 (m, 2 H); ^{13}C NMR δ 163.5, 157.9 (2 s), 131.6, 130.5, 121.2 (3 d), 119.1 (s), 111.7, 80.3 (2 d), 55.9 (q), 47.1 (d), 26.2, 25.5, 22.6, 20.8 (4 t). HRMS (ESI+) calcd for $C_{14}H_{18}NO_2$ [M + H]$^+$ 232.1338, found 232.1331.

3-Phenyl-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6s). Yield 46%, a yellow viscous oil; ^{1}H NMR δ 7.71 (m, 2 H), 7.39 (m, 3 H), 4.49 (m, 1 H), 3.26 (m, 1 H), 2.26 (m, 1 H), 1.98 (m, 1 H), 1.76 (m, 1 H), 1.70−1.60 (m, 2 H), 1.55 (m, 1 H), 1.31−1.18 (m, 2 H); ^{13}C NMR δ 163.9 (s), 129.0, 128.8 (2 d), 128.7 (s), 126.9, 80.4, 44.4 (3 d), 26.5, 25.1, 22.4, 20.2 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 90/10/0.5, retention times 6.9 min (6s′) and 7.9 min (6s″). HRMS (ESI+) calcd for $C_{13}H_{16}NO$ [M + H]$^+$ 202.1232, found 202.1228 (6s′) and 202.1239 (6s″).

3-Naphthalen-1-yl-3a,4,5,6,7,7a-hexahydrobenzo[d]isoxazole (6t). Yield 14%, a colorless wax; ^{1}H NMR δ 8.78 (d, 1 H, J = 8.5), 7.86 (m, 2 H), 7.57 (m, 2 H), 7.52 (dd, 1 H, J = 7.0, 6.9), 7.48 (dd, 1 H, J = 7.9, 7.5), 4.65 (m, 1 H), 3.53 (m, 1 H), 2.20 (m, 1 H), 1.84 (m, 1 H), 1.61 (m, 1 H), 1.60−1.52 (m, 2 H), 1.40 (m, 1 H), 1.30−1.20 (m, 2 H); ^{13}C NMR δ 164.5, 134.5, 131.7 (3 s), 130.8 (d), 130.2 (s), 128.9, 128.1, 127.7, 127.1, 126.7, 125.3, 79.8, 48.1 (8 d), 26.1, 26.0, 22.7, 20.7 (4 t); Chiral separation in *n*-hexane/*i*-PrOH/AcOH 90/10/0.5, retention times 9.9 min (6t′) and 17.2 min (6t″). HRMS (ESI+) calcd for $C_{17}H_{18}NO$ [M + H]$^+$ 252.1388, found 252.1389 (6t′) and 252.1398 (6t″).

3-(4-Nitro-3-(trifluoromethyl)phenyl)-4,5,6,7,8,8a-hexahydro-3aH-cyclohepta[d]-isoxazole (7d). Yield 58%, a yellow wax; ^{1}H NMR δ 8.14 (d, 1 H, J = 1.5), 7.95 (dd, 1 H, J = 8.5, 1.5), 7.94 (d, 1 H, J = 8.5), 4.99 (m, 1 H), 3.77 (m, 1 H), 2.09 (m, 1 H), 2.01 (m, 1 H), 1.81 (m, 1 H) 1.79−1.60 (m, 2 H), 1.60−1.52 (m, 3 H), 1.51−1.42 (m, 2 H); ^{13}C NMR δ 157.9, 148.2, 134.8 (3 s), 131.2 (d), 126.6 (q), 126.2 (d), 124.5 (dq), 122.1 (q), 86.9, 50.9 (2 d), 31.3, 30.4, 28.3, 27.4, 24.0 (5 t). HRMS (ESI+) calcd for $C_{15}H_{16}F_3N_2O_3$ [M + H]$^+$ 329.1113, found 329.1117.

3-(4-Nitro-2-(trifluoromethyl)phenyl)-4,5,6,7,8,8a-hexahydro-3aH-cyclohepta[d]-isoxazole (7e). Yield 24%, yellow viscous oil ^{1}H NMR: δ 8.62 (d, 1 H, J = 2.1), 8.44 (dd, 1 H, J = 8.3, 2.1), 7.72 (d, 1 H, J = 8.3), 4.99 (m, 1 H), 3.80 (m, 1 H), 2.81 (m, 1 H), 2.08 (m, 1 H), 1.95 (m, 1 H), 1.87 (m, 1 H), 1.34−1.22 (m, 2 H); ^{13}C NMR δ 159.9, 149.0, 136.5 (3 s), 133.0 (d), 130.7 (q), 126.5 (d), 122.5 (q), 122.4 (dq), 85.9, 54.2 (2d), 31.1, 30.3, 28.1, 27.2, 24.1 (5 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 12.5 min (7e′) and 13.6 min (7e″). HRMS (ESI+) calcd for $C_{15}H_{16}F_3N_2O_3$ [M + H]$^+$ 329.1113, found 329.1109 (7e′) and 329.1125 (7e″).

3-(4-Fluoro-3-(trifluoromethyl)phenyl)-4,5,6,7,8,8a-hexahydro-3aH-cyclohepta[d]-isoxazole (7f). Yield 70%, a yellow wax; ^{1}H NMR δ 7.90 (dd, 1 H, J = 6.8, 1.8), 7.83 (dd, 1 H, J = 9.0, 6.9, 1.8), 7.24 (dd, 1 H, J = 9.0, 8.0), 4.91 (m, 1 H), 3.72 (m, 1 H), 2.05 (m, 1 H), 1.98 (m, 1 H), 1.83−1.47 (m, 6 H), 1.45−1.36 (m, 2 H); ^{13}C NMR δ 160.7 (dd), 157.0 (s), 132.7 (dd), 126.6 (d), 126.2 (qd), 122.7 (q), 119.3 (qd), 117.9 (dd), 85.9, 51.5 (2 d), 31.4, 30.5, 28.4, 27.4, 24.1 (5 t). HRMS (ESI+) calcd for $C_{15}H_{15}F_4NO$ [M + H]$^+$ 302.1168, found 302.1180.

3-(4-Fluoro-2-(trifluoromethyl)phenyl)-4,5,6,7,8,8a-hexahydro-3aH-cyclohepta[d]-isoxazole (7g). Yield 73%, a yellow wax; ^{1}H NMR δ 7.49−7.43 (m, 2 H), 7.29 (ddd, 1 H, J = 8.9, 8.3, 2.6), 4.92 (m, 1 H),

3.71 (m, 1 H), 2.07 (m, 1 H), 1.94 (m, 1 H), 1.85 (m, 1 H) 1.73−1.62 (m, 2 H), 1.59−1.45 (m, 3 H), 1.38 (m, 1 H), 1.27 (m, 1 H); ^{13}C NMR δ 163.0 (dd), 158.9 (s), 134 (dd), 131.5 (qd), 125.7 (d), 123.7 (qd), 119.4 (dd), 114.7 (ddq), 85.6, 55.0 (2 d), 31.6, 30.7, 28.6, 27.5, 24.6 (5 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 7.7 min (**7g′**) and 9.9 min (**7g″**). HRMS (ESI+) calcd for C$_{15}$H$_{15}$F$_4$NO [M + H]$^+$ 302.1168, found 302.1173 (**7g′**) and 302.1172 (**7g″**).

3-(4-Nitro-3-(trifluoromethyl)phenyl)-3a,4,5,6,7,8,9,9a-octahydrocycloocta[d]isoxazole (8d). Yield 17%, a yellow wax; ^{1}H NMR δ 8.10 (s, 1 H), 7.91−7.89 (m, 2 H), 4.58 (m, 1 H), 3.42 (m, 1 H), 2.12 (m, 1 H), 2.01 (m, 1 H), 1.82−1.70 (m, 4 H), 1.66 (m, 1 H), 1.55−1.44 (m, 2 H), 1.40−1.25 (m, 3 H); ^{13}C NMR δ 160.1, 148.3, 134.7 (3 s), 131.1 (d), 126.6 (q), 126.2 (d), 124.9 (dq), 122.1 (q), 87.6, 49.7 (2 d), 30.3, 25.8, 25.6, 25.6, 25.2, 24.9 (6 t). HRMS (ESI+) calcd for C$_{16}$H$_{18}$F$_3$N$_2$O$_3$ [M + H]$^+$ 343.1270, found 343.1283.

3-(4-Nitro-2-(trifluoromethyl)phenyl)-3a,4,5,6,7,8,9,9a-octahydrocycloocta[d]isoxazole (8e). Yield 16%, yellow viscous oil ^{1}H NMR δ 8.60 (d, 1 H, *J* = 2.2), 8.42 (dd, 1 H, *J* = 8.4, 2.2), 7.70 (d, 1 H, *J* = 8.4), 4.70 (dd, 1 H, *J* = 10.4, 10.3), 3.51 (t, 1 H, *J* = 10.4), 2.08−1.94 (m, 2 H), 1.75−1.43 (m, 6 H), 1.43−1.20 (m, 3 H), 1.13 (m, 1 H); ^{13}C NMR δ 159.7, 148.5, 135.5 (3 s), 133.4 (d), 130.7 (q), 126.5 (d), 122.6 (q), 122.4 (dq), 86.8, 53.3 (2d), 29.6, 26.2, 25.9, 25.3, 25.2, 24.1 (6 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 18.8 min. (**8e′**) and 21.6 min. (**8e″**). HRMS (ESI+) calcd for C$_{16}$H$_{18}$F$_3$N$_2$O$_3$ [M + H]$^+$ 343.1270, found 343.1284 (**8e′**) and 343.1286 (**8e″**).

3-(4-Fluoro-3-(trifluoromethyl)phenyl)-3a,4,5,6,7,8,9,9a-octahydrocycloocta[d]isoxazole (8f). Yield 63%, a yellow wax; ^{1}H NMR δ 7.92 (dd, 1 H, *J* = 7.0, 1.5), 7.84 (ddd, 1 H, *J* = 9.0, 6.8, 1.5), 7.26 (dd, 1 H, *J* = 9.2, 9.0), 4.54 (m, 1 H), 3.42 (m, 1 H), 2.06 (m, 1 H), 1.88−1.73 (m, 4 H), 1.68 (m, 1 H), 1.60−1.50 (m, 2 H), 1.42−1.25 (m, 3 H); ^{13}C NMR δ 160.6 (dd), 160.8 (s), 132.7 (dd), 126.6 (d), 126.2 (qd), 122.6 (q), 119.3 (qd), 117.9 (dd), 86.6, 50.3 (2 d), 30.4, 25.8, 25.7, 25.6, 25.2, 24.8 (6 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 7.6 min (**8f′**) and 8.5 min (**8f″**). HRMS (ESI+) calcd for C$_{16}$H$_{18}$F$_4$NO [M + H]$^+$ 316.1325, found 316.1315 (**8f′**) and 316.1312 (**8f″**).

3-(4-Fluoro-2-(trifluoromethyl)phenyl)-3a,4,5,6,7,8,9,9a-octahydrocycloocta[d]-isoxazole (8g). Yield 30%, a yellow wax; ^{1}H NMR δ 7.44 (dd, 1 H, *J* = 8.5, 5.5), 7.42 (dd, 1 H, *J* = 9.1, 2.7), 7.26 (ddd, 1 H, *J* = 9.1, 8.5, 2.7), 4.63 (m, 1 H), 3.41 (m, 1 H), 2.00−1.91 (m, 2 H), 1.70−1.41 (m, 6 H), 1.40−1.10 (m, 4 H); ^{13}C NMR δ 163.1 (dd), 160.5 (s), 134.4 (d), 131.5 (qd), 125.4 (d), 123.3 (qd), 119.3 (dd), 115.0 (ddq), 86.1, 53.7 (2 d), 30.1, 26.7, 26.4, 25.7, 25.6, 24.2 (6 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 7.3 min (**8g′**) and 9.6 min (**8g″**). HRMS (ESI+) calcd for C$_{16}$H$_{18}$F$_4$NO [M + H]$^+$ 316.1325, found 316.1321 (**8g′**) and 316.1325 (**8g″**).

Synthesis of Cycloalkane[*d*]isoxazolylbenzonitriles (compounds 5a, 5b, 6a−c, 6h, 7a, 7b, 8a, and 8b). *Procedure B (Scheme 1).* A stirred solution of an appropriate fluoro compound (1 mmol) and KCN (4 mmol) in dry DMSO (5 mL) was heated at 80−150 °C on a sand bath overnight. After the reaction, the mixture was diluted with water and extracted with diethyl ether. The organic phase was dried (MgSO$_4$) and evaporated to give crude product, which was purified by TLC using DCM as an eluent.

4-(4,5,6,6a-Tetrahydro-3aH-cyclopenta[d]isoxazol-3-yl)-2-(trifluoromethyl)benzonitrile (5a). Yield 82%, a yellow wax; ^{1}H NMR δ 8.14 (s, 1 H), 7.95 (d, 1 H, *J* = 8.5), 7.87 (d, 1 H, *J* = 8.5), 5.35 (dd, 1 H, *J* = 9.0, 4.5), 4.03 (td, 1 H, *J* = 9.0, 2.0), 2.23 (m, 1 H), 2.00−1.78 (m, 4 H), 1.52 (m, 1 H); ^{13}C NMR δ 156.9 (s), 135.5, 134.9 (2 d), 133.7 (q), 130.2 (d), 125.1, 122.6 (2 q), 115.6 (d), 110.6 (s), 90.0, 51.3 (2 d), 36.0, 31.8, 23.9 (3 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 27.1 min (**5a′**) and 29.8 min (**5a″**). HRMS (ESI+) calcd for C$_{14}$H$_{12}$F$_3$N$_2$O [M + H]$^+$ 281.0902, found 281.0905 (**5a′**) and 281.0904 (**5a″**).

4-(4,5,6,6a-Tetrahydro-3aH-cyclopenta[d]isoxazol-3-yl)-3-(trifluoromethyl)benzonitrile (5b). Yield 37%, a yellow wax; ^{1}H NMR δ 8.00 (s, 1 H), 7.85 (d, 1 H, *J* = 8.0), 7.60 (d, 1 H, *J* = 8.0), 5.27 (m, 1 H), 4.08 (m, 1 H), 2.15 (m, 1 H), 1.77−1.69 (m, 2 H), 1.69−1.57 (m,

2 H), 1.48 (m, 1 H); ^{13}C NMR δ 157.8 (s), 135.6 (d), 134.0 (s), 132.8 (d), 131.0, 130.6, 123.1 (3 q), 117.3 (d), 114.3 (s), 88.7, 55.3 (2 d), 36.1, 30.9, 23.5 (3 t). HRMS (ESI+) calcd for C$_{14}$H$_{12}$F$_3$N$_2$O [M + H]$^+$ 281.0902, found 281.0910.

4-(3a,4,5,6,7,7a-Hexahydrobenzo[d]isoxazol-3-yl)-2-(trifluoromethyl)benzonitrile (6a). Yield 93%, a yellow wax; ^{1}H NMR δ 8.12 (d, 1 H, *J* = 0.8), 7.97 (dd, 1 H, *J* = 8.1, 0.8), 7.88 (d, 1 H, *J* = 8.1), 4.59 (m, 1 H), 3.29 (m, 1 H), 2.31 (m, 1 H), 1.98 (m, 1 H), 1.81 (m, 1 H), 1.75−1.62 (m, 2 H), 1.55 (m, 1 H), 1.35−1.18 (m, 2 H); ^{13}C NMR δ 162.0 (s), 135.6, 134.8 (2 d), 133.8 (q), 130.2 (d), 125.1, 122.5 (2 q), 115.5 (d), 110.9 (s), 82.3, 43.9 (2 d), 26.6, 25.2, 22.3, 20.3 (4 t). C$_{15}$H$_{14}$F$_3$N$_2$O [M + H]$^+$ 295.1058, found 295.1051.

4-(3a,4,5,6,7,7a-Hexahydrobenzo[d]isoxazol-3-yl)-3-(trifluoromethyl)benzonitrile (6b). Yield 67%, a yellow wax; ^{1}H NMR δ 8.05 (s, 1 H), 7.89 (d, 1 H, *J* = 8.0), 7.69 (d, 1 H, *J* = 8.0), 4.67 (m, 1 H), 3.37 (m, 1 H), 2.13 (m, 1 H), 1.83 (m, 1 H), 1.69 (m, 1 H), 1.59−1.45 (m, 3 H), 1.32−1.24 (m, 2 H); ^{13}C NMR δ 162.1 (s), 135.6 (d), 134.0 (s), 132.6 (d), 130.9, 130.7, 123.1 (3 q), 117.4 (d), 114.5 (s), 81.3, 48.1 (2 d), 25.7, 25.5, 22.2, 20.4 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 16.5 min (**6b′**) and 18.8 min (**6b″**). HRMS (ESI+) calcd for C$_{14}$H$_{14}$F$_4$NO [M + H]$^+$ 295.1058, found 295.1067 (**6b′**) and 295.1070 (**6b″**).

3-(3a,4,5,6,7,7a-Hexahydrobenzo[d]isoxazol-3-yl)-2-(trifluoromethyl)benzonitrile (6c). Yield 17%, yellow viscous oil ^{1}H NMR δ 7.92 (d, 1 H, *J* = 7.5), 7.73 (dd, 1 H, *J* = 8.3, 7.5), 7.71 (d, 1 H, *J* = 8.3), 4.66 (m, 1 H), 3.25 (m, 1 H), 2.16 (m, 1 H), 2.01 (m, 1 H), 1.71 (m, 1 H), 1.66 (m, 1 H), 1.58 (m, 1 H), 1.51 (m, 1 H), 1.25−1.18 (m, 2 H); ^{13}C NMR δ 162.7 (s), 136.0 (q), 135.9, 135.5, 132.2 (3 d), 128.8, 122.4 (2 q), 115.4 (s), 112.0 (q), 80.7, 48.4 (2 d), 30.4, 25.3, 22.0, 20.1 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 90/10/0.5, retention times 20.8 min. (**6c′**) and 24.7 min. (**6c″**). HRMS (ESI+) calcd for C$_{15}$H$_{14}$F$_3$N$_2$O [M + H]$^+$ 295.1058, found 295.1048 (**6c′**) and 295.1044 (**6c″**).

4-(3a,4,5,6,7,7a-Hexahydrobenzo[d]isoxazol-3-yl)benzonitrile (6h). Yield 30%, a colorless viscous oil; ^{1}H NMR δ 7.81 (m, 2 H), 7.69 (m, 2 H), 4.56 (m, 1 H), 3.26 (m, 1 H), 2.30 (m, 1 H), 1.97 (m, 1H), 1.79 (m, 1 H), 1.75−1.64 (m, 2H), 1.55 (m, 1 H), 1.32−1.21 (m, 2H). ^{13}C NMR δ 163.6 (s), 135.0, 133.5, 128.3 (s), 127.5, 81.9, 44.3 (3 d), 27.1, 25.5, 22.7, 21.0 (4 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 90/10/0.5, retention times 13.3 min (**6 h′**) and 14.3 min (**6 h″**). HRMS (ESI+) calcd for C$_{14}$H$_{15}$N$_2$O [M + H]$^+$ 227.1184, found 227.1184 (**6 h′**) and 227.1180 (**6 h″**).

4-(4,5,6,7,8,8a-Hexahydro-3aH-cyclohepta[d]isoxazol-3-yl)-2-(trifluoromethyl)benzonitrile (7a). Yield 81%, a yellow wax; ^{1}H NMR δ 8.07 (d, 1 H, *J* = 0.8), 7.87 (dd, 1 H, *J* = 8.1, 0.8), 7.88 (d, 1 H, *J* = 8.1), 4.94 (m, 1 H), 3.71 (m, 1 H), 2.05 (m, 1 H), 1.98 (m, 1 H), 1.83−1.47 (m, 6 H), 1.45−1.36 (m, 2 H); ^{13}C NMR δ 158.2 (s), 135.6, 134.9 (2 d), 133.8 (q), 130.4 (d), 125.3, 122.5 (2 q), 115.6 (d), 110.7 (s), 86.9, 50.8 (2 d), 31.3, 30.4, 28.4, 27.4, 24.0 (5 t). HRMS (ESI+) calcd for C$_{16}$H$_{16}$F$_3$N$_2$O [M + H]$^+$ 309.1215, found 309.1224.

4-(4,5,6,7,8,8a-Hexahydro-3aH-cyclohepta[d]isoxazol-3-yl)-3-(trifluoromethyl)benzonitrile (7b). Yield 78%, a yellow wax; ^{1}H NMR δ 8.04 (s, 1 H), 7.88 (d, 1 H, *J* = 8.0), 7.64 (d, 1 H, *J* = 8.0), 4.97 (m, 1 H), 3.77 (m, 1 H), 2.07 (m, 1 H), 1.94 (m, 1 H), 1.85 (m, 1 H), 1.74−1.25 (m, 5 H); ^{13}C NMR δ 158.3 (s), 135.5 (d), 134.0 (s), 132.9 (d), 131.3, 130.9, 123.0 (3 q), 117.3 (d), 114.3 (s), 86.2, 54.6 (2 d), 31.5, 30.7, 28.5, 27.5, 24.5 (5 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 16.7 min (**7b′**) and 19.0 min (**7b″**). HRMS (ESI+) calcd for C$_{16}$H$_{16}$F$_3$N$_2$O [M + H]$^+$ 309.1215, found 309.1230 (**7b′**) and 309.1220 (**7b″**).

4-(3a,4,5,6,7,8,9,9a-Octahydrocycloocta[d]isoxazol-3-yl)-2-(trifluoromethyl)benzonitrile (8a). Yield 98%, a yellow wax; ^{1}H NMR δ 8.07 (s, 1 H), 7.86−7.82 (m, 2 H), 4.56 (m, 1 H), 3.40 (m, 1 H), 2.15−2.08 (m, 1 H), 2.01 (m, 1 H), 1.81−1.70 (m, 4 H), 1.65 (m, 1 H), 1.55−1.43 (m, 2 H), 1.35−1.21 (m, 3 H); ^{13}C NMR δ 160.4 (s), 135.6, 134.8 (2 d), 133.8 (q), 130.3 (d), 125.3, 122.5 (2 q), 115.5 (d), 110.7 (s), 87.6, 49.6 (2 d), 30.3, 25.8, 25.6, 25.6, 25.2, 24.9 (6 t). HRMS (ESI+) calcd for C$_{17}$H$_{18}$F$_3$N$_2$O [M + H]$^+$ 323.1371, found 323.1387.

4-(3a,4,5,6,7,8,9,9a-Octahydrocycloocta[d]isoxazol-3-yl)-3-(trifluoromethyl)benzonitrile (8b). Yield 18%, a yellow wax; ^{1}H NMR δ 8.01 (s, 1 H), 7.86 (d, 1 H, J = 8.0), 7.62 (d, 1 H, J = 8.0), 4.68 (m, 1 H), 3.48 (m, 1 H), 2.05−1.93 (m, 2 H), 1.75−1.44 (m, 6 H), 1.43−1.07 (m, 4 H); ^{13}C NMR δ 159.9 (s), 135.5 (d), 134.2 (s), 133.2 (d), 130.9, 130.7, 123.0 (3 q), 117.3 (d), 114.4 (s), 86.7, 53.8 (2 d), 30.0, 26.6, 26.3, 25.7, 25.6, 24.4 (6 t). Chiral separation in *n*-hexane/*i*-PrOH/AcOH 98/2/0.5, retention times 15.4 min (8b′) and 17.5 min (8b″). HRMS (ESI+) calcd for $C_{17}H_{18}F_3N_2O$ [M + H]$^+$ 323.1371, found 323.1378 (8b′) and 323.1370 (8b″).

Firefly Luciferase Enzyme Inhibition Assay. The HPLC-purified samples were dissolved in ethanol at 0.01 mol/L concentrations. They were further diluted to a concentration range from 10^{-3} to 10^{-7} mol/L in 1x Reporter Lysis Buffer (Promega). The purified luciferase protein was purchased from Sigma-Aldrich (FLuc from the firefly *Photinus pyralis*). The luciferase was diluted to 0.1 μg/mL concentration in 1x Reporter Lysis Buffer. To 30 μL of the luciferase solution was added 1.2 μL of different compound concentrations as three replicates. After incubation at rt for 20 min, 10 μL of the incubations was transferred to 96-well plates (Greiner Microlon lumitrac 200) and 30 μL of luciferase substrate solution was added (Promega Luciferase Assay Substrate). Also three blank (1x Reporter Lysis Buffer-containing) and control (vehicle, ethanol-exposed luciferase) samples were measured. Lumiscence produced by the luciferase was measured with Thermo Luminoscan Ascent scanner.

Cell Culture, Transfection, and Luciferase Reporter Assays. Testosterone was purchased from Steraloids Inc., bicalutamide from Molekula Ltd., and hydroxyflutamide from Chemos GmbH. One day before transfection, COS-1 cells (from ATCC) were seeded onto 24-well plates in 1 mL of DMEM (Dulbeccòs modified Eagle medium, Gibco) containing 10% dextran-charcoal-treated fetal bovine serum (FBS) and 0.25% (v/v) penicillin−streptomycin (Euroclone) at a density of 70×10^3 cells/well. After medium change to DMEM containing 2.5% FBS, the cells were transfected for 24 h with pSG5-hAR (10 ng/well), pProbasin(−285/+32)-Luc (100 ng/well), and internal control pCMVβ (10 ng/well) by using TransIT-LT1 Transfection Reagent (Mirus Bio LCC). After the transfection, the cells were exposed to vehicle (ethanol) alone, testosterone (100 nM) as a reference for a pure androgen agonist (100% activation), or test compounds at 10 μM concentration. In the antagonism reporter assays, the cells were exposed simultaneously to testosterone and test compounds. After 18 h, cells were processed, luciferase and β-galactosidase activities and protein concentrations were measured, and relative luciferase activities were calculated as described previously.[45] When studying mutated androgen receptors, pSG5-hARW741L or pSG5-hART877A was used instead of pSG5-hAR.

Whole Cell Binding Assay. The ability of the test compounds to bind to AR was measured by relative binding inhibition (RBI) assay which measures their ability to displace ^{3}H-labeled synthetic agonist R1881 from AR expressed in COS-1 cells.[46] One day before transfection, COS-1 cells were seeded into 12-well plates in 2 mL of DMEM (Dulbecco's modified Eagle medium, Gibco) containing 10% dextran-charcoal-treated fetal bovine serum and 0.25% (v/v) penicillin−streptomycin (Euroclone) at a density of 140×10^3 cells/well. After medium change to DMEM containing 2.5% FBS, the cells were transfected for 24 h with pSG5-hAR (100 ng/well) by using TransIT LT1 transfection reagent. After 36 h, the medium was aspirated, and the cells were washed with phosphate-buffered saline (PBS) and then treated with test compounds at several concentrations: 1, 10, 100, 1000, and up to 10000-fold molarities compared to labeled R1881 (1.34 nM) in 0.5 mL of DMEM. After 2 h incubation at 37 °C, the medium was aspirated, and cells were transferred to Eppendorf tubes with phosphate-buffered saline (PBS), centrifuged at 4 °C at 4000g for 5 min, and then washed twice with an excess of PBS. The cell pellets were dissolved in 50 μL of 0.5 M NaOH and incubated for 15 min at 56 °C, after which the samples (three replicates for each sample compound and concentration) were transferred to liquid scintillation tubes with 3 mL of OptiPhase HiSafe 3 solution (Perkin-Elmer), and radioactivity of AR-bound [^{3}H]R1881 was measured with

LKB WALLAC 1214 racbeta counter. Nonspecific binding (NSB) was subtracted from these data, using corresponding radioinert hormone at 200-fold compared to [^{3}H]R1881. The results (% inhibition) were calculated as % inhibition = 100 − [100 × (average compound/average [^{3}H]R1881)]. The dose−response log IC$_{50}$ values were analyzed with GraphPad Prism.[56]

Statistical Analysis. Student's *t* test was used for comparisons with the aid of a software package GraphPad Prism.[56] $p < 0.05$ is considered as significant.

Molecular Modeling. The crystal structure of AR in complex with DHT (PDB code 1T7T)[57] was used for AR LBD coordinates. Ligand docking was performed using the crystal structure of the AR−1 complex (PDB code 1Z95).[58] Enantiomers (R,R)-6a and (S,S)-6a were docked to the LBP by superimposing the A rings of the compounds and 1. After that, the moiety formed by rings B and C was rotated to fit to the binding pocket. This approach was chosen because the A ring of 6a is identical to the aromatic ring of 1 and was therefore assumed to bind similarly. In addition, binding of the aromatic ring of 1 is shared by other nonsteroidal AR agonists and antagonists having identical or similar aromatic rings.[59] The docked structures were solvated in a box of water molecules and energy-minimized using the AMBER 9.0 program[60] and the Amber ff99SB force field.[61] Accelrys discovery studio software[62] was used to model the new compounds in the AR LBP.

Calculation of ECD Spectra. ECD spectra of the *S,S*-enantiomers of 5a, 5d, 6e, and 7e were calculated using the TDDFT[63,64] method at the B3LYP/aug-cc-pVDZ level. The polarizable continuum model using the integral equation formalism (acetonitrile)[65] was included in the TDDFT calculations. For the TDDFT calculations, geometries of the molecules were optimized at the B3LYP/6-311G** level. SpecDis 1.53 program[66] was used to sum up and visualize the ECD spectra. The spectra were simulated by using the calculated velocity rotational strengths, Gaussian bandshapes, and bandwidth σ = 0.3 eV. Quantum mechanical calculations were perfromed using the Gaussian09 program revision C.01.[67]

■ ASSOCIATED CONTENT

ⓢ Supporting Information

A figure showing the concentration dependence of the agonistic and antagonistic effect of 1, 6a, and 6d, figures showing the comparison of the experimental and calculated ECD spectra of 5a, 5d, 6e, and 7e, and tables showing the atomic coordinates of the optimized conformations of (3aS,6aS)-5a, (3aS,6aS)-5d, (3aS,7aS)-6e, and (3aS,8aS)-7e. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ ABBREVIATIONS USED

3D, three-dimensional; AR, androgen receptor; COS-1, African green monkey kidney cells; CRPC, castration-resistant prostate cancer; DFT, density functional theory; DHT, 5α-dihydrotes-

tosterone; DMEM, Dulbecco's modified Eagle medium; ECD, electronic circular dichroism; ER, estrogen receptor; FBS, fetal bovine serum; FLuc, firefly luciferase; hAR, human androgen receptor; N.D, not detected; NSB, nonspecific binding; p, probability value of statistical significance; R1881, 17β-hydroxy-17-methylestra-4,9,11-trien-3-one; RBI, relative binding inhibition; SD, standard deviation; SE, standard error; SEM, standard error of mean; TDDFT, time-dependent density functional theory; TEA, triethylamine

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