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Synthesis of theophylline derivatives and study of their activity as antagonists at adenosine receptors

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ABSTRACT

The synthesis of oligo(ethylene glycol)-alkene substituted theophyllines in positions 7 and/or 8 is described. The binding activity at adenosine receptors of selected derivatives was studied. Compound **2** showed high affinity for human A_{2B} receptor (K_i = 4.16 nM) with a selectivity K_{iA2A}/K_{iA2B} of 24.1, and a solubility in water of 1 mM. The alkenyl substituent in some of the theophylline derivatives allows for covalent attachment of them onto hydrogen-terminated silicon substrate surfaces via hydrosilylation. Alternatively, an azido group was incorporated to an oligo(ethylene glycol)theophylline derivative as an anchor for tethering the molecules on ethynyl presenting surfaces via click reaction.

1. Introduction

The methylxanthine theophylline (**1a**, Fig. 1) and its derivatives (i.e., **1b**) have been extensively studied. For example, they have been used (i.e., **1c**) as templates in molecularly imprinting related research. Due to its multivalency with various hydrogen-bonding donor and acceptor sites, this base can be recognized by the binding sites of molecularly imprinted polymers (MIPs) with specific structures.[1] Also, theophylline and its derivatives (i.e., **1d**) have been used for the conjugation with different high-molecular weight poly(ethylene glycol)s.[2] Among the xanthine derivatives, theophylline shows greater binding efficacy with DNA than theobromine and caffeine. It has been demonstrated that theophylline forms complexes with DNA through hydrogen bonds, while serving as a strong antioxidant that prevents DNA damage.[3]

The biological effect of theophylline is associated with its affinity with a family of adenosine receptors known as A_1, A_{2A}, A_{2B}, and A_3.[4] These adenosine receptors (or P1 receptors) are a class of purinergic receptors, G-protein coupled receptors with adenosine as endogenous ligand. In the last few years several groups designed and studied the structure–activity relationship of xanthine derivatives in searching for more potent and highly A_{2B}-selective ligands. It has been found that substitution at position 8 of theophylline (i.e., **1b**, **1e**) plays an important role in the antagonistic activities.[5,6] For example, compound **1e** presents an A_{2B} K_i of 1.39 nM.[7] Recently, several 1,3-dialkyl deazaxanthines have been reported as potent A_{2B} adenosine receptor antagonists.[8]

Through the binding with the above adenosine receptors, theophylline derivatives are well known to influence neuronal activities.[9] We are interested in immobilizing them on silicon substrate surfaces and studying their interactions with and triggered activities of neuron cells immobilized on the adjacent regions. Therefore, in this work we also introduce handles for attaching the theophylline derivatives on silicon substrate surfaces. Specifically, we incorporate a vinyl group to the molecules, allowing them to be attached via hydrosilylation forming Si–C bonds with hydrogen-terminated silicon surfaces.[10,11] In addition, we also incorporate an azido group for tethering the molecules onto alkynyl-presenting monolayers on silicon surfaces. Furthermore, an oligo(ethylene glycol) spacer is used to connect the theophylline moiety with the handles. This oligo(ethylene glycol) spacer allows the theophylline to stand above the surface of a monolayer of oligo(ethylene glycol)-terminated thin film used to render the silicon substrate surface resistant to non-specific adsorption of proteins,[12–14] which is a key requirement for the applications. In this paper, we report the synthesis of several theophylline-oligo(ethylene glycol) based anchors. We also demonstrate that some of the compounds possess

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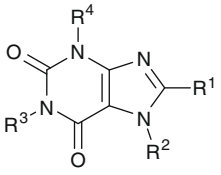


1a, R^1=R^2= H, R^3=R^4= CH_3, Theophylline
1b, R^1= Ph, R^2= H, R^3=R^4= CH_3, 8-Phenyltheophylline
1c, R^1= H, R^2= CH_2CH_2OH, R^3=R^4= CH_3
1d, R^1= H, R^2= CH_2OH, R^3=R^4= CH_3
1e, R^1= *p*-(C_6H_4)O(CH_2)CONH(*p*-C_6H_4)$COCH_3$), R^2= H, R^3=R^4= $(CH_2)_2CH_3$

Figure 1. Several active theophylline derivatives.

substantial activities at adenosine receptors, and thus are ready to be tested on silicon surfaces.

2. Results and discussion

2.1. Synthesis

The fact that substitution at the 8 position of theophylline, mainly with phenyl or cycloalkyl groups,[5,6] increases the activity at adenosine receptors prompted us to the synthesis of theophyllines modified at this position by introduction of a phenyl-tetra(ethylene glycol) terminated in alkene moiety in compound **2** and a di(ethylene glycol) substituent in derivative **3** (Fig. 2).

For the synthesis of **2**, starting material tosyl tetra(ethylene glycol) monoundec-9-enyl ether (**4**) has been prepared by treatment of tetra(ethylene glycol) with 11-bromoundec-1-yl to afford **5** in 83%[15] yield followed by tosylation to give **4** in 81% yield (Scheme 1).[16,17]

The preparation of **2** has been accomplished as shown in Scheme 2 with a good overall yield (20%). Condensation of the aldehyde **6** with 4,5-diamino-1,3-dimethyl uracil (**7**) gave the imine **8** which was oxidatively cyclized by treatment with diisopropyl azodicarboxylate (DIAD) instead of DEAD as reported in the literature for similar compounds,[18] to give the xanthine **2** in 77% yield (Scheme 2).

Compound **3** was synthesized in two steps. First, amide **9** was obtained by treating the diaminouracil **7** with 2-[(2-(2-methoxyethoxy)ethoxy]acetic acid in the presence of *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), and used in the next step without purification. Second, the 8-diethylene glycol derivative of theophylline **3** was obtained by ring closure reaction of the amide **9** with sodium methoxide in refluxing methanol in 52% yield (Scheme 3).[9]

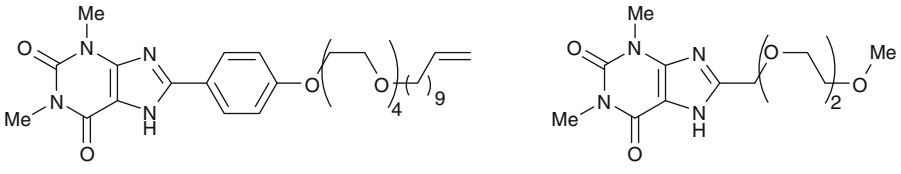
We also report the preparation of 7-substituted theophyllines **10a–c** shown in Scheme 4. The derivative **10a** was obtained from the sodium salt of **1a** in good yield (85%). 8-Phenyltheophylline (**1b**) was prepared from **7** by construction of the imidazole ring using benzaldehyde and DIAD as imine cyclization agent.[15] However, coupling of **1b** with **4** using 60% NaH as the base[19] afforded **10b** (<5%) in poor yields, probably due to the presence of the bulky phenyl group at position 8 of theophylline. Finally, reaction of 11-bromoundec-1-yl with **1a** in the presence of Et_3N gave compound **10c** in 20% yield.[20]

In addition to the direct attachment of the alkenyl-substituted theophylline derivatives on silicon surfaces via hydrosilylation, we are also interested in tethering theophylline via click chemistry based on Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction between azides and alkynes.[21–26] Recently we modified the copper catalysts reported by Chan et al.[27] to render them water soluble, and highly efficient for surface conjugation in aqueous solutions and in a microarray formate.[28]

The approach based on click chemistry in microarray format is highly efficient for optimizing the spacer and the density of theophylline derivatives on silicon surfaces for interaction with neurons. For this purpose, we need to introduce azido groups to the theophylline derivatives. The synthesis of the theophylline derivative **10e** with an azido handle is outlined in Scheme 5. Treatment of **1a** with tetra(ethylene glycol)ditosylate in the presence of 60% NaH afforded the 7-substituted theophylline **10d** in 50% yield. This product was converted to the azide **10e** in 33% yield by treatment with excess of NaN_3 in anhydrous DMF.

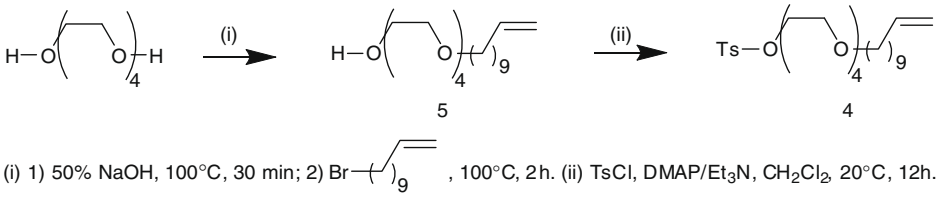
2.2. Binding assays

We report here the study on radioligand binding at adenosine receptors of some of the synthesized theophylline derivatives. Compounds **2**, **3**, **10a** and **10e** were assayed at human recombinant



2 3

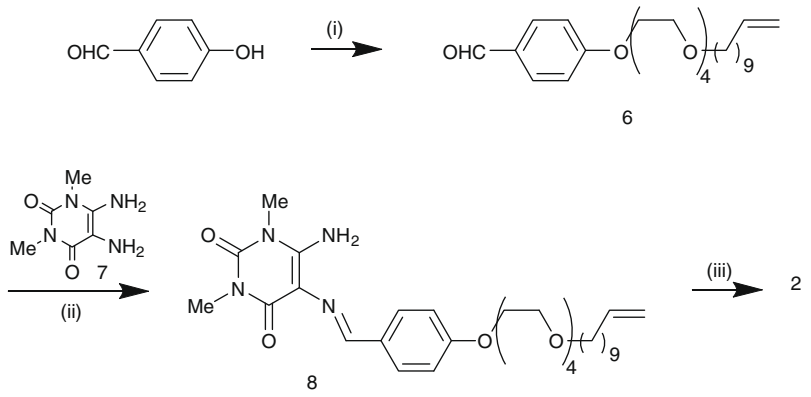
Figure 2. 8-Substituted theophylline derivatives.



5 4

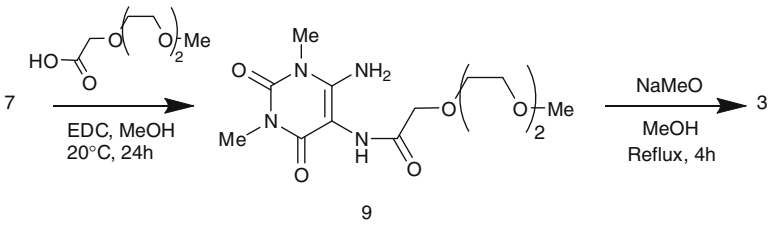
(i) 1) 50% NaOH, 100°C, 30 min; 2) Br–( )$_9$, 100°C, 2 h. (ii) TsCl, DMAP/Et_3N, CH_2Cl_2, 20°C, 12h.

Scheme 1. Preparation of the tetra(ethylene glycol) derivative **4**.

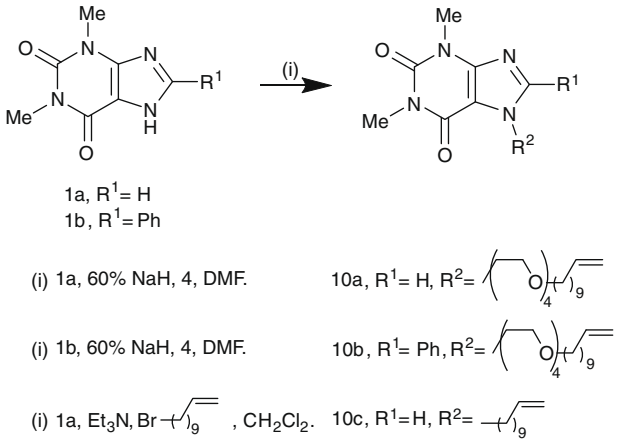


(i) 4, K_2CO_3/CH_3CN, reflux, 1h. (ii) 7, EtOH/AcOH, reflux, 12h. (iii) DIAD, DME, reflux, 12h.

Scheme 2. Synthesis of the 8-substituted theophylline derivative **2**.



Scheme 3. Synthesis of the theophylline-di(ethylene glycol) derivative **3**.

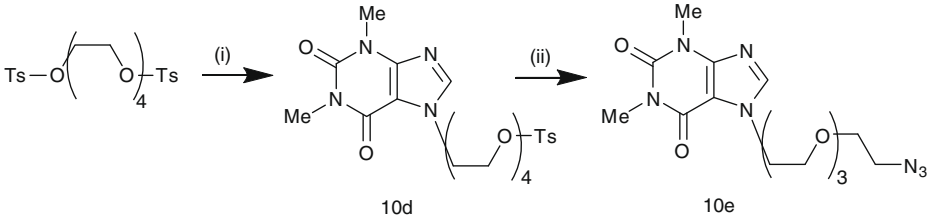


Scheme 4. Substitution at the theophylline N7.

A_1, A_{2A}, A_{2B} and A_3 receptors as described in the experimental section.[29] We choose these compounds for comparative structure–activity analysis, since compounds **2** and **3** present an ethylene glycol derivative substituent at position 8 of theophylline, while compounds **10a** and **10e** present the substitution over the N7 of the base. Radioligand assays were performed in vitro at such receptors by measuring the percent inhibition of specific binding at a single concentration (10 μM) and for compounds showing percentage values over 50% concentration–response curves in the range of 0.1 nM–1 mM for the affinity (K_i) calculation (Fig. 3).

The affinity results are summarized in Table 1. Compounds **3** and **10e** show percent inhibition values around or below 50% at 10 μM. We may therefore assume that these compounds have a low affinity for these receptors. However, compound **10a** shows some affinity for A_1 receptors and A_{2B}, for which the K_i could be calculated, and especially compound **2** shows a high affinity and selectivity for A_{2B} receptors, presenting a K_i in the nanomolar scale (4.16 nM) and it can be defined as a high affinity and selective A_{2B} receptor ligand. Selectivity of **2** between A_{2A} and A_{2B} receptors reach the ratio 24.1 for K_{iA2A}/K_{iA2B}.

As it can be seen in Table 1, low affinity for A_3 receptors has been found for these compounds, which do not present substitution at 1 and 3 positions of the base. This result was expected, since



(i) 1) 1a, 60% NaH, DMF; 2) Tetra(ethylene glycol)ditosylate, DMF, 20°C, 12h. (ii) NaN_3, DMF, 60°C, 12h.

Scheme 5. Preparation of the azide derivative **10e**.

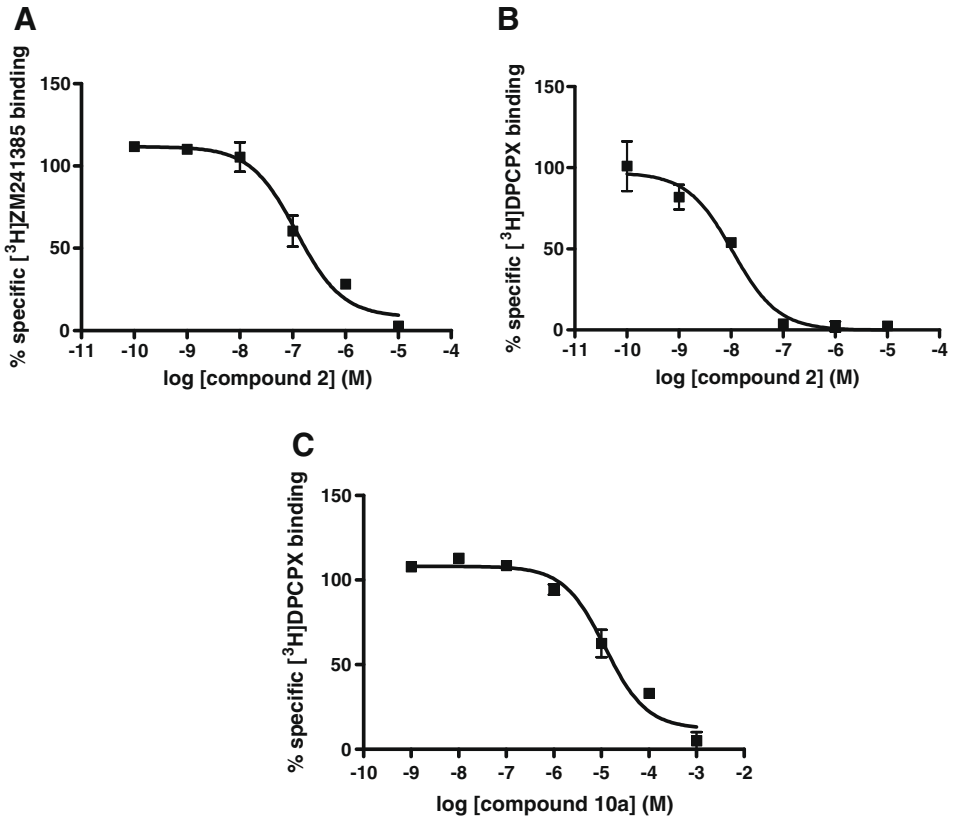


Figure 3. Binding competition experiments at cloned hA_1 hA_{2A} and hA_{2B} receptors. (A) Concentration–response curve for compound **2** at hA_{2A} receptors labeled with 3 nM [^{3}H]ZM241385. (B) Concentration–response curve for compound **2** at hA_{2B} receptors labeled with 25 nM [^{3}H]DPCPX. (C) Concentration–response curve for compound **10a** at *h*A1 receptors labeled with 2 nM [^{3}H]DPCPX. Values represent the mean ± sem (vertical bars) of three independent experiments.

Table 1
Affinity (K_i and percent displacement of specific binding) at A_1, A_{2A}, A_{2B} and A_3 receptors exhibited by the theophylline derivatives

Compound	A_1		A_{2A}		A_{2B}		A_3	
	% (10 μM)	K_i (nM)	% (10 μM)	K_i (nM)	% (10 μM)	K_i (nM)	% (10 μM)	K_i (nM)
1a	—	(10.1 ± 2.1) 10^3	—	(18.5 ± 0.1) 10^3	—	(2.7 ± 0.2) 10^3	—	(85.1 ± 1.5) 10^3
2	38 ± 1	—	—	100.4 ± 6.5	—	4.16 ± 0.71	7 ± 3	—
3	37 ± 5	—	20 ± 2	—	10 ± 3	—	1 ± 2	—
10a	53 ± 4	(5.0 ± 0.7) 10^3	18 ± 2	—	47 ± 3	—	19 ± 5	—
10e	24 ± 3	—	17 ± 3	—	3 ± 1	—	2 ± 1	—

Values show the mean ± sem of three independent assays with duplicate determinations.

the 1,3 unsubstituted caffeine or theophylline derivatives exhibit dramatically reduced affinity for the A_3 receptor in comparison, for example, with 1,3-dipropyl substituted derivatives.[30]

Chemical structure of compound **2** presents a *para*-3,6,9,12-tetraoxatricos-22-enyloxy substituted phenyl ring at position 8 of theophylline. The presence of this phenyl ring seems to be determinant for the affinity of this compound over A_{2A} and even more over A_{2B} receptors since the homologue compound **3**, lacking this ring, showed no affinity at those receptor subtypes. Structure–activity relationship (SAR) regarding the substitution at position 8 of theophylline derivatives and other xanthine bases has been extensively studied.[5,6,31,32] These studies show that the presence of a phenyl or cycloalkyl group at this position of theophylline increases the relative affinity at adenosine receptors, and mainly at the A_{2B} receptor.[31]

Theophylline and 3-*N*-(propyl)xanthine showed to be selective, albeit weak, antagonists at the A_{2B} adenosine receptors.[33] These findings prompted several groups to design and test a large number of xanthine derivatives in search for new, more potent and A_{2B}-selective ligands, and in the last few years some very potent and highly A_{2B}-selective xanthines have been discovered.[7] The affinities of these compounds are similar to that of the xanthine derivative **2**.

On the other hand, the presence of substituents at position N7 of the base in compounds **10a** and **10e** results in the reduction of its affinity for the receptors in comparison to that of **2**. This fact can be attributed to the hypothesized activity of this nitrogen atom as a hydrogen-bond acceptor at the binding pocket, which it is not allowed in tertiary N7.[5] However, compound **10a** still shows higher affinity at A_{2B} and A_1 receptors than **3** and **10e** (Table 1). This may be related to a favourable distal electronic interaction with the receptor due to the combination in **10a** of a long tetra(ethylene glycol) chain with a terminal electron-rich alkene group, similar to that in **2**.[6,32]

Finally, solubility in water and hydrophilic solvents is necessary for biological applications of active compounds. This physicochemical property has been a major issue with xanthine and non-xanthine antagonists of adenosine receptors, since the 8-phenyl

substitution in the xanthine pharmacophore increases receptor blocking activity, it also markedly decreases solubility.[34] In order to analyze this point, we measured the solubility of the oligo(ethylene glycol)-substituted theophyllines, compounds **2** and **3**, in water, di(ethylene glycol) and glycerin. While the solubility of both compounds in glycerin is poor, in water and di(ethylene glycol) is remarkably better to that of theophylline (**1a**) and 8-phenyltheophylline (**1b**). For instance, while **1a** and **1b** are not soluble in di(ethylene glycol), derivatives **2** and **3** displayed a solubility of 0.1 mM and 10 mM, respectively, in this solvent. Compound **3** reach 14 mM in water. In this sense, it is important to remark that the presence of the oligo(ethylene glycol) chain in compounds **2** and **3** increase the water solubility, and consequently the ratio solubility to receptor affinity.[34]

3. Conclusions

In summary, we have designed and synthesized two kinds of theophylline derivatives with a handle (vinyl or azido group) and an oligo(ethylene glycol) spacer for immobilization onto silicon substrate surfaces. The method for the synthesis of theophylline-oligo(ethylene glycol)-alkene derivatives is versatile, allowing for efficient preparation of the 7 and 8 substituted derivatives. We have also identified the theophylline derivative **2** with an oligo(ethylene glycol) side chain as adenosine antagonist which is potent and highly selective antagonist for human A_{2B} receptors, and also more soluble than the primary pharmacophore 8-phenyltheophylline. Unfortunately, the azido-modified theophylline derivative **10e** exhibited a low affinity to the receptors. However, we expect that introducing a phenylene unit between the ethylene glycol spacer and the theophylline, similar to **2**, should greatly improve the binding affinity. We are currently pursuing the synthesis of these compounds. The attachment of the alkenyl- and azido-substituted theophylline derivatives to silicon substrate surfaces at various densities and the study of the response of neuron cells to the immobilized neurotransmitters that are relevant to the field of machine brain interface[35] will be reported in due course.

4. Experimental

4.1. General

Melting points were determined with a Gallenkamp instrument and are given uncorrected. UV spectra were recorded with a Hewlett–Packard 8452A spectrophotometer, and IR spectra with Beckman Aculab IV and Perkin–Elmer 883 spectrophotometers. Mass spectrometry was carried out with a Thermo Finnigan instrument, using the direct injection and electron-impact (EI) modes. HRMS were recorded with a Micromass (Autospec-Q) spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on 400 MHz ARX 400 Bruker spectrometer, using the residual solvent peak in $CDCl_3$ (δ_H 7.24 ppm for ^{1}H and δ_C 77.0 ppm for ^{13}C), or CD_3SOCD_3 (δ_H 2.50 ppm for ^{1}H). Chemical shifts are given in ppm, and J values in hertz. TLC analyses were performed on Merck Silica Gel 60 F 254 plates, and column chromatography on Silica Gel 60 (0.040–0.063 mm).

4.2. Synthesis of 8-(4-(3,6,9,12-tetraoxatricos-22-enyloxy)phenyl)-theophylline (2)

4.2.1. 3,6,9,12-Tetraoxatricos-22-en-1-ol (5)

Under an Ar atmosphere, a mixture of 50% sodium hydroxide (1 mL) and tetra(ethylene glycol) (20.8 mL, 120 mmol) was stirred at 100 °C for 30 min. After this period, 11-bromoundec-1-yl (2.62 mL, 12.0 mmol) was added. The reaction was followed by tlc (EtOAc/cyclohexane 1:1) to completion. The reaction mixture was cooled to 20 °C and extracted with cyclohexane (3 × 20 mL), the organic phase was washed with brine, dried over $MgSO_4$ and concentrated to dryness. The residue was purified by column chromatography (cc) eluting with EtOAc/cyclohexane (1:1) to obtain **5** as a yellowish liquid (3.45 g, 83%);[15] ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.83–5.73 (m, 1H; =CH), 4.98–4.88 (m, 2H; $=CH_2$), 3.69 (d, J = 4.4 Hz, 2H; CH_2OH), 3.64–3.54 (m, 14H; 7 × OCH_2), 3.41 (t, J = 6.8 Hz, 2H; CH_2), 2.01–1.99 (m, 2H; =CH–CH_2), 1.56–1.53 (m, 2H; CH_2), 1.33–1.21 (m, 12H; 6 × CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 139.1 ppm (=CH), 114.0 ($=CH_2$), 72.5, 71.4, 70.46, 70.44, 70.42, 70.39, 70.1, 69.9, 61.57, 33.7, 29.42, 29.40, 29.31, 29.29, 29.0, 28.8, 25.9; IR (KBr) ν: 3466, 2923, 2854, 1104, 909 cm^{-1}.

4.2.2. 3,6,9,12-Tetraoxatricos-22-enyl 4-tosylate (4)

Under argon atmosphere, over a cooled (0 °C) solution of **5** (2.93 g, 8.47 mmol), Et_3N (5.9 mL, 42.4 mmol) and DMAP (15 mg) in dry CH_2Cl_2 (80 mL) was added a solution of tosyl chloride (3.38 g, 17.8 mmol) dry CH_2Cl_2 (20 mL). The reaction mixture was stirred at 20 °C for 12 h. After this period, the mixture was poured over ice-water (20 mL) and then extracted with CH_2Cl_2 (3 × 20 mL). The organic solution was washed with 10% NH_4Cl (20 mL), brine (20 mL), dried over $MgSO_4$ and concentrated to dryness. The residue was separated by cc eluting with EtOAc/cyclohexane 1:1) to afford compound **4** as a yellowish solid (3.44 g, 81%); mp 42–43 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.77 ppm (d, J = 8.4 Hz, 2H; 2 × ArH), 7.32 (d, J = 8 Hz, 2H; 2 × ArH), 5.84–5.73 (m, 1H; =CH), 4.99–4.89 (m, 1H; $=CH_2$), 4.15 (dd, J = 5.2, 6 Hz, 2H; CH_2OTs), 3.66 (t, J = 4.8 Hz, 2H; CH_2), 3.61–3.54 (m, 12H; 12 × OCH_2), 3.41 (t, J = 6.8 Hz, 2H; OCH_2), 2.42 (s, 3H; CH_3), 2.02 (m, 2H; CH_2), 1.54 (t, J = 7.2 Hz, 2H; CH_2), 1.36–1.25 (m, 12H; 6 × CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 144.7 ppm (C), 139.2 (=CH), 132.9 (C), 129.8 (2 × CH), 127.9 (2 × CH), 114.0 ($=CH_2$), 71.5, 70.7, 70.6, 70.51, 70.46, 70.0, 69.2, 68.6, 33.7, 29.6, 29.5, 29.41, 29.37, 29.1, 28.9, 26.8, 21.6, 26.0 (CH_3); IR (KBr) ν: 2924 cm^{-1}, 2854, 1357, 1176, 1097, 913; MS (EI, 70 eV) m/z: 500 (1) [M^+], 287 (7), 243 (9), 199 (100), 155 (22), 91 (17); HRMS m/z: 500.2808 (calcd for $C_{26}H_{44}O_7S$: 500.2808).

4.2.3. 4-(3,6,9,12-Tetraoxatricos-22-enyloxy)benzaldehyde (6)

A solution of p-hydroxybenzaldehyde (488 mg, 4 mmol), K_2CO_3 (553 mg, 4 mmol) and **4** (2.0 g, 4 mmol) in acetonitrile (30 mL) was refluxed under stirring for 1 h. After this period, the reaction mixture was cooled and 5% HCl (5 mL), followed by EtOAc (20 mL) were added. The organic phase was washed with water, dried over Na_2SO_4 and concentrated under vacuum to dryness. The residue was separated by cc eluting with cyclohexane/EtOAc 5:5 to obtain **6** as a yellowish syrup (1.06 g, 59%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.86 ppm (s, 1H; CHO), 7.81 (d, J = 9.2 Hz, 2H; 2 × ArH), 7.00 (d, J = 8.8 Hz, 2H; 2 × ArH), 5.82–5.75 (m, 1H; =CH), 4.99–4.89 (m, 2H; $=CH_2$), 4.19 (t, J = 4.8 Hz, 2H; OCH_2), 3.87 (t, 2H, J = 4.8 Hz, OCH_2), 3.72–3.40 (m, 12H; OCH_2), 3.41 (t, J = 6.4 Hz, 2H; OCH_2), 2.01 (dd, J = 6.8, 14 Hz, 2H; CH_2), 1.54 (q, J = 6.8 Hz, 2H; CH_2), 1.39–1.15 (m, 12H; 6 × CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 190.8 ppm (C=O), 163.8 (C), 139.2 (=CH), 131.9 (2 × CH), 129.8 (C), 114.8 (2 × CH), 114.1 ($=CH_2$), 71.5, 70.9, 70.6, 70.5, 70.0, 69.4, 69.2, 68.6, 67.7 (OCH_2), 33.8, 29.6, 29.5, 29.43, 29.39, 29.1, 28.9, 26.0; IR (KBr) ν: 2924 cm^{-1}, 2854, 1687, 1602, 1584, 1286, 1155, 1099, 836; UV (MeOH) λ_{max} (log ε): 284 nm (3.97), 222 (3.43), 204 (2.11); MS (EI, 70 eV) m/z (%): 450 (6) [M^+], 254 (17), 224 (30), 210 (23), 192 (8), 148 (100), 121 (38), 97 (22), 83 (32); HRMS m/z: 450.2988 (calcd for $C_{26}H_{42}O_6$: 450.2981).

4.2.4. 5-(4-(3,6,9,12-Tetraoxatricos-22-enyloxy)benzylideneamino)-6-amino-1,3-dimethyluracil (8)

Under an Ar atmosphere, a mixture of **6** (1.06 g, 2.4 mmol), 4,5-diamino-1,3-dimethyl uracil (**7**, 0.36 g, 2.1 mmol) and acetic acid

(0.1 mL) in ethanol (15 mL) was refluxed for 12 h. After this period, the reaction mixture was cooled at 20 °C, CH_2Cl_2 (20 mL) was added, and the solution was washed with water (20 mL) and brine (15 mL), dried over $MgSO_4$ and the solvent eliminated in vacuum. The crude of reaction was separated by cc eluting with EtOAc to obtain **8** as a yellowish syrup (0.55 g, 43%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.73 ppm (s, 1H; C=NH), 7.69 (d, J = 8.6 Hz, 2H; 2 × ArH), 6.92 (d, J = 8.6 Hz, 2H; 2 × ArH), 5.82–5.74 (m, 1H; =CH), 4.99–4.89 (m, 2H; $=CH_2$), 4.15 (t, J = 4.8 Hz, 2H; OCH_2), 3.85 (t, J = 4.8 Hz, 2H; OCH_2), 3.72–3.55 (m, 12H; 6 × OCH_2), 3.52 (s, 3H; NCH_3), 3.43 (t, J = 6.8 Hz, 2H; OCH_2), 3.37 (s, 3H; NCH_3), 2.03 (q, J = 7.2 Hz, 2H; CH_2), 1.55 (m, 2H; CH_2), 1.38 (m, 12H; 6 × CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 172.4 ppm (C=N), 160.2 (C), 158.2 (C=O), 152.9, 151.8 (C=O), 150.4 (=CNH_2), 139.2 (=CH), 131.4 (C), 128.8 (2 × CH), 114.6 (2 × CH), 114.1 (=CH_2), 71.5, 70.8, 70.6 (3 × CH_2), 70.5, 70.0, 69.6, 67.4, 33.8, 29.6, 29.5, 29.44, 29.40, 29.1, 28.9, 27.6, 26.0; IR (KBr) ν: 3164 cm^{-1}, 2923, 2854, 1688, 1649, 1106; UV (MeOH) λ_{max} (log ε): 282 nm (3.48), 204 (3.62); MS (EI, 70 eV) m/z (%): 603 (2) [M^+], 450 (12), 407 (7), 254 (20), 224 (39), 210 (27), 148 (100), 121 (28), 89 (31), 83 (40); HRMS m/z: 602.3672 (calcd for $C_{35}H_{50}N_4O_7$: 602.3679).

4.2.5. 8-(4-(3,6,9,12-Tetraoxatricos-22-enyloxy)phenyl)-theophylline (2)

Imine **8** (0.50 g, 0.83 mmol), was dissolved by refluxing in DME (10 mL). Then, DIAD (0.21 mL, 1.1 mmol) was added, and the mixture refluxed for 24 h. Then the mixture was cooled at 20 °C and the product **2** was obtained by crystallization from the reaction crude by addition of ethanol (5 mL) as a white solid (0.38 g, 77%), mp 195–196 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.18 ppm (d, J = 8.8 Hz, 2H; 2 × ArH), 6.97 (d, J = 8.8 Hz, 2H; 2 × ArH), 5.81–5.73 (m, 1H; =CH), 4.98–4.88 (m, 2H; $=CH_2$), 4.18 (t, J = 4.8 Hz, 2H; OCH_2), 3.88 (t, J = 4.6 Hz, 2H; OCH_2), 3.68 (s, 3H; NCH_3), 3.73–3.52 (m, 12H; 6 × OCH_2), 3.50 (s, 3H; NCH_3), 3.42 (t, J = 6.8 Hz, 2H; OCH_2), 2.00 (q, J = 7.2 Hz, 2H; CH_2), 1.54 (m, 2H; CH_2), 1.25 (m, 12H; 6 × CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 160.8 ppm (C), 155.5 (C=N), 151.8 (C-4), 151.5 (C=O), 149.9 (C=O), 139.2 (=CH), 128.5 (2 × CH), 121.4 (C), 114.8 (2 × CH), 114.0 (=CH_2), 107.4 (C-5), 71.5, 70.9, 70.61 (2 × OCH_2), 70.59, 70.56, 70.0, 69.6, 67.5, 33.8, 30.2, 29.6, 29.5, 29.43, 29.40, 29.1, 28.9, 28.4, 26.0; IR (KBr) ν: 3163 cm^{-1}, 2924, 2854, 1687, 1646, 1483, 1247, 1107; UV (MeOH) λ_{max} (log ε): 316 nm (3.20), 250 (2.04); MS (EI, 70 eV) m/z (%): 599 (2) [M^+], 355, 338, 323, 281, 238, 207; HRMS m/z: 623.3420 (calcd for $C_{32}H_{48}N_4NaO_7$: 623.3421).

4.3. Synthesis of 8-((2-(2-methoxyethoxy)ethoxy)methyl)theophylline (3)

4.3.1. *N*-(6-Amino-1,3-dimethyl-uracil-5-yl)-2-(2-(2-methoxyethoxy)ethoxy)acetamide (9)

Over a suspension of 4,5-diamino-1,3-dimethyl uracil (**7**, 715 mg, 4.2 mmol) in methanol (30 mL) was added 2-[2-(2-methoxyethoxy)ethoxy]acetic acid (0.69 mL, 4.5 mmol). The mixture of reaction was stirred at 20 °C for 30 min, and then *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC, 0.9 g, 4.7 mmol) was added and the mixture was stirred at 20 °C for 24 h. After this period, the solvent was removed under vacuum to obtain **9** (2.95 g, orange syrup). This compound was used in the next reaction without further purification; MS (EI, 70 eV) m/z (%): 330 (64) [M^+], 227 (30), 197 (100), 169 (85).

4.3.2. 8-((2-(2-Methoxyethoxy)ethoxy)methyl)theophylline (3)

A freshly prepared methanolic solution of MeONa in MeOH (Na 3.41 g, 148.1 mmol) was added to the oily amide **9**. The reaction mixture was kept at reflux for 4 h. It was then cooled to 40 °C, and water (5 mL) was added. The acidity was adjusted to acidic pH with 6 M HCl. The solid was filtered, the solution was concentrated to dryness under vacuum, and redissolved in $CHCl_3$ (40 mL). The organic solution was dried over $MgSO_4$ and concentrated to dryness. The solid residue was separated by cc eluting with CH_2Cl_2/MeOH 10:0.1 to obtain **3** as a yellowish solid (624 mg, 52%), mp 95–97 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.76–3.72 ppm (m, 10H; 5 × OCH_2), 3.56 (s, 3H; OCH_3), 3.45 (s, 3H; NCH_3), 3.40 (s, 3H; NCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 154.8 ppm (C=O), 151.9 (C=O), 151.0 (C-4), 148.8 (C-8), 107.0 (C-5), 71.7, 70.7, 70.6, 70.5, 66.4, 58.9, 30.0 (NCH_3), 28.1 (NCH_3); IR (KBr) ν: 3148 cm^{-1}, 1702, 1649, 1104, 993, 746; UV (MeOH) λ_{max} (log ε): 274 nm (1.82), 208 (3.81); MS (EI, 70 eV) m/z (%): 312 (18) [M^+], 209 (100), 193 (30); HRMS m/z: 312.1434 (calcd for $C_{13}H_{20}N_4O_5$: 312.1434).

4.4. Synthesis of 7-(3,6,9,12-tetraoxatricos-22-enyl)theophylline (10a)

60% NaH (84 mg, 2.1 mmol) was added over a solution of theophylline (**1a**, 342 mg, 1.9 mmol) in DMF (20 mL). After H_2 evolution ceased, a solution of **4** (1.90 g 3.8 mmol) in DMF (15 mL) was added, and the mixture of reaction was stirred at 20 °C for 12 h. After this period, the reaction was diluted with CH_2Cl_2 (40 mL), washed with water (3 × 30 mL) and brine (2 × 50 mL). The organic phase was dried over $MgSO_4$ and concentrated to dryness under vacuum. Compound **10a** was isolated by cc eluting with CH_2Cl_2/methanol 99:1 as a yellowish syrup (820 mg, 85%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.70 ppm (s, 1H; H-8), 5.81–5.74 (m, 1H; =CH), 4.98–4.88 (m, 2H; $=CH_2$), 4.74 (t, J = 4.6 Hz, 2H; OCH_2), 3.78 (t, J = 5 Hz, 2H; OCH_2), 3.62–3.53 (m, 12H; 6 × CH_2), 3.57 (s, 3H; NCH_3), 3.41 (t, J = 6.8 Hz, 2H; OCH_2), 3.37 (s, 3H; NCH_3), 2.01 (q, J = 6.8, 6.8 Hz, 2H; CH_2), 1.53 (m, 2H; CH_2), 1.29–1.24 (m, 12H; 6 × CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 152.0 ppm (C=O), 148.8 (C=O), 142.6 (C-8), 140.1 (C), 139.2 (=CH), 114.1 (=CH_2), 106.7 (C), 71.5, 70.59 (2 × CH_2), 70.57, 70.54, 70.4, 70.0, 69.4, 46.8, 33.8, 29.7, 29.6, 29.5, 29.43, 29.40, 29.1, 28.9, 27.9, 26.0; IR (KBr) ν: 2924 cm^{-1}, 2855, 1703, 1661, 1108; UV (MeOH) λ_{max} (log ε): 274 nm (1.25), 208 (3.98); MS (EI, 70 eV) m/z (%): 508 (14) [M^+], 312 (42), 282 (17), 250 (45), 224 (52), 207 (100), 180 (95); HRMS m/z: 508.2818 (calcd for $C_{26}H_{44}N_4O_6$: 508.2808).

4.5. Synthesis of 8-phenyl-7-(3,6,9,12-tetraoxatricos-22-enyl)theophylline (10b)

4.5.1. 8-Phenyltheophylline (1b)

Under an Ar atmosphere, a mixture of benzaldehyde (2.57 g, 24.2 mmol), 4,5-diamino-1,3-dimethyl uracil (**7**, 3.74 g, 22.0 mmol) and acetic acid (1 mL) in ethanol (60 mL) was refluxed for 12 h. After this period, the reaction mixture was cooled at 20 °C and a yellow solid appeared (needles). The solid was filtered off, washed with ethanol (2 × 5 mL) and ethyl ether (2 × 5 mL), and identified as 6-amino-5-(benzylideneamino)-1,3-dimethyluracil (4.61 g, 81%),[18] which was used in the next step without more purification; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.78 ppm (s, 1H; N=CH imine), 7.75 (d, J = 6.4 Hz, 2H; ArH), 7.38–7.36 (m, 3H; ArH), 5.71 (br s, 2H; NH_2), 3.50 (s, 3H; NCH_3), 3.37 (s, 3H; NCH_3); MS (EI, 70 eV) m/z (%): 258 (100) [M^+], 181 (17), 155 (32). Imine (1.24 g, 4.81 mmol) was dissolved by refluxing in DME (20 mL). Then, DIAD (1.24 mL, 6.25 mmol) was added, and after 5 min a white solid appeared. The reaction mixture was refluxed for 25 min more. After cooling at room temperature the solid was filtered and washed with cold EtOH (2 × 5 mL) and ethyl ether (2 × 5 mL) to obtain **1b** as a white solid (1.09 g, 82%);[14] ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.14 ppm (d, J = 6.4 Hz, 2H; ArH), 7.52–7.50 (m, 3H; ArH), 3.51 (s, 3H; NCH_3), 3.27 (s, 3H; NCH_3); MS (EI, 70 eV) m/z (%): 256 (100) [M^+].

4.5.2. 8-Phenyl-7-(3,6,9,12-tetraoxatricos-22-enyl)theophylline (10b)

The reaction was carried out as above for **10a** by using **1b** (0.90 g, 3.5 mmol), 60% NaH (0.16 mg, 3.9 mmol), **4** (5.27 g, 10.6 mmol) and DMF (70 mL). The reaction crude was purified by cc, eluting with CH_2Cl_2/methanol 99:1 to afford **10b** as a yellowish syrup (108.4 mg, 5%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.83–7.80 ppm (m, 2H; ArH), 7.49–7.47 (m, 3H; ArH), 5.83–5.73 (m, 1H; =CH), 4.99–4.89 (m, 2H; $=CH_2$), 4.47 (t, J = 5.2 Hz, 2H; OCH_2), 3.92 (t, J = 5.2 Hz, 2H; OCH_2), 3.62 (s, 3H; NCH_3), 3.64–3.50 (m, 12H; OCH_2), 3.42 (s, 3H; NCH_3), 3.40 (t, J = 6.8 Hz, 2H; OCH_2), 2.01 (q, J = 7.2 Hz, 2H; CH_2), 1.55–1.52 (m, 2H; CH_2), 1.36–1.24 (m, 12H; 6 × CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 155.2 ppm (C=N), 153.3 (C=O), 151.7 (C=O), 148.7 (C-4), 139.2 (=CH), 130.2 (CH), 129.9 (2 × CH), 128.7 (2 × CH), 128.6 (C), 114.1 ($=CH_2$), 107.6 (C), 71.4, 70.55, 70.54, 70.3, 70.1, 70.0, 69.9, 46.3, 33.7, 29.8, 29.55, 29.47, 29.40, 29.37, 29.1, 28.0, 26.0 (CH_2, NCH_3); IR (KBr) ν: 2924 cm^{-1}, 2854, 1700, 1660, 1106; UV (MeOH) λ_{max} (log ε): 292 nm (2.64), 228 (3.91), 208 (2.01); MS (EI, 70 eV) m/z (%): 585 (0.1) [M^+], 583 (0.5), 283 (15), 256 (38), 83 (79), 69 (85), 55 (100); HRMS m/z: 607.3476 (calcd for $C_{32}H_{48}N_4O_6Na$: 607.3472).

4.6. Synthesis of 7-(undec-10-enyl)theophylline (10c)

A solution of **1a** (1.80 g, 10 mmol), Et_3N (1.4 mL, 10 mmol) in dry CH_2Cl_2 (20 mL) was stirred under argon atmosphere at 20 °C for 20 min. Then 11-bromoundec-1-yl (2.4 mL, 11 mmol) was added dropwise. The mixture was refluxed for 4 h. After this period, dichloromethane (40 mL) was added, and the solution was washed successively with 1 M HCl (5 mL), satd $NaHCO_3$ (5 mL) and water (3 × 5 mL). Then the solution was dried over $MgSO_4$, filtered and the solvent eliminated in vacuum. The crude residue was separated by cc eluting with cyclohexane/CH_2Cl_2 6:4 and then CH_2Cl_2/MeOH 9.5:0.5, to obtain **10c** as a white solid (310 mg, 20%), mp 50–52 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.50 ppm (s, 1H; H-8), 5.82–5.72 (m, 1H; =CH), 4.98–4.88 (m, 2H; $=CH_2$), 4.25 (t, J = 7.0 Hz, 2H; NCH_2), 3.56 (s, 3H; NCH_3), 3.38 (s, 3H; NCH_3), 2.00 (q, J = 6.4, 6.4 Hz, 2H; CH_2), 1.84 (t, J = 6.4 Hz, 2H, CH_2), 1.28–1.23 (m, 12H; 6 × CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 154.8 ppm (C=O), 151.8 (C=O), 151.0 (C-8), 148.6 (C-4), 139.2 (=CH), 114.1 ($=CH_2$), 107.0 (C-5), 71.7, 70.66, 70.61, 70.5, 66.4, 58.9, 30.0 (NCH_3), 28.1 (NCH_3); IR (KBr) ν: 3100 cm^{-1}, 2919, 2850, 1644; UV (MeOH) λ_{max} (log ε): 274 nm (1.52), 212 (4.00); MS (EI, 70 eV) m/z (%): 332 (100) [M^+], 221 (20), 207 (24), 194 (34), 180 (90); HRMS m/z: 332.2210 (calcd for $C_{18}H_{28}N_4O_2$: 332.2212).

4.7. Synthesis of 7-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy) ethyl)theophylline (10e)

4.7.1. 7-(2-(2-(2-(2-Tosylethoxy)ethoxy)ethoxy)ethyl) theophylline (10d)

Compound **10d** was obtained by following the same procedure described for **10a** by using 60% NaH (0.244 g, 6.1 mmol), theophylline (1.0 g, 5.6 mmol) in DMF (55 mL) and tetra(ethylene glycol)ditosylate (5.0 g, 27.8 mmol) in DMF (70 mL). Compound **10d** was isolated by cc eluting with EtOAc/cyclohexanes 2:1 as a brown syrup (1.41 g, 50%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.77 ppm (d, J = 8.0 Hz, 2H; 2 × ArH), 7.69 (s, 1H; H-8), 7.31 (d, J = 8.0 Hz, 2H; 2 × ArH), 4.47–3.54 (m, 16H; 8 × OCH_2), 3.53 (s, 3H; NCH_3), 3.37 (s, 3H; NCH_3), 2.41 (s, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 155.7 ppm (C=O), 151.5 (C=O), 149.1 (C), 145.1, 142.9 (C-8), 142.0 (C), 130.1, 128.3 (CH), 106.8 (C-5), 71.0, 70.8, 70.7, 69.71, 69.69, 69.0.4 (CH_2), 47.1 (CH_2N), 30.1 (NCH_3), 28.2 (NCH_3), 21.9 (CH_3); IR (KBr) ν: 2869 cm^{-1}, 1701, 1656; UV (MeOH) λ_{max} (log ε): 274 nm (1.48), 208 (3.95); HRMS m/z: 533.1700 (calcd for $C_{22}H_{30}N_4NaO_8S$: 533.1682).

4.7.2. 7-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl)theophyl line (10e)

A solution of **10d** (1.4 g, 2.75 mmol), NaN_3 (0.218 g, 3.4 mmol) in DMF (30 mL) was stirred at 60 °C for 12 h. After this period, the reaction mixture concentrated to dryness under vacuum, and then CH_2Cl_2 (100 mL) was added. The solution was washed with water (3 × 100 mL) and brine (100 mL), dried over $MgSO_4$, filtered and the solvent removed in vacuum. The residue was purified by cc eluting with EtOAc, to obtain **10e** as a yellowish syrup (0.34 g, 33%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.69 ppm (s, 1H; H-8), 4.49–3.58 (m, 16H; 8 × OCH_2), 3.62 (s, 3H; NCH_3), 3.39 (s, 3H; NCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 155.4 ppm (C=O), 151.7 (C=O), 148.8, 142.5 (C-8), 106.4 (C-5), 70.7, 70.6, 70.53, 70.49, 70.0, 69.4 (6 × CH_2), 50.6 (CH_2N), 46.8 (CH_2N_3), 29.8 (NCH_3), 27.9 (NCH_3); IR (KBr) ν: 2869 cm^{-1}, 2099, 1701, 1654; UV (MeOH) λ_{max} (log ε): 272 (4.26), 224 nm (3.88); MS (EI, 70 eV) m/z (%): 381 (11) [M^+], 339 (60), 160 (100); HRMS m/z: 404.1667 (calcd for $C_{15}H_{23}N_7NaO_5$: 404.1658).

4.8. Radioligand binding competition assays

4.8.1. Human A_1 receptors

Adenosine A_1 receptor competition binding experiments were carried out in membranes from CHO-A_1 cells (Euroscreen, Gosselies, Belgium). On the day of assay, membranes were defrosted and re-suspended in incubation buffer 20 mM Hepes, 100 mM NaCl, 10 mM $MgCl_2$, 2 units/mL adenosine deaminase (pH 7.4). Each reaction well of a GF/C multiscreen plate (Millipore, Madrid, Spain), prepared in duplicate, contained 15 μg of protein, 2 nM [^{3}H]DPCPX and test compound. Non-specific binding was determined in the presence of 10 μM (*R*)-PIA. The reaction mixture was incubated at 25 °C for 60 min, after which samples were filtered and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain). A concentration–response curve of xanthin amino congener (XAC) was carried out in all the assays as an internal control (K_i = 23.2 ± 3 nM)

4.8.2. Human A_{2A} receptors

Adenosine A_{2A} receptor competition binding experiments were carried out in membranes from HeLa-A_{2A} cells. On the day of assay, membranes were defrosted and re-suspended in incubation buffer 50 mM Tris–HCl, 1 mM EDTA, 10 mM $MgCl_2$ and 2 UI/mL adenosine deaminase (pH 7.4). Each reaction well of a GF/C multiscreen plate (Millipore, Madrid, Spain), prepared in duplicate, contained 10 μg of protein, 3 nM [^{3}H]ZM241385 and test compound C0036E08. Non-specific binding was determined in the presence of 50 μM NECA. The reaction mixture was incubated at 25 °C for 30 min, after which samples were filtered and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain). A concentration–response curve of CGS15943 was carried out in all the assays as an internal control (K_i = 2.27 ± 0.5 nM).

4.8.3. Human A_{2B} receptors

Adenosine A_{2B} receptor competition binding experiments were carried out in membranes from HEK-293-A_{2B} cells (Euroscreen, Gosselies, Belgium) prepared following the provider's protocol. On the day of assay, membranes were defrosted and re-suspended in incubation buffer 50 mM Tris–HCl, 1 mM EDTA, 10 mM $MgCl_2$, 0.1 mM benzamidine, 10 μg/mL bacitracine and 2 UI/mL adenosine deaminase (pH 6.5). Each reaction well prepared in duplicate, contained 18 μg of protein, 35 nM [^{3}H]DPCPX and test compound.

Non-specific binding was determined in the presence of 400 μM NECA. The reaction mixture was incubated at 25 °C for 30 min, after which samples were filtered through a multiscreen GF/C microplate and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain). A concentration–response curve of ZM241385 was carried out in all the assays as an internal control (K_i = 19.3 ± 2.4 nM).

4.8.4. Human A_3 receptors

Adenosine A_3 receptor competition binding experiments were carried out in membranes from HeLa-A_3 cells. On the day of assay, membranes were defrosted and re-suspended in incubation buffer 50 mM Tris–HCl, 1 mM EDTA, 5 mM $MgCl_2$ and 2 UI/mL adenosine deaminase (pH 7.4). Each reaction well of a GF/B multiscreen plate (Millipore, Madrid, Spain), prepared in triplicate, contained 90 μg of protein, 30 nM [^{3}H]NECA and test compound. Non-specific binding was determined in the presence of 100 μM (*R*)-PIA. The reaction mixture was incubated at 25 °C for 180 min, after which samples were filtered and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain). A concentration–response curve of MRS1220 was carried out in all the assays as an internal control (K_i = 2.48 ± 0.37 nM).

Concentration–response binding competition curves were carried out by assaying different concentrations (range between 0.1 nm and 1 mM) of the compounds. Data were fitted by non-linear regression using GraphPad Prism v2.01 (GraphPad Software). The inhibition constant (K_i) of each compound was calculated by the Cheng–Prusoff expression $K_i = IC_{50}/(1 + (C/KD))$,[36] where IC_{50} is the concentration of compound that displaces the binding of radioligand by 50%, *C* is the free concentration of radioligand and KD is the apparent dissociation constant of the radioligand.

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