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1-Benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diols as novel affinity and photoaffinity probes for β-adrenoceptor subtypes

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Abstract—Trimetoquinol (TMQ, **1**) is a potent non-selective β-adrenoceptor (β-AR) agonist possessing a tetrahydroisoquinoline (THI) structure. The binding site for 1-trimethoxybenzyl group of **1**, which distinguishes it from classical catecholamines, is unknown. Affinity and photoaffinity labeled compounds are good tools to determine the exact interaction between a ligand and a specific amino acid(s) in a receptor. In this study, we designed and synthesized a series of affinity **6**, **12**, **18**, and photoaffinity **24**, **29** labeled analogues of TMQ. All of these compounds were full agonists and demonstrated an equal or greater binding affinity and functional activity as compared to TMQ on β_1-, β_2-, and β_3-AR. Washout experiments on Chinese hamster ovary (CHO) cells expressing hu β_2-AR were helpful in identifying the isothiocyanate **18** and the azide **24** as very effective affinity and photoaffinity labels at this receptor subtype.

1. Introduction

Trimetoquinol (TMQ, **1**, Chart 1) is a potent β-adrenoceptor (β-AR) agonist. The *S*(−) isomer of TMQ is currently used in Japan as a bronchodilatory agent.[1] The 3′,5′-diiodo derivative of TMQ compound **2** is a partial agonist on hu β_2-AR and a full agonist on hu β_1- and hu β_3-AR.[2] The binding affinities of **2** have been reported to be greater than those of **1**.[3] Like catecholamine adrenoceptor agonists such as norepinephrine (NE) and isoproterenol (ISO), the structure of TMQ contains both a catechol and a basic amino group, which comprise the pharmacophore of this structural class of β-AR agonists. Several unique structural features differentiate TMQ from other typical catecholamine β-AR agonists. Unlike flexible structures of NE and ISO, the catecholamine pharmacophore of TMQ is incorporated in a rigid THI ring system. Another distinguishing feature

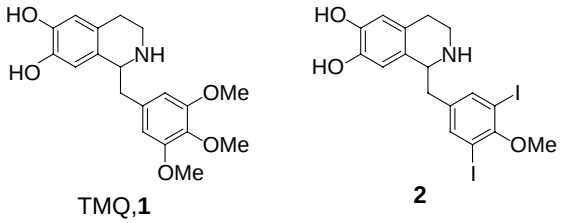

Chart 1.

of TMQ is the lack of a β-hydroxyl group, a substituent necessary for the potent stereoselective β-AR agonist activity of NE and ISO.[4] Despite the absence of a β-OH group, TMQ and related analogues exhibit potent β-AR activity. The trimethoxy-substituted benzyl group at the one position of TMQ is a more obvious discerning structural characteristic that distinguishes TMQ analogues from other catecholamines. This substituted benzyl group is a requirement for the potent β-AR activity of TMQ and structurally related analogues. Furthermore, the 1-benzyl substituent introduces a chiral center that differs from those of NE and ISO. Like NE and ISO, the β-AR agonist action of TMQ is highly

Keywords: β_3-Adrenergic receptor; Agonist; 1,2,3,4-Tetrahydroisoquinoline; Photoaffinity probe.
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stereoselective ($S \gg R$). The structural similarities and differences between TMQ and other catecholamines suggest that they interact with the same receptor binding site domains but differ in their specific binding interactions within the binding site area.

The β-ARs are classified into β_1, β_2,[5] and β_3 subtypes.[6,7] The β-AR subtypes obtained from different species were cloned and expressed as recombinants in cell lines including Chinese hamster ovary (CHO) cells. Traditionally, the therapeutic usefulness of β-AR drugs has been limited to cardiovascular (β_1-AR) and bronchorelaxant (β_2-AR) applications. Activation of the β_3-AR leads to lipolysis in white adipocytes, and both lipolysis and thermogenesis in brown adipocytes. Moreover, β_3-AR selective agonists decrease body fat and improve insulin sensitivity in animal models.[8,9] These studies suggest that β_3-AR selective agonists are promising candidates to manage human obesity and type II diabetes. Also they may be useful for the treatment of gastrointestinal hypermotility disorders.[10]

β-ARs belong to the superfamily of G-protein-coupled receptors and have seven transmembrane (TM)-spanning domains. TM regions are well conserved among the three β-AR subtypes. It is assumed that Asp^{113} in the TM3 of the β_2-AR forms an ionic bond with the amino group of ISO,[11] whereas catechol hydroxyl groups form hydrogen bonds with Ser^{204} and Ser^{207} in TM5.[12] We suggest that the same interactions exist for the TMQ analogues. However, the binding site for the 1-benzyl substituent of TMQ analogues is unknown, and therefore, the goal of this work is the preparation of analogues containing affinity labels in the 1-benzyl group. Labeling experiments on pure cloned receptor subtypes will give us an opportunity to determine the nature of the amino acid(s) that interact with the 1-benzyl substituent. This in turn will allow for an improved model and understanding of ligand–receptor interaction and might lead to creation of highly selective β-AR agonists with the ultimate goal of finding potent selective β_3-AR drugs.

A variety of affinity and photoaffinity β-AR antagonist analogues have been developed.[13–18] Most of these labels identify receptor proteins of M_r 58,000 (β_2-subtype) and of M_r 39,000 and M_r 45,000 (β_1-subtype). A photoaffinity analogue of NE labeled a protein band of M_r 65,000 in a guinea pig lung (β_2-subtype), which was identical to that which was observed with the antagonist photolabel [^{125}I]iodoazidobenzylpindolol.[16] Many of these studies employ mammalian tissues, which contain different ratios of β-subtypes, exhibit high background binding that may be dependent upon guanine nucleotides. Several photolabels of competitive antagonists (prenalterol, alprenolol, and CGP 12177) and agonist (NE) have been successfully used for the derivatization and isolation of recombinant hamster β_2-AR present in turkey erythrocytes and guinea pig lung membranes.[19,20] To overcome these problems, we continue to use CHO clones that express homogeneous populations of human β-AR subtypes. Our new procedures can be easily applied to the synthesis of analogues containing [^{125}I]. However, the search for a labeled amino acid could be performed without a radioactive label using electrospray ionization mass spectrometry.[21]

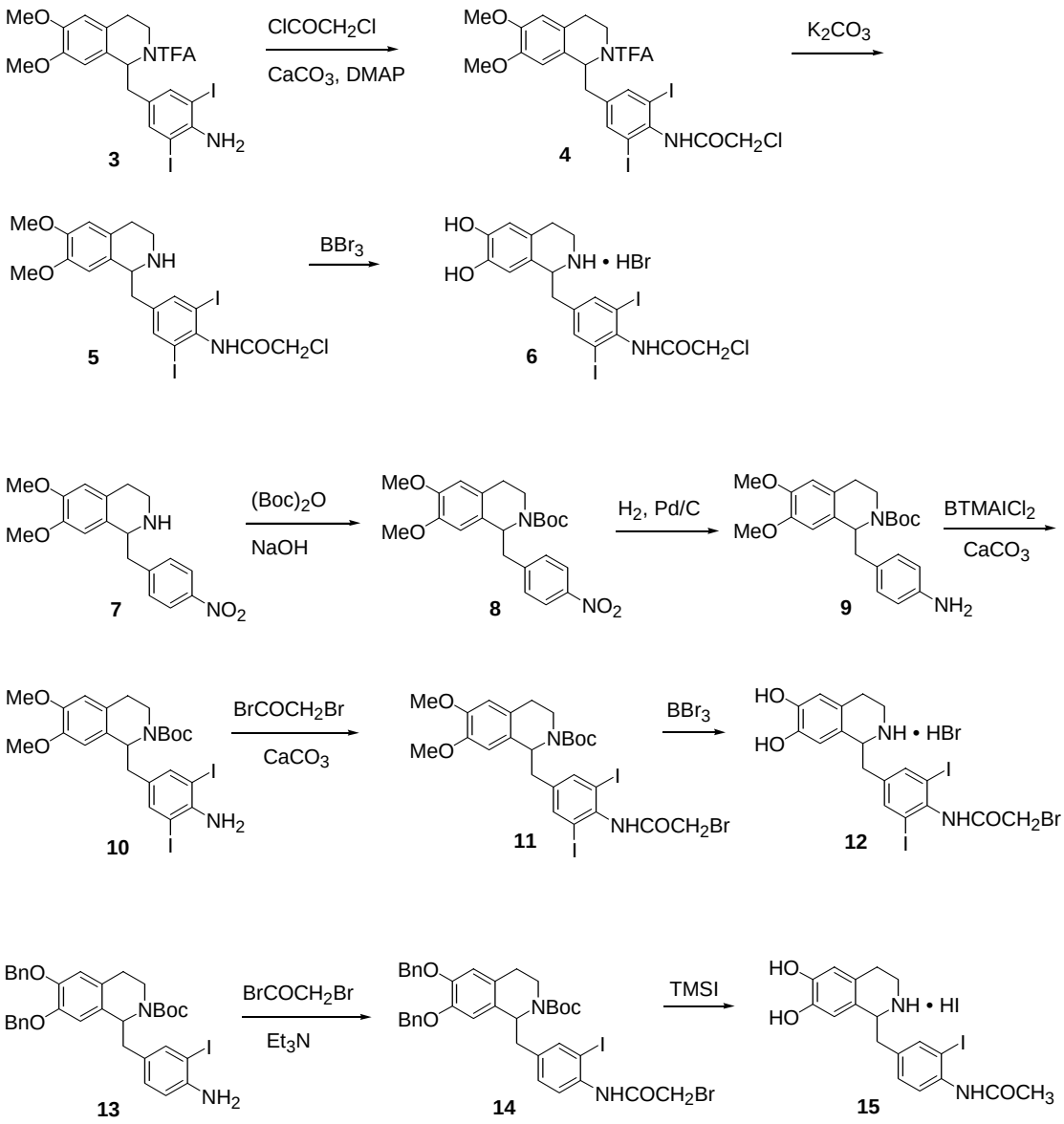
2. Chemistry

Chloroacetamide **6** was prepared starting from compound **3**, in which the synthesis was developed by our laboratory earlier (Scheme 1).[22] Treatment of isoquinoline **3** with chloroacetyl chloride in the presence of $CaCO_3$ and 4-(dimethylamino)pyridine (DMAP) resulted in chloroacetylation of diiodoaniline moiety. Trifluoroacetyl (TFA) protection in **4** was removed by mild basic hydrolysis using K_2CO_3. The resulting dimethoxy derivative **5** was demethylated with BBr_3 to afford the desired chloroacetamide **6** in 58% the overall yield.

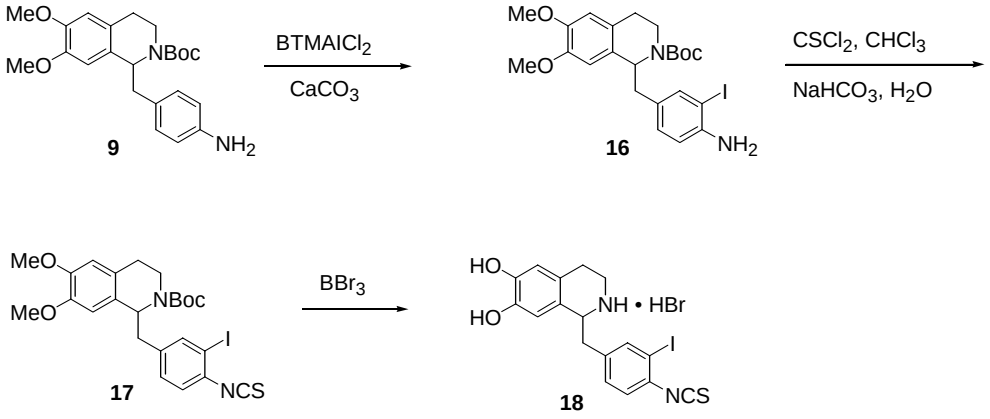
The bromoacetamido-labeling compound **12** was obtained using a single deprotection reagent approach to reduce the number of deprotection steps (Scheme 1). This approach required *tert*-butoxycarbonyl (Boc) protection of nitrogen in starting nitroisoquinoline **7**[22] with Boc_2O and 1 N NaOH. The nitro group in the resulting compound **8** was reduced with hydrogen over palladium on activated carbon to give aniline **9**, which in turn was diiodinated with an excess of benzyltrimethylammonium dichloroiodate ($BTMAICl_2$) in the presence of $CaCO_3$ according to the procedure by Kajigaeshi et al.[23] Acylation of **10** with bromoacetyl bromide in the presence of $CaCO_3$ gave triprotected intermediate **11**. The latter was deprotected in just one step using BBr_3 to form the desired bromoacetamide analogue **12** in 18% total yield.

We also attempted to synthesize a monoiodo analogue of **12**. *N*-Boc-diBnO-protected tetrahydroisoquinoline **13**[22] was acylated with bromoacetyl bromide in the presence of triethylamine to give compound **14**. It should be noted that *o*-iodoacetanilides (like **14**) behave as normal aromatic amides, while di-*ortho*-substituted acetanilides (e.g., **11**) have tendency to form diacetanilides and one should be very cautious to make monoacylated products. Similar *ortho* effect in acetanilides was studied and explained by Ayyangar and Srinivasan.[24] Second, di-*ortho*-substituted acetanilides are stable toward deprotection protocols with BBr_3. In contrast, the *o*-monoiodoacetanilides are cleaved with BBr_3.[22] Taking all these observations into consideration we tried deprotecting isoquinoline **14** with a milder reagent—namely trimethylsilyl iodide (TMSI). Unfortunately, we isolated only acetanilide **15**—the product of reduction of the bromoacetamido group.

The synthesis of isothiocyanato labeled isoquinoline **18** was started from the above-mentioned compound **9** (Scheme 2). Monoiodination of the latter compound with 1 equiv of $BTMAICl_2$ in the presence of $CaCO_3$ led to **16**, which was treated with thiophosgene in biphase media containing $NaHCO_3$ to obtain isothiocyanate **17**.[25] Standard deprotection of **17** with BBr_3 followed by recrystallization from cold methanol–ether gave the final isothiocyanate **18** with a 61% overall yield.



Scheme 1.

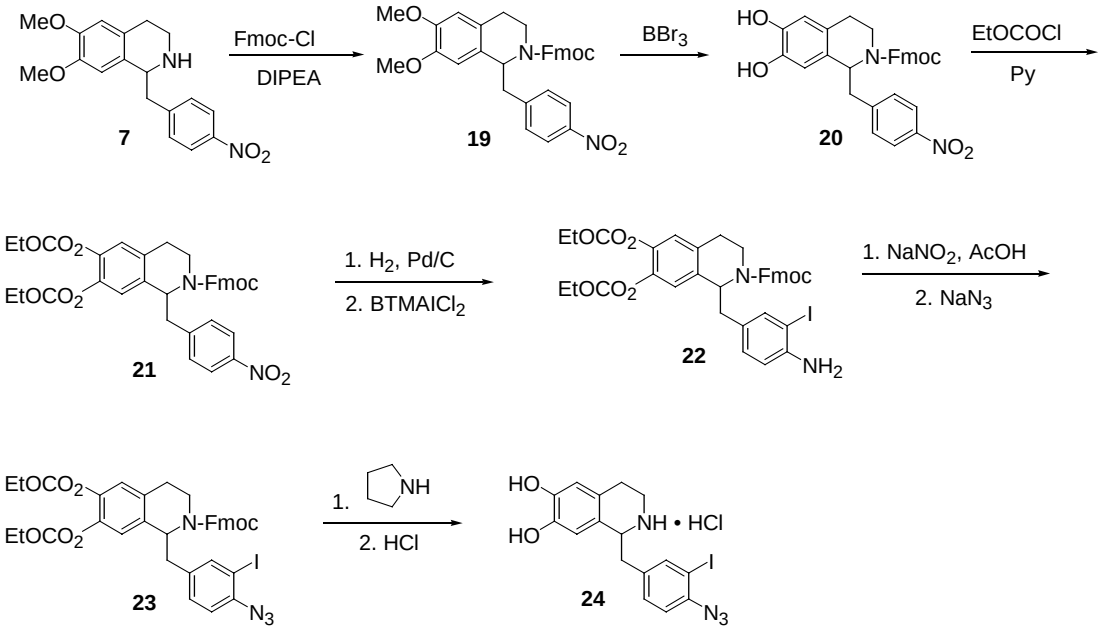


Scheme 2.

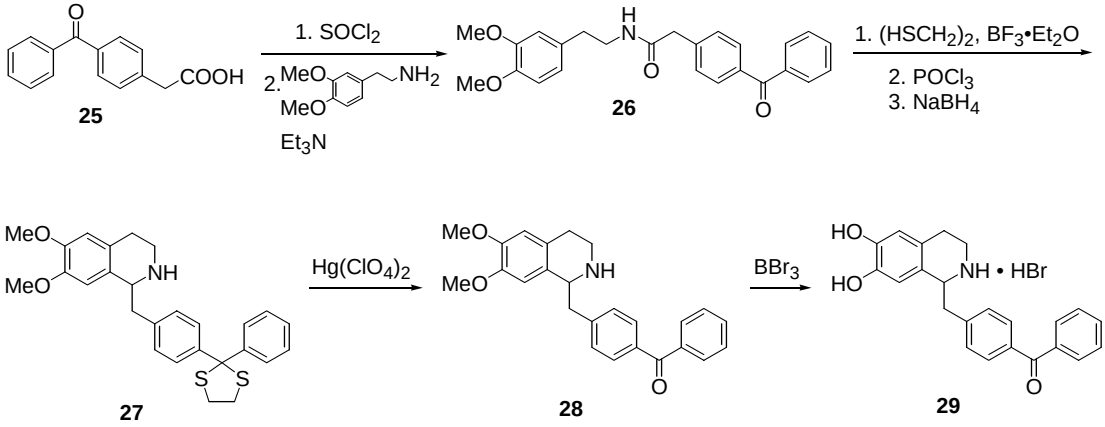
The most challenging project was the synthesis of azide **24**. The difficulties arise from lability of the azido group toward acids and Lewis acids and decomposition of catechol moiety in alkaline solutions. The synthesis of a linear catecholamine azide was described by Ruoho et al.[16,19] The strategy for the synthesis of

cyclic azidocatecholamines like tetrahydroisoquinolines should be different considering the very strenuous conditions of Bischler–Napieralski cyclization. We decided to reprotect the catechol fragment with carbonate groups and protect isoquinoline nitrogen with 9-fluorenylmethoxycarbonyl (Fmoc) group (Scheme 3). This strategy allowed us to use a basic amine as a single deprotecting reagent. Indeed, isoquinoline **7** was protected with Fmoc-Cl in the presence of *N*,*N*-diisopropylethylamine (DIPEA) to give derivative **19**, which was demethylated with BBr_3. Acylation of the resulting catechol **20** with ethyl chloroformate afforded triprotected analogue **21**. The nitro group in the latter compound was reduced with hydrogen in the presence of palladium on activated charcoal followed by monoiodination with 1.1 equiv of $BTMAICl_2$ in the presence of $CaCO_3$ to give *o*-iodoaniline **22**. A treatment of the diazonium salt, formed by the addition of aqueous solution of $NaNO_2$ to an acetic acid solution of compound **22**, with a solution of NaN_3 led to a protected azide **23**. The latter was treated with pyrrolidine, purified by flash chromatography, and the desired iodoazide **24** was isolated as HCl salt in 23% overall yield.

4-Benzoylphenylacetic acid **25** for the synthesis of benzophenone label **29** was obtained in three steps from *p*-methylbenzophenone according to the procedure by Zderic et al.[26] Acid **25** was transformed into acid chloride by heating with $SOCl_2$ followed by reaction with 3,4-dimethoxyphenethylamine in the presence of triethylamine (Scheme 4). The carbonyl group in the resulting amide **25** was protected with 1,2-ethanedithiol using $BF_3{\cdot}Et_2O$ as a catalyst.[27] Bischler–Napieralski cyclization of the resulting oily product in $POCl_3$/MeCN and reduction with $NaBH_4$ in methanol led to compound **27**. The dithiolane ring in compound **27** was smoothly cleaved with mercury (II) perchlorate to form ketone **28**.[27] The desired benzophenone containing photoaffinity label **29** was obtained after standard demethylation of compound **28** with BBr_3 in 31% total yield.



Scheme 3.



Scheme 4.

It should be noted that the ^{1}H and ^{13}C NMR spectra of N-Boc and N-Fmoc derivatives are quite complicated displaying signals corresponding to two major rotamers.

3. Biological results and discussion

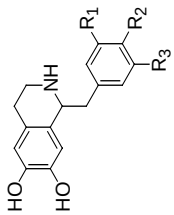
One objective of this research is to examine whether appropriately modified 1-benzyl TMQ analogues are suitable as affinity probes for β-AR subtypes. Biochemical and functional characterization of affinity labels **6**, **12**, and **18** on rat and human β_3-AR was discussed in our article recently.[28] Therefore, it was desirable to characterize the affinity and photoaffinity characteristics of the compounds we synthesized in CHO cells expressing the hu β_2-AR. 1-(4-Acetamido-3,5-diiodobenzyl) derivative of TMQ **30**, synthesized earlier,[22] was used for comparison.

Table 1 compares the binding affinities and functional activities of new analogues at human β_1-, β_2-, or β_3-AR to those of the reference compounds, ISO and TMQ. Both cAMP radioimmunoassay (cAMP-RIA) and cAMP response element-luciferase reporter gene (CRE-LUC) assay were employed to determine the receptor activities of the compounds. The non-selective β-AR agonist, (−)ISO, and 1-benzyl ring-substituted derivatives of TMQ competed for specific-bound [^{125}I]-(−)-3-iodocyanopindolol (ICYP) from the hu β-AR in a concentration-dependent manner. In studies with hu β_1- and hu β_2-ARs, the non-specific [^{125}I]ICYP binding was less than 5%, whereas with hu β_3-AR studies, non-specific binding was up to 30% of the total binding. At high concentrations, these ligands completely inhibited specific binding of ICYP to the hu β_1- and hu β_2-ARs, whereas in the hu β_3-AR system, only 60–90% of the specific binding of ICYP was inhibited by most TMQ analogues, an exception being azide **24** which completely abolished ICYP binding to this receptor subtype at high concentrations. In general, the compounds including ISO exhibited similar binding affinities for both hu β_1- and hu β_2-ARs. However, the TMQ derivatives possessed significantly higher (10- to 220-fold) binding affinities for the β-AR as compared to ISO.

Predictably, all the ligands exhibited significantly lower affinities for the hu β_3-AR as compared to the other two subtypes. Typically, the compounds showed 20- to 50-fold lower affinities for the hu β_3-AR subtype, with the exception of the azido derivative **24** which exhibited only 3- to 6-fold difference in binding affinities for the β-AR subtypes. The pK_is for azide **24** on all β-AR subtypes were over 6.70, which are greater than pK_is of 4–6 required for phenyl azides.[19]

Biochemical potencies expressed as pK_a indicated that all the TMQ analogues were more potent than ISO in CHO cells expressing all subtypes of hu β-ARs, the azide **24** being the most active in all cases, reaching the 13 pM level at β_2-AR. Also, the maximal intrinsic activities of the affinity and photoaffinity labels were 0.94–1.42 as compared to ISO (1.0), indicating these compounds to be full agonists in the hu β-AR systems.

Table 1. Binding affinities and activities of novel tetrahydroisoquinoline-6,7-diols affinity and photoaffinity labels on human β-AR expressed in CHO cells



Compound	R_1	R_2	R_3	hu β_1-AR	hu β_2-AR		hu β_3-AR		
				pK_i[a] ± SEM	pK_{act}[b] ± SEM (IA[c] ± SEM)	pK_i[a] ± SEM	pK_{act}[b] ± SEM (IA[c] ± SEM)	pK_i[a] ± SEM	pK_{act}[b] ± SEM (IA[c] ± SEM)
ISO				5.97 ± 0.19	8.93 ± 0.14 (100)	6.17 ± 0.12	8.41 ± 0.17 (100)	4.55 ± 0.14	7.99 ± 0.23 (100)
(*S*)-(−)-**1**	OMe	OMe	OMe	6.49 ± 0.05	8.70 ± 0.11 (109 ± 10)	7.36 ± 0.23	8.33 ± 0.24 (95 ± 3)	5.43 ± 0.28	8.60 ± 0.15 (95 ± 3)
30	I	NHAc	I	7.32 ± 0.07	9.49 ± 0.15 (113 ± 6)	8.22 ± 0.08	10.83 ± 0.12 (90 ± 4)	6.63 ± 0.14	8.96 ± 0.15 (101 ± 8)
6	I	$NHCOCH_2Cl$	I	8.31 ± 0.10	9.86 ± 0.08 (121 ± 9)	8.37 ± 0.18	9.88 ± 0.18 (94 ± 3)	6.77 ± 0.13	8.68 ± 0.14 (111 ± 8)
12	I	$NHCOCH_2Br$	I	8.23 ± 0.06	10.13 ± 0.19 (108 ± 3)	8.48 ± 0.14	10.54 ± 0.12 (101 ± 2)	6.94 ± 0.06	9.35 ± 0.16 (108 ± 4)
18	I	NCS	H	7.64 ± 0.04	9.42 ± 0.07 (129 ± 9)	7.35 ± 0.03	9.29 ± 0.18 (97 ± 4)	5.60 ± 0.15	8.02 ± 0.24 (114 ± 7)
24	I	N_3	H	7.45 ± 0.10	10.39 ± 0.18 (134 ± 11)	7.17 ± 0.05	10.88 ± 0.08 (97 ± 6)	6.70 ± 0.07	10.32 ± 0.09 (102 ± 6)
29	H	COPh	H	n.d.[e]	9.49 ± 0.46[d] (130 ± 30)	n.d.[e]	9.34 ± 0.18[d] (94 ± 6)	n.d.[e]	7.93 ± 0.17[d] (142 ± 17)

[a] K_i values were calculated using the following equation: K_i (M) = $IC_{50}/(1 + [L]/K_d)$, wherein IC_{50} is the molar concentration of a drug displacing 50% of specific bound radioligand, [L] is the concentration of radioligand, and K_d is the radioligand equilibrium dissociation constant. pK_i = −log K_i; SEM, standard error of mean; n = 4–7.

[b] cAMP-RIA assay. pK_{act} = −log EC_{50}; EC_{50}, concentration of agonist inducing a half-maximal rise in camp accumulation; n = 3–10.

[c] IA, intrinsic activity, maximal agonist response relative to maximal response of ISO.

[d] CRE-LUC assay.

[e] n.d., not determined.

3.1. Affinity labeling

In reversible competitive radioligand binding experiments, all of the selected 1-benzyl-substituted analogues of TMQ had inhibited [^{125}I]ICYP binding to the hu β_2-AR in CHO cells in a concentration-dependent manner (Table 1). In washout experiments, concentration-dependent inhibition of [^{125}I]ICYP binding to hu β_2-AR was observed in cells pretreated with isothiocyanate **18** or chloroacetamide **6**. In contrast, acetamide **30** pretreatment did not significantly affect radioligand binding after washout, irrespective of the concentration used (Fig. 1). Depending upon the concentration used, isothiocyanate **18** inhibited ICYP binding by 35–85%, whereas chloroacetamide **6** inhibited ICYP binding by a maximum of only 30–40% of the total radioligand binding. Non-specific radioligand binding to the receptors was around 5%. Thus, compound **8** was a more effective affinity ligand on the hu β_2-AR, whereas **6** exhibited weak irreversibly acting affinity properties on this system.

Figure 2 shows the effect of incubating acetamide **30**, chloroacetamide **6**, and isothiocyanate **18** over a range of 2–45 min at various concentrations with hu β_2-AR-CHO in these washout experiments. At concentrations up to 30K_i, the irreversible binding of all the three compounds to the receptor appeared to improve with incubation periods of up to 15 min, while slight recovery in ICYP binding occurred at an incubation period of 45 min. However, this trend was abolished when the compounds were incubated at higher concentrations

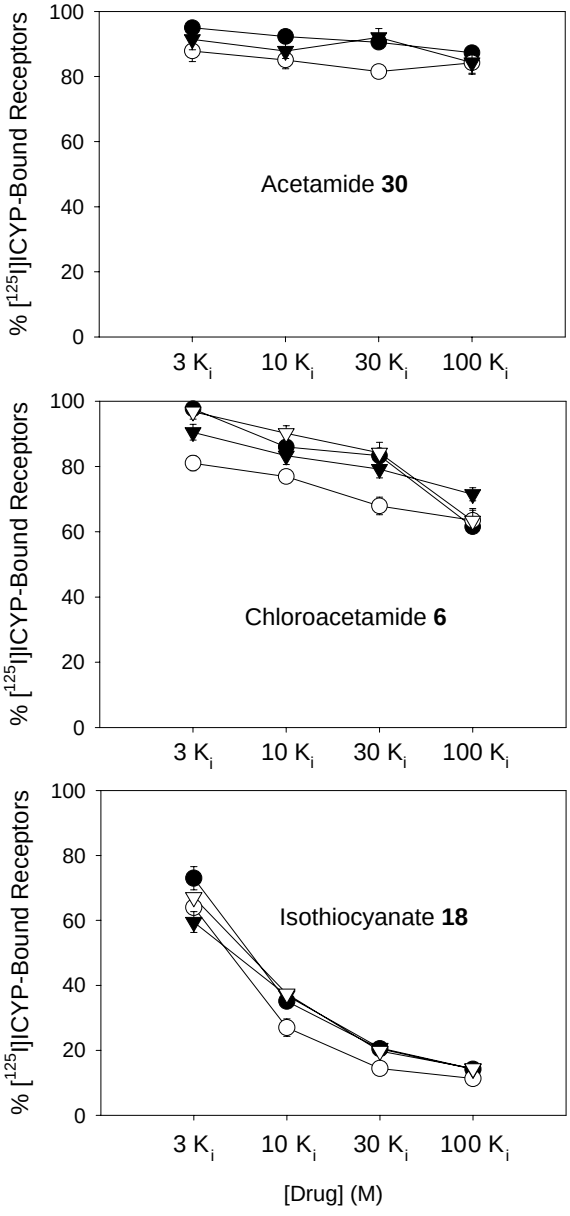


Figure 1. Concentration-dependent inhibition of [^{125}I]ICYP binding to hu β_2-AR in CHO cells in washout experiments after incubation with selected TMQ analogues for 2 min (▽), 5 min (●), 15 min (○) or 45 min (▼). Values are means ± SEM of three experiments, each in triplicate.

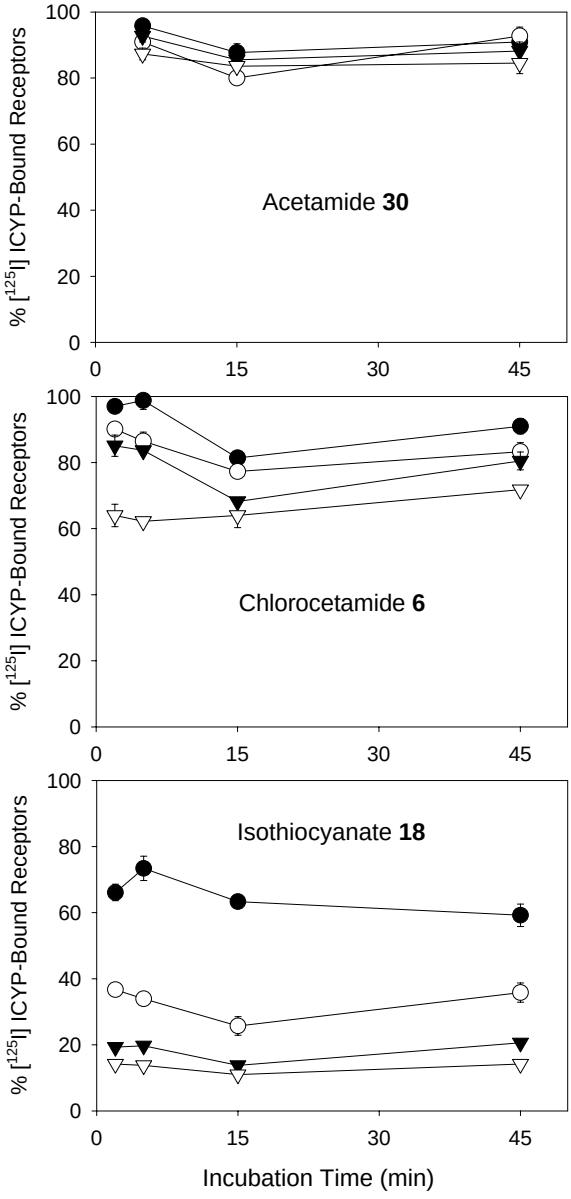


Figure 2. Effect of time of incubation with ligands at molar concentration of 3K_i (●), 10K_i (○), 30K_i (▼) and 100K_i (▽) in washout experiments, upon the inhibition of [^{125}I]ICYP binding to hu β_2-AR in CHO cells. The data are means ± SEM of three experiments, each in triplicate.

(100-fold of their K_i values) over this time range. Thus, there seems to be a more distinctive concentration dependency relationship for the irreversible binding of isothiocyanate **18** and chloroacetamide **6** analogues as compared to the relationship of binding to the period of incubation. In the case of the isothiocyanato **18** analogue, although a slightly greater effect was observed upon incubation for 15 min, shorter time periods of 2 and 5 min were sufficient to demonstrate 'irreversible' binding of the compound to the hu β_2-AR. Therefore, the binding by analogue **18** exhibits very rapid reaction kinetics. Collectively, the concentration and time data indicate that isothiocyanate **18** is an effective affinity analogue on the hu β_2-AR.

3.2. Photoaffinity labeling

When CHO cells expressing the hu β_2-AR were incubated with various concentrations of azide **24** under a red light for 1 h and exposed to UV light for a fixed amount of time (0.5 and 5 min), a concentration-dependent inhibition of ICYP binding to these cells was observed. This finding indicated that the azido analogue **24** was photolyzable and bound irreversibly to this receptor (see Fig. 3). At higher concentrations (10- and 100K_i) of the photoaffinity ligand **24**, inhibition of ICYP binding reached 47–87% of the total binding, and was dependent upon the sample exposure times to UV light of 350 nm. The extent of photoaffinity ligand irreversible binding was directly proportional to its concentration and time period of exposure to UV light. Samples incubated with the azido analogue **24** under the red light, but not exposed to UV light, did not exhibit irreversible binding which implies that the exposure of the azido analogue **24** to UV light is necessary for the formation of the highly reactive nitrene species under these photolytic conditions, which covalently binds to the receptor protein.

In control experiments where CHO cells were exposed to UV light for 5 min in the absence of the photoaffinity ligand, the radioligand binding of ICYP was not significantly affected (Fig. 3). Similarly, insignificant differences in the extent of ICYP binding were observed between samples incubated in complete dark environment and those under red light for 1 h.

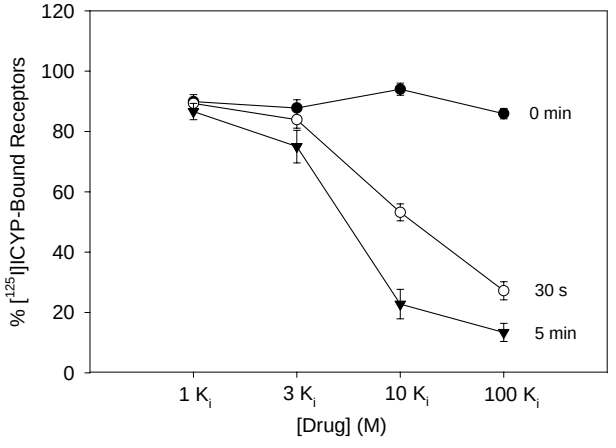


Figure 3. Concentration-dependent inhibition of [^{125}I]ICYP binding to hu β_2-AR in CHO cells in washout experiments by the azide **24** after incubation in red light for 1 h followed by exposure to UV light at 350 nm for 0 min, 30 s or 5 min. The data are means ± SEM of two–six experiments, each in triplicate.

4. Conclusions

We have developed the synthesis and evaluated the interaction of affinity and photoaffinity labels of the TMQ class for β-AR. Each compound showed high binding affinity and potency, and the compounds were full agonists on the three subtypes of human β-AR. Washout studies on β_2-AR demonstrated that isothiocyanate **18** reacted quickly to produce up to 85% of irreversible binding to the receptor subtype. Photoaffinity binding with the azide **24** reached up to 87% at the same receptor. Thus in this study, we have discovered one effective affinity agonist label and one photoaffinity agonist label for the β_2-AR belonging to the 1-benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol chemical class.

5. Experimental

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 FT-IR. Proton and Carbon-13 NMR spectra were obtained on a Bruker AX 300 spectrometer. Chemical shift values are reported as parts per million (δ) relative to tetramethylsilane (TMS) as an internal standard. Spectral data are consistent with assigned structures. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA, and found values are within 0.4% of the theoretical values. Flash chromatography was performed on silica gel (Merck, grade 60, 230–400 mesh, 60 Å). Anhydrous solvents were purchased from Aldrich.

5.1.1. 1-(4-α-Chloroacetamido-3,5-diiodobenzyl)-2-trifluoroacetyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (4). Chloroacetyl chloride (0.213 g, 1.88 mmol) was added to a cooled (0 °C) and stirred solution of **3** (0.44 g, 0.68 mmol), $CaCO_3$ (0.7 g, 6.99 mmol), and DMAP (1 mg) in dry CH_2Cl_2 (40 mL). The mixture was allowed to stir at room temperature overnight (15 h). Satd $NaHCO_3$ (20 mL) was added to the solution and the mixture was stirred for 1 h. The mixture was extracted with CH_2Cl_2 (3× 50 mL). The organic extract was dried over Na_2SO_4 then evaporated in vacuum to give a white solid. Recrystallization from ethyl acetate–hexanes mixture gave 0.37 g (69%) of the product as a white solid: mp 261–263 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (s, 1H, CONH), 7.65 (br m, 2H, ArH), 6.63 (s, 1H, ArH), 6.26 (s, 1H, ArH), 5.48 (t, 1H, CH), 4.25 (s, 2H, CH_2), 3.94–3.98 (m, 1H, CH), 3.87 (s, 3H, CH_3), 3.74 (s, 3H, CH_3), 3.64–3.67 (m, 1H, CH), 2.90–3.05 (m, 3H, CH), 2.65–2.80 (m, 1H, CH); IR (KBr) 3434 (NH), 1720 (C═O), 1684 (C═O) cm^{-1}.

Elemental analysis

Compound	Formula	Calculated (%)			Found (%)		
		C	H	N	C	H	N
6	$C_{18}H_{17}ClI_2N_2O_3{\cdot}HBr{\cdot}0.15\ Et_2O$	32.35	2.85	4.06	32.34	2.84	4.05
9	$C_{23}H_{30}N_2O_4$	69.32	7.59	7.03	69.21	7.61	7.05
10	$C_{23}H_{28}I_2N_2O_4{\cdot}0.4\ C_6H_{14}$	44.55	4.95	4.09	44.48	4.87	4.08
12	$C_{18}H_{18}N_2O_3Br_2I_2{\cdot}0.33\ Et_2O$	31.02	2.87	3.74	31.15	2.80	3.68
14	$C_{37}H_{38}BrIN_2O_5$	55.72	4.80	3.51	55.65	4.81	3.52
15	$C_{18}H_{19}IN_2O_3{\cdot}HI{\cdot}0.5\ EtOAc$	39.37	3.96	4.59	39.55	4.03	4.69
16	$C_{23}H_{29}IN_2O_4$	52.68	5.57	5.34	52.61	5.56	5.25
17	$C_{24}H_{27}IN_2O_4S{\cdot}0.1\ CHCl_3$	50.05	4.72	4.84	49.92	4.70	4.78
18	$C_{17}H_{15}\ IN_2O_2S{\cdot}HBr$	39.33	3.11	5.40	39.40	3.14	5.31
19	$C_{33}H_{30}N_2O_6{\cdot}0.45\ CH_2Cl_2$	68.23	5.29	4.76	68.20	5.40	4.66
20	$C_{31}H_{26}N_2O_6$	71.25	5.02	5.36	70.98	5.10	5.27
21	$C_{37}H_{34}N_2O_{10}$	66.66	5.14	4.20	66.53	5.20	4.14
22	$C_{37}H_{35}IN_2O_8$	58.27	4.63	3.67	58.32	4.67	3.64
23	$C_{37}H_{33}IN_4O_8$	56.35	4.22	7.10	56.21	4.18	7.04
24	$C_{16}H_{15}IN_4O_2{\cdot}HCl{\cdot}0.5\ Et_2O$	43.61	4.21	11.30	43.51	4.21	11.09
26	$C_{25}H_{25}NO_4{\cdot}0.05\ CH_2Cl_2$	73.79	6.21	3.44	73.75	6.30	3.43
27	$C_{27}H_{29}NO_2S_2{\cdot}(COOH)_2$	62.91	5.64	2.53	62.81	5.71	2.49
28	$C_{25}H_{25}NO_3{\cdot}(COOH)_2$	67.91	5.70	2.93	67.68	5.78	2.88
29	$C_{23}H_{21}NO_3{\cdot}HBr$	62.74	5.04	3.18	62.48	5.08	3.05

5.1.2. 1-(4-α-Chloroacetamido-3,5-diiodobenzyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (5). A solution of K_2CO_3 (1.4 g, 10 mmol) in 1:1 methanol/H_2O (40 mL) was added to a solution of **4** (0.37 g, 0.5 mmol) in methanol (30 mL). The mixture was stirred until completely dissolved (~7 h). The solution was concentrated in vacuum and extracted with ethyl acetate (3× 75 mL). The organic extract was dried with Na_2SO_4 and evaporated in vacuum to give 0.24 g (93%) of the product as a beige solid: mp 255 °C (dec.); ^{1}H NMR (300 MHz) δ 8.11 (s, 1H, CONH), 7.82 (s, 1H, ArH), 7.26 (s, 1H, ArH), 6.60 (s, 1H, ArH), 6.58 (s, 1H, ArH), 4.27 (s, 2H, CH_2Cl), 4.14 (m, 1H, CH), 3.86 (s, 3H, OMe), 3.84 (s, 3H, OMe) 3.08–3.17 (m, 2H, CH), 2.97 (m, 1H, CH), 2.7–2.84 (m, 2H, CH), 1.85 (br s, 1H, NH); IR (KBr) 1684 (C=O), 1518 (C=C, Ar) cm^{-1}.

5.1.3. 1-(4-α-Chloroacetamido-3,5-diiodobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrobromide (6). To a solution of **5** (0.523 g, 0.83 mmol) in dry CH_2Cl_2 (50 mL) at −78 °C was added dropwise 4 mL of 1 M solution of BBr_3 in CH_2Cl_2 under an argon atmosphere. The reaction mixture was allowed to warm to room temperature and stirring was continued overnight. The mixture was cooled (−78 °C) and quenched with methanol (20 mL). Stirring was continued at room temperature for 2 h. The solvents were evaporated in vacuum. A residue was dissolved in methanol (50 mL) and the solvent was removed in vacuum. This procedure was repeated four times to give a solid residue. Recrystallization from methanol–ethyl ether mixture gave 0.51 g (90%) of the product as an off-white solid: mp 202 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.24 (s, 1H, CONH), 9.15 (br m, 1H, OH), 8.05 (br m, 2H, NH^+), 8.54 (br m, 1H, OH), 7.99 (s, 1H, ArH), 7.95 (s, 1H, ArH), 6.72 (s, 1H, ArH), 6.56 (s, 1H, ArH), 4.66 (br m, 1H, CH), 4.27 (s, 2H, CH2), 3.3–3.38 (m, 2H, CH), 3.10–3.20 (m, 2H, CH), 2.80–3.0 (m, 4H, CH); IR (KBr) 1684 (C=O), 1518 (C=C, Ar) cm^{-1}. Anal. ($C_{18}H_{17}ClI_2N_2O_3{\cdot}HBr{\cdot}0.15\ Et_2O$) C, H, N.

5.1.4. 2-*tert*-Butoxycarbonyl-6,7-dimethoxy-1-(4-nitrobenzyl)-1,2,3,4-tetrahydroisoquinoline (8). To a cooled (0 °C) and stirred solution of **7** (2.0 g, 6.09 mmol) in THF (50 mL) was added 1 N NaOH (15 mL). Di-*tert*-butylcarbonate (1.33 g, 6.09 mmol) was added to the solution and stirring was continued for 30 min at 0 °C. The reaction mixture was stirred at room temperature for 24 h. The organic solvent was stripped in vacuum and the aqueous residue was diluted with water (20 mL). The cloudy solution was extracted with CH_2Cl_2 (3× 75 mL). The organic extract was dried (Na_2SO_4), then evaporated to give a glassy solid. Recrystallization from CH_2Cl_2–hexanes mixture gave 2.14 g (82%) of the product as light yellow crystals: mp 137–139 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.87 (m, 2H, ArH), 6.6 (m, 3H, ArH), 6.33 (s, 1H, ArH), 5.1 (t, J = 6.8 Hz, 1H), 4.15 (m, 1H, CH), 3.85 (s, 3H, OMe), 3.73 (s, 2H, Me), 3.64 (s, 1H, OMe), 3.25 (m, 1H, CH), 2.5–3.05 (m, 4H, CH), 1.45 (s, 3H, Me), 1.35 (s, 6H, Me); IR (KBr) 1682 (C=O) cm^{-1}; MS *m/e*: 428 (M^+, EI).

5.1.5. 1-(4-Aminobenzyl)-2-*tert*-butoxycarbonyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (9). A solution of **8** (2.0 g, 4.7 mmol) in ethyl acetate (100 mL) was hydrogenated (50 psi) over 10% Pd/C for 40 min. The catalyst was removed by filtration and the filtrate was concentrated to about 1/3 the original volume. Hexane was added until the solution became slightly cloudy.

The solution was allowed to stand at room temperature overnight. The white crystalline product (1.71 g, 91%) was collected by filtration: mp 170–172 °C; IR (KBr) 3432, 3353, 3241 (NH), 1668 (C=O) cm^{-1}; MS *m/e* 398 (M^+, EI). Anal. ($C_{23}H_{30}N_2O_4$) C, H, N.

5.1.6. 1-(4-Amino-3,5-diiodobenzyl)-2-*tert*-butoxycarbonyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (10). $CaCO_3$ (2.93 g, 29.3 mmol) and $BTMAICl_2$ (1.33 g, 3.76 mmol) were added to a solution of **9** (1.50 g, 3.76 mmol) in a mixture of CH_2Cl_2 (100 mL) and MeOH (40 mL). The reaction mixture was stirred at room temperature for 1 h. Additional $BTMAICl_2$ (3.35 g, 9.4 mmol) was added in two portions over a 2-day period. The solution was filtered, washed with 5% Na_2SO_3 (150 mL), and dried ($MgSO_4$). Evaporation of the solvent gave a glassy light red solid. Recrystallization from ethyl acetate–hexanes mixture gave 1.23 g (50%) of the product as peach colored crystals: mp 112–114 °C; IR (KBr) 3436 (NH), 3350 (NH), 1674 (C=O) cm^{-1}. Anal. ($C_{23}H_{28}I_2N_2O_4 \cdot 0.4\ C_6H_{14}$) C, H, N.

5.1.7. 1-(4-α-Bromoacetamido-3,5-diiodobenzyl)-2-*tert*-butoxycarbonyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (11). A mixture of **10** (0.70 g, 1.1 mmol) and $CaCO_3$ (1 g, 10 mmol) in CH_2Cl_2 (14 mL) was cooled to 0 °C in an ice bath. A solution of bromoacetyl bromide (0.232 g, 1.15 mmol) in CH_2Cl_2 (2 mL) was added dropwise with stirring. After the addition, the reaction mixture was stirred at room temperature for 45 min. The mixture was diluted with CH_2Cl_2 (40 mL) and filtered. The filtrate was washed with saturated $NaHCO_3$ solution (40 mL) and dried with Na_2SO_4. The solvent was evaporated in vacuum to give a white solid. Recrystallization of the crude product from ethyl acetate–hexanes mixture gave 0.49 g (58%) of the product: mp 172–174 °C (dec.); ^1H NMR (300 MHz, $CDCl_3$) δ 7.94 (s, 1H, NH), 7.64 (br s, 1H, ArH), 7.59 (br s, 1H, ArH), 6.60 (s, 1H, ArH), 6.28 and 6.23 (s, 1H, ArH), 5.2 and 5.05 (m, 1H, CH), 4.15 (m, 1H, CH), 4.05 (s, 2H, CH_2Br), 3.85 (s, 3H, OMe), 3.76 and 3.70 (s, 3H, OMe), 3.5–3.4 (m, 1H, CH), 2.65–3.05 (m, 4H, CH); IR (KBr) 3352 (NH), 1687 (C=O) cm^{-1}.

5.1.8. 1-(4-α-Bromoacetamido-3,5-diiodobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrobromide (12). A stirred solution of **11** (0.26 g, 0.34 mmol) in anhydrous CH_2Cl_2 (15 mL) was cooled in a dry ice-acetone bath and kept under an argon atmosphere. A 1 M solution of BBr_3 in CH_2Cl_2 (1.3 mL, 1.3 mmol) was slowly added via syringe. The reaction mixture was allowed to warm to room temperature and stirring was continued overnight. The mixture was cooled in an ice bath and carefully quenched with methanol (5 mL). Stirring was continued at room temperature for 2 h. The solvent was evaporated in vacuum and the oily residue was taken up in methanol (10 mL). The solvent was evaporated in vacuum. The methanol addition and evaporation were repeated three times to give an oily residue. The residue was taken up in a minimum amount of methanol. A small amount of anhydrous ether was added until formation of a precipitate. The cloudy solution was allowed to stand in a refrigerator overnight. The precipitated product was collected by filtration to give 0.21 g (85%) of the product as an off-white powdery solid: mp 210 °C (dec.); ^1H NMR (300 MHz, DMSO-d_6) δ 10.3 (s, 1H, CONH), 9.15 (br m, 1H, OH), 8.95 (br m, 2H, NH^+), 8.63 (br m, 1H, OH), 7.99 (s, 1H, ArH), 7.96 (s, 1H, ArH), 6.72 (s, 1H, ArH), 6.56 (s, 1H, ArH), 4.68 (br m, 1H, CH), 4.06 (s, 2H, CH_2), 3.28–3.38 (m, 2H, CH), 3.05–3.16 (m, 2H, CH), 2.70–2.97 (m, 4H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.03 (C=O), 147.05 (Ar), 145.88 (Ar), 142.04 (Ar), 141.89 (Ar), 141.45 (Ar), 140.40 (Ar), 123.69 (Ar), 123.04 (Ar), 116.31 (Ar), 114.11 (Ar), 100.00 (Ar), 99.99 (Ar), 57.40 (Al), 41.08 (Al), 39.87 (Al), 28.65 (Al), 25.66 (Al); IR (KBr) 1669 (C=O), 1522 (C=C, Ar) cm^{-1}; MS *m/e* 643, 645 (M+H, M+2+H, FAB). Anal. ($C_{18}H_{18}N_2O_3$-$Br_2I_2 \cdot 0.33\ Et_2O$) C, H, N.

5.1.9. 1-(4-α-Bromoacetamido-3-iodobenzyl)-6,7- dibenzyloxy-2-*tert*-butoxycarbonyl-1,2,3,4-tetrahydroisoquinoline (14). To a cold solution (0 °C) of isoquinoline **13** (0.68 g, 1 mmol) and Et_3N (0.34 g, 3 mmol) in CH_2Cl_2 (10 mL) was added $BrCOCH_2Br$ (0.40 g, 2 mmol). The cooling bath was removed and the mixture was stirred overnight. CH_2Cl_2 was added and the solution was washed with water, dried over $MgSO_4$, filtered, and concentrated. The resulting solution was purified by column chromatography (silica gel, hexane/EtOAc, 2:1). The solvents were evaporated under reduced pressure, ethyl ether was added, and evaporated to give a white glassy solid (0.44 g, 55%); ^1H NMR (300 MHz, $CDCl_3$) δ (mixture of conformers of 3:2 ratio) 8.54 (s, 1H, NH), 8.11 and 8.06 (s, 1H, H-5′), 7.60–7.25 (m, 11H, 2× Ph+H-2′), 7.08 and 7.00 (m, 1H, H-6′), 6.70 and 6.66 (s, 1H, H-5), 6.47 and 6.35 (s, 1H, H-8), 5.25–4.88 (m, 2× CH_2O+H-1), 4.11 and 3.73 (m, 1H, H-3e), 4.05 (m, 2H, CH_2Br), 3.28–3.10 (m, 1H, H-3a), 3.98–2.60 (m, 3H, CH_2 Ar+H-4a), 2.54 and 2.42 (m, 1H, H-4e), 1.43 and 1.31 (*t*-Bu); IR (KBr) 1688 (C=O), 1518 (C=C Ar) cm^{-1}. Anal. ($C_{37}H_{38}BrIN_2O_5$) C, H, N.

5.1.10. 1-(4-Acetamino-3-iodobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydroiodide (15). To a solution of isoquinoline **14** (0.40 g, 0.5 mmol) in anhydrous MeCN (5 mL) was added Me_3SiI (0.40 g, 2 mmol) via syringe under an argon atmosphere. The solution was stirred for 6 h followed by addition of MeOH (1 mL) and stirring was continued for 10 min. CH_2Cl_2 (50 mL) was added to reaction mixture and yellow crystals were filtered, yield 0.19 g (62%). Analytical sample was obtained by recrystallization from MeOH/MeCN/EtOAc mixture, mp 172–174 °C (dec.); ^1H NMR (300 MHz, DMSO-d_6) δ 9.39 (s, 1H, NH), 8.86 (br s, 1H, OH), 8.50 (br s, 1H, OH), 7.90 (d, J_m = 1.7 Hz, 1H, H-2′), 7.41 (d, J_o = 8.2 Hz, 1H, H-5′), 7.35 (dd, J_o = 8.2, J_m = 1.7 Hz, 1H, H-6′), 6.63 (s, 1H, H-5), 6.56 (s, 1H, H-8), 4.63 (m, 1H, H-1), 3.43–2.70 (m, 6H, H-3+H-4+CH_2Ar), 2.06 (s, 3H, Ac); ^{13}C NMR (75 MHz, methanol-d_4) δ 172.65 (s, C=O), 146.98 (s), 145.81 (s), 141.48 (d), 140.11 (s), 137.15 (s), 131.30 (d), 129.10 (d), 123.63 (s), 123.27 (s), 116.27 (d), 114.15 (d), 97.83 (s, C-3′), 57.60 (d, C-1), 41.01 (t), 39.98 (t), 25.68 (t), 23.09 (q, Me); IR (KBr) 3600–2400 (br, OH, NH), 1655 (C=O), 1624 (NH bend), 1522 (C=C Ar) cm^{-1}; MS *m/e* 439 (M^+). Anal. ($C_{18}H_{19}IN_2O_3 \cdot HI \cdot 0.5$ EtOAc) C, H, N.

5.1.11. 1-(4-Amino-3-iodobenzyl)-2-*tert*-butoxycarbonyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (16). $CaCO_3$ (1.7 g, 17 mmol) and $BTMAICl_2$ (0.76 g, 2.18 mmol) were added to a solution of **9** (0.87 g, 2.18 mmol) in a mixture of CH_2Cl_2 (50 mL) and MeOH (20 mL). The reaction mixture was stirred at room temperature for 1 h. The solution was filtered, washed with 5% Na_2SO_3 (100 mL) and dried over $MgSO_4$. Evaporation of the solvent gave a glassy light pink solid, yield 0.98 g (86%): mp 88–90 °C; IR (KBr) 3452 (NH), 3360 (NH), 1684 (C=O) cm^{-1}; MS *m/e* 525 (M+H, FAB). Anal. ($C_{23}H_{29}IN_2O_4$) C, H, N.

5.1.12. 2-(*tert*-Butoxycarbonyl)-1-(3-iodo-4-isothiocyanatobenzyl)-6,7-dimethoxy-1, 2,3,4-tetrahydroisoquinoline (17). To a mixture of isoquinoline **16** (0.52 g, 1 mmol) in $CHCl_3$ (10 mL) and $NaHCO_3$ (0.84 g, 10 mmol) in H_2O (10 mL) was added $CSCl_2$ (0.23 g, 2 mmol). The mixture was stirred at room temperature for 1 h. H_2O (40 mL) was added and the product was extracted with $CHCl_3$, washed with H_2O, dried over $MgSO_4$, and concentrated under reduced pressure. Ethyl ether was added to the residue and evaporated to give the isothiocyanate (0.47 g, 84%) as a white glassy solid; ^1H NMR (300 MHz, $CDCl_3$) δ (mixture of conformers of 3:2 ratio) 7.62 and 7.51 (s, 1H, H-2′), 7.03–7.20 (m, 2H, H-5′and H-6′), 6.62 and 6.60 (s, 1H, H-5), 6.41 and 6.29 (s, 1H, H-8), 5.21 and 5.07 (m, 1H, H-1), 4.2–2.1 (m, 6H, H-3, H-4 and CH_2Ar), 3.87, 3.81 and 3.72 (s, 6H, MeO), 1.58, 1.43 and 1.30 (s, 9H, *t*-Bu); IR (KBr) 2082 (NCS), 1686 (C=O), 1518 (C=C Ar) cm^{-1}. Anal. ($C_{24}H_{27}IN_2O_4S{\cdot}0.1CHCl_3$) C, H, N.

5.1.13. 1-(3-Iodo-4-isothiocyanatobenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrobromide (18). To a cold solution (−78 °C) of the isothiocyanate **17** (0.52 g, 0.92 mmol) in anhydrous CH_2Cl_2 (15 mL) was added 1 M solution of BBr_3 in CH_2Cl_2 (2.8 mL, 2.8 mmol) under argon atmosphere. The mixture was stirred overnight at room temperature followed by cooling it again (−78 °C) and dry MeOH (10 mL) was added. The mixture was stirred at room temperature for 1 h. Solvents were evaporated under reduced pressure at 25 °C and MeOH was added again and evaporated. This procedure was repeated three times. Ethyl ether was added to the oily residue. The off-white crystals formed were filtered and washed with ethyl ether yielding 0.47 g (84%) of the title compound: mp 246–247 °C; ^1H NMR (300 MHz, DMSO-d_6) δ 9.12 (br s, 1H, OH), 8.85 (br s, 2H, NH_2^+, 8.57 (br s, 1H, OH), 7.96 (d, J_m = 1.7 Hz, 1H, H-2′), 7.52 (d, J_o = 8.2 Hz, 1H, H-5′), 7.42 (dd, J_m = 1.7 Hz, J_o = 8.2 Hz, H-6′), 6.56 (s, 1H, H-5), 6.52 (s, 1H, H-8), 4.62 (br m, 1H, H-1), 3.5–2.7 (m, 6H, 3× CH_2); ^{13}C NMR (75 MHz, DMSO-d_6) δ 145.18, 144.05, 140.21, 137.77,134.11, 132.31,130.95, 127.04,122.31, 122.27, 115.18, 113.48, 96.44, 54.80, 39.00, 38.29, 24.19; IR (KBr) 3444 (OH), 3165, 2047 (NCS), 1589 (NH_2^+ bend), 1522 (C=C Ar) cm^{-1}. Anal. ($C_{17}H_{15}IN_2O_2S{\cdot}HBr$) C, H, N.

5.1.14. 2-(9-Fluorenylmethoxycarbonyl)-6,7-dimethoxy-1-(4-nitrobenzyl)-1,2,3,4-tetrahydroisoquinoline (19). A solution of **7** (3.28 g, 10 mmol) in 50 mL of CH_2Cl_2 was added dropwise to a cold (0 °C) solution of Fmoc-Cl (2.72 g, 10.5 mmol) in 50 mL of CH_2Cl_2. The mixture was stirred for 5 min followed by the addition of a solution of DIPEA (1.29 g, 10 mmol) in 10 mL of CH_2Cl_2 slowly. The cooling bath was removed and the solution was stirred for 2 h. The reaction mixture was washed twice with 1 N HCl, twice with water, dried over $MgSO_4$, filtered, concentrated, and recrystallized from AcOEt–hexanes. Filtration gave 5.67 g (96%) of yellow crystals: mp 140–141 °C; ^1H NMR (300 MHz, $CDCl_3$) δ (mixture of conformers of 10:9 ratio) 8.07 (d, J = 8.5 Hz, ArH), 7.97 (d, J = 8.4 Hz, ArH), 7.78 (d, J = 7.5 Hz, ArH), 7.73 (d, J = 7.4 Hz, ArH), 7.61 (d, J = 7.3 Hz, ArH), 7.56 (d, J = 7.3 Hz, ArH), 7.17–7.47 (m, ArH), 6.80 (d, J = 8.4 Hz, ArH), 6.60 (s, ArH), 6.55 (s, ArH), 6.29 (s, ArH), 6.02 (s, ArH), 5.32 (t, J = 6.5 Hz, CH), 4.73 (dd, J = 10.9, 4.8 Hz, CH), 4.64 (t, J = 6.8 Hz, CH), 4.36–4.50 (m, CH), 4.22 (t, J = 6.6 Hz, CH), 3.82–3.97 (m, CH), 3.86 (s, OMe), 3.70–3.80 (m, CH), 3.69 (s, OMe), 3.07–3.37 (m, CH), 2.61–2.88 (m, CH), 2.42–2.57 (m, CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ (conformers' mixture) 155.38 and 155.11 (C=O), 148.06, 147.32, 147.02, 146.73, 146.56, 145.94, 145.69, 144.07, 143.89, 143.88, 143.58, 141.48, 141.34, 130.55, 130.36, 127.71, 127.52, 127.41, 127.18, 127.01,126.53, 126.29, 124.87, 124.81, 124.59, 123.30, 123.20, 119.99, 119.83, 111.39, 111.29, 110.11, 109.93, 67.29 and 66.08 (OCH_2), 55.87 (OMe), 55.66 (C-1), 47.72 and 47.28 ($CHAr_2$), 42.50 and 42.42 (C-3), 39.40 and 38.59 (CH_2Ar), 27.94 and 27.50 (C-4); IR (KBr) 1688 (C=O), 1607, 1520 and 1347 (NO_2) cm^{-1}. Anal. ($C_{33}H_{30}N_2O_6{\cdot}0.45\ CH_2Cl_2$) C, H, N.

5.1.15. 2-(9-Fluorenylmethoxycarbonyl)-1-(4-nitrobenzyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (20). 1 M solution of BBr_3 in CH_2Cl_2 (28 mL, 28 mmol) was added to a cooled solution (−78 °C) of compound **19** (5.51 g, 9.36 mmol) in 100 mL of anhydrous CH_2Cl_2 under an argon atmosphere. The reaction mixture was stirred overnight at room temperature followed by 40 mL of MeOH added upon cooling (−78 °C) and stirred at room temperature for 5 h. The solution was then filtered and evaporated four times with MeOH. The product was recrystallized from MeOH–ethyl ether mixture, to give 4.65 g (95%) of yellow crystals: mp 216–218 °C; ^1H NMR (300 MHz, DMSO-d_6) δ (mixture of conformers of 1:1 ratio) 8.87 (br s, OH) 8.67 (br s, OH), 8.04 (d, J = 8.7 Hz, ArH), 7.87 (d, J = 7.5 Hz, ArH), 7.82 (d, J = 7.5 Hz, ArH), 7.52 (m, ArH), 7.12–7.43 (m, ArH), 6.54 (s, ArH), 6.46 (s, ArH), 6.35 (s, ArH), 5.15 (m, H-1), 4.76 (m, H-1), 4.02–4.34 (m, CH), 3.58–3.82 (m, CH), 2.83–3.40 (m, CH), 2.32–2.60 (m, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 154.47 and 154.38 (C=O), 146.87, 146.69, 145.99, 144.22, 144.05, 143.85, 143.75, 143.50, 143.40, 140.74, 140.70, 130.58, 127.55, 127.01, 126.60, 126.23, 124.86, 124.74, 124.54, 124.29, 124.24, 122.93, 120.08, 120.04, 115.19, 113.93, 66.38 and 66.06 (OCH_2), 55.17 and 54.78 (C-1), 46.72 and 46.60 ($CHAr_2$), 41.50 and 41.35 (C-3), 37.91 and 37.72 (CH_2Ar), 26.94 and 26.56 (C-4); IR (KBr) 3395 and 3204 (OH), 1649 (C=O), 1519 and 1346 (NO_2) cm^{-1}. Anal. ($C_{31}H_{26}N_2O_6$) C, H, N.

5.1.16. 6,7-Bis(ethoxycarbonyloxy)-2-(9-fluorenylmethoxycarbonyl)-1-(4-nitrobenzyl)-1,2,3,4-tetrahydroisoquinoline (21). Pyridine (0.55 g, 7 mmol) was added to a cold (0 °C) solution of the catechol **20** (1.05 g, 2 mmol) and ethylchloroformate (0.65 g, 6 mmol) in 20 mL of CH_2Cl_2. The mix-

ture was stirred overnight at room temperature. The solution was washed twice with water, twice with 1 N HCl, water (2×), dried over $MgSO_4$, filtered, and evaporated. Traces of solvent were removed on pump and drying unit to give 1.26 g (95%) of a white glassy solid, which starts melting at 58 °C. Long standing in hexanes gave crystals: mp 134–136 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (mixture of conformers of 5:6 ratio) 8.07 (d, J = 8.4 Hz, ArH), 7.95 (d, J = 8.4 Hz, ArH), 7.73 (t, J = 7.3 Hz, ArH), 7.16–7.57 (m, ArH), 7.05 (s, ArH), 6.99 (s, ArH), 6.77 (s, ArH), 6.76 (s, ArH), 6.73 (s, ArH), 6.55 (s, ArH), 5.37 (t, J = 6.4 Hz, H-1), 4.88 (dd, J = 11.0, 4.1 Hz, H-1), 4.17–4.54 (m, CH_2O+CH), 4.07 (m, CH), 3.93 (m, CH), 3.75 (m, CH), 3.07–3.33 (m, CH), 2.93–3.03 (m, CH), 2.65–2.83 (m, CH), 2.43–2.64 (m, CH), 1.40 (m, Me); ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.20 and 154.91 (NCO_2), 152.65 (OCO_2), 146.85, 146.62, 145.12, 145.00, 144.15, 143.81, 143.75, 143.02, 141.65, 141.33, 141.27, 141.13, 141.07, 140.50, 140.32, 134.13, 133.75, 133.23, 133.01, 130.44, 130.17, 127.73, 127.63, 127.13, 127.03, 126.96, 124.76, 124.46, 124.35, 123.51, 123.33, 123.17, 123.00, 121.71, 121.24, 120.04, 119.96, 67.28 and 66.05 (NCO_2CH_2), 65.32 (CH_2OCO_2), 55.60 and 55.37 (C-1), 47.92 and 47.25 ($CHAr_2$), 42.29 and 42.03 (C-3), 38.76 and 37.60 (CH_2Ar), 27.94 and 27.49 (C-4), 14.13 (Me); IR (KBr) 1772 (OCO_2), 1701 (NCO_2), 1520 and 1346 (NO_2), 1257 (C–O) cm^{-1}. Anal. ($C_{37}H_{34}N_2O_{10}$) C, H, N.

5.1.17. 1-(4-Amino-3-iodobenzyl)-6,7-bis(ethoxycarbonyloxy)-2-(9-fluorenylmethoxycarbonyl)-1,2,3,4-tetrahydroisoquinoline (22). (1) The solution of compound **21** (1.00 g, 1.3 mmol) in 50 mL of EtOAc was hydrogenated at 60 psi with 10% Pd/C (0.35 g) for 4 h. The reaction mixture was filtered through Celite and evaporated in vacuum. A glossy solid was stirred overnight with hexanes to give a white crystalline solid, yield 0.95 g (99%), mp 127–129 °C. The compound was used without further purification. (2) The solution of this aniline (0.83 g, 1.3 mmol), $BTMAICl_2$ (0.50 g, 1.43 mmol), and $CaCO_3$ (0.19 g, 1.95 mmol) in CH_2Cl_2 (30 mL) and MeOH (12 mL) was stirred for 2 h at room temperature, filtered, washed with satd Na_2SO_3 (2×), water (2×), dried over $MgSO_4$, filtered, concentrated, and purified by flash chromatography on silica gel (EtOAc/hexanes,1:2) to afford after stirring in hexanes a white glassy solid (0.64 g, 64%): mp 73–75 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (mixture of conformers of 5:7 ratio) 7.70–7.79 (m, ArH), 7.50–7.59 (m, ArH), 7.20–7.46 (m, ArH), 7.03 (d, J = 9.4), 6.78–6.86 (m, ArH), 6.53–6.63 (m, ArH), 5.30 (t, J = 6.6 Hz, H-1), 4.80 (m, H-1), 4.46–4.60 (m, CH), 4.19–4.40 (m, CH_2O+CH), 3.81–4.13 (m, CH), 3.32 (m, CH), 3.06 (m, CH), 2.93 (d, J = 6.6 Hz, CH_2Ar), 2.47–2.80 (m, CH), 1.40 (m, Me); ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.19 (NCO_2), 152.74 (OCO_2), 145.33, 144.39, 144.19, 143.84, 143.27, 141.40, 141.33,140.86, 140.34, 139.62, 139.40, 135.09, 134.82, 133.32, 133.04, 130.60, 130.47, 129.08, 127.58, 127.18, 127.08, 126.92, 125.06, 124.96, 124.82, 124.77, 123.12, 122.78, 121.80, 121.40, 120.00, 119.92, 114.60, 114.44, 83.93 and 83.84 (C-3′), 67.37 and 66.85 (NCO_2CH_2), 65.26 (CH_2OCO_2), 55.80 (C-1), 47.74 and 47.33 ($CHAr_2$), 40.96 (C-3), 38.61 and 37.23 (CH_2Ar), 28.08 and 27.75 (C-4), 14.16 (Me); IR (KBr) 3457 and 3371 (NH_2), 1770 (OCO_2), 1696 (NC=O), 1618, 1499, 1259 (C–O) cm^{-1}. Anal. ($C_{37}H_{35}IN_2O_8$) C, H, N.

5.1.18. 1-(4-Azido-3-iodobenzyl)-6,7-bis(ethoxycarbonyloxy)-2-(9-fluorenylmethoxycarbonyl)-1,2,3,4-tetrahydroisoquinoline (23). Isoquinoline **22** (0.153 g, 0.2 mmol) was dissolved in 4 mL of AcOH, 1 mL of water was added and stirred on an ice bath. A solution of $NaNO_2$ (0.028 g, 0.4 mmol) was added and stirred for 15 min, followed by 12 mL of cold water (0 °C) was added. A solution of NaN_3 (0.104 g, 1.6 mmol) in 1 mL of water was added and stirred for 2 h. The white precipitate was filtered, washed with water (4×), and dried in vacuum. Yield 0.154 g (97%): mp 61–63 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (mixture of conformers of 10:13 ratio) 7.70–7.80 (m, ArH), 7.20–7.60 (m, ArH), 6.93–7.10 (m, ArH), 6.81–6.90 (m, ArH), 6.68–6.77 (m, ArH), 5.32 (t, J = 6.5 Hz, H-1), 4.77 (dd, J = 10.8, 4.9 Hz, H-1), 4.62 (m, CH), 4.20–4.50 (m, CH_2O+CH), 3.94–4.13 (m, CH), 3.84 (m, CH), 3.32 (m, CH), 3.00 (m, CH), 2.44–2.82 (m, CH), 1.40 (m, Me); ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.20 and 155.01 (NCO_2), 152.71 (OCO_2), 144.23, 144.02, 143.78, 143.08, 141.52, 141.32, 141.05, 140.99, 140.75, 140.48, 140.38, 140.09, 140.00, 135.92, 135.87, 134.56, 134.22, 133.28, 132.98, 130.81, 130.61, 127.68, 127.66, 127.25, 127.08, 126.93, 124.94, 124.89, 124.66, 124.57, 123.20, 122.94, 121.77, 121.30, 120.07, 119.97, 118.19, 118.02, 87.44 and 87.36 (C-3′), 67.44 and 66.49 (NCO_2CH_2), 65.30 (CH_2OCO_2), 55.63 and 55.37 (C-1), 47.91 and 47.29 ($CHAr_2$), 41.27 and 41.03 (C-3), 38.63 and 37.30 (CH_2Ar), 28.04 and 27.58 (C-4), 14.16 (Me); IR (KBr) 2121 (N_3), 1771 (OCO_2), 1699 (NC=O), 1258 (C–O) cm^{-1}. Anal. ($C_{37}H_{33}IN_4O_8$) C, H, N.

5.1.19. 1-(4-Azido-3-iodobenzyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol hydrochloride (24). Pyrrolidine (3 mL) was added to a solution of isoquinoline **23** (0.279 g, 0.35 mmol) in 3 mL of CH_2Cl_2 under an argon atmosphere and stirred for 1 h at room temperature. The reaction mixture was concentrated and then dried on a vacuum pump. The resulting oil was purified by flash chromatography on silica gel (EtOAc/MeOH, 20:1, 10:1). Homogeneous fractions were evaporated, dissolved in MeOH, and 1 M HCl in ethyl ether (0.5 mL, 0.5 mmol) was added. More ether was added to initiate crystallization. Yield 0.072 g (44%): mp 188–190 °C (dec.); ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.14 (s, 1H, OH), 8.93 (br s, 1H, NH), 8.88 (s, 1H, OH), 8.74 (br s, 1H, NH), 7.87 (d, J = 1.8 Hz, 1H, ArH), 7.44 (dd, J = 8.2, 1.8 Hz, 1H, ArH), 7.35 (d, J = 8.2 Hz, 1H, ArH), 6.56 (s, 1H, ArH), 6.54 (s, 1H, ArH), 4.57 (m, 1H, CH), 2.30–3.30 (m, 6H, CH); ^{13}C NMR (75 MHz, methanol-d_4) δ 147.04 and 145.80 (C-6 and C-7), 142.81 (C-1′), 142.18 (C-2′), 135.24 (C-4′), 132.16 (C-6′), 123.68 and 123.19 (C-4a and C-8a), 120.10 (C-5′), 116.27 (C-5), 114.25 (C-8), 89.00 (C-3′), 57.48 (C-1), 40.88 (C-3), 39.92 (CH_2Ar), 25.63 (C-4); IR (KBr) 3600–2300 (br, OH, NH), 2122 (N_3), 1611, 1528, 1483 cm^{-1}. Anal. ($C_{16}H_{15}IN_4O_2$·HCl·0.5 Et_2O) C, H, N.

5.1.20. *N*-(3,4-Dimethoxyphenethyl)-4-benzoylphenylacetamide (26). A solution of *p*-benzoylphenylacetic acid[26] (4.81 g, 0.02 mol) and $SOCl_2$ (4.02 g, 0.034 mol) in benzene (40 mL) was heated at reflux for 3 h, evaporated

two times with benzene, dissolved in CH_2Cl_2 (50 mL), cooled on an ice bath, and 3,4-dimethoxyphenetylamine (3.99 g, 0.022 mol) was added dropwise. Et_3N (4.05 g, 0.04 mol) was added and the reaction mixture was stirred overnight at room temperature, washed with 1 N HCl (2×), H_2O, 1 N NaOH, and H_2O, dried over $MgSO_4$, and evaporated. The resulting oil was recrystallized from CH_2Cl_2–hexanes mixture. Yield 6.72 g (83%): mp 113–114 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.72–7.81 (m, 4H, ArH), 7.61 (m, 1H, ArH), 7.46–7.53 (m, 2H, ArH), 7.32 (d, J = 8.3 Hz, 2H, ArH), 6.73 (d, J = 8.1 Hz, 1H, ArH), 6.65 (d, J = 1.9 Hz, 1H, ArH), 6.56 (dd, J = 8.1, 1.9 Hz, 1H, ArH), 5.48 (br t, 1H, NH), 3.83 (s, 3H, MeO), 3.82 (s, 3H, MeO), 3.60 (s, 2H, CH_2CO), 3.48 (m, 2H, CH_2N), 2.71 (t, J = 6.9 Hz, 2H, CH_2Ar); ^{13}C NMR (75 MHz, $CDCl_3$) δ 196.13 (C=O), 169.85 (NCO), 149.08 (O–C, Ar), 147.75 (O–C, Ar), 139.53, 137.40, 136.55, 132.51, 130.93, 130.63, 129.94, 129.27, 128.32, 120.58, 111.75, 111.28, 55.87 (MeO), 55.83 (MeO), 43.73, 40.80, 35.02 (CH_2Ar); IR (KBr) 3327 (NH), 1659 (C=O), 1642 (C=O, amide), 1549, 1518 cm^{-1}. Anal. ($C_{25}H_{25}NO_4$·0.05 CH_2Cl_2) C, H, N.

5.1.21. 6,7-Dimethoxy-1-[4-(2-phenyl-1,3-dithiolane-2-yl)benzyl]-1,2,3,4-tetrahydroisoquinoline oxalate (27). A solution of the amide **26** (4.84 g, 12 mmol), 1,2-ethanedithiol (8.0 g, 82 mmol), and BF_3·Et_2O (7.2 mL, 8.3 g, 59 mmol) in 18 mL of anhydrous $CHCl_3$ was stirred overnight at room temperature under an argon atmosphere. $CHCl_3$ was added, washed three times with satd Na_2CO_3, brine, dried over $MgSO_4$, and concentrated. The resulting oil was dissolved in 50 mL of MeCN and reconcentrated, MeCN (85 mL) and $POCl_3$ (7.8 mL) were added and refluxed for 5 h. The solution was concentrated and evaporated with MeOH (50 mL) three times. The residue was dissolved in MeOH (125 mL) and $NaBH_4$ (9.6 g, 0.25 mol) was added in small portions. The reaction mixture was stirred overnight at room temperature. MeOH was evaporated, $CHCl_3$ added, and washed twice with 10% NaOH, H_2O, dried over $MgSO_4$, filtered, and concentrated. The product was dissolved in CH_2Cl_2 and MeOH, and solution of oxalic acid (1.8 g, 14 mmol) in minimal amount of MeOH was added followed by ethyl ether. The white crystals were filtered and washed with ethyl ether. Yield 3.93 g (59%): mp 151–152.5 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.43–7.54 (m, 4H, ArH), 7.20–7.36 (m, 5H, ArH), 6.77 (s, 1H, ArH), 6.17 (s, 1H, ArH), 4.63 (m, 1H, H-1), 3.71 (s, 3H, MeO), 3.39–3.50 (m, 1H, CH), 3.40 (s, 4H, SCH_2), 3.39 (s, 3H, MeO), 3.12–3.31 (m, 3H, CH), 2.85–2.98 (m, 2H, CH); ^{13}C NMR (75 MHz, methanol-d_4) δ 166.63 (HO_2CCO_2H), 150.51 (O–C, Ar), 149.00 (O–C, Ar), 146.23, 145.78, 136.11, 130.49, 129.91, 129.27, 128.93, 128.27, 124.93 and 124.56 (C-4a and C-8a), 112.98 and 111.54 (C-5 and C-8), 57.20 (C-1), 56.42 (MeO), 41.08 (SCH_2), 41.04 (SCH_2), 40.77 (C-3), 39.91 (CH_2Ar), 25.78 (C-4); IR (KBr) 3300–2300 (br, NH+OH), 1721 (C=O, acid), 1614, 1520 cm^{-1}. Anal. ($C_{27}H_{29}NO_2S_2$·$(COOH)_2$) C, H, N.

5.1.22. 4-(6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline-1-yl-methyl)benzophenone oxalate (28). Oxalic acid salt of **27** (3.81 g, 6.88 mmol) was mixed with 1 N NaOH, extracted with $CHCl_3$, washed with brine, dried over $MgSO_4$, filtered, and concentrated. The resulting oil was dissolved in $CHCl_3$ (75 mL) and a solution of $Hg(ClO_4)_2$·xH_2O (9.61 g, about 21 mmol) in MeOH (75 mL) was added and stirred for 1 h. A yellow precipitate was removed by filtering through Celite. The filtrate was extracted with $CHCl_3$, satd Na_2CO_3, brine, dried over $MgSO_4$, filtered, and concentrated to an approx. 10 mL volume. A solution of oxalic acid (1.26 g, 10 mmol) in MeOH (10 mL) was added followed by some ethyl ether. After cooling, the white crystals were filtered and washed with ethyl ether. Yield 2.35 g (72%): mp 201–202 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.24 (br s, NH), 7.65–77.6 (m, 5H, ArH), 7.49–7.61 (m, 4H, ArH), 6.79 (s, 1H, ArH), 6.49 (s, 1H, ArH), 4.73 (t, J = 7.0 Hz, 1H, H-1), 3.73 (s, 3H, MeO), 3.54 (s, 3H, MeO), 3.38–3.49 (m, 2H, CH), 3.19–3.32 (m, 2H, CH), 2.82–3.01 (m, 2H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 195.48 (C=O), 164.39 (HO_2CCO_2H), 148.20 and 146.99 (C-6 and C-7), 141.64, 137.05, 135.77, 132.68, 130.01, 129.89, 129.51,128.57, 124.19 and 124.01 (C-4a and C-8a), 111.74 (C-5), 110.14 (C-8), 55.44 (OMe), 55.27 (OMe), 54.77 (C-1), 38.38 (CH_2Ar), 24.67 (C-4); IR (KBr) 3300–2300 (NH, OH), 1721 (C=O, acid), 1657 (C=O), 1609, 1521 cm^{-1}. Anal. ($C_{25}H_{25}NO_3$·$(COOH)_2$) C, H, N.

5.1.23. 4-(6,7-Dihydroxy-1,2,3,4-tetrahydroisoquinoline-1-yl-methyl)benzophenone hydrobromide (29). The free base was obtained by extraction of a solution of isoquinoline salt **28** (0.478 g, 1 mmol) in 1 N NaOH with $CHCl_3$, dried over $MgSO_4$, evaporated, and dried in vacuum. The resulting oil was dissolved in anhydrous CH_2Cl_2 (20 mL) under an argon atmosphere and cooled down with dry ice bath. Solution of 1 N BBr_3 in CH_2Cl_2 (4.0 mL, 4 mmol) was added with syringe and stirred overnight at room temperature. Cooled down again followed by MeOH (10 mL) was added and stirred for 4 h at room temperature, and evaporated five times with MeOH almost to dryness. MeOH–ethyl ether mixture was added to crystals. After refrigeration, crystals were collected by filtration and washed with MeOH–ethyl ether. The product was recrystallized from MeOH–ether. Yield 0.388 g (88%): mp 239–241 °C; ^{1}H NMR (300 MHz, methanol-d_4) δ 7.79 (m, 4H, H-3′+H-5′+H-2″+H-6″), 7.65 (m, 1H, H-4″), 7.48-7.58 (m, 4H, H-2′+H-6′+H-3″+H-5″), 6.64 (s, 1H, ArH), 6.52 (s, 1H, ArH), 7.65 (m, 1H, H-1), 3.50–3.60 (m, 2H, H-3), 3.19–3.37 (m, 2H, CH), 2.88–2.32 (m, 2H, CH); ^{13}C NMR (75 MHz, methanol-d_4) δ 198.16 (C=O), 147.05 and 145.76 (C-6 and C-7), 141.95, 138.74, 138.19, 133.93 (C-4″), 131.77, 131.02, 130.89, 129.57, 123.71 and 123.30 (C-4a and C-8a), 116.30 (C-5), 114.35 (C-8), 55.45 (C-1), 41.11 (C-3), 40.87 (CH_2Ar), 25.63 (C-4); IR (KBr) 3600–2600 (OH+NH), 1646 (C=O), 1608 (C=C, Ar), 1527, 1282 cm^{-1}. Anal. ($C_{23}H_{21}NO_3$·HBr) C, H, N.

5.2. Pharmacological studies

5.2.1. Radioligand binding studies. The procedure for handling of CHO cells expressing β-AR subtypes and incubation with radioligand are as described previously.[28] Briefly, following growth of cells to 70% confluen-

cy, CHO cells expressing human β_1-, β_2-, or β_3-AR were trypsinized and harvested into Ham's F-12 solutions. Cells were pelleted and washed three times with Tris–EDTA buffer (pH 7.4; TRIZMA HCl, 50 mM; NaCl, 150 mM; disodium EDTA·$2H_2O$, 20 mM). Cells were then suspended in Tris–EDTA buffer after centrifugation. Competition binding assays were performed at 37 °C by incubating cells with varying concentrations of drugs in the presence of [^{125}I]ICYP ($(1.5–5 \times 10^4$ cells /18–70 pM for human β_1-, or β_2-AR, $(3–5) \times 10^5$ cells/ 200–500 pM for human β_3-AR). Non-specific binding was determined in the presence of (−)-propranolol (1 μM for human β_1-, or β_2-AR, 100 μM for human β_3-AR). The incubations were terminated by rapid filtration over Whatman GF/C (for human β_3-AR, presoaked in 0.1% polyethylenimine) glass fiber filters using a Brandel model 12-R cell harvester. The filters were washed three times with the Tris–EDTA buffer (4 °C) and dried under cell harvester vacuum. The radioactivity in the filters was measured by gamma scintillation counting using Beckmann gamma counter model 8000. K_i values were calculated from the obtained IC_{50} values by the method of Cheng and Prusoff.[29]

5.2.2. cAMP-RIA assay. The procedures for the handling, incubation ,and assay of cAMP in CHO cells expressing human β_1-, β_2-, or β_3-AR subtypes are as previously described.[30] Cells were grown to confluence in 60-mm dishes, washed with Hanks' balanced salt solution, and then incubated with Hanks' balanced salt solution (pH 7.4) containing 20 mM HEPES, 1 mM 3-isobutyl-1-methylxanthine (IBMX), and 1 mM L-ascorbic acid for 30 min at 37 °C. Varying concentrations (10^{-11}–10^{-4} M) of the compounds were added with incubation of an additional 30 min. After removal of the Hank's buffer, the cAMP generated within the cells was extracted by the addition of trichloroacetic acid (6% w/v). The precipitated protein was dissolved in 0.1 N NaOH. Protein content was determined by the method of Lowry et al.[31] using bovine serum albumin as a standard. cAMP levels in CHO cells were determined using the radioimmunoassay technique of Brooker et al.[32] The amount of cAMP was measured as the amount of ^{125}I-labeled succinyl-cAMP tyrosine methyl ester/antibody precipitated.

5.2.3. CRE-LUC assay. CHO cells stably expressing human β_1-, β_2-, or β_3-AR populations were transfected with a 6 CRE-LUC plasmid (gift from Dr. Himmler A. Vienna, Austria) using electroporation with a single 70 ms, 150 V pulse.[33] The transfected CHO cells were seeded at a density of 40,000/well in 96-well microtiter plates (culturplate, Packard) and allowed to grow for 20 h. After 20 h, the cells were treated with varying drug concentrations (10^{-11}–10^{-4} M) for 4 h. Following drug exposures, the cells were lysed and luciferase activity was measured using the LucLite® assay kit (Packard). Changes in light production were measured by a Topcount® luminometer (Packard). Data were analyzed in duplicate at each concentration and expressed as a percent luciferase response relative to the maximum response to (−)-isoproterenol (10^{-6} M). Results are expressed as means ± SEM of $n = 5–16$.

5.2.4. Time and concentration dependence of affinity binding. CHO cells expressing the human β_2-AR (about $30–40 \times 10^3$ cells/150 μL) were suspended in 1.2 mL Tris buffer in microfuge tubes and incubated at room temperature in a rotating shaker (Robbins Scientific) with the acetamide **30** (K_i = 6.0 nM), chloroacetamide **6** (K_i = 4.3 nM) or isothiocyanate **18** (K_i = 45 nM) at concentrations of 3-, 10-, 30-, and 100-fold of the K_i values for time periods ranging from 2 to 45 min. Incubations were stopped by centrifugation of the cell suspension at 1500*g* in microcentrifuge (Eppendorf Model 5415C). Cell pellets were resuspended in 1.2 mL of fresh buffer and the samples were placed again on the shaker for about 15 min to allow for drug equilibrium (bound and free drug), followed by recentrifugation and resuspension. This washing procedure was repeated three times for each sample. Protein contents of the final reconstituted cell suspensions were determined by the method of Lowry et al.[31]

Triplicate aliquots of normalized suspensions (20,000–30,000 cells/aliquot) were incubated with 60–240 pM of [^{125}I]ICYP in a final volume of 250 μL in buffer for 1 h at 37 °C. Binding reactions were terminated by rapid filtration (5 mL of ice-cold Tris buffer × 2 times) of the samples through Whatman GF/B filters on a Brandel Model 12-R cell harvester and the radioactivity present on filters was measured in a gamma counter (Model 1470 Wizard, Wallac Inc., Gaithersburg, MD). Non-specific binding for each sample aliquot was determined in the presence of 2×10^{-6} M (±) propranolol.

5.2.5. Time- and concentration-dependent photoaffinity binding. CHO Cells expressing the human β_2-AR (about $30–40 \times 10^3$ cells/150 μL) were suspended in 1.2 mL Tris buffer in microfuge tubes and incubated for 1 h at room temperature under red light in a rotating shaker (Robbins Scientific, 18 rpm) with the azide **24** (K_i = 68 nM) at concentrations of 1-, 3-, 10- and 100-fold of the K_i value. The suspensions were then transferred to quartz tubes and exposed to UV light at 350 nm for 30 s or 5 min. The photolyzed suspensions were then centrifuged for 2 min at about 1500*g* in microcentrifuge (Eppendorf Model 5415C). Cell pellets were resuspended in 1.2 mL of fresh buffer and the samples were placed again on the shaker for about 15 min in the dark to allow equilibrium of the drug between bound and free forms, which was followed by recentrifugation and resuspension. This washing procedure was repeated twice for a total of three times for each sample. Protein contents of the final reconstituted cell suspensions were determined by the method of Lowry et al.[31]

Triplicate aliquots of normalized suspensions (20,000–30,000 cells/aliquot) were incubated with 60–240 pM [^{125}I]ICYP in a final volume of 250 μL in buffer for 1 h at 37 °C. Binding reactions were terminated and radioactivity was measured as described earlier in Section 5.2. Non-specific binding for each sample aliquot was determined in the presence of 2×10^{-6} M (±) propranolol. In control experiments, untreated cell suspensions were subjected to identical photolysis procedure and evaluated for radioligand binding.

Non-photolyzed cells, in the absence of the photoaffinity ligand, were utilized for determining total binding of the radioligand.

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