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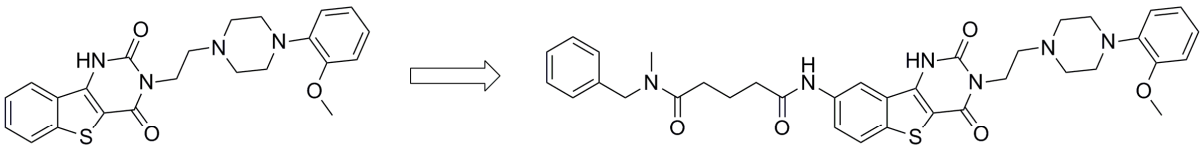

RX18 pK_i = 10.25 for α_{1D}-AR

High Affinity Ligands and Potent Antagonists for the α_{1D}-Adrenergic Receptor. Novel 3,8-Disubstituted [1]Benzothieno[3,2-*d*]pyrimidine Derivatives.

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Abstract

A new series of high affinity ligands and antagonists for the α_{1D}-adrenergic receptor (AR) has been discovered. New molecules present a [1]benzothieno[3,2-*d*]pyrimidin-2,4(1*H*,3*H*)-dione or a [1]benzothieno[3,2-*d*]pyrimidin-4(3*H*)-one scaffold and bear a 2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl moiety in the 3-position and various amide substituents in the 8-position. In binding assays at the three human cloned α_{1A}-, α_{1B}-, and α_{1D}-AR subtypes, they showed high affinity values, particularly for the α_{1D}-AR subtype. Compound **22** (RX18), N^1-methyl-N^5-[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl]-2,4-dioxo-1,2,3,4-tetrahydro[1]benzothieno[3,2-*d*]pyrimidin-8-yl]-N^1-(phenylmethyl)pentanediamide, was the most interesting in the series displaying very high affinity (pK_i = 10.25) and potent antagonism (pK_b = 9.15) when tested in a functional assay at the α_{1D}-AR.

Keywords

α_{1D}-Adrenergic Receptor; Antagonist; Ligand; [1]Benzothieno[3,2-*d*]pyrimidine.

Abbreviations

AR, adrenergic receptor; BPH, benign prostatic hypertrophy; CNS, central nervous system; TM, transmembrane domain; IL, intracellular loop; XL, extracellular loop, PP, phenylpiperazine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid; NA, noradrenaline.

1. Introduction

α_1-Adrenergic receptors (α_1-ARs) are class A (rhodopsin-like) members of the G protein-coupled receptor (GPCR) super-family. They are widely distributed in the human body and, along with α_2-ARs and β-ARs, mediate the functional effects of the endogenous catecholamines, noradrenaline and adrenaline. α_1-ARs are cell-surface receptors that are involved in several physiological functions (vascular smooth muscle contraction, cardiac inotropy and chronotropy, hepatic glucose metabolism) and in a number of pathological conditions such as myocardial hypertrophy and benign prostatic hypertrophy (BPH). They also represent the biological target of currently used drugs useful in the clinical treatment of arterial hypertension and male lower urinary tract symptoms associated to benign prostatic hypertrophy (BPH) [1].

α_1-ARs do not constitute a homogeneous population: three different subtypes are known, viz. α_{1A}-, α_{1B}-, and α_{1D}-ARs, encoded by distinct genes located on human chromosomes 8, 5, and 20, respectively. All three subtypes have been cloned and pharmacologically characterized [2]. Each subtype possesses a discrete tissue distribution, a fact suggesting unique physiological roles for each of them. In the CNS, all three α_1-AR subtypes are widely represented but with distinct patterns of distribution in the different brain and spinal cord areas [3].

From a topological point of view, α_1-ARs share with other GPCRs a common architecture based on a bundle of seven hydrophobic transmembrane α-helices (TM1–TM7) connected by alternating intracellular (IL1–IL3) and extracellular (XL1–XL3) loops; the N-terminal and C-terminal regions of the protein lie in the extracellular and in the intracellular environment, respectively. The sequence identity among the three α_1-AR subtypes is higher in TM regions (α_{1A}-α_{1B}: 75 %; α_{1A}-α_{1D}: 66 %; α_{1B}-α_{1D}: 73 %) and much lower in connecting loops and N- and C-termini [4].

As for other class A receptors, the so-called orthosteric binding site in α_1-ARs is believed to be located within the bundle of α-helices. This is strongly supported by site-directed mutagenesis studies, which identified within TMs specific amino acid residues involved in ligand recognition, and by the recent X-rays high-resolution structure of the complexes between the class A β_2-adrenergic receptor and agonists, inverse agonists or antagonists [5-7].

A conserved aspartate in TM3 (Asp3.32 in the Ballesteros-Weinstein notation [8]) of α_1-ARs is essential for ligand binding. Site-directed mutagenesis studies on α_{1B}-AR and α_{1D}-AR in which Asp3.32 has been mutated into alanine have shown that Asp3.32Ala receptor mutants are not able to bind either agonists or antagonists [9, 10]. These data strongly suggests the formation of an essential ionic interaction between the negatively charged side chain of Asp3.32 and the protonated amino group of the ligand.

In addition to the classic orthosteric binding site, topographically distinct allosteric binding sites have been identified in a number of class A receptors [11]. At the α_{1B}-AR subtype in particular, ρ-TIA, a small peptide from marine cone snail *Conus tulipa*, behaves as a subtype-selective allosteric antagonist and interacts with the extracellular receptor surface at the base of XL3 [12], defining an allosteric binding site different from that located within the bundle of α-helices. This suggests that subtype-selectivity, which can hardly be reached by ligands acting only within the orthosteric site, could be achieved exploiting the higher sequence dissimilarity of extracellular domains in α_1-AR subtypes and taking advantage of possible subtype-specific interactions between the ligand and amino acid residues belonging to such domains [13].

In the last decade, our research group has extensively worked on the development of new α_1-AR ligands containing a phenylpiperazine (PP) substructure as pharmacophoric moiety [14-19]. In most of these ligands, the PP moiety was linked, through an alkyl chain, to a heterobicyclic or

heterotricyclic planar scaffold. Notable examples of such a kind of molecules are RN17 and RC23, two potent α_1-AR ligands with antagonistic properties [14, 16, 20]. In particular, RN17 and RC23 possess a 4-(2-methoxyphenyl)piperazin-1-yl moiety connected through an ethylene chain to the 3-position of a tricyclic [1]benzothieno[3,2-*d*]pyrimidin-2,4(1*H*,3*H*)-dione or a [1]benzothieno[3,2-*d*]pyrimidin-4(3*H*)-one system, respectively (Figure 1). The two molecules act as high affinity ligands at all three human cloned α_{1A}-, α_{1B}, and α_{1D}-ARs with a slight preference for the last subtype (Table 1). In both structures, the protonatable amino group of the PP moiety can assure the ionic interaction with Asp3.32 side chain of the binding site.

In an effort to expand our previous work and with the aim to obtain α_1-AR ligands with higher affinity and greater subtype selectivity, here we now report the preparation of a series of new α_1-AR ligands (**7**-**10**, **20**-**23**, **26**, **27**, **33**-**36**, and **38**) based on structures of RN17 and RC23 (Figure 1). In particular, the 8-position of the two tricyclic systems in RN17 and RC23 was chosen for chemical manipulation. Through an amide linker, a number of side chains of increasing length and different natures were inserted at this position. At one end, these side chains bear a phenyl group or a protonatable amine function in order to take advantage of additional possible aromatic/aromatic, cation/aromatic, or ionic interactions that could take place between these groups and the extracellular surface of the receptor proteins, particularly the second loop (XL2). In fact, XL2 presents the highest divergence in sequence among the three human α_1-AR subtypes and it is rich in polar residues, especially negatively charged aspartate and glutamate residues.

The choice of the 8-position for the insertion of side chains was made taking into account the structure-activity relationships previously seen in a series of α_1-AR ligands designed using RN17 as template and developed at Abbott Laboratories [21]. These compounds, based on a pyrazino[2',3':4,5]thieno[3,2-*d*]pyrimidine-2,4(*1H,3H*)-dione tricyclic scaffold, bear different substituents (hydrogen, methyl, methoxy, chloro, phenyl) at the 7- and/or 8-positions. Compound

39 (Figure 1) is one of them: the presence in it of a bulky substituent such as a phenyl ring at the 8-position is well tolerated whereas the shifting of the same substituent to the 7-position causes a marked drop in affinity for all three α_1-AR subtypes [21]. This suggests that, *mutatis mutandis*, the 8-position could represent a site in the planar tricyclic systems of RN17 and RC23 useful for the introduction of additional structural moieties.

2. Chemistry

Synthetic pathways to the final [1]benzothieno[3,2-*d*]pyrimidin-2,4(1*H*,3*H*)-diones **7**-**10**, **20**-**23**, **26**, and **27** are shown in Schemes 1, 2, and 3, respectively.

Ethyl 3-amino-5-nitro[1]benzothiophene-2-carboxylate (**1**) [21] was treated with ethyl chlorocarbonate to give in good yield urethane **2**, which in turn was transformed into the tricyclic alcohol **3** by reaction with an excess of 2-aminoethanol (Scheme 1). Compound **4**, obtained by the action of thionyl chloride on alcohol **3**, was allowed to react with 1-(2-methoxyphenyl)piperazine to give tricyclic nitro derivative **5**. Successive reduction of the nitro group with hydrazine monohydrate in DMF in the presence of Raney Nickel afforded the 8-amino derivative **6** which represents the key intermediate in the preparation of all desired [1]benzothieno[3,2-*d*]pyrimidin-2,4(1*H*,3*H*)-diones. Reaction of **6** with benzoyl chloride or phenylalkanoyl chlorides gave final compounds **7**-**10** in good yields (Scheme 1).

In Scheme 2 is outlined the preparation of the final derivatives **20**-**23** bearing diamide side chains at the 8-position. The suitable acidic intermediates **16**-**19** were synthesized by reaction of amines **11**-**13** with succinic anhydride (**14**) or glutaric anhydride (**15**) in anhydrous toluene. Acids **16**-**19**, activated by ethyl chlorocarbonate, were treated with the 8-amino derivative **6** to afford the desired products **20**-**23**.

Worthy of note, due to the hindered rotation around the C(O)-N bond of the tertiary *N*-phenylmethyl-*N*-methylamide group, in ^{1}H NMR and ^{13}C NMR spectra of acid intermediate **18** and

of the corresponding final derivative **22,** it is possible to observe signals for the two rotational conformers *E* and *Z*, according to literature data for similar compounds [22].

Preparation of final compounds **26** and **27**, having basic side chains in the 8-position, was accomplished by reaction of amino acid hydrochloride **24**, obtained following the procedure of Gittos and Wilson [23], or the commercially available **25**, activated by 1,1'-carbonyldiimidazole, and the amino derivative **6** (Scheme 3).

The synthetic pathway for the preparation of the 8-amino derivative **32**, the key intermediate in the synthesis of the final [1]benzothieno[3,2-*d*]pyrimidin-4(3*H*)-ones **33-36** and **38** is presented in Scheme 4. Ethyl 3-amino-5-nitro[1]benzothiophene-2-carboxylate (**1**) was treated with 1,1,1-triethoxyethane to give the iminoether **28** which was then cyclized to the alcohol **29** by reaction with 2-aminoethanol. Conversion to alkyl chloride **30**, successive reaction with 1-(2-methoxyphenyl)piperazine to give the tricyclic nitro derivative **31**, and final reduction of the nitro group provided the 8-amino intermediate **32**.

As shown in Scheme 5, **32** was allowed to react with benzoyl chloride and phenylacetyl chloride to give **33** and **34** or with acids **17** and **19**, activated by ethyl chlorocarbonate, to provide diamides **35** and **36**, respectively.

Final compound **38** is an isomer of **35** and presents an inverse amide function on the side chain in the 8-position. Activation with ethyl chloroformate was also used in its preparation, which was accomplished starting from acid **37** and amino derivative **32** (Scheme 5). Acid **37** was synthesized by reaction of phenylacetyl chloride with 4-aminobutanoic acid in basic conditions [24].

3. Results and Discussion

3.1. Binding at the α_1-AR subtypes

Compounds **7-10**, **20-23, 26**, **27**, **33-36**, and **38** were evaluated in binding assays on human α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR subtypes stably expressed in HEK293 cells using [^{125}I]BE2254 as radioligand [18]. Their affinity values, expressed as pK_i, are summarized in Table 1. For sake of clarity, affinity values of parent compounds RN17 and RC23 are also reported.

All final compounds displayed good to excellent affinities for α_1-ARs, indicating that the introduction of long and bulky side chains in the 8-position of the tricyclic system is tolerated by receptor binding sites. Most of them showed affinities in the order α_{1D}- ≥ α_{1A}- > α_{1B}-AR and in some cases an α_{1A}-/α_{1D}-AR selectivity over the α_{1B}-AR subtype appeared.

In the series of [1]benzothieno[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-diones, derivatives **26** and **27**, bearing a basic tertiary amine as terminal group of the side chain, were the least interesting. They displayed no improvement in affinity with respect to parent compound RN17 and even selectivity among subtypes was completely absent; compound **27** showed the same pK_i value (8.78) for the three α_1-ARs (Table 1).

On the other hand, derivatives **7-10** and **20-23**, having neutral side chains with a terminal aromatic ring, gave better results, at least in terms of discrimination among receptor subtypes.

Compounds **7-10** bear simple side chains, constituted by a phenyl ring bound directly or through an alkyl chain to the amide linker in the 8-position. These compounds displayed receptor preferences in the order α_{1D}- > α_{1A}- > α_{1B}-AR and their affinity values were dependent on the distance between the phenyl ring and the tricyclic system. The best result was obtained with the benzoylamide derivative **7**, having the shortest side chain; lower affinities were displayed by homologues **8-10**, as the phenyl ring was moved farther away (Table 1).

Compound **7** showed higher pK_i values for both α_{1D}- and α_{1A}-AR than the parent ligand RN17 (9.24 and 8.96 *vs* 8.90 and 8.48, respectively) while it maintained the same pK_i value as RN17 for the α_{1B}-AR subtype (8.00 *vs* 8.05). These binding features make **7** a better ligand than RN17 with a selectivity α_{1D}-/α_{1A}-AR over α_{1B}-AR.

More promising results were obtained with the diamide derivatives **20**-**23**. They all proved to be better ligands than RN17 for the α_{1D}-AR with affinity values in the subnanomolar range. The length of the alkyl spacer between the two amide groups influences affinity, with pentanediamide derivative **21** being a better ligand for all three receptor subtypes than its succinamide homologue **20**. Unlike monoamides **7**-**10** (where the shorter the side chain is, the better is the affinity), in diamides **20**-**23,** longer alkyl spacer resulted in ligands with higher affinity. It is possible to speculate that the binding poses of the side chains in the two families of compounds could be different and this could explain the different SARs. In compounds **20**-**23** the second distal amide group in the side chain could exert possible additional interactions that are not available to monoamides **7**-**10**.

In pentanediamide derivatives **21**-**23**, the structure of the distal amide greatly affects the strength of the receptor/ligand interaction, in particular for the α_{1D}-AR. When the *N*-phenylmethylamide moiety in **21** was replaced by a *N*-phenylethylamide in **23**, a drop in affinity was observed (pK_i = 9.85 and 9.00, respectively). However, when the secondary amide moiety in **21** was transformed into the tertiary *N*-methyl-*N*-phenylmethylamide (**22**), affinity for the α_{1D}-AR increased to really high values (pK_i = 10.25) whereas those for the α_{1A}- and α_{1B}-AR subtypes underwent a decrease (Table 1). Thus, compound **22** (RX18) emerged as the most interesting molecule in the series; it displays very high affinity for the α_{1D}-AR coupled to selectivity over the other two subtypes.

Although 2-methyl[1]benzothieno[3,2-*d*]pyrimidin-4(3*H*)-ones **33**-**36** and **38** showed good affinities for the α_1-ARs, generally they are poorer ligand for the α_{1D}-AR than the corresponding [1]benzothieno[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione analogues (compare **7** *vs* **33**, **8** *vs* **34**, **21** *vs* **35**). Only the *N*-phenylethylamide derivative **36** maintained comparable affinities to its analogue **23**. This general trend was somewhat surprising because in RC23, the parent ligand of compounds **33**-

36 and **38**, the presence of a methyl group in 2-position of the tricyclic system had enhanced affinity for α_{1D}-AR (pK_i = 9.40) with respect to RN17 (pK_i = 8.90).

Worth of note and inversely to what has been seen in the [1]benzothieno[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione series, here the *N*-phenylethylamide derivative **36** possesses the highest affinity (pK_i = 9.12) at α_{1D}-AR whereas the *N*-phenylmethylamide derivative **35** displays the lowest (pK_i = 8.26).

In order to explore if the arrangement of the distal amide moiety in **35** could affect affinity, its isomer **38**, having an inverted amide function, was synthesized. With respect to **35**, it shows increased affinity for all three α_1-ARs with and a slight preference for the α_{1A}-AR subtype (Table 1).

The observation that the 2,4(1*H*,3*H*)-dione derivatives **7**, **8**, and **27** are better ligands than the corresponding 2-methyl-4(1*H*)-one analogues **33**, **34**, and **35** indicates that the carbonyl group in the 2-position of the tricyclic scaffold is important for an optimal binding at α_{1D}-AR site and suggests its probable influence in inducing the correct disposition of the flexible side chains in the ligands of the [1]benzothieno[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione series.

3.2. Functional activity at the α_1-AR subtypes

[1]Benzothieno[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-diones **21** and **22**, which, within the series, had showed the best affinities for the α_{1D}-AR, were also tested in functional assays for their activity at α_1-AR subtypes in isolated rat prostatic vas deferens (α_{1A}-AR), spleen (α_{1B}-AR) and thoracic aorta (α_{1D}-AR) [25]. Their pK_b values, along with those of the parent compound RN17, are summarized in Table 2.

Mimicking the functional activity of RN17, both compounds behaved as antagonists; in particular they displayed a competitive antagonism. In fact, the concentration-response curves of reference

agonists after and before incubation with compounds **21** and **22** were parallel without reduction of the maximal effect and the shift produced was proportional to the concentrations used.

Both compounds show a functional activity trend similar to that seen in binding studies; in fact, they are more active at α_{1D}-AR (pK_b = 9.15 and pK_b = 8.64) and display a lower and similar potency at α_{1A} (pK_b = 7.70 and pK_b = 7.82) and α_{1B}-AR (pK_b = 7.94 and pK_b = 7.77), respectively. Moreover, compound **22** is a more potent antagonist (pK_b = 9. 15) than **21** (pK_b = 8.64) at α_{1D}-AR. Thus, diamide **22** can be regarded as the most interesting compound in the series, being a high affinity ligand and a potent competitive antagonist for the α_{1D}-AR subtype.

4. Conclusions

Structure modifications of α_{1D}-AR ligands RN17 and RC23 gave a series of novel [1]benzothieno[3,2-*d*]pyrimidine derivatives. These compounds, bearing side chains in the 8-position, were tested for their affinity at the three α_1-AR subtypes. In binding assays on human cloned receptors, some derivatives showed very high affinity and a preference for the α_{1D}-AR subtype. Among them, compound **22** (RX18) emerged and revealed very high affinity (pK_i = 10.25) for the α_{1D}-AR; when tested in a functional assay, it displayed potent and competitive antagonism coupled to selectivity for the above-cited subtype.

In general, it can be stated that introduction of diamide side chains in the 8-position of the tricyclic scaffolds of RN17 and RC23 generates very high affinity α_{1D}-AR ligands with antagonistic properties. The nature of the distal amide group in the side chain influences affinity and additional investigations of the chemical space around this moiety can be imagined to further improve selectivity among α_1-AR subtypes.

Intriguingly, orthosteric ligands such as **22** (RX18) could represent a base for the future design, through an appropriate chemical manipulation of the distal amide group, of new

orthosteric/allosteric bitopic ligands which, as seen in other class A GPCRs [26, 27], can present enhanced binding and functional properties.

5. Experimental Section

5.1. Chemistry

Melting points were determined in a IA9200 Electrothermal apparatus equipped with a digital thermometer in glass capillary tubes and are uncorrected. Infrared spectra were recorded on a Perkin Elmer 281 FTIR spectrometer in KBr disks or NaCl crystal windows. Elemental analyses for C, H, N, and S were within ± 0.4% of theoretical values and were performed on a Carlo Erba Elemental Analyzer Mod. 1108 apparatus. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Inova Unity 200 spectrometer in DMSO-d_6 solution. Chemical shifts are given in δ values (ppm), using tetramethylsilane as the internal standard; coupling constants (*J*) are given in Hz. Signal multiplicities are characterized as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad signal). Microwave reaction were performed with a CEM Discovery instrument in sealed vessels. All the synthesized compounds were tested for purity on TLC (aluminium sheet coated with silica gel 60 F_{254}, Merck) and visualized by UV (λ = 254 and 366 nm). Purification of synthesized compounds by column chromatography was performed using silica gel 60 (Merck). All chemicals and solvents were reagent grade and were purchased from commercial vendors.

*5.1.1. Ethyl 3-amino-5-nitro-1-benzothiophene-2-carboxylate (**1**)*

This compound [21] was prepared following a published procedure [28] for the homologous methyl 3-amino-5-nitro-1-benzothiophene-2-carboxylate, starting from 2-chloro-5-nitrobenzonitrile and ethyl thioglycolate in the presence of K_2CO_3 in EtOH. Recristallization from EtOH gave **1** as a pure orange solid (93%), mp 200–201 °C; ^{1}H NMR (DMSO-d_6): δ 9.23 (d, *J* = 2.2 Hz, 1H, aromatic),

8.29 (dd, *J* = 9.0 Hz, *J* = 2.2 Hz, 1H, aromatic), 8.11 (d, *J* = 9.0 Hz, 1H, aromatic), 7.45 (br s, 2H, NH_2 which exchanges with D_2O), 4.29 (q, *J* = 7.0 Hz, 2H, $COOCH_2CH_3$), 1.31 (t, *J* = 7.0 Hz, 3H, $COOCH_2CH_3$). Anal. ($C_{11}H_{10}N_2O_4S$) C, H, N, S.

*5.1.2. Ethyl 3-[(ethoxycarbonyl)amino]-5-nitro-1-benzothiophene-2-carboxylate (**2**)*

Amino ester **1** (7.00 g, 26.29 mmol) was dissolved in hot toluene (150 mL). Solution was cooled to room temperature, ethyl chlorocarbonate (4.28 g, 39.44 mmol) was added and the mixture refluxed under stirring. After 12 h, ethyl chlorocarbonate (4.28 g, 39.44 mmol) was added again and the mixture refluxed for further 14 h. After being cooled, reaction mixture was poured into petroleum ether 40-60 °C (200 mL) and the precipitate was filtered off and dried to give crude **2** (7.66 g, 86 %). A small portion was recrystallized from ethanol affording **2** as a pure product: mp 144–145 °C; ^{1}H NMR (DMSO-d_6): δ 10.00 (s, 1H, NH which exchanges with D_2O), 8.81–8.79 (m, 1H, aromatic), 8.33–8.29 (m, 2H, aromatic), 4.32 (q, *J* = 7.2 Hz, 2H, $COOCH_2CH_3$), 4.16 (q, *J* = 7.2 Hz, 2H, $NHCOOCH_2CH_3$), 1.31 (t, *J* = 7.2 Hz, 3H, $COOCH_2CH_3$), 1.26 (t, *J* = 7.2 Hz, 3H, $NHCOOCH_2CH_3$). Anal. ($C_{14}H_{14}N_2O_6S$) C, H, N, S.

*5.1.3. 3-(2-Hydroxyethyl)-8-nitro[1]benzothieno[3,2-d]pyrimidine-2,4(1H,3H)-dione (**3**)*

Compound **2** (5.00 g, 14.78 mmol) was dissolved in 2-aminoethanol (100 mL) and the obtained solution was heated at 50 °C for 40 min. The reaction mixture was then poured into ice-water and acidified to pH 5 with HCl 0.5 M. The obtained precipitate was filtered off, thoroughly washed with water and dried. Recrystallization from ethanol gave **3** (3.95 g, 87 %) as a pure product: mp 290–291 °C; ^{1}H NMR (DMSO-d_6): δ 12.71 (s, 1H, NH which exchanges with D_2O), 9.46–9.38 (m, 1H, aromatic), 8.40–8.35 (m, 2H, aromatic), 4.80 (t, *J* = 5.8 Hz, 1H, OH), 4.05 (t, *J* = 6.4 Hz, 2H, CH_2CH_2OH), 2.56–2.46 (m, 2H, CH_2CH_2OH). Anal. ($C_{12}H_9N_3O_5S$) C, H, N, S.

*5.1.4. 3-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-nitro[1]benzothieno[3,2-d]pyrimidine-2,4(1H,3H)-dione (**5**)*

Alcohol **3** (0.50 g, 1.63 mmol) was dissolved in hot toluene (13 mL). The solution was allowed to cool to room temperature and thionyl chloride (0.58 g, 4.88 mmol) was added. The reaction mixture was refluxed under stirring for 12 h; then, a further amount of thionyl chloride (0.10 g, 0.84 mmol) was added and the mixture refluxed for further 10 h. After being cooled, liquids were eliminated in vacuo and solids were suspended in cyclohexane, then filtered off and dried. Obtained crude **4** (0.53 g, quantitative yield) was successively used without further purification.

A mixture of **4** (0.50 g, 1.53 mmol) and 1-(2-methoxyphenyl)piperazine (1.47 g, 7.65 mmol) was heated in an oil bath at 160 °C for 30 min. After being cooled, ethanol (10 mL) was added to the reaction mixture. Solids were filtered off, washed with cold ethanol and dried. The crude product was triturated in ethanol and then filtered off an dried to give **5** (0.62 g, 67 %) as a pure product: mp 249-251 °C; IR (KBr) cm^{-1} 3446, 3086, 2830, 1710, 1651, 1501, 1339, 1018; ^{1}H NMR (DMSO-d_6): δ 9.44–9.36 (m, 1H, aromatic), 8.45–8.30 (m, 2H, aromatic), 6.98–6.78 (m, 4H, aromatic), 4.70 (t, *J* = 6.6 Hz, 2H, CONCH_2CH$_2$), 3.77 (s, 3H, OCH$_3$), 3.05-2.88 (m, 4H, piperazine), 2.72–2.54 (m, 4H + 2H, piperazine + CONCH$_2$CH_2). Anal. ($C_{23}H_{23}N_5O_5S$) C, H, N, S.

*5.1.5. 8-Amino-3-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl][1]benzothieno[3,2-d]pyrimidine-2,4(1H,3H)-dione (**6**)*

A mixture of **5** (0.50 g, 1.04 mmol), hydrazine hydrate (0.26 g, 5.19 mmol), and Raney Ni (0.26 g) in DMF (12 mL) was stirred at room temperature for 24 h. Solids were eliminated by filtration and water (40 mL) was added to the filtrate. Obtained precipitate was filtered off, washed with water and dried. Recrystallization from ethanol gave **6** (0.35 g, 75 %) as a pure product: mp 249–251 °C; IR (KBr) cm^{-1} 3328, 2930, 1711, 1650, 1440, 1241, 745, 687; ^{1}H NMR (DMSO-d_6): δ 12.33 (s, 1H, NH which exchange with D_2O), 7.76–7.60 (m, 1H, aromatic), 7.50–7.38 (m, 1H, aromatic), 7.04–6.80 (m, 4H + 1H, aromatic), 5.32 (s, 2H, NH_2 which exchange with D_2O), 4.06 (t, *J* = 6.0 Hz, 2H,

$CONCH_2$), 3.76 (s, 3H, OCH_3), 2.98–2.88 (m, 4H, piperazine), 2.66-2.52 (m, 4H + 2H, piperazine + $CONCH_2CH_2$). Anal. ($C_{23}H_{25}N_5O_3S$) C, H, N, S.

*5.1.6. N-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2,4-dioxo-1,2,3,4-tetrahydro[1]benzothieno [3,2-d]pyrimidin-8-yl]benzamide (**7**)*

A mixture of compound **6** (0.20 g, 0.44 mmol), anhydrous potassium carbonate (0.31 g, 2.24 mmol) and benzoyl chloride (0.074 g, 0.53 mmol) in THF (10 mL) was stirred for 5 h at room temperature. An additional amount of benzoyl chloride (0.03 g, 0.21 mmol) was added and the mixture was stirred for further 24 h. Volatiles were eliminated under reduced pressure and the solid residue was suspended in water, then filtered off and dried. Recrystallization from DMF/water gave **7** (0.15 g, 61 %) as a pure compound: mp 285–287 °C; IR (KBr) cm^{-1} 3287, 3003, 1710, 1636, 1554, 1241, 749, 681; ^{1}H NMR (DMSO-d_6): δ 12.71 (br s, 1H, NH which exchanges with D_2O), 10.55 (s, 1H C_6H_5CONH which exchanges with D_2O), 9.02–8.98 (m, 1H, aromatic), 8.16–7.98 (m, 1H + 2H, aromatic), 7.78–7.52 (m, 1H, aromatic), 7.44–7.28 (m, 3H, aromatic), 6.98–6.80 (m, 4H, aromatic), 4.08 (t, J = 6.0 Hz, 2H, $CONCH_2$), 4.08 (s, 3H, OCH_3), 2.98–2.80 (m, 4H, piperazine), 2.68–2.45 (m, 4H + 2H, piperazine + $CONCH_2CH_2$). ^{13}C NMR (DMSO-d_6): δ 165.57, 159.00, 151.94, 151.50, 141.21, 140.07, 136.57, 135.45, 134.65, 131.76, 128.80, 128.49, 127.72, 124.05, 123.64, 122.32, 120.82, 117.87, 115.27, 111.84, 110.29, 55.28, 55.09, 53.15, 50.06, 37.68. Anal. ($C_{30}H_{29}N_5O_4S$) C, H, N, S.

*5.1.7. N-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2,4-dioxo-1,2,3,4-tetrahydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-2-phenylacetamide (**8**)*

A mixture of compound **6** (0.10 g, 0.22 mmol), anhydrous potassium carbonate (0.15 g, 1.08 mmol) and phenylacetyl chloride (0.04 g, 0.26 mmol) in THF (5 mL) was stirred for 4 h at room temperature. Volatiles were eliminated under reduced pressure, the solid residue was suspended in water, then filtered off and dried to give **8** (0.10 g, 79 %) as a pure compound: mp 227–229 °C. IR

(KBr) cm^{-1} 2940, 1703, 1646, 1446, 1241, 1025, 756, 690. ^{1}H NMR (DMSO-d_6): δ 12.65 (br s, 1H, NH which exchanges with D_2O), 10.44 (s, 1H, CH_2CONH which exchanges with D_2O), 8.84–8.75 (m, 1H, aromatic), 8.08–7.98 (m, 1H, aromatic), 7.65–7.58 (m, 1H, aromatic), 7.42-7.22 (m, 5H, aromatic), 6.98–6.78 (m, 4H, aromatic), 4.06 (t, J = 7.8 Hz, 2H, $CONCH_2$), 3.76 (s, 3H, OCH_3), 3.70 (s, 2H, $C_6H_5CH_2$), 3.01–2.88 (m, 4H, piperazine), 2.68–2.55 (m, 4H + 2H, piperazine + $CONCH_2CH_2$). ^{13}C NMR (DMSO-d_6): δ 169.54, 159.27, 152.13, 151.68, 141.32, 140.13, 136.75, 136.04, 135.24, 129.37, 128.99, 128.58, 126.87, 124.44, 122.65, 121.05, 118.11, 114.14, 112.02, 110.57, 55.48, 55.21, 53.27, 50.16, 43.35, 38.24. Anal. ($C_{31}H_{31}N_5O_4S$) C, H, N, S.

*5.1.8. N-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2,4-dioxo-1,2,3,4-tetrahydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-4-phenylbutanamide (**9**)*

A mixture of compound **6** (0.23 g, 0.51 mmol), anhydrous potassium carbonate (0.35 g, 2.53 mmol) and 4-phenylbutyryl chloride (0.10 g, 0.55 mmol) in THF (15 mL) was stirred for 6 h at room temperature. Volatiles were eliminated in vacuo and the solid residue was suspended in water, then filtered off and dried. Recrystallization from DMF/water gave **9** (0.18 g, 59 %) as a pure compound: mp 226–228 °C. IR (KBr) cm^{-1} 3529, 3025, 1774, 1710, 1240, 1179, 743, 684; ^{1}H NMR (DMSO-d_6): δ 12.68 (br s, 1H, NH which exchanges with D_2O), 10.16 (s, 1H, CONH which exchanges with D_2O), 8.82–8.74 (m, 1H, aromatic), 8.01–7.97 (m, 1H, aromatic), 7.62–7.57 (m, 1H, aromatic), 7.38–7.18 (m, 5H, aromatic), 6.98–6.78 (m, 4H, aromatic), 4.08 (t, J = 6.0 Hz, 2H, $CONCH_2$), 3.76 (s, 3H, OCH_3), 3.15–2.80 (m, 4H, piperazine), 2.63–2.53 (m, 4H + 2H + 2H, piperazine + $C_6H_5CH_2$ + $CONCH_2CH_2$), 2.40 (t, J = 7.2 Hz, 2H, CH_2CONH), 2.02–1.89 (m, 2H, $C_6H_5CH_2CH_2$). Anal. ($C_{33}H_{35}N_5O_4S$) C, H, N, S.

*5.1.9. N-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2,4-dioxo-1,2,3,4-tetrahydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-5-phenylpentanamide (**10**).*

A mixture of compound **6** (0.23 g, 0.51 mmol), anhydrous potassium carbonate (0.35 g, 2.53 mmol) and 5-phenylpentanoyl chloride (0.15 g, 0.76 mmol) in THF (15 mL) was stirred for 20 h at room temperature. Volatiles were eliminated in vacuo and the solid residue was suspended in a solution (25 mL) of potassium carbonate (5% w/v), then filtered off, washed with water and dried to give **10** (0.28 g, 90 %) as a pure compound: mp 214–216 °C. IR (KBr) cm^{-1} 3487, 2930, 1711, 1637, 1240, 1177, 741, 684; ^{1}H NMR (DMSO-d_6): δ 12.68 (br s, 1H, NH which exchanges with D_2O), 10.16 (s, 1H, CONH which exchanges with D_2O), 8.84–8.76 (m, 1H, aromatic), 8.02–7.78 (m, 1H, aromatic), 7.63–7.57 (m, 1H, aromatic), 7.38–7.15 (m, 5H, aromatic), 6.98–6.80 (m, 4H, aromatic), 4.08 (t, J = 6.0 Hz, 2H, $CONCH_2$), 3.76 (s, 3H, OCH_3), 3.02-2.80 (m, 4H, piperazine), 2.70-2.56 (m, 4H + 2H + 2H, piperazine + $C_6H_5CH_2$ + $CONCH_2CH_2$), 2.40 (t, J = 7.2 Hz, 2H, CH_2CONH), 1.70–1.57 (m, 2H + 2H, $CH_2CH_2CH_2CONH$). ^{13}C NMR (DMSO-d_6): δ 171.33, 159.11, 151.97, 151.56, 142.11, 140.99, 140.04, 136.80, 134.77, 128.85, 128.35, 128.33, 125.74, 124.15, 122.54, 122.41, 120,88, 117.98, 113.74, 111.90, 110.33, 55.33, 54.95, 49.72, 37.42, 36.11, 35.00, 30.70, 24.81. Anal. ($C_{34}H_{37}N_5O_4S$) C, H, N, S.

*5.1.10. 4-Oxo-4-[(phenylmethyl)amino]butanoic acid (**16**) [29].*

A solution of benzylamine (0.20 g, 1.87 mmol) in anhydrous toluene (6 mL) was added to a solution of succinic anhydride (0.19 g, 1.87 mmol) in anhydrous toluene (14 mL) at 0 °C and the mixture was stirred at the same temperature for 1 h. Then, solids were filtered off washed with cold toluene and dried. Obtained crude **16** (0.37 g, 94 %) was used successively without further purification: mp 127–130 °C. IR (KBr) cm^{-1} 3299, 2918, 1693, 1640, 1546, 1231**,** 734, 660; ^{1}H NMR (DMSO-d_6): δ 8.37 (t, J = 6.0 Hz, 1H, NH which exchanges with D_2O), 7.42–7.18 (m, 5H, aromatic), 4.26 (d, J = 6.0 Hz, 2H, $C_6H_5CH_2$), 2.44–2.38 (m, 2H + 2H, CH_2CH_2COOH).

*5.1.11. 5-Oxo-5-[(phenylmethyl)amino]pentanoic acid (**17**) [29].*

A solution of benzylamine (0.50 g, 4.67 mmol) in anhydrous toluene (15 mL) was added to a solution of glutaric anhydride (0.53 g, 4.67 mmol) in anhydrous toluene (40 mL) at 0 °C and the mixture was stirred at the same temperature for 30 min. Then, solids were filtered off, washed with cold toluene and dried. Obtained crude **17** (0.80 g, 78 %) was used successively without further purification: mp 84–85 °C. IR (KBr) cm^{-1} 3295, 3030, 1699, 1635, 1548, 1241, 746, 694; ^{1}H NMR (DMSO-d_6): δ 8.34 (t, J = 6.0 Hz, 1H, NH which exchanges with D_2O), 7.38–7.18 (m, 5H, aromatic), 4.25 (d, J = 6.0 Hz, 2H, $C_6H_5CH_2$), 2.22–2.08 (m, 2H + 2H, $CH_2CH_2CH_2COOH$), 1.80–1.62 (m, 2H, CH_2CH_2COOH).

*5.1.12. 5-[Methyl(phenylmethyl)amino]-5-oxopentanoic acid (**18**)*

A solution of *N*-benzylmethylamine (0.50 g, 4.12 mmol) in anhydrous toluene (5 mL) was added to a solution of glutaric anhydride (0.47 g, 4.12 mmol) in anhydrous toluene (40 mL) and the mixture was stirred at 40 °C for 42 h. Then, volatiles were eliminated under reduced pressure and the oily residue was dissolved in NaOH 1 M (9 mL). The alkaline phase was extracted with ethyl ether (2 × 20 mL) and the organic phase discharged. Aqueous phase was then acidified with HCl 1 M (14 mL) and extracted with ethyl ether (3 × 20 mL). Combined organic phases were dried on anhydrous sodium sulfate and evaporated at reduced pressure to give an oily residue. Obtained crude **18** (0.54 g, 53 %) was used successively without further purification. IR (KBr) cm^{-1} 2939, 1726, 1608, 1450, 1229, 1409, 734, 699; ^{1}H NMR (DMSO-d_6): δ 12.03 (s, 1H, COOH which exchanges with D_2O), 7.42–7.13 (m, 5H, aromatic), 4.55 (s, 2H, $C_6H_5CH_2$, conformer *E* (or *Z*)), 4.49 (s, 2H, $C_6H_5CH_2$, conformer *Z* (or *E*)), 2.88 (s, 3H, CH_3, conformer *Z* (or *E*)), 2.79 (s, 3H, CH_3, conformer *E* (or *Z*)), 2.45–2.18 (m, 2H + 2H, $COCH_2CH_2CH_2COOH$), 1.82–1.64 (m, 2H, CH_2CH_2COOH).

*5.1.13. 5-Oxo-5-[(2-phenylethyl)amino]pentanoic acid (**19**) [30]*

A solution of 2-phenylethylamine (0.50 g, 4.12 mmol) in anhydrous toluene (4 mL) was added to a solution of glutaric anhydride (0.47 g, 4.12 mmol) in anhydrous toluene (20 mL) and the mixture

was stirred under reflux for 3 h. Then, solids were filtered off, washed with cold toluene and dried to obtain **19** (0.32 g, 33 %): mp 87–90 °C. IR (KBr) cm^{-1} 3297, 2935, 1697, 1638, 1546, 863, 748, 698; ^{1}H NMR (DMSO-d_6): δ 7.91 (br t, J = 5.4 Hz, 1H, NH which exchanges D_2O), 7.35–7.16 (m, 5H, aromatic), 3.25–3.17 (m, 2H, CH_2NH), 2.69 (t, J = 7.4 Hz, 2H, $C_6H_5CH_2$), 2.20–2.00 (m, 2H + 2H, $CH_2CH_2CH_2$), 1.78–1.58 (m, 2H, $CH_2CH_2CH_2$). Anal. ($C_{13}H_{17}NO_3$) C, H, N.

*5.1.14. N^1-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2,4-dioxo-1,2,3,4-tetrahydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-N^4-(phenylmethyl)succinamide (**20**)*

Triethylamine (0.073 g, 0.72 mmol) was added to a solution of the acid **16** (0.15 g, 0.72 mmol) in THF (4 mL) and the mixture was placed under stirring in an ice bath. After five minutes ethyl chlorocarbonate (0.078 g, 0.72 mmol) was added. After further fifteen minutes, a solution of **6** (0.325 g, 0.72 mmol) in THF (21 mL) was added and then the mixture was left under stirring at room temperature for 24 h. Volatiles were eliminated under reduced pressure and the obtained residue was purified by flash chromatography on silica gel 60 using a mixture of chloroform/methanol (9/1, v/v) as eluent. Homogeneous fractions were combined and evaporated under reduced pressure to give **20** as a pure compound (0.033 g, 7 %): mp 251–253 °C. IR (KBr) cm^{-1} 3321, 2938, 1698, 1640, 1241, 1024, 747, 688; ^{1}H NMR (DMSO-d_6): δ 12.70 (br s, 1H, NH which exchanges with D_2O), 10.24 (s, 1H, NH which exchanges with D_2O), 8.76 (s, 1H, aromatic), 8.44 (t, J = 5.8 Hz, 1H, $C_6H_5CH_2NHCO$ which exchanges with D_2O), 8.08–7.98 (d, J = 9.2 Hz, 1H, aromatic), 7.66–7.58 (m, 1H, aromatic), 7.38–7.18 (m, 5H, aromatic), 7.00–6.82 (m, 4H, aromatic), 4.29 (d, J = 5.6 Hz, 2H, $C_6H_5CH_2NHCO$), 4.07 (t, J = 6.6 Hz, 2H, $CONCH_2$), 3.76 (s, 3H, OCH_3), 2.98–2.88 (m, 4H, piperazine), 2.72–2.58 (m, 4H + 2H, piperazine + $CONCH_2CH_2$). ^{13}C NMR (DMSO-d_6): δ 171.28, 170.61, 159.03, 151.97, 151.51, 141.24, 140.02, 139.60, 136.87, 134.64, 128.82, 128.27, 127.19, 126.73, 124.12, 122.35, 122.19, 120.86, 117.89, 113.42, 111.89, 110.27, 55.29, 55.11, 53.15, 50.07, 42.08, 37.66, 31.54, 30.20. Anal. ($C_{34}H_{36}N_6O_5S$) C, H, N, S.

*5.1.15. N^1-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2,4-dioxo-1,2,3,4-tetrahydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-N^5-(phenylmethyl)pentanediamide (**21**)*

A solution of the acid **17** (0.20 g, 0.90 mmol) in THF (15 mL) was prepared by gentle heating. Then, it was placed under stirring in an ice bath and triethylamine (0.091 g, 0.90 mmol) was added. After five minutes ethyl chlorocarbonate (0.098 g, 0.90 mmol) was added and the mixture was stirred for 3 h at 0 – 5 °C. The mixture was allowed to reach room temperature and a solution of **6** (0.33 g, 0.73 mmol) in THF (22 mL) was added; the mixture was left under stirring at room temperature for 40 h. Volatiles were eliminated under reduced pressure and the obtained residue was purified by flash chromatography on silica gel 60 using a mixture of chloroform/methanol (9/1, v/v) as eluent. Homogeneous fractions were combined and evaporated under reduced pressure to give **21** as a pure compound (0.14 g, 29 %): mp 232–235 °C. IR (KBr) cm^{-1} 3261, 2934, 1709, 1650, 1238, 1024, 753, 685; ^{1}H NMR (DMSO-d_6): δ 12.69 (br s, 1H, NH which exchanges with D_2O), 10.21 (s, 1H, CONH which exchanges with D_2O), 8.82–8.76 (m, 1H, aromatic), 8.41 (t, *J* = 6.0 Hz, 1H, $C_6H_5CH_2N\mathit{H}$), 8.08–7.98 (m, 1H, aromatic), 7.65–7.56 (m, 1H, aromatic), 7.38–7.18 (m, 5H, aromatic), 6.98–6.80 (m, 4H, aromatic), 4.30 (d, *J* = 5.6 Hz, 2H, $C_6H_5C\mathit{H}_2$), 4.08 (t, *J* = 6.0 Hz, 2H, $CONCH_2$), 3.76 (s, 3H, OCH_3), 3.05–2.90 (m, 4H, piperazine), 2.16–2.06 (m, 4H + 2H, piperazine + $CONCH_2C\mathit{H}_2$), 2.45 (t, *J* = 7.4 Hz, 2H, $C\mathit{H}_2CONH$), 2.25 (t, *J* = 7.4 Hz, 2H, $NHCOC\mathit{H}_2$), 1.98–1.72 (m, 2H, $CH_2C\mathit{H}_2CH_2$). ^{13}C NMR (DMSO-d_6): δ 171.79, 171.02, 159.05, 151.97, 151.52, 141.23, 140.03, 139.68, 136.81, 134.75, 128.87, 128.33, 127.22, 126.76, 124.12, 122.38 (2C), 120.87, 117.91, 113.69, 111.88, 110.30, 55.31, 55.11, 53.18, 50.09, 42.05, 37.71, 35.63, 34.64, 21.28. Anal. ($C_{35}H_{38}N_6O_5S$) C, H, N, S.

*5.1.16. N^1-Methyl-N^5-[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl]-2,4-dioxo-1,2,3,4-tetrahydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-N^1-(phenylmethyl)pentanediamide (**22**)*

Triethylamine (0.11 g, 1.10 mmol) was added to a solution of the acid **18** (0.26 g, 1.10 mmol) in THF (10 mL) and the mixture was placed under stirring in an ice bath. After five minutes ethyl chlorocarbonate (0.12 g, 1.10 mmol) was added. After 1 h, a solution of **6** (0.496 g, 1.10 mmol) in THF (33 mL) was added and then the mixture was left under stirring at room temperature for 30 h. Volatiles were eliminated under reduced pressure and the obtained residue was purified by flash chromatography on silica gel 60 using a mixture of chloroform/methanol (9/1, v/v) as eluent. Homogeneous fractions were combined and evaporated under reduced pressure to give **22** as a pure compound (0.16 g, 22 %): mp 207–208 °C. IR (KBr) cm^{-1} 3487, 3301, 2933, 1708, 1649, 1238, 743, 681; ^{1}H NMR (DMSO-d_6): δ 12.68 (br s, 1H, NH which exchanges with D_2O), 10.19 (s, 1H, CONH which exchanges with D_2O, conformer *Z* (or *E*)), 10.15 (s, 1H, CONH which exchanges with D_2O, conformer *E* (or *Z*)), 8.79 (s, 1H, aromatic, conformer *Z* (or *E*)), 8.76 (s, 1H, aromatic, conformer *E* (or *Z*)), 8.08-7.92 (m, 1H, aromatic), 7.68-7.56 (m, 1H, aromatic), 7.42-7.12 (m, 5H, aromatic), 7.01-6.80 (m, 4H, aromatic), 4.58 (s, 2H, $C_6H_5CH_2$, conformer *E* (or *Z*)), 4.52 (s, 2H, $C_6H_5CH_2$, conformer *Z* (or *E*)), 4.08 (t, *J* = 6.0 Hz, 2H, $CONCH_2$), 3.76 (s, 3H, OCH_3), 3.04–2.88 (m, 4H + 3H, piperazine + CH_3, conformer *Z* (or *E*)), 2.81 (s, 3H, CH_3, conformer *E* (or *Z*)), 2.70–2.55 (m, 4H + 2H + 2H, piperazine + $CONCH_2CH_2$ + CH_2CONH), 2.46–2.40 (m, 2H, $NHCOCH_2CH_2CH_2$), 1.90 (t, *J* = 8.8 Hz, $CH_2CH_2CH_2$). ^{13}C NMR (DMSO-d_6): (conformer *E* (or *Z*)) δ 172.04, 171.24, 159.09, 152.00, 151.57, 141.26, 140.05, 137.98, 136.82, 134.81, 128.90, 128.56, 127.50, 127.07, 124.16, 122.43 (2C), 120.91, 117.94, 113.78, 111.91, 110.36, 55.34, 55.15, 53.21, 50.12, 49.95, 37.74, 35.55, 34.76, 31.90, 20.69; (conformer *Z* (or *E*)) δ 172.04, 171.19, 159.09, 152.00, 151.57, 141.26, 140.05, 137.53, 136.82, 134.81, 128.90, 128.81, 127.28, 126.52, 124.16, 122.43 (2C), 120.91, 117.94, 113.78, 111.91, 110.36, 55.34, 55.15, 53.21, 52.40, 50.12, 37.74, 35.55, 33.50, 31.59, 20.89. Anal. ($C_{36}H_{40}N_6O_5S$) C, H, N, S.

*5.1.17. N^1-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2,4-dioxo-1,2,3,4-tetrahydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-N^5-(2-phenylethyl)pentanediamide (**23**)*

Triethylamine (0.086 g, 0.85 mmol) was added to a solution of the acid **19** (0.20 g, 0.85 mmol) in THF (15 mL) and the mixture was placed under stirring in an ice bath. After five minutes ethyl chlorocarbonate (0.092 g, 0.85 mmol) was added. After 2 h, a solution of **6** (0.38 g, 0.85 mmol) in THF (25 mL) was added and then the mixture was left under stirring at room temperature for 28 h. Volatiles were eliminated under reduced pressure and the obtained residue was purified by flash chromatography on silica gel 60 using a mixture of chloroform/methanol (9/1, v/v) as eluent. Homogeneous fractions were combined and evaporated under reduced pressure to give **23** as a pure compound (0.11 g, 20 %): mp 227–230 °C. IR (KBr) cm^{-1} 3298, 2934, 1708, 1649, 1239, 1023, 751, 699; ^{1}H NMR (DMSO-d_6): δ 12.69 (br s, 1H, NH exchanges with D_2O), 10.16 (s, 1H, NH which exchanges with D_2O), 8.78 (s, 1H, aromatic), 8.08–7.90 (m, 1H + 1H, aromatic + CH_2NHCO which exchanges with D_2O), 7.61 (d, J = 8.4 Hz, 1H, aromatic), 7.38–7.16 (m, 5H, aromatic), 6.98-6.80 (m, 4H, aromatic), 4.07 (t, J = 6.0 Hz, 2H, $CONCH_2$), 3.76 (s, 3H, OCH_3), 3.32-3.21 (m, 2H, CH_2NHCO), 3.00–2.88 (m, 4H, piperazine), 2.78–2.48 (m, 4H + 2H + 2H, piperazine + $CONCH_2CH_2$ + $C_6H_5CH_2$), 2.36 (t, J = 7.6 Hz, 2H, $CH_2CH_2CH_2$), 2.14 (t, J = 7.6 Hz, 2H, $CH_2CH_2CH_2$), 1.92–1.74 (m, 2H, $CH_2CH_2CH_2$). ^{13}C NMR (DMSO-d_6): δ 171.58, 170.95, 158.99, 151.93, 151.47, 141.20, 139.99, 139.51, 136.47, 134.69, 128.84, 128.61, 128.29, 126.05, 124.07, 122.31, 120.81, 117.85, 113.62, 111.84, 110.25, 55.27, 55.09, 53.14, 50.05, 40.14, 37.66, 35.56, 35.24, 34.64, 21.19. Anal. ($C_{36}H_{40}N_6O_5S$) C, H, N, S.

*5.1.18. N-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2,4-dioxo-1,2,3,4-tetrahydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-4-[methyl(phenylmethyl)amino]butanamide (**26**)*

1,1'-Carbonyldiimidazole (0.09 g, 0.55 mmol) was added to a solution of 4-[methyl(phenylmethyl)amino]butanoic acid hydrochloride **24** (0.07 g, 0.29 mmol) in anhydrous

THF (8 mL) and the mixture was stirred under reflux. After 1 h, compound **6** (0.10 g, 0.22 mmol) was added and the reaction mixture was stirred under reflux for 28 h. Then, the solvent was evaporated under reduced pressure and sodium carbonate 5 % (15 mL) was added to the residue. The mixture was extracted with $CHCl_3$ (3 × 15 mL), collected organic phase was dried on anhydrous sodium sulfate and solvent eliminated under reduced pressure. Obtained residue was purified by flash chromatography on silica gel 60 using a mixture of chloroform/methanol (8/2, v/v) as eluent. Homogeneous fractions were combined and evaporated under reduced pressure to give **26** as a pure compound (0.02 g, 14 %): mp 148–151 °C dec. ^{1}H NMR ($CDCl_3$): δ 10.20 (br s, 1H, NH exchanges with D_2O), 8.70 (s, 1H, aromatic), 7.61 (d, *J* = 8.6 Hz, 1H, aromatic), 7.40–7.22 (m, 5H, aromatic), 7.14 (d, *J* = 8.6 Hz, 1H, aromatic), 7.04–6.78 (m, 4H, aromatic), 4.35 (t, *J* = 7.4 Hz, 2H, $CONCH_2$), 3.81 (s, 3H, OCH_3), 3.58 (s, 2H, $C_6H_5CH_2$), 3.10–2.94 (m, 4H, piperazine), 2.86–2.68 (m, 4H + 2H, piperazine + $CONCH_2CH_2$), 2.66–2.50 (m, 2H + 2H, $CH_2CH_2CH_2$), 2.27 (s, 3H, NCH_3), 2.05–1.88 (m, 2H, $CH_2CH_2CH_2$). ^{13}C NMR (DMSO-d_6): δ 172.47, 159.34, 152.62,152.14, 141.24, 139.17, 137.41, 136.34, 136.18, 129.44, 128.83, 128.52, 127.57, 123.71, 122.76, 121.88, 120.89, 118.08, 113.23, 111.06, 62.56, 57.34, 55.59, 55.26, 53.46, 50.52, 41.62, 38.24, 36.86, 22.53. Anal. ($C_{35}H_{40}N_6O_4S$) C, H, N, S.

*5.1.19. 4-(Dimethylamino)-N-[3-[2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl]-2,4-dioxo-1,2,3,4-tetrahydro[1]benzothieno[3,2-d]pyrimidin-8-yl]butanamide (**27**)*

1,1'-Carbonyldiimidazole (0.26 g, 1.60 mmol) was added to a solution of 4-(dimethylamino) butanoic acid hydrochloride **25** (0.14 g, 0.83 mmol) in anhydrous 1,4-dioxane (23 mL) and the mixture was stirred under reflux. After 1 h, compound **6** (0.30 g, 0.66 mmol) was added and the reaction mixture was stirred under reflux for 4 h. Then, the solvent was evaporated under reduced pressure and sodium carbonate 5 % solution (45 mL) was added to the residue. The mixture was extracted with $CHCl_3$ (3 × 20 mL), collected organic phase was dried on anhydrous sodium sulfate and solvent eliminated under reduced pressure. Obtained residue was purified by flash

chromatography on silica gel 60 using a mixture of methanol/NH_4OH 30 % (9.9/0.01, v/v) as eluent. Homogeneous fractions were combined and evaporated under reduced pressure to give **27** as a pure compound (0.16 g, 43 %): mp 187–189 °C dec. IR (KBr) cm^{-1} 3241, 2942, 1707, 1647, 1501, 1242, 754, 684; ^1H NMR ($CDCl_3$): δ 10.84 (br s, 1H, NH which exchanges with D_2O), 8.82 (d, *J* = 1.6 Hz, 1H, aromatic), 7.69 (m, *J* = 8.8 Hz, 1H, aromatic), 7.22 (dd, *J* = 8.8 Hz, *J* = 1.6 Hz, 1H, aromatic), 7.00–6.78 (m, 4H, aromatic), 4.32 (t, *J* = 6.6 Hz, 2H, $CONCH_2$), 3.76 (s, 3H, OCH_3), 3.06-2.94 (m, 4H, piperazine), 2.82–2.68 (m, 4H + 2H, piperazine + $CONCH_2CH_2$), 2.57 (t, *J* = 6.6 Hz, 2H, CH_2CONH), 2.45 (t, *J* = 5.8 Hz, 2H, $CH_2CH_2CH_2CONH$), 2.30 (s, 3H + 3H, CH_3 + CH_3), 1.96–1.78 (m, 2H, CH_2CH_2CO). Anal. ($C_{29}H_{36}N_6O_4S$) C, H, N, S.

*5.1.20. Ethyl 3-[(1-ethoxyethylidene)amino]-5-nitro-1-benzothiophene-2-carboxylate (**28**)*

Amino ester **1** (1.00 g, 3.75 mmol) and 1,1,1-triethoxyethane (6.98 g, 43.02 mmol) were inserted in. a 10 mL vessel. The vessel was sealed with the appropriate stopper septum and irradiated under stirring in a CEM Discovery microwave instrument set at power = 60 W and temperature = 200 °C, for 60 min. After being cooled, the reaction mixture was transferred in a flask, cyclohexane (20 mL) was added and the obtained suspension was gentle heated under stirring for 30 min. The warm mixture was filtered and the filtrate collected. Volatiles were eliminated under reduced pressure and the obtained residue was purified by flash chromatography on silica gel 60 using a mixture of cyclohexane/ethyl acetate (9/1, v/v) as eluent. Homogeneous fractions were combined and evaporated under reduced pressure to give **28** as a pure compound (1.09 g, 86 %): mp 113 °C. IR (KBr) cm^{-1} 2983, 2931, 1711, 1513, 1441, 1257, 1051, 836; ^1H NMR (DMSO-d_6): δ 8.41–8.22 (m, 3H, aromatic), 4.38 (q, *J* = 7.2 Hz, 2H, CH_2CH_3), 4.28 (q, *J* = 7.2 Hz, 2H, CH_2CH_3), 1.82 (s, 3H, CH_3), 1.38 (t, *J* = 7.2 Hz, 3H, CH_2CH_3), 1.28 (t, *J* = 7.2, 3H, CH_2CH_3). Anal. ($C_{15}H_{16}N_2O_5S$) C, H, N, S.

*5.1.21. 3-(2-Hydroxyethyl)-2-methyl-8-nitro[1]benzothieno[3,2-d]pyrimidin-4(3H)-one (**29**)*

A mixture of **28** (0.75 g, 2.23 mmol) and 2-aminoethanol (3.00 g, 49.1 mmol) was heated at 50 °C for 2 h. Then, water was added and the precipitate was filtered off, washed with water and dried. Obtained crude **29** (0.52 g, 76 %) was used in the successive synthetic step without any further purification. An analytical sample was recrystallized from ethanol: mp 235 °C. IR (KBr) cm^{-1} 3382, 1650, 1550, 1461, 1334, 1181, 1049, 830; ^{1}H NMR (DMSO-d_6): δ 8.86–8.84 (m, 1H, aromatic), 8.45–8.40 (m, 2H, aromatic), 5.09 (t, *J* = 5.8 Hz, 1H, OH which exchanges with D_2O), 4.23 (t, *J* = 5.2 Hz, 2H, C*H*$_2$CH$_2$OH), 3.80–3.68 (m, 2H, CH$_2$C*H*$_2$OH), 2.81 (s, 3H, CH_3). Anal. ($C_{13}H_{11}N_3O_4S$) C, H, N, S.

*5.1.22. 3-(2-Chloroethyl)-2-methyl-8-nitro[1]benzothieno[3,2-d]pyrimidin-4(3H)-one hydrochloride (**30**)*

Thionyl chloride (0.53 g, 4.45 mmol) was added to a solution of **29** (0.45 g, 1.47 mmol) in toluene (12 mL) and the mixture was refluxed under stirring for 3 h. After being cooled, volatiles were eliminated under reduced pressure and cyclohexane (20 mL) was added to the residue. The obtained suspension was filtered under vacuum and the solid residue was washed with cyclohexane and dried. Obtained compound **30** (0.49 g, 92 %) was used successively without further purification: mp 210–212 °C. IR (KBr) cm^{-1} 3068, 1785, 1629, 1564, 1381, 1133, 955; ^{1}H NMR (DMSO-d_6): δ 8.88–8.85 (m, 1H, aromatic), 8.49–8.44 (m, 2H, aromatic), 5.05 (br s, 1H, NH which exchanges with D_2O), 4.50 (t, *J* = 6.8 Hz, 2H, C*H*$_2$CH$_2$Cl,), 4.01 (t, *J* = 6.8 Hz, 2H, CH$_2$C*H*$_2$Cl,), 2.82 (s, 3H, CH_3).

*5.1.23. 3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2-methyl-8-nitro[1]benzothieno[3,2-d]pyrimidin-4(3H)-one (**31**)*

A mixture of **30** (0.49 g, 1.36 mmol) and 1-(2-methoxyphenyl)piperazine (1.31 g, 6.81 mmol) was heated in an oil bath at 160 °C for 3 h. After being cooled, ethanol (10 mL) was added to the

reaction mixture. Solids were filtered off, washed with cold ethanol and dried. The crude product was triturated in ethanol and then filtered off and dried to give **31** (0.53 g, 81 %) as a pure product: mp 210 °C; IR (KBr) cm^{-1} 2951, 1676, 1555, 1341, 1245, 1035, 929; ^{1}H NMR (DMSO-d_6): δ 9.00–8.82 (m, 1H, aromatic), 8.52–8.40 (m, 1H, aromatic), 7.00–6.81 (m, 4H + 1H, aromatic), 4.30 (t, *J* = 7.2 Hz, 2H, $CONCH_2$), 3.77 (s, 3H, OCH_3), 3.00–2.93 (m, 4H, piperazine), 2.85 (s, 3H, CH_3), 2.78–2.60 (m, 4H + 2H, piperazine + $CONCH_2CH_2$). Anal. ($C_{24}H_{25}N_5O_4S$) C, H, N, S.

*5.1.24. 8-Amino-3-]2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl]-2-methyl[1]benzothieno[3,2-d]pyrimidin-4(3H)-one (**32**)*

A mixture of **31** (0.53 g, 1.10 mmol), hydrazine hydrate (0.28 g, 5.59 mmol), and Raney Ni (0.26 g) in DMF (40 mL) was stirred at room temperature. Two further amounts of hydrazine hydrate were added after 24 h (0.14 g, 2.80 mmol) and 48 h (0.055 g, 1.10 mmol) from the starting of the reaction. After further 48 h, solids were eliminated by filtration and water (60 mL) was added to the filtrate. The mixture was extracted with ethyl acetate (3 × 25 mL). Combined organic phases were dried on anhydrous sodium sulfate and evaporated at reduced pressure to give a residue which was suspended in diethyl ether. Solids were filtered off, washed with diethyl ether and dried to give **32** (0.43 g, 87 %) as a pure product: mp 230 °C; IR (KBr) cm^{-1} 3448, 2816, 1671, 1553, 1449, 1335, 1144, 1011, 926; ^{1}H NMR (DMSO-d_6): δ 7.70 (d, *J* = 8.4 Hz, 1H, aromatic), 7.40–7.34 (m, 1H, aromatic), 7.05–6.84 (m, 4H + 1H, aromatic), 5.44 (br s, 2H, NH_2 which exchange D_2O), 4.25 (t, *J* = 7.2 Hz, 2H, $CONCH_2$), 3.77 (s, 3H, OCH_3), 3.10–2.90 (m, 4H, piperazine), 2.76 (s, 3H, CH_3), 2.70–2.58 (m, 4H + 2H, piperazine + $CONCH_2CH_2$). Anal. ($C_{24}H_{27}N_5O_2S$) C, H, N, S.

*5.1.25. N-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2-methyl-4-oxo-3,4-dihydro[1]benzothieno[3,2-d]pyrimidin-8-yl]benzamide (**33**)*

A mixture of compound **32** (0.20 g, 0.44 mmol), anhydrous potassium carbonate (0.31 g, 2.24 mmol) and benzoyl chloride (0.074 g, 0.53 mmol) in THF (10 mL) was stirred for 24 h at room

temperature. Then, water was added and the mixture extracted with ethyl acetate (3 × 20 mL). Combined organic phases were dried on anhydrous sodium sulfate and evaporated at reduced pressure to give a residue which was suspended in water, then filtered off and dried. Recrystallization from ethanol gave **33** (0.11 g, 45 %) as a pure compound: mp 202 °C; IR (KBr) cm^{-1} 3254, 2946, 1674, 1454, 1236, 1027, 927, 805, 745; ^{1}H NMR (DMSO-d_6): δ 10.55 (br s, 1H, NH which exchanges with D_2O), 8.90–8.82 (m, 1H, aromatic), 8.14–7.94 (m, 2H + 2H, aromatic), 7.68–7.54 (m, 3H, aromatic), 6.95–6.82 (m, 4H, aromatic), 4.28 (t, *J* = 6.0 Hz, 2H, $CONCH_2$), 3,77 (s, 3H, OCH_3), 2.99–2.90 (m, 4H, piperazine), 2.81 (s, 3H, CH_3), 2.74–2.60 (m, 4H + 2H, piperazine + $CONCH_2CH_2$). ^{13}C NMR (DMSO-d_6): δ.169.22, 165.87, 158.18, 157.95, 152.04, 151.35, 141.18, 137.22, 135.44, 134.76, 134.47, 131.90, 128.59, 127.80, 124.04, 122.58, 120.95, 118.04, 113.91, 111.95, 55.59, 55.38, 53.34, 50.13, 42.25, 23.00. Anal. ($C_{31}H_{31}N_5O_3S$) C, H, N, S.

*5.1.26. N-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2-methyl-4-oxo-3,4-dihydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-2-phenylacetamide (**34**)*

A mixture of compound **32** (0.10 g, 0.22 mmol), anhydrous potassium carbonate (0.15 g, 1.08 mmol) and phenylacetyl chloride (0.040 g, 0.26 mmol) in THF (5 mL) was stirred for 4 h at room temperature. Then, volatiles were eliminated under reduced pressure and sodium carbonate 5 % solution (10 mL) was added. After stirring for 30 min, suspension was filtered under vacuum, solids washed with water and dried. Obtained solid product was suspended again in petroleum ether (40-60 °C, 15 mL). After stirring for 30 min, suspension was filtered under vacuum and the obtained solid product was dried to give **34** (0.075 g, 59 %) as a pure compound: mp 187–189 °C; IR (KBr) cm^{-1} 3240, 2938, 2817, 1667, 1512, 1453, 1239, 1019, 747; ^{1}H NMR (DMSO-d_6): δ 10,50 (br s, 1H, NH which exchanges with D_2O), 8.72-8.69 (m,1H, aromatic), 8.04 (d, *J* = 8.8 Hz, 1H, aromatic), 7.75–7.67 (m, 1H, aromatic), 7.40–7.20 (m, 5H, aromatic), 6.95-6.80 (m, 4H, aromatic), 4.25 (t, *J* = 6.0 Hz, 2H, $CONCH_2$), 3.76 (s, 3H, OCH_3), 3.70 (s, 2H, CH_2CO), 3.00–2.90 (m, 4H,

piperazine), 2.78 (s, 3H, CH_3), 2.74–2.58 (m, 4H + 2H, piperazine + $CONCH_2CH_2$). ^{13}C NMR (DMSO-d_6): δ 169.50, 158.10, 157.86, 151.98, 151.21, 147.38, 141.13, 137.15, 135.87, 134.95, 134.48, 129.19, 128.40, 126.67, 124.13, 122.49, 121.48, 120.87, 117.98, 112.66, 111.92, 55.54, 55.34, 53.28, 50.07, 43.42, 42.16, 22.92. Anal. ($C_{32}H_{33}N_5O_3S$) C, H, N, S.

*5.1.27. N^1-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2-methyl-4-oxo-3,4-dihydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-N^5-(phenylmethyl)pentanediamide (**35**)*

A solution of the acid **17** (0.21 g, 0.95 mmol) in THF (15 mL) was prepared by gentle heating. Then, it was placed under stirring in an ice bath and triethylamine (0.096 g, 0.95 mmol) was added. After five minutes ethyl chlorocarbonate (0.103 g, 0.95 mmol) was added and the mixture was stirred for 3 h at 0–5 °C. The mixture was allowed to reach room temperature and a solution of **32** (0.35 g, 0.78 mmol) in THF (30 mL) was added dropwise; the mixture was left under stirring at room temperature for 5 days. Then, solids were filtered off; on the filtrate, volatiles were eliminated under reduced pressure and the obtained residue was dissolved in ethyl acetate. The organic phase was washed with sodium carbonate 5 % solution, then with brine and dried on anhydrous sodium sulfate. Volatiles were eliminated under reduced pressure and the obtained residue was purified by flash chromatography on silica gel 60 using a mixture of ethyl acetate/methanol (9/1, v/v) as eluent. Homogeneous fractions were combined and evaporated under reduced pressure to give **35** (0.20 g, 39 %) as a pure compound: mp 180 °C. IR (KBr) cm^{-1} 3305, 2955, 1581, 1453, 1234, 1146, 1023, 829; ^{1}H NMR (DMSO-d_6): δ 10.23 (s, 1H, CONH which exchanges with D_2O), 8.76–8.73 (m, 1H, aromatic), 8.39 (t, J = 6.0 Hz, 1H, $ArCH_2NHCO$ which exchanges with D_2O), 8.02 (d, J = 8.8 Hz, 1H, aromatic), 7.75–7.65 (m, 1H, aromatic), 7.36-7.17 (m, 5H, aromatic), 6.93–6.87 (m, 4H, aromatic), 4.30–4.25 (m, 2H + 2H, $ArCH_2NH$ + $CONCH_2$), 3.77 (s, 3H, OCH_3), 2.98–2.90 (m, 4H, piperazine), 2.80 (s, 3H, CH_3), 2.72–2.60 (m, 4H + 2H, piperazine + $CONCH_2CH_2$), 2.40 (t, J = 7.2 Hz, 2H, $CH_2CH_2CH_2$), 2.24 (t, J = 6.8 Hz, 2H, $CH_2CH_2CH_2$), 1.93-1.84 (m, 2H, $CH_2CH_2CH_2$). ^{13}C

NMR (DMSO-d_6): δ 171.72, 171.19, 158.07, 157.82, 151.97, 151.23, 141.14, 139.65, 137.24, 134.65, 134.46, 128.29, 127.18, 126.71, 123.99, 122.43, 121.44, 120.84, 117.95, 112.56, 111.89, 55.58, 55.31, 53.29, 50.09, 42.19, 42.01, 35.78, 34.56, 22.92, 21.21. Anal. ($C_{36}H_{40}N_6O_4S$) C, H, N, S.

*5.1.28. N^1-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2-methyl-4-oxo-3,4-dihydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-N^5-(2-phenylethyl)pentanediamide (**36**)*

To a solution of the acid **19** (0.22 g, 0.93 mmol) in THF (15 mL), placed under stirring in an ice bath, triethylamine (0.094 g, 0.93 mmol) was added. After five minutes ethyl chlorocarbonate (0.101 g, 0.93 mmol) was added and the mixture stirred for 3 h at 0–5 °C. The mixture was allowed to reach room temperature and a solution of **32** (0.35 g, 0.78 mmol) in THF (30 mL) was added dropwise; the mixture was left under stirring at room temperature for 5 days. Then, solids were filtered off and the filtrate kept. Volatiles were eliminated under reduced pressure and the obtained residue was dissolved in ethyl acetate. The organic phase was washed with sodium carbonate 5 % solution, then with brine and dried on anhydrous sodium sulfate. Volatiles were eliminated under reduced pressure and the obtained residue was purified by flash chromatography on silica gel 60 using a mixture of ethyl acetate/methanol (9/1, v/v) as eluent. Homogeneous fractions were combined and evaporated under reduced pressure to give **36** (0.06 g, 12 %) as a pure compound: mp 195–197 °C. IR (KBr) cm^{-1} 3675, 3311, 2931, 2819, 2362, 1674, 1554, 1454, 1238, 1145, 1021, 751; ^{1}H NMR ($CDCl_3$): δ 8.64 (s, 1H, NH which exchanges with D_2O), 8.52–8.46 (m, 1H, aromatic), 7.83–7.72 (m, 2H, aromatic), 7.35–7.12 (m, 5H, aromatic), 7.05–6.81 (m, 4H, aromatic), 5.85 (br t, 1H, CH_2NH which exchanges with D_2O), 4.31 (t, J = 6.9 Hz, 2H, $CONCH_2$), 3.86 (s, 3H, OCH_3), 3.63–3.50 (m, 2H, CH_2NH), 3.16-3.03 (m, 4H, piperazine), 2.90–2.72 (m, 4H + 3H + 2H + 2H, piperazine + CH_3 + $CONCH_2CH_2$ + $C_6H_5CH_2$), 2.46 (t, J = 6.8 Hz, 2H, $CH_2CH_2CH_2$), 2.31 (t, J = 6.8 Hz, 2H, $CH_2CH_2CH_2$), 1.95–1.85 (m, 2H, $CH_2CH_2CH_2$). Anal. ($C_{37}H_{42}N_6O_4S$) C, H, N, S.

*5.1.29. 4-[(Phenylacetyl)amino]butanoic acid (**37**) [24]*

A solution of phenylacetyl chloride (0.50 g, 3.23 mmol) in diethyl ether (1.5 mL) was added to a mixture of 4-aminobutanoic acid (0.37 g, 3.59 mmol), NaOH 4% solution (7.5 mL) and sodium carbonate (0.76 g, 7.18 mmol). The mixture was vigorously stirred at room temperature for 3 h. Then the reaction mixture was acidified with HCl 1 M and the obtained precipitate was filtered off, washed with water and dried. The solid was triturated in petroleum ether (40–60 °C, 15 mL), then filtered off and dried. Recrystallization from ethyl acetate gave **37** (0.22, 31 %) as a pure compound: mp 92–93 °C. IR (KBr) cm^{-1} 3750, 3290, 2957, 1698, 1555, 1431, 1310, 1204, 1068, 908, 709; ^{1}H NMR (DMSO-d_6): δ 12.06 (br s, 1H, COOH which exchanges with D_2O), 8.08 (br t, 1H, NH which exchanges with D_2O), 7.38–7.18 (m, 5H, aromatic), 3.38 (s, 2H, CH_2CONH), 3.11–2.98 (m, 2H, NHCH_2), 2.20 (t, J = 7.6 Hz, 2H, CH_2COOH), 1.70–1.52 (m, 2H, $CH_2CH_2CH_2$). Anal. ($C_{12}H_{15}NO_3$) C, H, N.

*5.1.30. N-[3-[2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl]-2-methyl-4-oxo-3,4-dihydro[1]benzothieno[3,2-d]pyrimidin-8-yl]-4-[(phenylacetyl)amino]butanamide (**38**)*

To a solution of the acid **37** (0.18 g, 0.81 mmol) in THF (13 mL), placed under stirring in an ice bath, triethylamine (0.081 g, 0.80 mmol) was added. After five minutes ethyl chlorocarbonate (0.087 g, 0.80 mmol) was added and the mixture stirred for 3 h at 0 – 5 °C. The mixture was allowed to reach room temperature and a solution of **32** (0.30 g, 0.67 mmol) in THF (26 mL) was added dropwise; the mixture was left under stirring at room temperature for 5 days. Then, solids were filtered off and the filtrate kept. Volatiles were eliminated under reduced pressure and the obtained residue was dissolved in ethyl acetate. The organic phase was washed with sodium carbonate 5 % solution, then with brine and dried on anhydrous sodium sulfate. Volatiles were eliminated under reduced pressure and the obtained residue was purified by flash chromatography on silica gel 60 using a mixture of ethyl acetate/methanol (9/1, v/v) as eluent. Homogeneous

fractions were combined and evaporated under reduced pressure to give **38** (0.20 g, 46 %) as a pure compound: mp 186–188 °C. IR (KBr) cm^{-1} 1648, 1516, 1453, 1240, 1185, 1149, 880, 742; ^{1}H NMR ($CDCl_3$): δ 9.31 (s, 1H, CON*H* which exchanges with D_2O), 8.65–8.56 (m, 1H, aromatic), 7.82–7.75 (m, 2H, aromatic), 7.39–7.22 (m, 5H, aromatic), 7.07–6.81 (m, 4H, aromatic), 6.00 (t, *J* = 6.0 Hz, 1H, CON*H*CH_2 which exchanges with D_2O), 4.31 (t, *J* = 6.4 Hz, 2H, $CONCH_2$), 3.86 (s, 3H, OCH_3), 3.62 (s, 2H, $C_6H_5CH_2$), 3.42–3.30 (m, 2H, CONHC*H*$_2$), 3.15–3.06 (m, 4H, piperazine), 2.85–2.72 (m, 4H + 3H + 2H, piperazine + CH_3 + $CONCH_2CH_2$), 2.39 (t, *J* = 6.0 Hz, 2H, CH_2CH_2CONH), 1.93–1.78 (m, 2H, $CH_2CH_2CH_2$). Anal. ($C_{36}H_{40}N_6O_4S$) C, H, N, S.

5.2. Binding experiments on human cloned α_1-AR subtypes

5.2.1. Transfection and cell culture

HEK293 cells were transfected with the constitutively active pRSVICAT vectors containing the human α_{1A}-AR [31], α_{1B}-AR [32], or α_{1D}-AR [33] cDNAs by calcium phosphate transfection [34]. Cells were propagated for several weeks in the presence of 400 μg/mL geneticin, and subclones screened by radioligand binding for high receptor expression. Transfected HEK293 cells were propagated in 75 cm^2 flasks at 37 °C in a humidified 5 % CO_2 incubator in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, 1.4% glutamine, 20 mM HEPES, 100 mg/L streptomycin, 10^5 units/L penicillin, and 10 % calf serum. The cells were detached by trypsinization and subcultured at a ratio of 1:4 upon reaching confluency.

5.2.2. Radioligand Binding.

Confluent 100-mm plates were washed with phosphate buffered saline (18 mM Na_2HPO_4, 2 mM NaH_2PO_4, 154 mM NaCl, pH 7.6) and harvested by scraping. Cells were collected by centrifugation and homogenized with a Polytron. Cell membranes were collected by centrifugation at 30,000 x *g* for 10 min and resuspended by homogenization. Receptor density was determined by saturation

analysis of the α_1-AR specific antagonist radioligand [^{125}I]BE 2254 (20-800 pM) [35] For analysis of competition by selective drugs, 50 pM radioligand was used. Curves were analyzed by nonlinear regression analysis using GraphPad Prism [36]. Nonspecific binding was determined in the presence of 10 μM phentolamine.

5.3. Functional Antagonism in Isolated Tissues.

Male Wistar rats (275-300 g) were killed by cervical dislocation and the required organs were isolated, freed from adhering connective tissue, and set up rapidly under a suitable resting tension in 20 mL organ baths containing physiological salt solution kept at 37 °C and aerated with 5 % CO_2:95 % O_2 at pH 7.4. Concentration-response curves were constructed by cumulative addition of reference agonist. The concentration of agonist in the organ bath was increased approximately 3-fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Contractions were recorded by means of a transducer connected to the MacLAb system PowerLab/800.

At first the compounds under study were added in the organ bath in order to construct a concentration-response curve such as that for the reference agonist, but no response was obtained; after this, the compounds were treated as antagonists. In particular, after construction of concentration-response curves of the reference agonist following 30 min of washing, tissues were incubated with the compound under study for 30–60 min and a new dose-response curve to the agonist was recorded. In all cases, parallel experiments in which tissues received only the reference agonist were run in order to check any variation in sensitivity.

All animal testing was carried out according to European Communities Council Directive of 24 November 1986 (86/609/EEC).

The antagonist potency was expressed by pK_b at a single concentration [37]. pK_b values were calculated from the equation pK_b = log(DR−1) − log[B], where DR is the ratio of ED_{50} values of

agonist after and before treatment with one or two antagonist concentrations [B]. Data are presented as the mean ± SE of 4–5 experiments. Differences between mean values were tested for significance by Student's t-test.

5.3.1. Rat Vas Deferens Prostatic Portion

This tissue was used to assess α_{1A}-AR antagonism [38]. Prostatic portions of 2-cm length were mounted under 0.35 g tension at 37 °C in a Tyrode solution of the following composition (mM): NaCl, 130; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 0.89; NaH_2PO_4, 0.42; $NaHCO_3$, 25; glucose, 5.6. Cocaine hydrochloride (10 μM) was added to the Tyrode to prevent the neuronal uptake of (-)-noradrenaline (NA). After the equilibration period, tissues were primed twice by addition of 10 μM of the agonist (-)-NA in order to obtain a constant response. After another washing and equilibration period of 45 min, a cumulative (-)-NA concentration-response curve was constructed isotonically to determine the relationship between agonist concentrations and the contractile response (basal response). When measuring the effect of the antagonist, it was allowed to equilibrate with the tissue for 60 min before constructing a new concentration-response curve to the agonist. (-)-NA solution contained 0.05 % $Na_2S_2O_5$ to prevent oxidation.

5.3.2. Rat Spleen

This tissue was used to assess α_{1B}-AR antagonism [39]. The spleen was removed and bisected longitudinally into two strips which were suspended in tissue baths containing Krebs solution of the following composition (mM): NaCl, 120; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.5; KH_2PO_4, 1.2; $NaHCO_3$, 20; glucose, 11; K_2EDTA, 0.01. (±)-Propranolol hydrochloride (4 μM) was added to block β-adrenoceptors. The spleen strips were placed under 1 g resting tension and equilibrated for 2 h. The cumulative concentration-response curves to phenylephrine were measured isometrically and obtained at 30 min intervals, the first one being discarded and the second one taken as control.

The antagonist was allowed to equilibrate with the tissue for 30 min; then a new concentration-response curve to the agonist was constructed.

5.3.3. Rat aorta

This tissue was used to assess α_{1D}-AR antagonism [40]. Thoracic aorta was cleaned from extraneous connective tissue and placed in Krebs solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 1.9; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11.7. Cocaine hydrochloride (10 μM), normetanephrine hydrochloride (1 μM) and (±)-propranolol hydrochloride (1 μM) were added to prevent the neuronal and extraneuronal uptake of (-)-NA and to block β-adrenoceptors, respectively. Two helicoidal strips (15 x 3 mm) were cut from each aorta beginning from the end most proximal to the heart. The endothelium was removed by rubbing with filter paper: the absence of acetylcholine (100 μM)-induced relaxation to preparations contracted with (-)-NA (1 μM) was taken as an indicator that vessel was denuded successfully. Vascular strips were then tied with surgical thread and suspended in a jacketed tissue bath containing Krebs solution. Strip contractions were measured isometrically. After at least a 2 h equilibration period under an optimal tension of 1 g, cumulative (-)-NA concentration-response curves were recorded at 1 h intervals, the first two being discarded and the third one taken as control. The antagonist was allowed to equilibrate with the tissue for 60 min before the generation of the fourth cumulative concentration-response curve to (-)-NA. (-)-NA solutions contained 0.05 % $Na_2S_2O_4$ to prevent oxidation.

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Table 1. Binding properties of benzothieno[3,2-*d*]pyrimidine derivatives.

Compound	pK_i (M)[a]		
	α_{1A}-AR	α_{1B}-AR	α_{1D}-AR
7	8.96 ± 0.06	8.00 ± 0.04	9.24 ± 0.06
8	8.83 ± 0.05	7.55 ± 0.04	9.04 ± 0.10
9	8.15 ± 0.05	7.47 ± 0.06	8.49 ± 0.11
10	8.28 ± 0.06	7.44 ± 0.06	8.93 ± 0.06
20	8.96 ± 0.06	8.57 ± 0.06	9.43 ± 0.08
21	9.49 ± 0.06	9.08 ± 0.05	9.85 ± 0.11
22 (RX18)	9.03 ± 0.19	8.72 ± 0.10	10.25 ± 0.17
23	8.79 ± 0.14	8.19 ± 0.15	9.00 ± 0.06
26	8.16 ± 0.16	8.13 ± 0.28	8.39 ± 0.11
27	8.77 ± 0.23	8.78 ± 0.06	8.78 ± 0.20
33	8.41 ± 0.14	7.41 ± 0.18	8.30 ± 0.12
34	8.47 ± 0.05	7.52 ± 0.06	8.48 ± 0.08
35	8.45 ± 0.15	7.72 ± 0.16	8.26 ± 0.21
36	9.04 ± 0.12	8.19 ± 0.06	9.12 ± 0.08
38	9.00 ± 0.08	8.12 ± 0.05	8.64 ± 0.07
RN17[b]	8.48 ± 0.09	8.05 ± 0.08	8.90 ± 0.12
RC23[b]	8.83 ± 0.03	7.76 ± 0.05	9.40 ± 0.05

[a]Each value is the mean ± SE for data from three different experiments conducted in duplicate.

[b]Data from ref. 14.

Table 2. Antagonist potency of compounds **21** and **22** in isolated rat prostatic vas deferens (α_{1A}-AR), spleen (α_{1B}-AR) and thoracic aorta (α_{1D}-AR).

Compound	**pK_b ± SE**		
	α_{1A}-AR	**α_{1B}-AR**	**α_{1D}-AR**
21	7.70 ± 0.09[a]	7.94 ± 0.02[a]	8.64 ± 0.02[a]
22 (RX18)	7.82 ± 0.16[b]	7.77 ± 0.13[a]	9.15 ± 0.07[a]
RN17[c]	8.61 ± 0.14	8.44 ± 0.15	8.89 ± 0.04

[a]At 0.1 μM a decreasing of 100 % in the dose response curve was obtained.

[b]At 0.1 μM a decreasing of 90 % in the dose response curve was obtained.

[c]Data from ref. 16.

Figure Captions

Figure 1. Structures of α_1-AR antagonists RN17, RC23, **39**, and general structures of the new 8-substituted [1]benzothieno[3,2-*d*]pyrimidine derivatives.

Scheme 1[a]

[a]Reagents and conditions: (a) ethylchlorocarbonate, toluene, reflux; (b) 2-aminoethanol, 50 °C, 40 min; (c) thionyl chloride, toluene, reflux; (d) 1-(2-methoxyphenyl)piperazine,160 °C, 30 min; (e) hydrazine hydrate, DMF, Raney Ni, r.t., 24 h; (f) RCOCl, THF, potassium carbonate.

Scheme 2[a]

[a]Reagents and conditions: (a) toluene; (b) ethyl chlorocarbonate, TEA, THF then **6**.

Scheme 3[a]

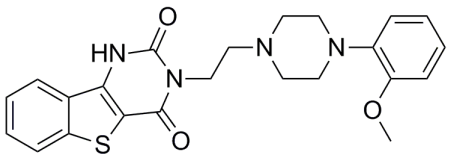
[a]Reagents and conditions: (a) 1,1'-carbonyldiimidazole, 1,4-dioxane, then **6**.

Scheme 4[a]

[a]Reagents and conditions: (a) 1,1,1-triethoxyethane, MW; (b) 2-aminoethanol, 50 °C, 2 h; (c) thionyl chloride, toluene, reflux, 3 h; (d) 1-(2-methoxyphenyl)piperazine,160 °C, 3 h; (e) hydrazine hydrate, DMF, Raney Ni, r.t., 4 days;

Scheme 5[a]

[a]Reagents and conditions: (a) RCOCl, THF, toluene, 4 h; (b) **17** or **19**, ethyl chlorocarbonate, TEA, THF, 0° C → r.t.; (c) ethyl chlorocarbonate, TEA, THF, 0° C → r.t.



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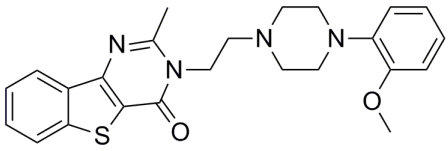

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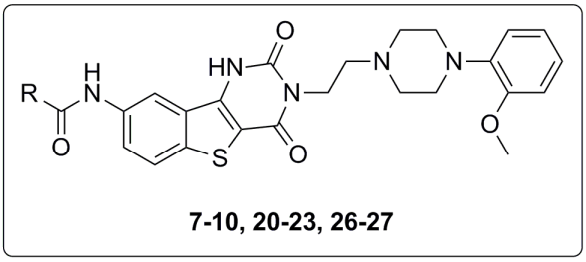

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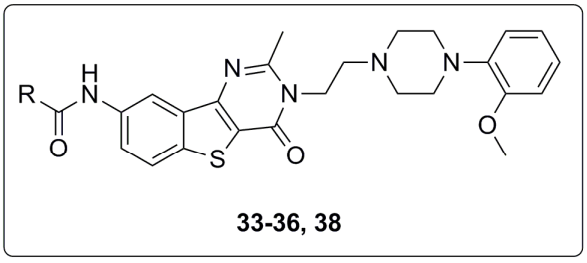

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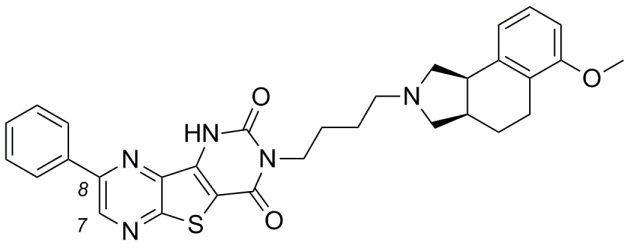

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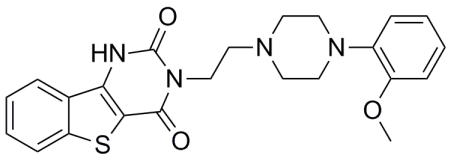

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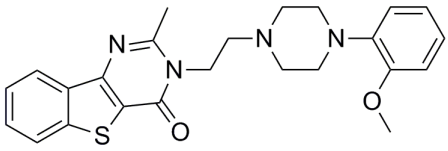

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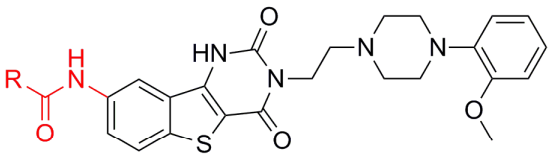

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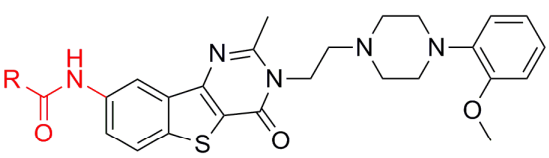

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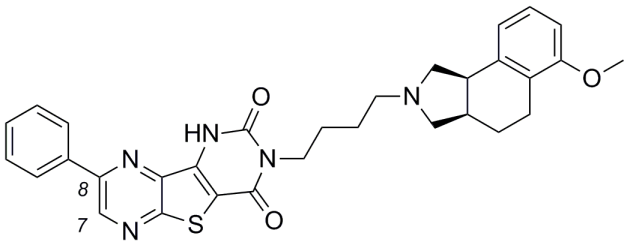

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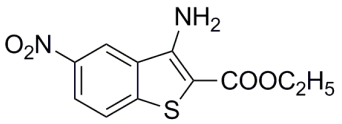

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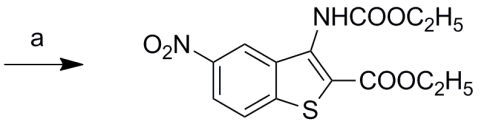

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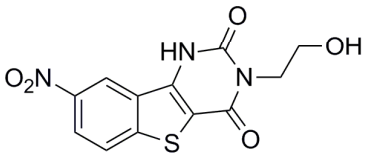

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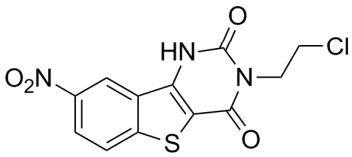

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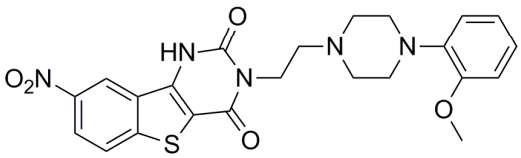

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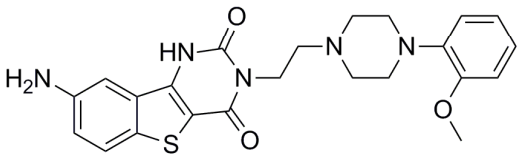

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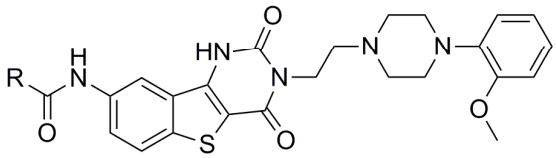
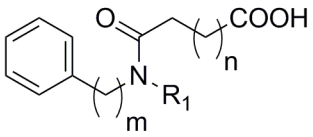


7: R = C_6H_5;
8: R = $CH_2C_6H_5$;
9: R = $(CH_2)_3C_6H_5$;
10: R = $(CH_2)_4C_6H_5$;

11: m = 1; R_1 = H;
12: m = 1; R_1 = CH_3;
13: m = 2; R_1 = H;

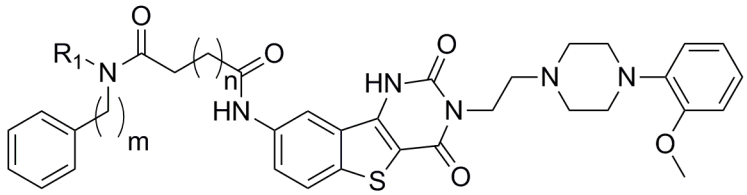
14: n = 1;
15: n = 2;

a

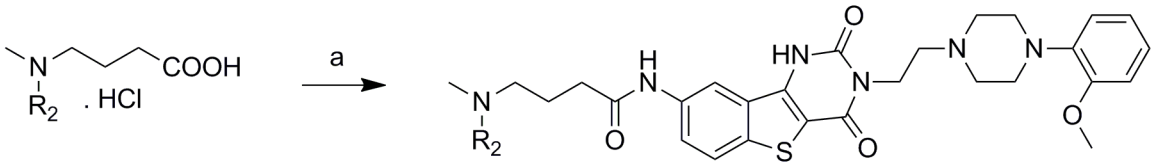


16: m = 1; n = 1; R_1 = H;
17: m = 1; n = 2; R_1 = H;
18: m = 1; n = 2; R_1 = CH_3;
19: m = 2; n = 2; R_1 = H;

b

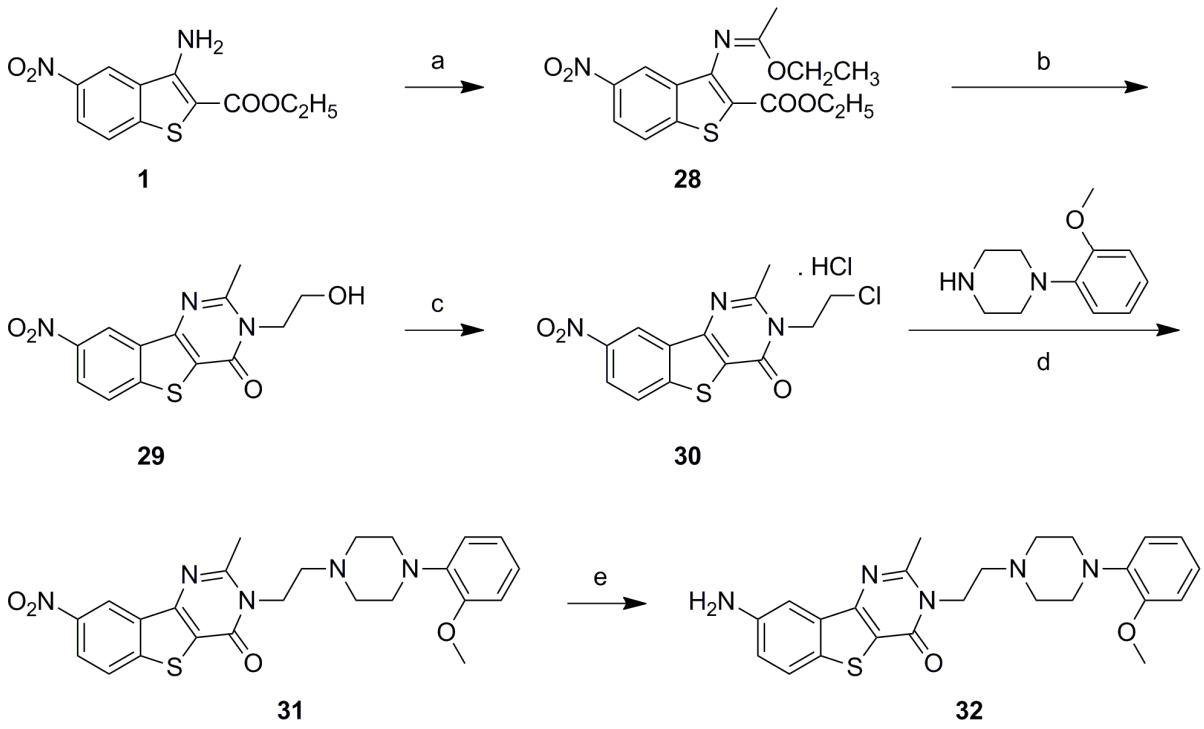


20: m = 1; n = 1; R_1 = H;
21: m = 1; n = 2; R_1 = H;
22: m = 1; n = 2; R_1 = CH_3;
23: m = 2; n = 2; R_1 = H;

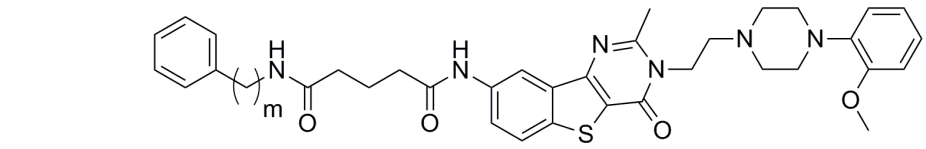


24: $R_2 = CH_2C_6H_5$;
25: $R_2 = CH_3$;

26: $R_2 = CH_2C_6H_5$;
27: $R_2 = CH_3$;

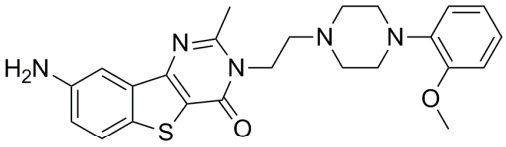


1 **28** **29** **30** **31** **32**



35: m = 1;
36: m = 2;

b



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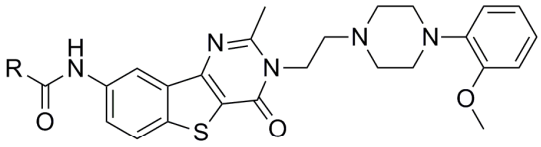
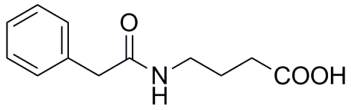


33: R = C_6H_5;
34: R = $CH_2C_6H_5$;



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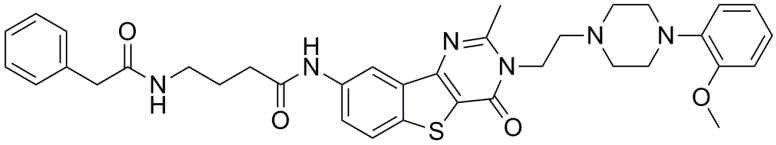

38

Highlights

1. Novel benzothieno[3,2-*d*]pyrimidine derivatives as ligands for the α_1-AR subtypes
2. Receptor affinity was strongly influenced by nature of the chain at the 8-position
3. Diamide derivative RX18 showed a very high affinity for the α_{1D}-AR subtype
4. New derivatives behaved as potent α_{1D}-AR antagonists in a functional assay