Published in final edited form as:
Chembiochem. 2012 October 15; 13(15): 2277–2289. doi:10.1002/cbic.201200427.

Development of Benzophenone-Alkyne Bifunctional Sigma Receptor Ligands

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Abstract

Sigma (σ) receptors represent unique non-opioid binding sites that are associated with a broad range of disease states. Sigma-2 receptors provide a promising target for diagnostic imaging and pharmacological interventions to curb tumor progression. Most recently, the progesterone receptor (PGRMC1, 25 kDa) has been identified to contain σ2 receptor-like binding properties, highlighting the need to understand the biological function of an 18-kDa protein that exhibits σ2-like photoaffinity labeling (herein denoted as σ2-18k) but the amino acid sequence of which is not known. In order to provide novel tools for the study of the σ2-18k protein, we have developed bifunctional sigma receptor ligands that bear a benzophenone photo-crosslinking moiety and an alkyne group, to which an azide-containing biotin affinity tag can be covalently attached *via* click chemistry following photo-crosslink. While several compounds showed favorable σ2 binding properties, compound **22** exhibited the highest affinity (2 nM) and the greatest potency in blocking photolabeling of the σ2-18k by a radioactive photoaffinity ligand. Thus, these benzophenone-alkyne sigma receptor ligands may be amenable for studying the σ2-18k protein *via* chemical biology approaches. To our knowledge, these compounds represent the first reported benzophenone-containing clickable sigma receptor ligands, which may potentially serve broad applications by “plugging” in various tags.

[e]These two authors contributed equally to this work.

Keywords

Sigma-2 receptor ligand; σ2-18k; benzophenone; alkyne; click chemistry

INTRODUCTION

The sigma receptors were originally proposed to be opioid receptors but were later identified as unique, non-opioid binding sites (see review [1]). Pharmacological studies identified at least two subtypes, the sigma-1 and sigma-2 receptors [2]. These two subtypes are distinct not only in their pharmacological profiles, but also in their tissue distribution as well as pathophysiologic involvement. Interest has been growing rapidly in pursuit of therapeutic interventions targeting the sigma receptors in various diseases.

The σ1 receptor, which does not share sequence homology to any known mammalian protein [3], has been discovered to be a unique ligand-operated chaperone [4]. Progesterone and pregnenolone were earlier proposed to be natural ligands for the σ1 receptor [5]. Our recent studies further identified dimethyltryptamine [6] and sphingosine [7] also as endogenous σ1 ligands. Many studies have proposed selective σ1 agonists as therapeutic agents for anxiety, depression, psychosis, and learning and memory improvement, and recently, neuroprotective effects of σ1 agonists have garnered increasing attention [8].

Although much less is known about the σ2 subtype, these receptors have been shown to play an important role in the suppression of tumor growth. Sigma-2 (σ2) receptors are known to be abundant in various tumor cells [9], and in particular, σ2 expression in proliferating tumor cells is 10-fold higher than in quiescent cells [10]. Importantly, σ2 receptor agonists have been found to induce apoptosis in various tumor cell lines [11]. Growing evidence strongly advocates σ2 receptors as a biomarker for solid proliferating tumors, and considerable effort is being invested in developing σ2 agonists as potential anti-cancer drugs or diagnostic imaging agents [12].

The lack of understanding of σ2 receptors is attributable primarily to the fact that the molecular identity of this subtype has evaded elucidation since its first pharmacological classification [2]. Most recently, a major breakthrough was reported that the progesterone receptor membrane component 1 (PGRMC1) was identified to contain σ2 receptor-like binding characteristics [13]. This discovery has greatly stimulated further enthusiasm to gain better knowledge of the molecular properties of σ2 receptors, which is essential for effectively exploiting the therapeutic potential of this drug target.

The key feature of the σ2 receptor binding sites is their strong interaction with DTG (azido-di-o-tolylguanidine), a drug that binds both σ2 and σ1 receptors equally well, but very low affinities for (+)-pentazocine which is a prototypical ligand for the σ1 receptor [2]. While meeting this basic σ2 pharmacological characteristic, PGRMC1 has also been found to be highly expressed in proliferating tumor cells [13], consistent with the previously identified biological function of σ2 binding sites [10].

It is interesting to note that PGRMC1 has a molecular size (25kDa) [13] distinct from σ2 binding sites initially observed by Bowen *et al* through ^{3}H-Az-DTG photoaffinity labeling (21.5 and/or 18 kDa) [14]. Moreover, photolabeling with [^{125}I]-IAF (1-N-(2',6'-dimethyl-morpholino)-3-(4-azido-3-[^{125}I]iodo-phenyl propane) has also repeatedly displayed a band of 18 kDa (denoted as σ2-18k throughout this report) that meets the characteristics of σ2 receptors [6, 15]. IAF is a photoactivatable ligand that binds both σ1 and σ2 receptors with high affinities. In these studies, whereas DTG blocked the [^{125}I]-IAF photolabeling of both

the σ1 receptor (26 kDa) and the σ2-18k protein which were separated on a SDS gel, (+)-pentazocine could readily diminish the labeling of the σ1 receptor but not the σ2-18k band. Thus, intriguing questions arise with regard to the molecular relationship between the σ2-18k and PGRMC1 and the biological function(s) of the σ2-18k protein.

Without an amino acid sequence available for the σ2-18k, the most convenient means for studying this protein may be chemical biology. In the present study, we have introduced two functional groups into σ2 ligands, a benzophenone photoreactive moity and an alkyne group. Benzophenone is known to be superior in term of photo-crosslinking efficiency [16]. The alkyne group, which is not naturally present in biological systems, provides a unique "handle" for attaching desired tags *via* click chemistry [17]. Since the discovery of click chemistry, this reaction has been widely applied in chemical biology because of the high yield that can be attained under mild conditions.

Interestingly, some of the novel benzophenone-alkyne bifunctional ligands we have developed, such as compounds **9** and **22**, exhibited excellent σ2-binding affinities (Ki < 5 nM) in the RT-4 cell membranes. Moreover, these compounds specifically blocked the [^{125}I]-IAF photolabeling of the σ2-18k receptor. Thus, these new compounds appear to be useful for future σ2-18k studies by photo-crosslinking followed by click chemistry to attach various azide-containing tags such as biotin.

RESULTS AND DISCUSSION

The purpose of this study is to develop high-affinity σ2 ligands that are amenable for photo-crosslinking and also for covalently attaching an affinity tag. Benzophenone has become the choice of photoreactive moity because of its stability in ambient light and excellent crosslink efficiency when exposed to UV [16]. Recently, click chemistry has been broadly applied in chemical biology to join two functional groups *via* the cycloaddition reaction between an alkyne and an azide group [17]. Thus, the σ2-binding compounds reported here are ideal for our purpose since they contain both a benzophenone and an alkyne group.

We first set out to identify benzophenone-containing lead compounds that show reasonable σ2-binding affinities. It is interesting to note that in previous reports several high-affinity σ2 ligands such as PB28[11c], siramesine[11d], SW-120 and some benzamide-isoquinoline derivatives [13, 15b, 18] share a general composition of two ring structures linked by an alkyl chain. With this in mind, we have used benzophenone with an alkyl chain as a module to "plug in" various ring groups on the opposite side.

It has been proposed that the cyclohexylpiperazine moity affords σ2 affinity/selectivity [19]. In a recent report from the McCurdy group, a series of compounds containing the cyclohexylpiperazine group showed excellent σ2 affinities (Ki ~1 nM) [20]. Here as shown in Table 1, adding the cyclohexylpiperazine group to the benzophenone module (compound **6**) generated a novel compound with an affinity of 33 nM for σ2 receptors and a 40-fold selectivity over the σ1 receptor. Similarly, adding a fluoro-benzoyl piperazine group (compound **7**) afforded a Ki of 34 nM for σ2 receptors and a 70-fold selectivity versus σ1.

In some compounds the isoquinoline group has been reported to confer good σ2 affinity when linked to a benzamide ring structure through a butyl chain [13, 15b, 18]. The combination of isoquinoline with benzophenone, however, did not result in appreciable binding to σ2 receptors (see compound **13** in Table 1). Although in our previous study the phthalimide/isoquinoline pair linked with a butyl chain afforded reasonable σ2 affinity of 21 nM [15b], the phthalimide group did not facilitate strong σ2 binding when paired with benzophenone (compound **12**), neither with the fluoro-benzoyl piperazine group (**4b**). It is

noteworthy that an alkyne group directly linked to benzophenone afforded a 120 nM affinity for the σ1 receptor (compound **8**). The binding of this compound to σ2 receptors, however, was very weak (Ki >10 μM), thus its selectivity for the σ1 receptor is high (over 100 fold). In our previous report, having two butyl groups on the same nitrogen in a σ1 ligand, nitro-C29, has dramatically enhanced ligand affinity for the σ1-receptor as compared to the compound with a single butyl [21]. Based on this result, we propose that adding a second alkyne, which is hydrophobic like the butyl group, to the same nitrogen where an alkyne is attached in compound **8**, may substantially improve its σ1-binding affinity thus converting compound **8** into a highly σ1-selective bi-functional ligand.

Therefore, through this primary screening, compounds **6** and **7** presented themselves as suitable lead compounds. We then used these two compounds to test the impact of adding an alkyne group on their binding with σ2 receptors (Table 2). Remarkably, with an alkyne group added to the secondary amine in the alkyl chain between benzophenone and cyclohexylpiperazine (compound **9**), the affinity for σ2 binding was improved nearly 10 fold (3.8 nM) as compared to the lead compound without alkyne (compound **6**). Interestingly however, adding the alkyne group to compound **7** did not increase its affinity for σ2 receptors but rather decreased the affinity by 2 fold (70 nM). This opposite effect of the alkyne group is surprising given that compounds **6** and **7** are similar in their structures as well as their affinities for σ2 receptors. The distinct outcomes caused by the alkyne adduct in compounds **10** and **9** may imply the importance of the steric relationship between benzopheone and the ring system on the opposite side of the alkyl chain. In other words, the alkyne interference may result in a different packing of the two ring systems (linked with an alkyl chain) in the σ2-binding pocket through differential interactions with cyclohexylpiperazine and fluoro-benzoyl piperazine.

Based on the structure of compound **9**, we attempted to further optimize its ability to bind to the σ2 receptor sites. Several variations were made such as replacing the nitro group on the benzophenone, changing the length of the alkyl chain or the location of the alkyne group. Neither of the resultant derivatives improved σ2 affinity, but rather, they showed impaired binding with σ2 receptors (see compounds **15**, **19**, and **17**). This result may serve as additional evidence for the speculation that an appropriate spatial arrangement of the two ring systems linked by an alkyl chain of a certain length is important for a tight binding of the ligand to the σ2-18k receptor. Interestingly, in comparison to compound **17**, the insertion of an amide next to the benzophenone (**22**) increased its σ2 affinity (2 nM) by nearly 40 fold. This effect mirrors the presence of an amide in some benzamide-isoquinoline compounds that have shown high affinities for σ2 receptors [21, 27, 28].

Using this high-affinity benzophenone compound (**22**) as a template, we replaced the alkyne group with a butyl-biotin group in an effort to create a benzophenone-containing σ2 ligand with biotin directly attached (see compound **27** in Table 2). The resultant new compound showed reduced affinities for both σ2 (*Ki* = 87 nM) and σ1 binding sites. It is likely that the bulkiness of the biotin group imposed steric hindrance to the ligand-σ2 interaction or to the interaction between the two ring systems. Similarly, substituting alkyne with butyl-iodine, which is relatively bulky, also impaired the σ2 binding of the benzophenone-cyclohexylpiperazine ligand (see compound **25**, *Ki* = 45 nM). Nevertheless, this iodinated benzophenone ligand may serve as a lead compound in the future for development of radioiodinated sigma receptor ligands for the purpose of photoaffinity labeling. One great attraction of this type of sigma receptor photoreactive ligand is the ease of radioiodination using a triflate as a starting material (Scheme 10). In addition, we also explored the possibility of placing biotin on the piperazine side. As a result, the affinity for σ2 receptors was greatly reduced (data not shown) compared with the alkyne-containing compound **9** (Table 2).

Importantly, we confirmed that these novel photoactivatable and clickable ligands indeed targeted the σ2-18k binding site, as shown by [^{125}I]-IAF (1-N-(2',6'-dimethyl-morpholino)-3-(4-azido-3-[^{125}I]iodo-phenyl propane) photoaffinity labeling (Figure 1). Consistent with the affinity data obtained by competitive radioligand binding assays (Tables 1–2), compound **9** at 100 nM blocked [^{125}I]-IAF labeling of the σ2-18k significantly while compound **6** which has a lower affinity did so to a lesser extent (Figure 1A). Compound **22** showed the most profound potency against [^{125}I]-IAF labeling of the σ2-18k. Comparable levels of protection against the [^{125}I]-IAF labeling were obtained by **15** and **10** at a concentration of 1 μM. Further analysis showed that 1 μM compound **9** and compound **22**, both high-affinity σ2 ligands, completely reduced the [^{125}I]-IAF labeling of the σ2-18k to the background level that was indicated by DTG protection (Figure 1B). The lack of protection of the non-specifically labeled higher molecular weight bands manifested the [^{125}I]-IAF labeling specificity for the σ2-18k receptor.

Even though these novel bifunctional σ receptor ligands did not show reduced selectivity for the σ1 receptor, this will not likely limit their use for studying the σ2-18k because the σ1 receptor and the σ2-18k can be readily separated on an SDS-polyacrylamide gel (Figure 1). Rather, the reasonable affinities of **22** and **9** for the σ1 receptor are expected to be advantageous for future experiments using the pure σ1 receptor as a positive control [22]. Moreover, when using these compounds which are nearly 10 fold selective toward σ2 versus σ1 in the σ2-rich RT-4 cell membranes, which are known to contain approximately 10 fold more σ2 binding sites than the σ1 receptor [23], the presence of σ1 binding site is not expected to be problematic.

Finally, as further proof of photoactivatability and clickability, we tested whether an azide-biotin affinity tag could be covalently linked to the alkyne group compound **22** by click chemistry following derivatization of BSA by photoactivation of the benzophenone moity (Figure 2). The far-western blot indicated that biotin was readily linked to the alkyne group *via* compound **22** that was pre-attached to BSA through photo-crosslinking. Thus, the biotin derivatization indeed occurred through click chemistry, as manifested by the lack of biotin signal either from the alkyne-free control (**6**) or in the absence of $CuSO_4$, a key catalyst in click chemistry.

CONCLUSION

We have developed several benzopheone/alkyne-containing compounds and characterized their sigma receptor-binding properties. Compounds **22** and **9** exhibited excellent affinities for σ2 receptors. Photolabeling data indicated that these compounds bound potently to the σ2-18k binding site in RT-4 cell membranes. Thus these novel bifunctional ligands appear to be potentially useful for studying the σ2-18k protein using a chemical biology strategy of photocrosslinking followed by click chemistry.

EXPERIMENTAL SECTION

Chemistry

All yields refer to isolated products after purification. All products were characterized by their spectra (IR, ^{1}H-NMR, ^{13}C NMR, CHN, high resolution mass spectroscopy and GC) and melting points, and tested as free bases. All melting points were taken on a Gallenkamp melting apparatus. ^{1}H-NMR spectra and ^{13}C-NMR spectra were recorded at 300 MHz and 75 MHz, respectively, in $CDCl_3$ or d6DMSO (TMS was used as an internal standard) on a Varian 300 NMR spectrometer. FT-IR spectra were recorded on a spectrophotometer (Jasco-680, Japan). Spectra of solids were carried out using KBr pellets. Vibrational transition frequencies are reported in wave numbers (cm^{-1}). In addition we used a GC

BEIFIN 3420 Gas Chromatograph equipped with a Varian CP SIL 5CB column (30 m, 0.32 mm, 0.25 μm) for examination of reaction completion and yields. CHN analyses were measured at Isfahan University of Technology using an Elemental Vario ELIII (Germany). EMS was recorded using Waters (Micromass) LCT®: electrospray ionization, time-of-flight (TOF). All the starting materials were purchased from Sigma-Aldrich.

Synthesis of 4-Methyl-4'-nitrobenzophenone (1a) and 4-Methyl-benzophenone (1b, Scheme 1)—In a 500 mL round-bottomed flask containing a stirred solution of toluene (300 ml) and 4-nitrobenzoyl chloride or benzoyl chloride (108 mmol) was added aluminum chloride (140 mmol, 18.7 g). The resulting red solution was stirred at room temperature for 90 min, 5 ml of water was then added drop-wise and the mixture was stirred for 20 min. The reaction mixture was washed with water (2 × 100 ml) and 10% $NaHCO_3$ (2 × 100 ml) and dried (with $MgSO_4$). The solvent was evaporated to pale yellow oil under reduced pressure with a rotary evaporator. The resulting residue was recrystallized from CH_2Cl_2/hexane to produce compound **1a** or **1b**.

4-Methyl-4'-nitrobenzophenone (1a)—80% Yield as light yellow needles. Mp: 119–120°C. ^{1}H NMR: δ 2.50 (s, 3 H), 7.31 (d, J = 8.0 Hz, 2 H), 7.70 (d, J = 8.0 Hz, 2 H), 7.9 (d, J = 8.1 Hz, 2 H), 8.36 (d, J = 8.8 Hz, 2 H). Anal. Calcd for $C_{14}H_{11}NO_3$: C, 69.70; H, 4.60; N, 5.81. Found: C, 69.80; H, 4.70; N, 5.80.

4-Methyl-benzophenone (1b)—74% Yield as light yellow needles. Mp: 56–58°C. ^{1}H NMR: δ 2.50 (s, 3 H), 7.78-7.34 (m, 9 H). EMS [MH^+] for $C_{14}H_{12}O$, Calcd. 106.24, Found, 197.1916.

Synthesis of 2a and 2b (Scheme 1)—In a 250 ml round-bottomed flask containing a stirred solution of CCl_4 (100 ml) and compound **1a** or **1b** (10 mmol) was added *N*-bromo succinimide (NBS) (11 mmol, 2.0 g) and 2,2'-azobisisobutyronitrile (AIBN) (10 mg). The resulting mixture was refluxed for 24 h. After cooling, the white precipitate of succinimide was filtered and the solvent was evaporated to a brown solid. The resulting residue was purified by column chromatography on silica gel (CH_2Cl_2/hexane, 1:1 v/v) to produce compound **2a** or **2b**.

4-Bromomethyl-4'-nitrobenzophenone (2a)—Light yellow crystals, yield 66%, mp: 124–125°C. ^{1}H NMR δ 4.54 (s, 2 H), 7.55 (d, J = 8.1 Hz, 2 H), 7.9 (d, J = 7.9 Hz, 2 H), 8.40 (d, J = 8.8 Hz, 2 H). Anal. Calcd for $C_{14}H_{10}BrNO_3$: C, 52.52; H, 3.15; N, 4.38. Found: C, 52.60; H, 3.20; N, 4.30.

4-Bromomethyl-benzophenone (2b)—White crystals, yield 90 %, mp: 113–114°C. ^{1}H NMR δ 4.53 (s, 2 H), 7.46–7.52 (m, 4 H), 7.57–7.81 (m, 5 H). ^{13}C NMR: δ 32.2, 128.3, 128.9, 130.0, 130.6, 132.5, 136.4, 137.4, 142.1, 190.0. MS (EI): *m/z* 274 and 276 (M^+, 1:1 ratio), 195 (100 %), 167 (52 %), 77 (14 %).

Synthesis of (4-(3-bromopropyl)phenyl)(p-tolyl)methanone (2c, Scheme 1)—4-Methy1benzoyl chloride (7.30 ml, 42.8 mmol) was added dropwise to a suspension of aluminum chloride (42.8 mmol, 5.70 g) in CS_2 (40 ml). 3-Bromopropylbenzene (5.0 ml, 32.9 mmol) was then added drop-wise to the solution. The reaction mixture was refluxed for 10 h and poured into a mixture of concentrated HC1 (22 ml) and ice (80 g). The product was extracted with CCl_4. The CCl_4 layer was washed with water and 2% NaOH solution and dried with $MgSO_4$. After filtration and evaporation of the solvent, the crude product was chromatographed over silica gel by using a mixture of hexane: ethyl acetate (9:l v/v) as an

eluent to give a yellow liquid **2c**, yield 58.1%; ^{1}H NMR δ 1.9–2.5 (m, 5 H), 2.8 (t, *J* = 4.8, 2 H), 3.3 (t, *J* = 4.8, 2 H), and 7.0–7.8 ppm (m, 8 H).

Preparation of Compounds 5a-5c—A mixture of *N*-(3-bromobutyl)-phthalimide (**3**) (2.8 g, 10 mmol), the appropriate amines (10 mmol) and K_2CO_3 (2.8 g, 20 mmol) in ethanol (100 ml) was refluxed for 12 h. The reaction mixture was then filtered and the solvent was evaporated to give crude products **4a-4c** which were used without purification. A solution of **4a-4c** (free bases, 1.2 mmol) and hydrazine monohydrate (0.05 ml, 15 mmol) in ethanol (95 %, 15 ml) was refluxed for 1 h. The reaction mixture was cooled and treated with an additional amount of ethanol (95 %, 15 ml) and concentrated HCl (1.3 ml). The reaction mixture was then refluxed for 4 h and left overnight in a refrigerator. The precipitate was collected by filtration, and the solvent was evaporated. The residue was treated with *n*-hexane (20 ml) and aqueous NH_4OH (15 ml). The solution was extracted with $CHCl_3$ (3 × 15 ml), the organic layer was dried over anhydrous K_2CO_3, and the solvents were evaporated to give **5a**-**5c** as pure products.

3-(4-cyclohexylpiperazin-1-yl)propan-1-amine (5a)—Yellow semi-solid 86% yield, ^{1}H NMR δ 1.02–1.22 (m, 4 H), 1.55–1.86 (m, 8 H), 2.13–2.21 (m, 5 H), 2.34–2.65 (m, 10 H), 3.30 (s br, 2 H). GC-MS *m/z* (M^+), 225, 195, 181(94 %), 71 (100 %). IR cm^{-1}, 3281, 2930, 1640.

3-(4-(4-Fluorophenyl)piperazin-1-yl)propan-1-amine (5b)—Yellow oil, 89% yield, ^{1}H NMR δ 1.68–1.76 (m, 4 H), 2.48 (t, *J* = 7.2 Hz, 2 H), 2.62 (t, *J* = 4.8, 4 H), 2.81 (s br, 2 H), 3.12 (t, *J* = 4.8 Hz, 4 H), 6.85–6.90 (m, 2 H), 6.96 (t, J = 8.4 Hz, 2 H), IR cm^{-1}, 3281, 2930, 1640.

3-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)propan-1-amine (5c)—Yellow oil, yield 85%; ^{1}H NMR, δ 1.72 (m, 2 H), 2.46 (t, *J* = 4.8 Hz, 2 H), 2.63 (t, *J* = 4.8 Hz, 2 H), 2.78 (t, *J* = 7.6 Hz, 2 H), 2.95 (t, *J* = 7.6 Hz, 2 H), 3.83 (s, 6 H), 3.70 (s, 2H), 5.11 (s br, 2 H), 6.92 (t, *J* = 8.6 Hz, 2 H). ^{13}C NMR, δ: 23.5, 27.4, 40.1, 56.1, 56.8, 59.7, 63.8, 111.4, 126.9, 146.7, 148.2 . EMS [MH^+] for $C_{14}H_{22}N_2O_2$, Calcd. 250.34, Found, 251.3613.

General procedure for preparation of compounds 6–8 (Scheme 2)—To a solution of **5a, 5b**, or propargyl bromide (free bases, 1 mmol) and **2a** or **2b** (1 mmol) in ethanol (15 ml) was added K_2CO_3 (0.22 g, 2 mmol). The reaction mixture was refluxed for 12 h and then cooled. The precipitate was filtered off and the solvent was evaporated. The residue was purified using column chromatography (silica gel, CH_3Cl: MeOH, 95:5) to give compounds **6–8**.

(4-((3-(4-cyclohexylpiperazin-1-yl)propylamino)methyl)phenyl)(4-nitrophenyl)methanone (6)—Yellow oil, 83 % yield; ^{1}H NMR *δ* 1.02–1.22 (m, 4H), 1.55–1.86 (m, 8H), 2.13–2.21 (m, 4H), 2.34–2.65 (m, 10H), 3.30 (br s, 1 H), 3.76 (s, 2H), 7.31 (d, *J* =8.0 Hz, 2 H), 7.72 (d, *J* =8.0 Hz, 2 H), 7.9 (d, *J* =8.1 Hz, 2 H), 8.35 (d, *J* =8.8 Hz, 2 H). ^{13}C NMR *δ* 24.7, 27.2, 28.6, 36.4, 45.9, 51.2, 58. 63.9, 72.1, 123.2, 130.6, 136.7, 143.2, 151.8, 194.6. EMS [MH^+] for $C_{27}H_{36}N_4O_3$, Calcd. 464.60, Found, 465.2932.

(4-((3-(4-(4-fluorophenyl)piperazin-1-yl)propylamino)methyl)phenyl)(4-nitrophenyl)methanone (7)—Yellow oil, 84 % yield, ^{1}H NMR *δ* 1.68–1.76 (m, 4H), 2.48 (t, *J* = 7.2 Hz, 2 H), 2.62 (t, *J* = 4.8 Hz, 4 H), 2.95 (s br, 1 H), 3.12 (t, *J* = 4.8 Hz, 2 H) , 3.76 (s, 2H),6.85–6.90 (m, 2 H), 6.96 (t, *J* = 8.2 Hz, 2 H), 7.31 (d, *J* =8.0 Hz, 2 H), 7.72 (d, *J* =8.0 Hz, 2 H), 7.9 (d, *J* =8.1 Hz, 2 H), 8.35 (d, *J* =8.8 Hz, 2 H). ^{13}C NMR *δ* 27.1, 36.2, 46.8,

51.1, 51.9, 55.9, 115.2, 116.8, 123.2, 130.6, 136.7, 143.2, 15 1.8, 156.9, 194.61. EMS [MH^+] for $C_{27}H_{29}FN_4O_3$, Calcd. 476.54, Found, 477.6824.

(4-nitrophenyl)(4-((prop-2-ynylamino)methyl)phenyl)methanone (8)—Light yellow oil, 88% yield; ^{1}H NMR δ 2.69 (s, 1H), 2.85 (s br, 1H), 3.30 (s, 2 H), 3.76 (s, 2H), 7.31 (d, J=8.0 Hz, 2 H), 7.72 (d, J=8.0 Hz, 2 H), 7.9 (d, J=8.1 Hz, 2 H), 8.35 (d, J=8.8 Hz, 2 H). ^{13}C NMR δ 41.2, 53.9, 73.5, 81.8, 123.2, 130.6, 136.7, 143.2, 15 1.8, 156.9, 194.61. EMS [MH^+] for $C_{17}H_{14}N_2O_3$, Calcd. 294.30, Found, 295.4213.

General procedure for preparation of compounds 9–10 (Scheme 2)—To a solution of **6** or **7** and propargyl bromide (1 mmol, 0.12g) in ethanol (15 ml) was added K_2CO_3 (2 mmol, 0.22 g). The reaction mixture was refluxed for 8 h and then cooled down, and the precipitate was filtered off and the solvent was evaporated. The residue was dissolved in CH_2Cl_2 (30 ml) and washed with water (3 × 30 ml). The organic phase was dried with $MgSO_4$ and the solvent was evaporated. The residue was then purified using column chromatography (silica gel, CH_3Cl: MeOH, 95:5) to give compounds **9–10**.

(4-((3-(4-cyclohexylpiperazin-1-yl)propyl)(prop-2-ynyl)amino)methyl)phenyl)(4-nitrophenyl) methanone (9)—Dark yellow oil, quantitative yield; ^{1}H NMR δ 1 (m, 4H), 1.55–1.86 (m, 7H), 2.13–2.26 (m, 2 H), 2.34–2.65 (m, 10H), 2.68–2.85 (m, 3H), 3.87 (s, 2 H), 3.66 (s, 2H), 7.31 (d, J=8.0 Hz, 2 H), 7.72 (d, J=8.0 Hz, 2 H), 7.9 (d, J=8.1 Hz, 2 H), 8.35 (d, J=8.8 Hz, 2 H). ^{13}C NMR δ 24.7, 27.2, 28.6, 6.4, 45.9, 53.2, 58. 3, 60.9, 72.1, 73.4, 78.0, 123.2, 130.6, 136.7, 143.2, 151.8, 194.6. EMS [MH^+] for $C_{30}H_{38}N_4O_3$, Calcd. 502.65, Found, 503.4292.

(4-((3-(4-(4-fluorophenyl)piperazin-1-yl)propyl)(prop-2-ynyl)amino)methyl)phenyl)(4-nitrophenyl) methanone (10)—Brown oil, 91% yield; ^{1}H NMR δ 1.68–1.76 (m, 4H), 2.26 (s, 1 H), 2.48 (t, J = 7.2 Hz, 2 H), 2.62 (t, J = 4.8 Hz, 4 H), 2.81 (t, J = 6.4 Hz, 2 H), 3.12 ((t, J = 4.8 Hz, 2 H) , 3.67 (s, 2H), 3.87 (s, 2 H), 6.85–6.90 (m, 2 H), 6.96 (t, J = 8.2 Hz, 2 H), 7.31 (d, J=8.0 Hz, 2 H), 7.72 (d, J=8.0 Hz, 2 H), 7.9 (d, J=8.1 Hz, 2 H), 8.35 (d, J=8.8 Hz, 2 H). ^{13}C NMR δ 27.1, 36.2, 41.5, 46.8, 51.1, 51.9, 53.4, 552, 82.3, 73.1, 115.6, 116.8, 123.2, 130.6, 136.7, 143.2, 15 1.8, 156.9, 194.61. EMS [MH^+] for $C_{30}H_{31}FN_4O_3$, Calcd. 514.23, Found, 515.3641.

2-(3-(4-benzoylbenzylamino)propyl)isoindoline-1,3-dione (12)—To a solution of **11** (1 mmol, 0.19 g) and **2b** (1 mmol, 0.23 g) in ethanol (15 ml) was added K_2CO_3 (2 mmol, 0.22 g). The reaction mixture was refluxed for 8 h and then cooled down, and the precipitate was filtered off and the solvent was evaporated. The residue was purified using column chromatography (silica gel, CH_3Cl: MeOH, 95:5) to give **12**. Yellow oil, 77% yield, ^{1}H NMR δ 1.81 (m, 2 H), 2.68 (br s, 1 H), 2.55 (t, J = 4.8 Hz, 2 H), 3.28 (t, J = 4.8 Hz, 2 H), 3.73 (s, 2H), 7.16 (m, 2H), 7.33–7.85 (m, 9H), 8.13(m, 2H). ^{13}C NMR δ 28.6, 32.9, 46.7, 54.9, 123.7, 128.3, 128.9, 130.0, 130.6, 132.5, 132.6, 136.4, 137.4, 142.1, 167.9, 196.0. EMS [MH^+] for $C_{25}H_{22}N_2O_3$, Calcd. 398.44, Found, 399.2317.

N-(3-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)propyl)-4-(4-nitrobenzoyl)benzamide (13)—To a solution of 4-(4-nitrobenzoyl)benzoic acid (**2d**, 1.1 mmol, 0.30 g) in anhydrous THF (50 ml) was added dicyclohexylcarbodiimide (DCC, 1.1 mmol, 023 g) in anhydrous THF (5 ml) and the reaction mixture was stirred at room temperature. After 5 min, a solution of 3-[3,4-dihydro-6,7-dimethoxyisoquinolin-2(1*H*)-yl]propan-1-amine (**5c**) in THF (5 ml) was added and stirred at room temperature overnight. The precipitate was filtered off, and the solvent was evaporated. The residue was dissolved in CH_2Cl_2 (30 ml) and washed with water (3 × 30 ml). The organic layer was dried over

anhydrous $MgSO_4$ and the solvents were evaporated to give **13**, which was purified using column chromatography (silica gel, $CHCl_3$: MeOH, 95:5). Oil, 96% yield; ^{1}H NMR δ 1.58–2.36 (m, 4H), 2.56 (t, J = 4.7 Hz, 2H), 2.62–2.96 (m, 4 H), 3.52 (t, J = 4.7 Hz, 2 H), 3.78 (s, 6H), 6.51 (s, 1H), 6.57 (s, 1H), 7.31 (d, J =8.0 Hz, 2 H), 7.72 (d, J =8.0 Hz, 2 H), 7.9 (d, J =8.1 Hz, 2 H), 8.21 (br s, 1 H), 8.35 (d, J =8.8 Hz, 2 H). ^{13}C NMR δ 27.3, 27.6, 37.1, 56.4, 60.1, 63.4, 108.3, 111.6, 126.2, 127.4, 130.6, 136.7, 143.2, 146.7, 148.6, 151.8, 167.8, 194.6. EMS [MH^+] for $C_{28}H_{29}N_3O_6$, Calcd. 503.55, Found, 504.6123.

Preparation of (4-(3-(3-(4-cyclohexylpiperazin-1-yl)propylamino)propyl)phenyl)(p-tolyl)methanone (14, Scheme 3)—To a solution of (4-(3-bromopropyl)phenyl)(p tolyl)methanone (**2c**, 1 mmol, 0.32 g) and 3-(4-cyclohexylpiperazin-1-yl)propan-1-amine (**5a**, 1 mmol, 0.23 g) in ethanol (15 ml) was added K_2CO_3 (2 mmol, 0.22 g). The reaction mixture was refluxed for 12 h and then cooled. The precipitate was filtered off, and the solvent was evaporated. The residue was purified using column chromatography (silica gel, CH_3Cl: MeOH, 95:5) to give **14**. Dark yellow oil, 85% yield. ^{1}H NMR δ 1.02–1.58 (m, 10 H), 1.95–2.12 (m, 3 H), 2.35–2.38 (m, 14 H), 2.55–2.68 (m, 8 H), 6.92 (d, J = 8.2, 2 H), 7.33 (d, J = 8.0, 2 H), 7.68–7.73 (m, 4 H). ^{13}C NMR δ 21.3, 24.4, 25.7, 29.5, 30.7, 46.6, 48.2, 51.9, 56.4, 63.4, 72.8, 127.8, 130.4, 135.6, 143.6, 145.1, 194.3. EMS [MH^+] for $C_{30}H_{43}N_3O$, Calcd. 461.68, Found, 462.5818.

(4-(3-((3-(1,4'-bipiperidin-1'-yl)propyl)(prop-2-ynyl)amino)propyl)phenyl)(p-tolyl)methanone (15)—To a solution of **14** (1 mmol, 0.46 g) and propargyl bromide (1 mmol, 0.12 g) in ethanol (15 ml) was added K_2CO_3 (2 mmol, 0.22 g). The reaction mixture was refluxed for 8 h and then cooled. The precipitate was filtered off, and the solvent was evaporated. The residue was purified using column chromatography (silica gel, CH_3Cl:MeOH, 95:5) to give **15**. Yellow oil, 96% yield. ^{1}H NMR δ 1.02–1.58 (m, 10 H), 1.95–2.12 (m, 3 H), 2.35–2.38 (m, 14 H), 2.55–2.68 (m, 8 H), 3.83 (s, 2H), 6.92 (d, J = 8.2, 2 H), 7.33 (d, J = 8.0, 2 H), 7.68–7.73 (m, 4 H). ^{13}C NMR δ 21.3, 24.4, 25.7, 29.5, 30.7, 46.6, 48.2, 49.3, 51.9, 56.4, 63.4, 72.8, 73.6, 77.4, 127.8, 130.4, 135.6, 143.6, 145.1, 194.3. EMS [MH^+] for $C_{33}H_{45}N_3O$, Calcd. 499.36, Found, 500.6221.

Preparation of 1-(3-bromopropyl)-4-cyclohexylpiperazine. A mixture of 4-cyclohexylpiperazine (10 mmol, 1.68 g), 1,3-diboropropane (10 mmol, 2.0 g) and K_2CO_3 (20 mmol) in ethanol (100 ml) was refluxed for 12 h. The reaction mixture was then filtered and the solvent was evaporated to give crude product which was purified using column chromatography (silica gel, CH_3Cl:MeOH, 95:5). Yellow oil, 90% yield. ^{1}H NMR δ 1.02–1.58 (m, 10 H), 1.95–2.12 (m, 3 H), 2.35–2.38 (m, 10 H), 3.38 (t, 2 H). ^{13}C NMR δ 21.3, 25.4, 29.5, 30.7, 32.6, 49.3, 51.9, 53.4, 61.4. Anal. Calcd for $C_{13}H_{25}BrN_2$: C, 53.98; H, 8.71; N, 9.68. Found: C, 53.60; H, 8.90; N, 9.50.

Preparation of (4-(3-(4-cyclohexylpiperazin-1-yl)propylamino)phenyl)(phenyl)methanone (16)—To a solution of 4-aminobenzophenone (1 mmol, 0.20 g) and 1-(3-bromopropyl)-4-cyclohexylpiperazine (1 mmol, 0.30 g) in ethanol (15 ml) was added K_2CO_3 (2 mmol, 0.22 g). The reaction mixture was refluxed for 16 h and then cooled down. The precipitate was filtered off, and the solvent was evaporated. The residue was purified using column chromatography (silica gel, CH_3Cl:MeOH, 95:5) to give **16**. Yellow oil, 74% yield. ^{1}H NMR δ 1.02–1.58 (m, 5 H), 1.55–1.87 (m, 7 H), 2.13–2.21 (m, 1 H), 2.34–2.85 (m, 12 H), 4.17 (s br, 1 H), 6.68 (d, J = 8.8, 2 H), 7.43–7.56 (m, 3 H), 7.71–7.74 (m, 4 H). ^{13}C NMR δ 21.3, 24.4, 25.7, 29.5, 30.7, 46.6, 48.2, 51.9, 56.4, 63.4, 72.8, 113.7, 127.5, 128.1, 129.5, 131.4, 133.1, 138.9, 150.3. EMS [MH^+] for $C_{26}H_{35}N_3O$, Calcd. 405.58, Found, 406.3928.

Preparation of (4-((3-(4-cyclohexylpiperazin-1-yl)propyl)(prop-2-ynyl)amino)phenyl)(phenyl)methanone (17)—To a solution of **16** (1 mmol, 0.40 g) and propargyl bromide (1 mmol, 0.12 g) in ethanol (15 ml) was added K_2CO_3 (2 mmol, 0.22 g). The reaction mixture was refluxed for 12 h and then cooled. The precipitate was filtered off, and the solvent was evaporated. The residue was purified using column chromatography (silica gel, CH_3Cl:MeOH, 95:5) to give **17**. Yellow oil, 81% yield. ^{1}H NMR δ 1.02–1.58 (m, 5 H), 1.55–1.87 (m, 7 H), 2.13–2.21 (m, 1 H), 2.34–2.85 (m, 13 H), 4.02 (s, 2 H), 6.68 (d, *J* = 8.8, 2 H), 7.43–7.56 (m, 3 H), 7.71–7.74 (m, 4 H). ^{13}C NMR δ 21.3, 24.4, 25.7, 29.5, 30.7, 46.6, 48.9, 48.2, 51.9, 56.4, 63.4, 72.8, 73.4, 76.0, 113.7, 127.5, 128.1, 129.5, 131.4, 133.1, 138.9, 150.3. EMS [MH^+] for $C_{26}H_{37}N_3O$, Calcd. 443.62, Found, 444.3246.

Preparation of *N*-(3-(4-(3-aminopropyl)piperazin-1-yl)propyl)-4-(4-nitrobenzoyl)benzamide (18)—To a solution of 4-(4-nitrobenzoyl)benzoic acid (**2d**, 1.1 mmol, 0.30 g) in anhydrous THF (50 ml) was added dicyclohexylcarbodiimide (DCC, 1.1 mmol, 023 g) in anhydrous THF (5 ml) and the reaction mixture was stirred at room temperature. After 5 min, a solution of 3,3'-(piperazine-1,4-diyl)dipropan-1-amine in THF (5 ml) was added to this reaction mixture and stirred at room temperature overnight. The precipitate was filtered off, and the solvent was evaporated. The organic layer was dried over anhydrous $MgSO_4$ and the solvents were evaporated to give **18**, which was purified using column chromatography (silica gel, $CHCl_3$: MeOH, 95:5). Yellow oil, 76% yield; ^{1}H NMR δ 1.73–1.82 (m, 4H), 2.41 (s, 8 H), 2.51 (t, *J* = 4.6, 4H), 2.73 (t, *J* = 4.6, 2 H), , 4.86 (s br, 23.47 (t, *J* = 4.6, 2 H) H), 7.31 (d, *J* =8.1 Hz, 2 H), 7.68 (d, *J* =8.1 Hz, 2 H), 7.96 (d, *J* =8.7 Hz, 2 H), 8.04 (s, 1 H), 8.31 (d, *J* =8.7 Hz, 2 H). ^{13}C NMR δ 27.2, 28.3, 37.3, 41.3, 46.8, 52.3, 58.7, 73.6, 82.9, 108.3, 111.6, 126.2, 127.4, 130.6, 136.7, 143.2, 146.7, 148.6, 151.8, 167.8, 194.6. EMS [MH^+] for $C_{24}H_{31}N_5O_4$, Calcd. 453.24, Found, 454.2405.

Preparation of 4-(4-nitrobenzoyl)-N-(3-(4-(3-(prop-2-ynylamino)propyl)piperazin-1-yl)propyl)benzamide (19, Scheme 3)—To a solution of N-(3-(4-(3-aminopropyl)piperazin-1-yl)propyl)-4-(4-nitrobenzoyl)benzamide (**18**, 1.0 mmol, 0.45 g) and propargyl bromide (1 mmol, 0.12 g) in ethanol (15 ml) was added K_2CO_3 (2 mmol, 0.22 g). The reaction mixture was refluxed for 8 h and then cooled. The precipitate was filtered off, and the solvent was evaporated. The residue was purified using column chromatography (silica gel, CH_3Cl:MeOH, 95:5) to give **19**. Light brown oil, 76% yield; ^{1}H NMR δ 1.53–1.82 (m, 4H), 2.43 (s br, 1 H), 2.41 (s, 8 H), 2.41–2.58 (m, 7H), 3.31 (s, 2 H), 3.49 (t, *J* =4.6 Hz, 2 H), 7.31 (d, *J* =8.1 Hz, 2 H), 7.68 (d, *J* =8.1 Hz, 2 H), 7.96 (d, *J* =8.7 Hz, 2 H), 8.04 (s, 1 H), 8.31 (d, *J* =8.7 Hz, 2 H). ^{13}C NMR δ 23.6, 28.3, 37.3, 51.9, 58.7, 108.3, 111.6, 126.2, 127.4, 130.6, 136.7, 143.2, 146.7, 148.6, 151.8, 167.8, 194.6. EMS [MH^+] for $C_{27}H_{33}N_5O_4$, Calcd. 491.58, Found, 492.2848.

Preparation of *N*-(4-Benzoyl-phenyl)-2-bromo-acetamide (20, Scheme 4)—To a solution of 4-aminobenzophenone (2.15 mmol) and 2-bromo-acetyl bromide (2.15 mmol, 0.43g) in methylene chloride (5 ml) stirred at 0°C was added pyridine (2.15 mmol, 0.17 g). The reaction mixture was warmed to ambient temperature and stirred for 1 h. The reaction mixture was then diluted with EtOAc (25 ml), washed with 1M HCl (25 ml), and saturated with aqueous $NaHCO_3$ (25 ml) and brine (25 ml). The organic layer was dried (with $MgSO_4$), filtered and concentrated in vacuum to obtain compound **20** as brown oil. 72% yield, FTIR (CHCl3) 3285 (br m), 3056 (w), 1702 (m), 1653 (s), 1596 (s), 1532 (s), 1408 (w), 1318 (m), 1281 (m), 1252 (w), 1176 (w), 1151 (w) cm^{-1}; ^{1}H NMR δ 4.05 (s, 2 H), 7.49 (dd, *J* = 7.3 and 8.1 Hz, 2 H), 7.59 (m, 4 H), 7.68 (d, *J* = 7.3 Hz, 2 H), 7.85 (d, *J* = 8.7 Hz, 1 H), 8.33 (br s, 1 H); ^{13}C NMR δ 29.3, 119.0, 128.3, 129.9, 131.6, 132.4, 134.0, 137.6, 140.7, 163.6, 195.8, 163.6, 140.7, 137.6, 134.0, 132.4, 131.6, 129.9, 128.3, 119.0, 29.3.

Anal. Calcd for $C_{15}H_{12}BrNO_2$: C, 56.62; H, 3.80; N, 4.40. Found: C, 56.80; H, 3.70; N, 4.80.

Preparation of *N*-(4-benzoylphenyl)-2-(3-(4-cyclohexylpiperazin-1-yl)propylamino)acetamide (21, Scheme 4)—To a solution of *N*-(4-Benzoyl-phenyl)-2-bromo-acetamide (**20**, 1 mmol, 0.32 g) and 3-(4-cyclohexylpiperazin-1-yl)propan-1-amine (**5a**, 1 mmol, 0.23 g) in ethanol (15 ml) was added K_2CO_3 (2 mmol, 0.22 g). The reaction mixture was refluxed for 16 h and then cooled. The precipitate was filtered off, and the solvent was evaporated. The residue was purified using column chromatography (silica gel, CH_3Cl:MeOH, 95:5) to give **21**. Yellow oil, 89% yield. ^{1}H NMR δ 1.02–1.52 (m, 5 H), 1.55–1.87 (m, 7 H), 2.13–2.21 (m, 1 H), 2.34–2.85 (m, 12 H), 3.22 (s br, 1 H), 3.31 (s, 2 H), 7.49 (dd, *J* = 7.3 and 8.1 Hz, 2 H), 7.59 (m, 4 H), 7.68 (d, *J* = 7.3 Hz, 2 H), 7.85 (d, *J* = 8.7 Hz, 1 H), 8.33 (br s, 1 H). ^{13}C NMR δ 21.3, 24.4, 25.7, 29.5, 30.7, 46.6, 48.2, 51.3, 51.9, 56.4, 63.4, 72.8, 119.0, 128.3, 129.9, 131.6, 132.4, 134.0, 137.6, 140.7, 163.6, 195.8, 163.6, 140.7, 137.6, 134.0, 132.4, 131.6, 129.9, 128.3, 119.0, 29.3. EMS [MH^+] for $C_{28}H_{38}N_4O_2$, Calcd. 462.63, Found, 463.4526.

Preparation of *N*-(4-benzoylphenyl)-2-((3-(4-cyclohexylpiperazin-1-yl)propyl)(prop-2-ynyl)amino)acetamide (22, Scheme 4)—To a solution of *N*-(4-benzoylphenyl)-2-(3-(4-cyclohexylpiperazin-1-yl)propylamino)acetamide (**21**) (1 mmol, 0.46g) and propargyl bromide (1 mmol, 0.12 g) in ethanol (15 ml) was added K_2CO_3 (2 mmol, 0.22 g). The reaction mixture was refluxed for 5 h and then cooled. The precipitate was filtered off and the solvent was evaporated. The residue was purified using column chromatography (silica gel, CH_3Cl:MeOH, 95:5) to give **22**. Yellow oil, 96% yield. ^{1}H NMR δ 1.02–1.52 (m, 5 H), 1.55–1.87 (m, 7 H), 2.13–2.21 (m, 1 H), 2.34–2.85 (m, 13 H), 3.36 (s, 2 H), 3.92 (s, 2 H), 7.49 (dd, *J* = 7.3 and 8.1 Hz, 2 H), 7.59 (m, 4 H), 7.68 (d, *J* = 7.3 Hz, 2 H), 7.85 (d, *J* = 8.7 Hz, 1 H), 8.33 (br s, 1 H). ^{13}C NMR δ 21.3, 24.4, 25.7, 29.5, 30.7, 46.6, 47.8, 48.2, 51.3, 52.7, 58.5, 63.4, 72.8, 73.6, 77.8, 119.0, 128.3, 129.9, 131.6, 132.4, 134.0, 137.6, 140.7, 163.6, 195.8, 163.6, 140.7, 137.6, 134.0, 132.4, 131.6, 129.9, 128.3, 119.0, 29.3. EMS [MH^+] for $C_{31}H_{40}N_4O_2$, Calcd. 500.67, Found, 501.6491.

Preparation of *N*-(4-benzoylphenyl)-2-((3-(4-cyclohexylpiperazin-1-yl)propyl)(4-hydroxybutyl)amino)acetamide (23, Scheme 4)—To a solution of *N*-(4-benzoylphenyl)-2-(3-(4-cyclohexylpiperazin-1-yl)propylamino)acetamide (**21**) (1 mmol, 0.46g) and 4-bromobutanol (1 mmol, 0.15 g) in ethanol (15 ml) was added K_2CO_3 (2 mmol, 0.22 g). The reaction mixture was refluxed for 16 h and then cooled. The precipitate was filtered off and the solvent was evaporated. The residue was purified using column chromatography (silica gel, CH_3Cl:MeOH, 95:5) to give **23**. Yellow oil, 73 % yield. ^{1}H NMR δ 1.02–1.52 (m, 9 H), 1.55–1.87 (m, 7 H), 2.13–2.21 (m, 1 H), 2.34–2.85 (m, 14 H), 3.31 (s, 2 H), 3.65 (t, *J* = 4.9 Hz, 2 H), 3.71 (s br, 1 H), 7.49 (dd, *J* = 7.3 and 8.1 Hz, 2 H), 7.59 (m, 4 H), 7.68 (d, *J* = 7.3 Hz, 2 H), 7.85 (d, *J* = 8.7 Hz, 1 H), 8.33 (br s, 1 H). ^{13}C NMR δ 21.3, 23.9, 24.4, 24.8, 25.7, 29.5, 30.7, 46.6, 48.2, 51.3, 51.9, 56.4, 56.9, 62.1, 63.4, 72.8, 119.0, 128.3, 129.9, 131.6, 132.4, 134.0, 137.6, 140.7, 163.6, 195.8, 163.6, 140.7, 137.6, 134.0, 132.4, 131.6, 129.9, 128.3, 119.0, 29.3. EMS [MH^+] for $C_{32}H_{46}N_4O_3$, Calcd. 537.73, Found, 538.6806.

Preparation of *N*-4-((2-(4-benzoylphenylamino)-2-oxoethyl)(3-(4-cyclohexylpiperazin-1-yl)propyl)amino)butyl trifluoromethanesulfonate (24, Scheme 4)—To a solution of *N*-(4-benzoylphenyl)-2-((3-(4-cyclohexylpiperazin-1-yl)propyl)(4-hydroxybutyl)amino)acetamide (**23**) (0.5 mmol, 0.27 g) and trifluoromethanesulfonyl chloride (1 mmol, 0.17 g) in CH_2Cl_2 (15 ml) stirred at 0°C was added pyridine (1 mmol, 0.16 g). The reaction mixture was then stirred at room temperature

for 10 h. The reaction mixture was filtered off, washed with water (2 × 15 ml), the organic phase dried over $MgSO_4$ and the solvent was evaporated. The residue was purified using column chromatography (silica gel, CH_3Cl) to give **24**. Yellow oil, 81 % yield. ^{1}H NMR δ 1.02–1.52 (m, 9 H), 1.55–1.87 (m, 7 H), 2.13–2.21 (m, 1 H), 2.34–2.85 (m, 14 H), 3.31 (s, 2 H), 4.38 (t, J = 5.0 Hz, 2 H), 7.49 (d, *J* = 7.3 and 8.1 Hz, 2 H), 7.59 (m, 4 H), 7.68 (d, *J* = 7.3 Hz, 2 H), 7.85 (d, *J* = 8.7 Hz, 1 H), 8.33 (br s, 1 H). ^{13}C NMR δ 21.3, 23.9, 24.4, 24.8, 25.7, 29.5, 30.7, 46.6, 48.2, 51.3, 51.9, 56.4, 56.9, 63.4, 70.2, 72.8, 118.5, 119.0, 128.3, 129.9, 131.6, 132.4, 134.0, 137.6, 140.7, 163.6, 195.8, 163.6, 140.7, 137.6, 134.0, 132.4, 131.6, 129.9, 128.3, 119.0, 29.3. EMS [MH^+] for $C_{33}H_{45}F_3N_4O_5S$, Calcd. 666.7900, Found, 667.8764.

Preparation of *N*-(4-benzoylphenyl)-2-((3-(4-cyclohexylpiperazin-1-yl)propyl)(4-iodobutyl)amino)acetamide (25, Scheme 4)—The reaction mixture of *N*-4-((2-(4-benzoylphenylamino)-2-oxoethyl)(3-(4-cyclohexylpiperazin-1-yl)propyl)amino)butyl trifluoromethanesulfonate (**24**) (0.25 mmol, 0.17 g) and NaI (0.5 mmol, 0.08 g) in 1,4-dioxane (15 ml) was stirred at 50°C overnight. The reaction was then filtered off and the solvent was evaporated. The residue was dissolved in DCM and washed with water (2 × 15 ml) and K_2CO_3 (5 %, 2 × 10 ml), the organic phase was dried over $MgSO_4$ and purified using column chromatography (silica gel, CH_3Cl:MeOH) to give **25**. Yellow oil, 73% yield. ^{1}H NMR δ 1.02–1.52 (m, 9 H), 1.55–1.87 (m, 7 H), 2.13–2.21 (m, 1 H), 2.34–2.85 (m, 14 H), 3.31 (s, 2 H), 3.19(t, J = 5.0 Hz, 2 H), 7.49 (d, *J* = 7.3 and 8.1 Hz, 2 H), 7.59 (m, 4H), 7.68 (d, *J* = 7.3 Hz, 2 H), 7.85 (d, *J* = 8.7 Hz, 1 H), 8.33 (br s, 1 H). ^{13}C NMR δ 6.9, 21.3, 24.4, 25.7, 28.8, 29.5, 30.7, 31.2, 46.6, 48.2, 51.3, 51.9, 55.8, 56.4, 63.4, 72.8, 119.0, 128.3, 129.9, 131.6, 132.4, 134.0, 137.6, 140.7, 163.6, 195.8, 163.6, 140.7, 137.6, 134.0, 132.4, 131.6, 129.9, 128.3, 119.0, 29.3. Anal. Calcd for $C_{32}H_{45}IN_4O_2$: C, 59.60; H, 7.0; N, 8.9. Found: C, 59.33; H, 7.21; N, 8.78.

Synthesis of *N*-(3-bromopropyl)-5-((4R)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (26, Scheme 4)—To a solution of 2,5-dioxopyrrolidin-1-yl 5-((4R)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (0.3 mmol, 0.102 g) in DMF (1.5 ml) was added 3-bromobutan-1-amine (3 mmol, 0.042 g) in dichloromethane (10 ml). After 3 h reaction at room temperature the mixture was washed with water and dried over $MgSO_4$. The solvent was evaporated (89.4 mg; 82 %) and the solid obtained was purified by HPLC (H_2O/CF_3COOH, 0.1% MeOH). ^{1}H NMR (d_6DMSO) δ 1.42–1.98 (m, 6 H), 2.11 (m, 2 H), 2.39 (t, *J* = 7.7 , 2 H), 2.71 (d, *J* = 12.1 , 1 H), 2.92 (d, *J* = 12.1 and 5 Hz, 1 H), 3.18 (t, *J* = 8.1 and 7.0 Hz, 1 H), 3.31 (t, *J* = 4.6 Hz, 2 H), 3.67 (t, *J* = Hz, 2 H), 4.64 (t, *J* = 8.3, 1 H), 4.50 (d, *J* = 8.1 and 5.0 Hz, 1 H), 5.65 (s br, 2 H) 8.12 (s, 1 H). EMS [MH+] for $C_{13}H_{22}BrN_3O_2S$, Calcd. 363.10, Found, 365.1092 and 366.2115 (1:1 ratio).

Synthesis of N-(3-((2-(4-benzoylphenylamino)-2-oxoethyl)(3-(4-cyclohexylpiperazin-1-yl)propyl)amino)propyl)-5-((4R)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (27, Scheme 4)—To a solution of *N*-(3-bromopropyl)-5-((4R)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (**26**) (0.1 mmol, 0.036 g) in DMF (1 ml) was added *N*-(4-Benzoyl-phenyl)-2-bromo-acetamide (**21**) (0.1 mmol, 0.032 g) in DCM (5 ml) and K_2CO_3 (0.2 mmol, 0.022 g). The reaction mixture was refluxed for 16 h and then cooled. The precipitate was filtered off, washed with water and dried over $MgSO_4$. The solvent was evaporated (38.1 mg; 52 %) and the obtained oil was purified by HPLC (H_2O/CF_3COOH, 0.1% MeOH). ^{1}H NMR (d_6DMSO) δ 1.02–1.98 (m, 18 H), 2.11–2.21 (m, 3 H), 2.34–2.89 (m, *J* = 7.7 , 14 H), 2.31–2.71 (m, 3 H), 2.92 (d, *J* = 12.1 and 5 Hz, 1 H), 3.18 (dt, *J* = 8.1 and 7.0 Hz, 1 H), 3. 31 (t, *J* = 4.6 Hz, 2 H), 3.67 (t, *J* = Hz, 2 H), 4.64 (t, *J* = 8.3, 1 H), 4.50 (dd, *J* = 8.1 and 5.0 Hz, 1 H), 5.65 (s br, 2

H) 8.12 (s, 1 H), 7.49 (dd, J = 7.3 and 8.1 Hz, 2 H), 7.59 (m, 4 H), 7.68 (d, J = 7.3 Hz, 2 H), 7.85 (d, J = 8.7 Hz, 1 H), 8.33 (br s, 1 H). EMS [MH^+] for $C_{40}H_{59}N_7O_4S$, Calcd. 745.43, Found, 746.8598.

Preparation of rat liver membranes and RT-4 cell membranes

Rat livers (65 g, Pel-Freez Biologicals) were minced in 100 ml of homogenization buffer (20 mM Tris-HCl pH 8.0, 0.32M sucrose) that contained a Protease Inhibitor Cocktail (Sigma-Aldrich P8340-5ML, for use with mammalian cell and tissue extracts) and then homogenized on ice using a Brinkman Polytron Homogenizer (setting 6, 4 bursts of 10 sec each) followed by a glass homogenizer (Teflon pestle with 6 slow passes at 3000 rpm). The homogenized tissue was first centrifuged at 1,000 ×g for 10 min and the supernatant was then centrifuged at 100,000 ×g for 1 h at 4°C. The membrane pellets were resuspended in the homogenization buffer, and used for competitive sigma receptor binding assays.

Human urinary bladder transitional papilloma RT-4 cells (HTB-2, ATCC) were harvested from the cell culture, and lysed using a sonicator (Branson Sonifier, output 50%, duty cycle 50%, 6×10 sec) in the homogenization buffer. The cell homogenates were centrifuged at 1,000 ×g for 10 min, and the supernatant was then centrifuged at 100,000 ×g for 1 h at 4°C. The pelleted membranes were resuspended by brief sonication and used fresh for the competitive radioligand binding assays and photolabeling assays.

Sigma receptor radioligand binding assays

Competitive binding assays were performed to determine binding affinities of the new compounds for the σ2 and σ1 receptors as previously described[21, 24]. Assays for σ2 binding were performed using RT-4 cell membranes since this cell line is highly enriched in the σ2 receptor [23]. [^{3}H]-DTG of 10 nM (PerkinElmer, 58.1 Ci/mmol) was incubated for 1.5 h at 32°C with ~15 μg (per reaction) of total proteins in the membranes in 50 mM Tris-HCl, pH 8.0 in a 96-well plate. To assess the σ2 binding affinities of the compounds listed in Table 1, serial dilutions of a given compound were added to the reactions to compete with the binding of [^{3}H]-DTG with the σ2 receptor. Non-radioactive (+)-pentazocine was added (to a 100 nM final concentration) to block σ1 binding sites. Haloperidol (Sigma-Aldrich) at 10 μM was used to determine non-specific binding. The σ1 binding properties of these compounds were assayed similarly in rat liver membranes by using 10 nM [^{3}H]-(+)-pentazocine (PerkinElmer, 34.8 Ci/mmol). Following the incubation, the reactions were filtered through a Brandel GF/B Fired Membrane (pretreated with 0.5% polyethyleneimine) using a Brandel Cell Harvester (M-48T). Radioactivity on the filter discs was determined using a Beckman Scintillation Counter (LS-6500) in an NEN formula-989 scintillation cocktail (Ultima Gold MV, PerkinElmer). Values were fit to a one-site competition non-linear regression curve using Graphpad Prism Version 4.0c. *Ki* was calculated using the Cheng-Prusoff equation [25]. For calculation of *Ki*, a *Kd* of 8.3 nM was used for [^{3}H]-DTG in RT-4 cell membranes [23] and a *Kd* of 20 nM was used for (+)-pentazocine in rat liver membranes.

Photoaffinity labeling of sigma receptors

Radiochemical synthesis of the sigma receptor photolabel [^{125}I]-IAF (1-N-(2',6'-dimethyl-morpholino)-3-(4-azido-3-[^{125}I]iodo-phenyl propane) was performed as previously described [15a]. Fresh RT-4 cell membranes were used for [^{125}I]-IAF photoaffinity labeling of sigma receptors. To test the σ2 or σ1 binding specificity of the new compounds, Compounds **22** and **9** were first pre-incubated with RT-4 membranes (200 μg total proteins per reaction) in 50 mM Tris (pH 7.4) for 30 min at 32°C. [^{125}I]-IAF was then added to the membranes to a final concentration of 1 nM (final 1% ethyl acetate) and incubated for another 50–60 min at 32°C. Following incubation, the [^{125}I]-IAF photoreactive label was

activated by exposure to UV for 5 sec using a high-pressure AH-6 mercury lamp (10 cm distance). The photolysis reactions were then quenched immediately with the sample buffer containing 200 mM β-mercaptoethanol and 1% SDS. Proteins were separated on a 15% SDS gel and the radiolabeled proteins were visualized using a PhosphorImager (Molecular Dynamics).

Photo-crosslinking and click chemistry

In order to test whether an affinity tag could be covalently linked to the alkyne-containing sigma receptor ligands, we performed proof-of-principle experiments using BSA as a carrier protein. To conjugate the compounds to BSA, 100 μM compound **22** or compound **6** (as the non-alkyne negative control) was incubated with 5 μM BSA in 100 μl of PBS buffer and exposed to UV light for 50 min. The UV light was provided by a Rionet Photoreactor as described previously [16]. Following photolysis, unreacted compound was removed by filtering the BSA solution 3 times using a Microcon filter (Milipore). Into the BSA conjugates, 1% SDS, 10 μM biotin azide, 1 mM TCEP, 200 μM TBTA (tris-(benzyltriazolylmethyl)amine), and 1 mM $CuSO_4$ were added sequentially and incubated for 1 h at 50°C. The reactions products were resolved on a 15% SDS polyacrylamide gel. Biotin-tagged BSA molecules were visualized through far-Western blotting using streptavidin-conjugated HRP according to our previously described method [26].

Acknowledgments

This work was supported by NIH Grants MH065503 and DA027191, and a UW ERI Retina Research Foundation Edwin & Dorothy Gamewell Professorship (to A.E.R). We thank Dr. Uyen B. Chu for assistance in the radiochemical synthesis of [^{125}I]-IAF.

ABBREVIATIONS

PGRMC1	progesterone receptor membrane component 1
RT	room temperature
DCC	dicyclohexylcarbodiimide
THF	tetrahydrofuran
Ar	aromatic ring
OMe	methoxy
EtOH	ethanol
DTG	1,3-di(2-tolyl)guanidine
[^{125}I]-IAF	1-N-(2',6'-dimethyl-morpholino)-3-(4-azido-3-[^{125}I]iodo-phenyl propane
TBTA	tris-(benzyltriazolylmethyl)amine
TCEP	tris(2-carboxyethyl)phosphine

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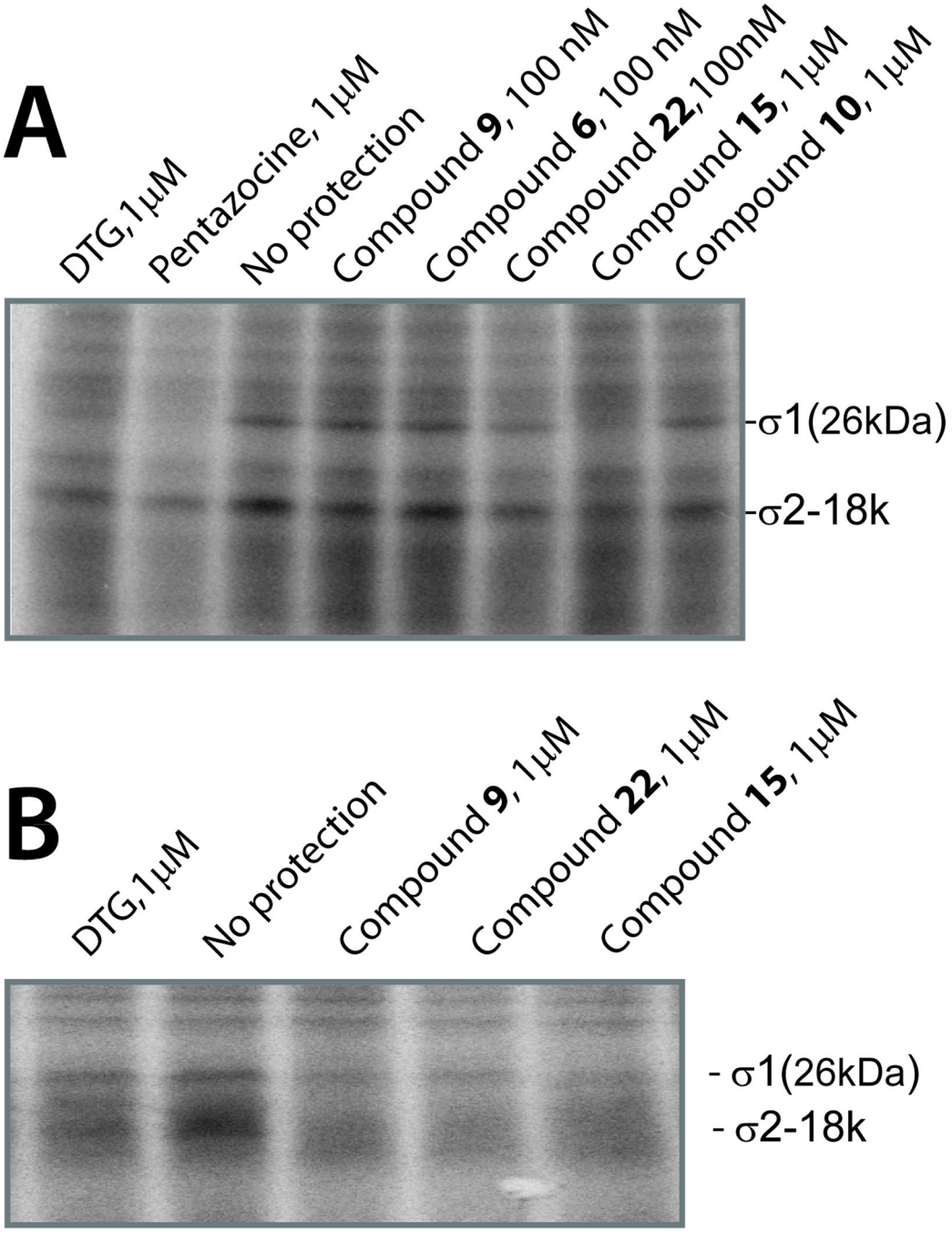


Figure 1.

Protection against [^{125}I]-IAF photolabeling of the σ2-18k receptor band by compounds **9** and **22**. Photoaffinity labeling of RT-4 cell membranes with 1 nM [^{125}I]-IAF was performed in the absence or presence of compounds **9** and **22** at 100 nM (A) or 1 μM (B).

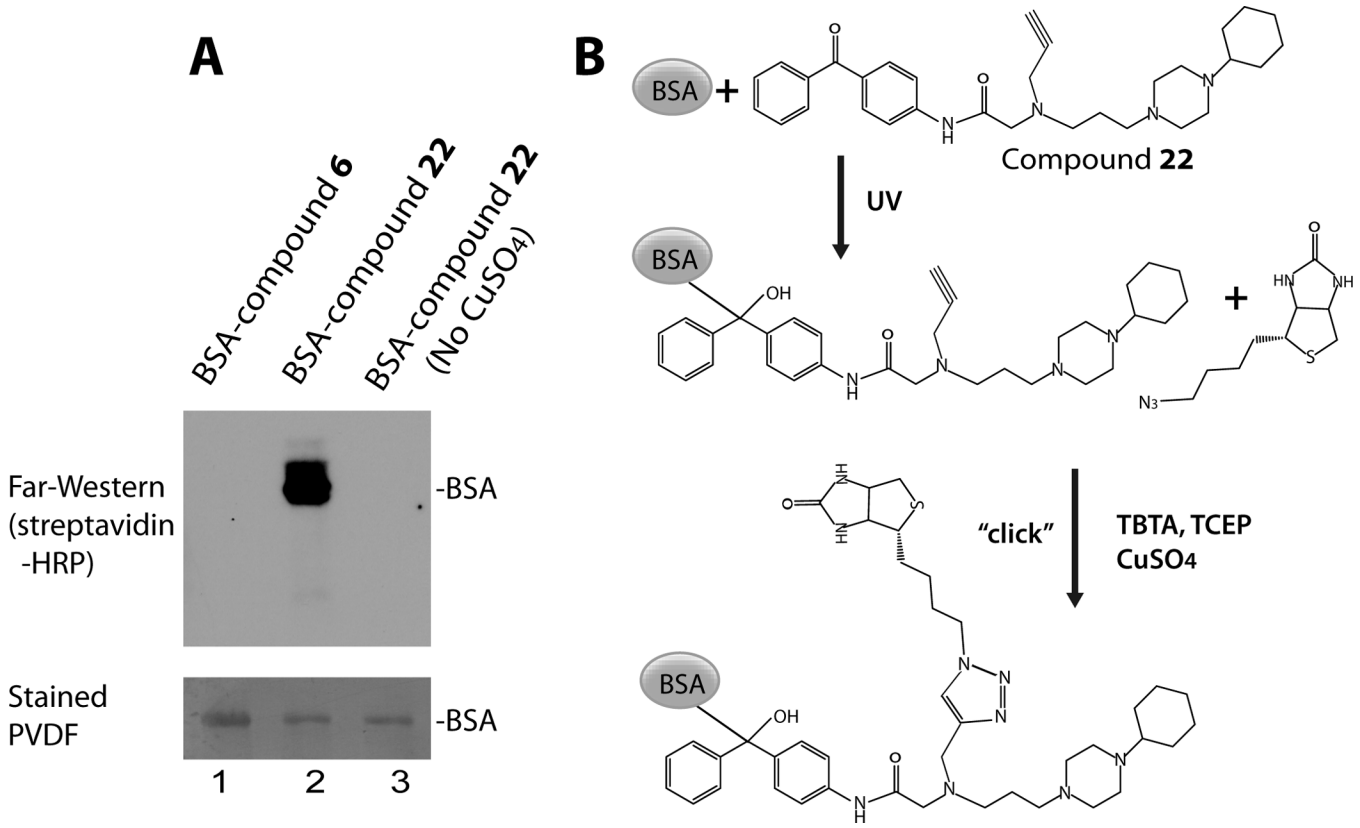
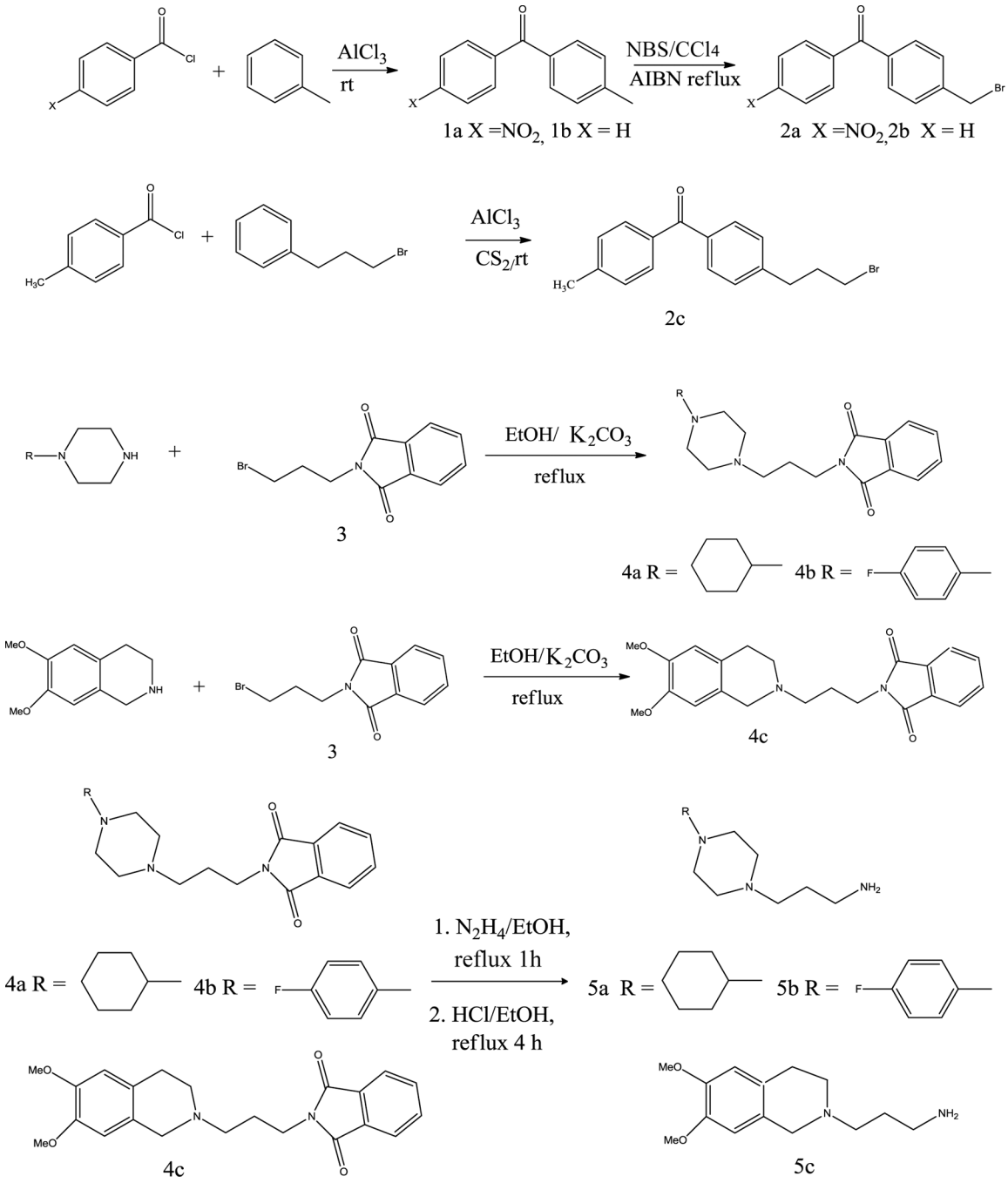
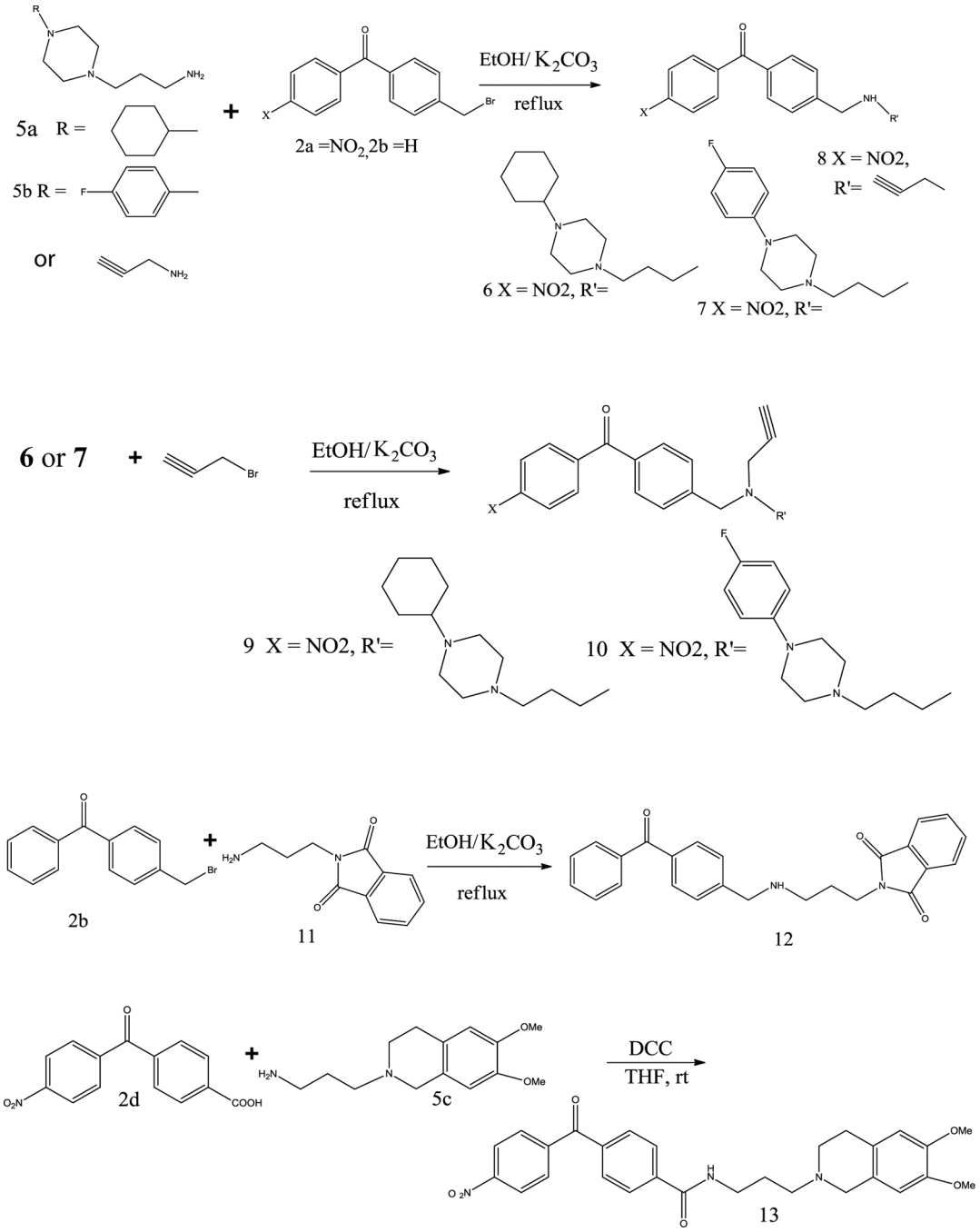


Figure 2.

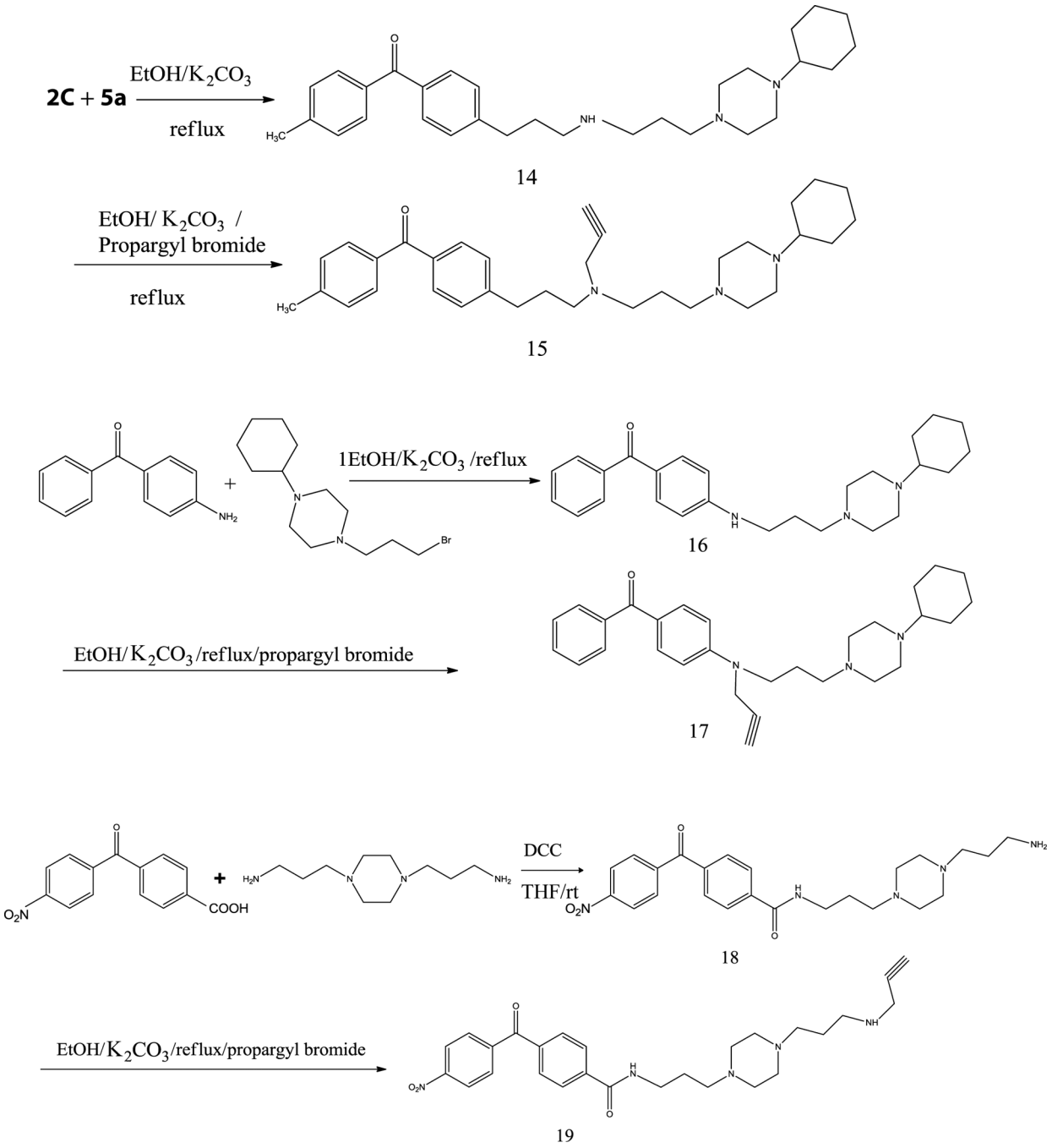
Proof-of-principle for the clickability of the alkyne-benzophenone compound **22** A). Biotin-streptavidin far-Western blotting. BSA was first labeled by nonspecific photo-crosslinking with either compound **6** which does not carry an alkyne group (lane 1) or its alkyne-containing equivalent, compound **22** (lanes 2 and 3), and then tagged with azido-biotin through click chemistry in the presence (Lanes 1 and 2) or absence of $CuSO_4$ (lane 3). Covalent linking of biotin to BSA was detected by far-Western blotting (upper panel). BSA of 2 μg was loaded in each lane, as shown on the Coomassie-stained PVDF membrane (lower panel). B). Diagram of the photo-crosslinking/click chemistry experiments in A.



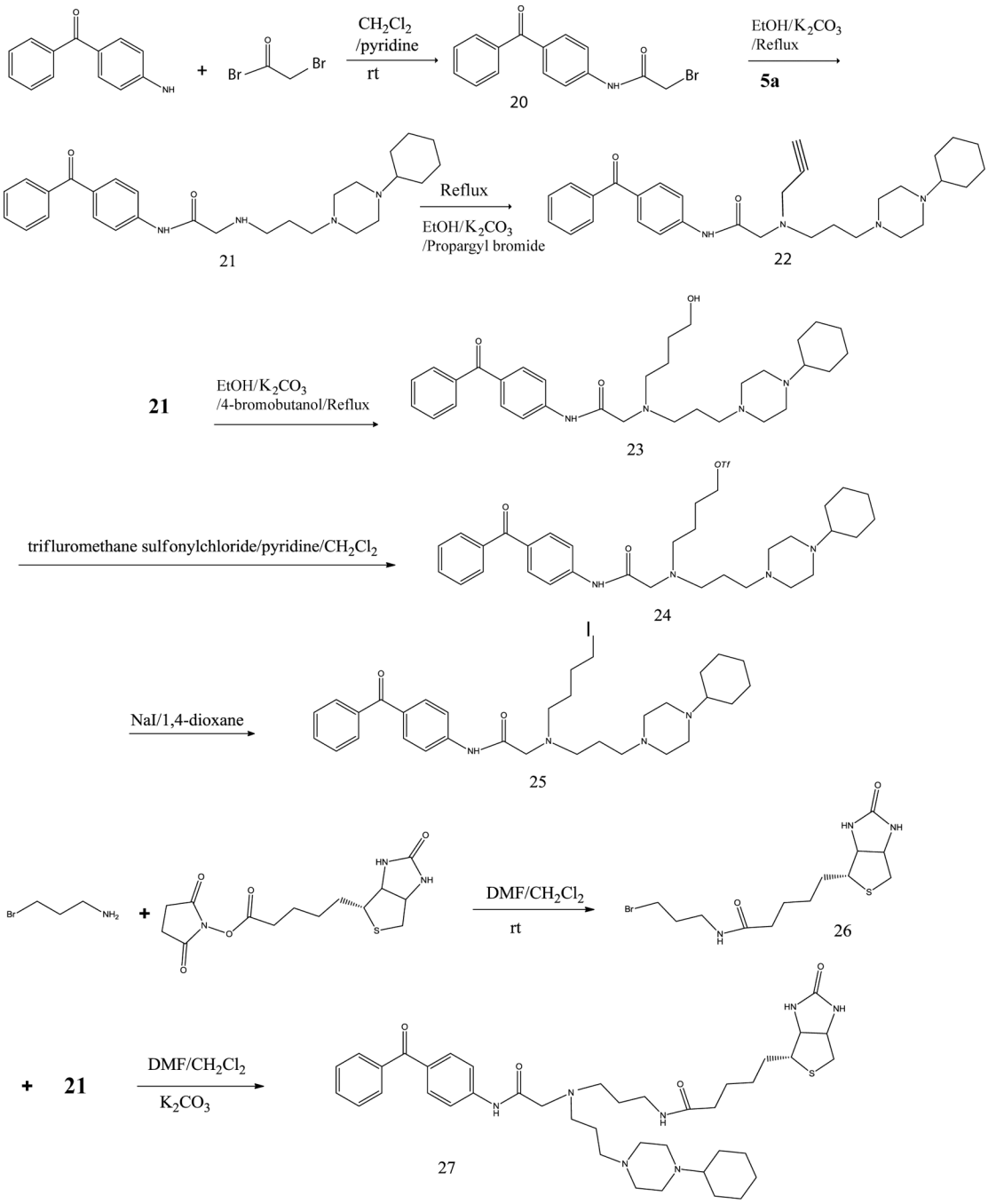
Scheme 1.
Synthesis of compounds **1a–5c**



Scheme 2.
Synthesis of compounds **6–13**



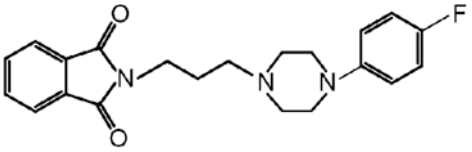
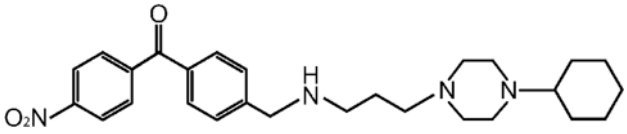
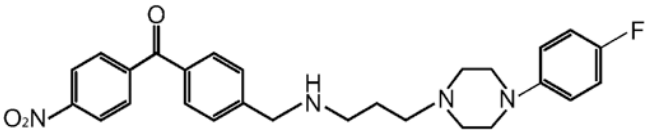
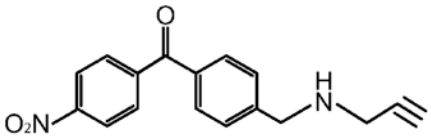
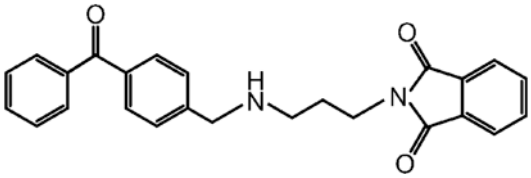
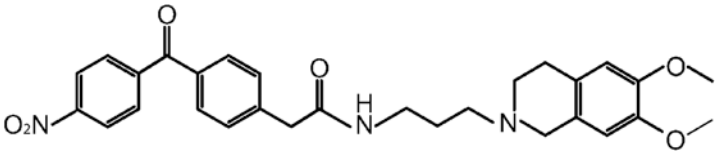
Scheme 3.
Synthesis of compounds **14–19**



Scheme 4.
Synthesis of compounds **20–27**

Table 1

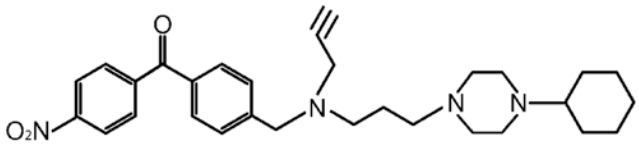
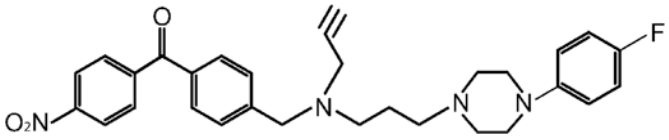
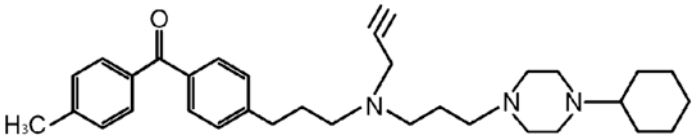
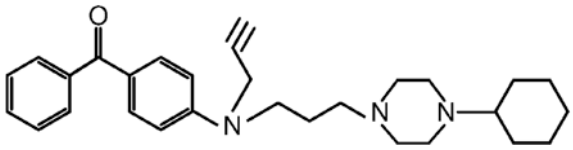
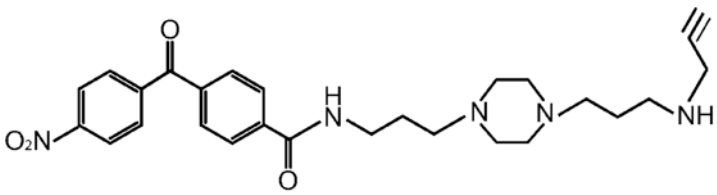
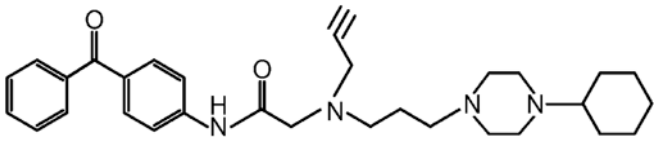
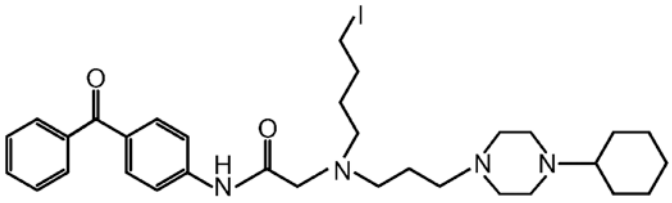
Screening of the benzophenone-containing sigma receptor ligands

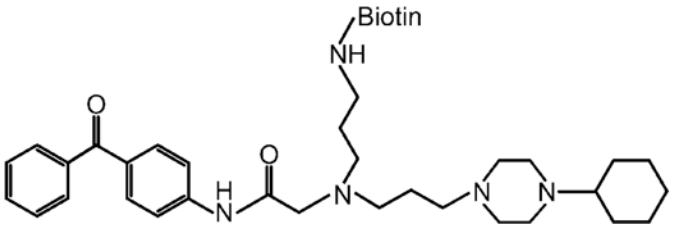
	Compounds	σ2*Ki* (nM)	σ1*Ki* (nM)	σ1/σ2
4b		741.67 (±83.12)	1,600 (±500)	2.16
6		33.31 (±16.01)	1,325 (±360.06)	39.78
7		33.97 (±5.80)	2,407 (±135.32)	70.86
8		>10^4	120.05	<0.01
12		>10^4	311.2	<0.03
13		1,202	>10^4	>8.31

Ki is presented as a mean (±SEM) of 2–3 independent determinations, in each determination duplicate samples were used at each concentration.

Table 2

Characterization of the alkyne-containing benzopheone-piperazine sigma receptor ligands

	Compounds	σ2 *Ki* (nM)	σ1 *Ki* (nM)	σ1/σ2
9		3.78 (±1.21)	29.80 (±0.90)	7.88
10		69.43 (±5.25)	290.00 (±39.88)	4.18
15		17.37 (±4.06)	25.25 (±5.45)	1.45
17		77.71	652.00	8.39
19		60.14 (±33.57)	444.50 (±179.50)	7.39
22		2.11 (±0.17)	16.95 (±3.05)	8.03
25		44.71 (±3.29)	171.50 (±44.50)	3.84

	Compounds	σ2 *Ki* (nM)	σ1 *Ki* (nM)	σ1/σ2
27		86.54 (±1.97)	221.23 (±14.63)	2.56

Ki is presented as a mean (±SEM) of 2–3 independent determinations, in each of which duplicate samples were used at each concentration.