SYNTHESIS AND CHARACTERIZATION OF DMAP-MODIFIED NPY Y_1 RECEPTOR ANTAGONISTS AS ACYL-TRANSFER CATALYSTS

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Dedicated to Professor Pavel Kočovský on the occasion of his 60th birthday.

Starting from the working hypothesis that specific chemical labelling may be an attractive approach to detect and study G protein-coupled receptors (GPCRs) we synthesized catalytically active antagonists of the neuropeptide Y_1 receptor (Y_1R). An argininamide-type Y_1R antagonist scaffold was combined with a DMAP moiety via spacers of different length and chemical nature. These hybrid compounds have Y_1R affinities in the two-digit nanomolar range and are capable of catalysing acyl-transfer reaction to surrogates of bionucleophiles, as demonstrated in the absence of cells by using esters of fluorescent dyes as substrates in buffer. By contrast, selective staining of Y_1Rs on living MCF-7 cells was not achieved due to significant non-catalysed (Y_1R ligand independent) reaction with biomolecules and the limited density of Y_1R on the cell surface. Although this may also depend on insufficient selectivity of the staining reagents, the results of this study suggest that the general applicability of catalytic staining to GPCRs has to be reconsidered, as this approach is hampered by a very low portion of receptor of interest compared to the total amount of membrane proteins.
Keywords: Medicinal chemistry; Acylation; Molecular recognition; Neuropeptide Y; Antagonist.

G protein-coupled receptors (GPCR) represent one of the major classes of biological targets in drug research[1]. Therefore, there is a need not only for selective agonists and antagonists of special GPCRs but also for novel strategies to detect and study such receptors. Apart from eliciting intracellular responses, stimulation of a GPCR with an agonist results in desensitization. After phosphorylation of serine and threonine residues, arrestin binding, internalisation and down-regulation of receptors may occur[2]. Thus, sus-

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tained drug-induced receptor activation can result in a significant decrease or complete loss of the cellular response. Fluorescent antibodies against extracellular epitopes of the receptor protein were applied to visualize internalisation[3]. However, appropriate antibodies are not always available, and these macromolecules can interfere with ligand binding and receptor functionality[3]. Expression of fusion proteins comprising the receptor of interest and, e.g., the yellow fluorescent protein may alter structure and function of the protein significantly[4]. The introduction of specific tags that are recognized with high specificity by functionalized dyes leading to non-covalent labelling is another successfully applied strategy, which again requires protein modification[5–7].

A chemical reaction in close proximity to the active site of a receptor leading to labelling of the protein with a fluorescent dye would be a convenient approach, provided that such a reaction takes places with high selectivity and efficiency. Recently, Hamachi et al. reported the chemical labelling of lectins using a catalytically active carbohydrate ligand[8]. In an attempt to apply such an approach to membrane receptors, we selected neuropeptide Y (NPY) receptors as a model system of a class A GPCR. Four subtypes of NPY receptors (Y_1, Y_2, Y_4 and Y_5) are functionally expressed in humans. Here we report the synthesis of antagonists for the NPY Y_1 receptor that catalyse acylation reactions of nucleophiles in buffers.

4-Dimethylaminopyridine (DMAP) is a well known nucleophilic acyltransfer catalyst that was successfully applied for target-specific acylations[8,9]. In our investigations, DMAP was connected to the potent argininamide-type Y_1R antagonist BIBP 3226 [10] (Table I, compound **1**) to obtain catalyti-

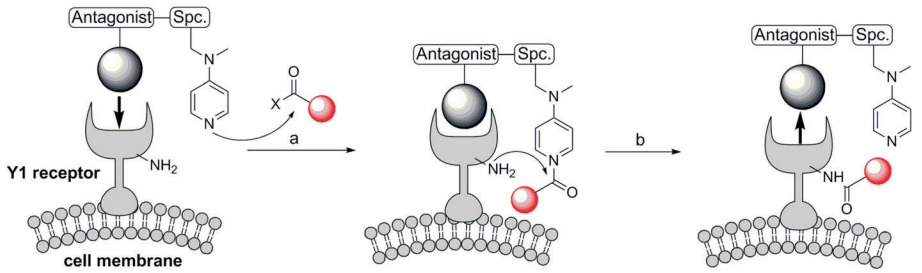
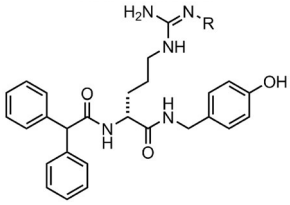


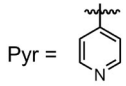
FIG. 1
Illustration of the working hypothesis aiming at a catalytic staining of the Y_1R. The Y_1R structure is simplified in this scheme; it is a heptahelical GPCR with a transmembrane binding pocket for nonpeptidic antagonists such as BIBP 3226. In a, the DMAP moiety of a catalytic antagonist activates a fluorescent active ester. A nucleophilic amino acid (e.g. lysine) of the receptor is acylated in step b

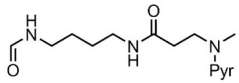
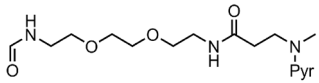
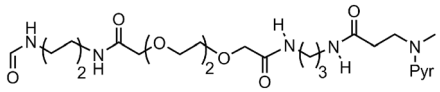
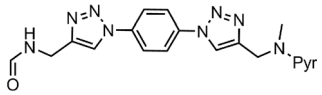
cally active Y_1R ligands. The BIBP 3226 skeleton tolerates acylation at the terminal guanidine nitrogen (N^G) with moderate decrease or even gain of affinity[11–14]. Catalytic Y_1R specific staining should be achieved by addition of a fluorescent active ester (Fig. 1)[8].

TABLE I

Structures and Y_1R binding data of compounds **1a–1e**. K_i values were determined from the displacement of [^{3}H]-UR-MK114 [16] (1.5 nM) using Y_1R expressing SK-N-MC cells; K_i values represent mean values ± SEM from two or three independent experiments



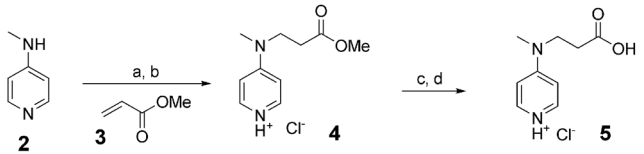


No.	R	K_i, nM
1	H	1.3 ± 0.2
1a		53 ± 13
1b		96 ± 20
1c		90 ± 6
1d		78 ± 0.4
1e		400 ± 19

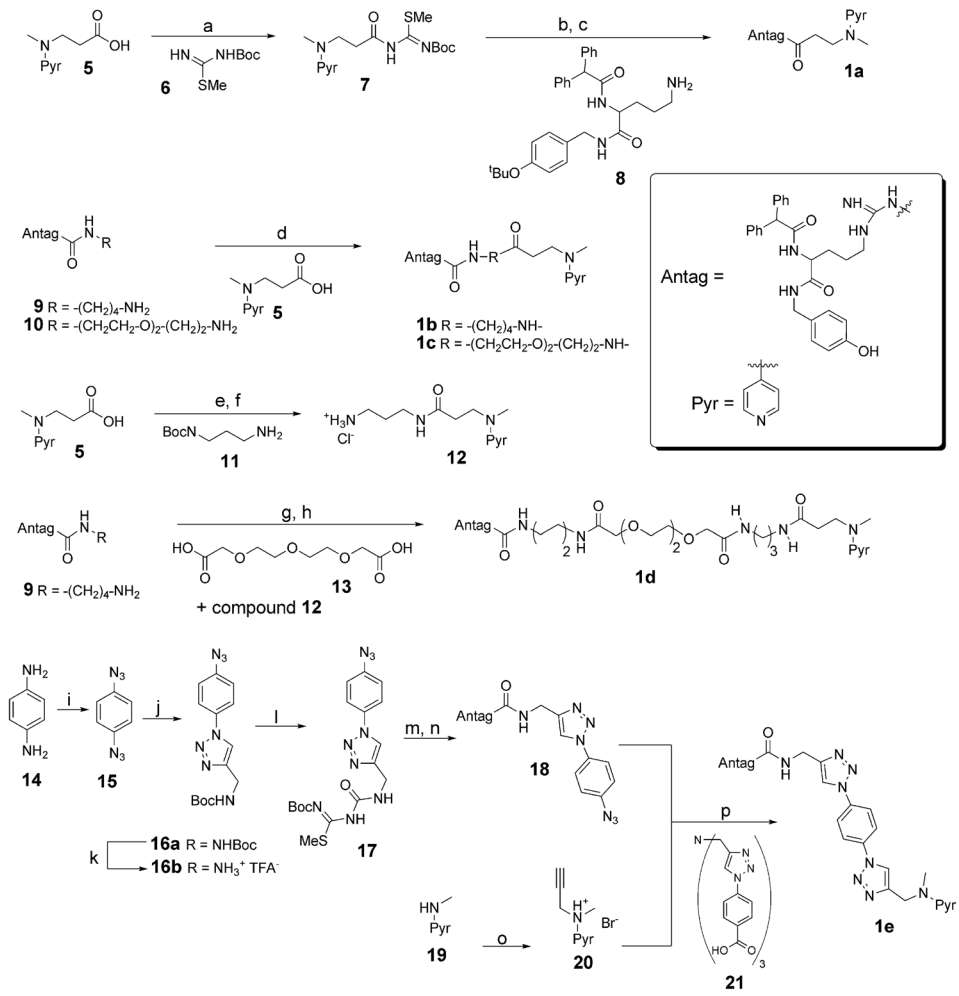
RESULTS AND DISCUSSION

Synthesis and Binding Affinity of Putative Catalytically Active Y_1R Antagonists

Previously, we demonstrated that N^G-acylation of BIBP 3226 (**1**) is tolerated in terms of Y_1R affinity[12]. In addition, the catalytic activity of *N*-substituted DMAP derivatives is comparable to that of the parent compound[15]. Therefore, hybrid compounds (Table I) combining Y_1R antagonism with catalytic activity were constructed by connecting the guanidine group in **1** with DMAP by linkers of different length and chemical nature. Synthesis started from 4-aminopyridine **2** which was converted to the acid **5** in two steps (Scheme 1). Acid **5** was converted to a guanidinylation reagent (**7**) or coupled to amino-functionalized acylated argininamides via different spacers as depicted in Scheme 2. Compound **1a** was synthesized from *N*-Boc-*S*-methylisothiourea **6** via acylation affording **7**, followed by guanidinylation of the ornithine derivative **8**. The synthesis was performed in analogy to a previously described procedure[13,14]. Compounds **1b** and **1c** were obtained from the precursor amines **9** and **10** by coupling with the acid **5** in moderate yield. The compound **1d** was also obtained from precursor amine **9**, which was coupled in a one pot reaction with the dicarboxylic acid **13** and amine **12**. Amine **12** was synthesized from acid **5** and the mono-Boc protected propane-1,3-diamine **11**. The conformationally constrained ligand **1e** was synthesized starting from 1,4-phenylenediamine, which was converted to the diazide **15** and subjected to a copper catalysed "click-reaction" yielding amine **16b**. Coupling to *N*-Boc-*S*-methylisothiourea and guanidinylation of compound **8** resulted in azide **18**. The latter was allowed to react with the propargyl derivative **20** to give the conformationally constrained Y_1R antagonist **1e**. The copper(I) ligand **21** was used to form the active catalyst for the reaction.



SCHEME 1
Synthesis of the precursor **5** of the catalytic substructure: methyl acrylate, reflux (a); HCl, 76% (b); NaOH, MeOH (c); HCl, 30% (d)



SCHEME 2

Synthesis of Y_1R antagonists **1a–1e**: EDC, HOBt, DIPEA, DCM, 75% (a); DMF, $HgCl_2$, NEt_3, 36% (b); TFA/DCM 1:1, quantitative yield (c); DMF, DIPEA, TBTU, 67% (**1b**), 47% (**1c**) (d); MeCN, DIPEA, DCC (e); MeOH/HCl 3:1, 36% (f); DMF, DIPEA, TBTU (g); MeCN, TFA, 16% (h); $NaNO_2$, H_2SO_4, NaN_3, 66% (i); Boc-propargylamine, $CHCl_3$/MeOH/H_2O, ascorbate, copper sulfate pentahydrate, 79% (j); DCM/TFA, quantitative (k); triphosgene, DCM, DIPEA; *N*-Boc-*S*-methyl-isothiourea, DCM, 46% (l); Compound **8**, DMF, NEt_3, $HgCl_2$, 57% (m); DCM/TFA, quantitative (n); THF, n-BuLi, propargylbromide, 10% (o); DMSO, MeOH, H_2O, ascorbate, copper sulfate pentahydrate, 51% (p). Compound **21** is added as a ligand for the copper-catalysed cycloaddition reaction

Synthesis and Hydrolytic Stability of Active Esters

For the catalytic staining reaction of the receptors we prepared active esters that are easily activated with DMAP. Carboxyfluorescein and cyanine dyes were modified for their application as acyl-donors (Fig. 2). Compound **23** was prepared according to the procedure of Hamachi and coworkers[8]. The phenyl thioester **26** and the *p*-nitrophenyl ester **27** were synthesized from the commercially available cyanine dye **24** in one coupling step.

The hydrolysis kinetics of the active esters **23**, **25** and **27** and the ability of the Y_1R ligands to catalyse acyl-transfer to simple nucleophiles was investigated in buffered aqueous solution at pH 7.4. For monitoring of the hydrolysis kinetics the dyes were incubated at 50 μM for 3 h in phosphate buffer (30 mM) at pH 7.4. In a second series they were incubated with additional 50 μM of DMAP. Samples were taken RF 30, 90 and 150 min. The amount of decomposition was determined with HPLC (Fig. 3).

The acceleration of the hydrolysis in the presence of DMAP was most significant for phenyl thioester **23** (about 400% RF 150 min) and only moderate for *p*-nitrophenyl ester **27** (60%) and succinimidyl ester **25** (48%). Although hydrolysis rates are not directly comparable with acyl-transfer reaction rates to other nucleophiles, the results indicate that the thioester is more suitable than the *p*-nitrophenol and the NHS ester. The active ester reactivity correlates with the acidity of the corresponding acids: pK_a(NHS): 6.0 [17], pK_a(*p*-nitrophenol): 7.4 [17], and pK_a(thiophenol): 8.3 [18].

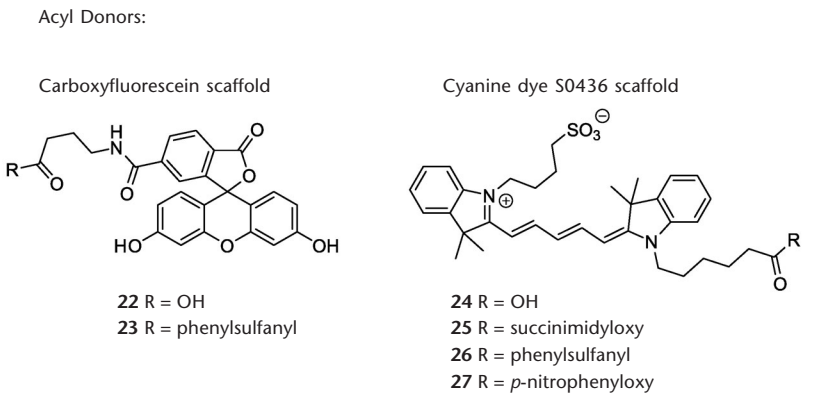


FIG. 2
Structures of the carboxyfluorescein and cyanine dyes used as carboxylic acid derivatives for catalytic staining

Acyl-Transfer Studies

Intermolecular interactions at the cell surface are rather complex. Therefore we studied first the catalysed acyl-transfer to surrogates of bionucleophiles in buffered solution to optimize the reaction conditions. Incubation of catalyst **1a** and active ester **25** in aqueous solution and subsequent HPLC analysis showed the complete decomposition of **1a** to compound **30** RF an incubation time of 3 h. As described in literature certain acyl-guanidines are labile against intra-molecular cleavage[14,19]. We assume the formation of

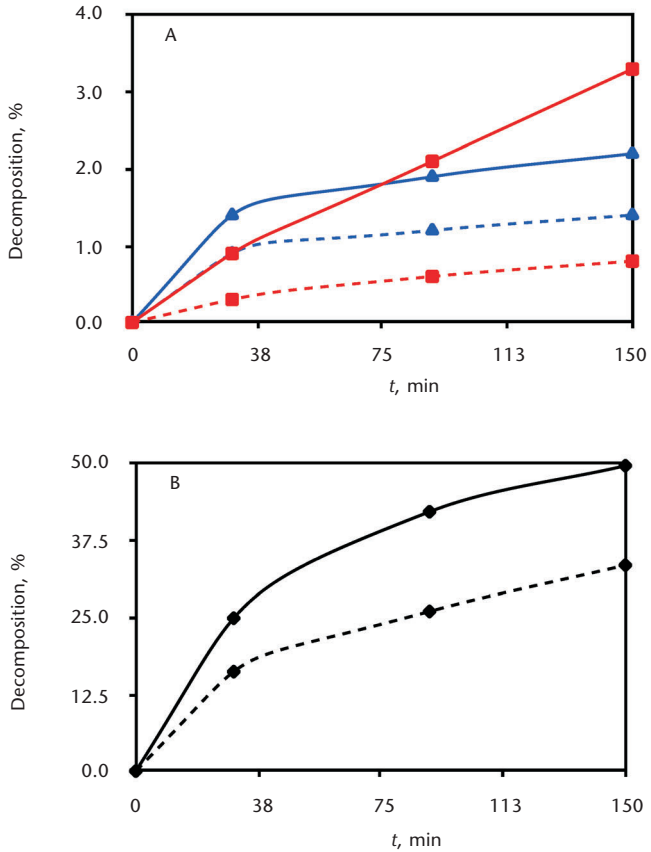
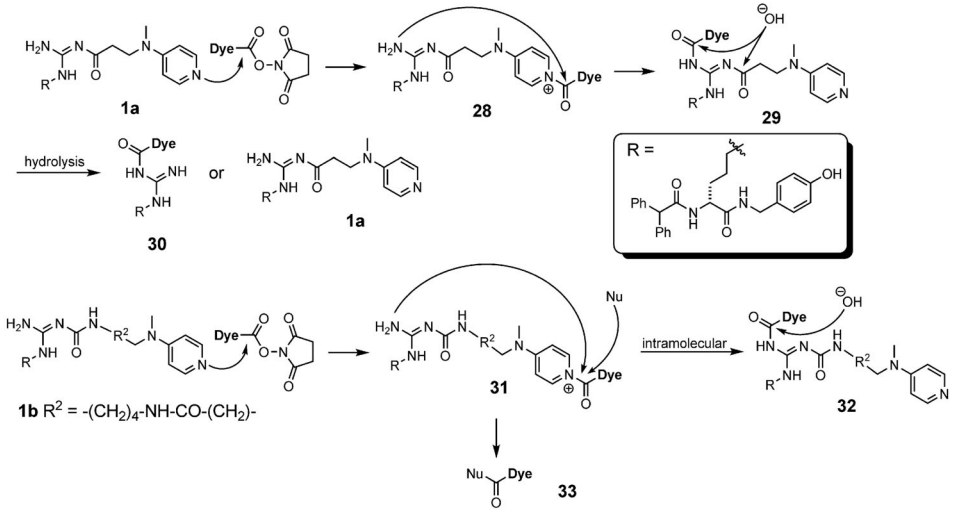


FIG. 3
Diagram A: Hydrolysis of phenyl thioester **23** (red) and *p*-nitrophenyl ester **27** (blue), both 50 μM in presence (solid line) and absence (dashed line) of 50 μM DMAP. Diagram B: Hydrolysis graph of succinimidyl ester **25** (50 μM) in presence (solid line) and absence (dashed line) of DMAP

diacylguanidine **29** (Scheme 3) and fast hydrolysis of this labile intermediate[20]. In contrast, compound **1b** was not degraded during incubation with the active ester. The carbamoylguanidine substructure is more resistant against cleavage, because of the electron donating effect of the amide N-atom. After an incubation period of 3 h, compounds **1b** and **32** were detected by LC-MS. Thus acyl-guanidine **1a** was not applicable for the staining reaction, but the more stable carbamoylguanidines **1b–1e** are promising compounds. However, the fast intramolecular self-acylation of these compounds competes with the acyl-transfer to other nucleophiles and increases the rate of hydrolysis of the active ester. The catalytic Y_1R antagonist **1e** was designed to circumvent this problem. The conformationally constrained spacer connecting the DMAP moiety and the BIBP 3226 scaffold prevents intramolecular self-acylation.

Catalytic acyl-transfer to small nucleophiles was investigated for the Y_1R antagonists **1b–1e**. 2-(Dimethylamino)ethylamine (DMAEA), butanol and BIBP 3226 (BIBP) were used as mimics of nucleophilic residues in proteins (DMAEA for lysine, butanol for serine and threonine, BIBP 3226 for arginine and tyrosine). As an example of an acyl-donor, the succinimidyl ester **25**, was incubated with catalyst and nucleophile for 30 min. The acylation products were detected by LC-MS coupling. The acyl-transfer experiments were carried out with the succinimidyl ester **25**, because the



SCHEME 3
Acylation of the Y_1R antagonists **1a** and **1b** with succinimidyl ester **25**

thioesters were not suitable for the subsequent receptor staining investigations on cells in section Catalytic Staining of MCF-7 Cells.

The rate of hydrolysis of active ester **25** (cf. entries 1–15 in Table II) was accelerated when catalysed (entries 2–6) compared the uncatalysed reaction (entry 1). Antagonists **1b–1e** were even more active than DMAP and consumed succinimidyl ester 25 completely (entries 3–5) or almost completely (entry 6). Acyl-transfer to BIBP 3226 was increased by adding the catalysts (entries 8–11) compared to the uncatalysed reaction (entry 7). The sterically hindered catalyst **1e** still forms an appreciable amount of the acylated cata-

TABLE II
Catalysed and non-catalysed acyl-transfer of succinimidyl ester **25** to small nucleophiles (product yields were not quantified, the peak heights show only detected products and reaction tendencies)

Entry	Active ester	Catalyst	Nucleophile	% rel. peak height after 30 min incubation time[a]			
				free acid (hydrolysis product)	active ester	acylated catalyst (**32**)	nucleophile-dye (product) structure **33**
1	**25**	–	water	79	21	–	–
2	**25**	DMAP	water	92	8	–	–
3	**25**	**1b**	water	78	0	22	–
4	**25**	**1c**	water	83	0	17	–
5	**25**	**1d**	water	83	0	17	–
6	**25**	**1e**	water	75	4	21	–
7	**25**	–	BIBP	57	19	–	24
8	**25**	**1b**	BIBP	55	0	11	34
9	**25**	**1c**	BIBP	47	0	13	40
10	**25**	**1d**	BIBP	45	0	15	40
11	**25**	**1e**	BIBP	42	0	8	50
12	**25**	–	DMAEA	58	13	–	29
13	**25**	**1e**	DMAEA	74	0	13	13
14	**25**	–	n-butanol	77	23	–	–
15	**25**	**1e**	n-butanol	82	4	14	–

[a] Relative fraction of the compounds in percent (the sum of all four peak heights is 100).

lyst (entries 6, 11, 13, 15). The primary alcohol n-butanol was not attacked by the active ester (entry 14, 15) due to its weakly nucleophilic properties.

The catalysts **1b–1e** are promising prototypical ligands for the catalytic staining of GPCRs despite BIBP acylation in the absence of the biological target, as the BIBP 3226 scaffold binds tightly to the Y_1R binding pocket, which should prevent this process.

Catalytic Staining of MCF-7 Cells

In principle, the active esters **23**, **25–27** can react with cellular nucleophiles in the absence of a DMAP moiety. Therefore, we first determined the extent of non-catalysed staining of MCF-7 cells. The cells were treated with 10 μM of active ester for 15–60 min in PBS (pH 7.4). The thioesters **23** and **26** were not applicable for the staining of the Y_1R of MCF-7 cells under these conditions, because they pass the cell membrane and accumulate inside the cell. The *p*-nitrophenyl ester **27** penetrated the membrane to a significantly lower extent, and the succinimidyl ester **25** proved to be the best suited active ester, as intracellular accumulation was not observed.

For the catalytic staining reaction the cells were pre-incubated with the corresponding Y_1R antagonist, washed with buffer and incubated with the succinimidyl ester **25**. The Y_1R retained their functional activity as confirmed by a FURA-2 functional assay (data not shown). However, compared to the uncatalysed staining reaction, there was no significant difference detectable by confocal microscopy (data not shown). Obviously, Y_1R-independent unselective, i.e. uncatalysed, staining prevails due to a very high abundance of nucleophilic groups other than those of Y_1Rs at the cell membrane.

CONCLUSION

The results of this study suggest that more selective staining reagents are required for further investigations. Although MCF-7 cells express a rather high number of Y_1Rs (approximately 300,000 sites/cell), the portion of Y_1Rs related to the total amount of membrane proteins is very low (<0.1% of the total membrane protein). Therefore, a very high ratio of the catalysed acylation reactions compared to the non-catalysed background reaction must be achieved to allow for the envisaged selective staining. The currently available DMAP-modified receptor antagonists do not provide the required selectivity.

EXPERIMENTAL

General Experimental Conditions

Unless otherwise noted, solvents (analytical grade) were purchased from commercial suppliers and used without further purification. Ethyl acetate (EA), petrol ether (PE; 60–70 °C), methanol and dichloromethane were obtained in technical grade and distilled before application. Acetonitrile (MeCN) for HPLC was obtained from Merck (Darmstadt, Germany). Cyanine dye S0436 (**24**) and NHS ester S0536 (**25**, FEW Chemicals, Bitterfeld–Wolfen, Germany), 4-(methylamino)pyridine (Alfa Aesar, Karlsruhe, Germany), methyl acrylate (Merck, Darmstadt, Germany), 3,6,9-trioxaundecanedioic acid (Fluka, Sigma–Aldrich Chemie GmbH, Munich, Germany), 1,4-diaminobenzene (Fluka, Sigma–Aldrich Chemie GmbH, Munich, Germany), butyl lithium (Fluka, Sigma–Aldrich Chemie GmbH, Munich, Germany) and propargyl bromide (Fluka, Sigma–Aldrich Chemie GmbH, Munich, Germany) were purchased. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Nucleodur 100-5 C18 (250 × 21 mm, 5 μm; Macherey–Nagel, Germany) and a Eurospher-100 C18 (250 × 32 mm, 5 μm; Knauer, Germany) served as RP-columns at flow rates of 20 and 38 ml/min, respectively. Mixtures of MeCN and diluted aqueous TFA (0.1%) were used as mobile phase. ^{1}H NMR spectra were recorded at 300 MHz on a Bruker Avance 300 spectrometer or at 600 MHz on a Bruker Avance III 600 with cryogenic probehead (Bruker, Karlsruhe, Germany). ^{13}C NMR spectra were recorded at 75 MHz on a Bruker Avance 300 spectrometer. All chemical shifts values are reported in ppm (δ-scale), coupling constants *J* in Hz. UV/VIS spectra were recorded with a Varian Cary BIO 50 UV/VIS/NIR spectrophotometer (Varian Inc., CA, USA). Mass spectra: Finnigan SSQ 710A (EI), Finnigan MAT 95 (CI), Finnigan MAT TSQ 7000 (Thermo FINNIGAN, USA) (ES/LC-MS). LC-system for LC-MS: Agilent 1100 (Palo Alto, USA). LC-MS method I (LC-MS-I): Column: Phenomex Luna C18, 3.0 μm, 100 × 2 mm HST (Phenomenex, Aschaffenburg, Germany); flow: 0.30 ml/min; solvent A (water + 0.1% TFA), solvent B (MeCN); gradient: 0 min [A/B 95/5], 1 min [A/B 95/5], 11 min [A/B 2/98], 18 min [A/B 2/98], 19 min [A/B 95/5], 24 min [A/B 95/5]. LC-MS method II: Column: Phenomex Luna C18, 2.5 μm, 50 × 2 mm HST (Phenomenex, Aschaffenburg, Germany); flow: 0.40 ml/min; solvent A (water + 0.1% TFA), solvent B (MeCN); gradient: 0 min [A/B 95/5], 8 min [A/B 2/98], 11 min [A/B 2/98], 12 min [A/B 95/5], 15 min [A/B 95/5]. Melting points were determined with a Lambda Photometrics Optimelt MPA100 apparatus (Lambda photometrics, Harpenden, UK), they are not corrected. Thin layer chromatography (TLC) was performed on alumina plates coated with silica gel (Merck silica gel 60 F_{245}, thickness 0.2 mm). Column chromatography (CC) was performed with Merck Geduran SI 60 silica gel as the stationary phase.

Compounds **4** [15], **5** [15], **6** [13], **8** [16], **9** [20], **10** [20], **11** [21], **21** [22,23], **22** [8], **23** [8] and Boc-propargylamine[24] were prepared according to literature procedures. The synthesis of literature known compound **15** [25] was improved.

tert-Butyl-{3-[methyl(pyridin-4-yl)amino]propanamido}(methylthio)-methylenecarbamate (**7**)

Acid **5** (216 mg, 1.00 mmol), DIPEA (258 mg, 2.00 mmol) and HOBt (149 mg, 1.10 mmol) were dissolved in 10 ml cold DCM and EDC (171 mg, 1.10 mmol) was added. After 15 min, compound **6** (190 mg, 1.00 mmol) was added. The mixture was stirred overnight. Next day

the reaction mixture was concentrated and the crude product was purified with column chromatography (EA/EtOH 1:1, R_F 0.1). Compound **7** (323 mg, 75%) was obtained as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): 1.48 s, 9 H; 2.36 s, 3 H; 2.79 t, 2 H, *J* = 6.87; 3.14 s, 3 H; 3.83 t, 2 H, *J* = 6.88; 6.65–6.75 m, 2 H; 8.10–8.20 m, 2 H; 9.00–13.00 m, 2 H. ^{13}C NMR (75 MHz, $CDCl_3$): 14.7, 27.9, 31.7, 38.6, 48.0, 79.9, 106.8, 141.5, 141.8, 143.5, 156.0, 170.8. $C_{16}H_{24}N_4O_3S$: MS (LC-MS-I): *m/z* (%) [t_r = 8.1 min]: 353 (100, MH^+), 705 (5).

(*R*)-4-((3-((4-(2,2-Diphenylacetamido)-5-(4-hydroxybenzylamino)-5-oxopentyl)guanidino)-3-oxopropyl)(methyl)amino)pyridinium 2,2,2-Trifluoroacetate (**1a**)

Compound **7** (123 mg, 0.35 mmol) was dissolved in 4 ml DMF and mixed with compound **8** (170 mg, 0.35 mmol). NEt_3 (0.5 ml) was added and then a solution of $HgCl_2$ (95 mg, 0.35 mmol) in 1 ml DMF was dropped into the reaction mixture. The mixture was stirred overnight. The next day DMF was evaporated under high vacuum and DCM (5 ml) was added. The insoluble mercury salts were filtered off and the solution was concentrated. The crude material was purified with column chromatography (EA/EtOH 1:1).

The product was deprotected without further characterisation: a mixture of DCM/TFA (1:1; 4 ml) was added and the solution was stirred for 2 h. Then it was concentrated and purified with HPLC. A white solid was obtained (109 mg, 36%), m.p. > 190 °C (decomp.). ^{1}H NMR (300 MHz, CD_3CN): 1.40–1.90 m, 4 H; 2.78 t, 2 H, *J* = 7.05; 3.15 s, 3 H; 3.15–3.30 m, 2 H; 3.85 t, 2 H, *J* = 7.06; 4.10–4.30 m, 2 H; 4.30–4.40 m, 1 H; 5.02 s, 1 H; 6.60–7.20 m, 4 H; 6.71 d, 2 H, *J* = 8.54; 7.03 d, 2 H, *J* = 8.50; 7.20–7.35 m, 10 H; 7.96 bs, 2 H; 9.47 bs, 1 H; 9.95 bs, 1 H; 12.13 bs, 1 H; 13.37 bs, 1 H. $C_{36}H_{43}N_7O_4$: MS (LC-MS-I): *m/z* (%) [t_r = 7.6 min]: 318 (100), 339 (35), 636 (50, MH^+).

(*R*)-4-((4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-9-imino-3,11,18-trioxo-2,8,10,12,17-pentaazaicosan-20-yl)(methyl)amino)pyridinium 2,2,2-Trifluoroacetate (**1b**)

The acid **5** (5.6 mg, 26 μmol) was dissolved in DMF (2 ml) and DIPEA (28 mg, 0.21 mmol) and TBTU (8.3 mg, 26 μmol) were added. After 10 min, amine **9** (20 mg, 22 μmol) was added. The mixture was stirred overnight. The next day DMF was removed under high vacuum and the oily residue was purified with HPLC. A white solid was obtained (14 mg, 67%), m.p. > 190 °C (decomp.). ^{1}H NMR (300 MHz, CD_3CN): 1.30–1.80 m, 8 H; 2.00–2.80 m, 2 H; 3.10 s, 3 H; 3.10–3.30 m, 6 H; 3.80 t, 2 H, *J* = 6.45; 4.10–4.40 m, 3 H; 5.03 s, 1 H; 6.50–7.90 m, 8 H; 6.67 d, 2 H, *J* = 8.45, 2 H; 7.00 d, 2 H, *J* = 8.45; 7.20–7.35 m, 10 H; 7.90–8.05 m, 2 H; 9.00–9.20 bs, 1 H; 11.90–13.00 m, 1 H. $C_{41}H_{51}N_9O_5$: MS (LC-MS-II): *m/z* (%) [t_r = 4.9 min]: 375 (100), 750 (30, MH^+).

(*R*)-4-((4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-9-imino-3,11,22-trioxo-15,18-dioxa-2,8,10,12,21-pentaazatetracosan-24-yl)(methyl)amino)pyridinium 2,2,2-Trifluoroacetate (**1c**)

Acid **5** (3.0 mg, 13.7 μmol) was dissolved in DMF (2 ml) and DIPEA (14.7 mg, 114 μmol) and TBTU (4.4 mg, 13.7 μmol; dissolved in 0.2 ml MeCN) were added. After 10 min, amine **10** (10 mg, 11.4 μmol) was added. The solution was stirred overnight. Next day DMF was removed completely and the oily residue was suspended in some MeCN (1 ml). TFA was added until pH < 2. MeCN was evaporated and the crude material was purified with HPLC. A white solid was obtained (5.5 mg, 47%), m.p. > 190 °C (decomp.). ^{1}H NMR (300 MHz,

D_2O): 1.20–1.80 m, 4 H; 2.38 t, 2 H, J = 6.59; 2.93 s, 3 H; 3.03 t, 2 H, J = 7.28; 3.13 t, 2 H, J = 5.41; 3.25 t, 2 H, J = 5.16; 3.34 t, 2 H, J = 5.39; 3.40–3.55 m, 6 H; 3.64 t, 2 H, J = 6.61; 4.00–4.10 m, 1 H; 4.10–4.25 m, 2 H; 5.01 s, 1 H; 6.55–6.85 m, 6 H; 6.92–7.00 d, 2 H, J = 8.53; 7.00–7.10 m, 2 H; 7.10–7.30 m, 8 H; 7.82 d, 2 H, J = 7.29. $C_{43}H_{55}N_9O_7$: MS (LC-MS-II): *m/z* (%) [t_r = 4.98 min]: 405 (100), 810 (10, MH^+).

4-((3-(3-Ammoniopropylamino)-3-oxopropyl)(methyl)amino)pyridinium Chloride (**12**)

Acid **5** (216 mg, 1.00 mmol) was dissolved in MeCN (10 ml) and DIPEA (271 mg, 2.10 mmol) was added. The mixture was cooled in an icebath and DCC (227 mg, 1.10 mmol) was added. After 15 min, amine **11** (174 mg, 1.00 mmol) was added. The turbid reaction mixture was stirred at room temperature for 5 h and then the mixture was heated to 50 °C for 30 min. After that time, MeCN was evaporated completely and the residue (viscous oil) was redissolved in DCM. The precipitate (DCU) was filtered off and the crude material was purified with column chromatography (EA/MeOH 9:1 + 1% NEt_3, R_F 0.05). The Boc-protected, purified white solid (134 mg, 40%) was deprotected with a 3:1 mixture of methanol and concentrated hydrochloric acid (4 ml). After complete deprotection (10 min, room temperature), the solvent was evaporated, water was added (2–3 ml) and the solution was lyophilized overnight. A white solid was obtained (110 mg, 36% overall), m.p. > 190 °C (decomp.). ^{1}H NMR (300 MHz, D_2O): 1.60–1.75 m, 2 H; 2.51 t, 2 H, J = 6.61; 2.82 t, 2 H, J = 7.62; 3.00–3.15 m, 5 H; 3.78 t, 2 H, J = 6.61; 6.70–7.00 bs, 2 H; 7.93 d, 2 H, J = 7.48. $C_{12}H_{20}N_4O$: MS (LC-MS-II): *m/z* (%) [t_r = 0.31 min]: 119 (5), 139 (65), 160 (100), 237 (65, MH^+).

(*R*)-4-((4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-9-imino-3,11,18,28,34-pentaoxo-20,23,26-trioxa-2,8,10,12,17,29,33-heptaazahexatriacontan-36-yl)(methyl)amino)pyridinium 2,2,2-Trifluoroacetate (**1d**)

Acid **13** (6.6 mg, 30 μmol) was dissolved in DMF (2 ml) and DIPEA (35 mg, 270 μmol) and TBTU (18.9 mg, 59 μmol) were added. After 10 min, the reaction was started with the addition of the compounds **9** (25.0 mg, 27 μmol) and **12** (8.3 mg, 27 μmol). The reaction mixture was stirred overnight. Next day DMF was removed completely and the oily residue was suspended in some MeCN (1 ml). TFA was added until pH < 2. MeCN was evaporated and the crude mixture was purified with HPLC. A white solid was obtained (5.5 mg, 16%), m.p. > 190 °C (decomp.). ^{1}H NMR (300 MHz, D_2O): 1.25–1.90 m, 10 H; 2.44 t, 2 H, J = 6.48; 2.90–3.20 m, 13 H; 3.50–3.60 m, 8 H; 3.72 t, 2 H, J = 6.48; 3.89 d, 4 H, J = 1.70; 4.05–4.15 m, 1 H; 4.15–4.30 m, 2 H; 5.05 s, 1 H; 6.60–6.90 m, 4 H; 6.95–7.30 m, 12 H; 7.87 d, 2 H, J = 7.52. $C_{52}H_{71}N_{11}O_{10}$: MS (LC-MS-II): *m/z* (%) [t_r = 4.99 min]: 505 (100), 1010 (5, MH^+).

1,4-Diazidobenzene (**15**)

$NaNO_2$ (1.45 g, 21 mmol) was suspended in 10 ml concentrated sulfuric acid at 10 °C in small amounts. The mixture was stirred 10 min at 20 °C and then heated to 70 °C until a clear solution was obtained. The mixture was cooled to 10 °C and 1,4-diaminobenzene (1.08 g, 10 mmol) dissolved in 10 ml glacial acetic acid was dropped slowly into the mixture. A yellow solid precipitated. The mixture was stirred one hour at 15 °C. After that time, the mixture was dropped in a 500 ml flask containing 150 ml ice-cold water (with ice).

A clear, yellow solution was obtained. NaN_3 (1.43 g, 22 mmol) dissolved in 10 ml water was added dropwise at 5 °C under vigorous stirring (caution: HN_3 gas can develop). Nitrogen was formed and a yellow solid precipitated. After 2 h, the product was extracted with EA (2 × 75 ml). The organic layer was separated and dried over $MgSO_4$. The solvent was concentrated and the crude material was recrystallized in PE. Yellow crystalls were obtained (1.06 g, 66%), m.p. 155 °C (decomp.).

tert-Butyl-[(1-(4-azidophenyl)-1*H*-1,2,3-triazol-4-yl)methyl]carbamate (**16a**)

1,4-Diazidobenzene (500 mg, 3.13 mmol) was dissolved in 5 ml of $CHCl_3$ and was added to a solution of Boc-propargylamine (484 mg, 3.13 mmol) in 5 ml of MeOH. Then ascorbic acid (55 mg, 0.31 mmol, dissolved in 0.5 ml of H_2O) and $CuSO_4 \cdot 5H_2O$ (40 mg, 0.16 mmol; dissolved in 0.5 ml of H_2O) were added. The mixture was stirred at 60 °C for 1 h. EA (50 ml) and water (50 ml) were added and the organic layer was separated. The mixture was concentrated and a white solid precipitated (double coupled side product). The precipitate was filtered off and the remaining solution was concentrated and purified with column chromatography (PE/EA 2:3, R_F 0.5). The product was obtained as a white solid (782 mg, 79%), m.p. 155 °C (160 °C decomp.). ^{1}H NMR (300 MHz, $CDCl_3$): 1.45 s, 9 H; 4.47 d, 2 H, J = 6.03; 5.20 bs, 1 H; 7.10–7.20 m, 2 H; 7.65–7.75 m, 2 H; 7.94 s, 1 H. ^{13}C NMR (75 MHz, $CDCl_3$): 28.4, 36.1, 79.9, 120.2, 122.0, 132.1, 133.8, 140.7, 146.4, 155.9. $C_{14}H_{17}N_7O_2$: MS (LC-MS-II): *m/z* (%) [t_r = 3.57 min]: 216 (100, M*H$^+$), 257 (20), 431 (25). The compound was completely Boc-deprotected during LC-MS.

(1-(4-Azidophenyl)-1*H*-1,2,3-triazol-4-yl)methanaminium 2,2,2-Trifluoroacetate (**16b**)

Compound **16a** (287 mg, 0.91 mmol) was deprotected with a 2:1 mixture of DCM and TFA (6 ml). The mixture was stirred for 1 h and then the solvent was removed. Water was added (5 ml) and the solution was lyophilized. A white solid was obtained (300 mg, quantitative), m.p. 157 °C (decomp.). ^{1}H NMR (300 MHz, D_2O): 4.36 s, 2 H; 7.14–7.17 m, 2 H; 7.63–7.66 m, 2 H; 8.44 s, 1 H. ^{13}C NMR (75 MHz, D_2O): 33.9, 120.0, 122.1, 123.4, 132.6, 140.3, 140.9. $C_9H_9N_7$: MS (LC-MS-II): *m/z* (%) [t_r = 3.57 min]: 216 (100, MH$^+$), 257 (20), 431 (25). IR (ν, cm^{-1}): 3166, 2757, 2136 (N_3), 2095 (N_3), 1711, 1618, 1555, 1514.

tert-Butyl-(3-((1-(4-azidophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)ureido)-(methylthio)methylenecarbamate (**17**)

Triphosgene (71 mg, 0.24 mmol) was dissolved in icecold DCM (10 ml) and a solution of compound **16b** (200 mg, 0.61 mmol) and DIPEA (285 mg, 2.21 mmol) was dropped slowly (20 min) at 0 °C into the triphosgene solution. After complete addition, the reaction was allowed to warm up to ambient temperature and a solution of Boc-*S*-methyl-isothiourea **6** (116 mg, 0.61 mmol) in DCM (10 ml) was added over a period of 10 min. The mixture was stirred overnight at ambient temperature. The next day, the reaction mixture was concentrated and the crude material was purified with column chromatography (PE/EA 3:1 → 2:3) [R_F 0.3 (PE/EA 3:2)]. A light yellow solid was obtained (122 mg, 46%), m.p. 126 °C (162 °C decomp.). ^{1}H NMR (300 MHz, $CDCl_3$): 1.48 s, 9 H; 2.29 s, 3 H; 4.58 d, 2 H, J = 6.19; 6.23 t, 1 H, J = 5.49; 7.10–7.20 m, 2 H; 7.75–7.75 m, 2 H; 7.97 s, 1 H; 12.19 bs, 1 H. $C_{17}H_{21}N_9O_3S$: MS (LC-MS-II): *m/z* (%) [t_r = 7.83 min]: 432 (100, MH$^+$), 863 (40).

(*R*)-1-(1-(4-Azidophenyl)-1*H*-1,2,3-triazol-4-yl)-10-(4-hydroxybenzylcarbamoyl)-3,12-dioxo-13,13-diphenyl-2,4,6,11-tetraazatridecan-5-iminium 2,2,2-Trifluoroacetate (**18**)

Compound **17** (101 mg, 0.23 mmol), compound **8** (114 mg, 0.23 mmol) and NEt_3 (230 mg, 2.3 mmol) were dissolved in 3 ml DMF. $HgCl_2$ (64 mg, 0.23 mmol) was dissolved in 0.5 ml of DMF and added to the mixture. The mixture was stirred overnight. Next day DMF was removed completely and the residue was dissolved in 5 ml DCM. Hg-salts were filtered off and the crude material was purified with column chromatography (PE/EA 7:3, R_F 0.4). The compound was deprotected without further characterization. DCM/TFA was added (1:1 mixture, 4 ml) and the mixture was stirred for 2 h. Then it was concentrated and the crude material was purified with HPLC. A white solid was obtained (112 mg, 57%), m.p. > 190 °C (decomp.). ^{1}H NMR (600 MHz, DMSO-d_6): 1.35–1.48 m, 2 H; 1.48–1.70 m, 2 H; 3.10–3.25 m, 2 H; 4.05–4.20 m, 2 H; 4.30–4.35 m, 1 H; 4.46 d, 2 H, J = 5.29; 5.12 s, 1 H; 6.67 d, 2 H, J = 8.45; 7.00 d, 2 H, J = 8.45; 7.15–7.30 m, 10 H; 7.30–7.35 m, 2 H; 7.88–7.92 m, 2 H; 8.13 bs, 1 H; 8.36 t, 1 H, J = 5.80; 8.37–8.46 bs, 1 H; 8.47 d, 1 H, J = 8.11; 8.67 s, 1 H; 8.89 bs, 1 H; 9.28 s, 1 H; 9.73 bs, 1 H. ^{13}C NMR (HSQC, HMBC, ROESY) (151 MHz, DMSO-d_6): 24.55, 29.42, 34.82, 41.58, 52.23, 55.88, 114.97, 120.47, 121.10, 121.59, 126.56, 128.13, 128.17, 128.38, 128.46, 128.48, 129.11, 133.46, 139.70, 140.24, 140.42, 156.23, 170.93, 170.98. $C_{37}H_{38}N_{12}O_4$: MS (LC-MS-II): *m/z* (%) [t_r = 6.01 min]: 715 (100, MH^+). UV (MeCN): λ (ε) 272 (20×10^3). IR (ν, cm^{-1}): 3277, 2130 (N_3), 2100 (N_3), 1686, 1638, 1542, 1512, 1446, 1364.

4-[Methyl(prop-2-ynyl)amino]pyridinium Chloride (**20**)

N-Methylpyridin-4-amine **19** (200 mg, 1.85 mmol) was dissolved in 5 ml of dry THF. Then a BuLi solution (1.6 M in hexanes; 1.4 ml, 2.22 mmol) was added at room temperature under nitrogen. After 10 min, when no more gas was formed, a propargylbromide solution (80 wt.% in toluene; 330 mg, 2.22 mmol) was added slowly at –5 °C over a period of 1 h. The mixture was stirred overnight. The next day water was added (5 ml) and then THF was removed completely under reduced pressure. NaOH (1 M, 20 ml) and EA (20 ml) were added and the organic layer was collected. Organic phase was acidified with $NaHSO_4$ solution (20 ml, 5 wt.%) and the aqueous layer was collected. NaOH (1 M, 40 ml) and EA (40 ml) were added and the organic layer was collected. The organic layer was washed several times with water until not converted *N*-methylpyridin-4-amine **19** was removed completely. The organic layer was dried over $MgSO_4$ and the solvent was removed. A brown oil was obtained. Diluted hydrochloric acid (10%) was added dropwise until pH < 2. A yellow precipitate was formed. It was collected and recrystallized with $CHCl_3$/PE. Light yellow-brown crystals were obtained (33 mg, 10%), m.p. 193 °C (decomp.). ^{1}H NMR (300 MHz, D_2O): 2.63 t, 1 H, J = 2.47; 3.15 s, 3 H; 4.26 d, 2 H, J = 2.47; 6.92 d, 2 H, J = 7.26; 8.00 d, 2 H, J = 7.67. ^{13}C NMR (75 MHz, D_2O): 38.0, 41.5, 74.1, 77.0, 107.8, 138.9, 157.3. $C_9H_{10}N_2$: MS (LC-MS-II): *m/z* (%) [t_r = 0.56 min]: 147 (100, MH^+), 188 (10), 293 (10).

(*R*)-4-(((1-(4-(4-(10-(4-Hydroxybenzylcarbamoyl)-5-imino-3,12-dioxo-13,13-diphenyl-2,4,6,11-tetraazatridecyl)-1*H*-1,2,3-triazol-1-yl)phenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-(methyl)amino)pyridinium 2,2,2-Trifluoroacetate (**1e**)

Compound **18** (10 mg, 12.1 μmol) was dissolved in 0.5 ml of DMSO and alkyne 20 (2.2 mg, 12.1 μmol) was added. The solution was diluted with a of 3:1 mixture of MeOH and H_2O (0.5 ml). Then ascorbic acid (1.1 mg, 6.1 μmol [stock solution 100 mg/ml in 1 M NaOH])

and $CuSO_4 \cdot 5H_2O$ (0.91 mg, 3.6 μmol [stock solution 100 mg/ml in H_2O]) were dropped into the mixture. Copper(I) stabilizing ligand **21** (0.8 mg, 1.21 μmol) was added and the mixture was heated to 65 °C for 1 h. After that time, TLC control demonstrated complete conversion. TFA was added until pH < 2 and the crude material was purified with HPLC. A white solid was obtained (6.7 mg, 51%), m.p. > 190 °C (decomp.). ^{1}H NMR (300 MHz, DMSO-d_6): 1.35–1.75 m, 4 H; 3.15–3.25 m, 2 H; 3.32 s, 3 H; 4.05–4.25 m, 2 H; 4.25–4.40 m, 1 H; 4.40–4.55 m, 2 H; 5.02 s, 2 H; 5.12 s, 1 H; 6.67 d, 2 H, J = 8.45; 7.00 d, 2 H, J = 8.45; 7.05–7.40 m, 12 H; 8.05–8.20 m, 5 H; 8.25–8.60 m, 6 H; 8.81 s, 1 H; 8.91 bs, 1 H; 8.96 s, 1 H; 9.30 s, 1 H; 9.86 bs, 1 H; 13.44 bs, 1 H. $C_{46}H_{58}N_{14}O_4$: MS (LC-MS-II): *m/z* (%) [t_r = 5.18 min]: 431 (100), 861 (5, MH^+), 975 (2).

Phenyl Thioester **26**

Compound **24** (3.00 mg, 4.96 μmol) was mixed with DIPEA (1.92 mg, 14.9 μmol), DMAP (0.06 mg, 0.49 μmol) and TBTU (1.91 mg, 5.95 μmol). After 10 min, the reaction was started with the addition of thiophenol (0.65 mg, 5.95 μmol). Next day, DMF was removed completely and the crude material was purified with HPLC. A blue solid was obtained (2.40 mg, 69%). $C_{41}H_{49}N_2O_4S_2$: MS (LC-MS-II): *m/z* (%) [t_r = 7.87]: 697 (100, M^+), 1395 (15).

p-Nitrophenyl Ester **27**

Compound **24** (2.70 mg, 4.46 μmol) was dissolved in in 1 ml of DMF and DIPEA (2.7 mg, 21 μmol) was added. Then bis(4-nitrophenyl) carbonate (3.1 mg, 10.20 μmol) was added. The mixture was stirred overnight at room temperature. The next day, DMF was removed completely and the residue was purified with HPLC. A blue solid was obtained (2.90 mg, 91%). $C_{41}H_{48}N_3O_7S$: MS (LC-MS-II): *m/z* (%) [t_r = 7.61]: 726 (100, M^+).

Radioligand Competition Binding Assay

Radioligand competition experiments at SK-N-MC neuroblastoma cells using the radioligand [^{3}H]UR-MK114 (1.5 nM) were performed as described elsewhere[16].

Fura-2 Assay on HEL Cells

The Fura assay was performed with HEL cells as previously described using a Perkin–Elmer LS50 B spectrofluorimeter (Perkin–Elmer, Überlingen, Germany)[26].

Hydrolytic Stability of the Active Esters

The studies were performed in 2 ml Eppendorf reaction vessels using 285 μl phosphate buffer (30 mM, pH 7.4) per vessel (cup). Stock solutions of the active esters (1 mM) and DMAP (10 mM) in DMSO were prepared. Then 1.5 μl of the DMAP solution was added to the corresponding cups. Reaction was started with the addition of 15 μl of the corresponding active ester solution (vortexed). Active ester concentration was 50 μM. After 30, 90 and 150 min, 75 μl aliquots were taken and put into 75 μl quenching solution [25 vol.% MeCN, 62.5 vol.% H_2O, 37.5 vol.% TFA_{aq} (1%)]. 5 μl of internal standard (1 mM in MeCN) was added to every cup and the samples were analyzed with HPLC. The integrals of active ester and free acid were determined and the total amount of active ester was calculated (calibration with different concentrations of the free acids from 0.5 to 25 μM).

Acyl-Transfer Studies

The acyl-transfer studies were performed in 2 ml Eppendorf reaction vessels using 200 µl phosphate buffer (30 mM, pH 7.4) per vessel. Stock solutions of the catalysts, nucleophiles and active esters in DMSO were prepared (10 mM). According to the particular experiment 1 µl of catalyst and 1 µl of nucleophile were added to the buffer and the mixture was vortexed. The acyl-transfer was started with the addition of 1 µl of the active ester (vortexed). Then it was incubated for 30 min. After that time, the reaction was quenched with the addition of 10 µl AcOH. 1 µl of a 10 mM solution of an internal standard was added and the samples were analyzed with LC-MS. The internal standard peak height was set as 100% and the relative peak intensities were determined.

Cell Culture and Confocal Microscopy

MCF-7 cells were cultivated in EMEM medium (Sigma, Deisenhofen, Germany), containing 5% FCS (Biochrom AG, Berlin, Germany), in 8 well µ-slides (Ibidi GmbH, Munich) at 37 °C for two days. Estradiol (1 nM) was added to the medium to stimulate Y_1R expression[27]. Before the experiment the medium was replaced by Leibovitz L15 medium (LM). Cells were incubated with 200 nM catalyst (in LM, 200 µl per well) at room temperature for 15 min. Then the medium was removed and the cells were washed once with PBS. Cells without catalyst were also washed with PBS. Thereafter, a 10 µM solution of NHS ester **25** in PBS was added (150 µl per well), and the cells were incubated in the dark at room temperature for 30 min. The cells were washed carefully three times with LM to remove non-covalently bound NHS ester and the free acid **24** from the membranes. To determine non-specific binding 100 nM pNPY (in LM, 200 µl) were added, and cells were incubated at room temperature. Images were taken 90 min after pNPY incubation.

Confocal microscopy was performed with a Zeiss Axiovert 200M microscope, equipped with a LSM 510 laser scanner. The dye S0436 was excited with a 633 nm laser, carboxyfluorescein was excitated at 488 nm. The emission of S0436 dyes was detected >650 nm (long pass filter) and the emission of carboxyfluorescein >505 nm (long pass filter). An immersion objective (Plan-Neofluar 40×/1.3) was used.

Data Processing

Data from radioligand competition experiments were analyzed by 4 parameter sigmoidal fits (SigmaPlot 9.0, Systat Software). IC_{50} values from radioligand competition studies were converted to K_i values according to the Cheng–Prusoff equation[28] using the respective K_D value of the radioligand.

For the determination of Y_1R antagonistic activities in fura-2 assays on HEL cells, three data points (between 20 and 80% inhibition) served for the calculation of IC_{50} values after logit-log transformation. IC_{50} values were converted to K_b values according to the Cheng–Prusoff equation using an $EC5_0$ value of 1.8 nM for pNPY (mean value from 4 independently determined concentration-effect curves on HEL cells).

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